MAST CELLS IN ITCH, PAIN AND NEURO-INFLAMMATION

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MAST CELLS IN ITCH, PAIN AND NEURO-INFLAMMATION

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EDITORIAL
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Editorial: Mast Cells in Itch, Pain and Neuro-Inflammation

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Keywords: mast cells, pain, itch, neuroinflammation, neuro-immune synapse

Editorial on the Research Topic

Mast Cells in Itch, Pain and Neuro-Inflammation

The present e-book contains a collection of articles on the emerging role of mast cells in various immunological, neurological, and cerebrovascular diseases. The growing interest to this cell type is based on the accumulating findings that mast cells are the key players in the so called "*neuro-immune synapse*" underlying pathologic processes both in the periphery and the central nervous system (CNS). This concept suggests that in addition to providing immune surveillance and triggering the inflammatory response, mast cells can also provide a fast-bidirectional crosstalk with neurons (**Figure 1**).

It has been known for a long time that mast cells are ubiquitously present in many tissues, located close or even in direct contact with blood vessels and nerve fibers. The tissues where mast cells are most abundant are body surfaces exposed to the environment, such as skin, lungs, and gut. However, mast cells have a significant presence in the meninges and certain brain regions, especially the diencephalon, under normal and/or pathological conditions. The latter fact is of special interest, as these brain mast cells could be increased or stimulated in certain neurological diseases. Mast cells have been increasingly reported as important contributors to pain conditions such as fibromyalgia, headache, itch, sickle cell disease and brain disorders, stroke, traumatic brain injury, and various neurodegenerative disorders. However, mast cells display tremendous heterogeneity in their location, structure, content of active molecules, responsiveness to the surrounding environment and function. Hence, the mast cell phenotype cannot be generalized and varies in different pathophysiological conditions, including nociception and neurodegenerative disorders. Mast cells may also have a disease modifying effects including the pathobiology of pain, the time-course and the outcome of neurodegenerative diseases such as Alzheimer's and Parkinson disease as well as stroke and traumatic brain injury.

This premise requires establishing the disease-specific role of mast cells. Many of these interesting novel lines of research are presented in the current collection.

The present e-book consists of 17 articles including one brief research report, one hypothesis and theory article, seven original research articles, five reviews, and three mini-reviews.

Although the idea of the *neuro-immune synapse* is presented in direct or indirect manner in most of the papers, some of them are specifically devoted to this concept. Thus, Mittal et al. present the concept of *neuro-immune synapse* and the critical role of mast cells in neuroinflammation. In particular, they discuss the novel aspect of mast cell interaction with the nervous system through extracellular vesicles, tunneling nanotubes, and extracellular traps that may contribute to various pathological brain conditions.

The paper by Magadmi et al. is devoted to the organization of the neuro-immune synapse and suggests the contribution of the CADM1-dependent mechanism for adhesion of the key partners involved in the *neuro-immune synapse*.

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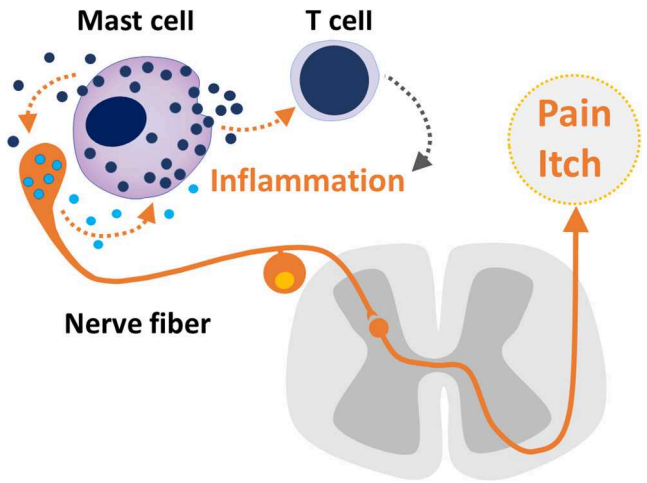

FIGURE 1 | The bidirectional crosstalk between sensory neuron and mast cells, mast cell - T cell communication in inflammation and nociceptive signaling underlaying pain and itch.

Siiskonen and Harvima present the concept of the *neuro-immune synapse* for skin disorders. They reviewed the role of crosstalk between mast cells with sensory nerves as the contributors to neurogenic inflammation and pruritus in chronic skin inflammation.

The most widely accepted role of mast cells is the promotion of pain states. Theoharides et al. proposed that thalamic mast cells contribute to inflammation and pain in fibromyalgia by releasing multiple pro-inflammatory molecules which could either stimulate thalamic nociceptive neurons directly or activate microglia in the diencephalon. They also suggest stabilization of mast cells as the novel approach in treating this painful state.

Richards et al. demonstrate the discogenic back pain and mast cell/Proteinase Activated Receptor 2-mediated interactions in this disorder.

In this collection, the role of meningeal mast cells as the triggers of migraine pain and local inflammation, originally proposed by the co-Editor of this e-book, T. Theoharidis, is presented in several papers. The group of Jansen-Olesen, in the original study, explored the role of the newly emerged migraine mediator PACAP as the trigger of degranulation of meningeal mast cells via the atypical orphan MRGB3-receptor (Pedersen et al.).

The experimental study for Koroleva et al. shows, by using mice deficient in mast cells, that the putative migraine mediator extracellular ATP strongly activates nociceptive firing in meningeal trigeminal afferents via degranulation of resident mast cells and release of serotonin together with the direct excitatory action on the nerve terminals via purinergic receptors.

Irmak et al. review the role of meningeal mast cells in migraine pathology by further developing the concept of couplings between the sensory nerve fibers and meningeal mast cells. They highlight the bidirectional signaling between these key partners of the *neuro-immune synapse* and shared fate of them in generation of the persistent pain state specific for migraine.

The study by Nurkhametova et al. demonstrates the important contribution of P2X7 receptors to ATP-driven activation of meningeal mast cells, suggesting these purinergic mechanisms as potential triggers of neuroinflammation and pain sensitization in migraine.

Traina presents an interesting view on the contribution of intestinal microbiota to mood and behavioral disorders via microbiota–gut–brain axis.

Several papers analyze the role of mast cells in brain disorders and suggest the beneficial role of their stabilization as a promising therapeutic approach. Thus, the study by Dong et al. shows that the stabilization of brain mast cells alleviated neuroinflammation by inhibiting microglia activation.

The paper by Jones and Gupta provides the general overview of the field of mast cells in neuroinflammation and neurodegeneration with the focus on development and progression of four prominent neurodegenerative diseases: Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis, and Huntington's Disease.

Laurino et al. shows that 3-iodothyroacetic acid degranulate hypothalamic mast cells to provide the antidepressant effect in the brain circuits via histamine signaling.

Arac et al. in the "Hypothesis and Theory" article, which is based on their recent original study, further develop the idea that meningeal mast cells are the key effectors in stroke pathology.

Landucci et al. present an interesting hypothesis that thyroid function may affect mast cell function, consequently, and they suggest that mast cell degranulation in the brain may impact thyroid function.

Several papers highlight the phenomenon and explore the underlaying mechanisms of mast cell-induced modulation of the blood-brain barrier permeability. Thus, Kempuraj et al. focuses on the pathogenic stress as the initial trigger in development of neurodegenerative diseases. Specially, they propose the important role of the stress associated corticotropin-releasing hormone (CRH) in activation of mast cells ultimately leading to neuroinflammation.

Tran et al. provide novel mechanistic insights into mast cell-induced blood-brain barrier damage in cerebrovascular diseases via endoplasmic reticulum stress in the endothelium.

In summary, this e-book presents the current state of knowledge about the involvement of mast cells in pain, itch, neuroinflammation, and associated neurological disorders. This collection identifies treatable targets for the development of novel pharmacologic agents and approaches for the treatment of pain and neuro-inflammatory disorders.

AUTHOR'S NOTE

The editorial is devoted to the memory of our colleague Stephen Skaper who passed away in 2018 soon after start of this project. Dr. Skaper's contribution to the field of mast cells will continue to guide the field, and his contribution to the editorial efforts of this issue are highly appreciated.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Activation of P2X7 Receptors in Peritoneal and Meningeal Mast Cells Detected by Uptake of Organic Dyes: Possible Purinergic Triggers of Neuroinflammation in Meninges

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Extracellular ATP activates inflammasome and triggers the release of multiple cytokines in various immune cells, a process primarily mediated by P2X7 receptors. However, the expression and functional properties of P2X7 receptors in native mast cells in tissues such as meninges where migraine pain originates from have not been explored. Here we report a novel model of murine cultured meningeal mast cells and using these, as well as easily accessible peritoneal mast cells, studied the mechanisms of ATP-mediated mast cell activation. We show that ATP induced a time and dose-dependent activation of peritoneal mast cells as analyzed by the uptake of organic dye YO-PRO1 as well as 4,6-diamidino-2-phenylindole (DAPI). Both YO-PRO1 and DAPI uptake in mast cells was mediated by the P2X7 subtype of ATP receptors as demonstrated by the inhibitory effect of P2X7 antagonist A839977. Consistent with this, significant YO-PRO1 uptake was promoted by the P2X7 agonist 2′,3′-O-(benzoyl-4-benzoyl)-ATP (BzATP). Extracellular ATP-induced degranulation of native and cultured meningeal mast cells was shown with Toluidine Blue staining. Taken together, these data demonstrate the important contribution of P2X7 receptors to ATP-driven activation of mast cells, suggesting these purinergic mechanisms as potential triggers of neuroinflammation and pain sensitization in migraine.

Keywords: mast cells, ATP, P2X7 receptor, degranulation, neuroinflammation, migraine

INTRODUCTION

Mast cells are well-known players in allergic responses and essential contributors to inflammation in various tissues (Galli and Tsai, 2012). When activated, mast cells release multiple substances such as biogenic amines, histamine and serotonin, enzymes β-hexosaminidase, chymase and tryptase, and a number of pro-inflammatory cytokines and growth factors (Wernersson and Pejler, 2014). The particular profile of these secreted agents determines the type of inflammatory responses in

surrounding tissues. Notably, mast cells are tissue resident and their morphology and functional role are tissue-dependent according to the local microenvironment and triggering stimuli (Galli et al., 2011). Therefore, the data obtained from one population of mast cells cannot be simply extrapolated to another type of mast cells.

Recently, much attention has been paid to the role of meningeal mast cells as the triggering actor in migraine attack. It has been suggested that degranulation of mast cells located in meningeal tissues contributes to pain signaling in migraine (Levy, 2009, 2012; Kilinc et al., 2017). However, the main missing piece of information in this hypothesis concerns the nature of the endogenous trigger for degranulation of mast cells in meninges *in situ*.

A purinergic hypothesis of migraine, originally proposed by Burnstock (1981), was complimented by a more recent hypothesis suggesting the role of ATP-gated P2X3 receptors in generation of migraine pain (Giniatullin et al., 2008; Yegutkin et al., 2016; Zakharov et al., 2016). However, given the presence of multiple types of ATP receptors in meningeal tissues, the full spectrum of ATP driven mechanisms in migraine remains incomplete. For instance, it is well established that extracellular ATP activates ligand-gated P2X7 receptors present in the majority of immune cells thus leading to a release of multiple pro-inflammatory cytokines and activation of inflammasome (Sperlágh and Illes, 2014; Franceschini et al., 2015; Burnstock, 2016; Karmakar et al., 2016). Consistent with this, P2X7 receptor knockout animals have a blunted inflammatory response and failed to develop certain types of pain (Chessell et al., 2005). In contrast to other types of immune cells, the role of P2X7 receptors in mast cells is little explored. Nevertheless, there is evidence for the role of ATP-mediated mast cell dependent inflammation through P2X7 receptors in the intestine (Kurashima et al., 2012). P2X7 receptors are also characterized in human LAD2 mast cells, derived from a patient with mast cell leukemia (Wareham and Seward, 2016). However, there is still lack of information regarding the role of P2X7 receptors in activation of meningeal mast cells, which are potential players in migraine.

In this study, we developed a new model of cultured meningeal mast cells, and using a combination of various techniques, including time-lapse flow cytometry measurements, we show that mast cells obtained from the peritoneal cavity and from meninges, express ATP-gated P2X7 receptors and are permeable to organic molecules. ATP mediated degranulation of meningeal mast cells may be responsible for the activation of trigeminal nerve fibers and local neuroinflammation in the trigeminovascular system associated with migraine attack.

MATERIALS AND METHODS

Animals

Experiments were performed on 10-12 week-old male C57BL mice obtained from the Animal Facilities of the University of Eastern Finland (UEF). The animal treatment procedures were approved by the Committee for the Welfare of Laboratory Animals of the University of Eastern Finland and the Provincial Government of Kuopio. All experiments were conducted in accordance with the guidelines of the European Community Council (Directives 86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Isolation and Identification of Mast Cells

To obtain meningeal mast cells, we adapted the method of dural immune cell isolation described by McIlvried et al. (2015). Animals under deep Avertin (tribromoethanol) anesthesia were perfused through the ascending aorta with phosphate buffer saline (PBS), pH 7.2. After decapitation, the head was cut along the sagittal suture and the brain was gently removed from hemispheres leaving intact meninges. For meningeal mast cell isolation, hemiskulls were gently scraped with pestles into PBS. The obtained cell suspension was transferred to ice-cold PBS supplied with 2% of heat inactivated fetal bovine serum (FBS) and centrifuged at 300 *g* for 5 min at 4°C. The pellet was resuspended in PBS, filtered through 70 μm pre-separation filters (Miltenyi Biotec, Germany) and used for mast cell identification.

Peritoneal mast cells were isolated as described previously by Jensen et al. (2006) with slight modifications to improve cell viability and minimize baseline mast cell activation: lavage procedure was performed using ice-cold PBS with 2% FBS and all following steps were conducted at 4°C. The obtained pellet was resuspended in PBS and filtered through 50 μm filters (Sysmex CellTrics®, Germany).

For flow cytometry characterization, peritoneal or meningeal cells were stained with anti-mouse FcεRI conjugated with Alexa Fluor® 647 (clone MAR-1, BioLegend, USA), and CD117 conjugated with tandem dye APC/Cy7 (clone 2B8, Biolegend) antibodies for 15 min at room temperature, washed with PBS with 2% FBS (300 g for 5 min) and resuspended in 300 μl of fresh PBS. Cell viability was determined using SYTO 16 Green Fluorescent Nucleic Acid Stain (Thermo Fisher Scientific, Waltham, MA, USA).

The data were acquired using BD FACSAria™ III cell sorter (BD Biosciences, San Jose, CA, USA) equipped with 488 and 633 nm lasers. SYTO 16 is excited by the 488 nm laser and detected through 530/30 filter. Phenotyping marker fluorochromes are excited by the 633 nm laser and detected through 660/20 and 780/60 filters for Alexa Fluor® 647 and APC/Cy7, respectively. Compensation for the spillover of fluorochromes into other channels was made using single stained cells.

Culturing of Peritoneal and Meningeal Mast Cells

Unfractionated peritoneal cells or cells obtained by hemiskull scraping were centrifuged at 300 *g* for 5 min at 4°C. The pellet was re-suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% antibiotics (penicillin/streptomycin), 2 mM L-glutamine, 50 μM B-mercaptoethanol, 10 ng/ml murine recombinant stem cell factor (SCF; PeproTech, NJ, USA), and 10 ng/ml murine recombinant interleukin (IL)-3 (PeproTech, NJ, USA). After 2–3 weeks of culture, more than 98% of cells were identified as

mast cells by Toluidine Blue staining. Cells were kept in culture for up to 5 weeks.

Toluidine Blue Staining of Meningeal Mast Cells

Whole mount meninges on hemiskulls were pre-treated with or without 1 mM ATP in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 115, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 25 and glucose 11; bubbled with 95% O_2/ 5% CO_2) for 10 min at room temperature. Then samples were fixed with 4% paraformaldehyde at 4°C overnight. After rinsing with PBS, meninges were carefully dissected from the skull, and put on a glass coated with poly-L-lysine (Polysine® Thermo-Scientific, USA). Staining with Toluidine Blue (pH 2.0) was performed according to the standard protocol we described previously (Levy et al., 2007; Kilinc et al., 2017). Images were captured using Olympus AX-TFSM microscope (Olympus, Japan). The number of granulated and degranulated mast cells in each meninges (n = 5) was counted in five random areas containing the main branches of the middle meningeal artery by an observer blinded to treatment groups. Mast cells were classified as degranulated if they were pale, poorly stained, had distorted cytoplasmic boundaries, and surrounding positively stained granules (Shelukhina et al., 2017).

Stimulation of Peritoneal and Meningeal Mast Cells With ATP

To study P2X7 receptor activation in freshly isolated peritoneal and meningeal mast cells, the cells were treated with different concentrations of ATP and 2′,3′-O-(benzoyl-4-benzoyl)-ATP (BzATP; both from Sigma-Aldrich, Germany). Notably, BzATP is more potent than ATP as an agonist at P2X7 receptors whereas it is equally or less potent than ATP at other P2X receptors (North and Surprenant, 2000).

ATP-induced mast cell activation was evaluated using the fluorescent dye YO-PRO1 (Thermo Fisher Scientific, Waltham, MA, USA) which enters the cells through the dilated P2X7 receptor ion channel (Michel et al., 1999; Browne and North, 2013; Browne et al., 2013). ATP at final concentrations 100 μM, 1 mM or 5 mM or BzATP to a final concentration of 100 μM were added and samples were incubated for 20 min in the dark at room temperature, followed by addition of 1 μM of YO-PRO1. After incubation, 200 μl of fresh PBS was added.

Samples were run on a BD FACSAria III cell sorter (BD Bioscience). YO-PRO1 is excited by the 488 nm laser and detected through 530/30 filter. The data were shown as a percentage of YO-PRO1 positive cells in each sample as previously reported (Karmakar et al., 2016).

Cultured mast cells, before stimulation, were washed once with Dulbecco's PBS, and then centrifuged at 300 g for 5 min at 4°C, and the pellet was resuspended in 1 ml of PBS. A cell suspension (5×10^5 cells/ml) was plated onto 24-well plates (100 μl per well). Meningeal cell-derived mast cells (MDMCs) were stimulated with ATP at final concentration 1 mM for 5 min at room temperature. Peritoneum-derived mast cells were stimulated with ATP (100 μM, 1 or 5 mM) or BzATP (100 μM) at room temperature. For inhibitory experiments, peritoneum-derived mast cells were pre-treated with P2X7 antagonist A839977 (10 μM) for 5 min followed by 1 mM ATP stimulation. Application of PBS was used as the control. After incubation, mast cells were transferred onto glass microscope slides, dried at 37°C, and stained with Toluidine Blue. The number of intact and degranulated mast cells was counted randomly and blindly in five fields on each slide. Mast cells were defined as stated above (Shelukhina et al., 2017).

Time-Lapse Analysis of DAPI Fluorescence

It has been recently shown that P2X7 receptors are also permeable to the DNA dye 4,6-diamidino-2-phenylindole (DAPI; Bukhari et al., 2016). We used flow cytometry to determine the time-course of DAPI uptake (excitation/emission 405/450 nm) by mast cells. Peritoneal mast cells were identified based on FcεRI and CD117 expression as described above. Samples were analyzed using the Cytoflex flow cytometer equipped with 405, 488, and 638 nm lasers (Beckman Coulter Inc., CA, USA). A peristaltic pump in this device allowed the addition of the agonist ATP during on-line acquisition of data. ATP at final concentrations 100 μM or 1 mM was added at 20 s after the beginning of the recording. Up to 25,000 peritoneal mast cells per sample were acquired during 120 s. All flow cytometric data were analyzed using CytExpert Software v 1.3 or Kaluza Software v 1.5 (Beckman Coulter Inc., CA, USA). DAPI (1 μg/ml) incorporation was measured by using median fluorescence intensity (MFI) of single cells after ATP application or control. The data from independent experiments were tested for normality of distribution by the Kolmogorov-Smirnov test ($n > 50$) at each time point.

Statistical Analysis

Data were analyzed using Statistica 8 Software (Quest Software Inc., Aliso Viejo, CA, USA), Origin (Origin labs, MS, USA) and GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed using nonparametric Mann-Whitney U test, Student t-test or one-way ANOVA, followed by Dunnett's multiple comparisons test when appropriate. Differences with p values of less than 0.05 were considered statistically significant. The data are presented as mean ± SEM.

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

RESULTS

Identification of Mast Cells

In order to distinguish a population of murine mast cells from other cell types localized in meninges or in the peritoneal cavity, the cell surface expression of FcεRI and CD117 was determined. FcεRI is a mast cell membrane receptor specific for IgE, which is a potent inducer of mast cell activation and degranulation (Rivera and Gilfillan, 2006) whereas CD117 (also named c-kit) is a receptor for the SCF important for mast cell migration, survival and proliferation (Yamazaki et al., 2015). **Figure 1** shows

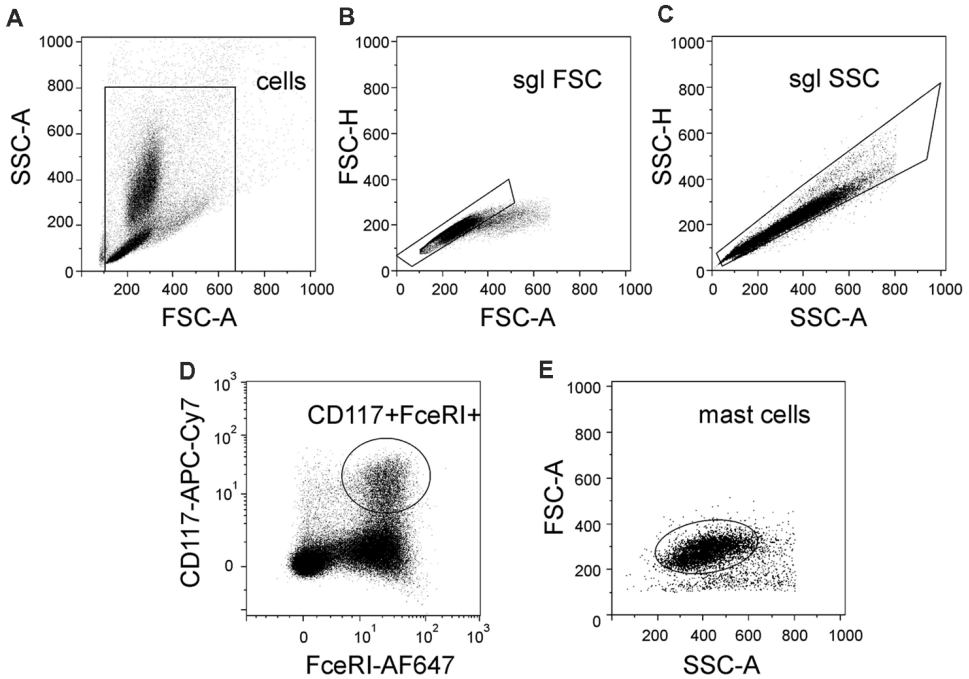

FIGURE 1 | Flow cytometric gating strategy used to identify mast cells. **(A)** Light scatter profile for cells based on forward scatter (FSC-A) and side scatter (SSC-A) (the region is set to discriminate between cells and debris). **(B,C)** Singlet gating based on FSC-H vs. FSC-A and then SSC-A vs. SSC-H, respectively (the regions are set to discriminate cell doublets). **(D)** Cells were divided into subpopulations based on Alexa Fluor 647-FcεRI and APC-Cy7-CD117 (the region is set to discriminate between double-positive cells and all other subpopulation of cells). **(E)** Back-gating of double positive cells onto the light scatter plot FSC-A vs. SSC-A. This shows relative size and granularity of mast cells compared to those in **(A)**. Cells from the region of "mast cells" were used further to assess the action of ATP on mast cell functionality.

the gating strategy of our protocol to obtain the final fraction of mast cells. Thus, there are light scatter dot plots for isolated cells based on forward scatter (FSC-A) related to light refraction and cell size and side scatter (SSC-A) reflecting cell granularity (**Figure 1A**). The selected region from the light scatter plot, which eliminates debris, was set to remove doublet cell aggregates (**Figures 1B,C**). Cells were further divided into subpopulations based on expression of FcεRI and CD117 (**Figures 1D,E**) to isolate the fraction of "mast cells." Cells with double positive expression of both FcεRI and CD117 were identified as mast cells. This approach allowed us to identify a pure fraction of mast cells, which were further tested with the purinergic agonists.

ATP-gated P2X7 Receptor Mediates Peritoneal Mouse Mast Cell Degranulation

First, to optimize our technical approach, we evaluated the ability of ATP to activate ATP-gated P2X7 receptors in easily accessible peritoneal mouse mast cells by using YO-PRO1 which is able to penetrate the cell membrane during the activation of P2X7 receptors (Browne et al., 2013). As expected, unstimulated mast cells failed to take up YO-PRO1, as only 8.2 ± 1.3% (n = 11) of the cells contained YO-PRO1 (**Figure 2A**). Application of 1 mM ATP for 15 min increased the uptake up to 37.9 ± 9.2% (n = 8, $p < 0.01$; **Figure 2B**). P2X7 specific antagonist A839977 (Honore et al., 2009) prevented the ATP induced increase in YO-PRO1 signal (8.7 ± 0.6%, n = 4; **Figure 2C**) whereas application of P2X7 agonist BzATP (Bianchi et al., 1999) effectively enhanced the YO-PRO1 loading of the cells by 32.2 ± 10.9% (n = 5, $p < 0.05$; **Figure 2D**). Next, we demonstrated the dose-dependent action of ATP on YO-PRO1 uptake (**Figure 2E**). Stimulation of mast cells with increasing concentrations of ATP led to increased YO-PRO1 uptake. Pre-treatment with P2X7 antagonist A839977 (5 μM) prevented the ATP-induced YO-PRO1 incorporation (**Figure 2E**).

Next, we reconstructed a time-course for ATP-induced responses of P2X7 allowing us to characterize the early events in the activation of P2X7 receptors. Application of 100 μM ATP on murine peritoneal mast cells induced a slight increase in DAPI fluorescence by 120 s (**Figures 3A,D,E**) whereas in samples treated with 1 mM ATP, a robust enhancement of DAPI fluorescence was observed during the 120 s recording time (**Figures 3B,D,E**). Specific P2X7 receptor antagonists prevented the stimulatory effect of 1 mM ATP on DAPI fluorescence (**Figures 3C–E**).

In addition to the flow cytometry approach, we confirmed the ATP action on cultured peritoneal mast cells by morphological analysis (**Figure 4**). In control conditions, most mast cells were intact, and exhibited dense, compact, unbroken cytoplasmic boundaries and did not have many surrounding granules (**Figure 4A**). Application of 1 mM ATP (**Figure 4B**) or 100 μM BzATP (**Figure 4C**) increased the number of mast cells with

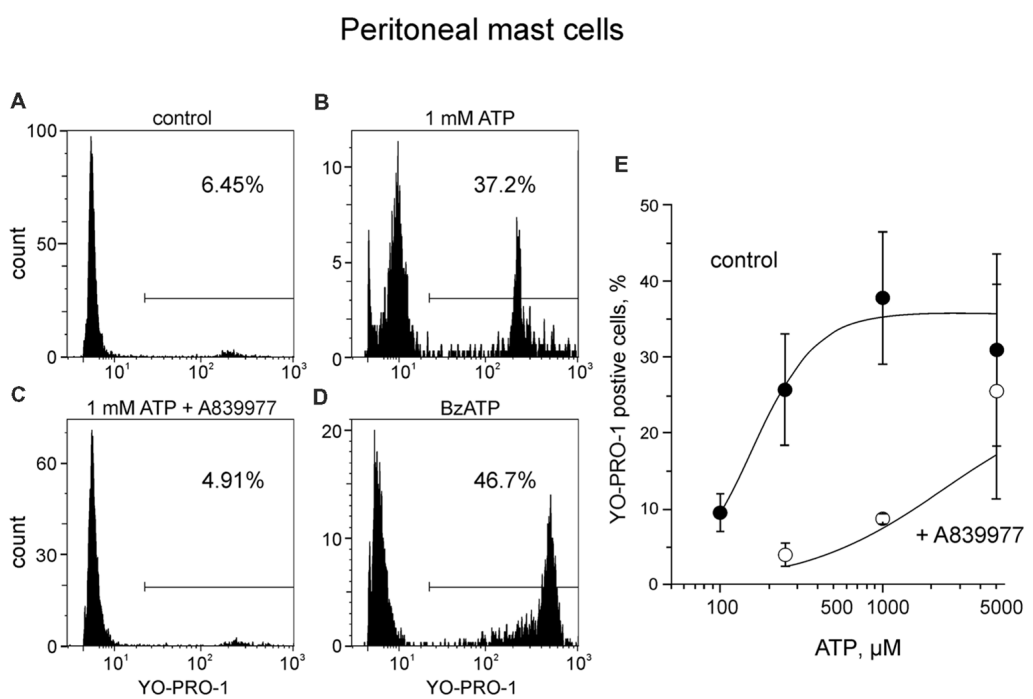

FIGURE 2 | ATP induces the uptake of YO-PRO1 by peritoneal mast cells. **(A–D)** Representative histograms (fluorescence intensity vs. number of cells) of YO-PRO1 incorporation into murine peritoneal mast cells. **(A)** Negative control of untreated murine peritoneal mast cells incubated with 1 μM of YO-PRO1 for 15 min ($n = 11$). **(B)** Mast cells pre-treated with 1 mM of ATP for 15 min and stained with 1 μM of YO-PRO1 ($n = 8$, $p < 0.01$). **(C)** Mast cells in the presence of P2X7 antagonist A839977 (5 μM), then stimulated with 1 mM of ATP for 15 min and stained with 1 μM of YO-PRO1 ($n = 4$). **(D)** Mast cells pre-treated with 100 μM of 2′,3′-O-(benzoyl-4-benzoyl)-ATP (BzATP) for 15 min and stained with 1 μM of YO-PRO1 ($n = 5$, $p < 0.05$). **(E)** The dose-response curve showing percent of mast cells which incorporated YO-PRO1 after stimulation with increasing doses of ATP and in the presence of the P2X7 antagonist A839977 (5 μM). Mean ± SEM (one-way ANOVA, followed by Dunnett's multiple comparison test).

blurred contours and numerous granules around the cells which is indicative of degranulation. Notably, even 100 μM ATP significantly increased mast cell degranulation (by 37.1 ± 1.5%, $n = 4$, $p = 0.0013$). However, a much higher level of degranulation was observed with 1 mM and 5 mM ATP. Consistent with this the P2X7 agonist BzATP (100 μM) effectively degranulated most mast cells. Pre-treatment with the P2X7 antagonist A839977 (10 μM) suppressed the ATP-induced degranulation (**Figure 4D**). Thus, consistent with flow cytometry data, two agonists (ATP and BzATP) induced significant degranulation of peritoneal mast cells mediated by the P2X7 receptors.

Long-Term Culturing Enriched Mature Meningeal Mast Cells

In order to evaluate P2X7-receptor activation on a more relevant model of mast cells, we developed a method to culture mouse meningeal mast cells. Freshly isolated meningeal cells were identified in Toluidine Blue stained slides by their rounded shape and average size of 12 μm (**Figure 5A**). The granules of meningeal mast cells were always stained metachromatically in violet by Toluidine Blue. All other meningeal cells, their nuclei and the nuclei of mast cells were stained orthochromatically in blue (**Figure 5A**). During 1 month of observations, the cultures were significantly enriched by mast cells (**Figures 5A–D**). After 1 week in culture, all cells showed similar morphological features: a rounded shape with an average size of 7.95 ± 1.1 μm and poorly visualized granules in the cytoplasm. The cytoplasm was stained in tones from light blue to blue in the presence of Toluidine Blue (**Figure 5B**). After 2 weeks of cultivation, the cultured cells retained a round shape and had an average size of 7.33 ± 0.96 μm. After two and three weeks, granules were seen within the cytoplasm of these cells (**Figure 5C**, red arrows). After 3 weeks of cultivation, cells exhibited a rounded shape and the cytoplasm was filled with metachromatically (violet) stained granules (**Figure 5D**). Mature mast cells obtained from the culture were heterogeneous in size (average cell size was 7.3 ± 0.97 μm) and density of metachromatic granules. The MDMCs maintained such morphology up to 3 months of culture.

Murine mast cells were identified from other cell types localized in meninges or in the peritoneal cavity based on their cell surface expression of FcεRI and CD117 (**Figures 5E–H**). **Figure 1** shows the gating strategy to identify a pure fraction of mast cells. Less than 1% of the freshly isolated cells from meningeal tissues were FcεRI+CD117+ positive. By the end of the first week of culturing the percentage of FcεRI+CD117+ cells increased up to 27%, reaching over 95% by the second week in culture (**Figures 5E–H**). The percentage of viable FcεRI+CD117+ mast cells remained over 95% up to fifth week of culture. The culture viability remained between 81% and 100% for the first

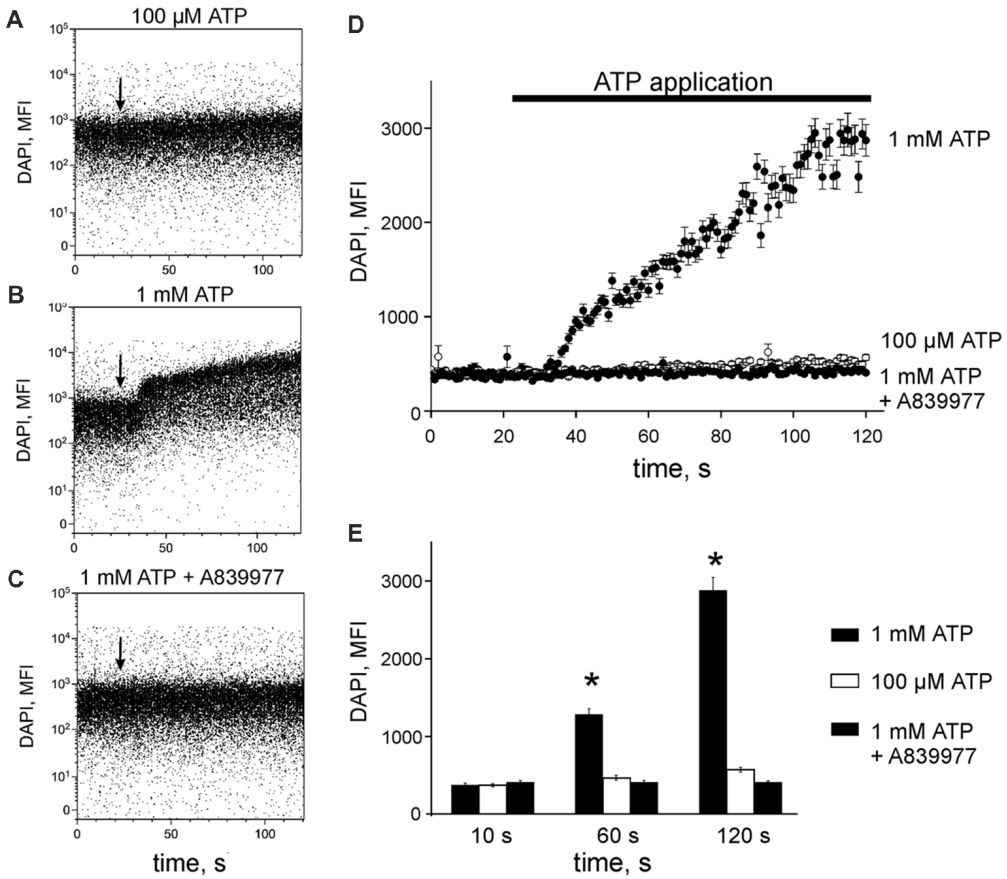

FIGURE 3 | ATP induces 4,6-diamidino-2-phenylindole (DAPI) uptake by peritoneal mast cells. **(A–C)** Representative dot-plots [time vs. DAPI (1 μg/ml) fluorescence intensity] obtained from murine peritoneal mast cells stimulated with 100 μM and 1 mM ATP, 1 mM ATP in the presence of 150 nM P2X7 antagonist A839977 (dot-plots **A**, **B** and **C**, respectively). The X-axis represents the time (seconds) from the beginning of sample acquisition, ATP was added at 20 s; the Y-axis represents the relative fluorescence of DAPI (notice log scale). **(D)** Pooled DAPI fluorescence intensity data for murine peritoneal mast cells ($n = 3$) stimulated with 100 μM and 1 mM ATP, and 1 mM ATP + 150 nM P2X7 antagonist A839977, respectively. **(E)** Histograms showing DAPI fluorescence at three different time points before and after application of 100 μM ATP (white), 1 mM ATP (black) alone or 1 mM ATP in the presence of 150 nM P2X7 antagonist A839977 (gray). Mean ± SEM, $n = 3$, $^*p = 0.049$ (paired sample Student t-test).

1–4 weeks and decreased to 62.4% during the fifth week in culture (data not shown).

P2X7 Receptors Are Expressed in Meningeal Mouse Mast Cells

Next, in order to investigate the tissue specific properties of mast cells in the dura mater, where they are likely to be involved in triggering of migraine attack (Levy, 2009; Kilinc et al., 2017), we explored P2X7 receptor activation in meningeal mast cells. Mouse meningeal mast cells were identified based on labeling with CD117 and FcεRI (**Figure 6A**) and further separated from debris based on their light scatter characteristics (FCS-A vs. SSC-A, **Figure 6B**). The final population contained at least 95% of viable meningeal mast cells (**Figure 6C**) which were tested for ATP-induced P2X7 activation. In control conditions, in a population of freshly purified cells obtained from meninges, the percentage of YO-PRO1 uptake was low, approximately 14.4 ± 1.9% (**Figure 6D**). Incubation with 1 mM ATP significantly enhanced the uptake of the dye (**Figure 6D**). Similar to the peritoneal cells, the treatment with the P2X7 antagonist A839977 (5 μM) prevented the ATP induced YO-PRO1 uptake (**Figure 6D**).

To explore if the purinergic challenge has a functional impact on the release of active components from granules we tested the degranulation ability of 1 mM ATP on mouse meningeal mast cells in whole mount meningeal tissues as identified by Toluidine Blue staining (Levy et al., 2007; Kilinc et al., 2017). In naïve isolated meninges most mast cells were intact (**Figure 7A**), whereas ATP triggered degranulation of multiple mast cells localized near the meningeal artery (**Figure 7B**). **Figure 7C** shows pooled data obtained from five mice, indicating the ability of ATP to induce significant

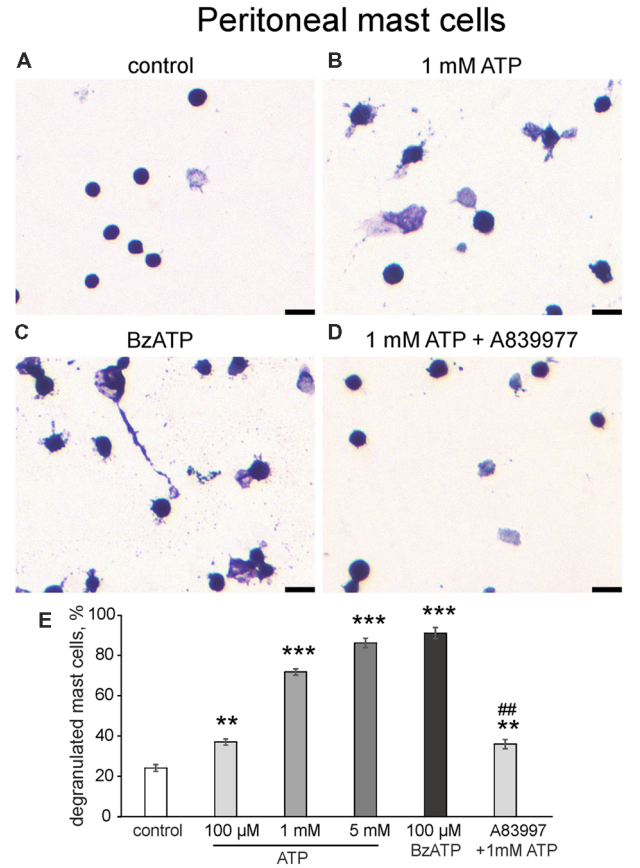

FIGURE 4 | Degranulation of cultured peritoneal mast cells induced by ATP and BzATP. **(A–D)** Toluidine Blue staining of cultured peritoneal mast cells (20×). **(A)** Control conditions, **(B)** application of 1 mM ATP, **(C)** application of 100 μM BzATP, **(D)** pre-treatment with P2X7 antagonist A839977 (10 μM) followed by 1 mM ATP stimulation. **(E)** Histograms showing a percent of degranulated mast cells in control, after application of ATP (100 μM, 1 mM, 5 mM) and BzATP (100 μM), and after pre-treatment with A839977 (10 μM) followed by application of 1 mM ATP. Calibration bar 100 μm. Mean ± SEM, $n = 4$, $^{**}p \leq 0.005$ vs. control, $^{***}p < 0.0001$ vs. control, $^{\#\#}p < 0.0001$ vs. 1 mM ATP group (one-way ANOVA, followed by Dunnett's multiple comparison test).

degranulation of mast cells in the dura mater. These findings were further confirmed in cultured meningeal mast cells by morphological analysis. In control conditions most mast cells were intact (**Figure 7D**), and the application of 1 mM ATP significantly increased the number of degranulated mast cells (**Figures 7E,F**).

DISCUSSION

Here, we show for the first time that rodent mast cells derived from meninges can be grown, matured and enriched in long-term culture. The ability of mast cell granules to show metachromasia, that is, to display a color different from that of the applied dye, is a key feature of mature mast cells. In mammalian mast cells, the distinctive property of metachromasia is accounted for the presence of heparin, a sulfur-rich glycosaminoglycan, in mast cell secretory granules (Härmä and Suomalainen, 1951). Based on these data, blue (orthochromatic) staining of mast cell granules, which was observed after 1 week of cultivation, may indicate immaturity. The appearance of violet (metachromatic) granules in the cell cytoplasm after 2 weeks of cultivation is evidence of active maturation. The presence of metachromatic granules in almost all cells after 3 weeks of cultivation indicates the functional maturity of the mast cells at this time point.

The high-affinity IgE receptor (FcεRI) and CD117 (c-Kit) we used for flow cytometry are classical mast cell markers. Both mast cell progenitors and mature mast cells express these cell surface markers (Dahlin et al., 2015). This explains the high percentage of double positive cells already after 2 weeks of cultivation when only a few cells were identified to contain metachromatic stained granules in the presence of Toluidine Blue. Comparing the obtained flow cytometry data with the Toluidine Blue staining data, it can be concluded that the MDMCs have reached maturity after 3 weeks of cultivation. At this time point, more than 95% of the living cells in culture express FcεRI and CD117 and contain metachromatically violet stained (mature) granules in the cytoplasm. Based on these results, we recommend the use of MDMC culture 3–5 weeks after the start of cultivation.

By using these and the easily accessible peritoneal mast cells, we characterized the role of ATP-gated P2X7 receptors associated with the uptake of organic dyes. The ability of ATP to degranulate meningeal mast cells suggests that this extracellular purinergic messenger could act as an endogenous trigger of neuroinflammation in various neurological disorders, including meningitis and migraine. ATP-gated P2X7 receptors are important triggers of neuroinflammation in different tissues. The activation of P2X7 receptors in mast cells is associated with release of pro-inflammatory cytokines such as IL-1β, IL-18 (Ferrari et al., 2006) and IL-6 (Shieh et al., 2014) which are essential contributors to neuropathic and inflammatory pain (Chessell et al., 2005; Sperlágh and Illes, 2014). The role of the NLRP3 inflammasome and release of IL-1β have been shown also in pneumococcal meningitis (Zwijnenburg et al., 2003) suggesting the involvement of P2X7 receptors in this pathology. However, recent testing with P2X antagonists did not reveal a significant change in the time-course of the disease which the authors explained by down-regulation of ATP receptors expression and decreased concentration of endogenous ATP (Zierhut et al., 2017). Unlike bacterial meningitis, in aseptic form of this disorder, so called drug-induced aseptic meningitis, headache is the leading symptom (Holle and Obermann, 2015), thus closely linking trigeminal pain and meningeal neuroinflammation.

P2X7 receptors are expressed in the majority of immune cells (Junger, 2011; Burnstock and Boeynaems, 2014). For instance, they have been found in macrophages (Moore and MacKenzie, 2007), monocytes (Humphreys and Dubyak, 1998; Grahames et al., 1999), neutrophils (Chen et al., 2004; Christenson et al., 2008), and different subtypes of T cells (Frascoli et al., 2012; Rissiek et al., 2015). However, P2X7 receptors are less studied in mast cells, which are often implicated in allergic reactions and in neuroinflammation. Nevertheless, one study

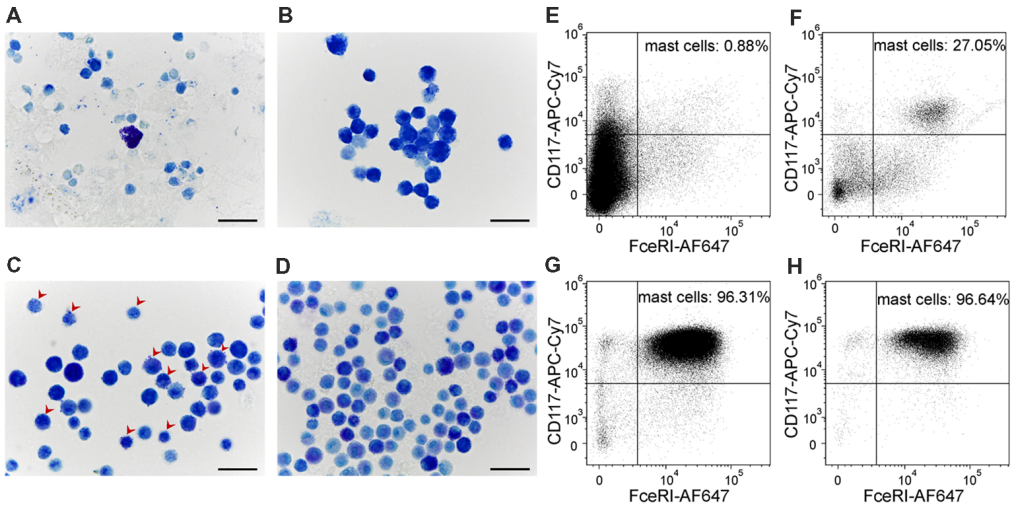

FIGURE 5 | Cultured meningeal mast cells exhibit typical mast cell morphology. **(A–D)** Toluidine Blue staining of **(A)** freshly isolated meningeal mast cells, **(B)** cells kept in culture for 1 week, **(C)** 2 weeks (red arrows showing the presence of both blue and violet granules in the cytoplasm of mast cells), and **(D)** 3 weeks. **(E–H)** Cultured mast cells were identified by their surface expression of FcεRI and CD117. The percentage of FcεRI+CD117+ cells increased upon time in culture. **(E)** freshly isolated cells, **(F)** cells after 1 week in culture, **(G)** cells after 2 weeks in culture, **(H)** cells after 3 weeks in culture.

reported that P2X7 receptors in mast cells play a role in gut inflammation (Kurashima et al., 2012). Another recent very detailed study, using a calcium imaging technique, demonstrated the functional expression of different P2X receptors, including P2X7 subtype, in human LAD2 mast cells (Wareham and Seward, 2016). The advantage of our study is that we focused on techniques, which allowed us to test the function of P2X7 receptors that is critical for initiation of neuroinflammation and compared two different populations of native mast cells.

Activation of P2X7 receptors in different cells is often followed by uptake of relatively large organic molecules such as the fluorescent dye YO-PRO1, which normally does not penetrate the cell membrane (Michel et al., 1999; Jindrichova et al., 2015; Bukhari et al., 2016). It is still a matter of debate whether these dyes penetrate the dilated ion channel of P2X7 receptor or enter through other P2X7 receptor associated proteins (Rassendren et al., 1997; Jiang et al., 2005; Pelegrin and Surprenant, 2006). Recent studies, however, showed that the P2X7 receptor permeability to organic cations such as YO-PRO1 is the intrinsic property of the ion channel itself determined by the long COOH-terminal tail reviewed recently by Di Virgilio et al. (2018). Thus, our data with the measurement of the fluorescence of YO-PRO1 and DAPI reflect, actually, the function of the ion channel of the P2X7 receptor opened by BzATP or ATP.

In the current project, using flow cytometry, we found that stimulation of mast cells with ATP led to P2X7 receptor mediated influx of YO-PRO1 in murine peritoneal and meningeal mast cells. This uptake was inhibited by the P2X7 antagonist A839977 suggesting either direct or indirect involvement of P2X7 receptors. A similar effect was observed using the P2X7 agonist BzATP and with relatively high concentrations of ATP. Taken together, these findings indicate a key role for P2X7 receptor in the activation of mast cells.

One novelty of our study was to use a flow cytometry technique to assess the permeability of mast cell membrane for the dye DAPI in real time after stimulation with 1 mM ATP. We found that stimulation with ATP caused DAPI influx into murine peritoneal mast cells in tens of seconds, and this effect was completely prevented by the P2X7 antagonist A839977. These data indicated the key role of P2X7 receptors in activation of mast cells.

It has been reported that human P2X7 receptor has essentially a higher affinity for several agonists than the mouse equivalent (Chessell et al., 1998). This suggests that the processes, which we observed in mouse cells, could be better presented in human tissues. Moreover, in humans, there are differences in dye uptake properties of the P2X7 receptor due to high polymorphism typical for this receptor type. Interestingly, this single nucleotide polymorphism can be linked (or probably even determine) lower pain sensitivity (Sorge et al., 2012). The latter observation, essential for personified pain medicine, highlights the need for further investigation of native P2X7 receptors in individual patients in order to evaluate the risk of pain state formation.

Mast cells are best known for their ability to release a plethora of various active substances. The early phase of mast cell activation leads to a release of pre-formed pro-inflammatory mediators from secretory granules followed by synthesis of lipid messengers, cytokines and chemokines (Boyce, 2005; Lorentz et al., 2012; Wernersson and Pejler, 2014). Classical mediators such as histamine and serotonin are released by different mechanisms such as degranulation (Dvorak, 1992; Moon et al., 2014) and constitutive or regulated exocytosis (Lacy and Stow, 2011; Lorentz et al., 2012; Moon et al., 2014). Degranulation of

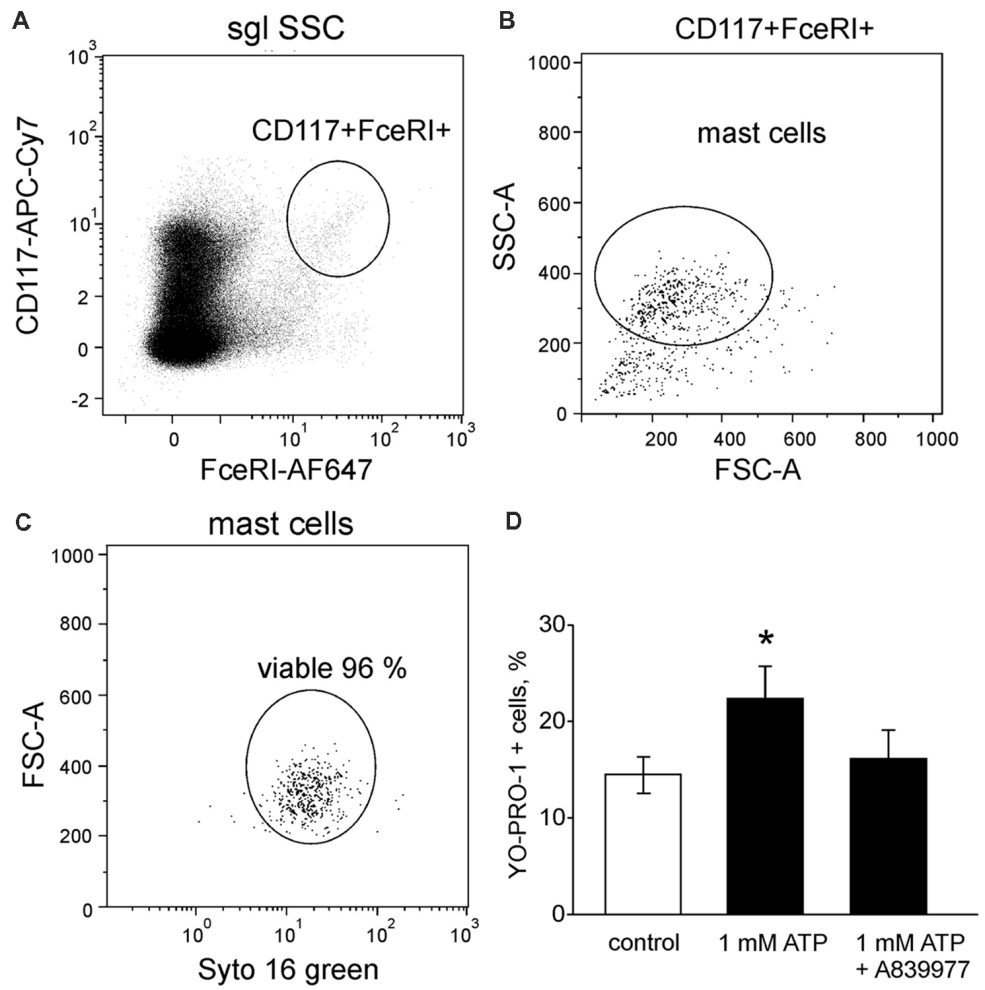

FIGURE 6 | YO-PRO1 uptake in meningeal mast cells in response to ATP. **(A)** Flow cytometric gating strategy used to identify mouse meningeal mast cells is first based on CD117 and FcεRI labeling. **(B)** Next elimination of debris by gating of mast cells based on light scattering properties FSC and SSC. **(C)** Viable meningeal mast cells are gated on the viability dye SYTO 16 Green Fluorescent Nucleic Acid Stain. **(D)** Stimulation with ATP led to increase of YO-PRO1 positive cells (n = 8) whereas pre-treatment with the P2X7 antagonist A839977 inhibited incorporation of YO-PRO1 (n = 5). Mean ± SEM, (n = 10 in control), *p = 0.016 (Mann-Whitney U test).

mast cells may be provoked by various stimuli such as antigens, monomeric IgE, neuropeptides (substance P, CGRP) and viruses involving different receptors and various signaling mechanisms (Moon et al., 2014). Among a number of stimuli, ATP emerged recently as an important trigger of mast cell activation (Wareham and Seward, 2016). These authors showed P2X7 mediated mast cell activation and degranulation in LAD2 mast cells by assessing calcium fluxes and β-hexosaminidase release (Wareham and Seward, 2016). In our study, we demonstrated not only that ATP activates native P2X7 receptors in meningeal mast cells but also showed that the application of ATP induces the release of granules from these cells.

According to common view, migraine pain is initiated by sensitized trigeminal nerve terminals in meninges within the so-called trigeminovascular system (Moskowitz, 1993; Levy, 2012; Zakharov et al., 2015). Meninges are occupied by a plethora of mast cells, which are localized at "strategic loci" close to main meningeal vessels and nerve fibers suggesting a functional interaction (Levy, 2009; Kilinc et al., 2017). Although we do not have direct evidence that mast cells degranulation causes activation of trigeminal nerve endings, there are data which provide evidence that activation of mast cells plays a triggering role in the underlying sensitization process (Levy et al., 2007; Kilinc et al., 2017). In a previous study, we developed the purinergic hypothesis of migraine originally proposed by Burnstock (1981), by showing that extracellular ATP activates primary afferents in meninges (Yegutkin et al., 2016). One open issue still remains: what is the source of extracellular

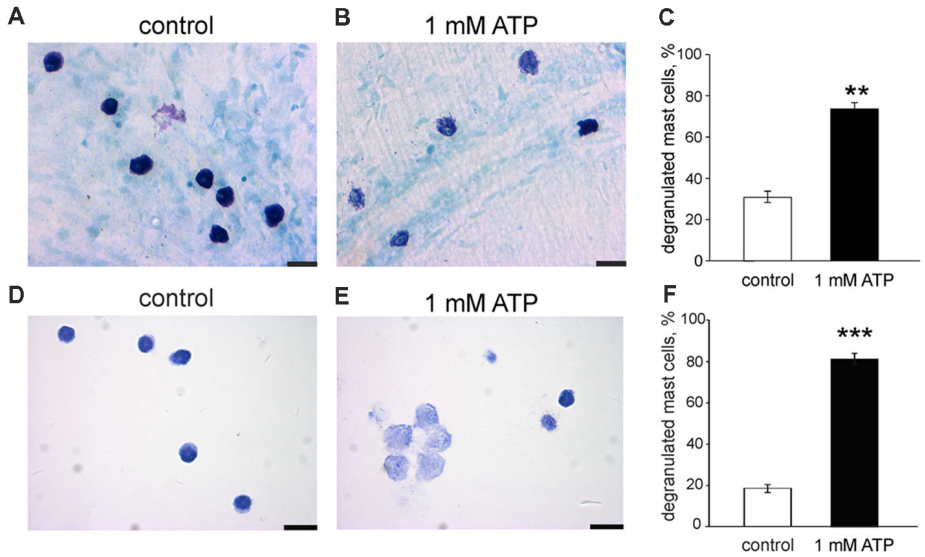

FIGURE 7 | Degranulation of native and cultured meningeal mast cells with ATP. **(A,B)** Toluidine Blue staining of mast cells in whole mount meninges (20×). Mast cells are mostly intact in control conditions **(A)**. After treatment with 1 mM ATP the number of degranulated mast cells significantly increased **(B)**. **(C)** Histograms showing a fraction of degranulated cells in control and after application of 1 mM ATP. Calibration bar 20 μm. Mean ± SEM, $n = 5$, $^{**}p = 0.008$. **(D,E)** Toluidine Blue staining of cultured mast cells (40×). **(D)** Control conditions, **(E)**, application of 1 mM ATP. **(F)** Histograms showing a percentage of degranulated mast cells in control and after application of 1 mM ATP. Calibration bar 50 μm. Mean ± SEM, $n = 4$, $^{***}p < 0.0001$ vs. control (Mann-Whitney *U* test).

ATP in migraine? There are plenty of potential sources to release ATP in the nervous system including astrocytes, neurons, platelets, and endothelial cells (Pangršič et al., 2007; Burnstock and Ralevic, 2014). In meninges, the main sources of ATP could be vessels, nerves and mast cells themselves. ATP-driven degranulation of mast cells is likely happening in migraine with aura since the cortical spreading depression is itself an inducer of meningeal mast cells degranulation and opening of pannexin1 channels (Karatas et al., 2013), which are permeable to ATP (Dahl, 2015). Notably, there is a positive feedback loop providing ATP-induced ATP release *via* pannexins (Dahl, 2015). This loop can amplify the initial signal to provide a level of extracellular ATP high enough to activate P2X7 receptors. A recent study indicated that the complex of P2X7 receptors and pannexins determines not only neuroinflammation but also the development of the cortical spreading depolarization, which is a key process underlying migraine aura (Chen et al., 2017).

We propose that the released extracellular ATP acts through the P2X7 subtype of purinergic receptors thus leading to both mast cells' activation and degranulation. The main actor after degranulation of meningeal mast cells appears to be serotonin robustly exciting nerve terminals *via* ligand gated 5-HT3 receptors (Kilinc et al., 2017), whereas ATP can also act directly on nerve terminals *via* P2X3 receptors (Yegutkin et al., 2016; Zakharov et al., 2016). Taken together, these mechanisms contribute both to meningeal neuroinflammation and lasting pain formation in migraine.

In conclusion, we show the leading role of ATP-gated P2X7 receptors in activation and degranulation of mast cells that naturally reside in two different body compartments. Given the emerging appreciation of the role of mast cells in neuroinflammation, the present data could help to identify new therapeutic strategies to alleviate peripheral and central neurological disorders, including migraine.

AUTHOR CONTRIBUTIONS

DN, RRG and VG contributed to the data collection, analysis, interpretation and the manuscript writing. MS contributed to the data collection and analysis. SW contributed to the data collection and the manuscript editing. FT, AR and JK supervised the study. IK contributed to the study design, data collection, analysis, interpretation and the manuscript writing. TM and RG contributed to the study design and supervision, manuscript writing and final editing.

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Mast Cells Induce Blood Brain Barrier Damage in SCD by Causing Endoplasmic Reticulum Stress in the Endothelium

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Endothelial dysfunction underlies the pathobiology of cerebrovascular disease. Mast cells are located in close proximity to the vasculature, and vasoactive mediators released upon their activation can promote endothelial activation leading to blood brain barrier (BBB) dysfunction. We examined the mechanism of mast cell-induced endothelial activation *via* endoplasmic reticulum (ER) stress mediated P-selectin expression in a transgenic mouse model of sickle cell disease (SCD), which shows BBB dysfunction. We used mouse brain endothelial cells (mBECs) and mast cells-derived from skin of control and sickle mice to examine the mechanisms involved. Compared to control mouse mast cell conditioned medium (MCCM), mBECs incubated with sickle mouse MCCM showed increased, structural disorganization and swelling of the ER and Golgi, aggregation of ribosomes, ER stress marker proteins, accumulation of galactose-1-phosphate uridyl transferase, mitochondrial dysfunction, reactive oxygen species (ROS) production, P-selectin expression and mBEC permeability. These effects of sickle-MCCM on mBEC were inhibited by Salubrinal, a reducer of ER stress. Histamine levels in the plasma, skin releasate and in mast cells of sickle mice were higher compared to control mice. Compared to control BBB permeability was increased in sickle mice. Treatment of mice with imatinib, Salubrinal, or P-selectin blocking antibody reduced BBB permeability in sickle mice. Mast cells induce endothelial dysfunction *via* ER stress-mediated P-selectin expression. Mast cell activation contributes to ER stress mediated endothelial P-selectin expression leading to increased endothelial permeability and impairment of BBB. Targeting mast cells and/or ER stress has the potential to ameliorate endothelial dysfunction in SCD and other pathobiologies.

Keywords: blood brain barrier, endoplasmic reticulum stress, endothelial cell, mast cell, P-selectin, sickle cell disease

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Abbreviations: ATF4, activating transcription factor 4; BBB, blood brain barrier; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; GalT, galactose-1-phosphate uridylyltransferase; GRP78, glucose regulated protein 78; HMGB1, high mobility group 1B protein; mBECs, mouse brain endothelial cells; MCCM, mast cell conditioned medium; NOX4, NADPH oxidase 4; phos-eIF2α, phosphorylated eukaryotic initiation factor 2α; phos-PERK, phosphorylated protein kinase-R-like ER kinase; ROS, reactive oxygen species; SCD, sickle cell disease; SP, substance P; sXBP1, spliced X-box binding protein; VOC, vaso-occlusive crisis.

INTRODUCTION

Neurovascular networks and blood brain barrier (BBB) play a critical role in cerebrovascular dysfunction underlying many pathobiological conditions including cognitive impairment and stroke (Zhang J. H. et al., 2012; Zhao et al., 2015; Raja et al., 2018). One of the genetically inherited conditions with high childhood mortality due to stroke and cognitive impairment is sickle cell disease (SCD; Gold et al., 2008). SCD is a genetic disorder arising from a substitution of valine for glutamic acid at position six on the β-globin gene (Ingram, 1956). A unique hallmark of SCD is vaso-occlusive crisis (VOC) during which sickle red blood cells (RBCs) cluster and occlude blood vessels leading to impaired oxygen supply to the organs, causing end organ damage and acute pain (Platt et al., 1994; Frenette and Atweh, 2007; Tran et al., 2017). Vascular dysfunction is a common feature of several comorbidities of SCD including lung injury, impaired BBB permeability and stroke (Hebbel et al., 2004; Manci et al., 2006; Jordan and Debaun, 2018). Endothelial activation plays a key role in the pathobiology of SCD replete with inflammation and oxidative stress (Hebbel et al., 2004). One known feature of endothelial activation in SCD is the overexpression of cell adhesion molecules (CAMs), which also contributes to VOC (Embury et al., 2004; Manwani and Frenette, 2013).

Amongst the CAMs, P-selectin expressed on endothelial cell (EC) membranes plays a critical role in microvascular blood flow and VOC in SCD (Embury et al., 2004; Kutlar and Embury, 2014). Anti-P-selectin antibody, Crizanlizumab reduced VOC in patients with SCD (Ataga et al., 2017). However, mechanisms causing endothelial P-selectin expression and endothelial activation in SCD remain poorly understood.

Mast cells have been shown to increase E- and P-selectin expression on the endothelium (Kubes and Granger, 1996). Mast cells, the tissue-resident granulocytes, release vasoactive, inflammatory, and neuromodulatory mediators including histamine, proteases, cytokines, and neuropeptides such as substance P (SP) upon activation (Aich et al., 2015). Histamine, a mediator released by mast cells, upregulates endothelial P-selectin *in vitro* and *ex vivo*, and participates in regulating P-selectin-mediated extravasation of immune cells (Jones et al., 1993). Mast cells are located in close proximity to the vasculature, and can cause endothelial activation, plasma extravasation, vasodilatation, and vascular dysfunction (Gupta and Harvima, 2018). We have previously observed that mast cell activation in HbSS-BERK mice contributes to neurogenic inflammation, resulting in increased vascular permeability (Vincent et al., 2013). HbSS-BERK mice express human α and βS globin chains with >99% human sickle hemoglobin, but no murine α or β globins (Paszty et al., 1997). Similar to patients with sickle cell anemia, HbSS-BERK mice demonstrate hemolysis, extensive organ damage, shortened life span and pain (Kohli et al., 2010; Cain et al., 2012). Increased permeability in the BBB has been observed in HbSS-BERK mice (Manci et al., 2006). Moreover, IL1β, TNFα and SP are significantly increased in transgenic humanized sickle mice and patients with SCD compared to non-sickle controls (Michaels et al., 1998; Hebbel et al., 2004; Vincent et al., 2013; Brandow et al., 2016; Campbell et al., 2016; Douglas, 2016; Wang et al., 2016; Solovey et al., 2017). Recent elegant studies have demonstrated that SP and IL33 stimulate TNFα and IL1β release from mast cells (Taracanova et al., 2017, 2018). Mast cells have been observed in brain parenchyma in some pathological conditions (Gupta and Harvima, 2018). It is therefore likely that mast cell activation may contribute to increased BBB permeability in SCD.

One of the known triggers of endothelial dysfunction, inflammation, and oxidative stress is endoplasmic reticulum (ER) stress (Lenna et al., 2014). We hypothesized that in a sickle microenvironment, mediators derived from activated mast cells contribute to endothelial dysfunction and impaired BBB by stimulating ER stress. Disturbance of the equilibrium between ER protein load and folding capacity can lead to the accumulation of misfolded proteins (Lenna et al., 2014). This accumulation of misfolded proteins activates one of the ER-stress sensors, protein kinase RNA-like ER kinase (PERK), which subsequently phosphorylates the eukaryotic translation initiation factor 2 alpha (eIF2α) and attenuates global protein synthesis. The translation of activating transcription factor-4 (ATF-4), however, is increased in response to eIF2α phosphorylation. In chronic ER stress, PERK-ATF4 pathway promotes the transcription of C/EBP homologous protein (CHOP), resulting in increased inflammatory cytokines and reactive oxygen species (ROS; Scheuner and Kaufman, 2008; Lenna et al., 2014). We therefore examined the ability of mast cells to stimulate ER-stress mediated endothelial P-selectin expression leading to impaired BBB permeability in transgenic sickle mice. We have shown earlier that mast cells are activated in the skin of sickle mice, and continue to actively degranulate following isolation *in vitro* (Vincent et al., 2013). Here, we demonstrate that mast cell activation in sickle mice stimulates P-selectin expression, increases endothelial permeability and compromises BBB permeability by inducing ER stress.

We used normal mouse brain ECs (mBEC) and transgenic BERK mice expressing either human sickle hemoglobin (called HbSS-BERK or *sickle* mice henceforth) or normal human hemoglobin A (called HbAA-BERK or *control* mice henceforth) to obtain cutaneous mast cells and examine BBB permeability.

MATERIALS AND METHODS

Mice

Transgenic HbSS-BERK mice feature homozygous knockout of both α and β murine globins and possess transgenes for human α and β^S (hemoglobin S). Control HbAA-BERK mice are also knockout for both α and β murine globins but carry normal human α and β^A globins (hemoglobin A). Heterozygous HbAS-BERK mice are homozygous for normal human α globin, and heterozygous for human sickle β^S globin and human normal β^A globin. HbSS-BERK mice are characterized with similar pathology to human SCD, including hemolysis, reticulocytosis, anemia, extensive organ damage, reduced life span and pain (Paszty et al., 1997; Kohli et al., 2010).

It is challenging to use HbSS-BERK female mice for breeding. Therefore, HbSS-BERK male mice are mated with heterozygous HbAS females. Both sickle parents and offspring are maintained on the Sickle Diet (59M3, TestDiet, St Louis, MO, USA) up to 4–5 weeks of age and eventually changed to the regular Rodent Diet (Harlan Laboratories, Hayward, CA, USA). Litters were weaned 3 weeks after birth. Mice were housed in our AAALAC-approved, pathogen-free, climate-controlled (12 h light-to-dark cycle at 23°C) facility at the University of Minnesota. Mice were genotyped to verify the knockout of mouse globins and presence of human globins (Transnetyx, Cordova, TN, USA), and phenotyped by isoelectric focusing for the presence of HbS and/or HbA as described by us (Sagi et al., 2018). All procedures followed approved protocols from the University of Minnesota's Institutional Animal Care and Use Committee (IACUC) and complied with the statutes of the Animal Welfare Act and the guidelines of the Public Health Service as stated in the Guide for the Care and Use of Laboratory Animals. Cannabinoid-based therapy and approaches to quantify pain in sickle cell disease; IACUC Protocol # 1306-30698A, approval date: June 24, 2013; renewed as IACUC Protocol # 1603-33542A, approval date: May 24, 2016; annual continuing review: May 10, 2018.

Reagents

Roswell Park Memorial Institute 1640 Medium (RPMI; 72400047), Dulbecco's Modified Eagle Medium (DMEM; 11995065), fetal bovine serum (FBS; 10438026), and cell culture supplements were from Life Technologies (Grand Island, NY). Salubrinal (SML0951), collagenase Type II (6885), hyaluronidase (H3506), protease (P8811), deoxyribonuclease I (DN25), Percoll (P1644), recombinant mouse stem cell factor (S9915) and general chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Growth and Treatment Media

Complete mast cell growth medium (RPMI with 10% FBS, 1.2 mg/mL sodium bicarbonate, 2 mM L-glutamine, 25 mM HEPES, and 10 ng/mL recombinant mouse stem cell factor) was used to incubate sickle and control mast cells as described (Vincent et al., 2013). After 24 h of incubation, sickle and control mast cell conditioned medium (MCCM) were collected. Complete mast cell growth medium was incubated in parallel without mast cells to obtain unconditioned medium.

Mast Cells

As described earlier mast cells were isolated from freshly collected shaved dorsal skin (1–2 g dissected into 1-cm^3 pieces) of sickle and control mice, washed twice with RPMI and digested with 15 ml collagenase Type II (0.2 mg/mL), hyaluronidase (0.1 mg/mL), and 0.2 mg/mL protease (0.2 mg/mL) in RPMI at 37°C for 1 h with end-over-end mixing (Vincent et al., 2013). After sedimentation by gravity, the supernatant was collected and placed on ice. The tissue pellet was suspended in 15 ml of the same enzyme solution, followed by incubation at 37°C for 30 min with mixing; then 10 ml of RPMI with 0.015 mg/ml DNase was added to the tissue/enzyme solution and the incubation continued for an additional 30 min with mixing. Again, the supernatant was collected and placed on ice; the remaining tissue was rinsed with 5 ml RPMI. The combined supernatants were passed through a 70 μm filter and concentrated by centrifugation for 10 min at 200× *g* at 4°C. The cell pellet was resuspended in 1 ml RPMI medium with 0.015 mg/ml DNase and layered on 5 ml of 70% isotonic Percoll followed by centrifugation for 20 min at 500× *g* at 4°C. Mast cells in the pellet were suspended in complete mast cell growth medium. Purity of mast cells was validated with toluidine blue and staining for c-kit (CD117, sc-1493; RRID:AB_631031, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and FcεR1 (sc-68943; RRID:AB_2103020, Santa Cruz Biotechnology; Metcalfe, 2001; Vincent et al., 2013). After 5 days, mast cells were sub-cultured, and MCCM was collected after 24 h of incubation.

Endothelial Cells

mBECs, a kind gift from Dr Robert Auerbach (University of Madison, WI, USA) were cultured in EC medium (DMEM supplemented with 10% FBS, sodium pyruvate, 0.02 mg/ml heparin, and 0.1% growth factor (EG-5, Vec Technologies, Rensselaer, NY, USA). Cells were characterized as endothelial on the basis of cobblestone morphology, uptake of acetylated LDL (BT-902, Biomedical Technologies, Inc, Stoughton, MA, USA) and the presence of VEGFR2/KDR (Clone JH121; MS-350-P0; RRID:AB_61321, Neomarkers-Thermo Fisher, Fremont, CA, USA; Gupta et al., 1997; Chen et al., 2006).

Treatment of Endothelial Cells With Mast Cell Conditioned Medium and/or Salubrinal

Immortalized mBECs were treated with MCCM from HbSS-BERK-mast cells and HbAA-BERK-mast cells, or unconditioned medium diluted 1:1 with EC medium with 1% FBS without growth factors and incubated for 24 h, in the absence/presence of 5 μM Salubrinal (Boyce et al., 2005), an inhibitor of eIF-2α, which prevents downstream protein synthesis, and therefore lessens the burden on the ER, as applicable.

Transmission Electron Microscopy for Endothelial Cells

Unconditioned medium or sickle or control MCCM-treated mBECs cultured on cover slips (Thermanox, Nunc 174950, Thermo Fisher, Waltham, MA, USA) was fixed in a solution of 3% paraformaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose in 0.1 M sodium cacodylate buffer with 5 mM calcium chloride and 5 mM magnesium chloride (pH 7.4) for 1 h at room temperature, rinsed three times in 0.1 M sodium cacodylate buffer for 5 min each, and then placed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer overnight at 4°C. The following day cells were rinsed with ultrapure water three times for 5 min each, and post-stained in 1% aqueous uranyl acetate for 1–2 h. Subsequently, the coverslips were rinsed in ultrapure water, dehydrated in a graded series of ethanol solutions up to 100% ethanol, and embedded in Embed 812 resin (14120, Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections (65 nm) were stained with uranyl acetate and lead citrate, and then examined at 75 K, with a JEOL 1200EX II electron microscope (Peabody, MA, USA).

Immunofluorescence Microscopy of Endothelial Cells

Unconditioned medium or sickle or control MCCM-treated mBECs cultured in eight well chamber slides (ibidi USA, Madison, WI, USA) were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, for 10 min, washed with PBS containing 0.01% Tween-20 (PBS-Tw) and blocked by incubation in 3% normal donkey serum/PBS-Tw. The mBECs were incubated for 1 h at room temperature with primary antibodies diluted in 3% normal donkey serum/PBS-Tw. The following antibodies were used: ER-specific rabbit anti-galactose-1-phosphate uridylyltransferase antibody (GalT, 1:100; ab178406, Abcam, Cambridge, MA, USA), Golgi-specific rabbit anti-Giantin antibody (1:100; ab24586; RRID:AB_448163, Abcam), goat anti-P-selectin (1:100; AF737; RRID:AB_2285644, R&D, Minneapolis, MN, USA). Slides were washed and then incubated for 1 h at room temperature with species-specific donkey secondary antibodies conjugated with Cy2 and Cy3 (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in 3% normal donkey serum/PBS-Tw to visualize the immunoreactive proteins. Samples were mounted with Vectashield (H-1000, Vector Labs, Burlingame, CA, USA) and fluorescence images were captured using Olympus IX 70 inverted microscope (Olympus Corporation, Center Valley, PA, USA).

Mitochondrial Membrane Potential

Unconditioned medium or sickle or control MCCM-treated mBECs were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide (JC-1, MitoProbe M34152, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Measurement of red fluorescence (excitation/emission at 535 nm/595 nm) and green fluorescence (excitation/emission at 485 nm/535 nm) was performed using images captured with Olympus IX 70 inverted microscope (Olympus Corporation). Fluorescence was quantitated according to the total of fluorescence pixels divided by the total area, and analyzed with Photoshop (Adobe, San Jose, CA, USA). The ratio of red to green fluorescence was calculated and this ratio increases as mitochondrial membrane potential increases (Kimura and Murakami, 2014).

Reactive Oxygen Species Assay

ROS formation was detected using a cell permeable fluorescent compound, 2′,7′-dichlorofluorescein diacetate (DCFDA) according to the manufacturer's instruction (ab113851, Abcam). In brief, mBECs were seeded on a 96 well plate (clear bottom/black microplates, Nunc 165305, Thermo Fisher) and grown to 80%–85% confluence. After treatment with unconditioned medium or MCCM, the mBECs were washed twice with assay buffer and stained with 20 μM of DCFDA in assay buffer for 45 min at 37°C. Following staining with DCFDA, the cells were washed twice with PBS and the fluorescence was read immediately at 485 nm excitation and 535 nm emission on a fluorescent plate reader (Synergy HT, Biotek, Winooski, VT, USA) with Gen5^{TM} 1.0 software (Biotek). Changes in ROS were determined as fold change in fluorescence as compared to vehicle treated control. All analyses and calibrations were performed at least in triplicate (Ye et al., 2015).

Western Blotting

Unconditioned medium or MCCM-treated mBECs in 6-well plates were lysed with 25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.1% Triton X-100, 5 mM DTT, pH 7.6 with protease inhibitors. Whole cell lysates (15–40 μg of protein) were resolved by 3%–15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, IPVH00010, Millipore, Bedford, MA, USA). The membrane was blocked and then probed with primary antibodies overnight at 4°C. The antibodies used were antibodies against phospho-eIF2α (Ser51; 9721; RRID:AB_330951), PERK (3192; RRID:AB_2095847), phospho-PERK (Thr980; 3179; RRID:AB_2095853; all from Cell Signaling Technology, Beverly, MA, USA); eIF2α (sc-11386; RRID:AB_640075), glucose regulated protein 78 (GRP78; sc-13968; RRID:AB_2119991), NADPH oxidase 4 (NOX4; sc-30141; RRID:AB_2151703), spliced X-box binding protein (sXBP1; sc-7160; RRID:AB_794171; all from Santa Cruz Biotechnology); CHOP (MA1-250; RRID:AB_2292611, Pierce-Thermo Fisher); ATF4 (ARP37017_p050; RRID:AB_593104, Aviva Systems Biology, San Diego, CA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; G9545; RRID:AB_796208, Sigma-Aldrich). After incubation with an alkaline phosphatase-conjugated donkey secondary antibody (sc-2083, Santa Cruz Biotechnology) for 60 min at room temperature the membranes were washed and the immuno-reactive proteins were detected with the ECF Western blotting system (RPN5785, GE Healthcare Life Sciences, Piscataway, NJ, USA). Chemifluorescence signals were acquired using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Chemifluorescence of each lane was quantified by densitometry in arbitrary units using ImageJ software (National Institutes of Health).

Determination of Endothelial Permeability

The mBECs were cultured on 0.2% gelatin treated Transwell inserts (Corning 3384) in 96 well plates (Corning 3382). At 80%–85% confluence the cells were made quiescent by incubation with EC medium with 1% FBS and no growth factor for 18–20 h. Quiescent mBECs were incubated with 100 μl of 5 μM Salubrinal or vehicle control for 30 min in phenol red free EC medium (phenol red free DMEM, 21063029, Life Technologies) with 1% FBS in the luminal (upper) chamber. The pretreatment was replaced with 100 μl solution of unconditioned medium or MCCM diluted 1:1 with phenol red free EC medium with 1% FBS which also contained the inhibitor or vehicle and incubation was continued up to 8 h. Then, the Transwell inserts were washed briefly with PBS containing Ca^{2+}/Mg^{2+}. Next 100 μl Evans blue (0.6 mg/ml) bound to 0.4% BSA in PBS with Ca^{2+}/Mg^{2+} was added to the upper chamber and 200 μl PBS with Ca^{2+}/Mg^{2+} was added to the lower chamber. After 30 min of incubation, the absorbance of the lower chamber was determined at 620 nm with a microplate reader (Synergy HT, Biotek; Friedl et al., 2002; Garcia et al., 2011).

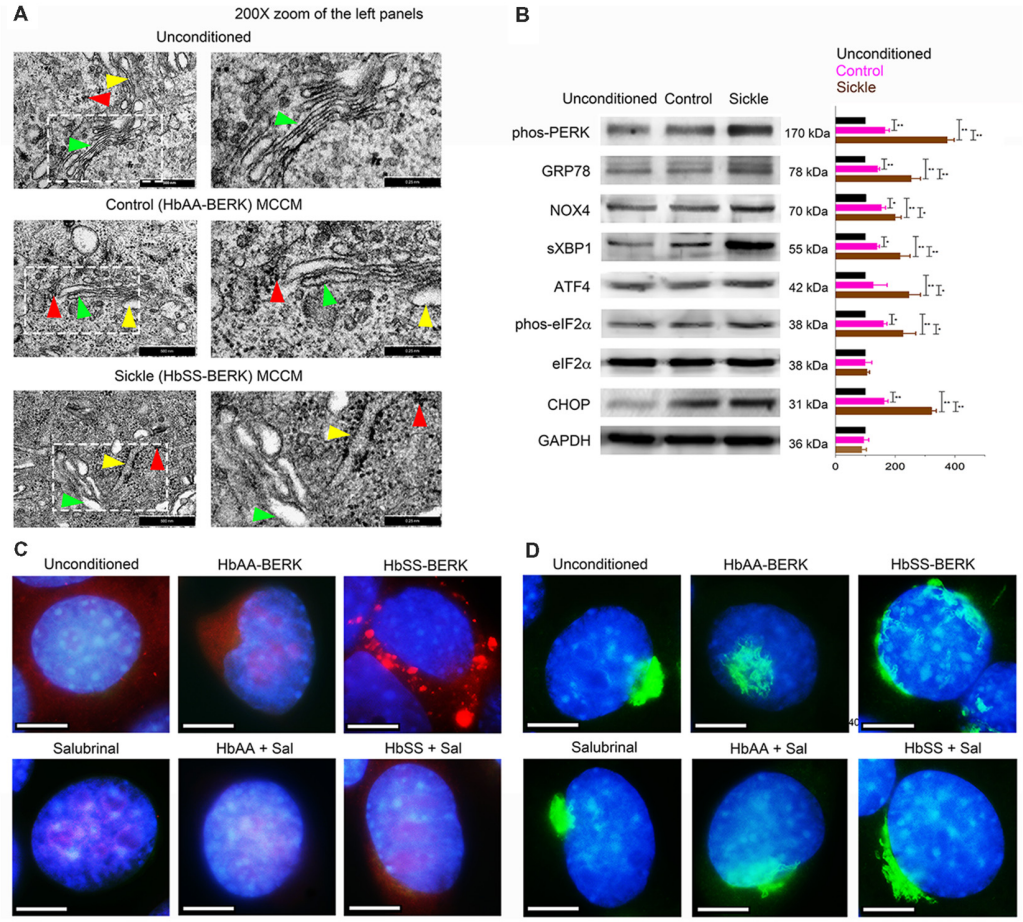

FIGURE 1 | Mast cells from sickle mice induce endoplasmic reticulum (ER) stress in endothelial cells (ECs). Mouse brain ECs (mBECs) were treated for 24 h with mast cell conditioned medium (MCCM) from cultures of mast cells isolated from the skin of sickle or control mice. mBECs were pre-treated with Salubrinal (5 μM) or vehicle for 30 min before addition of the MCCM, where applicable. **(A)** Transmission electron microscopic images of ER (yellow arrows), Golgi (green arrows), and ribosomes (red arrows) in mBECs. Left column, Magnification ×75,000; scale bar = 500 nm. Right column shows 200× magnification of the inset in the left column; scale bar = 0.25 nm. Each image represents five separate and reproducible experiments. **(B)** Sickle and control MCCM stimulate an increase in ER stress markers. Western blot analysis of phos-protein kinase-R-like ER kinase (PERK), glucose regulated protein-78 (GRP-78), NADPH oxidase 4 (NOX4), spliced X-box binding protein (sXBP1), activating transcription factor 4 (ATF4), phos-eukaryotic translation initiation factor 2 alpha (eIF2α), eIF2α, C/EBP homologous protein (CHOP) and protein-loading control GAPDH. Each band is a representative of three independent and reproducible experiments. Each bar is the mean ± SEM of percentage of band density of indicated protein to that of GAPDH from three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. **(C)** Sickle MCCM-induced accumulation of proteins in mBEC is abrogated by Salubrinal. mBEC stained for the ER marker, Galactose-1-phosphate uridylyltransferase (GalT; red) and nuclei (DAPI, blue). Each image represents images from five separate and reproducible experiments. Scale bar = 10 μm; magnification 150×. **(D)** Loss of Golgi organization is attenuated by Salubrinal in mBEC treated with sickle MCCM. Representative images of Golgi (Giantin; green) and nuclei (DAPI, blue). Magnification 150× and scale bar = 10 μm. Each image represents reproducible images from five separate experiments. Sal, Salubrinal.

Treatment of Mice With Endoplasmic Reticulum Stress Inhibitor, Salubrinal

Salubrinal was reconstituted in DMSO to make the stock solution of 20 mg/ml which was subsequently diluted in saline (0.9% NaCl) to prepare injection solution of 100 μg/ml. The mice were intraperitoneally injected with 1 mg/kg Salubrinal.

Treatment of Mice With Imatinib

Mice were treated daily for 5 days with 100 mg/kg body weight imatinib mesylate (Gleevec, NDC 0078-0401034; Novartis) *via* gavage.

Treatment of Mice With P-selectin Blocking Antibody

Mice were treated with 1 mg/kg body weight P-selectin blocking antibody (RB40.32, BD Biosciences) for 3 days *via* intravenous injection.

Determination of Blood Brain Barrier Permeability

After respective treatments, mice were injected with 16.7 mg/kg FITC-Dextran 10 kDa (FD10S, Sigma-Aldrich) 1 h before euthanasia (Egawa et al., 2013). Brains were collected, homogenized in 50 mM Tris-Cl (pH = 7.6; 1 μl/mg

brain) and centrifuged at 16,000 *g* for 30 min at 4°C. Fifty microliter of supernatant was transferred to a 96 well black polystyrene assay plate (Corning 3915) for analysis. A series of standards containing 0.005, 0.02, 0.1, 0.5, 2.5, 5 and 10 μg/ml FITC-Dextran 10 kDa in 50% Tris-HCl/50% absolute methanol were used. The concentration of FITC was determined by spectrofluorometry with an excitation of 485 nm (20 nm bandwidth) and an emission wavelength of 528 nm (20 nm bandwidth).

Histamine Analysis

Skin punch biopsies (4 mm) were collected from the dorsal skin of mice immediately after euthanasia. Biopsies were incubated in DMEM plus antibiotics with 2 mM L-glutamine and 10 mM HEPES (Thermo Fisher) for 24 h at 37°C in a 5% CO_2 incubator. The conditioned media was snap-frozen and stored at −80°C until analyzed.

Whole blood was collected by cardiac puncture into EDTA tubes (T-MQK, Terumo Medical Corp., Somerset, NJ, USA). Plasma was separated by centrifugation of whole blood at 1,200 *g* and 4°C for 10 min. Equal volumes of plasma and methanol were added to a 1.5 ml microcentrifuge tube, mixed, and incubated on ice for 3 min. Supernatant was collected after the mixture was centrifuged at 5,000 *g* for 5 min. The extract was then dried in a centrifugal concentrator for 2–3 h at room temperature and reconstituted with assay buffer prior to analysis.

The processed sample was analyzed using the Histamine ELISA kit (ENZ-KIT140-0001, Enzo LifeScience). Assay results

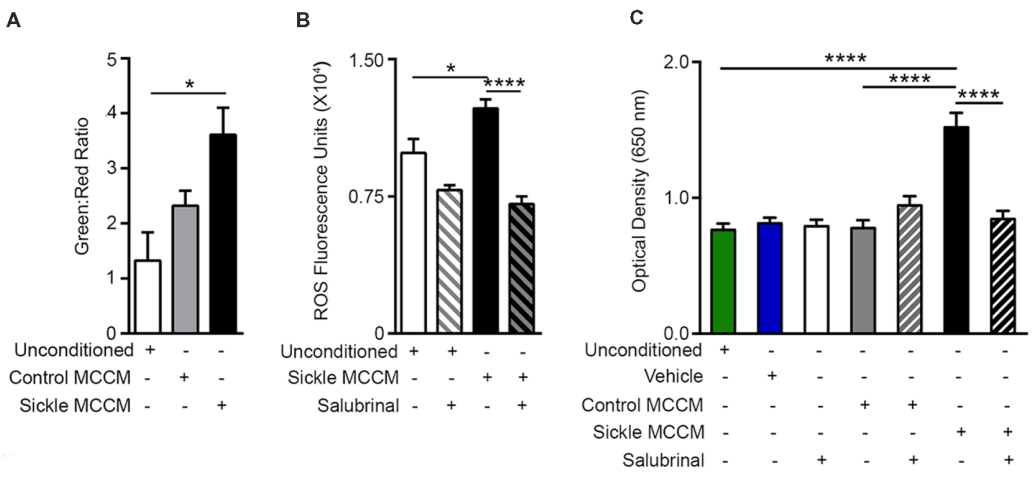

FIGURE 2 | Mast cell induced endothelial ER stress is accompanied by mitochondrial dysfunction, reactive oxygen species (ROS) production, and increased permeability. mBECs were treated for 24 h with MCCM from cultures of mast cells isolated from the skin of sickle or control mice. mBECs were pre-treated with Salubrinal (5 μM) or vehicle for 30 min before addition of MCCM, where applicable. **(A)** Sickle MCCM significantly decreased mitochondrial membrane potential detected by JC-1. An increased green to red fluorescence ratio is indicative of decreased membrane potential. $^{*}p < 0.05$. **(B)** Sickle MCCM significantly increases the production of ROS, which is ameliorated by pre-treatment with Salubrinal. ROS in mBEC is shown as fluorescence units of oxidized 2′,7′-dichlorodihydrofluorescein. **(C)** Sickle MCCM significantly increases the endothelial permeability, which is ameliorated by pre-treatment with Salubrinal. Evans blue leakage through mBEC monolayer following incubation with control or sickle MCCM, measured at 650 nm is shown. $^{*}p < 0.05$, $^{****}p < 0.0001$.

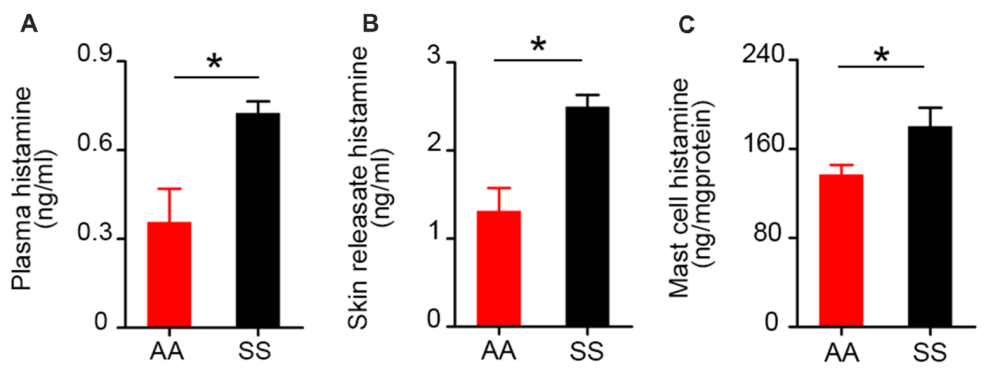

FIGURE 3 | Histamine levels in plasma, skin secretagogue, and mast cells of sickle mice are higher than control mice. Histamine concentration in **(A)** plasma ($n = 6$). **(B)** Skin releasate/secretagogue ($n = 3$). **(C)** Cutaneous mast cells in culture ($n = 6$). $^{*}p < 0.05$. All specimens were from ∼3.5-month-old female mice. AA, HbAA-BERK control mice and SS, HbSS-BERK sickle mice.

were collected and calculated using the Synergy HT plate reader and Gen5^{TM} 1.0 data analysis software (BioTek).

Statistical Analysis

All data were analyzed using Prism software (v 6.0a, GraphPad Software Inc., San Diego, CA, USA). A two-way repeated measures analysis of variance (ANOVA) with Bonferroni's correction was used to compare the responses among treatments. A p-value of < 0.05 was considered significant. All data are presented as mean ± SEM.

RESULTS

Mast Cells From Sickle Mice Induce Endoplasmic Reticulum Stress in Endothelial Cells

Normal mBECs were incubated with MCCM from mast cell cultures derived from sickle and control mice. In parallel, mBECs were also incubated with unconditioned mast cell culture medium incubated without mast cells. Transmission electron microscopy of mBECs incubated with unconditioned medium showed the normal presentation of the ER (yellow arrowheads) and Golgi (green arrowheads) with well-organized stacks of cisternae and few sparsely scattered ribosomes (red arrowheads; **Figure 1A**). MCCM from control mice induced partial disruption and swelling of ER and Golgi cisternae with prominent dark granular ribosomes (**Figure 1A**). mBECs treated with sickle-MCCM exhibited pronounced swelling and structural disorganization of Golgi and ER, accompanied by aggregation of dense clusters of ribosomes around the ER and throughout the cytoplasm (**Figure 1A**). Extensive ribosomal aggregates indicate accumulation of misfolded proteins due to malfunctioning of the ER and Golgi (Hiramatsu et al., 2015; Oakes and Papa, 2015). Therefore, these observations suggest that mast cell released mediators stimulate ER stress in mBECs which was higher with MCCM from sickle mouse mast cells compared to that of control mice.

Complementary to transmission electron microscopy, Western blotting demonstrated significantly enhanced expression of ER stress markers, phos-PERK, GRP78, NOX4, sXBP1, ATF4, phos-eIF2α, and CHOP in mBECs incubated with sickle MCCM, compared to those incubated in control MCCM or unconditioned MCCM (**Figure 1B**). We also observed accumulation of galactose-1-phosphate uridylyltransferase (GalT; red) in mBECs treated with sickle MCCM, but not in those treated with control MCCM or unconditioned medium (**Figure 1C**). GalT accumulation in the ER, instead of being secreted and distributed throughout the cell, is indicative of accumulation of unfolded proteins under ER stress. Similarly, complete loss of structural integrity of Golgi (Giantin; green) was observed in mBECs treated with sickle MCCM, but not in those treated with control MCCM or unconditioned medium (**Figure 1D**). Salubrinal has been shown to increase levels of phos-eIF2α, which prevents downstream protein

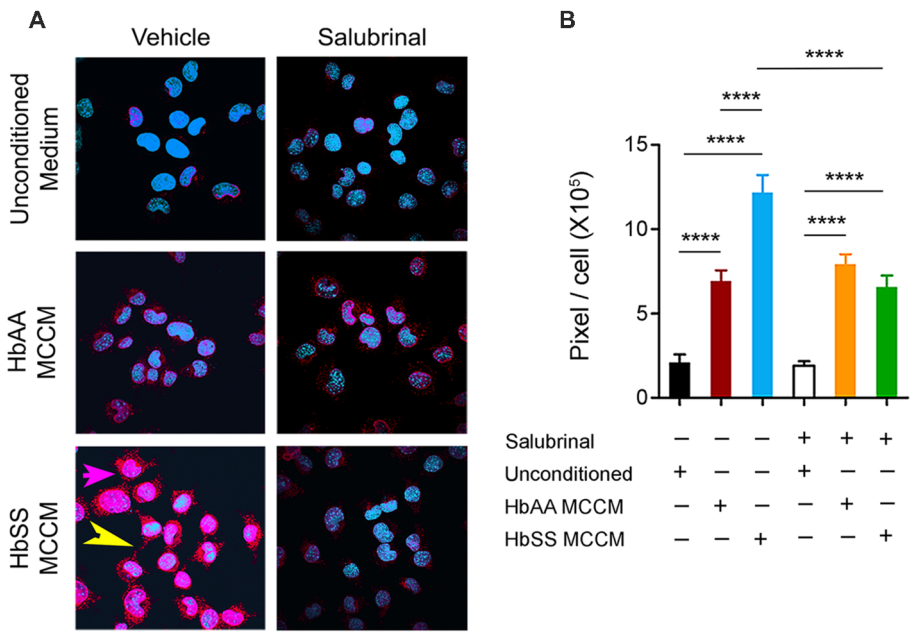

FIGURE 4 | Mast cells induce P-selectin expression in ECs. mBECs were treated for 24 h with MCCM from cultures of mast cells isolated from the skin of sickle or control mice. **(A)** Representative images of P-selectin in mBECs, magnification 60× showing blue nuclei and red P-selectin staining. In bottom left panel for mBEC incubated with sickle-MCCM please note magenta-pink staining (pink arrow), indicating cell surface expression covering the entire cell surface and also red extracellular P-selectin granules (yellow arrow; **B**). Quantification of P-selectin expression on mBEC. mBECs treated with unconditioned medium, $n = 14$, black; mBECs treated with HbAA MCCM, $n = 12$, maroon; mBECs treated with HbSS MCCM, $n = 16$, blue; mBECs pre-treated with Salubrinal followed by treatment with unconditioned medium, $n = 15$, white; mBECs pre-treated with Salubrinal followed by treatment with HbAA MCCM, $n = 12$, orange; mBECs pre-treated with Salubrinal followed by treatment with HbSS MCCM, $n = 11$, green. ****$p < 0.0001$. HbAA, HbAA-BERK control and HbSS, HbSS-BERK sickle mice.

synthesis, by blocking dephosphorylation after ER stress induced phosphorylation (Boyce et al., 2005; Lewerenz and Maher, 2009). We performed a functional assay to examine the impact of Salubrinal on unfolded protein accumulation. Salubrinal abrogated the accumulation of GalT and reduced the changes in Golgi (**Figures 1C,D**). Therefore, sickle MCCM contributes to ER stress in mBECs by inducing structural changes in the ER-Golgi complex *via* functionally activating unfolded protein response pathways. These processes are attenuated by reducing ER stress with Salubrinal.

Mast Cells Contribute to Endothelial Mitochondrial Dysfunction and Oxidative Stress

ER stress is also accompanied by increased oxidative stress due to mitochondrial dysfunction and dysregulated antioxidant homeostasis (Lenna et al., 2014). Correspondingly, we observed decreased mitochondrial potential (**Figure 2A**; $p = 0.0347$) and increased ROS (**Figure 2B**; $p = 0.0135$) in mBECs treated with sickle MCCM as compared to those treated with unconditioned medium. Salubrinal inhibited ROS production in mBECs induced by sickle MCCM (**Figure 2B**; $p < 0.0001$). This result supports previous findings where Salubrinal promotes homeostasis by decreasing ROS production, ER stress, and mitochondrial dysfunction (Dou et al., 2012; Wu et al., 2012; Zhu et al., 2012). Salubrinal acts by preventing dephosphorylation of eIF2α and sustaining PERK-ATF4 signaling during ER stress (Boyce et al., 2005; Tsaytler et al., 2011). The mBEC monolayer treated with sickle MCCM *in vitro* demonstrated increased Evans blue leakage relative to those treated with control MCCM ($p < 0.0001$) or unconditioned medium ($p < 0.0001$), indicative of increased endothelial permeability (**Figure 2C**), likely caused by the action of vasoactive substances such as SP and/or histamine or other substances released from mast cells.

Mast Cell Activation Contributes to Increased Histamine in Sickle Mice

Histamine is one of the potent inflammatory mediators released from mast cells. Mast cell histamine release is considered to be one of the major cellular mechanisms underlying histamine-induced barrier dysfunction (Kumar et al., 2009). Besides systemic inflammation, histamine is known for its critical role in neurogenic inflammation and transmission of pain throughout the nervous system (Rosa and Fantozzi, 2013). We compared control and sickle plasma, skin secretagogue, and mast cell histamine levels. We found a significant increase in histamine levels in sickle mice in the plasma (**Figure 3A**; $p = 0.0119$), skin secretagogue (**Figure 3B**; $p = 0.0156$) and mast cells (**Figure 3C**; $p = 0.0368$) isolated from the skin when compared to control mice.

Sickle Mast Cell Mediators Induce P-selectin Expression on Endothelial Cells

P-selectin is known to be upregulated by mast cell activation (Torres et al., 2002), and contributes significantly to the recruitment and rolling of leukocytes and neutrophils (Jones et al., 1993; Mayadas et al., 1993), and participates in the attachment of sickle RBCs to the endothelium (Matsui et al., 2001). mBECs incubated with control and sickle MCCM exhibited about 3- and 6-fold fold increase, respectively, in P-selectin expression compared to unconditioned culture medium (**Figures 4A,B**; $p < 0.0001$ and $p < 0.0001$, respectively). P-selectin expression induced by sickle-MCCM appeared to be associated with cell membrane as well as dense intracellular granules (**Figure 4A**). In contrast P-selectin expression induced by control-MCCM appeared to be confined as dense red intracellular staining. Pre-incubation of mBECs with Salubrinal significantly inhibited sickle MCCM-induced P-selectin expression on the cell membrane of mBECs and intracellularly (**Figure 4B**; $p < 0.0001$) to the level induced by control MCCM. In contrast, Salubrinal did not inhibit control MCCM-induced P-selectin expression on mBECs. It is therefore likely that mast cells from sickle mice release substances that promote translocation of P-selectin to endothelial surface which is mediated by ER stress.

Blood Brain Barrier Permeability in Sickle Mice Is Attenuated by Reducing Endoplasmic Reticulum Stress or Blockade of P-selectin or Inhibiting Mast Cells

We next examined the effects of Salubrinal (to reduce ER stress), imatinib (to inhibit mast cell activation), and P-selectin

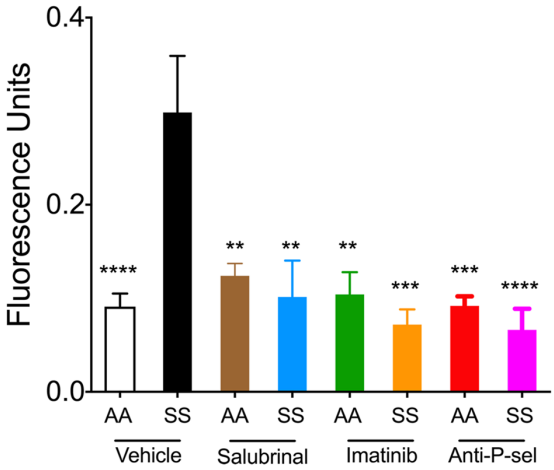

FIGURE 5 | Salubrinal, imatinib, and P-selectin blocking antibody reduce blood brain barrier (BBB) permeability in sickle mice. Mice were treated with either a single dose of 1 mg/kg body weight Salubrinal for 48 h, 100 mg/kg body weight imatinib for 5 days, or 1 mg/kg body weight P-selectin blocking antibody for 3 days. After each of the respective treatments, mice were injected with 16.7 mg/kg FITC-Dextran 10 kDa through the tail vein 1 h prior to euthanasia at the end of the study. FITC-dextran leakage in the brain is shown. Control mice treated with vehicle, $n = 8$, white; sickle mice treated with vehicle, $n = 6$, black; control mice treated with Salubrinal, $n = 4$, brown; sickle mice treated with Salubrinal, $n = 6$, blue; control mice treated with imatinib, $n = 4$, green; sickle mice treated with imatinib, $n = 3$, orange; control mice treated with P-selectin blocking antibody, $n = 4$, red; sickle mice treated with P-selectin blocking antibody, $n = 6$, purple. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to sickle mice treated with vehicle. Female sickle (SS) or control (AA) mice at ∼3.5 months of age were used.

blocking antibody on BBB permeability in mice by examining the leakage of FITC-dextran. Sickle mice showed significantly increased extravasation of FITC-dextran compared to control mice (**Figure 5**; $p < 0.0001$). After 48 h of treatment with Salubrinal, a reduction in BBB permeability was observed in sickle mice when compared to the vehicle group (**Figure 5**; $p = 0.017$). A similar decrease was observed in sickle mice treated with imatinib for 5 days (**Figure 5**; $p = 0.0008$) and P-selectin blocking antibody for 3 days (**Figure 5**; $p < 0.0001$).

DISCUSSION

Our data demonstrate that mast cells contribute to upregulation of endothelial P-selectin expression *via* an ER stress mediated mechanism, which leads to increased endothelial permeability and impairment of the BBB in sickle mice. For the first time we show the novel role of mast cells on endothelial activation which could have implications in multiple consequences of SCD including VOC and stroke. Mast cells are constitutively activated in sickle mice (Vincent et al., 2013). Our observations therefore evince the significance and the pivotal role of mast cells in SCD pathobiology (**Figure 6**).

P-selectin is a CAM known to initiate and mediate the binding of leukocytes to anchor and roll on vessel walls (Mcever et al., 1995). In sickle mice, EC surface P-selectin facilitates the adhesion of sickle RBCs to the vessel wall leading to vascular occlusion (Embury et al., 2004). Absence and/or blockade of cell surface P-selectin leads to reduced adhesion of leukocytes and sickle RBCs to the endothelium, and has an inhibitory effect on vaso-occlusion in sickle mice (Turhan et al., 2002; Embury et al., 2004; Gutsaeva et al., 2011). We noted that ECs express and maintain strong P-selectin expression on the cell surface of mBECs when incubated with MCCM from mast cells derived from sickle mice but not from MCCM from control mice mast cells. Histamine, which is a prominent mast-cell derived mediator, has been demonstrated to stimulate P-selectin expression on the endothelium, which is accompanied by increased adhesion of neutrophils to vessel walls (Sun et al., 2012). Histamine has also been shown to rapidly disrupt cell-cell and cell-extracellular matrix interaction, which affects the stability of endothelium-basal connective tissue (Moy et al., 2000). Plasma, skin releasate, and mast cell histamine levels were significantly higher in sickle mice when compared to control mice. Therefore, in SCD, histamine and other mast cell-derived mediators lead to functional overexpression of P-selectin on ECs, exaggerating the immune response, leading to inflammation and a more adherent lumen. ROS have been shown to upregulate adhesion molecules by increasing the transport of molecules such as P-selectin to the cell surface and the circulation (Lum and Roebuck, 2001). High mobility group 1B protein (HMGB1) has also been implicated in increasing the expression of P-selectin *via* ER stress (Luo et al., 2013). HMGB1 has been known to activate Toll-like receptor 4, inducing organ injury and pain in SCD (Xu et al., 2014). Mast cells have been shown to release HMGB1 after injury (Cai et al., 2011). Therefore, mast cells and their mediators increase production of P-selectins by increasing ROS and provoking ER stress as observed in this study. Thus, mast cells play a cardinal role in functional endothelial P-selectin expression in SCD. Mast cells release several mediators including cytokines, proteases, and neuropeptides such as SP upon activation (Vincent et al., 2013; Aich et al., 2015). Thus, it may be challenging to target each mediator individually, and an upstream approach inhibiting the activity of mast cells may be more appropriate. Inhibitors of mast cells as well as P-selectin have been tested clinically leading to reduced VOC in SCD without known adverse events (Kutlar et al., 2012; Kutlar and Embury, 2014; Ataga et al., 2017).

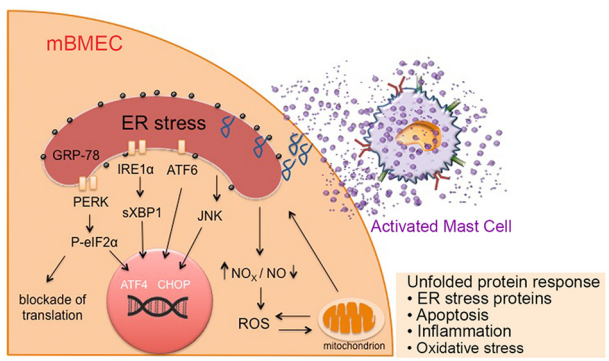

FIGURE 6 | Mast cell activation in sickle cell disease (SCD) leads to ER stress, mitochondrial dysfunction, and associated oxidative stress, leading to increased endothelial permeability. Mediators released from activated mast cells in sickle mice stimulate ER stress in endothelium. These factors may directly act on the ER or increase ROS production and mitochondrial dysfunction leading to the activation of chaperone proteins involved in ER homeostasis maintenance, resulting in the unfolded protein response and accumulation of unfolded proteins in the ER. This further augments mitochondrial dysfunction and subsequent oxidative stress, resulting in inflammation, leading to a vicious cycle of inflammation, oxidative stress and mast cell activation. Prolonged ER stress may result in apoptosis. ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; GRP-78, glucose regulated protein-78; IRE1, inositol-requiring enzyme 1; mBECs, mouse brain microvascular endothelial cells; NO, nitric oxide; NOX, nicotinamide adenine dinucleotide phosphate oxidase; P-eIF2α; phosphorylated eukaryotic initiation factor 2α; PERK, protein kinase-R-like ER kinase; ROS, reactive oxygen species; sXBP1, spliced X-box binding protein.

ER stress contributes to both vascular and neural pathobiology of SCD. ER stress is activated by p38 mitogen-activated protein kinases (p38MAPK) and may even suppress endothelial nitric oxide synthase, thus depleting nitric oxide (Galan et al., 2014; Santos et al., 2014). Reduced nitric oxide bioavailability is a critical feature of sickle pathobiology (Rees and Gibson, 2012). Our laboratory observed increased p38MAPK phosphorylation in the whole tissue lysates of spinal cords of HbSS-BERK mice correlative to central sensitization of spinal dorsal horn neurons, which contribute to chronic pain (Cataldo et al., 2015). Elegant studies on diabetic mice show the contribution of ER stress to neuropathic pain and inhibition with soluble epoxide hydrolase inhibitors (Inceoglu et al., 2015). ER stress contributes to hypoxia/reperfusion-induced brain damage in growing rats, which is ameliorated by Salubrinal (Cai et al., 2014). Mast cell-mediated ER stress leading to endothelial dysfunction, observed herein, may also

underlie many vascular dysfunction associated complications including acute lung injury and stroke—critical co-morbidities in SCD. Mast cell activation has been shown to contribute to stroke (Arac et al., 2014) and acute lung injury in preclinical studies (Zhao et al., 2014). Silent infarcts and overt strokes are common in children with SCD (Gold et al., 2008; Rees et al., 2010). However, the mechanistic understanding of cerebral vascular dysfunction in SCD remains an enigma (Hillery and Panepinto, 2004). Our results demonstrate a fundamental mechanism of mast cell-orchestrated endothelial dysfunction *via* ER stress. Therefore, ER stress may represent a therapeutic target to ameliorate vascular dysfunction using novel pharmacologics such as Salubrinal, in addition to mast cell stabilization. Salubrinal may also have beneficial off-target effects because it has been shown to promote bone healing in rat femurs (Zhang P. et al., 2012) and increase fetal hemoglobin expression in primary human erythroid cells (Hahn and Lowrey, 2014).

Both internal and external ROS contribute to loss of EC—cell interactions (van Wetering et al., 2002), altered BBB integrity and disruption of tight junctions (Schreibelt et al., 2007; Lehner et al., 2011). In sickle mice, ROS is increased in the spinal cords and underlies the ischemia reperfusion injury (Osarogiagbon et al., 2000; Valverde et al., 2016). BBB permeability is also compromised in sickle mice (Manci et al., 2006). Our observation of increased ROS in ECs treated with sickle mast cell secretagogue and mast cell activation in sickle mice (Vincent et al., 2013) suggests that mast cell activation could compromise the BBB and allow further entry of inflammatory substances into the brain. Our findings of increased BBB permeability and mast cell-induced ER stress provide another perspective of SCD pathobiology and therapy.

In conclusion, these observations on the involvement of mast cell-induced endothelial ER stress have wide-ranging translational potential in developing therapies to co-treat organ damage and pain in SCD and cerebrovascular dysfunction in other conditions.

AUTHOR CONTRIBUTIONS

HT performed experiments and wrote the manuscript. AM performed experiments, analyzed and interpreted the data, and prepared figures. VS wrote the manuscript, analyzed and interpreted the data, and prepared figures. KL, AN, JN, YL and JL performed experiments. MG developed experimental plan, analyzed the data and edited the manuscript. AG advised on ER stress, interpreted the data and edited the manuscript. KG conceived, designed, planned, and supervised the entire study, analyzed and interpreted data, and edited the manuscript.

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frontiers in Cellular Neuroscience

MINI REVIEW
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Mast Cells in Stress, Pain, Blood-Brain Barrier, Neuroinflammation and Alzheimer's Disease

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Mast cell activation plays an important role in stress-mediated disease pathogenesis. Chronic stress cause or exacerbate aging and age-dependent neurodegenerative diseases. The severity of inflammatory diseases is worsened by the stress. Mast cell activation-dependent inflammatory mediators augment stress associated pain and neuroinflammation. Stress is the second most common trigger of headache due to mast cell activation. Alzheimer's disease (AD) is a progressive irreversible neurodegenerative disease that affects more women than men and woman's increased susceptibility to chronic stress could increase the risk for AD. Modern life-related stress, social stress, isolation stress, restraint stress, early life stress are associated with an increased level of neurotoxic beta amyloid (Aβ) peptide. Stress increases cognitive dysfunction, generates amyloid precursor protein (APP), hyperphosphorylated tau, neurofibrillary tangles (NFTs), and amyloid plaques (APs) in the brain. Stress-induced Aβ persists for years and generates APs even several years after the stress exposure. Stress activates hypothalamic-pituitary adrenal (HPA) axis and releases corticotropin-releasing hormone (CRH) from hypothalamus and in peripheral system, which increases the formation of Aβ, tau hyperphosphorylation, and blood-brain barrier (BBB) disruption in the brain. Mast cells are implicated in nociception and pain. Mast cells are the source and target of CRH and other neuropeptides that mediate neuroinflammation. Microglia express receptor for CRH that mediate neurodegeneration in AD. However, the exact mechanisms of how stress-mediated mast cell activation contribute to the pathogenesis of AD remains elusive. This mini-review highlights the possible role of stress and mast cell activation in neuroinflammation, BBB, and tight junction disruption and AD pathogenesis.

Keywords: Alzheimer's disease, amyloid plaques, chronic stress, corticotropin releasing hormone, mast cells, neurodegenerative disease, neuroinflammation

INTRODUCTION

Stress is our body's normal physiological response to any adverse changes in our environment to deal and overcome these challenges (Bisht et al., 2018). Chronic diseases can disrupt the quality of normal life and day-to-day life activities that can lead to psychological stress. Stress induces the onset and progression of pain, cognitive disorder, and psychiatric disorders. Stress induces disease(s) and the disease (s) in turn can exacerbate the stress severity in a vicious cycle (Justice, 2018). Chronic stress due to continuous wars, military service, chronic diseases, dementia, neurotrauma, poor sleep habits, immobilization, isolation, noise, high workload, unstable job, annoying work environment, difficult spouse; acute stress due to chronic diseases, modern life conditions, immobilization, isolation, noise, physical, visual, emotional, social, environmental, temperatures (hot and cold), odors, certain foods, new challenges, competitions, presentation at work, and intermittent fasting can induce several unwanted changes in the central nervous system (CNS). These changes include cognitive disorders, neuroinflammation, altered secretion of growth factors, high proinflammatory cytokines and chemokines secretion, increased oxidative stress, blood-brain barrier (BBB) disruption, ultrastructural and molecular changes in tight junctions, neurovascular unit (NVU), gliovascular unit (GVU), changes in the brain volume, and neuroinflammation (Kempuraj et al., 2017a; Lurie, 2018). Stress can also induce the changes in the peripheral system, the CNS immune components, and affect immune cells such as mast cells.

Blood-brain barrier disruption is associated with the entry of proinflammatory cytokines, chemokines, immune and inflammatory cells in to the brain, neuroinflammation and neurodegeneration (Patel and Frey, 2015; Kempuraj et al., 2017b). Stress and immune system interact bi-directionally and enhance stress response even in the CNS (Holzer et al., 2017). Mast cell activation induces glial cells activation, neuroinflammation, stress response, and pain signals (Theoharides et al., 2012; Kempuraj et al., 2017b; Skaper, 2017; Skaper et al., 2017; Gupta and Harvima, 2018; Theoharides and Kavalioti, 2018). Stress conditions inhibit immune response, but can worsen inflammatory conditions including neuroinflammation (Esposito et al., 2001a; Karagkouni et al., 2013). In fact, most of the CNS disorders show disruption of BBB and tight junction proteins. Mast cells and neurons are closely associated both anatomically and functionally throughout the body including the CNS (Forsythe, 2019). The number, distribution and the activation status of mast cells in the brain is not constant, but varies due to environment, behavioral changes and physiological state (Forsythe, 2019). Neuroinflammation induces NVU and GVU dysfunctions in many neuroinflammatory diseases (Li et al., 2017). Increased levels of inflammatory cytokines, chemokines and microglial activation contribute to the activation of pain mechanisms (Lurie, 2018). The initial stress and pain responses protect the body, however, chronic stress and chronic pain can induce many health problems. In this mini-review, we highlight the recent knowledge on the possible role of stress, and mast cell activation in neuroinflammation, BBB and tight junction disruption, onset, progression and severity of Alzheimer's disease (AD).

MAST CELLS, PAIN, AND NEUROINFLAMMATION

Mast cells are implicated in neuroprotection, pain, neuroinflammation and neurodegenerative diseases by releasing several preformed and preactivated inflammatory mediators, as well as release of newly synthesized cytokines, chemokines, and neurotoxic molecules (Gordon and Galli, 1990; Kempuraj et al., 2017b; Conti et al., 2018; Kempuraj et al., 2018a,b,c; Ocak et al., 2018; Skaper et al., 2018). IL-1 family cytokines such as IL-1β and IL-33 can activate mast cells and are implicated in inflammation including neuroinflammation but IL-37 is anti-inflammatory cytokine that can be used to treat inflammatory conditions (Tettamanti et al., 2018; Varvara et al., 2018). Neuroinflammation further leads to the release of additional inflammatory cytokines, chemokines, prostaglandins, activation of nociceptors, acute and chronic pain, headache, BBB dysfunction, neuronal excitability, and glial and neuronal damage in the CNS (Skaper et al., 2012, 2017; Skaper, 2016). Patients with neurodegenerative diseases such as AD, and Parkinson's disease (PD), Huntington's disease (HD) show painful symptoms, but the origin of pain is variable in these patients (de Tommaso et al., 2017; Matsuda et al., 2018).

About 38–75% of AD patients and 40–86% of PD patients also show painful symptoms in addition to other clinical disorders (de Tommaso et al., 2016). The International Association for the Study of Pain (IASP) describe, "pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (de Tommaso et al., 2016). The origin of pain in neurodegenerative diseases is multifactorial involving either nociceptive or neuropathic and sometimes both. The prevalence of dementia and pain increases with aging (Defrin et al., 2015). However, severe dementia and AD dementia patients are unable to report the full extent and the severity of pain, and therefore pain symptoms are not properly treated in these patients. About 50% of community dwelling patients and about 45–83% of dementia patients living in nursing homes suffer from pain due to various causes including infections. It is not clear if the drugs such as L-dopa or riluzole used in the patients are effective in significant pain suppression (de Tommaso et al., 2017). Therefore, pain management needs careful evaluation in these neurodegenerative patients (de Tommaso et al., 2017).

Mast cells may either directly influence nociceptive neurons or through glial cells, based upon the location of mast cells, and pain pathways in the brain (Caraffa et al., 2018; Gupta and Harvima, 2018). Mast cell-derived TNF is known to sensitize meningeal nociceptors and induce neuroinflammation (Caraffa et al., 2018). Because of the presence of vicious positive-feedback mechanism of mast cells and glial cells activation with inflammatory mediators' release, even a small number

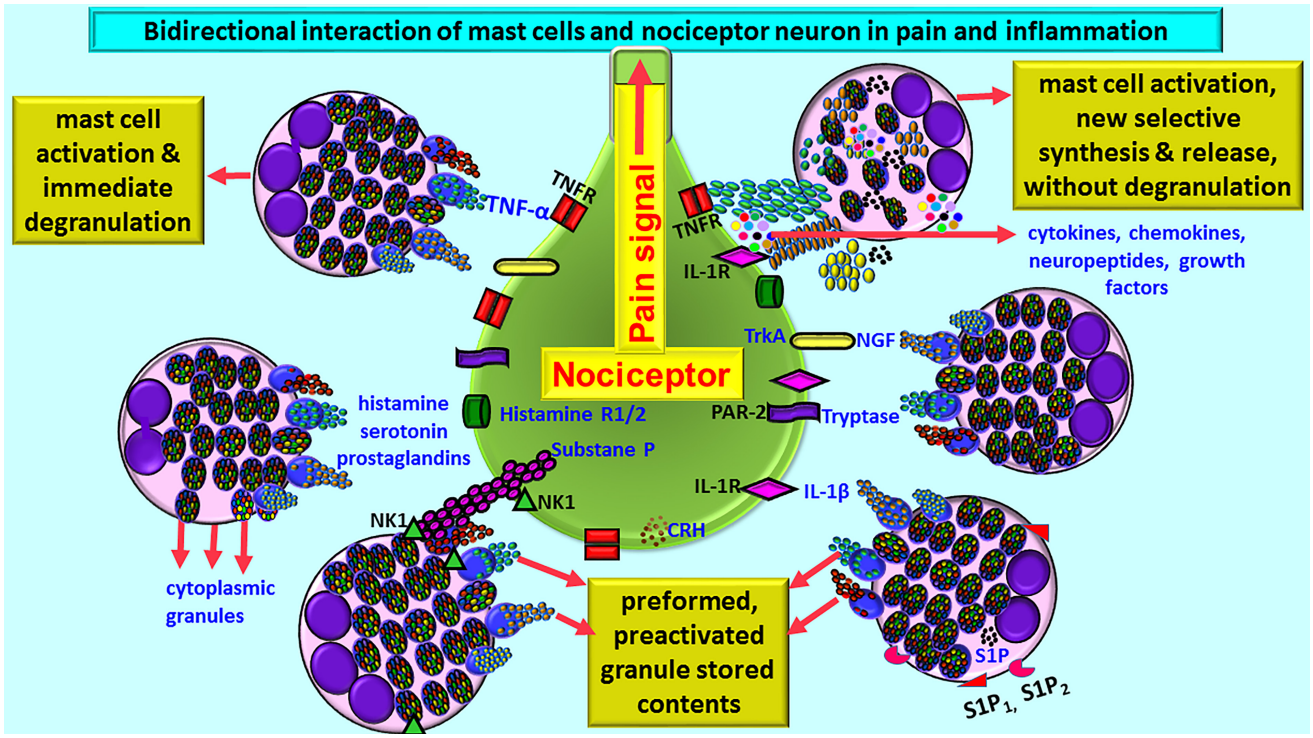

FIGURE 1 | Schematic diagram shows bidirectional communications between nociceptor neuron and mast cells during pain and inflammation. Mast cells are located close to nociceptor/neurons. Several conditions activate mast cells to release preformed preactivated and granule stored neuroactive inflammatory mediators and growth factors by degranulation or release many newly synthesized neuroactive and neuroinflammatory mediators. Mast cells express several receptors including CRHR, NK1, S1P1, and S1P2 for the mediators released from the neurons and for the cytokines/chemokines and various growth factors. Similarly, nociceptor neurons also express receptors including PAR-2, TNF-R, IL-1R, histamine R1/2, and NK-1for mast cell released mediators. Histamine, serotonin, and prostaglandins released from mast cells induce pain signals. Mast cell-released inflammatory mediators and growth factors induce pain signals and inflammation in many neuroinflammatory and neurodegenerative diseases. CRHR, corticotropin-releasing hormone receptor; IL-1R, IL-1 receptor; NK-1, neurokinin-1; NGF, nerve growth factor; PAR-2, protease-activated receptor-2; S1P, sphingosine-1 phosphate; TNFR, TNF receptor; TrkA, Tropomyosin receptor kinase A.

of mast cells can induce significant neuroinflammation in the brain. In fact, about 50% of histamine and 25% of tumor necrosis factor-alpha (TNF-α) are from the mast cells in the rat brain that can cause nociception and pain signals in the brain (Hendriksen et al., 2017; Gupta and Harvima, 2018). Mast cell activation leads to the release of many neuropeptides and inflammatory mediators including histamine, tryptase, and prostaglandins that can act on nociceptor on sensory neurons for the pain sensation (Schwartz, 1990; Levy et al., 2012; Aich et al., 2015; Kempuraj et al., 2017b). Neurons in turn release various neuropeptides, neuroinflammatory and analgesic mediators that can activate mast cells in a vicious cycle. This continuous process leads to an increased vascular permeability, chronic pain, itch, inflammation, and neuroinflammation (Gupta and Harvima, 2018; **Figure 1**). Mast cells are present at the nerve terminals in the periphery, meninges, and vasculature in the brain (Gupta and Harvima, 2018). Mast cells-associated histamine, tryptase, nerve growth factor (NGF), sphingosine-1 phosphate (S1P) are involved in the pain sensation. Mast cell-released histamine acts on nerve endings through histamine 1 receptor (H1R), H2R, H3R, and H4R. Therefore, anti-histaminergic drugs show significant reduction in the pain sensation in the humans. Nociceptive C and A-delta nerve fibers respond to histamine in the peripheral system and in the CNS, and transmit nociceptive signals to the thalamus and then to the cortical and subcortical areas including amygdala and striatum regions. Neuronal calcitonin gene related peptide (CGRP) induces mast cells to release histamine. Mast cell proteinase tryptase acts on nerve endings through protease-activated receptor-2 (PAR-2) and increases the release of substance P and CGRP, which in turn induce mast cell activation and release inflammatory mediators (**Figure 1**). Thus, increased mast cell activation is associated with high levels of tryptase associated with severe pain (Gupta and Harvima, 2018). Increased mast cell activation also increases tryptase levels in the blood. Mast cells synthesize and secrete NGF that can act again on mast cells as well as nerve endings through its receptor tropomyosin receptor kinase A (TrkA) and further release histamine and NGF from mast cells. NGF level has been shown to be increased in various inflammatory and painful conditions that are associated with increased mast cell activation. Mast cells also release S1P that can act on mast cells through S1P1 and S1P2 receptors and induce mast cell activation and degranulation

(Gupta and Harvima, 2018). These mechanisms induce pain, mast cells recruitment to the site of inflammation, and chemokine release. Both NGF and S1P receptor antagonists are shown to be useful in reducing the severity of pain in inflammatory disorders. All these findings show that mast cells are involved in pain sensation including headache associated with neuroinflammation.

STRESS, PAIN, AND NEUROINFLAMMATION

Chronic diseases can disrupt normal life and day-to-day life activities that may lead to psychological stress. Psychological stress, diet, hormonal fluctuations, and post-traumatic stress disorder (PTSD) can induce inflammation including sterile inflammation, oxidative stress, pain, and neuroinflammation (Ferdousi and Finn, 2018; Garfin et al., 2018; Ramachandran, 2018; Rometsch-Ogioun El Sount et al., 2018). Increased BBB permeability causes edema, increased S100B expression, and neuroinflammation (Koh and Lee, 2014). Activated mast cells cause both neuronal response and vascular response, as they are close to BBB structure and neurons. Stress-induced mast cell activation in dural vasculature plays an important role in the acute and chronic headaches (Kandere-Grzybowska et al., 2003; Shelukhina et al., 2017). Psychological stress conditions activate neurons to release CGRP, substance P, and neurokinins that activate mast cells and release many inflammatory mediators. These inflammatory mediators activate nociceptors and pain mechanisms (Forsythe, 2019). Recent reports indicate that stress induces inflammation in many diseases such as allergic diseases, eczema, fibromyalgia, mast cell activation syndrome, irritable bowel syndrome, chronic fatigue syndrome, and autism, and that the intranasal administration of natural flavonoid compounds such as tetramethoxyluteolin, and luteolin with Ashwaganda could inhibit inflammation, neuroinflammation and the severity of neurodegenerative diseases (Theoharides and Kavalioti, 2018; Theoharides and Tsilioni, 2018). Further, this report also suggests that interaction of mast cells and microglia in the hypothalamus could induce stress-mediated neuroinflammation (Theoharides and Kavalioti, 2018). Other natural plant products such as thymoquinone is known to improve cognitive disorders and neuroprotective effects in cerebral small vessel disease and can protect from stress effects (Guan et al., 2018).

Chronic stress and chronic pain conditions are considered as two sides of the same coin due to the similarities between them, though they are actually different (Abdallah and Geha, 2017). Hypothalamus, hippocampus, amygdala and pre-frontal cortex (limbic system) are important in learning process. These regions process incoming nociceptive pain signals as well as the signals from stress, and make signals for the specific decision making. Limbic system and hypothalamic-pituitary adrenal (HPA) axis are interconnected and regulate stress response of the body. Both chronic stress and chronic pain affect these regions and impair the functions. Factors such as low income, poverty, uncompleted education and unsuccessful occupation account for the socioeconomic stress-mediated adverse behavior, depression, substance use, crime, and obesity (Abdallah and Geha, 2017). Both chronic stress and pain can reduce the hippocampal volume and the stress is a risk factor for developing pain in the human (Chen et al., 2010; Mutso et al., 2012; Abdallah and Geha, 2017). Dark microglia, a newly identified microglia phenotype has been associated with stress and AD (Bisht et al., 2018). These dark microglia are structurally different from ionized calcium-binding adapter molecule 1 (Iba1) expressed microglia, and they are increased at the site of microglial alteration and activation such as around amyloid plaques (APs), dystrophic neurons, triggering receptor expressed on myeloid cells 2 (TREM2), in stress, aging, and AD (Heneka et al., 2013; Zheng et al., 2017). Chronic stress can induce BBB disruption and increase neuroinflammation that can induce and worsen AD pathogenesis. BBB dysfunction causes decreased beta amyloid (Aβ) entry from brain to blood circulation. AD induces ultrastructural changes in the endothelial cells, tight junction proteins, pericytes and astrocytes, increase oxidative stress, neuroinflammation, and enhance Aβ level by increasing β and γ-secretase activities. These changes continue as positive-feedback loop and cause dementia and cognitive disorders (Cai et al., 2011, 2018). Several acute stress conditions are associated with severe headache. Migraine headaches and neuroinflammation are worsened by stress conditions (Ramachandran, 2018). Migraine is also known to induce BBB permeability (Dreier et al., 2005). Previous study has shown that acute immobilization stress induces the activation of dura mast cells in C57BL/6 mice, but not in neurokinin-1 receptor deficient (NK-1R KO) mice. Moreover, stress-induced vascular permeability was reduced in mast cell deficient mice (Kandere-Grzybowska et al., 2003). These studies show that mast cells are important in stress-mediated adverse effects in the CNS.

Corticotropin-releasing hormone (CRH)/corticotrophin releasing factor (CRF) is expressed in neocortex, basal ganglia, amygdala and hippocampus in the CNS (Zhang et al., 2018). CRH released from the brain and peripheral system can activate mast cells to release neuroinflammatory mediators that can induce BBB permeability, neuroglial activation and neuroinflammation (Esposito et al., 2002; Theoharides and Konstantinidou, 2007; **Figure 2**). Mast cells express functional CRH-receptor1 (CRH-R1) and CRH-R2 receptors for CRH (Cao et al., 2005; Papadopoulou et al., 2005; Kritas et al., 2014). Mast cells can synthesize and release CRH that can activate mast cells and glial cells to release inflammatory mediators (Kempuraj et al., 2004; Yang et al., 2005). CRH-associated CRHR activation leads to neuronal death through protein kinase A (PKA), PKC, Ca^{++} and nuclear factor-kappa B (NF-kB) pathways in the neuroglia in the CNS disorders (Chen et al., 2014; Kritas et al., 2014). Stress-mediated CRH induces spine loss and inhibit synapse formation and inhibiting the dural secretion of chemokine (C-C motif) ligand 5 (CCL5) from glia (Zhang et al., 2018). CRH can directly affect brain endothelial cells and BBB permeability

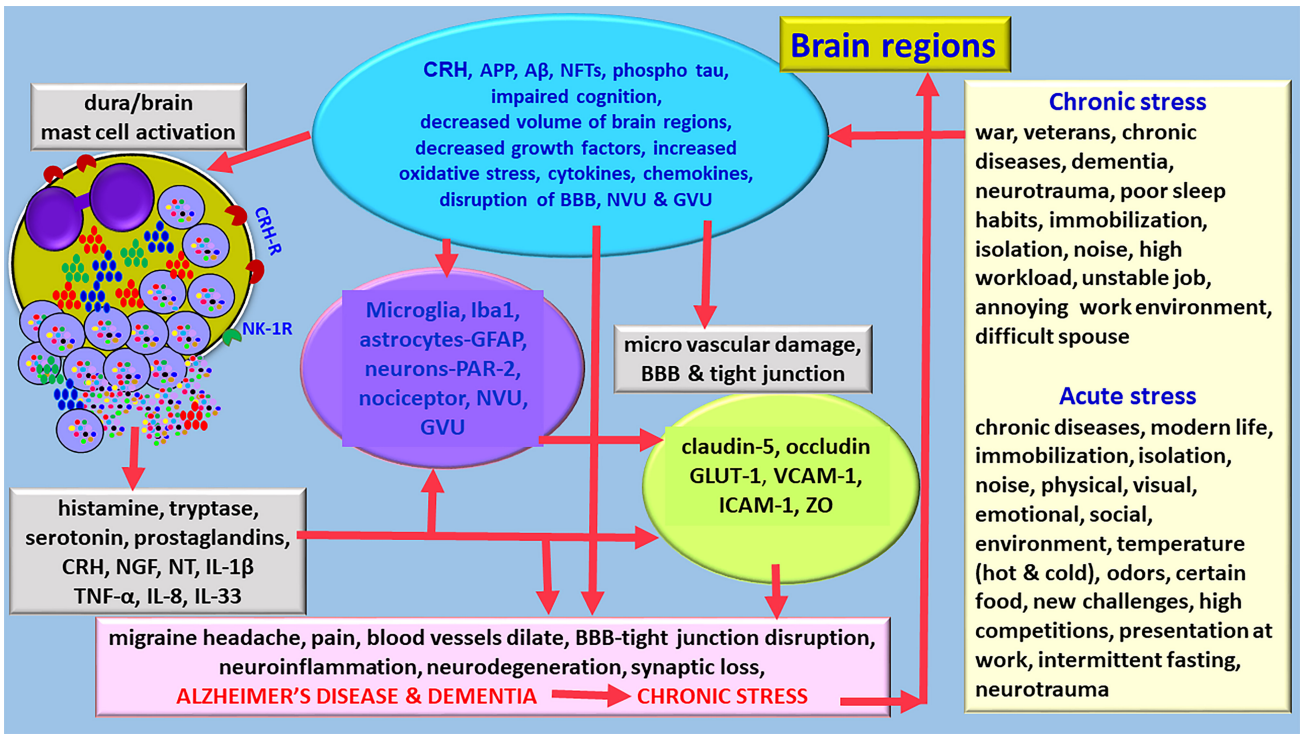

FIGURE 2 | Diagram showing stress can exacerbate neuroinflammation and neurodegeneration and accelerates the pathogenesis of AD. Various chronic stress and acute stress conditions differentially activates hypothalamus and release CRH in the brain. Stress also activates immune and inflammatory response in the brain that leads to the activation of immune and inflammatory cells, and glia associated with neuroinflammatory mediator release and pain. Stress increases the generation of APP, Aβ, hyperphosphorylated tau, induces cognitive disorders, reduce brain volume, decrease growth factor expression, increases oxidative stress, and activates mast cells and neuroglia. Several mast cell-derived inflammatory mediators induce severe migraine/headache in stress conditions. Stress affects BBB functions, induces tight junction damage and tight junction protein rearrangements. Chronic stress can shorten the duration to develop AD and dementia and increases its severity. APP, amyloid precursor protein; Aβ, beta amyloid; BBB, blood-brain barrier; GLUT-1, glucose transporter-1; IL, interleukin; NFTs, GVU, gliovascular unit; neurofibrillary tangles; NT, neurotensin; NVU, neurovascular unit; ZO, zonula occluden; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

(Esposito et al., 2003). These reports indicate that stress can induce neuroinflammatory pathways.

STRESS ASSOCIATED CHANGES IN THE BBB, NEUROVASCULAR UNIT, GLIOVASCULAR UNIT, TIGHT JUNCTION, AND ADHERENS JUNCTION

About 600 km length of capillaries and micro vessels supply blood to the brain that consists of about 100 billion neurons. BBB is a special semi-permeable barrier that prevents, restricts and selectively allows the movement of cells and substances from the peripheral blood to the brain and brain to the blood (Stamatovic et al., 2016). BBB mainly maintains and protects healthy microenvironment in the brain, in addition to blood-cerebrospinal fluid barrier (BCSFB) and arachnoid barrier. Studies have shown the effects of acute and chronic stress on BBB dysfunctions, but the studies on specific molecular and ultrastructural changes at the tight junction proteins and adherens junction are insufficient, and thus not clearly understood (Santha et al., 2015). Oxygen, carbon dioxide, glucose, and amino acids can pass through BBB, but not any foreign objects, microorganism and toxins. BBB and tight junction proteins that exist between the vascular endothelial cells regulate the passage of large, negatively charged molecules through paracellular diffusion method, but the transcellular transportation across the endothelial cells is regulated by many transporter proteins, by endocytosis, and diffusion methods (Kealy et al., 2018).

Blood-brain barrier consists of non-fenestrated special type of endothelial cells, astrocytes, pericytes, innate immune cells, and basement membrane. BBB with neurons and astrocytes constitute NVU and GVU, respectively. Both neuroinflammation and stress conditions can affect NVU and GVU in the brain. The adjacent endothelial cells contact each other through tight junction that consists of occludin, claudin-1, claudin-3, claudin 5, claudin-12; zonula occludens (ZO) ZO-1, ZO-2, ZO-3; junctional adhesion molecule-A (JAM-A), JAM-B and JAM-C; adherens junction, and gap junction. Claudin-5 is the predominant type among claudins (Lochhead et al., 2017). BBB also includes adherens junction and gap junctions. Adherens junction consists of transmembrane proteins such as cadherens (Ve-cadherens, E-cadherens) and catenins (α catenin, β-catenin).

Gap junction consists of many connexins such as connexin 37, connexin 40, and connexin 43. Additionally, endothelial cell-selective adhesion molecule (ESAM), partitioning defective-3 (Par-3), and Par-6 are other junction proteins similar to the JAMs (Stamatovic et al., 2016; Lochhead et al., 2017). Tight junction complexes such as claudins and occludins are connected intracellularly to the actin filaments. Both tight junction and adherens junction play different roles in BBB functions. Tight junction provides barrier functions and the adherens junction connects adjacent endothelial cells, promotes maturation of these cells, and provides plasticity to the endothelial cells. The junctional proteins can move and loose network connectivity in BBB dysfunctions in neurological and neuroinflammatory disorders. However, specific changes and relocation of junctional proteins in neurological disorders including AD and in stress is not yet clearly explored.

This emerging new field of research on BBB junctional complexes could provide useful information to understand the mechanism of neurological disorders such as in stress, stroke, dementia, and AD, and to develop disease specific and efficient therapeutic options. Pericytes are considered as the gatekeepers of the BBB and play role in angiogenesis and BBB integrity (Presta et al., 2018). Tight junctions prevent the flow of solutes through paracellular route. Substances such as glucose move across BBB through transcellular route by glucose transporter-1 (GLUT-1). GLUT-1 is highly expressed in the endothelial cells in the brain (DeStefano et al., 2018). Chronic social stress can cause BBB dysfunction associated with the loss of tight junction proteins such as claudin 5, and the entry of immune and inflammatory cells and cytokines from the peripheral system to the brain parenchyma (Menard et al., 2017).

Stress primarily affects hippocampus and frontal cortex in the brain. Study has shown that restraint stress significantly decreased claudin-5 and occludin in the hippocampus and frontal cortex in rats, at different periods of stress exposure (Santha et al., 2015). The same study also reported that restraint stress increased GLUT-1 and decreased astrocytic glial fibrillary acidic protein (GFAP) immunofluorescence in the frontal cortex. No neuronal changes were observed after immobilization stress, as determined by NeuN staining. Immobilization stress induces structural alterations of BBB endothelial cells. These endothelial cells show protrusions and detachment from the basement membrane (Santha et al., 2015). Immobilization stress increases the number of open junctions and damaged tight junctions, increases the thickness of the basal membrane, and edema of astrocytes in the hippocampus (Santha et al., 2015). Stress and aging contribute to the cognitive decline and hippocampal neurogenesis (Grilli, 2017).

Innate immune cells including granulocytes, macrophages, microglia and mast cells are important in the regulation of barrier functions of the BBB (Presta et al., 2018). Astrocytes, pericytes, and microglia release cytokines and chemokines that influence immune cells adhesion to the endothelial cells and migrate into the brain. However, the exact details of interactions and functions of these cells, and the ultrastructural and molecular mechanisms involved are not yet clearly understood. Acute stress can activate mast cells and increase the permeability of BBB (Kempuraj et al., 2017a). However, deficiency of mast cells or inhibition of mast cell activation by mast cell stabilizer disodium cromoglycate (Cromolyn) show reduced BBB permeability indicating mast cells play an important role in stress-induced BBB disruption (Esposito et al., 2001a,b). Activated mast cells release TNF-α that can downregulate the expression of the tight junction proteins such as occludin, claudin-5, ZO-1 and adherens junction VE-cadherin (Rochfort and Cummins, 2015). Other studies show that inhibition of TNF-α protects *in vitro* model of BBB that consists of endothelial cells and astrocytes, indicating the role of TNF-α in the BBB and tight junction dysfunctions (Abdullah et al., 2015; Rochfort and Cummins, 2015). A recent study show decreased expression of occludin and claudin 5 in the brain endothelial cells *in vitro* when incubated with mast cell tryptase (Zhou et al., 2018). Stress conditions alter BBB endothelial cells, tight junction proteins as well as the astrocytic end feet in neurodegenerative diseases including PD (Dodiya et al., 2018). Stress activates HPA axis through CRH and increases the release of glucocorticoids that inhibit immune response in the body (Esposito et al., 2001a). BBB dysfunction has been reported in many psychiatric disorders (Kealy et al., 2018). Mind and body practice such as yoga, exercise, nutritional supplement from plant products can reduce the level of pro-inflammatory mediators and improve the severity of pain, depression, anxiety, and cognition (Gu et al., 2018; Lurie, 2018). Stress is known to accelerate the onset and clinical severity of the experimental autoimmune encephalomyelitis (EAE) in mice in which mast cells are activated (Chandler et al., 2002; Brown and Hatfield, 2012). From the above reports, it is clear that stress affects BBB, NVU, and GVU in the brain.

STRESS AND ALZHEIMER'S DISEASE

AD is an irreversible neurodegenerative disease characterized by the presence of extracellular APs, intracellular neurofibrillary tangles (NFTs) and hyperphosphorylated tau, neuronal loss, loss of synapses, NVU and GVU changes, and oxidative stress in the specific brain regions. About 5.7 million AD patients are currently living in the United States. AD is the sixth leading cause of death, and AD and AD dementia will cost $277 billion in the United States in 2018 (Alzheimer's association, Chicago, IL, United States). Several chronic inflammatory conditions are associated with AD. There is no disease specific treatment option for AD, as the disease mechanism, risk factors, and the comorbid conditions are not yet clearly understood. Neuroinflammation, activation of glia, elevation of neuroinflammatory molecules and neuronal death are implicated in Alzheimer's disease (Zaheer et al., 2008, 2011; Ahmed et al., 2017; Raikwar et al., 2018; Thangavel et al., 2018). Although the deposition of extracellular APs and intracellular formation of NFTs are traditionally considered as hallmarks of AD pathology over a long period, extensive recent findings indicate that several other factors including excessive neuroimmune and neuroinflammatory components significantly contribute to the pathogenesis of AD (Liberman et al., 2018; Saito and Saido, 2018). Therefore, the current drugs that target Aβ and NFTs did not show disease modifying beneficial effects, though they

improve cognitive dysfunctions to some extent in AD patients (Fish et al., 2018). Newer approaches that target neuroimmune and neuroinflammatory components along with NVU and GVU are currently very active to treat neurodegenerative diseases including AD.

Chronic stress is one of the risk factors associated with dementia and AD pathogenesis (Rothman and Mattson, 2010; Bisht et al., 2018). It has been reported that mild and moderate stress conditions increase the level of amyloid precursor protein (APP), generation of Aβ peptide, intracellular NFTs, intracellular hyperphosphorylated tau, loss of synaptic plasticity, and extracellular APs that are associated with AD pathogenesis in the animals (Rothman and Mattson, 2010; Bisht et al., 2018; Justice, 2018; **Figure 2**). Chronic mild stress in $APP_{swe}/PS1_{de9}$ mice show depressive behaviors, reduced sociability, excessive Aβ level, glial cell activation and neuroinflammation in the brain (Gao et al., 2018). Another recent study showed that chronic noise stress altered gut microbiota, cognitive impairment, Aβ deposition in young senescence-accelerated mouse prone 8 (SAMP8) (Cui et al., 2018). Stress can exacerbate cognitive dysfunction and affect the functions of the hippocampus in the brain. Increased levels of Aβ is reported, even after 1 h of restraint stress. Further, stressful conditions such as modern life stress, chronic isolation stress, chronic social stress, chronic immobilization stress, and stress at early age show increased level of APs in the animals, indicating that these stressors are clearly associated with the pathogenesis of AD (Justice, 2018). Increased phosphorylation of tau and NFTs formation in many stress conditions lead to the damage of neurons and neuronal loss in AD and dementia (Sierra-Fonseca and Gosselink, 2018). The level of cortisol (corticosterone in rodents) is increased in stress conditions as well as in patients with dementia and cognitive impairment, indicating the relationship of stress and AD (Justice, 2018). One long term study, for over 50 years, with thirteen thousand patients reported that late-life depression increases the risk of dementia and AD (Barnes et al., 2012). Prolonged glucocorticoid levels in chronic stress can induce Aβ and tau deposition in AD pathogenesis in humans (Dong and Csernansky, 2009). Chronic stress also activates microglia that contribute to AD pathogenesis (Satoh et al., 2017). Gender and brain region-specific effects of stress has been reported previously (Devi et al., 2010; Bisht et al., 2018). Chronic restraint stress or repeated social defeat stress affects the release of neurotrophins and decreases the level of brain-derived neurotrophic factor (BDNF) that are important in neuronal growth, prevention of synaptic loss and maintenance of neuronal plasticity (Roth et al., 2011; Chiba et al., 2012). Pre-clinical stages in AD patients show reduced levels of pro-BDNF and BDNF (Peng et al., 2005). BBB dysfunction can activate β and γ secretase and generate and increase Aβ level in AD (Cai et al., 2018). Aβ transport through BBB is regulated by low density lipoprotein-1 (LRP-1) and receptor for advanced glycation end products (RAGE) expressed on the surface of the endothelial cells (Fei et al., 2018). Loss of pericytes and astrocyte abnormalities increases Aβ level in AD brains. Neurodegenerative diseases including AD show structural alterations in the tight junction proteins. Increased levels of RAGE associated Aβ toxicity induce damage to tight junctions in AD. BBB tight junction proteins ZO-1, occludin, claudin-1, claudin-3, claudin-5, claudin-12 and claudin-19 are implicated in AD pathogenesis. Occludin expression is increased in dementia and AD. Matrix metalloproteinases (MMPs) and apolipoproteinE4 (ApoE4) affect tight junction integrity in AD. Loss of tight junction integrity leads to increased permeability, edema, micro hemorrhage, and neuronal death (Yang et al., 2018). However, molecular and ultrastructural changes in BBB and tight junction proteins in AD is not yet clearly studied. Additionally, these changes in stress associated AD pathogenesis is much more complicated and currently not clearly understood. Moreover, studies also report that there is no association between Aβ and BBB dysfunctions. Aβ increases the expression of vascular adhesion molecules that are associated with the recruitment of inflammatory cells into the brain in AD. Increased hyperphosphorylation of tau generates NFTs that promote neuroinflammation, neuronal damage and BBB dysfunctions in AD. Childhood stress is associated with the development of dementia, cognitive impairment and neurodegeneration in late life in men (Donley et al., 2018). Further, a recent report indicate that early life stress is associated with late-onset-AD dementia (Lemche, 2018). However, it is not clear how the childhood stress continues to influence the body to develop neurological disorders in the late life. It is interesting to know if this effect is gender based or any population specific.

It has been shown that stress also increases cognitive dysfunctions in animals. Though several reports from animal studies support the concept that stress induces and worsens neuroinflammatory conditions including neurodegenerative diseases, the exact mechanism and the direct evidence are not yet clearly demonstrated, especially in the human diseases. This is because the exact mechanism of stress and AD pathogenesis is not yet clearly understood. Moreover, there are also significant differences in the stress response in the humans. Additionally, transgenic AD animal models show abnormal and aggressive behaviors with different degrees/severity of stress effects (Justice, 2018). Thus, animal models are not very suitable models to assess the stress effects that are much different in humans. It is very difficult to compare the results obtained from animal models of stress with the human patients due to these differences. Recently, it has been suggested that stress hormone CRH can be manipulated to reduce the risk of AD pathogenesis (Justice, 2018). Physical activities are associated with decreased risk of developing many chronic diseases in the aged. A recent study demonstrated that physical activity can reduce the chronic effects of restraint stress and the severity of AD in the animal model of AD (Yuede et al., 2018). There are several hypotheses and mechanisms proposed to explain how stress can accelerate AD pathogenesis. Aβ can activate neurons in the HPA axis that can induce stress effects and AD pathogenesis through cortisol. Mast cell activation-mediated inflammatory mediators play an important role in neuroinflammation (Kempuraj et al., 2016, 2017b; Hendriksen et al., 2017). Activation of neuroglia, inflammatory mediator release and neuroinflammation induce cognitive disorders, neurodegeneration and AD (Dansokho and Heneka, 2017; Swanson et al., 2018). We and others have previously reported that acute and chronic stress conditions can

activate mast cells and that the increased mast cell activation can induce the onset and progression of neurodegenerative diseases including AD through the activation of neuroglia and increased BBB permeability (Shaik-Dasthagirisaheb and Conti, 2016; Kempuraj et al., 2017a). In fact, mast cell inhibitor drug Masitinib used as an adjunct therapy for mild to moderate AD in clinical trial has been shown to improve cognitive functions (Piette et al., 2011).

CONCLUSION AND POTENTIAL FUTURE DEVELOPMENT

Mast cells are associated with inflammation and pain. Stress conditions can activate mast cells and augment neuroinflammation through the activation of glial cells and neurons. Stress can induce HPA activation and mast cell activation that lead to neuroinflammation, BBB disruption and tight junction damage in the brain. Stress can induce the generation of APP, hyperphosphorylation of tau, NFTs, Aβ peptide, APs, oxidative stress, cognitive dysfunction, synaptic loss, neuronal loss, inflammatory mediator expression, and dementia in AD pathogenesis. Though several studies have shown the association of stress with BBB dysfunction, and tight junction protein alterations, the exact ultrastructural and molecular changes in these structures are not yet clearly known. Therefore, no effective therapeutic options are currently available to treat these conditions. Elaborate and sustained studies are needed to better understand these changes in stress associated AD pathogenesis in humans.

AUTHOR CONTRIBUTIONS

DK wrote and edited the manuscript. AZ critically edited the manuscript and acquired the funding. SM, RT, MA, GS, SPR, ID, SZ, and SI edited the manuscript.

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REVIEW
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Mast Cell Neural Interactions in Health and Disease

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Mast cells (MCs) are located in the periphery as well as the central nervous system (CNS). Known for sterile inflammation, MCs play a critical role in neuroinflammation, which is facilitated by their close proximity to nerve fibers in the periphery and meninges of the spinal cord and the brain. Multifaceted activation of MCs releasing neuropeptides, cytokines and other mediators has direct effects on the neural system as well as neurovascular interactions. Emerging studies have identified the release of extracellular traps, a phenomenon traditionally meant to ensnare invading pathogens, as a cause of MC-induced neural injury. In this review article, we will discuss mechanisms of MC interaction with the nervous system through degranulation, *de novo* synthesis, extracellular vesicles (EVs), tunneling nanotubes, and extracellular traps with implications across a variety of pathological conditions.

Keywords: blood brain barrier, mast cell, endothelial cell, pain, inflammation, nervous system

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INTRODUCTION

Mast cells (MCs) are proinflammatory cells that are the first responders of the immune system (Galli et al., 2005; Gupta and Harvima, 2018). MCs localize in proximity to afferent fibers innervating the periphery, visceral organs and meninges. MC proximity to the external environment makes them prime candidates for a rapid response against external stimuli and internal microenvironment. In addition to their function against the external environment, MCs are also sensitive to the endogenous environment and may thereby contribute to multiple pathobiologies, including pain, itch, and disorders of the nervous system (Mattila et al., 2011; Xanthos et al., 2011; Arac et al., 2014; Kempuraj et al., 2017; Gupta and Harvima, 2018). It is suggested that MCs contribute to pathology through interaction with the vasculature and the central nervous system (CNS). Upon activation, MCs quickly release substances from preformed granules including proteoglycans, proteases, leukotrienes, biogenic amines, and cytokines (Vukman et al., 2017). In addition to degranulation, MCs have a delayed response leading to the release of cytokines, neuropeptides, and chemokines by *de novo* synthesis. Noxious (toxic or injurious) substances released include but are not limited to histamine (Sjoerdsma et al., 1957),

Abbreviations: Ags, exogenously encountered antigens; BBB, blood brain barrier; c-kit, receptor tyrosine kinase kit; CNS, central nervous system; DRG, dorsal root ganglion; EV, extracellular vesicle; FcεR1, receptors with high affinity for IgE; IgE, immunoglobulin E; IL, interleukin; MC, mast cell; MCET, mast cell extracellular trap; MS, multiple sclerosis; NK1, neurokinin 1; PAD, peptidylarginine deiminase; PBK/Akt, protein kinase B; ROS, reactive oxygen species; SCF, stem cell factor; SCD, sickle cell disease; SK2, small conductance Ca^{2+}-activated K^{+}; TLR, toll-like receptor; TNFα, tumor necrosis factor α; TNT, tunneling nanotubules; TRPA1, transient receptor potential ankyrin 1.

tryptase (Glenner and Cohen, 1960), chymase (Benditt and Arase, 1959), tumor necrosis factor α (TNFα; Gordon and Galli, 1990), and interleukin (IL-6), IL1β (Bradding et al., 1993; Nakamura et al., 2012), monocyte chemoattractant protein 1 (Vincent et al., 2013), nerve growth factor (Leon et al., 1994), brain-derived neurotrophic factor (Yuan et al., 2010), gonadotropin-releasing hormone (Khalil et al., 2003) and substance P (SP; Vincent et al., 2013; Taracanova et al., 2018). In this review article, we will examine the different ways in which MC activation interacts with the nervous system and their pathological implications.

LOCALIZATION OF MAST CELLS

MCs are specifically located in the dura mater/meninges of the spinal cord and brain (Khalil et al., 2007). Within the CNS, MCs are located on the abluminal side of the blood brain barrier (BBB) in apposition to astrocytes and neurons (Manning et al., 1994; Florenzano and Bentivoglio, 2000; Silverman et al., 2000). Increased BBB permeability can lead to MCs crossing into the CNS (Silverman et al., 2000). In addition, MCs can cross through the blood-spinal cord barrier (Dong et al., 2014). MCs are found in close proximity to sensory nerve endings, and their degranulation can modulate the excitability of nociceptors. In the spinal cord, white matter separates the dura from the lumbar dorsal horn allowing MC mediators to reach the superficial laminae, which is a key relay station that modulates synaptic transmission and nociception (Xanthos et al., 2011). It has been found that MCs can induce persistent nociception and long-term potentiation at spinal C-fiber synapses (Xanthos et al., 2011). This is mediated through SP release, which mediates pain sensation through unmyelinated C fibers (Tore and Tuncel, 2009). In most tissues, MCs and nerves have a gap of 20 nm allowing MCs to immediately act on peripheral nerves following degranulation. A correlation has been shown between MC proximity to nerve fibers and complex regional pain syndrome (Morellini et al., 2018). MCs also co-localize with astrocytes and may modulate the behavior of astrocytes to release more mediators through the release of histamine from MCs (Skaper and Facci, 2012).

MAST CELL ACTIVATION AND THE NERVOUS SYSTEM

Mast Cell Degranulation

Degranulation occurs within minutes of activation and results in the rapid release of substances from pre-formed granules. Degranulation begins with the activation of receptors with high affinity for IgE (FcεR1) and rearrangement of F-actin and microtubule formation. FcεR1 activates Fyn/Gab2/RhoA tyrosine kinases and leads to microtubule polymerization and the shuttling of secretory granules to the plasma membrane (**Figure 1**; Nishida et al., 2005). Tyrosine kinases Lyn and Syk of the activated FcεR1 cause calcium mobilization, vesicle fusion, and exocytosis of the granule through the protein kinase B (PBK)/Akt pathway and upregulation of peptidylarginine deiminase-4 (PAD-4) activation (**Figure 1**; Doyle et al., 2013; Aich et al., 2015). A variant of degranulation called transgranulation is associated with neuropathic pain (Keith et al., 1995; Wilhelm et al., 2005). Transgranulation occurs when MCs are in direct contact with other cells and thereby transfer the granules into nearby cells through exocytosis of the MCs and intake by the recipient cell. The effect is furthered through MC cytoplasmic extensions similar to pseudopodia that increase the range a MC can act through transgranulation (Barbara et al., 2004; Wilhelm et al., 2005). Because MCs are located closely to vasculature and nerve fibers, transgranulation can have a disruptive effect on those cells contributing to vascular dysfunction and neuropathic pain, respectively.

In the bladder, bidirectional communication between nerves and MCs using lamellipodia that enclosed fibers within a cell have been observed (Keith et al., 1995). Retrograde transport of MC mediators has also been shown in dorsal root ganglia (DRG; Murphy et al., 1999). In this study, a nerve injected with a MC degranulation product increased the expression of IL-6 mRNA in sensory neurons, and blockade of retrograde axonal transport attenuated the induction of IL-6 mRNA in primary sensory neurons.

De novo Synthesis

As a late response, MCs release cytokines and chemokines that are synthesized *de novo*. The mechanism for activation is thus complex and involves multiple receptors. Immunoglobulin E (IgE) receptors are involved in mediating response in cooperation with the toll-like receptor (TLR). TLRs also recruit CD14 or CD48 for the effect of TLR ligands (Marshall, 2004).

IgE independent receptors also regulate MC *de novo* synthesis. This involves the receptor tyrosine kinase kit (*c-kit)*, which is a receptor tyrosine kinase for stem cell factor (SCF; Mitsui et al., 1993). SCF promotes the development of more MCs. The large number of cytokines, chemokines, and neuropeptides released from MCs along with their proximity to axonal processes may be a large contributor to neuropathy and inflammation.

Extracellular Vesicles

Extracellular vesicle (EV) release is common to many different cells. EVs may be formed in endosomes and released through exocytosis. MC exosomes are known to interact with sensory nerves through the release of SP (Azimi et al., 2017). In turn, SP interacts with Mas-related G protein-coupled receptors (Mrgprs) to activate DRG neurons in mice (Azimi et al., 2017). Neurokinin 1 (NK1) receptors and Mrgprs were activated in mice and then pain behaviors were measured *in vivo* and DRG activation was measured in culture leading to the conclusion that SP activates DRG in culture through Mrgprs not NK-1 receptors (Azimi et al., 2017).

Because of their small size, EVs can travel long distances and influence synaptic transmission in the CNS. EVs may also participate in reuptake by local neurons as EVs can be localized as MC granules (Groot Kormelink et al., 2016). Groot Kormelink et al. (2016) found that upon activation of MCs a CD63-positive subset of EVs is released. Upon phospholipid and proteome

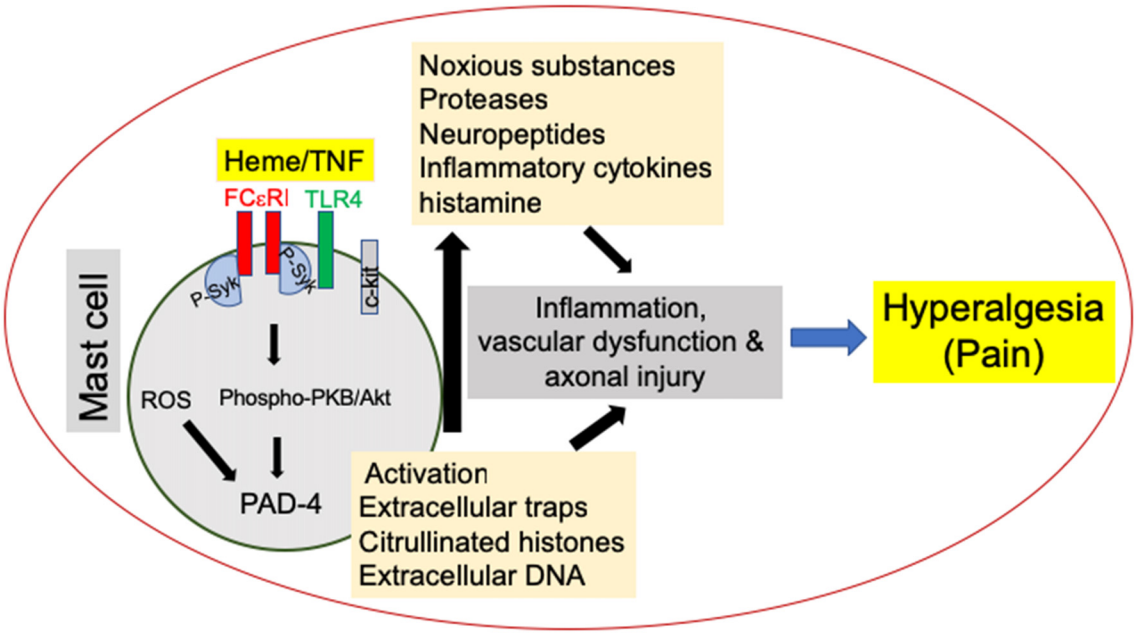

FIGURE 1 | Mast cell (MC) activation promotes hyperalgesia in sickle cell disease (SCD). MC activation leads to the release of noxious substances such as proteases, neuropeptides and cytokines as well as the release of MC extracellular traps (MCETs) with citrullinated H3 histones and DNA, which cause noxious as well as physical injury to the vasculature and nerve bundles, leading to hyperalgesia in SCD. Emerging data suggest that this process is mediated by endoplasmic reticulum (ER) stress/reactive oxygen species (ROS) production and may engage FcεR1 and toll-like receptor 4 (TLR4) leading to Syk associated downstream protein kinase B (PKB/Akt) signaling. Consequent peptidylarginine deiminase-4 (PAD-4) activation may stimulate the release of extracellular traps, while inflammasome signaling may further augment inflammation.

analysis of these EVs, it was found that the EVs contain MC-specific mediators (Groot Kormelink et al., 2016).

MC-released EVs can influence dendritic cell maturation *via* immunomodulatory exogenously encountered antigens (Ags; Skokos et al., 2003). Ags induce phenotypic changes in dendritic cell maturation by up-regulating MHC class II, CD80, CD86, and CD40 molecules. These molecules stimulate T lymphocytes and induce Ag-specific immune responses which could impact the function of dendritic cells.

MicroRNAs contained in EVs may also participate in neuropathy. MiRNA-let-7b causes rapid excitation in DRG neurons *via* TLR7 and transient receptor potential ankyrin 1 (TRPA1; Park et al., 2014). MiRNA-let-7b causes rapid inward currents and exciting the DRG neurons and inducing pain *via* TLR7 and TRPA1 (Park et al., 2014).

Tunneling Nanotubules

MC form tunneling nanotubules (TNTs), which are F-actin structures that form in response to reticulation. TNTs are similar to pseudopodia described earlier, except they are not adherent, and can span distances longer than pseudopodia. MC-microglia interactions have been found to be involved in brain inflammation (Skaper and Facci, 2012). MCs cultured in medium containing MC activators were found to rapidly form TNTs to transport mitochondrial and secretory granule particles to other MCs and glioblastoma, implicating TNTs in MC-microglia interactions (Weng et al., 2016).

Mast Cell Extracellular Traps

Extracellular trap formation is a process to ensnare external organisms for self-defense. This process involves citrullination of histone proteins, resulting in disassembly of DNA and ejection of web-like contents (Jorch and Kubes, 2017).

MC extracellular trap (MCET) formation is dependent on reactive oxygen species (ROS) formation and engagement of TLR4 (**Figure 1**; Stoiber et al., 2015). In addition to DNA, MCETs also contain fibers with tryptase. MCETs have been suggested to contain chemokines and cytokines necessary for an inflammatory response, which could lead to tissue damage, inflammation, and neuronal activation (Schauer et al., 2014; Möllerherm et al., 2016). IL-17 and IL-8 have been shown to be released by MCETs. MCs are the majority of IL-17-containing cells in control and psoriatic skin (Lin et al., 2011). Interestingly, MCETs showed bright costaining for IL-17. MCs and neutrophils have been reported to release IL-17, which contributes to the pathology of psoriasis.

NEUROVASCULAR INTERACTIONS AND PATHOLOGICAL OUTCOMES

It is well documented that MC mediators contribute to endothelial dysfunction in the vasculature (Kunder et al., 2011). Excess of MC mediators can cause an increase in BBB permeability. It is known that activation of MCs locally increases BBB permeability (Zhuang et al., 1996). Zhuang et al. (1996)

found that upon MC degranulation by C48/80 treatment, BBB permeability was increased.

MCs operate in a feed-forward mechanism. Inflammation caused by MCs can further activate the MCs in an autocrine manner. Mediators released by MCs such as IL33 and tumor necrosis factor alpha (TNFα) have been known to activate MCs (Taracanova et al., 2017). SP along with IL33 causes MCs to increase secretion and gene expression of IL-1β (Taracanova et al., 2018). These responses were mediated by SP and IL-33 receptors, NK1 and small conductance Ca^{2+}-activated K^{+} (SK2), respectively on MCs. Receptors were inhibited by methoxyluteolin inhibiting IL33 decreased IL-1β release (Taracanova et al., 2017).

MCs have been found to increase vascular permeability in the skin of mice with sickle cell disease (SCD; Vincent et al., 2013). Treatment of mice with a MC inhibitor cromolyn or imatinib or cannabinoids reduced vascular permeability in these mice (Vincent et al., 2016). These observations led to the suggestion that MC activation leads to the release of SP which activates protease-activated receptor 2 on the peripheral nerve endings, which in turn release more neuropeptides including SP leading to vascular dilatation and increased permeability. Thus MC activation leads to neurogenic inflammation involving neurovascular interactions.

Increased vascular permeability has been found in the CNS where MC degranulation compromises the BBB and allows further entry of inflammatory substances into the brain (Zhuang et al., 1996). Acute stress has pro-inflammatory effects that are mediated through the activation of MCs *via* corticotropin-releasing hormone (Esposito et al., 2001). Additionally, external and internal ROS formation contributes to changes in endothelial cell-cell interactions (van Wetering et al., 2002), BBB integrity (Lehner et al., 2011), and the disruption of tight junctions (Schreibelt et al., 2007). Increase in BBB permeability is associated with higher levels of neuroinflammation and brain dysfunction. Additionally, BBB disruption may further changes by systemic inflammation (Dénes et al., 2011; Knowland et al., 2014). Dénes et al. (2011) found that systemic inflammation compromises survivability after stroke which also augments BBB damage. Activation of meningeal MCs has been shown to worsen stroke pathology in mice (Arac et al., 2014). Therefore, activation of MCs in the periphery, as well as CNS, has implications in altering the neural activity and function directly and/or *via* neurovascular interactions.

CONCLUSION

MCs contribute to neural and vascular injury directly as well as induce neurovascular interactions. The complex milieu of multiple mediators released from MCs *via* diverse mechanisms alters the microenvironment leading to a hypersensitized system. Recent advances in MC-CNS interactions demonstrate that MC-induced hypersensitivity also contributes to the CNS disorders and pain. Thus, targeting of MCs provides a potentially treatable target for the disorders of the CNS and pain.

AUTHOR CONTRIBUTIONS

AM wrote the manuscript and prepared for submission. VS edited the manuscript. MG wrote and edited the manuscript. KG conceived, designed, and supervised the manuscript writing and editing and prepared the figure.

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PACAP-38 and PACAP(6–38) Degranulate Rat Meningeal Mast Cells *via* the Orphan $MrgB_3$-Receptor

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Infusion of pituitary adenylate cyclase activating peptide-38 (PACAP-38) provokes migraine attacks in migraineurs and headache in non-migraineurs. Adverse events like long-lasting flushing and heat sensation can be terminated with oral antihistamine treatment, indicating the involvement of mast cell activation after PACAP-infusion. Degranulation of rat peritoneal mast cells was provoked by several isoforms of PACAP *via* previously unknown receptor pharmacology. The effect might thus be mediated either *via* specific splice variants of the PAC_1-receptor or *via* an unknown receptor for PACAP-38. In the present study, we characterize degranulation of rat meningeal mast cells in response to PACAP-receptor ligands. Furthermore, we investigate if PACAP-38-induced mast cell degranulation is mediated *via* PAC_1-receptor splice variants and/or *via* the orphan Mas-related G-protein coupled member B3 ($MrgB_3$)-receptor. To address this, the pharmacological effect of different PACAP isoforms on meningeal mast cell degranulation was investigated in the hemisected skull model after toluidine blue staining followed by microscopic quantification. Presence of mRNA encoding PAC_1-receptor splice variants and the $MrgB_3$-receptor in rat mast cells was investigated by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis. The effect of PACAP isoforms on PAC_1- and $MrgB_3$-receptor-expressing *Xenopus laevis* oocytes were performed by two-electrode voltage-clamp (TEVC) electrophysiology. PACAP-38 is a more potent mast cell degranulating agent than Pituitary Adenylate Cyclase Activating Peptide-27 (PACAP-27) in the meninges. Presence of mRNA encoding the PAC_1-receptor and its different splice variants could not be detected in peritoneal mast cells by RT-PCR, whereas the orphan $MrgB_3$-receptor, recently suggested to be a mediator of basic secretagogues-induced mast cell degranulation, was widely present. In PAC_1-receptor-expressing *Xenopus laevis* oocytes both PACAP-38, PACAP-27 and the specific PAC_1-receptor agonist maxadilan were equipotent, however, only PACAP-38

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Abbreviations: BSA, Bovine Serum Albumin; MrgB3, Mas-related G-protein coupled receptor member B3; MrgX2, Mas-related G-protein coupled receptor member X2; PACAP-27, Pituitary Adenylate Cyclase Activating Peptide-27; PACAP-38, Pituitary Adenylate Cyclase Activating Peptide-38; PACAP(6–38), Pituitary Adenylate Cyclase Activating Peptide(6–38); PLC, Phospholipase C; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction; TEVC, Two-Electrode Voltage Clamp; VIP, Vasoactive Intestinal Peptide.

showed a significant degranulatory effect on mast cells. We confirmed Pituitary Adenylate Cyclase Activating Peptide(6–38) [PACAP(6–38)] to be a PAC_1-receptor antagonist, and we demonstrated that it is a potent mast cell degranulator and have an agonistic effect on $MrgB_3$-receptors expressed in oocytes. The present study provides evidence that PACAP-induced mast cell degranulation in rat is mediated through a putative new PACAP-receptor with the order of potency being: PACAP-38 = PACAP(6–38) > > PACAP-27 = maxadilan. The results suggest that the observed responses are mediated *via* the orphan $MrgB_3$-receptor.

Keywords: migraine, pituitary adenylate cyclase activating peptide, dura mater, *Xenopus laevis* oocytes, mast cell, Mas-related G-protein coupled receptor member B3, PAC1-receptor, two-electrode voltage clamp

INTRODUCTION

Pituitary adenylate cyclase-activating peptide-38 (PACAP-38) is a 38-amino acid neuropeptide located in both sensory and parasympathetic perivascular nerve fibers (Moller et al., 1993; Mulder et al., 1994). A C-terminal truncated 27-amino acid (PACAP-27) version is endogenously present as well but is less abundant (Miyata et al., 1990; Arimura et al., 1991; Ogi et al., 1993). A 20-min intravenous infusion of PACAP-38 provokes migraine attacks in migraine patients as well as headache in non-migraineurs (Schytz et al., 2009). At present, three PACAP-receptors have been identified: PAC_1, $VPAC_1$ and $VPAC_2$. The neurotransmitter vasoactive intestinal peptide (VIP) shares high amino acid sequence homology with PACAP and its affinity to $VPAC_1$ and $VPAC_2$ equals that of PACAP (Spengler et al., 1993; Pantaloni et al., 1996) whereas binding to the PAC_1-receptor is 1,000 times lower (Miyata et al., 1989, 1990; Harmar et al., 1998). Interestingly, VIP only induces a mild headache and no migraine-like attacks in migraineurs (Rahmann et al., 2008), which leads to the suggestion that PACAP and the PAC_1-receptor are key targets for future migraine treatment.

Infusion of PACAP-38 caused not only migraine attacks but also heat sensation and long-lasting flushing (Schytz et al., 2009). This is in line with PACAP-38 being a mast cell degranulator and mast cells have been suggested to play a role in migraine pathogenesis (Moskowitz, 1993; Levy et al., 2006, 2007). Degranulation of mast cells can be induced either by an allergen-IgE-dependent mechanism or *via* an IgE-independent mechanism. The latter mechanism can be activated by a group of molecules known as basic secretagogues. These molecules only share one physicochemical nature, their cationic property (Ferry et al., 2002). Several of these molecules are endogenous peptides and high concentrations are required for initiation of mast cell degranulation, an effect that involves pertussis toxin-sensitive G-proteins coupled to phospholipase C (PLC) activation (Ferry et al., 2002).

Inspired by clinical findings, we have previously characterized the degranulating effect of various PACAP-analogues on isolated rat peritoneal mast cells. Based on the expectation that degranulation is mediated through the PAC_1-receptor, we found an unpredicted order of potency (Baun et al., 2012). In peritoneal mast cells, the PAC_1-receptor antagonist Pituitary Adenylate Cyclase Activating Peptide(6–38) [PACAP(6–38)] caused mast cell degranulation that was as potent as PACAP-38 (Robberecht et al., 1992; Baun et al., 2012). Furthermore, the PAC_1-receptor agonist maxadilan was ineffective (Baun et al., 2012).

Several PAC_1-receptor splice variants have been cloned and characterized by ligand binding and signal transduction (Spengler et al., 1993; Pantaloni et al., 1996; Pisegna and Wank, 1996; Dautzenberg et al., 1999; Lutz et al., 2006). In 2006, Tatemoto et al. (2006) found the Mas-related G-protein coupled receptor member X_2 ($MrgX_2$) to be present in human mast cells. Mast cell degranulation induced by basic secretagogues appeared in the same concentrations as responses found in $MrgX_2$-expressing cells. The rat counterpart of $MrgX_2$ was found to be the Mas-related G-protein coupled receptor member B_3 ($MrgB_3$; Tatemoto et al., 2006). In the present study, we hypothesized that PACAP mediated degranulation by rat peritoneal and dural mast cells were either caused by a splice variant of the PAC_1-receptor or *via* $MrgB_3$-receptors.

MATERIALS AND METHODS

Animals

A total of 115 male Sprague-Dawley rats weighing 320–440 g (Taconic Europe, Ejby, Denmark) were used in this study. The rats were group-housed under a 12-h light/dark cycle and allowed *ad libitum* access to a standard rodent diet and water. All rats were euthanized by inhalation of a CO_2/O_2-mixture followed by CO_2 asphyxiation. Experimental procedures were approved by the National Danish Animal Experiments Inspectorate (License number 2014-15-0201-00256) and carried out in accordance with Danish legislation.

Mast Cell Degranulation in Hemi-skull Preparations

Mast cell degranulation was performed as previously described (Pedersen et al., 2015). In brief, skulls were cut mid-sagittal and the brain halves were removed, leaving the dura mater undisturbed. This was followed by immediate addition of either 350 µl 0.1, 1 or 10 µM PACAP-38 (custom synthesis by Caslo Laboratory ApS, Lyngby, Denmark) or vehicle (saline) in phosphate buffered saline (PBS). After 30 s incubation, the reactions were terminated, and the skulls were fixated in 4% paraformaldehyde in phosphate buffered saline (PBS;

TABLE 1 | Primers designed for detection of the housekeeping gene β-actin, the N-terminal part and the hip-hop variants of the PAC_1-receptor and the $MrgB_3$-receptor.

Primer	Forward (5′→3′)	Reverse (5′→3′)	Product size
β-actin	tca aca ccc cag cca tgt acg	cag gaa gga agg ctg gaa gag	422 bp
N-terminal	tct gac tgc atc ttc aag aag	acc gac agg tag taa taa tcc	392 bp
Hip + Hop	ctt gta cag aag ctg cag tcc cca gac atg	ccg gtg ctt gaa gtc cat agt gaa gta acg gtt cac ctt	471bp / 387 bp
$MrgB_3$	ccc ctg gaa tgt tct ttt gtg tag	aca gtg aaa aat gca gga act tcg	259 bp

Each set of forward and reverse primer was designed in Primer3 (Broad Institute) against the Adcyap1r1 gene (NM_001270582.1), RGD1560730 (XM_006229262.3) encoding the $MrgB_3$-receptor, and β-actin and tested for specificity by BLAST alignment tool (NCBI). The sizes of the amplified sequences are shown as base pair (bp).

Glostrup Hospital Pharmacy, Denmark). The dura mater was dissected from the skull, whole mounted on slides, and mast cells were visualized by staining with 0.1% acidified toluidine blue (Sigma Aldrich, Germany). Tissues were dehydrated in graded alcohols prior to cover slip mounting. The level of mast cell degranulation was evaluated by 400× magnification (Nikon Eclipse Ni microscope) by a researcher blinded to the treatment and was counted in 10 consecutive fields along the stem part of the middle meningeal artery. Mast cells were considered degranulated if an extensive dispersion of more than 10 extruded granules were localized outside the cell or if an extensive loss of staining gave the cell a "ghostly" look.

Peritoneal Mast Cell Isolation and RNA-Extraction

Peritoneal mast cells were harvested from three rats by injecting 20 ml oxygenated buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.5 nM $CaCl_2$, 0.4 mM NaH_2PO_4, 10 mM HEPES and 5.6 mM glucose, pH 7.6) in the peritoneal cavity of asphyxiated rats. The cavity was then gently massaged and subsequently opened by midline incision for the lavage to be removed by pipetting. Cells were washed three times by sedimentation at 13°C by a 7 min centrifugation at 400 *g*. The pellet was re-suspended in 5 ml oxygenated buffer and layered on top of a BSA-Percoll® density gradient (81%) containing 162 µl 35% bovine serum albumin (BSA), 8.1 ml Percoll® (GE Healthcare, Buckinghamsure, UK), 580 µl distilled water and 1.16 ml salt solution (1.54 M NaCl, 27 mM KCl, 3.8 mM $CaCl_2$). Cell types were separated by centrifugation at 225 *g* for 25 min at 13°C. The density gradient was discarded, and the pellet was again washed three times. The purity of mast cells was determined by histological characterization of the percentwise mast cell fraction. Only samples with a purity >90% were used for further analysis. Peritoneal mast cell RNA was extracted using the Isolation of Small and Large RNA Kit (Macherey-Nagel, Germany) in combination with TRIzol® (Qiagen) according to manufacturer's recommendations.

Reverse Transcriptase-Polymerase Chain Reaction

cDNA was synthesized from 500 ng peritoneal mast cell RNA using the iScript cDNA Synthesis kit (BioRad) according to instructions. PAC_1-receptor splice variants were identified in peritoneal mast cells and spinal cord using HotStarTaq® DNA polymerase (QIAGEN) with 10 µM primer. The $MrgB_3$-receptor was only tested in mast cell RNA. Primers against the Adcyap1r1 gene (NM_001270582.1, encoding the PAC_1-receptor), RGD1560730 (XM_006229262.3, encoding the $MrgB_3$-receptor), and β-actin were designed in Primer3 (Broad Institute) and tested for specificity by BLAST alignment tool (NCBI) and ordered from DNA Technology, Aarhus, Denmark (**Table 1**). PAC_1-primers were specifically designed to span the extracellular N-terminal (exon 3 to exon 8), exon 14 (known as "hip") and exon 15 (known as "hop"). β-actin was included as a positive control. The amplification protocol was as follows: initial heat activation at 95°C for 15 min, followed by 45 cycles with denaturation at 95°C for 1 min, annealing at variable temperatures depends on the primer set (50°C for β-actin and N-terminal, 56°C for $MrgB_3$ and 66°C for Hip + Hop) for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 10 min. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) products were visualized by agarose gel electrophoresis.

In vitro Transcription

DNA of the coding region of *Rattus Norvegicus* Adcyap1r1 variant 5 (NM_001270582.1) encoding the null-splice variant (short N-terminal and neither exon 14 or 15) of the PAC_1-receptor (Vector: EX-Rn10199-M03) was ordered from GeneCopoeia. DNA encoding the RGD1560730 gene (XM_006229262.3, GenScript), was cloned into the pXOOM vector as previously described (Jespersen et al., 2002). DNA was purified using Plasmid DNA Purification NucleoBond Xtra Midi-kit (Macherey-Nagel). The inserts were fully sequenced (MWG Operon) to confirm the expected sequence (data not shown). Extracted plasmids were linearized down-stream the poly(A) segment using the *XhoI* restriction enzyme (New England Biolabs, Ipswich, MA, USA). RNA was *in vitro* transcribed by synthetization from the T7 RNA polymerase promoter using the mMessenger mMachine kit (Ambion) according to manufacturer's protocol. Messenger RNA was purified using the MEGAclear kit (Ambion). Transcribed RNA integrity was assessed by agarose gel electrophoresis.

Expression in *Xenopus laevis* Oocytes and Two-Electrode Voltage Clamp

Stage V and VI defolliculated *Xenopus laevis* oocytes were purchased from EcoCyte Bioscience (Dortmund, Germany) and kept in Kulori medium (90 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Hepes, pH 7.4). Oocytes were micro-injected with 50 nl mRNA solution containing 30 ng RNA per oocyte and incubated at 19°C. Currents were

TABLE 2 | The number of degranulated meningeal mast cells in % of total number of mast cells after 30 s incubation with either vehicle, PACAP-38 or PACAP-27 in concentrations ranging from 0.1 to 10 μM.

Conc. (μM)	Number of exp.	Vehicle to PACAP-38 (% degranulation)	PACAP-38 (% degranulation)	Vehicle to PACAP-27 (% degranulation)	PACAP-27 (% degranulation)
0.1	5	8.5 ± 3.0	12.4 ± 3.8	4.7 ± 1.9	7.1 ± 3.2
1.0	6	7.2 ± 1.3	17.9 ± 3.7	7.2 ± 2.0	16.9 ± 5.5
10.0	6	6.2 ± 1.7	48.4 ± 6.2****	5.9 ± 1.3	12.1 ± 3.8

*Values are presented as means ± SEM. Statistical analysis (Two-way ANOVA followed by Sidak's test) was performed to find significant differences in mast cell degranulation induced by each substance in each concentration. Significance is given as ****$p < 0.0001$.*

measured after 2–5 days using a conventional two-electrode-voltage clamp (TEVC). The oocytes were placed in a 200 μl chamber and continuously exposed to a flow of Kulori medium with or without ligands (1 ml/min) while impaled with both a current and a voltage electrode filled with 3 M KCl and connected to an Oocyte Clamp Amplifier [Warner Instruments Corp. (OC-725 B) and a PC-interface (Digidata1440A, Molecular Devices)]. Current amplitude in absence or presence of ligands were analyzed using pClamp 10.2 software (Molecular Devices). All experiments were performed with oocyte membrane voltages constantly clamped to −70 mV and the temperature was kept between 19 and 22°C. In activation experiments, non-responding as well as low-responding (<10 nA) oocytes were excluded from the dataset. Ligands PACAP-38, PACAP-27, PACAP(6–38) were custom synthetized by Caslo (Lyngby, Denmark) while maxadilan was purchased from Bachem (Bubendorf, Switzerland).

Due to receptor desensitization, it was not possible to repeat measurements on individual oocytes, so only one dose could be tested per egg. Different batches of oocytes showed different expression levels and all figures are based on several batches of oocytes.

Statistical Analysis

Concentration-response curves for both oocytes and dural mast cells were analyzed for overall effects of PACAP-38 and -27 by two-way analyses of variance (ANOVA) followed by Sidak's test for multiple pairwise comparisons. Effects of PAC_1-receptor ligands on mast cell degranulation or on receptor-expressing oocytes were analyzed with a one-way ANOVA followed by Tukey's multiple comparisons test. The effect of PACAP-38 and PACAP(6–38) on $MrgB_3$-expressing oocytes were analyzed by an unpaired two-tailed *t*-test. Differences between groups were considered significant when $p < 0.05$ and data are presented as mean with standard error of the mean (±SEM). GraphPad Prism 7 (GraphPad Prism Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Effects of PACAP-38 and PACAP-27 on Dura Mast Cell Degranulation

PACAP-provoked mast cell degranulation was characterized by stimulating the dura mater with PACAP-38, PACAP-27 or saline in concentrations ranging from 0.1 to 10 μM (n = 5–6; **Table 2**). PACAP-38 stimulation resulted in an eight-fold increase and highly significant mast cell degranulation at the 10 μM concentration ($p < 0.0001$). However, even at the highest tested concentration, PACAP-27 did not induce mast cell degranulation that was significantly different from saline treatment.

Splice Variants of PAC_1-Receptor in Rat Peritoneal Mast Cells

Because mast cell degranulation induced by increasing concentrations of the different PACAP-isoforms did not follow the known order of potencies for PAC_1-, $VPAC_1$- or $VPAC_2$-receptors, we investigated the presence of PAC_1-receptor mRNA and possible splice variants by using RT-PCR (**Figure 1**). As a positive control, we included rat spinal cord tissue in which the PAC_1-receptor previously has been localized (Dickinson et al., 1999). We performed RT-PCR analysis targeted to several areas involved in splice variation of the PAC_1-receptor (N-terminal, exon 14 and exon 15) and found the PAC_1-receptor to be absent in mast cells and present in spinal cord (**Figure 1**). The lack of PAC_1-receptor mRNA expression in rat peritoneal mast cells indicates mast cell degranulation to be mediated *via* a non-PAC_1-receptor.

mRNA Expression of $MrgB_3$-Receptor in Rat Peritoneal Mast Cells

The PAC_1-receptor was not expressed in rat peritoneal mast cells. Thus, we investigated a possible expression of the $MrgB_3$-receptor as previously shown (Tatemoto et al., 2006). Using primers directed towards $MrgB_3$-receptor mRNA, we found it to be expressed in rat peritoneal mast cells (**Figure 2**). Therefore, we decided to study the effect of PACAP isoforms on $MrgB_3$-receptors and to compare the pharmacological profile on the PAC_1-receptor using TEVC in the *Xenopus laevis* oocyte expression system.

Effects of PACAP-38 and -27 on PAC_1- and $MrgB_3$-Receptors Expressed in *Xenopus laevis* Oocytes

Effects of PACAP-38 and PACAP-27 on PAC_1- and $MrgB_3$-receptors expressed in *Xenopus laevis* oocytes were investigated by TEVC. Upon addition of PACAP-38 and/or PACAP-27 at concentrations ranging from 0.01 to 1 μM to PAC_1-receptor-expressing *Xenopus laevis* oocytes, a rapid concentration-depended inward current was observed (**Figure 3A**) consistent with activation of an endogenous Cl^- current following receptor activation. These findings confirmed the aforementioned studies

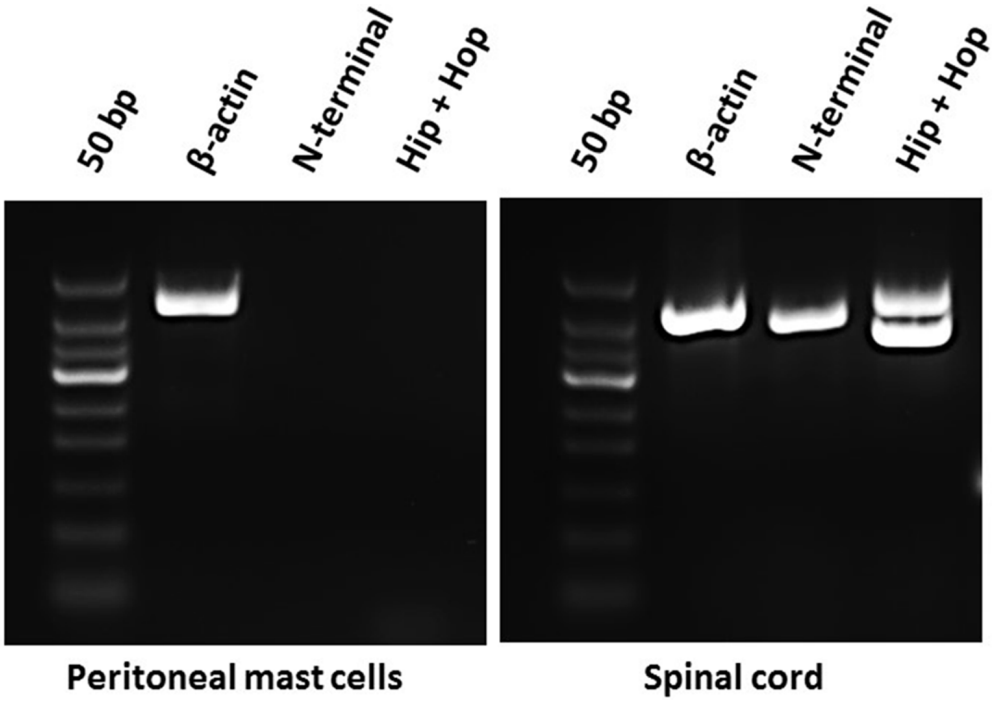

FIGURE 1 | Agarose gel electrophoresis showing the absence of mRNA-expression of PAC_1-receptor splice variants in rat peritoneal mast cells (left) and their presence in rat spinal cord (right) using primers directed towards the N-terminal part of the PAC_1-receptor (392 bp) and exon 14 and 15 of the Hop-Hop variant of the PAC_1-receptor (upper amplicon 471 bp and lower 387 bp). Primers detecting β-actin (422 bp) was used as a positive control and was present in both tissues. The experiment was performed in peritoneal mast cells from three rats.

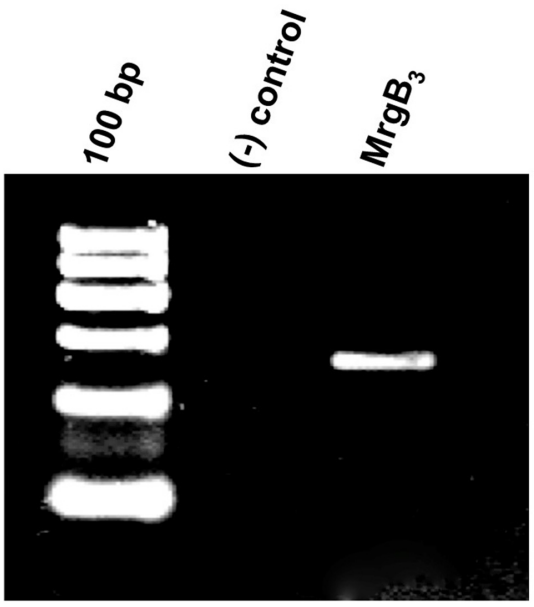

FIGURE 2 | Agarose gel electrophoresis showing the RT-PCR-product corresponding the presence of mRNA encoding the $MrgB_3$ receptor (259 bp) in rat peritoneal mast cells. No band is seen in the negative control [(–) control] where mRNA was not reverse transcribed to cDNA prior to amplification. The experiment was performed in peritoneal mast cells from three rats.

of PACAP-38 and PACAP-27 being equipotent on the PAC_1-receptor (Shivers et al., 1991; Pisegna and Wank, 1993). At 0.1 μM, PACAP-38 and PACAP-27-induced currents of -0.93 ± 3 μA ($n = 19$) and -1.04 ± 3 μA ($n = 15$), respectively. Stimulation with 1 μM of PACAP-38 and PACAP-27 resulted in currents of -2.27 ± 4 μA ($n = 15$) and -1.96 ± 5 μA ($n = 13$), respectively.

In $MrgB_3$-receptor-expressing oocytes, PACAP-38, but not PACAP-27, induced a rapid concentration-depended inward current in the concentration range of 1–10 μM ($n = 3$–24). The maximum current induced by PACAP-38 was -1.87 ± 5 μA ($n = 12$) at 3 μM (**Figure 3B**). The maximum response to PACAP-27 was found at 10 μM resulting in a current of -0.14 ± 0.4 μA ($n = 10$), which was not significantly different from baseline. These rapid responses were not seen in un-injected oocytes. In concentrations between 0.1 and 3 μM no effect was observed in un-injected oocytes, indicating that *Xenopus laevis* oocytes do not endogenously express PAC_1- or $MrgB_3$-receptors. However, concentrations at 10 μM PACAP-38 (15 out of 19 oocytes) but not PACAP-27 occasionally caused a delayed long-lasting response in un-injected oocytes, which was distinct from the above described fast responses. Thus, 10 μM PACAP-38 was not included in the experiments. Taken together, expression of the PAC_1-receptor and the $MrgB_3$-receptor in *Xenopus laevis* oocytes shows that PAC_1 is activated by PACAP-27 as well as PACAP-38, whereas the $MrgB_3$-receptor is activated by PACAP-38 only. Thus, the activation profile

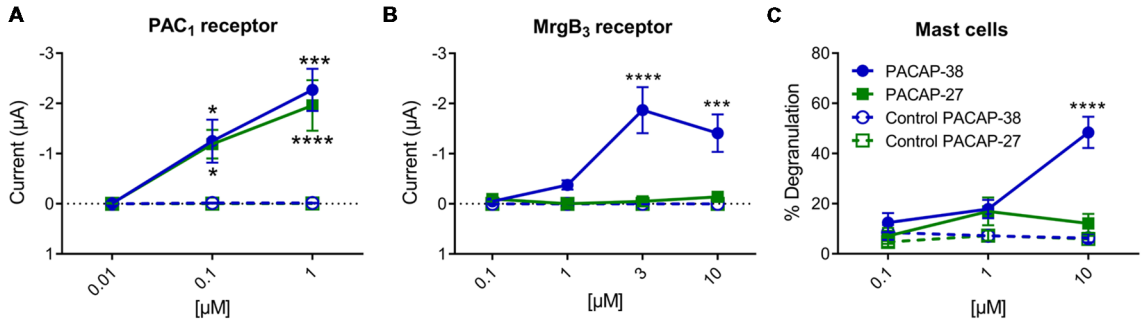

FIGURE 3 | (A) Two-electrode voltage clamp performed on *Xenopus laevis* oocytes expressing the PAC_1-receptor (n = 8–19, $n_{control}$ = 6–16) showed that increasing concentrations (0.01–10 μM) of PACAP-38 and PACAP-27 cause a significant increase in membrane current. The inward currents are measured at a holding potential of −70 mV. In **(B)** the *Xenopus laevis* oocytes is expressing the $MrgB_3$-receptor (n = 4–24, $n_{control}$ = 3–13). In these oocytes only PACAP-38 induces a significant change in current (Vm = −70 mV). **(C)** Concentration-response curves of PACAP-38 and PACAP-27 on rat meningeal mast cell degranulation after 30 s stimulation at concentrations ranging from 0.1 to 10 μM. It was found that a significant mast cell degranulation only was observed after stimulation with PACAP-38. Values are given as percentage of degranulated mast cells of total number of counted mast cells (n = 5–6). *Represent $p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ [two-way analysis of variance (ANOVA), Sidak's multiple comparisons test]. All values are given as mean ± standard error of the mean (SEM).

found for $MrgB_3$ resembles the effects of ligands observed for mast cell degranulation (**Figure 3C**). Taken together, mast cell degranulation induced by PACAP-38 and PACAP-27 in the meninges resembled mostly the current changes evoked in $MrgB_3$-receptor transfected oocytes.

Pharmacological Characterization of $MrgB_3$- and PAC_1-Receptor-Expressing Oocytes as Compared to Mast Cell Degranulation

Effect of PAC_1-Receptor Agonist Maxadilan on $MrgB_3$- and PAC_1-Receptors

Next, we characterized mast cell degranulation in response to the specific PAC_1-receptor agonist maxadilan. We found that neither PACAP-27 nor maxadilan caused mast cell degranulation (12 ± 4%, n = 6 and 5 ± 2%, n = 5, respectively) despite the high concentration (10 μM) tested (**Figure 4A**). When we compared the potencies of the same agonists in a relevant concentration (0.1 μM) in PAC_1-expressing *Xenopus laevis* oocytes, we observed that PACAP-27 and maxadilan induced changes in currents of −0.26 ± 1 μA (n = 14) and −0.21 ± 0.8 μA (n = 15), respectively. These responses were not significantly different (p = 0.8669) from currents observed after application of PACAP-38 (−0.27 ± 0.6 μA, n = 21; **Figures 4B**, **5A–C**). In $MrgB_3$-expressing oocytes, 3 μM PACAP-38 caused currents of −1.87 ± 5 μA (n = 12) that was significantly stronger than the effect induced by either 3 μM PACAP-27 (−0.06 ± 0.2 μA, n = 8) or up to 10 μM maxadilan (−0.03 ± 0 μA, n = 9; **Figures 4C**, **5D–F**). Currents were not observed when PACAP-38, PACAP-27 or maxadilan were added to un-injected oocytes in the same concentrations (*data not shown*). Thus, this series of experiments conclude that maxadilan, PACAP-27 and PACAP-38 activates the PAC_1-receptor with apparently equal potencies, whereas maxadilan does not activate $MrgB_3$-receptors.

Effect of PAC_1-Receptor Antagonist PACAP(6–38) on $MrgB_3$- and PAC_1-Receptors

Pharmacological characterization of PACAP-mediated mast cell degranulation was studied using the PAC_1-receptor antagonist PACAP(6–38). As previously shown in peritoneal mast cells this antagonist showed agonistic properties in meningeal mast cells by inducing a significant ($p < 0.0001$) and almost complete degranulation (93 ± 2%, n = 7) when administered in a concentration of 10 μM (**Figure 6A**). This response was very similar to degranulation induced by PACAP-38 (96 ± 3%, n = 5). In PAC_1-receptor-expressing oocytes PACAP(6–38) in a concentration of 0.1 μM had, as expected, no effect (−5 ± 6 nA, n = 9). However, the effect of PACAP-38 (−0.18 ± 0.5 μA, n = 11) was significantly antagonized when PACAP(6–38) was administered together with PACAP-38 in 0.1 μM (−0.05 ± 0.02 μA, n = 10; **Figure 6B**). In $MrgB_3$-receptor-expressing oocytes, 3 μM PACAP(6–38) induced a change in the current of −2.17 ± 3 μA (n = 27), which was not significantly different from the response induced by PACAP-38 (−1.62 ± 4 μA, n = 21; **Figure 6C**).

DISCUSSION

PACAP-38, but not the related peptide VIP, induces migraine headache in migraineurs suggesting the specific PACAP-receptor, PAC_1, as a potential target for migraine treatment (Rahmann et al., 2008; Schytz et al., 2009). Furthermore, all participants in the clinical provocation studies experienced long-lasting flushing, especially on the face and trunk, which could be terminated by antihistamine treatment, suggesting the involvement of mast cell degranulation (Schytz et al., 2009). In a previous series of experiments performed on rat peritoneal mast cells, we found that PACAP-38, but not PACAP-27 and VIP, caused degranulation. In addition, we showed that the selective PAC_1-receptor agonist maxadilan had no effect on mast cell degranulation. It was also found that

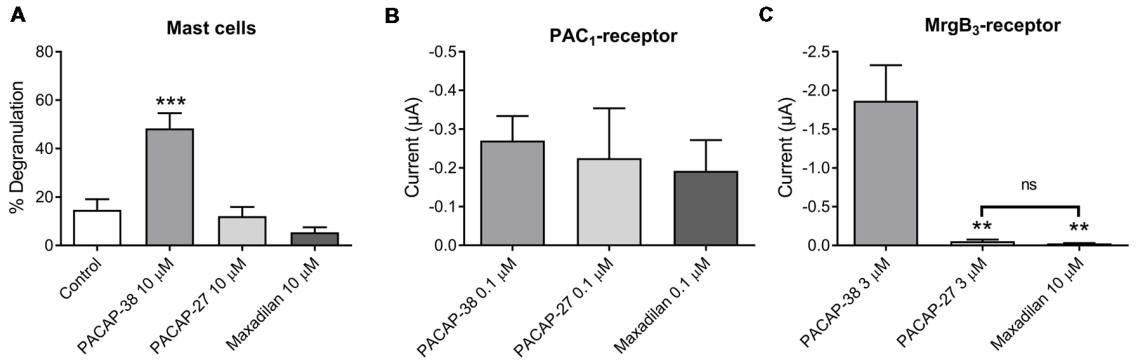

FIGURE 4 | Effect of PACAP-38, PACAP-27 and maxadilan (PAC_1-receptor agonist) on **(A)** rat meningeal mast cell degranulation following 30 s of 10 μM ligand stimulation. PACAP-38-induced a strong degranulation of meningeal mast cells. The mast cells were unresponsive to PACAP-27 and maxadilan. Values are given as percentage of degranulated mast cells from the total number of counted mast cells, $n = 5–11$. **(B)** Measurements on PAC_1-receptor-expressing *Xenopus laevis* oocytes using two-electrode voltage clamp showed similar changes in current during 60 s perfusion of PACAP-38, PACAP-27 or maxadilan (all ligands 0.1 μM, $n = 14–21$). **(C)** In $MrgB_3$-receptor-expressing oocytes PACAP-38 and PACAP-27 were perfused in a concentration of 3 μM and maxadilan at 10 μM ($n = 8–12$). Only PACAP-38 caused a change in current. All measurements were done at a holding potential of −70 mV. In **(A)**. ***represent $p < 0.001$ as compared to control **(A)**. In **(C)** ns, $p = 0.2879$ (Mann-Whitney non-parametric *t*-test). **Represent $p < 0.01$ as compared to PACAP-38 (one-way ANOVA, Tukey's multiple comparisons test). Values are given as mean ± SEM.

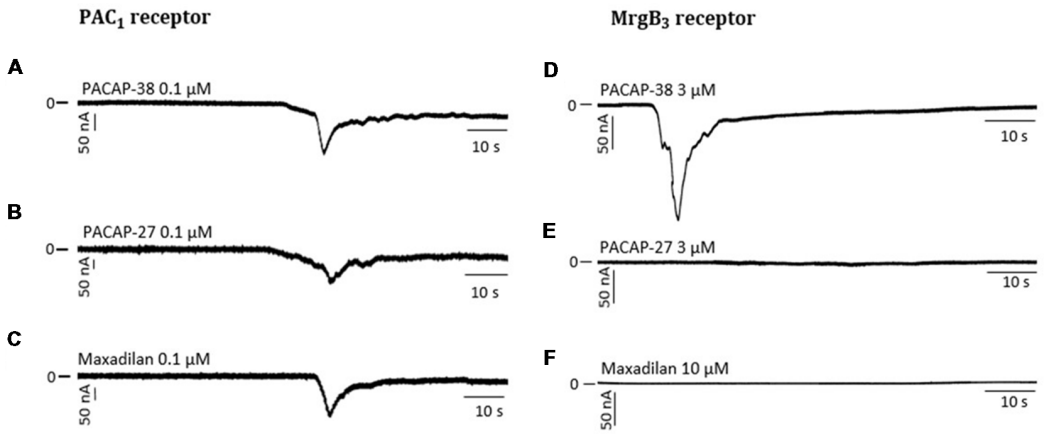

FIGURE 5 | Representative traces showing a change in current after perfusion with PACAP-38 **(A)**, PACAP-27 **(B)** and maxadilan **(C)** on PAC_1-receptor-expressing *Xenopus laevis* oocytes (**A** to **C**). In $MrgB_3$-receptor-expressing *Xenopus laevis* oocytes (**D** to **F**) PACAP-38 caused a change in current **(D)** while there was no change after perfusion with PACAP-27 **(E)** and maxadilan **(F)**. The membrane potential was clamped to −70 mV. Scale bars represent 50 nA and 10 s.

the selective PAC_1-receptor antagonist, PACAP(6–38), induced a pronounced mast cell degranulation (Baun et al., 2012). Based on these observations, we suggested that the PACAP-provoked meningeal mast cell degranulation is mediated through another receptor than the PAC_1-receptor (Baun et al., 2012). Our results suggest that PACAP-induced degranulation of rat peritoneal and meningeal mast cells is mediated *via* the orphan $MrgB_3$-receptor.

Pharmacology of PACAP on Mast Cell Degranulation

The weak degranulating effect of VIP and PACAP-27 compared to the strong degranulation effect of PACAP-38 in rat mast cells is inconsistent with the previously reported equipotent profiles of PACAP-38, PACAP-27, and VIP on $VPAC_1$- and $VPAC_2$-receptors (Harmar et al., 2012). In migraineurs, VIP does not provoke migraine headache, which suggests $VPAC_1$- and $VPAC_2$-receptors to be of minor importance in comparison to the PAC_1-receptor (Rahmann et al., 2008). Furthermore, the PAC_1-receptor antagonist PACAP(6–38) has not been shown to have an affinity to $VPAC_1$- and $VPAC_2$-receptors (Harmar et al., 2012). Taken together, this leads us to rule out the possible involvement of $VPAC_1$- and $VPAC_2$-receptors in PACAP-mediated mast cell degranulation.

Expression of PAC_1-Receptor Splice Variants

Several different splice variants of the PAC_1-receptor have been identified in rats. Splice sites in the extracellular N-terminal

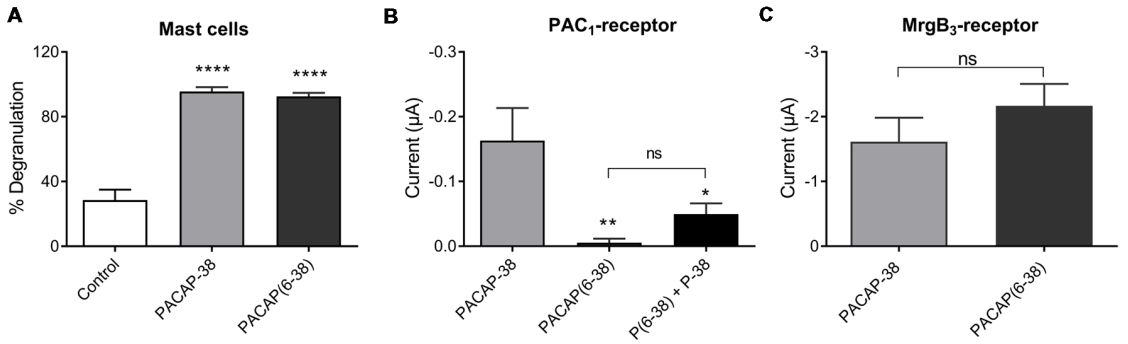

FIGURE 6 | (A) The PAC_1-receptor antagonist PACAP(6–38; 10 μM) is as potent as PACAP-38 (10 μM) to induce degranulation of rat meningeal mast cells. Values are given as percentage of degranulated mast cells (****$p < 0.0001$, one-way ANOVA followed by Tukey's multiple comparisons test), $n = 4–7$. **(B)** PACAP(6–38; 0.1 μM) is unable to induce currents in PAC_1-receptor-expressing *Xenopus laevis* oocytes using two-electrode voltage-clamp (TEVC) after 1 min perfusion of ligand. However, PACAP(6–38) caused a significant inhibition of PACAP-38 induced responses (*$p < 0.05$, **$p < 0.01$ one-way ANOVA followed by Tukey's multiple comparisons test), $n = 8–13$. **(C)** In $MrgB_3$-receptor-expressing *Xenopus laevis* oocytes PACAP(6–38; 3 μM) was equipotent to PACAP-38 (3 μM) to elicit currents (ns, $p = 0.2944$, unpaired *t*-test), $n = 21–27$. All measurements were done at a holding potential of −70 mV. All values are given as mean ± SEM.

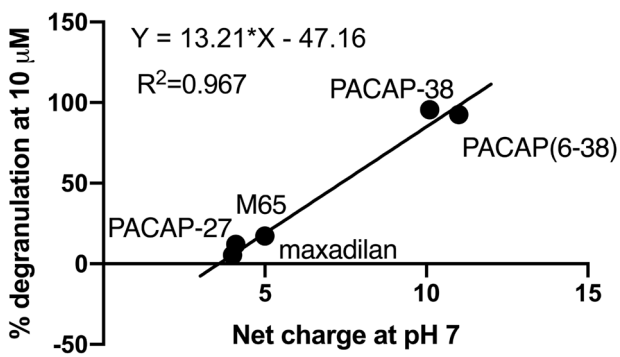

FIGURE 7 | A correlation of 0.967 is obtained between the mean amount of degranulation induced by five PACAP isoforms (PACAP-38, PACAP-27, PACAP(6–38), maxadilan and M65), $n = 5–11$ in meningeal mast cells and their net charge at neutral pH. Calculations are performed using the Bachem Peptide Calculator (http://www.bachem.com/service-support/peptide-calculator/).

domain and the third intracellular loop account for fine tuning of ligand affinity and signal transduction through adenylate cyclase or PLC activation (Deutsch and Sun, 1992; Spengler et al., 1993). The presence of a 21-amino acid domain in the extracellular N-terminal domain (PAC_1-full, short) impairs PACAP-27 binding (Pantaloni et al., 1996). Hip and hop (exon 14 and 15, respectively) insertions into the third intracellular loop are suggested to modulate G-protein coupling and favor PACAP-38 induced PLC activation *via* Gq-proteins as compared to PACAP-27 (Spengler et al., 1993; Blechman and Levkowitz, 2013). This could explain the difference found in PACAP-38 and PACAP-27 provoked mast cell degranulation. We, therefore, designed primers directed towards the N-terminal part and towards exon 14 and 15. However, using the RT-PCR analysis we were unable to identify PAC_1-receptor expression in the mast cell transcriptome. To further confirm the validity of the primers, we made parallel RT-PCR experiments on mRNA from spinal cord showing the presence of both the N-terminal part and the hip-hop variants of the PAC_1-receptor. Based on these findings, we suggest PACAP-38-provoked mast cell degranulation to act *via* a target distinct from PAC_1-receptors.

PACAP as a Basic Secretagogue

Degranulation of mast cells induced *via* the IgE-independent pathway is mediated by a variety of compounds collectively designated as basic secretagogues. This is a mechanism highly conserved among mammals and birds, which appoints it to be ancient and fundamental (Halpern and Wood, 1950; Taneike et al., 1988). In general, basic secretagogues are positively charged, although hydrophobic structured compounds causing rapid mast cell degranulation [within ∼10–20 s through PLC stimulation, which is sensitive to Gi-protein inhibition, e.g., pertussis toxin (Ferry et al., 2002; Tatemoto et al., 2006)]. PACAP-38 induced an almost total mast cell degranulation within the first 10–20 s after application. The degranulation was impaired by the PLC-inhibitor U-73122, whereas adenylyl cyclase inhibitor SQ22536 was ineffective (Baun et al., 2012), indicating PLC activation as the responsible transduction pathway for PACAP-induced mast cell degranulation.

Interestingly, basic secretagogues seem to activate connective type mast cells independent of their putative receptor but only when applied in high concentrations (Ferry et al., 2002). Plotting the net charge of PACAP related molecules at neutral pH towards the level of degranulation induced by these PACAP analogues at 10 μM (**Figure 7**), we found a linear relationship with an R^2 value close to 1. Thus, a high net charge of the molecules correlates with a high mast cell degranulating effect of the PACAP analogous tested, and several factors indicate that PACAP may act as a basic secretagogue to cause mast cell degranulation despite the absence of PAC_1-receptors.

Expression of $MrgB_3$-Receptors

The exact mechanism of basic secretagogue-mediated mast cell degranulation remains unclear. The ability of basic secretagogues to act as direct activators of purified G-proteins as well as numerous failed attempts to identify an endogenously expressed receptor could suggest a receptor-independent mechanism of action (Mousli et al., 1990, 1994; Seebeck et al., 1998). However, this would require the ability of peptides, despite their positive charge, to diffuse across the membrane in order to reach intracellular G-proteins, and as this has not been shown, the hypothesis seems unlikely (Tatemoto et al., 2006). The identification of a basic secretagogue receptor has for a long time been sought but without success (Ferry et al., 2002). In 2006, Tatemoto et al. (2006) studied members of the Mrg family. These are G-protein coupled receptors and expressed in a subset of nociceptive sensory neurons, thus making them interesting targets (Dong et al., 2001; Lembo et al., 2002). Expression of $MrgX_2$-receptors in humans and $MrgB_3$-receptors in rats were shown to be present in connective type mast cells and with affinity to various peptides like PACAP(6–27), mast cell depleting peptide and [D-$Trp^{7,9,10}$]-substance P could link these receptors to PACAP-mediated mast cell degranulation (Tatemoto et al., 2006). In a recent study, $MrgX_2$-receptor (human) and $MrgB_2$-receptor (which is the mouse orthologue of the human $MrgX_2$-receptor and the rat $MrgB_3$-receptor) was convincingly demonstrated to be mast cell-specific and responsible for inflammatory activation by basic secretagogues (McNeil et al., 2015). By RT-PCR, we found the $MrgB_3$-receptor to be present in rat peritoneal mast cells and decided to study the effect of selected PACAP analogues on *Xenopus laevis* oocytes expressing $MrgB_3$-receptors and to compare the responses to the effects obtained by the same PACAP analogues on PAC_1-receptor-expressing oocytes.

Experiments on $MrgB_3$-Receptor and PAC_1-Receptor-Expressing Oocytes as Compared to Mast Cell Degranulation

In rat $MrgB_3$-receptor-expressing oocytes, we found that PACAP-38, but not PACAP-27 and maxadilan induces currents. The effect mediated by the different PACAP agonists in the oocytes had the same characteristics as those previously found to induce a significant degranulation of rat meningeal mast cells. The findings were also in line with our previous results from peritoneal mast cells (Baun et al., 2012). Importantly, the concentrations required for effects were in the 1–10 μM range. In PAC_1-receptor-expressing oocytes, PACAP-38, PACAP-27 and maxadilan were equipotent and responses were found to be significant at 10 times lower concentrations.

In line with our results from peritoneal mast cells (Baun et al., 2012), we found the PAC_1-receptor antagonist PACAP(6–38) both to be a potent degranulator of rat meningeal mast cells and to induce a significant current in $MrgB_3$-receptor-expressing oocytes. Contrarily, PACAP(6–38) showed the predicted antagonistic effect on PACAP-38-induced currents in PAC_1-receptor-expressing oocytes (Harmar et al., 2012). Taken together, our studies suggest mast cell degranulation to be mediated *via* $MrgB_3$-receptors and not *via* the PAC_1-receptor. However, the conclusion is limited by the fact that currently no selective antibodies or antagonists directed towards the $MrgB_3$-receptor are available to provide the final pharmacological evidence.

Interestingly, a similar finding of PAC_1-receptor pharmacology and functional observations in the rat trigemino-vascular system was reported. In these studies, PACAP-38, but neither VIP, PACAP-27 nor maxadilan, mediated the release of the sensory vasodilator peptide, calcitonin gene-related peptide (CGRP) from the trigeminal nucleus caudalis. Furthermore, the response to PACAP-38 seemed not to be mediated *via* PAC_1-receptors due to lack of inhibition by the PAC_1-receptor antagonist M65 (Jansen-Olesen et al., 2014). Future studies will have to rule out if $MrgB_3$-receptors are involved in PACAP-38-induced CGRP release in trigeminal nucleus caudalis.

CONCLUSION

In the present study, we found PACAP agonists and antagonists to have the same pharmacological effect in meningeal mast cells as previously found in peritoneal mast cells. By RT-PCR, we showed that there was no PAC_1-receptor transcription in peritoneal mast cells thus excluding the possibility of the degranulating effect to be mediated *via* PAC_1-receptor splice variants. However, we found mRNA encoding the rat $MrgB_3$-receptor to be expressed in mast cells. This receptor was previously suggested to mediate mast cell degranulation in rat after application of basic secretagogues. This finding led us to investigate the effect of different PACAP analogues on *Xenopus laevis* oocytes expressing either PAC_1- or $MrgB_3$-receptors. The expressed $MrgB_3$-receptor but not the PAC_1-receptor share the same order of potency for PACAP analogues as found in rat peritoneal and meningeal mast cells. Thus, we hereby suggest the $MrgB_3$-receptor to be a mediator for PACAP-induced mast cell degranulation.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

IJ-O, SP and DK designed the research. KC and FH contributed with valuable intellectual input for improvement of the study. SP and SC performed the experiments. IJ-O, SP, CK, DK and SC performed data analysis. IJ-O and SP drafted the manuscript. JO, DK, KC, FH and SC read and corrected the manuscript. JO, IJ-O, SP and DK received financial support for the study. All authors read and approved the final manuscript.

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Thyroid Hormone, Thyroid Hormone Metabolites and Mast Cells: A Less Explored Issue

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Mast cells are primary players in immune and inflammatory diseases. In the brain, mast cells are located at the brain side of the blood brain barrier (BBB) exerting a crucial role in protecting the brain from xenobiotic invasion. Furthermore, recent advances in neuroscience indicate mast cells may play an important role in glial cell-neuron communication through the release of mediators, including histamine. Interestingly, brain mast cells contain not only 50% of the brain histamine but also hormones, proteases and lipids or amine mediators; and cell degranulation may be triggered by different stimuli activating membrane bound receptors including the four types of histaminergic receptors. Among hormones, mast cells can store thyroid hormone (T3) and express membrane-bound thyroid stimulating hormone receptors (TSHRs), thus suggesting from one side that thyroid function may affect mast cells function, from the other that mast cell degranulation may impact on thyroid function. In this respect, the research on hormones in mast cells is scarce. Recent pharmacological evidence indicates the existence of a non-genomic portion of the thyroid secretion including thyroid hormone metabolites. Among which the 3,5 diiodothyronine (3,5-T2), 3-iodothyroanamine (T1AM) and 3-iodothyroacetic acid (TA1) are the most studied. All these compounds are endogenously occurring and found to be increased in inflammatory-based diseases involving mast cells. T1AM and TA1 induce, as T3, neuroprotective effects and itch but also hyperalgesia in rodents with a mechanism largely unknown but mediated by the release of histamine. Due to the rapid onset of their effectiveness they may trigger histamine release from a cell where it is "ready-to-be released," i.e., mast cells. Following a very thin path which passes through old experimental and clinical evidence, at the light of novel acquisitions on endogenous T3 metabolites, we aim to stimulate the attention on the possibility that mast cell histamine may be the connector of a novel (neuro) endocrine pathway linking the thyroid with mast cells.

Keywords: mast cells, thyroid hormone, T3, histamine, 3-iodothryonamine, T1AM, 3-iodothryoacetic acid, TA1

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BACKGROUND

Mast cells are ubiquitous cells of hematopoietic origin, disseminated in the periphery and in the nervous system. In the brain, mast cells are few in number, ideally located to the meninges and perivascular area on the brain side of the blood-brain barrier (BBB), a strategic localization to initiate the detoxification of xenobiotics and/or vascular and immunological effects.

In most species studied, mast cells show a typical localization allowing an ideal interaction with neurons. The mast cell population of the brain is not static and it increases in numbers, distribution and activation in response to a variety of environmental stimuli that are associated with altered behaviors and physio-pathological conditions (Cirulli et al., 1998). Furthermore, emerging experimental evidence indicates that mast cells play pivotal roles in directing brain cell differentiation and plasticity including sexual maturation and behavior (Lenz et al., 2018) and in neuroprotection. In respect of this latter, stabilization of mast cells might represent a novel neuroprotective strategy during excitotoxicity (Ocak et al., 2018). The understanding of the mechanisms underlying the roles of mast cells in the brain is one of the challenges of the modern neurosciences. The hope is that the definition of such issues may help to comprehend how the mismatching of pro-inflammatory/anti-inflammatory factors levels, among which those derived from mast cells, may drive behaviors and neuron activation/survival possibly indicating novel drug targets. The relationship between mast cells and neurons is also reproduced in somatosensory neurons.

Irrespective of their tissue distribution, the characteristic feature of mast cells is the presence of cytoplasmic secretory granules which, upon activation, spread in the microenvironment lipid mediators, amines and hormones other than proteoglycans including heparin. Actually, the composition of mast cell granules shows some kind of heterogeneity depending on their tissue localization (Beil et al., 2000) and it looks that the presence of charged compounds in the granules is essential for the storage of other mediators (Grujic et al., 2013). As to say that there is a sort of chemical essential requisite for filling mast cell granules. Irrespective of this, mast cell granules always contain histamine, a charged amine which can be secreted together with the other mediators.

Sporadic evidence indicates that cells of the immune system, including mast cells can synthesize and store hormones among which are thyroid stimulating hormone (TSH) and the thyroid hormone T3 (Csaba and Pállinger, 2009a,b; Thangam et al., 2018). T3 levels were found increased following cell exposure to low histamine concentrations (Csaba and Pállinger, 2009a). Furthermore, evidence also indicates that mast cells express T3 receptors and that tissue mast cells population increased in hypothyroidism (Siebler et al., 2002), thus suggesting that the health or sick thyroid could condition mast cell hormone levels and/or that mast cells may represent an alternative source of packaged T3 locally deliverable (Thangam et al., 2018). Actually, even if evidence that T3 content decreased in peritoneal mast cells from hypothyroid rats (Csaba, 2014), it could further sustain this dual role of mast cells, though a definitive proof of this duality is lacking. In this respect, the progress in understanding the roles of T3 metabolites might open a new scenario.

MAST CELLS AND THE THYROID

The potential relationship, if any, between the thyroid and mast cells is certainly complex and likely two-fold. There is evidence that T3 effects can be modulated by mast cells, and that mast cells can modulate thyroid function. This interaction is clinically relevant in autoimmune disorders such as rheumatoid arthritis or intestinal inflammatory diseases (Costela-Ruiz et al., 2018) or in autoimmune thyroiditis disease such as chronic urticaria, characterized by recurrent episodes of mast cell-driven wheal and flare-type skin reactions, as well as in Grave's and Hashimoto disease (Ruggeri et al., 2013). In patients with chronic urticaria and angioedema, the presence of thyroid autoantibodies (Leznoff et al., 1983) and IgE antibodies against thyroid peroxidase were identified. By binding on the surface of mast cells these anti-TPO-IgE autoantibodies cause mast cell activation and degranulation, thus playing an active role in the pathogenesis of chronic urticarial (Altrichter et al., 2011). Mast cells also play a pivotal role in a complication associated to Grave's disease. van Steensel et al. (2012) demonstrated that in patients with Grave's ophthalmopathy, the number of mast cells in orbital tissue increased and these cells were capable to activate, through the PDGF pathway, the orbital fibroblast causing the onset of ophthalmopathy. These data suggest mast cells not only as regulators of fibroblast activation, but also as a possible therapeutic target (van Steensel et al., 2012). In fact, in a small cohort of patients, a little clinical improvement after treatment with cetirizine was observed (Lauer et al., 2008). Another interaction between thyroid and mast cells takes place in the bone and in particular, in bone remodeling and endochondral bone formation. The effect of thyroid on these processes are well known but the specific mechanism is partially elucidated. T3 exerts direct actions on chondrocyte growth and mast cells located in the bone marrow closed to epiphyseal plate are able to interact with osteoblasts and chondrocytes were found involved in processes that modify cartilage matrix and influence mineralization, further supporting the link between thyroid function and mast cells in bone metabolism. In fact, the number and the distribution of bone marrow-derived mast cells was found influenced by the thyroid status. Interestingly, bone marrow mast cells express thyroid hormone receptors with cytoplasmic localization which limits receptor trafficking towards the nuclei. Interestingly, such evidence indicates that mast cells represent a conjunction ring between T3 and bone remodeling and opens to non-genomic effects of T3 in bone metabolism and differentiation (Siebler et al., 2002). Data identifying an influence of mast cells on the thyroid support the bivalence of the relationship between thyroid and mast cells. Rocchi et al. (2007) demonstrated a specific role of mast cells on thyroid function in non-thyroidal illness (NTI). NTI are defined as changes of the hypothalamic—pituitary—thyroid axis in patients suffering from illnesses not primarily originating in the thyroid. These clinical conditions are characterized by: (a) decreased T3 levels; (b) decreased T4 due to a reduced binding capacity and/or affinity of serum carrier proteins for T4; and (c) inappropriately normal or low levels of TSH with respect to the decreased thyroid hormone levels (Faber et al., 1981; Kaptein et al., 1982; Docter et al., 1993; Wilcox et al., 1994). In a mouse model of NTI, a specific link between mast cells and bacterial infection-induced hypothyroidism was also found. This study identifies a role for mast cells as sensors capable of controlling the homeostatic

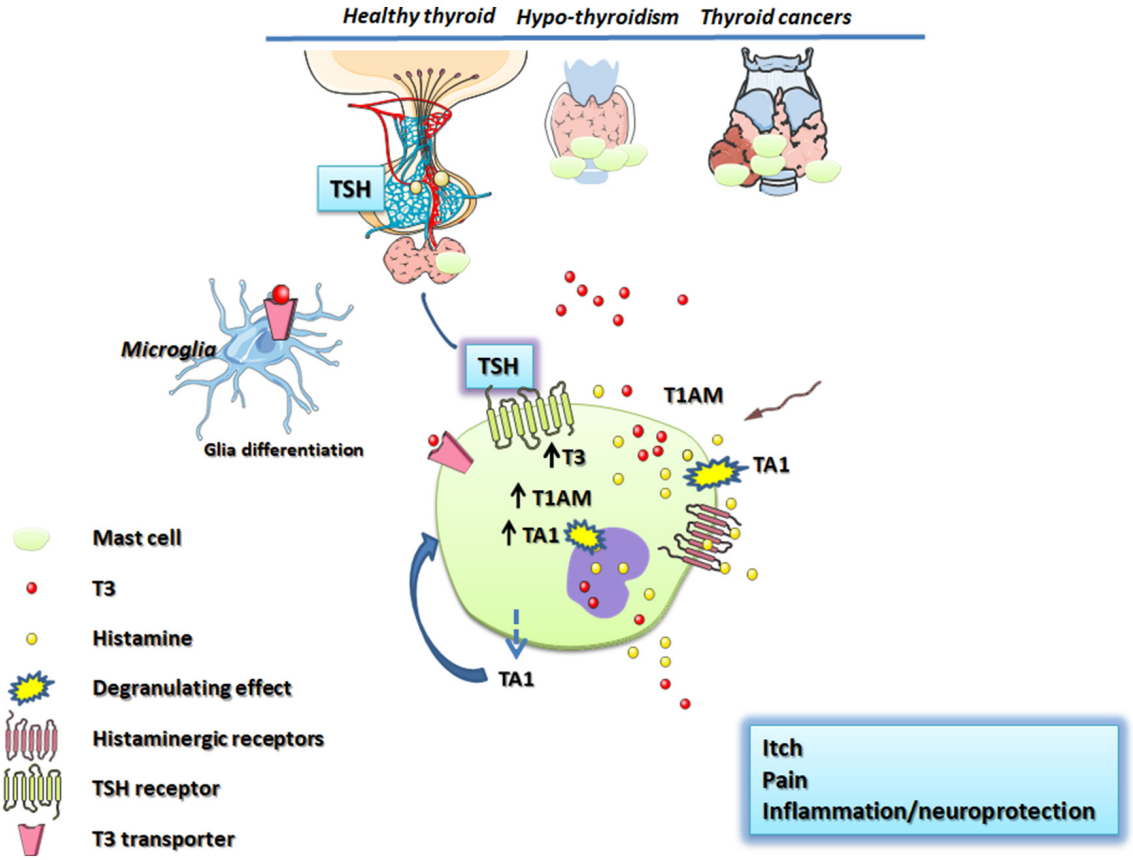

FIGURE 1 | Schematic representation of thyroid and mast cell connections. The hypothalamus, throughout the release of the TSH, stimulates mast cells increasing the T3 content. T3 is co-stored with histamine in mast cell granules or is degraded to T1AM and/or TA1. T1AM and TA1 derived from circulation or produced inside mast cells trigger mast cell degranulation releasing T3 and histamine which mediates pain, itch and central effects including neuroprotection/neuroinflammation. Thyroid stimulating hormone (TSH); TSH receptor (TSHR); thyroid hormones (T3, T4); 3-iodothyronamine (T1AM); 3-iodothyroacetic acid (TA1); monoamine oxidases (MAO).

responsiveness of the hypothalamus—pituitary—thyroid axis through the activation of specific crystallizing fragment (Fc) receptors (FCgR3). The lack of response in mast cells deficient mice confirmed the pivotal role of these cells (Rocchi et al., 2007).

This link might be stronger in case of heterogeneous diseases related to abnormal mast cell activation or increased tissue mast cell number, including cutaneous and/or their systemic presentations whose diagnosis is based on the monitoring of serum tryptase, elevated 24-h urinary histamine metabolite (methylhistamine). Among these is mastocytosis, a pathology characterized by increased tissue mast cell number. Among clinical manifestations of mastocytosis are dermatological symptoms, including itch and urticaria, and also neurological and psychiatric disorders (Georgin-Lavialle et al., 2016) mainly supported by increased histamine levels.

HISTAMINE AND THE THYROID

Histamine is essentially a pro-inflammatory mediator and if a low-grade inflammation is considered to be beneficial, sustained inflammation may generate abnormal cell behaviors. Histamine has different fate and effects in neuronal and not neuronal tissues. In not neural tissues, histamine deriving from mast cells, basophiles or enterochromaffin-like cells is recognized as a charged amine which enters in the calculation of the diamine (polyamine) tissue levels and it is implicated in local inflammation, pain, itch and vasodilatation. In periphery it is preferentially scavenged by the diamine oxidase (DAO) producing hydrogen peroxide and promotes its effects activating mainly the for types of receptors. In the central nervous system, histamine is produced and released by histaminergic neurons and by mast cells (Li et al., 2018) and the discrimination of the two possible sources can be achieved using genetic models or stabilizing pharmacologically mast cells. In the central nervous system, histamine is not considered a polyamine, the DAO is absent, and the enzymes involved in its metabolism, include the histamine methyl-transferase and the type B monoamine oxidase (MAO-B). Brain histamine is retained as a signal of the cell-to-cell communication with particular respect to microglia and neurons and the modulation of the brain histaminergic system may be afforded by drugs promoting histamine release

acting at receptors, working somehow as "neuromodulators" or by triggering mast cell degranulation. To note, mast cells possess receptors for aminergic mediators including the four types of histaminergic receptors, a condition which allows histamine to establish a sort of paracrine control on mast cells degranulation (Thangam et al., 2018). Thus, the role of mast cells, where histamine is ready-to-be released, may became relevant in generating high local histamine levels and immune-mediated inflammatory milieus (Fang et al., 2014).

Brain histamine (neuronal and not neuronal) is part of the mediators involved in the control of hypothalamic governed behaviors and it is also endowed of neuroprotective effects against excitotoxic damage (Kukko-Lukjanov et al., 2006). Furthermore, brain histamine is also part of the mechanisms of the neuroprotection offered by T3 metabolites (Cao et al., 2009; Laurino et al., 2018a,b). On the other hand, it is well assessed that histamine also controls the release of TSH (Roberts and Calcutt, 1983), a finding potentially linking the histaminergic system to the control of thyroid function. Actually, data regarding the effect of some histaminergic type 2 receptor antagonists on patient T3 serum levels seem to support, at least in part, the role of histamine in the control of TSH (Pasquali et al., 1981; Corinaldesi et al., 1987), thus suggesting a possible role of histamine in thyroxine regulation.

The T3 levels are critical regulators of the prenatal and neonatal development of the nervous system and of post-natal brain plasticity. A part of the well-known role of T3 on neurons, T3 is also a critical regulator of glial cell functions with not only genomic but also non-genomic mechanisms. Glial cells, as mast cells, express T3 transporters (Mori et al., 2015).

Histamine levels were found high during the embryonic brain development (Pearce and Schanberg, 1969) and Sabria et al. (1987) reported T3 as possible candidate for controlling brain mast cells number and, consequently, the levels of brain histamine during development. Till now there is no evidence that brain mast cells may contain T3 but there are evidence that T3 metabolites activate the histaminergic system in the brain as well as in periphery (Laurino et al., 2018a,b).

Upadhyaya et al. (1993) demonstrated that in L-thyroxine-treated rats, histamine levels were found increased in the hypothalamus, thalamus and cortex of the rats, and that there was a positive correlation between circulating T3 and T4 levels and histamine. Csaba and Pállinger (2009a) demonstrated that very low histamine concentrations, not active on pain and inflammation, increased T3 mast cell content.

Inflammatory-based diseases of the thyroid are named thyroiditis. Banovac and De Forteza (1992) demonstrated that mast cells degranulation plays a pivotal role in promoting the early stage of thyroiditis. More recently, experimental data from Visciano et al. (2015) demonstrated that mast cells histamine promotes a pro-tumorogenic effect on the thyroid and, in thyroid tumors and in experimental secondary hypothyroidism as, the mast cell population of the gland increased (Melander et al., 1971; Melillo et al., 2010), thus increasing histamine content of the gland. Furthermore, supporting the role of inflammation and autoimmune diseases in thyroid cancers, evidence indicate that mast cells play a pro-tumorogenic role in human thyroid cancer promoting neoangiogenesis and invasiveness (Melillo et al., 2010). Furthermore, mast cell number increased in thyroid cancer particularly in the follicular variant of papillary thyroid carcinoma, where their localization could represent a diagnostic marker of this kind of tumor (Proietti et al., 2011). Furthermore, assuming that mast cells of the healthy or sick thyroids behave as peritoneal mast cells, their role would become relevant in the economy of T3 (Melander et al., 1975).

Among thyroid diseases manifestations are skin symptoms among which are including chronic urticaria, alopecia and atopic dermatitis (Artantas et al., 2009). Interestingly, in all these complex diseases the brain-skin connection and the pivotal role of stress-induced mast cell degranulation has been recently reviewed (Shimoda et al., 2010; Alexopoulos and Chrousos, 2016). In this respect, the local release of T3 or of T3 metabolites might participate in the clinical symptoms of these kin diseases.

To note, T3 been detected in peritoneal mast cells, a finding suggesting that these mobile cells may deliver the hormone systemically. Furthermore, Csaba and Pállinger (2009a,b) demonstrated that secondary to TSH receptor activation, mast cells T3 content was found increased, without however indicating the mechanism. Unfortunately, the knowledge on the physio-pathological significance of T3 presence in peritoneal mast cells did not improve further from the evidence of Csaba and Pállinger (2009a,b). Thus, the possibility that mast cells may function as an "alternative" thyroid gland remains only a hypothesis.

THYROID HORMONE METABOLITES AND HISTAMINE

Recent experimental evidence suggest that T3 metabolism generates compounds that are not only able to reproduce similar but also opposite effects to those of T3 without activating nuclear receptors. T3 metabolisms is carried out by decarboxylase, deiodinases and MAOs which can work on T3 in sequence or alternatively producing three main families of derivatives namely thyronines, thyronamines, thyroacetic and thyropropionic acids at different degree of iodination. To note, the activity of MAO allows the transformation of thyronamines into thyroacetic acids while deiodinase activity removes iodide ions without transforming the chemical family. Despite of this, the synthetic pathway of these compounds is not fully elucidated yet.

Interestingly, several experimental and clinical evidence suggest that T3 metabolites may be part of the thyroid homeostasis and that they could be implicated in thyroid diseases. Among the main derivatives studied are the 3,5 diiodothyronine (3,5-T2), the 3-iodothyronamine (T1AM) and 3-iodothyroacetic acid (TA1; Scanlan et al., 2004; Chiellini et al., 2012; Galli et al., 2012; la Cour et al., 2018). All of them were found endogenously in rodents and in humans with a tissue distribution of T1AM and TA1 mirroring that of the T3 (Chiellini et al., 2012). Some studies reported an increased T1AM levels in pathological conditions including diabetes (Galli et al., 2012) and heart failure (la Cour et al., 2018). Interestingly enough, 3,5-T2 circulating levels were found increased in

cirrhosis, in brain tumors and in patients with non-thyroidal illnesses. In these latter cohort of patients, 3,5-T2 circulating levels were found correlated with the onset of post-surgical atrial fibrillation episodes (Dietrich et al., 2015). At the level of the heart, Frascarelli et al. (2011) demonstrated that T1AM exerts cardioprotective effects in an isolated rat heart model of ischemia-reperfusion injury, as to say that thyroid function on the heart is complex involving T3 and T3 metabolites.

Also, pharmacological evidence indicate T1AM is a regulator of body temperature (Scanlan et al., 2004) of mice feeding (Manni et al., 2012), it promotes memory acquisition and retrieval (Manni et al., 2013; Bellusci et al., 2017), it is anti-amnestic (Laurino et al., 2017), induces hyperalgesia and also has the potential to behave as an anti-obesity drug (Assadi-Porter et al., 2018). T1AM has a short half-life being rapidly degraded to its oxidative metabolite, the 3-iodothyroacetic acid (TA1). Since the oxidative deamination is carried on by MAO activity, TA1 is considered part of the pharmacological profile of T1AM, being MAO ubiquitously expressed (Laurino et al., 2015a,b). As a fingerprint of both T1AM and TA1, pharmacological effects is the rapid onset of their rapid onset, within 15 min from administration. Furthermore, they are active at very low doses always showing inverted bell-shaped dose-effect curves.

Laurino et al. (2015a,b, 2018a,b) demonstrated that T1AM and TA1 behavioral effects were dependent on the activation of the brain or peripheral histaminergic system, with a mechanism which, however, remains to be clarified.

T1AM is a multi-target compound (Bräunig et al., 2018) able to interact at G-protein coupled receptors, including the trace amine associated receptors, and also ion channels but not with T3 receptors. However, if we accept the trace amine associated receptors, the affinity of T1AM for such targets is much lower than its *in vivo* potency, thus making unlikely the participation of such targets in T1AM *in vivo* effects. Notwithstanding this, all the behavioral effects of T1AM (and of TA1) including the pro-learning effect, hyperalgesia and the neuroprotection were abolished by anti-histaminergic drug treatment of mice including type 1 receptor antagonists, a strategy which however does not allow to recognize the source of histamine which consists of neuronal and mast cell derived histamine. Considering T3 metabolites can pass the BBB reproducing most of the effects described for histamine, the timing of their effects, the localization of brain mast cells at the BBB, the possibility that mast cells, other than histaminergic neurons, are among the targets of T3 metabolites become a plausible hypothesis. This source of histamine would also explain the bell-shaped curves observed following T1AM (and TA1) administration where a slow re-synthesis does not allow a fast refilling of the granules. Furthermore, the link between T3 metabolites and their possible degranulating effect on mast cells might be more stringent in the case of peripheral histamine-mediated effects. In fact, T3 supplementation is one among the cause of systemic itch (Reamy et al., 2011) and pruritus is one among the clinical symptoms of hyperthyroidism (Ward and Bernhard, 2005). Similarly, T3 metabolites induce itch (Laurino et al., 2015a,b) activating, histamine-dependent, pERK in the dorsal root ganglia. This pathway is considered selective for mast cell-derived histamine-induced itch sensation (Dong and Dong, 2018; Huang et al., 2018). Even if the definitive proof is lacking, T3 metabolites, by activating mast cells, might be the mediators of T3-induced itch. Furthermore, confirming that itch and pain sensation have some common neuronal pathways, T1AM and TA1 also induce histamine-dependent hyperalgesia to thermal stimuli (Manni et al., 2013), a condition typically activating mast cells (Zhang et al., 2012).

In conclusion, the relationship between the thyroid and mast cells is scarcely studied but we strongly believe it merits to be investigated further from the clinical and mechanistic point of view. In this respect in this article, we tried to point the attention on the non-canonical portion of the thyroid secretion constituted by T3 metabolites, as possible activators of mast cells and releaser of histamine (**Figure 1**).

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All the authors participated in collecting and discussing the literature data.

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Meningeal Mast Cells as Key Effectors of Stroke Pathology

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Stroke is the leading cause of adult disability in the United States. Because post-stroke inflammation is a critical determinant of damage and recovery after stroke, understanding the interplay between the immune system and the brain after stroke holds much promise for therapeutic intervention. An understudied, but important aspect of this interplay is the role of meninges that surround the brain. All blood vessels travel through the meningeal space before entering the brain parenchyma, making the meninges ideally located to act as an immune gatekeeper for the underlying parenchyma. Emerging evidence suggests that the actions of immune cells resident in the meninges are essential for executing this gatekeeper function. Mast cells (MCs), best known as proinflammatory effector cells, are one of the long-term resident immune cells in the meninges. Here, we discuss recent findings in the literature regarding the role of MCs located in the meningeal space and stroke pathology. We review the latest advances in mouse models to investigate the roles of MCs and MC-derived products *in vivo*, and the importance of using these mouse models. We examine the concept of the meninges playing a critical role in brain and immune interactions, reevaluate the perspectives on the key effectors of stroke pathology, and discuss the opportunities and challenges for therapeutic development.

Keywords: meninges, mast cells, ischemic stroke, meningeal mast cells, stroke pathology

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INTRODUCTION

Every year, ∼800,000 people suffer from stroke in the United States (Benjamin et al., 2018). The currently available therapies for acute stroke focus on removal of the blood clot, either pharmacologically or mechanically (Fisher and Saver, 2015). With advances in careful patient selection for these therapies, the time window for therapy initiation can be extended up to 24 h (Albers et al., 2018; Nogueira et al., 2018). However, the majority of patients are still not eligible for these therapies. Ischemic stroke occurs when the blood supply to the brain is interrupted by a blood clot. This initiates a cascade of events that includes excitotoxicity, free radical release, mitochondrial changes, and various degrees of immune response that leads to neuronal and glial cell death, blood-brain barrier dysfunction and ultimately the clinical symptoms (Moskowitz et al., 2010; Knowland et al., 2014; George and Steinberg, 2015). The timing of these events differ, and additionally, the post-stroke immune response has different phases that can be detrimental or beneficial (Anrather and Iadecola, 2016). Thus, a detailed understanding of the post-stroke immune response is necessary in order to better utilize its therapeutic potential.

The brain is long considered to be an immune-privileged organ (Louveau et al., 2015a). This is in part due to its anatomical isolation from the rest of the body, having its own unique resident immune cells (microglia), and having restricted access for circulating immune cells under homeostatic conditions. This is important for homeostatic functions but becomes especially important in pathological conditions. More recently, meninges—the membranes surrounding the brain—have been proposed to play important roles in the regulation of brain-immune interactions (Rua and McGavern, 2018). Emerging evidence suggests that the actions of immune cells resident in the meninges are important for the immunoregulatory role of the meninges (Rua and McGavern, 2018). Mast cells (MCs) reside in high numbers within the meninges. These tissue-resident immune cells have proinflammatory and immunoregulatory roles (Grimbaldeston et al., 2007; Biggs et al., 2010; Galli et al., 2011; Tsai et al., 2011), and play key functions in both innate and adaptive immune responses. They act as constitutive or inducible sources of many cytokines, chemokines and proteases (Mukai et al., 2018). This makes them potential targets for therapeutic intervention, depending on their roles in the pathophysiology. Fortunately, there are several methodologies to study the role of MCs and MC-derived products *in vivo* (Galli et al., 2015).

Here, we discuss the evidence that MCs located in the meningeal space can worsen stroke pathology. We also review some of the latest advances in mouse models to investigate the roles of MCs and MC-derived products *in vivo*, and the importance of using these mouse models. We examine the concept of the meninges playing a critical role in brain and immune interactions, reevaluate perspectives on key effectors of stroke pathology, and discuss the opportunities and challenges for therapeutic development.

MENINGES AS KEY SITES FOR BRAIN IMMUNE ACCESS

Meninges are the connective tissue that surrounds the brain and spinal cord. They consist of three layers (**Figure 1**): an outer thick layer, dura mater, that is attached to the skull, an inner thin layer, pia mater, that is attached to the brain and spinal cord parenchyma, and a spider net-like structure, arachnoid mater, in between the dura and pia maters. The blood vessels and the cerebrospinal fluid exist within the space between the arachnoid mater and pia mater (subarachnoid space). The meninges have long been considered as just an anatomical barrier; however, accumulating evidence suggests that they are important for brain–immune communications in health and disease (Androdias et al., 2010; Derecki et al., 2010; Shechter et al., 2013; Kwong et al., 2017; Benakis et al., 2018). All the arteries that penetrate the brain parenchyma first travel through the subarachnoid space before entering into the brain. The venous blood then travels along the sinuses within the dura mater before exiting the skull. Moreover, the recent discovery of the central nervous system (CNS)'s lymphatic vessels within the meninges highlights their potential role in brain–immune interactions (Aspelund et al., 2015; Louveau et al., 2015b). These meningeal lymphatics have been shown to be important in controlling both neuroinflammatory events and immune cell trafficking (Louveau et al., 2018b). Additionally, direct vascular channels that traverse the meninges have been discovered recently between the bone marrow in the skull and the

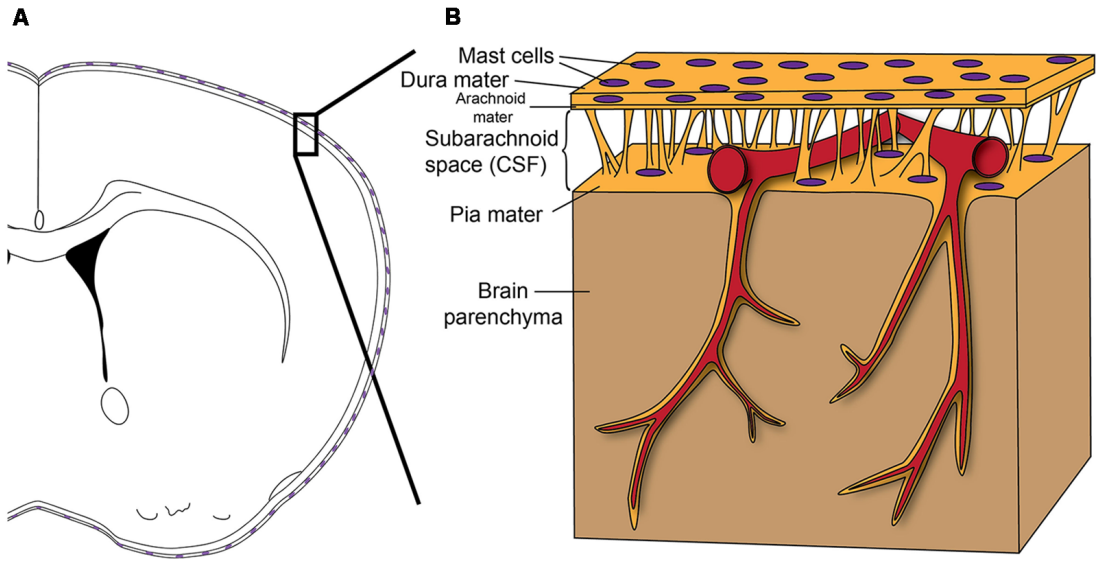

FIGURE 1 | (A) Scheme shows how the brain is enveloped by the meninges that contain Mast cells (MCs) in both the dura mater and pia mater. **(B)** Before entering the brain parenchyma, blood vessels course on the surface of the brain between the dura mater and pia mater. Therefore, as a resident immune cell in the meninges, the MC has the potential to influence blood vessels and to function as a gatekeeper to influence brain inflammation and pathology. Reprinted from Arac et al. (2014) with permission from Elsevier.

brain surface (Herisson et al., 2018). These direct vascular channels help enable myeloid cell migration into the brain parenchyma in stroke (Herisson et al., 2018). All these vascular structures (the arteries and veins, meningeal lymphatic vessels, and direct vascular channels) travel through the meninges, making the meninges ideal for a possible gatekeeper role (Rua and McGavern, 2018).

While some immune cells locate to dura mater after stroke (Benakis et al., 2018) or aseptic inflammation (Kwong et al., 2017), there are also tissue-resident immune cells normally residing in the meninges. One such immune cell type is the MC (Dimlich et al., 1991; Arac et al., 2014; **Figure 1**). MCs are tissue-resident immune cells. Mature MCs do not circulate in the blood but exist especially in tissues exposed to the exterior, such as skin, lungs, and gut (Galli et al., 2011; Tsai et al., 2011). These regions are generally critical for host defense and require strong immune regulation, as they represent the interface between the body and the external environment (Galli et al., 2008). In this respect, it is not surprising that MCs exist at high numbers in meninges as the meninges separate blood and an immune-privileged brain parenchyma (Louveau et al., 2015a). Given the meninges' potential gatekeeper role in orchestrating brain–immune interactions, meningeal MCs can play significant roles in such interactions.

CELLULAR ELEMENTS OF POST-STROKE INFLAMMATION

Inflammation is critically important in stroke pathology. Several elements of the immune system play roles at different time points after stroke. The first immune cells to respond to interruption of blood flow to the brain are the resident microglia. There is evidence that microglia show signs of activation within 30 min to 1 h after stroke (Ito et al., 2001; Clausen et al., 2008); these changes evolve during the 12 h following stroke and result in decreased microglial numbers within 24 h (Ito et al., 2001). These changes persist several weeks after stroke onset (Lambertsen et al., 2005; Perego et al., 2011). The microglia secrete several pro- and anti-inflammatory cytokines and chemokines (Clausen et al., 2008; Lambertsen et al., 2009), and can be either detrimental (Neher et al., 2013) or beneficial (Lambertsen et al., 2009; Neumann et al., 2009) through secreted factors or direct phagocytosis.

One of the first immune cells that infiltrate the brain after stroke is the neutrophil. They start infiltrating around 6 h after stroke onset and reach peak numbers at 24 h (Perez-de-Puig et al., 2015). The predominant effect of neutrophils appears to be increased ischemic injury as inhibition of their trafficking results in better outcomes (Allen et al., 2012; Jickling et al., 2015; Neumann et al., 2015). However, there is also evidence that neutrophils play role in resolution of inflammation (Cuartero et al., 2013) and promotion of remodeling (Christoffersson et al., 2012) after stroke. Thus, the role of neutrophils in stroke pathology is more complex, which may in part account for the failure of translational approaches targeting neutrophils as stroke therapy (Jickling et al., 2015).

Monocytes follow neutrophils in parenchymal infiltration after a stroke, starting to infiltrate around 6–48 h after stroke onset and staying in the brain weeks thereafter (Lambertsen et al., 2005; Perego et al., 2011). The $CCR2^{+}Ly6C^{high}$ monocytes/macrophages are thought to be involved with the acute inflammation, thus worsening the damage, whereas $CX3CR1^{+}Ly6C^{low}$ macrophages are considered as the immune cells involved in the repair process (Garcia-Bonilla et al., 2016; Tsuyama et al., 2018). However, monocyte response after stroke is very complicated. For example, the so-called inflammatory $CCR2^{+}Ly6C^{high}$ monocytes enter first, then are converted to regulatory $CX3CR1^{+}Ly6C^{low}$ macrophages (Garcia-Bonilla et al., 2016). Interestingly, efforts to target these monocytes populations have shown mixed results; reducing monocyte infiltration reduced stroke recovery in one study (Wattananit et al., 2016), while another study concluded that targeting monocyte subsets did not change the outcome after stroke (Schmidt et al., 2017).

The response of lymphocytes (T and B cells) after stroke is quite different from that of the other immune cells as they tend to infiltrate the brain at later time points (days) and stay longer (weeks to months) after stroke onset (Liesz et al., 2013a). The role of these cells depends on the subtype of lymphocytes involved, for example, T cells can worsen the injury (Yilmaz et al., 2006; Clarkson et al., 2014; Mracsko et al., 2014). More specifically, regulatory T cells have been shown to be protective *via* secretion of interleukin-10 (Liesz et al., 2009, 2013b), whereas gamma-delta-T cells can worsen the injury by secreting interleukin-17 (Shichita et al., 2009). Moreover, targeting T cell infiltration by inhibiting their trafficking into the brain parenchyma protected the brain against deleterious neuroinflammation (Liesz et al., 2011b). However, amplifying regulatory T cells with a CD28 superagonist in preclinical studies have elicited conflicting outcomes, and resulted in reduced brain damage in one study (Na et al., 2015), vs. increased brain damage in another (Schuhmann et al., 2015). Moreover, treatment with another immunomodulatory drug, FTY720, despite reducing the post-stroke lymphocyte infiltration after stroke did not improve the post-stroke outcomes (Liesz et al., 2011a). The role of B cells in stroke pathology is also very unclear. Although in some studies B cells did not have any direct effect on the stroke pathology (Yilmaz et al., 2006; Schuhmann et al., 2017), in others they were shown to be beneficial (Ren et al., 2011; Chen et al., 2012). However, B cells were also shown to have delayed deleterious effects such as cognitive impairment after stroke (Doyle et al., 2015).

MCs have been proposed to play important roles in stroke pathology. Specifically, cerebral MCs were shown to worsen the brain swelling and neutrophil accumulation after stroke (Strbian et al., 2006) and stroke induced the degranulation of brain parenchymal MCs (Biran et al., 2008; Jin et al., 2009; Lindsberg et al., 2010). Moreover, a MC stabilizer in rats, cromolyn, was shown to be protective in stroke (Strbian et al., 2007; Jin et al., 2009). Cerebral MCs were also shown to mediate blood-brain barrier disruption after stroke (Mattila et al., 2011; McKittrick et al., 2015). As opposed to these studies,

which proposed a role for cerebral MCs in worsening stroke pathology, another study (reviewed in detail below), proposed that meningeal, rather than cerebral, MCs play important roles in exacerbating stroke pathology, in part by secreting IL-6 (Arac et al., 2014).

Overall, despite being a major participant in stroke pathology, inflammatory responses after stroke are complex, and attempts should be made to understand their detailed pathophysiological mechanisms before attempting therapeutic interventions.

In vivo MODELS TO STUDY MC FUNCTION ("MC KNOCK-IN MICE")

MCs are tissue-resident immune cells derived from bone marrow. Small numbers of MC progenitors exist in the blood, but they complete their differentiation and maturation in tissue microenvironments (Galli et al., 2008, 2011). MCs can be activated by diverse mechanisms including *via* binding of antigen to antigen-specific IgE (Galli and Tsai, 2012), as well as by physical agents, innate danger signals (Supajatura et al., 2002), venoms (Metz et al., 2006), complement activation (Schäfer et al., 2013), and exposure to certain chemokines and cytokines. Upon activation, MCs can secrete either stored mediators (such as histamine and heparin) or *de novo* synthesized cytokines, chemokines, and growth factors (Mukai et al., 2018). These MC-derived products have been shown to have positive or negative effects on inflammation (Galli et al., 2008). However, many of these MC-derived products are also produced by a variety of other immune cells.

In order to identify a role for MCs in different biological settings, one can ablate MCs selectively (either by a drug or antibody, or genetically) to support their necessity, and then replace their function selectively to support claims of sufficiency. Pharmacological approaches such as MC stabilizers (cromolyn) or activators (c48/80) have commonly been used to infer a role for MCs. However, both of these drugs can have MC-independent effects on other immune cells (Arumugam et al., 2006; Oka et al., 2012; Schemann et al., 2012). There are also other approaches using recombinant MC proteases, such as tryptase, chymase, and tyrosine kinase inhibitors. However, all of these approaches have potential off-target effects limiting the interpretation of the results (Galli et al., 2015).

As these approaches lack true specificity for MCs, genetic approaches represent a more definitive way to assess the functions of MCs *in vivo*. Two commonly used genetically MC-deficient mice are the $Kit^{W/W\text{-}v}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice. These mice have different types of mutations affecting the c-*kit* gene that result in a profound deficiency of MCs and melanocytes in both mice. However, both WBB6F$_1$-$Kit^{W/W\text{-}v}$ and C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ also have several other abnormalities within and outside the immune system, including effects on hematopoietic cells other than MCs (Galli et al., 2015). Thus, the differences in the biological responses in these $Kit^{W/W\text{-}v}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice compared with wild type (WT) mice may, in principle, reflect any of the abnormalities in these mice and are not necessarily due to their MC deficiency. However, the MC deficiency in these mice can be selectively "repaired" by the adoptive transfer of *in vitro*-derived WT or mutant MCs (Nakano et al., 1985; Galli et al., 2005; Grimbaldeston et al., 2005). These *in vitro*-grown, bone marrow-derived, cultured MCs (BMCMCs) can be administered systemically or locally to create the so-called "MC knock-in mice" (Galli et al., 2015). These engrafted BMCMCs were shown to survive, and function normally, up to 18 weeks in lungs (Yu et al., 2006), 12 weeks in the skin (Biggs et al., 2010), and 10 weeks in the meninges (Arac et al., 2014). Moreover, the numbers of MCs in the MC knock-in mice are generally equivalent to those of the WT animals (Yu et al., 2006; Biggs et al., 2010; Arac et al., 2014). MC knock-in mice have been widely used to assess the roles of MCs in several biological events *in vivo*.

Despite the power of MC knock-in mice, these mice still carry potential problems inherent to c-*kit* related abnormalities (and other abnormalities in the $Kit^{W\text{-}sh/W\text{-}sh}$ mice) in the mutant mice. To overcome this issue, MC-deficient mice with normal c-*kit* function were developed by using MC-specific Cre recombinase approach (Dudeck et al., 2011; Feyerabend et al., 2011; Lilla et al., 2011). Such mice profoundly lack MCs (and to a lesser extent, basophils), but have normal c-*kit* function and lack the known abnormalities that the c-*kit* mutant mice have. Moreover, inducible models of MC deficiency were also developed by using the MC-specific Cre recombinase and inducible diphtheria toxin receptor expression (Dudeck et al., 2011; Otsuka et al., 2011; Reber et al., 2014). Injection of diphtheria toxin locally to the site of interest results in profound depletion of local MCs in these mice.

Given all these *in vivo* models to study MC function (with different pros and cons), one recommended approach has been to use more than one of these models to assess the initial biological responses, and if there are consistent results between the models, then proceeding to further studies (Galli et al., 2015). For additional discussion of these models, we recommend reading this review (Galli et al., 2015).

MENINGEAL MCs EXACERBATE STROKE PATHOLOGY

We utilized some of these various *in vivo* models in order to assess the role of MCs in stroke pathology (Arac et al., 2014). WBB6F$_1$-$Kit^{W/W\text{-}v}$ mice had significantly smaller infarcts and less brain swelling compared to WT controls at 3 and 14 days after stroke. Systemic engraftment of WT MCs in these c-*kit* mutant mice resulted in the same extent of injury as the WT mice. In parallel to these results, the MC-deficient *Cpa3-Cre; Mc1–1$^{fl/fl}$* mice (Lilla et al., 2011; MC-deficient mice with normal c-*kit* function) also had a smaller extent of stroke pathology when compared to their corresponding WT counterparts. Both mouse models also showed a similar MC-dependent pattern for myeloid, but not lymphoid, cell numbers in the brain at 3 days after stroke. Together, these data from two different types of *in vivo* mouse models of genetically-determined MC deficiency provide strong support that MCs play an important role in worsening the brain injury after stroke.

Because the MC deficiencies in both of these models are in all examined tissues, it is hard to discern which population of MCs might be critical for this effect. Thus, we compared

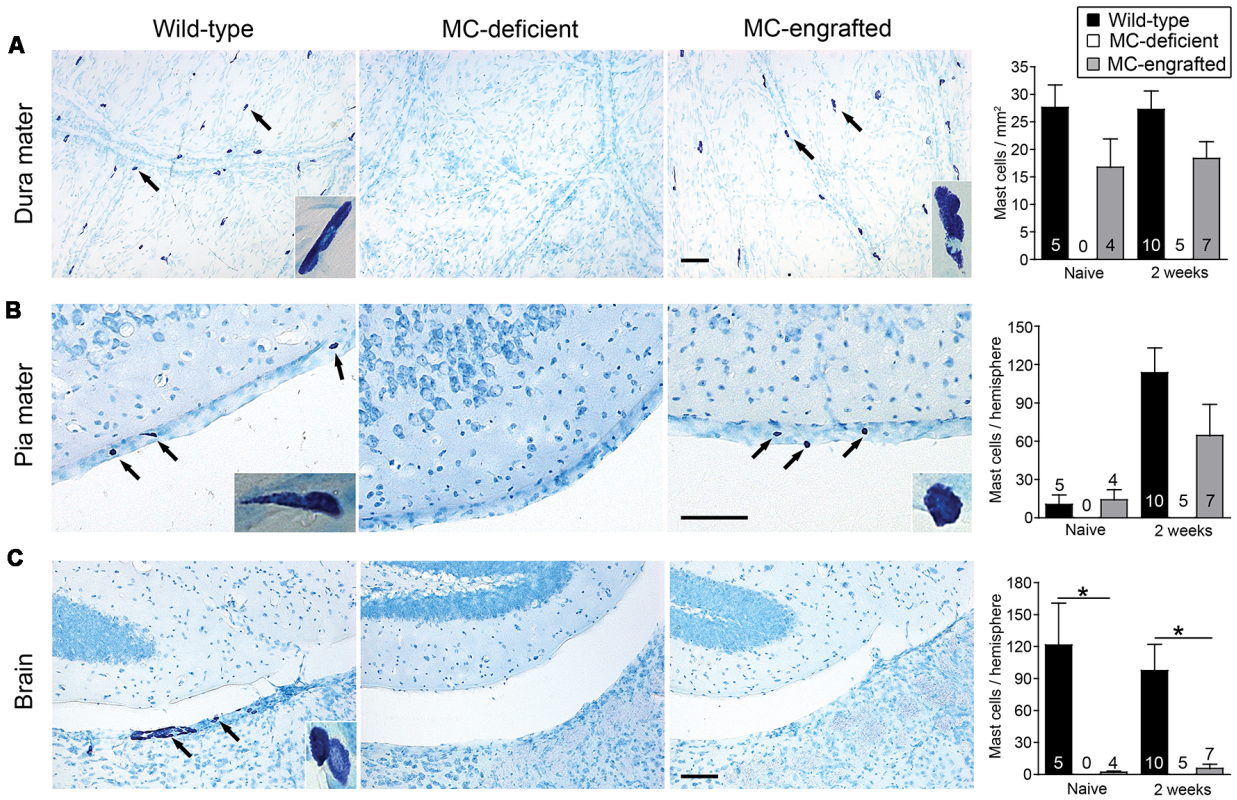

FIGURE 2 | Location of MCs in the central nervous system (CNS). Representative toluidine blue-stained images of and quantification of MCs in dura mater **(A)**, pia mater **(B)**, and brain parenchyma **(C)** of MC-deficient WBB6F1-*Kit*$^{W/W\text{-}v}$ mice and the corresponding wild type (WT) mice and MC-engrafted *Kit*$^{W/W\text{-}v}$ mice. MCs stain purple with toluidine blue; arrows indicate representative MCs. Insets show magnified images of MCs in each tissue. Data are expressed as means+SEM. The number of mice in each group is indicated in (or over) each bar. *$P < 0.05$. Scale bars: 100 mm **(A–C)**. Reprinted from Arac et al. (2014) with permission from Elsevier.

the number of CNS MCs in the MC-engrafted WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice to those of WT (WBB6F$_1$-*Kit*$^{+/+}$) mice. Both WT mice and MC-engrafted mice had similar numbers of MCs in the dura and pia mater both before and 2 weeks after stroke (**Figures 2A,B**). In contrast, the brain parenchyma of the MC-engrafted mice had either no or substantially fewer MCs (**Figure 2C**). These data strongly suggest that brain parenchymal MCs are not responsible for the MC-dependent worsening of the stroke pathology observed in this mouse model. It instead suggests a potential role for meningeal MCs in modulating this response. Moreover, the density of MCs in the dura mater of both WT and MC-engrafted mice (15–27 cells/mm^2) is similar to that in humans (Varatharaj et al., 2012; 11–23 cells/mm^2). In order to calculate these dural MC densities, we used dura mater whole mount preparations (**Figure 2A**). This whole mount preparation of dura mater was also later used to identify the meningeal lymphatics (Louveau et al., 2015b, 2018a).

To test whether the meningeal MCs are sufficient in modulating the MC-dependent stroke pathology, we engrafted BMCMCs locally into the meninges. With a modified intracranial injection method, the MCs engraft only in the meninges and the number of meningeal MCs in MC-engrafted animals are similar to those of the WT mice (Sayed et al., 2010; Arac et al., 2014). After meningeal engraftment of MCs, MC-engrafted mice developed significantly worse injury after stroke (Arac et al., 2014). This provides strong evidence that the meningeal MCs are sufficient to elicit the MC-dependent effects in stroke pathology. Moreover, we found by microarray analysis of the dura of MC-deficient WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice, the corresponding WT (WBB6F$_1$-*Kit*$^{+/+}$) mice, and WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice which had been engrafted in the meninges with WT BMCMCs (unpublished data), that the meninges are a site of inflammation-related activity after stroke and that the meningeal inflammatory gene response to stroke is modulated, at least in part, by MCs (**Figure 3A**). We found that many stroke-activated genes in the meninges are involved in the regulation of inflammatory and immune system processes. These include cytokines, chemokines, cell adhesion molecules, immune signaling receptors and extracellular matrix remodeling molecules, all of which are known to contribute to leukocyte migration and activation (**Figure 3B**). Notably, we found that many of the stroke-induced gene changes involved in inflammation were either absent or exhibited a smaller response in MC-deficient mice, but were recapitulated when MCs were engrafted

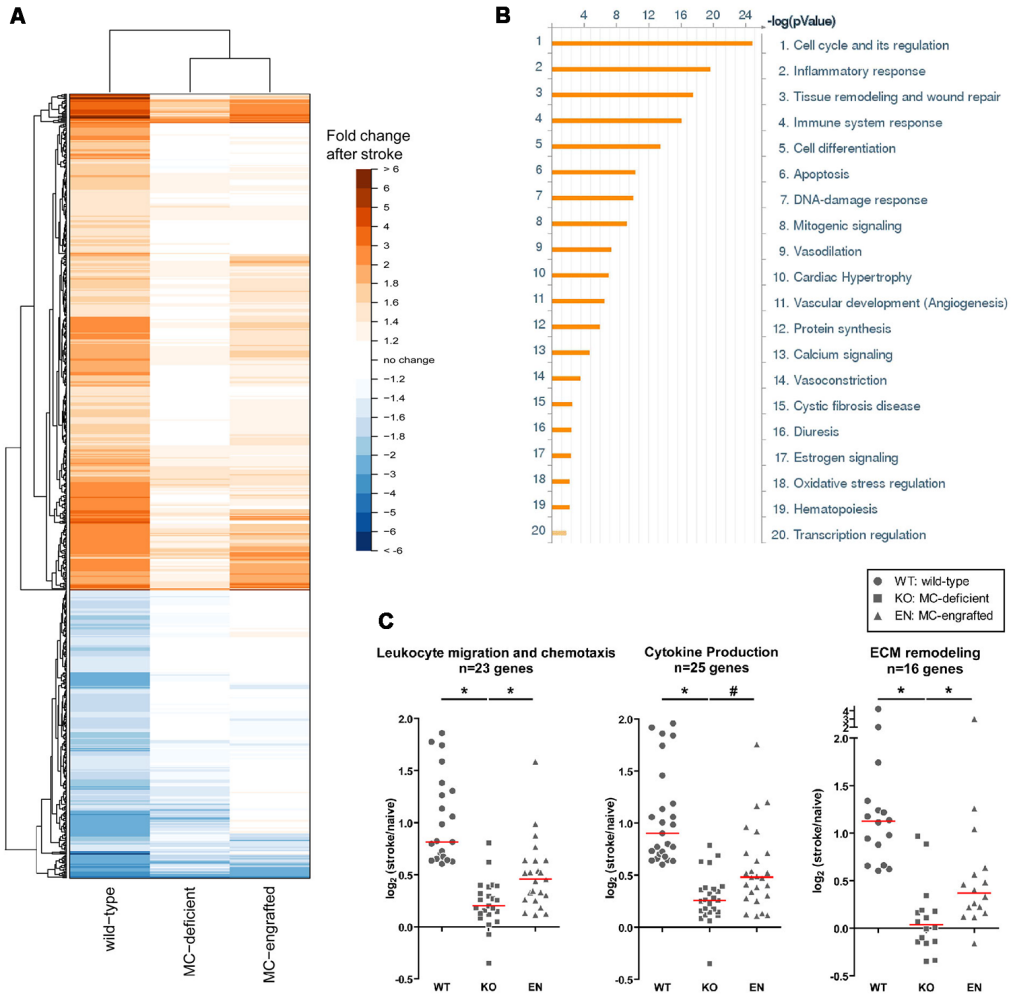

FIGURE 3 | Meningeal gene expression changes after stroke. **(A)** Heatmap showing the fold-change in expression of genes significantly changed ($p < 0.05$) after stroke in WT (WBB6F$_1$-*Kit*$^{+/+}$) mice with the corresponding change in expression in MC-deficient (WBB6F$_1$-*Kit*$^{W/W\text{-}v}$) and MC-engrafted [WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice which had been engrafted in the meninges with WT bone marrow-derived, cultured MCs (BMCMCs)] mice (irrespective of whether the gene changes in these two groups after stroke reach significance). The genes represented are those with an absolute fold-change after stroke of ≥1.5 in WT mice. **(B)** Pathway map of the functional grouping of genes that were significantly upregulated after stroke in WT mice. **(C)** Dot plots showing the $\log_2$ fold-change in expression after stroke of genes within the indicated gene ontology groups in each of three mouse groups. The genes represented in each gene ontology group were significantly upregulated after stroke in WT mice, with a fold-change of ≥1.5. Each data point within a mouse group represents a different gene. Red line indicates median. $^*p < 0.05$; $^{\#}p = 0.05$.

into these mice, consistent with the idea that MCs can modulate the meningeal inflammatory gene changes after stroke (**Figure 3C**).

In order to identify the MC-derived products that influence stroke pathology, BMCMCs derived from two candidate factor-deficient mice (IL-6 and CCL7) were injected into the meninges of MC-deficient mice (Arac et al., 2014). We found that mice meningeally engrafted with BMCMCs which lack IL-6 failed to demonstrate MC-dependent responses in stroke pathology compared to the WT BMCMC engrafted mice. By contrast, MC-derived CCL7 had less of an effect on stroke pathology (Arac et al., 2014). This demonstrates how meningeal MC-derived IL-6 can, in part, explain the MC-dependent effects in stroke pathology.

There are several questions that require further studies. For example, how does the meningeal MC-derived IL-6 execute its function to exacerbate stroke pathology? What downstream pathways are involved in this biological response? Does it alter the meningeal access of immune cells to the brain? What are its effects on brain meningeal lymphatics? Likewise, what are the mechanisms involved in stimulating the meningeal MCs to release IL-6 after a stroke? Are there other MC-derived factors that also might be involved in worsening the stroke pathology? Contrary to these, could anti-coagulative effects of MC-derived heparin proteoglycan and/or proteases have effects in dissolving the blood clot in ischemic stroke? Moreover, there could also be other, yet to be identified, meningeal MC-derived products that are important in the

repair process after stroke. Thus, one should be cautious in directly targeting MCs as a therapeutic for stroke (Ocak et al., 2018). These and many other questions need to be investigated to better understand the role of meningeal MCs in stroke pathology.

OPPORTUNITIES AND CHALLENGES FOR FUTURE

Immune responses after stroke are complex with many elements involved, and it is not the focus of this article to comprehensively review that literature; interested readers can find up-to-date review articles on this topic (McCombe and Read, 2008; Iadecola and Anrather, 2011; Macrez et al., 2011; Lambertsen et al., 2018; Rayasam et al., 2018). The post-stroke immune response potentially holds very promising targets for therapeutic interventions for stroke (Arac et al., 2011; Macrez et al., 2011). However, these post-stroke immune events have not been well characterized, with many of the immune responses potentially having dual roles. Development of new and effective therapeutics for stroke will require detailed, mechanistic studies before consideration of specific approaches for therapeutic intervention. Here, we have discussed recent advances in post-stroke immune response with a particular focus on the role of meningeal MCs. Given the increasing importance and key roles of meninges in the regulation of brain–immune interactions (Rua and McGavern, 2018), meningeal MCs are ideally located to play potentially key roles in these interactions. As MCs have diverse roles in many immune responses (Galli et al., 2005, 2008), their key location in the meninges makes them potentially important players in the regulation of immune responses in the brain during health and disease.

Identifying the roles of several immune elements in stroke pathology is critical for the development of effective therapeutics. Fortunately, there are many advanced immunological tools to perform mechanistic studies. Without such detailed characterization of post-stroke immune responses, performing clinical tests of potential targets will likely result in failures, and may decrease the enthusiasm for future studies.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AA wrote the initial draft of the review. All authors contributed to revising the manuscript, reading and approving the submitted version.

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frontiers in Cellular Neuroscience

REVIEW
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Shared Fate of Meningeal Mast Cells and Sensory Neurons in Migraine

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Migraine is a primary headache disorder which has complex neurogenic pathophysiological mechanisms still requiring full elucidation. The sensory nerves and meningeal mast cell couplings in the migraine target tissue are very effective interfaces between the central nervous system and the immune system. These couplings fall into three categories: intimacy, cross-talk and a shared fate. Acting as the immediate call-center of the neuroimmune system, mast cells play fundamental roles in migraine pathophysiology. Considerable evidence shows that neuroinflammation in the meninges is the key element resulting in the sensitization of trigeminal nociceptors. The successive events such as neuropeptide release, vasodilation, plasma protein extravasation, and mast cell degranulation that form the basic characteristics of the inflammation are believed to occur in this persistent pain state. In this regard, mast cells and sensory neurons represent both the target and source of the neuropeptides that play autocrine, paracrine, and neuro-endocrine roles during this inflammatory process. This review intends to contribute to a better understanding of the meningeal mast cell and sensory neuron bi-directional interactions from molecular, cellular, functional points of view. Considering the fact that mast cells play a *sine qua non* role in expanding the opportunities for targeted new migraine therapies, it is of crucial importance to explore these multi-faceted interactions.

Keywords: migraine, neuroinflammation, mast cells, sensory neurons, ATP, CGRP, PACAP, autonomic nervous system

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INTRODUCTION

Migraine headache has been known for 6,000 years but has not been completely cured yet. Human descriptions of migraine headache date from the earliest recorded history of man, in Mesopotamia, the cradle of civilization in 4000 B.C. In the 5th century, Hippocrates, the Father of Medicine, described in detail a headache that would be called migraine with aura. The Turkish philosopher physician Ibn-i Sina (930–1,037) also known as Avicenna, in his book The Canon of Medical Sciences, asserted that food and sounds provoked pain and that the patient could not tolerate light and should be rest alone in the darkness. This is still valid today for migraineurs (Daniel, 2010).

Today, migraine is listed in the top 20 conditions by the World Health Organization (WHO); a debilitating neurological condition with a high prevalence approximately 6%–8% of men and 15%–25% of women in western countries (Pietrobon and Striessnig, 2003). The Headache Classification Committee working under the International Headache Society, complete the following diagnostic criteria for migraine: episodic headache lasting from 4 to 72 h,

concomitant with two of the following, throbbing, unilateral pain, aggravation on movement, or pain of at least moderate severity, and at least one of the following, nausea or vomiting, or photophobia and phonophobia (Headache Classification Subcommittee of the International Headache Society [IHS], 2004). Migraine is a condition that significantly impairs patients' quality of daily life. The Global Burden of Disease Study ranked migraine as the seventh most common disabling pathology among 289 diseases, referred to as the 7th disabler (Wöber-Bingöl, 2013; Malone et al., 2015). Migraine has a co-morbidity in a number diseases which is mostly associated with mast cells (Graziottin et al., 2014; Xu and Chen, 2015; Eller-Smith et al., 2018; Graif et al., 2018).

To understand the pathophysiology of migraine, several important steps have been taken in the last decades. Today, it is known that the aura is caused by the activation of a neurophysiological phenomenon called cortical spreading depression (CSD) and the trigeminovascular system (Boran and Bolay, 2013; Alstadhaug, 2014). The mechanisms for triggering and ending the attacks are still largely mysterious.

In this review article, we aimed to compile the information about the migraine pathophysiology and the use of this data in efforts that have been made to find the best therapeutical options. In this regard, we focused on neurogenic inflammation which is crucial in the pathophysiology of migraine, mast cells, sensory neuronal axis and bi-directional interactions of these from molecular, cellular, functional and clinical points of views.

MAST CELLS

A medical student Paul Ehrlich (1878), coined the name "mastzellen" stemming from the Greek word mastos (μασδός), meaning breast (Crivellato et al., 2003). Today, we know that they originate from pluripotential hematopoietic cells in bone marrow and they are deeply involved in the trophism of tissues (Tore and Tuncel, 2009). Mast cells (MCs) are tissue-resident granulocyte that originate from CD34+/CD117+ and circulate in the blood during their immature stage (Tore and Tuncel, 2011; Theoharides et al., 2012). Stem cell growth factor (SCF) and other cytokines (Interleukin 3, 4, 9) help the maturation of MCs in the tissue (Varatharaj et al., 2012). MCs lodge in all vascularized tissues (3,000–25,000 mast cells/mm^3), such as intestines, respiratory tract and skin as well as in the dura mater (Galli and Tsai, 2008; Theoharides et al., 2012).

Their vicinity enables MCs to be an "immediate call center" for exposure to pathogens and allergens. When activated, MCs degranulate and release mediators. They participate in tissue repair and interact with other immune cells (Galli and Tsai, 2008; Theoharides et al., 2012; Graziottin et al., 2014).

MCs are commonly round or elongated in shape, with a diameter of approximately 10–20 μm. They do not represent a homogeneous population. The recruitment and differentiation of MCs is a complex process in which tissue-specific microenvironmental factors such as cell types and cytokines, neuropeptides available around them are highly involved. Such cytokines and neuropeptides work through many up-and-down regulating mechanisms and are crucial in the recruitment, differentiation, maturation and even apoptosis of mast cells. The effect of a cytokine or neuropeptide on undifferentiated or mature mast cells might be different (Tore and Tuncel, 2011; Aich et al., 2015; Gupta and Harvima, 2018). MCs have proven to have fundamental differences in size, staining, sensitivity to stimuli/drugs, species, and function. The findings on these enigmatic cells are difficult to interpret.

Classical MC mediators are proteases (e.g., tryptase, chymase, etc.), bio-organic amines (e.g., histamine and serotonin), proteoglycans (e.g., heparin, etc.), many cytokines [e.g., tumor necrosis factor-α (TNF-α) nitric oxide (NO), prostaglandins, leukotrienes and kinins; Nilsson et al., 1999; Theoharides and Kalogeromitros, 2006; Gri et al., 2012; Sismanopoulos et al., 2012], neuropeptides [e.g., corticotropin-releasing factor, endorphins, somatostatin, substance P (SP), vasoactive intestinal peptide, Pituitary adenylate cyclase activating polypeptide (PACAP); Metcalfe et al., 1997; Luger and Lotti, 1998; Kempuraj et al., 2004; Theoharides and Kalogeromitros, 2006; Hildebrand et al., 2008; Lennerz et al., 2008; Tore and Tuncel, 2009; Okragly et al., 2018] as well as growth factors [e.g., transforming growth factor, vascular endothelial growth factor, granulocyte-monocyte colony stimulating factor, nerve growth factor (NGF); Metcalfe et al., 1997; Tore and Tuncel, 2009]. Each mast cell mediator function in a number of ways, and they overlap in their effects in the body. These mediators modify mast cell behaviors *via* autocrine effects, modulate neighboring nerves, endothelia and vessel smooth muscle functions *via* paracrine effects and regulate remote organ functions *via* endocrine effects (**Figure 1**).

Meningeal MCs and sensory nerve relation consist of proximity, communication and a shared fate. Proven not to be a random configuration, mast cell–nerve membrane-to-membrane contacts are highly common and in some cases, they even exchange granules (Rozniecki et al., 1999; Tore and Tuncel, 2011). This is likely to be by fate rather than an accident since such spatial distributions mostly indicate a functional relationship. MCs sometimes release their contents without degranulation; they also transport extracellular vesicles (30–150 nm) which contain several mediators, including micro RNAs and major histocompatibility complex-II, to the neighboring cells (Gupta and Harvima, 2018). MCs settle close to nerve fibers, which makes them strong candidates for modulating neural activity and nociception. Increased MC counts in proximity to the neural system (Barbara et al., 2004) and NGF are related with nerve fiber structure, which is responsible for hyperalgesia (Watson et al., 2008). MC degranulation also contributes to hyperalgesia in experimental settings (Vincent et al., 2013).

Sicuteri, in 1963 was one of the first to suggest a role for MCs in migraine pathophysiology by MC degranulating agent, compound 48/80 induced headache (Sicuteri, 1963). Plasma histamine, 5-hydroxy tryptamine (5-HT) and urinary histamine and tryptase levels are elevated during migraine attacks (Heatley et al., 1982; Ferrari et al., 1989; Olness et al., 1999). Histamine infusion also promotes a migraine-like headache (Lassen et al., 1995). MCs are not the only source for histamine in brain; specifically 90% of thalamic histamine and up to 50% of total brain histamine are produced by MCs

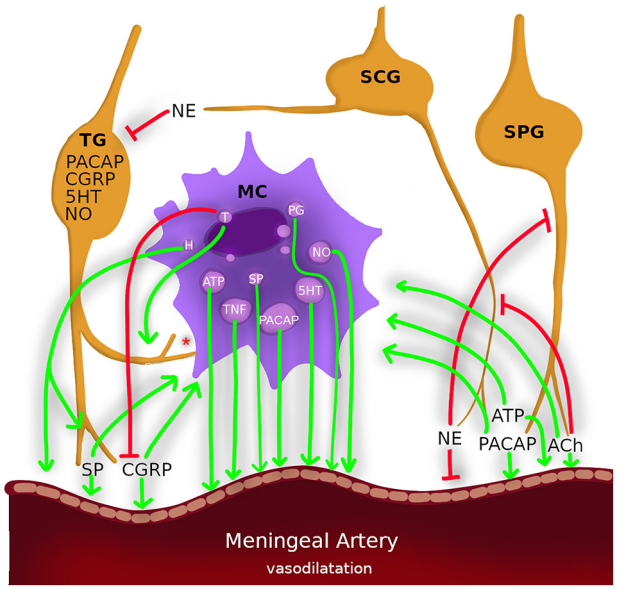

FIGURE 1 | Nerves, mast cells and meningeal arteries constitute a triangle of paracrine/autocrine/endocrine interactions which involve the common fate in neurogenic inflammation in migraine. Nerves: trigeminal ganglion (TG), superior cervical ganglion (SCG), sphenopalatine ganglion (SPG). Neurotransmitters: calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase activating polypeptide (PACAP), acetylcholine (ACh) and adenosine triphosphate (ATP) and substance P (SP) degranulate mast cells and causes vasodilatation (arrows). Mast cell mediators: histamine (H), nitric oxide (NO), and tumor necrosis factor-α (TNF-α) sensitizes sensory neurons and causes vasodilatation (arrows). Tryptase (T) also sensitizes sensory neurons (arrows) also cleavages CGRP (blinded lines). Norepinephrine (NE) inhibits trigeminal activation also causes vasoconstriction. *Mast cell have membrane-membrane contacts with sensory neurons.

in rats (Dong et al., 2014). Histamine receptors (H1, H2, H3, and H4) are expressed in microglias, astrocytes, sensory neurons, smooth muscle cells of vessels, and also in MCs itself. These receptors mediate or prevent degranulation of MCs in certain conditions in related venues (Rosa and Fantozzi, 2013; Alstadhaug, 2014). Cromolyn and antihistaminics were used for migraine prophylaxis (Rossi et al., 2003). But only a small proportion of migraineurs have benefited from them, possibly due to the variety of mast cell responses and mast cell mediator contents. As illustrated in **Figure 1**, not only histamine but also many mast cell mediators are involved, directly or indirectly, in the activation of meningeal nociceptors (Levy et al., 2007; Zhang et al., 2007; Zhang and Levy, 2008; Rosa and Fantozzi, 2013). Recently Kilinc et al. (2017). showed that compound 48/80 induced persistent nociceptive firing in the trigeminal nerve endings was blocked by 5-HT3 receptor antagonist.

NEUROGENIC INFLAMMATION

In recent decades, emerging data from animal and human research brought the integrated theory which implicates vascular and neural components. In particular, the activation of the meningeal afferent neurons, neuropeptide release, and neurogenic inflammation play key and complex roles in migraine headache (Buzzi and Moskowitz, 2005; Peroutka, 2005; Burgos-Vega et al., 2015). This concept has been supported by the large amount of experimental evidence accumulated. The studies were mainly performed by the activation of primary afferent neurons, either in disease or disease mimicking condition or experimentally with electrical stimulus, or by the activation of polymodal nociceptive receptors expressed on the peripheral nerve terminal that causes the release of proinflammatory neuropeptides. In this framework, these neuropeptide mediators interact with endothelial cells, mast cells, immune cells, and vascular smooth muscle cells, initiating a cascade of inflammatory responses (Geppetti et al., 2012).

However, there is not a full consensus on the pathophysiology of migraine, though it has been agreed that this disorder can be said mainly to result from the activation and sensitization of the trigeminovascular system (Goadsby et al., 2017a,b). Activated trigeminal nociceptive afferents release calcitonin gene-related peptide (CGRP) and SP, which subsequently cause sterile neurogenic inflammation in the meninges. Neurogenic inflammation is characterized by vasodilatation of meningeal vessels, increased vascular permeability, plasma protein extravasation, and mast cell degranulation (Boran and Bolay, 2013; Erdener and Dalkara, 2014). Although CGRP or some other vasodilatators of meningeal arteries do not induce nociceptive activation, vasodilatation of extracranial vessels can activate nociceptive afferents (Levy et al., 2005; Shevel, 2011). It has been proposed that the inflammatory mediators further activate meningeal nociceptors and induce peripheral and central sensitization (Levy, 2012).

Under experimental conditions, neurogenic inflammation can be induced by inflammatory agents applied topically to the dura mater. An inflammatory cocktail containing histamine, serotonin, bradykinin, and prostaglandin E2, was used to elucidate migraine pathophysiology and to predict the effectiveness of the treatments that have been developed for migraine (Zhang and Levy, 2008; Yan et al., 2018). A functional magnetic resonance imaging study showed that dural application of the inflammatory cocktail in awake rats demonstrated similar responses to migraine patients (Becerra et al., 2017). Meningeal inflammation arises as a result of CSD. During CSD, mediators, such as potassium-ions and glutamate are released and can cause the activation of nociceptors on meningeal sensory neurons and mast cells (Waeber and Moskowitz, 2005; Bogdanov et al., 2011; Karatas et al., 2013; Pietrobon and Moskowitz, 2013). This framework suggests a cross-talk of the actors such as MCs and neurons in meningeal inflammation. Ramachandran has recently outlined the mechanistic hypothesis of neurogenic inflammation in the dura mater (Ramachandran et al., 2014). He proposed that CSD or stress factors lead events along two separate paths. Either the trigeminal system is activated and neuropeptides are released, or the MCs are degranulated and sensitize the nociceptors. In both cases, neuropeptides such as CGRP and many MC mediators induce meningeal vasodilatation (**Figure 1**). In neurogenic inflammation, where MCs, sensory nerves and blood vessels form a multifaceted triangle (**Figure 1**).

MC releases algogenic and vasoactive mediators, which activate sensory nerve fibers and cause vasodilatation *via* paracrine, autocrine and neuroendocrine interactions (Aich et al., 2015; Tore and Tuncel, 2011; Theoharides et al., 2012; Gupta and Harvima, 2018). Then, nerve fibers release inflammatory and vasoactive neuropeptides; they activate mast cells which results in a vicious cycle of mast cell and nociceptor activation leading to neurogenic inflammation and pain. Like a chicken and egg situation, we still do not know which comes first: nociceptor activation or mast cell activation. Is there a key molecule that activates both of them at the same time? This situation plays an important role not only in the pathogenesis of migraine but also of numerous mast cell-associated diseases including asthma, fibromyalgia, eczema, psoriasis, interstitial cystitis, liver fibrosis, inflammatory bowel diseases, colitis, periodontitis, and arthritis (Tore and Tuncel, 2011; Theoharides et al., 2012). Surprisingly, the literature on the etiopathogenesis of these diseases discuss the same cells, the same receptors, and the same clinical management recommendations; however, there is a gap in the correlation of all this data concerning specific aspects of MCs involvement.

FROM FUNCTIONAL CROSS-TALK TOWARD THERAPEUTICAL TARGET

Autonomic Nervous System, the Oldest

MCs are found mostly in cranial dura mater which is innervated densely by both autonomic and sensory nerves. Autonomic parasympathetic nerves originate from the sphenopalatine and otic ganglia, whereas sympathetic nerves originate from the superior cervical ganglion (SCG). These neurons express PACAP, NO, vasoactive intestinal polypeptide (VIP), norepinephrine (NE), acetylcholine (ACh), and neuropeptide Y (NPY; Artico and Cavallotti, 2001; Goadsby, 2013; Levy et al., 2018). It has been long suspected that the autonomic nervous system might play a role in the pathophysiology of migraine (Peroutka, 2004; Alstadhaug, 2009; Goadsby, 2013). An autonomic dysfunction has been repeatedly described in headache sufferers. ACh was the first discovered neurotransmitter and the first postulated neurotransmitter in migraine pathophysiology because of its vasodilatory and pain-inducing effects. Almost a hundred years ago, for the first time, Kunkle showed increased ACh levels in the cerebrospinal fluid (CSF) of migraine patients (Kunkle, 1959). Later, this approach was displaced from the attention of the researchers by the rise of the serotonin theory. Recently, some studies have recalled attention to the parasympathetic system in migraine. Parasympathetic contributions to the peripheral and central sensitization during migraine have been reported (Yarnitsky et al., 2003). CSD induces trigeminal and parasympathetic activation (Bolay et al., 2002). Giniatullin group showed that ACh, carbachol and nicotine significantly increased nociceptive firing in the peripheral terminals of the meningeal trigeminal nerves (Schytz, 2010; Mikhailov et al., 2016; Shelukhina et al., 2017). Carbachol, but not nicotine, induced massive degranulation of meningeal mast cells. In a clinical study report, sphenopalatine ganglion blockage prevented migraine attacks (Binfalah et al., 2018).

The sympathetic nervous system inhibits the trigeminal system (Peroutka, 2004). Superior cervical ganglionectomy increased dura mater NO levels, c-fos expression in the spinal trigeminal nucleus caudalis (TNc) and induced degranulation of meningeal mast cells (Tore et al., 2010; Kilinc et al., 2015). Yildiz et al. (2007) measured a facial sympathetic skin response that indicated the activation of the sympathetic nervous system. They found a sympathetic hypofunction on the symptomatic side in attack and interictal periods and contrary in the post-attack period in migraineurs (Yildiz et al., 2008). Sympathetic hypofunction might lead to an increase in parasympathetic activation, or vice versa, because of reciprocal innervations of the autonomic nervous system in many organs. Recently, the meta-analysis showed a major catecholamine metabolite homovanillic acid (HVA) increase in CSF obtained from migraineurs compared to controls (van Dongen et al., 2017). While the roles of serotonin and CGRP in migraine treatment are popularly considered, the involvement of the autonomic nervous system has been ignored in clinical trials. However, we suggest that it should be kept in mind as a new therapeutic approach.

CGRP, the Most Popular

CGRP is a 37-amino acid neuropeptide neurotransmitter that was first identified in 1982 (Amara et al., 1982). CGRP and its receptors are found in all organs, especially sensory neurons. CGRP is highly expressed in the central terminals of the trigeminal nerve and the trigeminal ganglion (TG) where CGRP is often coreleased with SP. CGRP is multifunctional peptide mediating pain as well as a growth factor for primitive cells, Schwann cells and endothelial cells (Lennerz et al., 2008; Recober and Russo, 2009; Messlinger, 2018).

Previously, the effects of CGRP on the cardiovascular system were studied intensively. CGRP is known to regulate cardiac excitability, microvascular permeability, vascular smooth muscle tone, and angiogenesis. CGRP is a potent dilator of cerebral and dural vessels (Brain and Grant, 2004). A meta-analysis showed increased concentrations of CGRP in the CSF and blood of migraineurs. The plasma CGRP level is proposed as a biochemical biomarker for migraine (Cernuda-Morollón et al., 2013). Infusion of CGRP can trigger a migraine attack (van Dongen et al., 2017). Stimulation of human TG increases CGRP levels in cranial circulation (Goadsby et al., 1988). Additionally, CGRP can also cause degranulation and subsequent release of inflammatory mediators from meningeal mast cells, whereas MC mediator histamine does not induce CGRP release in meningeal sensory afferents (Theoharides et al., 2005; Russo and Dickerson, 2006; Schwenger et al., 2007; Lennerz et al., 2008). There is a complex bidirectional relationship between MCs and CGRP. Mast cell tryptase not only activates proteinase-activated receptor 2 (PAR2) in trigeminal nociceptive afferent nerves which results in the release of NO, SP and CGRP but also cleavages CGRP (Zhang and Levy, 2008; Dux et al., 2009; Tore et al., 2010). PAR2 and transient receptor potential vanilloid 1 (TRPV1) receptors are colocalized in dural afferents and the sensitization of TRPV1 receptors by PAR2 mediates CGRP

release (Dux et al., 2009; Zakharov et al., 2015). NO sources can be neurons, mast cells, and endothelial cells. Wherever it comes from, NO triggers perivascular neurogenic inflammation by facilitating the synthesis and release of CGRP and SP from dural nociceptive afferent fibers (Olesen, 2008). NO promotes CGRP gene activity in trigeminal neurons, and it works by signaling *via* a mitogen-activated protein (MAP) kinase pathway and T-type calcium channels. This suggests that endogenous NO could have a modulatory role in neurogenic inflammation (Ramachandran et al., 2014).

Recently Kilinc et al. (2017) indicated that 5-HT induced CGRP release and nociceptive activity in peripheral nerve terminals *via* 5-HT3 receptors. 5-HT is pro-nociceptive peripherally and anti-nociceptive centrally (Kilinc et al., 2017). The same author showed that calcitonin administration prevented CGRP release, trigeminal activation and mast cell degranulation in a glyceryltrinitrate-induced migraine model and *ex vivo* meningeal preparations (Kilinc et al., 2018). CGRP cannot easily pass the blood-brain barrier. Thus, it may induce the generation of pronociceptive substances and receptors in the trigeminal ganglion, transported along the central terminals. In this way, peripherally acting therapeutics can have a central antinociceptive effect (Messlinger, 2018). The rationale constituting the basis for this target can be at least based on CGRP receptor locations, the activation mechanism of these receptors and the change in the levels of this peptide in relevant venues.

PACAP, the Newest

PACAP, which was first isolated from ovine hypothalamic extracts, is a new player in the migraine arena. It was named for its action, which is to stimulate cAMP formation in anterior pituitary cells (Dogrukol-Ak et al., 2004; Eftekhari et al., 2015). Two amidated forms with PACAP38 and PACAP27 residues exist, but the major form in tissues is PACAP38, with high concentrations found in the trigeminal ganglion, hypothalamus, cerebral cortex, hippocampus, posterior pituitary, testes and adrenal gland (Eftekhari et al., 2015). PACAP38 (10 pmol/kg/min) induces migraine-like attacks in patients with migraine without aura (Schytz, 2010). In migraineurs, the level of PACAP in the peripheral blood is increased during a migraine attack (Tuka et al., 2013). PACAP induces marked vasodilation and degranulation of dural mast cells (Baun et al., 2012). MCs release PACAP itself (Okragly et al., 2018). Körtési et al. showed that electrical stimulation of the TG increased mRNA expression of PACAP38 which was inhibited by N-methyl-d-aspartate (NMDA) glutamate (NMDA) receptor inhibitor, kynurenic acid or MK-801 (Tuka et al., 2012; Körtési et al., 2018). PACAP is transported into the brain by transmembrane diffusion, a non-saturable mechanism (Dogrukol-Ak et al., 2004). Not only neuronal PACAP but also mast cell-derived PACAP can be involved in migraine pathophysiology.

ATP, the Last but Not the Least

The nucleotide adenosine triphosphate (ATP) is a promising candidate for migraine pathophysiology. ATP is well known as an intracellular energy source, however accumulating data proved that ATP is also a neurotransmitter (Burnstock, 1981, 2006; Burnstock et al., 2011). Extracellular ATP exhibits its effects *via* the two main types of purinergic receptors: ionotropic P2X (P2X1-7) and metabotropic P2Y (P2Y1-14). Rat mast cells express both ATP and cell-surface purinergic receptors of the P2 class; therefore, they are both the source and target of extracellular ATP (Bulanova and Bulfone-Paus, 2010; Burnstock and Boeynaems, 2014; Idzko et al., 2014). It was demonstrated that the activation of P2X7 receptors with both ATP and BzATP (P2X/agonist) increases calcium in human mast cells and induces degranulation of mast cells (Bulanova and Bulfone-Paus, 2010; Arandjelovic et al., 2012; Wareham and Seward, 2016). It was also shown that extracellular ATP evoked nociceptive spikes through P2X3 receptors in trigeminal nerve fibers innervating meninges at the origin site of migraine pain (Yegutkin et al., 2016). Additionally, CGRP upregulates P2X3 receptors in trigeminal sensory neurons (Fabbretti et al., 2006). Recently, Giniatullin's group has demonstrated that extracellular ATP promotes the activation of trigeminal neurons and degranulation of meningeal mast cells *via* P2X7 receptors (Nurkhametova et al., 2019). ATP might be a key molecule responsible for the vicious cycle between meningeal mast cells and the nervous system. The antagonists of P2X3 and P2X7 receptors maybe promising potential targets for migraine treatment.

CLINICAL TRIALS AND RECENT APPROACHES

Migraine undoubtedly has a severely disabling nature, thus there is an inevitable unmet medical need here as we still do not have a fully effective and safe treatment. Existing therapies are often non-specific, poorly tolerated, not fully effective or have cardiovascular contraindications, resulting in limitations on the use of these treatments. Among the issues seen, in turn, at patients' level, it is notable that half of the patients are not satisfied with current therapies in terms of pain recurrence, the same percentage complainants of requiring supplementary dosing, almost 80% of the patients consider acute alternative immediate therapies, and medication overuse headache also accompanies the therapies (Tepper, 2018).

In the 1990s, serotonin 5-HT1B/1D receptor agonists were introduced for improvement in the management of acute migraine, and there have been new trials investigating new agonists that are effective and result in fewer or no adverse events. LY-334370, a selective serotonin-1F-receptor agonist, has been reported to be efficacious in the abortive treatment of migraine (Shepheard et al., 1999) as it acts by inhibiting neurogenic inflammation. Moreover, new compounds like 4991W93 (Earl et al., 1999) and PNU-14263 (Gomez-Mantilla et al., 2001) are selective serotonin-1D-receptor agonists.

As a promising agent, NGF-targeted therapies using NGF-sequestering antibodies were highly effective in pain control, but as these compounds led the adverse events involving the sympathetic nervous system and bones, this treatment option was stopped by the Food and Drug

Administration (FDA; Kelleher et al., 2017; Skaper, 2017; Gupta and Harvima, 2018).

Some trials have demonstrated the nonselective NO synthase inhibitor, L-N-monomethylarginine (L-NMMA), to be highly successful in treating both migraine attacks and chronic tension-type headache (Ashina, 2002). NO synthase inhibitors may open the way to new avenues in the pharmacological treatment of migraine as they act by inhibiting NO production and neuropeptide release and pharmacological inhibition of several steps of the NO-signaling cascade. Cotreatment with the serotonergic, antimigraine drug sumatriptan suppresses the stimulatory effects of NO on CGRP promoter activity and release. Similarly, the application of nonselective and neuronal nitric oxide synthase (nNOS) inhibitors was able to partially attenuate neurogenic vasodilation (Klede et al., 2003).

Acting as a pivotal player in migraine pathophysiology, CGRP has been defined as a therapeutic target for migraine therapy. This attraction towards CGRP comes from its role both in onset and probably in the progress of the disease (Tepper, 2018). The translation of the CGRP acting mechanism at the beginning and during the course of the migraine into a therapeutical approach led to two paths: the development of small molecule CGRP receptor antagonists (Gepants), and the development of monoclonal antibodies. The gepants which have been the subject of published trials for acute treatment of migraine are as follows: Olcegepant (Olesen et al., 2004), Telcagepant (Ho et al., 2008; Connor et al., 2009), Rimegepant (Marcus et al., 2014), Ubrogepant (Voss et al., 2016), BI 44370 TA (Diener et al., 2011), MK-3207 (Hewitt et al., 2011). A severe adverse event liver toxicity appeared as a major challenge in the development programs of the gepants which was well-documented in the clinical trials, mostly tested through phase II, and phase III trials. The said drug development program was terminated due to the liver toxicity signals (Connor et al., 2011). Gepants are still important in identifying CGRP as key in migraine treatment and a potential target for acute treatment and, maybe for prophylaxis. It is well-understood that gepants are effective in the acute treatment of episodic migraine. The evidence also reveals that they are well tolerated. Ongoing and future studies on gepant safety, tolerability, and efficacy in migraine prevention are currently being evaluated. The data obtained from phase II and III trials of eptinezumab, erenumab, fremanezumab, and galcanezumab demonstrated that these monoclonal antibodies targeting the CGRP pathway demonstrate favorable effects in the preventive treatment of episodic and chronic migraine. A number of phase II and III trials are being conducted to further determine or prove the efficacy and safety of this new drug option. The cardiovascular effects of long-term CGRP blockade should be taken as a priority (Khan et al., 2019; www.ClinicalTrials.gov).

In particular, the most popular large phase III studies demonstrated that treatment with erenumab was associated with a substantive decrease in migraine frequency and the requirement for acute migraine-specific medication use in patients with episodic migraine. These trials are well reported as follows: ARISE (NCT02483585; Dodick et al., 2018), STRIVE (NCT02456740; Goadsby et al., 2017a,b) and LIBERTY (NCT03096834; Reuter et al., 2018). STRIVE and ARISE were completed within the first half of 2017. Positive results for erenumab constituted the basis for its recent approval in the US for the preventive treatment of migraine in adults. It has also received a favorable opinion in the Europen Union (EU) for the prophylaxis of migraines.

There are a few trials investigating the corticosteroid treatments that can be associated with MC involvement in this context, i.e., NCT02903680, NCT03220113, NCT03066544 (www.ClinicalTrials.gov).

CONCLUSION

Mast cell-sensory nerve relationship in migraine pathophysiology is versatile and not fully mapped, yet. The main difficulty in understanding these complex interactions that constitute the bi-directional cross-talk of the mast cells, nerves and the vessel components comes from multi-dimensional channels of communication working in harmony or disharmony. The diverse interpretation of the messages that are released from dose-dependent ligand response constitutes a wide spectrum of commentary remarks. Thus, the clinical evidence suggests that there is no one-fit-all treatment choice for migraine.

We suggest hereby, to further understand the etiopathogenesis and molecular aspects of migraine, specifically neurogenic inflammation. More detailed identification of the disease sub-types, plus a better understanding of the individual conditions will help scientists to find and the physicians to decide on the treatment choices tailored for the patients. In order to investigate the effective and safe treatment options which entail the combination of the existing monoclonal antibodies and mast cell stabilizers and triptans for the proper combat with the least side effects and the most efficacious cure methods could be evaluated.

AUTHOR CONTRIBUTIONS

All authors have equal role in literature search, writing and revising the manuscript.

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Mast Cells in Neurodegenerative Disease

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Neurodegenerative diseases affect millions of people worldwide, yet there are currently no effective treatments. Because risk of neurodegenerative disease substantially increases with age, greater life expectancy with a concomitant aging population means more individuals will be affected in the coming decades. Thus, there is an urgent need for understanding the mechanisms driving neurodegenerative diseases in order to develop improved treatment strategies. Inflammation in the nervous system, termed "neuroinflammation," has become increasingly recognized as being associated with neurodegenerative diseases. Early attention focused primarily on morphological changes in astrocytes and microglia; however, brain and CNS resident mast cells are now receiving attention as a result of being "first responders" to injury. Mast cells also exert profound effects on their microenvironment and neighboring cells including behavior and/or activation of astrocytes, microglia, and neurons, which, in turn, are implicated in neuroinflammation, neurogenesis and neurodegeneration. Mast cells also affect disruption/permeability of the blood brain barrier enabling toxin and immune cell entry exacerbating an inflammatory microenvironment. Here, we discuss the roles of mast cells in neuroinflammation and neurodegeneration with a focus on development and progression of four prominent neurodegenerative diseases: Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis, and Huntington's Disease.

Keywords: mast cells, neuroinflammation, neurodegenerative disease, Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, Huntington's

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INTRODUCTION

Mast cells are "first responders" that become activated with exposure to a diverse array of stimuli, from allergens and antigens to neuropeptides, trauma and drugs (Hendriksen et al., 2017). Activated mast cells are multifunctional effector cells that exert a variety of both immediate and delayed actions. Within minutes of stimulation, mast cells release granules containing preformed cytokines, biogenic amines, proteoglycans, proteases, leukotrienes, and lysosomal enzymes. Subsequent *de novo* synthesis and release of lipid mediators (e.g., leukotrienes, growth factors, prostaglandins) as well as cytokines and chemokines may sustain or oppose the early effects (Gupta and Harvima, 2018). Mast cells may also release extracellular vesicles, extracellular traps, and form nanotubes (Weng et al., 2016) that enable interactions with neighboring cells and structures including vessels and nerve fibers (Gupta and Harvima, 2018).

Myeloid progenitor cells from the bone marrow form immature mast cell precursors that migrate through the bloodstream to different tissues, where they undergo differentiation into mature mast cells and persist for long periods (Gupta and Harvima, 2018). Signals from the surrounding microenvironment and any attendant pathological conditions critically influence local mast cell size, structure, secretagog, sensitivity to stimuli and response to inhibitory signals/drugs. Mast cells may thus display substantial phenotypic heterogeneity between and within different organs including the nervous system (Metcalfe et al., 1997).

Chronic and acute inflammation in the nervous system, termed "neuroinflammation," have been associated with several neurodegenerative diseases, including those discussed in this review. Acute and chronic inflammation are also involved in neuropathic pain (Gupta and Harvima, 2018). Hence, although its close proximity to, and extensive communication with, the immune system provides the nervous system with substantial protection, this same relationship also makes the nervous system highly vulnerable to severe pathologies that significantly impact quality of life. The role of mast cells in neurodegenerative diseases is being increasingly recognized. In this review, we present an overview of mast cell function within the central and peripheral nervous systems with specific attention to neuroinflammation and neurodegeneration. We then focus on the roles of mast cells in the development and progression of four prominent and devastating neurodegenerative diseases: Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis and Huntington's Disease.

MAST CELL LOCALIZATION IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

Mast cells populate the brain during both development (Skaper et al., 2014) and adulthood, when they may migrate from the periphery to the brain (Nautiyal et al., 2011). The healthy human brain contains small numbers of mast cells located primarily in the abluminal perivascular areas and meninges (Banuelos-Cabrera et al., 2014; Dong et al., 2014), whereas mice have higher numbers of mast cells populating diverse regions of the brain (Nautiyal et al., 2012). Mast cells have been identified in the area postrema of the dorsal medulla, choroid plexus, and parenchyma of the thalami and hypothalamus (Ribatti, 2015; Hendriksen et al., 2017). The number and distribution of mast cells in the brain may change during infection, trauma, or stress (Bugajski et al., 1994; Maslinska et al., 2005; Silver and Curley, 2013).

Mast cells are also present the dura of the spinal cord, but not in the cord parenchyma under normal conditions. Nonetheless, mast cell mediators may still be able to modulate synaptic transmission and nociception at the level of the dorsal horn due to the close apposition of dura and white matter in this compartment (Michaloudi et al., 2008; Xanthos et al., 2011). Mast cells are also found in close proximity to peripheral nerves in tissues throughout the body (Schemann and Camilleri, 2013; Kritas et al., 2014a; Forsythe, 2015; Gupta and Harvima, 2018).

MAST CELL ACTIVATION, NEUROINFLAMMATION, AND NEURODEGENERATION

Hendriksen et al. (2017) have suggested a framework for characterizing the role of mast cells in neuroinflammation:

(1) Reciprocal interactions with microglia, astrocytes and neurons (Skaper et al., 2014)
(2) Effects on blood-brain barrier permeability (Hendriksen et al., 2017)
(3) Effects on neurogenesis: proliferation, differentiation, and migration (Molina-Hernandez and Velasco, 2008; Borsini et al., 2015)
(4) Effects on neurodegeneration: neuronal death, synaptic dysfunction, excitotoxicity (Kempuraj et al., 2017b)

A full discussion of any/all of these phenomena is beyond the scope of this review. Selected processes most relevant to neurodegenerative diseases are described below.

Mast Cell-Microglia Interactions

In the brain and CNS, microglial cells are the guardian immune surveillance effectors that constantly monitor the surrounding microenvironment for injury and pathogen entry, which elicit microglial activation encompassing the release of cytokines/chemokines, phagocytosis of cellular debris and antigen presentation to T cells (Colonna and Butovsky, 2017). Cross-talk between microglial cells and other cells of the immune system enable complex, multifaceted communication between the brain, CNS and "first responders" that affords neural protection. Nevertheless, such homeostatic and protective responses are prone to dysregulation, particularly as a consequence of aging, which gives rise to chronic inflammation, resulting in tissue damage with a concomitant impaired ability to heal (Di Benedetto et al., 2017).

While microglial cells provide immune surveillance to the brain and CNS, other immune cells have recently been recognized for their contributions to neuronal degenerative diseases. Notably, tissue-resident mast cells have garnered much attention as primary communicators and mediators between the peripheral immune system and the nervous system during inflammatory responses (Gupta and Harvima, 2018). Mast cells have long been recognized for their roles in allergic inflammation and anaphylaxis; however, their localization within the CNS has led to recent exploration into their possible roles in neuroinflammation and neurodegenerative disease (Hendriksen et al., 2017). In fact, mast cells within the CNS, as opposed to microglial cells, are now recognized as the primary first responders to injury as conferred by their secretory granule arsenal of preformed/stored immunomodulators, neuromodulators, proteases, amines and growth factors (Gupta and Harvima, 2018). Mast cell progenitors are able to traverse the blood-brain-barrier (BBB) and blood-spinal cord-barrier under states of inflammation and infection (Nautiyal et al., 2011). In response to localized microenvironment perturbation, mast cells undergo activation in which pre- and newly synthesized

mediators such as GnRH, monoamines, specific proteases (e.g., chymases, tryptases, and carboxypeptidase A), cytokines, and histamine are secreted by the process of degranulation (Metcalfe et al., 1997; Vukman et al., 2017). Release of these compounds elicits profound effects on neighboring cells including T cells, which are able to enter the brain via compromised BBB. Microglial cells are also activated in response to mast cell release of tryptase and histamine, resulting in a pro-inflammatory state mediated by microglial secretion of cytokines/chemokines into the microenvironment (Hendriksen et al., 2017). In addition, mast cells release chemoattractants, which recruit eosinophils, monocytes, and neutrophils further exacerbating an inflammatory environment (Jolly et al., 2004).

Interactions between mast and microglial cells involves complex cross-communication that can be both unidirectional and bidirectional. Activated microglia release IL-6 and chemokine (C-C motif) ligand 5 (CCL5), which affect surface expression levels of toll-like receptors, TLR2 and TLR4, on mast cells thus modulating the ability of mast cells to respond to endotoxins (Pietrzak et al., 2011). Conversely, release of CCL5/RANTES (regulated on activation, normal T cell expressed and secreted) by mast cells induces proinflammatory responses in microglial cells (Skuljec et al., 2011). Tryptase released from mast cells cleaves and activates protease activated receptor 2 (PAR2) on microglial cells resulting in the upregulation and release of brain-derived neurotrophic factor (BDNF); while IL-6 and TNF-α released from microglial cells upregulates PAR2 expression on mast cells, resulting in mast cell activation (Zhang and Levy, 2008; Zhang et al., 2010). C-X-C chemokine receptor type 4 (CXCR4) expression promotes migration and activation of microglial cells and also acts as a mast cell chemotaxin (Juremalm et al., 2000; Wang et al., 2008). ATP stimulates IL-33 release from microglial cells, which in turn induces IL-6, IL-13, and monocyte chemoattractant protein 1 secretion from mast cells, resulting in modulatory responses in microglial cells (Osipchuk and Cahalan, 1992; Bulanova and Bulfone-Paus, 2010; Taracanova et al., 2017). From these few examples, it is clear that mast cell-microglia interactions encompass highly complex paracrine mechanisms by which these cells, as a result of close proximity, influence each other's behavior and responses to their microenvironment.

Mast Cell Effects on Blood-Brain Barrier Permeability

The blood-brain barrier (BBB) plays a critical role in controlling the entry of molecules, pathogens, and toxins into the CNS. The primary barrier units of the BBB are tight junctions between endothelial cells (ECs) that limit paracellular transport. Tight junctions are composed of transmembrane proteins such as claudin and occludin (Strbian et al., 2009). BBB-specific receptors on ECs modulate trafficking of molecules into and out of the brain (Keaney and Campbell, 2015). Surrounding pericytes, astroglia and neurons communicate with ECs and impart further integrity and complexity to the BBB (Banuelos-Cabrera et al., 2014; Ribatti, 2015). Disruption and breakdown of the BBB is associated with a variety of neoplastic, infectious, inflammatory, and neurodegenerative diseases (Daneman and Prat, 2015; Ribatti, 2015). Mast cells are believed to influence BBB integrity through release of proteases that can degrade tight junction proteins and extracellular matrix components, as well as release of vasoactive mediators including histamine and TNF-α (Strbian et al., 2009; Mattila et al., 2011).

Mast Cell Effects on Neurodegeneration

As a result of their ability to quickly release diverse immuno- and neuromodulators, the relatively small numbers of mast cells in the brain and CNS have a substantial influence on the behaviors of neurons and glial cells. Direct influences via release of TNF-α, histamine and proteases empower mast cells with the ability to potentiate neuroinflammation, neurogenesis, neurodegeneration and BBB disruption/permeability. Nevertheless, to date all evidence regarding the direct influence of mast cells on neurodegeneration derive from animal studies (Secor et al., 2000). It therefore remains to be determined the extent to which such findings hold true human relevance. With respect to neurodegeneration, mast cell release of TNF-α and other cytokines can increase neuroinflammation and the formation of neurotoxic nitric oxide by astrocytes. Cytokines such as IL-6, IL-1β, and TNF-α can elicit protective as well as detrimental effects and these differential outcomes are likely dependent on the concentration and duration with which these cytokines are expressed and released. TNF-α and IL-6 can affect the expression and function of tight junction proteins; and, therefore, the release of these cytokines is likely involved in the capacity of mast cells to modulate BBB permeability and the entry of immune cells and other molecules that do not have access under physiological conditions. The potential roles of histamine in neurogenesis and neurodegeneration have remained somewhat controversial since conflicting reports indicate that histamine is both neuroprotective and can also increase neurotoxicity (Skaper et al., 2001; Fang et al., 2014). Moreover, reports indicate that histamine can potentiate both increased BBB integrity and increased BBB permeability. It is likely that different histamine receptors may have diverse effects, and their presentation may thus guide context-specific histamine activity. The roles of mast cells in neurogenesis and neurodegeneration thus involve a high level of nuanced complexity.

MAST CELLS IN ALZHEIMER'S DISEASE

Alzheimer's Disease (AD) is the most common cause of dementia among the elderly population, with 5–7 million new diagnoses annually (Robinson et al., 2017). Patients experience progressive, disabling cognitive deficits and decline in learning and memory (Bianchetti et al., 2006). Although the cardinal histopathological features of extracellular amyloid β-peptide (Aβ) deposition, intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein and synaptic loss have been extensively described, the exact etiology of AD remains unclear (Zlomuzica et al., 2016). The causative role of amyloid toxicity in AD pathogenesis has long been questioned (Yankner, 1989). Contemporary understanding

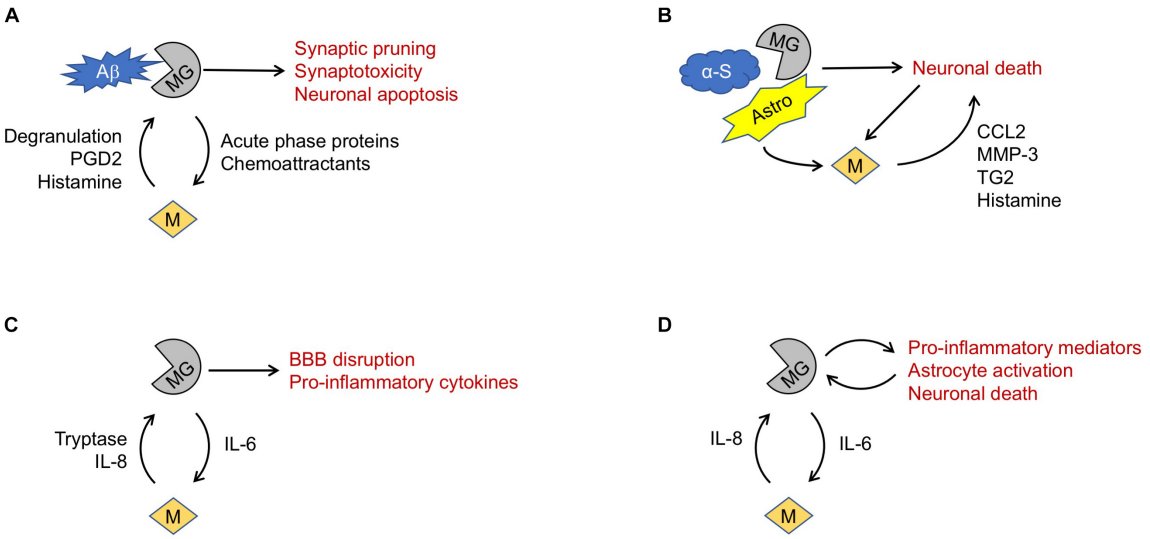

FIGURE 1 | Proposed mechanisms of mast cell involvement in neurodegenerative diseases. **(A)** In Alzheimer's Disease, microglia (MG) phagocytosis of amyloid β (Aβ) fragments may trigger release of mediators causing mast cell (M) degranulation. Mast cell products may in turn promote microglia-mediated neurotoxicity. **(B)** In Parkinson's Disease, misfolded α-synuclein (α-S) may trigger microglia-mediated death of dopaminergic neurons. Astrocytes (Astro), microglia and dying neurons may all promote mast cell recruitment and release of mediators that exacerbate neuronal death. **(C)** In amyotrophic lateral sclerosis, TNF-α and IL-6 elaborated by microglia may drive mast cell recruitment, activation, and degranulation. Release of mediators such as tryptase and IL-8 can reciprocally activate microglia, exacerbating blood-brain barrier (BBB) disruption and release of pro-inflammatory cytokines. **(D)** Similarly, in Huntington's Disease a feed-forward interaction between microglia and mast cells may promote a pro-inflammatory and neurotoxic milieu.

suggests that Aβ oligomers may impair synaptic function and plasticity (Selkoe, 2008), with attendant derangement of multiple neurotransmitter systems and neuronal networks (Zlomuzica et al., 2016).

Recent studies increasingly implicate neuroinflammation in AD neurodegeneration (Heneka et al., 2015). For example, microglia have been found to surround Aβ plaques in postmortem AD brain specimens as well as mouse models of amyloid deposition (Skaper et al., 2018). Phagocytosed Aβ peptides have been shown to regulate microglia phenotype (Manocha et al., 2016), induce production of trophic factors (Rivest, 2009) and synaptotoxic compounds (Skaper et al., 2018), and trigger early widespread synaptic pruning (Hong et al., 2016) (**Figure 1**).

Autopsy studies have shown mast cells surround amyloid plaques in Alzheimer's patients in higher numbers than corresponding brain regions of control patients (Maslinska et al., 2007). It has been suggested that inflammation in the AD brain may trigger CNS glia to produce acute phase proteins and mast cell chemoattractants such as serum amyloid A that home mast cells to sites of amyloid deposition (Skaper et al., 2018). It is also possible that mast cells themselves are early detectors of amyloid peptides; indeed, Harcha et al. (2015) showed increased mast cell numbers in the cortex and hippocampus of a mouse model of AD prior to amyloid detection. The authors also demonstrated that amyloid peptides can activate membrane Panx1 hemichannels on mast cells, leading to degranulation. Importantly, the authors demonstrated attenuation of these responses by Panx1 inhibitors *in vitro* as well as in the mast cells of *Panx1−/−* mice (Harcha et al., 2015). Subsequent release of mediators including histamine and prostaglandin D2 (PGD2) may exacerbate local inflammatory processes including microglial activation (Shaik-Dasthagirisaheb and Conti, 2016).

The oral tyrosine kinase inhibitor masitinib modulates mast cell degranulation, differentiation and survival through c-kit and Lyn targeting (Dubreuil et al., 2009). A phase 2 randomized, placebo-controlled trial of masitinib as add-on therapy for patients with mild-to-moderate AD showed reduction in the rate of cognitive decline over a 24-week period. The ability of mast cells to disrupt integrity of the BBB has been implicated in the stress-induced neuropathological processes involved in the development and progression of AD (Esposito et al., 2001; Mravec et al., 2018). Because masitinib is unlikely to cross the BBB, this was potentially achieved by inhibiting mast cells in close proximity to the BBB from releasing mediators that would impair BBB permeability, leading to a decrease in local proinflammatory molecules and further migration of mast cells into the brain (Piette et al., 2011). Masitinib is currently in further phase 2 and 3 clinical trials (Folch et al., 2016).

MAST CELLS IN PARKINSON'S DISEASE

Parkinson's Disease (PD) is characterized by progressive motor deficits including rigidity, bradykinesia and resting tremor, alongside non-motor deficits that may evolve later in the disease course (Shulman et al., 2011). Recent studies have linked neuroinflammation, particularly microglial activation, to PD pathogenesis (Stojkovska et al., 2015). Misfolded α-synuclein can activate microglia via several receptors including

major histocompatibility complex II (Harms et al., 2013) and signaling cascades involving NF-kB and MAPKs. These events promote migration, phagocytosis and lymphocyte recruitment by microglia, and trigger increased expression of mediators including TNF-α, IL-6, and cyclooxygenase-2 (COX-2), ultimately exacerbating death of dopaminergic neurons (Zhang et al., 2017).

Parkinson's Disease pathogenesis is also thought to involve down-regulation of nuclear receptor Nurr1 in microglia and astrocytes, leading to increased production of mediators such as chemokine CCL2 that may promote apoptosis of dopaminergic neurons (Liu et al., 2017). BBB dysfunction has also been shown in animal models and validated in PD patients (Gray and Woulfe, 2015). In a PD mouse model with impaired BBB function, matrix metalloproteinase-3 (MMP-3) was shown to play a critical role in death of nigrostriatal dopaminergic neurons (Chung et al., 2013). In spite of the known effects of mast cells on BBB permeability and disruption, there is currently little direct evidence for a role of mast cells in the pathogenesis of PD. Nevertheless, initial presumptive findings suggest that mast cells may contribute to PD via neural cell mediated activation. Kempuraj et al. (2016) demonstrated that exposure to dopaminergic toxin triggers release of CCL2 and MMP-3 by human umbilical cord blood-derived cultured mast cells and mouse bone marrow-derived mast cells (BMMCs) *in vitro*. The authors further characterized this phenomenon by exposing mast cells to glial activating factors in co-culture with fetal mouse brain-derived astrocytes, neurons, and/or mixed glia/neurons. Release of specific inflammatory mediators and neurite outgrowth were both quantified. By comparing all possible combinations of cell types, the authors demonstrated that mast cells specifically release mediators including tryptase. Furthermore, mast cell-specific mediators were shown to trigger CCL2 and MMP-3 release by astrocytes and glia in this model system. Taken together, these results suggest mast cell interactions with neurons and glial cells may play a role in PD pathogenesis (Kempuraj et al., 2018).

Hong et al. (2018) recently demonstrated that CCL2 production by microglia and astrocytes may recruit mast cells into the substantia nigra in a PD mouse model. Recruited mast cells were shown to express the cross-linking enzyme tissue transglutaminase 2 (TG2) in an NF-kB dependent manner, with subsequent release of pro-inflammatory mediators including histamine, leukotrienes, and TNF-α that are implicated in dopaminergic neuronal death. Although the comparison against TG2 knockout mice supported these findings, validation was not carried out in mast cell knockout controls. The authors also found increased TG2 expression in the serum of PD patients compared to control patients (Hong et al., 2018).

These results suggest intriguing roles for mast cells in PD pathogenesis, but should be interpreted cautiously when considering disease in humans. Animal and *in vitro* model systems may not fully recapitulate complex local microenvironments that substantially alter mast cell phenotype. Furthermore, postmortem studies of PD brain specimens have not consistently shown increased numbers of mast cells by conventional detection methods (Hurley et al., 2015).

MAST CELLS IN AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) involves progressive degeneration of both upper and lower motor neurons, often accompanied by cognitive and/or behavioral symptoms. ALS is the most common and aggressive form of motor neuron degeneration in adults, with a heterogenous but invariably progressive and fatal disease course (Hardiman et al., 2017). The underlying etiology remains unknown. Although some patients have inherited familial disease, the majority of cases are sporadic. The common pathologic feature is accumulation of ubiquitylated cytoplasmic protein inclusions in motor neurons. These inclusions are composed of transactive response (TAR) DNA binding protein 43 (TDP43) aggregates in the majority of cases (Neumann et al., 2006). Rodent models of ALS predominantly overexpress superoxide dismutase 1 (SOD1), which has also been implicated in some human cases (Hardiman et al., 2017).

Microglial activation has been shown in *SOD1*-transgenic mice (Liao et al., 2012) as well as human postmortem brain specimens and *in vivo* imaging in ALS patients (Turner et al., 2004; Corcia et al., 2012; Brites and Vaz, 2014). Accumulation of degranulating mast cells associated with macrophages at the neuromuscular junction has been shown to occur after onset of motor weakness in a rat model of ALS and correlate with denervation. Although a mast cell knockout control was not employed, treatment with the mast cell inhibitor masitinib reduced mast cell numbers and progression of motor symptoms, suggesting a cell type-specific effect (Trias et al., 2017). The mast cell chemoattractant IL-15 is elevated in the serum and cerebrospinal fluid of ALS patients (Rentzos et al., 2010), and mast cells expressing IL-17 have been found in the spinal cord of ALS patients (Fiala et al., 2010).

Cytokines including IL-12 are elevated in the serum and cerebrospinal fluid (CSF) of ALS patients (Rentzos et al., 2010). High mobility group box 1 protein and other damage-associated host biomolecules that can trigger an inflammatory response through TLR2/TLR4 signaling are elevated in the spinal cords of ALS patients (Casula et al., 2011). Yang et al. (2010) demonstrated that IL-12 can upregulate mast cell expression of TLR2/TLR4 pattern recognition receptors; although this was carried out in a mouse mastocytoma cell line *in vitro*, the findings support a possible role for mast cell autocrine signaling in the neuroinflammatory cascade.

Elevated IL-6 and IL-8 levels were found in the peripheral blood of ALS patients (Ehrhart et al., 2015). TNF-α and IL-6 elaborated by microglia have been shown to drive mast cell recruitment, activation, and degranulation, releasing mediators such as tryptase, which can reciprocally activate microglia in a feed-forward cycle in a rat model of PD (Zhang et al., 2010, 2011; Skaper et al., 2018). Mast cell tryptase has also been shown to stimulate microglial protease-activated receptors (PARs) that can cause disruption of the BBB in wild-type as

compared to PAR-deficient mice (Bunnett, 2006). Human mast cell lines have been observed to release IL-8 (Chen et al., 2016; Yu et al., 2016). IL-8 enhances production of pro-inflammatory cytokines by microglia exposed to Aβ peptide *in vitro* (Franciosi et al., 2005). Taken together, these findings suggest further involvement of mast cells in cross-talk with microglia in the neuroinflammatory milieu of ALS.

Human and animal studies have also demonstrated impairment of the BBB and blood-spinal cord barriers in ALS (Rodrigues et al., 2012). As a result of pre-synthesized vasoactive mediators, mast cells affect the permeability and integrity of both the BBB (Ribatti, 2015) and blood-spinal cord-barrier. Therefore, in the case of ALS, mast cells have the potential to cross the blood-spinal cord-barrier and release neuropeptides, proteases, cytokines, histamine etc., via degranulation resulting in localized neuroinflammation and dysregulated neuronal function. Support for this, as stated above, comes from the finding that mast cells expressing IL-17 were present in the spinal cords of ALS patients. Further studies are needed to demonstrate and characterize the degree to which mast cells may be involved in these processes via the inflammatory and vasoactive mediators described above. Studies in animal models of ALS will be critical for validating the findings from different disease and *in vitro* contexts.

MAST CELLS IN HUNTINGTON'S DISEASE

Huntington's Disease (HD) is an autosomal dominant disorder caused by expansion of a CAG triplet in the *HTT* gene that leads to expression of a mutant form of the Huntington protein, HTT. Mutant HTT (mHTT) causes excitatory neurotoxicity in inhibitory medium spiny neurons (MSNs) in the striatum and cortex. Patients experience involuntary jerking movements as well as dystonia, rigidity, cognitive, and neuropsychiatric symptoms (Zuccato and Cattaneo, 2014).

Although the role of mast cells in HD pathogenesis has not yet been established, substantial evidence supports a strong role of neuroinflammation via interactions between neurons, microglia and astrocytes. Expression of mHTT in astrocytes downregulates production of neuronal growth factors, while mHTT expression in microglia promotes expression of proinflammatory cytokines (IL-6, TNF-α) and toxic metabolites. These processes converge to cause neurodegeneration, exacerbated by mHTT expression in neurons that triggers cell-autonomous apoptosis and degeneration. Components of dead neurons may be detected and phagocytosed by microglia in a similar fashion to Aβ detection in AD, resulting in further production of proinflammatory mediators, astrocyte activation, and a feed-forward loop of neuronal damage (Crotti et al., 2014; Crotti and Glass, 2015).

Plasma levels of IL-6 and IL-8 correlate with functional scores in HD patients (Bouwens et al., 2017). Several signaling cascades were found to drive microglia overexpression of IL-8 in a porcine model of HD (Valekova et al., 2016). Similar to findings in ALS, it is possible that these represent previously unexplored evidence of mast cell involvement in HD pathogenesis, and will require rigorous validation in animal models of HD.

FUTURE DIRECTIONS

Emerging studies implicate chronic stress in a variety of neuroinflammatory processes (Machado et al., 2014) that may increase the risk of developing neurodegenerative disease (Hoeijmakers et al., 2017; Piirainen et al., 2017). Mast cells release and respond to molecules such as corticotropin releasing hormone during stress and neuroinflammation, suggesting a role in the pathogenesis of stress-related neurodegeneration and neuroinflammation (Kritas et al., 2014b). However, these mechanisms have yet to be definitively elucidated in the setting of neurodegenerative disease (Kempuraj et al., 2017a; Skaper et al., 2018). Similarly, the immune modulatory and neuroprotective role of gut microbiota has also been hypothesized to involve mast cells (Girolamo et al., 2017), suggesting another fruitful avenue for integrative mechanistic studies.

There are additional challenges and opportunities for future studies that are beyond the scope of this review. Possible neuroprotective roles of mast cells warrant further investigation. The dependence of mast cell phenotype on tissue- and pathology-specific microenvironment necessitates careful selection of animal and *in vitro* model systems, as well as validation in human tissue specimens. Further studies are also needed to elucidate the multifaceted cross-talk between mast cells and microglia, astrocytes and neurons. The scale of this problem may require informatics-based approaches; for example large genomic datasets derived from experimental mouse models may enable *in silico* discovery of target genes and guide rational validation in appropriate model systems (Khayer et al., 2017).

CONCLUSION

A complete understanding of the mechanisms driving the development of neurodegenerative diseases is lacking. This has impeded the advancement of effective therapeutic strategies aimed at preventing both disease onset and progression. Although the many neurodegenerative diseases thus far identified have diverse characteristics and etiologies, many contributing factors are likely shared in common, which offers the possibility of identifying novel targets for intervention. Neuroinflammation, which is now recognized as a primary pathological component of diseases such as multiple sclerosis, is gaining acceptance as an underlying component of most, if not all, neurodegenerative diseases. Whereas past focus has predominantly centered on glial cells of the CNS, recently mast cells have emerged as potential key players in both neuroinflammation and neurodegenerative diseases. Mast cells are well positioned for such a role owing to their ability to affect both their microenvironment and neighboring cells including T cells, astrocytes, microglia, and neurons. The secretory granules of mast cells contain an arsenal of preformed/stored immunomodulators, neuromodulators, proteases, amines and growth factors that enable complex cross-communication, which can be both unidirectional and bidirectional. Mast cells can also affect disruption/permeabilization of the BBB and this has the potential for dramatically altering the neuroinflammatory state.

With respect to AD, PD, ALS, and HD, discussed in the present review, mast cell perturbation of the BBB appears to share a commonality. Moreover, mast cells have been found to home to sites of amyloid deposition in AD; and, an inhibitor of mast cell function was shown to reduce cognitive decline in AD patients. Mast cell interactions with neurons and glial cells have also been implicated in PD pathogenesis. Emerging evidence suggests that mast cell autocrine signaling may contribute to ALS: The mast cell chemoattractant, IL-15, is elevated in the serum and cerebrospinal fluid of ALS patients; and, mast cells expressing IL-17 have been found in the spinal cord of ALS patients. Plasma levels of cytokines (IL-6, IL-8), known to affect mast cell activation, have been correlated with functional scores in HD patients suggesting the possible involvement of mast cells in the pathogenesis of HD. Future considerations include validation of animal and *in vitro* models, which incorporates microenvironment-specific influences and the complex, multifaceted cross-talk between mast cells and microglia, astrocytes and neurons. In addition to the potential role(s) of mast cells in neuroinflammation and neurodegenerative diseases, the possible neuroprotective roles of mast cells also warrant further investigation.

AUTHOR CONTRIBUTIONS

All authors participated in the conceptualization, literature review, drafting, and editing of the manuscript.

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Stabilization of Brain Mast Cells Alleviates LPS-Induced Neuroinflammation by Inhibiting Microglia Activation

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Background: The functional aspects of mast cell-microglia interactions are important in neuroinflammation. Our previous studies have demonstrated that mast cell degranulation can directly induce microglia activation. However, the role of mast cells in Lipopolysaccharide (LPS)-induced microglia activation, neuroinflammation and cognitive impairment has not been clarified.

Methods: This study investigated the interaction between brain microglia and mast cells *in vivo* through site-directed injection of cromolyn into rat right hypothalamus using stereotaxic techniques. Cognitive function was subsequently assessed using trace fear conditioning and Y maze tests. Mast cells in rat brain were stained with toluidine blue and counted using Cell D software. Microglia activation was assessed by Iba1 immunohistochemistry both in rat brain and in mast cell-deficient $Kit^{W-sh/W-sh}$ mice. Receptor expression in rat microglia was determined using flow cytometry analysis. Cytokine levels in rat brain tissue and cell supernatant were measured using high-throughput ELISA. Western blotting was used to analyze Cell signaling proteins.

Results: In this study, intraperitoneal injection of 1 mg/kg LPS induced mast cell activation in hypothalamus and cognitive dysfunction in rats, and that this process can be repressed by the mast cell stabilizer cromolyn (200 μg). Meanwhile, in mice, LPS IP injection induced significant microglia activation 24 h later in the hypothalamus of wild-type (WT) mice, but had little effect in $Kit^{W-sh/W-sh}$ mice. The stabilization of mast cells in rats inhibited LPS-induced microglia activation, inflammatory factors release, and the activation of MAPK, AKT, and NF-κB signaling pathways. We also found that LPS selectively provokes upregulation of H_1R, H_4R, PAR2, and TLR4, but downregulation of H_2R and H_3R, in ipsilateral hypothalamus microglia; these effects were partially inhibited by cromolyn. In addition, LPS was also found to induce activation of P815

cells *in vitro*, consistent with findings from *in vivo* experiments. These activated P815 cells also induced cytokine release from microglia, which was mediated by the MAPK signaling pathway.

Conclusion: Taken together, our results demonstrate that stabilization of mast cells can inhibit LPS-induced neuroinflammation and memory impairment, suggesting a novel treatment strategy for neuroinflammation-related diseases.

Keywords: mast cells, LPS, microglia activation, neuroinflammation, migtation

INTRODUCTION

Neuroinflammation has been recognized as the chief culprit in multiple neurodegenerative diseases. An increasing number of studies suggest that communications between microglia, immune cells, and neurons might promote the acceleration of neuroinflammation and the exacerbation of neurodegenerative disorders (Block and Hong, 2005). However, the association between microglia and immune cells still remains to be fully explored.

Emerging evidence indicates that prolonged inflammatory responses involving astrocytes and microglia promote neurodegenerative disease exacerbation (Block and Hong, 2005). Microglia are the resident immune cells in central nervous system (CNS), providing immune surveillance. Under physiological conditions, microglia exhibit a resting state associated with the secretion of neurotrophic and anti-inflammatory factors (Streit, 2002). In pathological situations, microglia switch to an activated phenotype that initiates an inflammatory response. In most cases, this response is temporary and has beneficial effects in eradicating injured CNS cells. However, prolonged and inappropriate activation of microglia can lead to brain injury and neuronal apoptosis (Dauer and Przedborski, 2003). Hence, modulation and inhibition of the over-activation of microglia may provide a novel therapeutic target to improve treatment of neurodegenerative diseases.

Mast cells, notorious for their role in allergic diseases, reside close to microglia and neurons in the CNS, mainly present in the leptomeninges, blood vessels, thalamus and hypothalamus (Florenzano and Bentivoglio, 2000; Khalil et al., 2007). Mast cells store numerous proinflammatory mediators, such as histamine and proteases, in secretory granules, and can secrete them upon activation (Schwartz, 1987; Serafin and Austen, 1987; Galli, 1993; Dvorak, 1997; Galli et al., 2005). Notably, as the "first responder" cells of the CNS, mast cells can store preformed TNF-a in the secretory granules (Zhang B. et al., 2012). They can induce the secretion of pro-inflammatory cytokines from activated microglia via signaling through the H_1R, H_4R and PAR2 receptors (Zhang S. et al., 2012; Dong et al., 2014). As mast cells participate in the opening of the blood-brain barriers (BBB) and in neuroinflammation, meningeal mast cells are able to recruit an early wave of neutrophils and T cells to the CNS, leading to further inflammatory cell influx and exaggerated neuroinflammation (Sayed et al., 2010; Zhang S. et al., 2016). Owing to this pivotal role of mast cells in the pathobiology of neuroinflammation, it is important to determine the exact regulatory effects of activated mast cells on microglia in neuroinflammation.

We have previously reported that mast cell degranulation could directly induce microglia activation (Dong et al., 2017). However, whether mast cells affect LPS-induced microglia activation has not been reported. In the present study, we demonstrate that mast cell degranulation can aggravate LPS-induced neuroinflammation by evoking microglia activation. In addition, we also find that stabilization of mast cells can inhibit LPS-induced neuroinflammation and memory impairment.

MATERIALS AND METHODS

Animals

Eighty-four adult male SD rats (each weighing about 250 g), 12 adult mast cell-deficient $Kit^{W-sh/W-sh}$ mice and 12 littermate controls were used in this experiment, which were obtained from the Mode Animal Research Center of Nanjing University. All animals were housed under conditions previously described (Dong et al., 2017): five per cage, 50–60% humidity, 22°C constant room temperature and free access to food and water. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication no. 85-23, revised 1985) and the Guidelines for the Care and Use of Animals in Neuroscience Research by the Society for Neuroscience. They were approved by IACUC (Institutional Animal Care and Use Committee of Nanjing Medical University, No: 14030126).

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco–BRL (Grand Island, NY, United States). Lipopolysaccharide (LPS, from Escherichia coli 0111:B4), disodium cromoglycate (cromolyn) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Fluorescenc mounting medium with DAPI and anti-mast cell Tryptase antibody were purchased from Abcam (HK, China). Rabbit anti-Iba1 antibody was purchased from Wako Chemicals USA, Inc. Rabbit anti-H_1R and anti-H_2R antibodies were purchased from Alomone Labs (Jerusalem, Israel). Rabbit anti-H_3R antibody was purchased from Abcam (Hong Kong, China). And rabbit anti-H_4R was purchased from Santa Cruz (CA, United States). Fluorescein isothiocyanate (FITC) – conjugated mouse anti-OX-42 antibody and isotype control, phycoerythrin (PE)-conjugated goat anti-rabbit secondary antibody were

purchased from BD (BD Biosciences, United States). Cytokines (TNF-α, IL-6, IL-1β, INF-γ, RANTES, and IL-10) measured kit was purchased from MERCK Millipore Corporation (Billerica, MA, United States). Specific rabbit anti-p38, anti-Phospho-p38, anti-JNK, anti-Phospho-JNK, anti-ERK, anti-Phospho-ERK, anti-AKT, anti-Phospho-AKT, and anti-Phospho-NF-κB p65 antibodies, and goat anti-rabbit secondary antibody were obtained from Cell Signaling (Beverly, MA, United States).

In vivo Studies

Surgery and Drug Administration

Sixty rats were randomly assigned to five groups (groups A–E) with 12 rats in each group. This study was performed double-blind. Rats in groups D–E were pretreated with site-directed injection of the mast cell stabilizer cromolyn (200 μg/μl) into the hypothalamus, while rats in groups A–C were pretreated with 0.9% NaCl in the hypothalamus. After 30 min, rats in groups B to E were given intraperitoneal injection of LPS (1 mg/kg) while rats in group A were injected with 0.9% NaCl intraperitoneally. Rats in groups B and D were sacrificed 30 min after LPS injection, while rats in groups A, C, and E were sacrificed 24 h after LPS injection.

Mast cells are plentiful in hypothalamus. Therefore mast cell stabilizer cromolyn was centrally site-injected into the ipsilateral hypothalamus to determine whether mast cells are involved in LPS-induced neuroinflammation. As described in our previous report (Dong et al., 2017), the rats were anaesthetized by 50 mg/kg of pentobarbital sodium given intraperitoneally, then placed in a stereotaxic apparatus (Stoelting Instruments, United States). Guide cannulas (Plastic One) were inserted into the right hypothalamus of rats at 1.80 mm lateral and 1.90 mm posterior from Bregma, with a depth of 8 mm and at a 10° angle. After implantation, the rats were given 14 days to recover, with daily handling to check on the guide cannula. For the experiments involved, 1 μl of 200 μg/μl cromolyn (200 μg) or 1 μl of 0.9% NaCl was injected directly into the ipsilateral hypothalamus through the implanted guide cannulas. These rats were kept in their cages for 30 min without other restraint. Then, the rats were injected intraperitoneally with either LPS or 0.9% NaCl (control group). After drug administration, the rats were sacrificed and their brains were collected for morphological ($n = 6$) and biochemical ($n = 6$) analyses.

To evaluate the effects of LPS on microglia activation in mast cell-deficient mice, 12 $Kit^{W-sh/W-sh}$ and 12 wild-type (WT) mice were each divided into two equal groups, of which one received intraperitoneal LPS ($n = 6$) and the other received 0.9% NaCl ($n = 6$).

Mast Cell Staining and Counting

Rats were anesthetized with chloral hydrate, then perfused with 0.9% NaCl followed by 4% cold paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. The brains were dissected out and maintained overnight in 4% paraformaldehyde, then cryopreserved in PBS containing 30% sucrose before being stored at −70°C until use. Free-floating sections encompassing the entire brain were prepared using a cryostat, then stained with 0.05% toluidine blue and counted as previously described (Dong et al., 2017). Briefly, a 1% stock solution of toluidine blue in 70% ethanol was dissolved in 0.5% NaCl (pH 2.2–2.3). The slides were immersed in this staining solution for 30 min, then washed twice with distilled water and dehydrated using a series of increasing concentrations of ethanol, and finally immersed in butyl acetate ester. Cover slips were applied using Eukitt® mounting medium and the slides were allowed to dry overnight.

The entire surface area of the ipsilateral and contralateral thalamus was scanned manually using a light microscope at 200× magnification. Mast cells were counted under double-blind conditions with the help of the Cell D software (Olympus) and expressed as the number of cells in the high power field. Mast cells were considered degranulated based on the following criteria: loss of purple staining, fuzzy appearance, distorted shape, or multiple granules visible near the cell.

Immunohistochemical and Immunofluorescence Analysis

Manual immunochemical and immunofluorescence analyses of brain sections were performed as previously described (Dong et al., 2017). Brain sections of rat and mice were obtained by the method described above. Then, rat brain sections were processed for immunohistochemistry, and mouse brain sections were processed for immunofluorescence.

For immunochemistry analyses, rat brain tissue section (30 μm) were incubated for 1 h in 10% bovine serum albumin (BSA) with 0.3% Triton X-100 in 0.01 M phosphate-buffered saline, then overnight with primary antibodies at 4°C. The primary antibodies used in this experiment were rabbit anti-Iba1 (1:200) and mouse anti-tryptase (1:100). Tissue sections were washed and incubated in the following day with goat anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. Immunostaining was visualized with 3, 3′- diaminobenzidine, after which sections were counterstained with hematoxylin. The slides were scanned using a Leika 2500 (Leica Microsystems, Wetzlar, Germany) at 200× magnification.

For immunofluorescence analysis, mouse brain sections were incubated overnight with rabbit anti-Iba1 monoclonal antibody (1:200) in blocking solution at 4°C. Tissue sections were washed three times with PBS, then incubated with PE-conjugated goat anti-rabbit secondary antibody (1:200) at 37°C for 1 h. Cell nuclei were stained with DAPI. Fluorescent images were acquired using a confocal microscope (Leica, Frankfurt, Germany). Counts of Iba-positive cells were determined with the help of the Cell D software (Olympus) and expressed as the number of cells in the high power field.

Behavioral Analysis

Y Maze

The Y maze was previously described (Lu et al., 2015). To adapt to the environment, one rat was placed at the end of a randomly-chosen arm and allowed to move for 3 min without stimulation. Then the test started, with foot stimulation given until the rat reached the illuminated arm (safe region). During each test, we used a randomized method to vary the orientation of the safe and stimulation regions. The test was considered correct (learned) if it reached the safe region within 10 s, and nine

correct responses out of 10 consecutive foot stimulations (9/10 standard) were required to consider the rat as having reached the learning criterion. All rats reached the learning criterion in the present study. Learning ability was defined as the total number of stimulations needed to reach the criterion during training.

Trace Fear Conditioning (TFC)

Hippocampal–dependent memory in rats was assessed as previously described (Feng et al., 2013). Twenty-four rats were trained to associate an unconditional stimulus (foot shock) and a conditional stimulus (tone) with environment. The training model as followed: shock duration of 2 s, and shock intensity of 0.8 mA; a tone duration of 20 s, and sound level of 80 dB (Sun et al., 2015). Cromolyn was given immediately after the fear conditioning paradigm, and IP LPS injection was performed 30 min later. The training was comprised of an initial exploratory phase (100 s), followed by two trials with a 100 s interval. The trials included a 20 s auditory cue (80 dB, 5 kHz), followed by a 2 s foot shock (0.8 mA). Rats anticipate the shock by "freezing," which is defined as the absence of all movement except respiration; this defensive posture reflects learned fear. When placed in the same context on a subsequent occasion, the learned fear is recalled and the degree of learning and recall can be determined from the extent of freezing. Contextual memory of the learned fear was assessed 1 day after the LPS injection, and freezing behavior in the absence of the tone and shock was automatically scored by video tracking software (Xeye Fcs, Beijing MacroAmbition S&T Development Co., Ltd., Beijing, China) over the course of 300 s. Freezing scores for each subject were expressed as a percentage of the total testing time.

Flow Cytometry Analysis

Flow cytometry was employed to determine microglial activation in mice and receptor expression in rat microglia. As previously described (Dong et al., 2017), the dissociated cells from ipsilateral hypothalamus tissues were incubated with appropriate primary antibody overnight at 4°C, then incubated with 1 μg/ml of FITC-conjugated goat anti-rabbit secondary antibody for 1 h at 37°C. FACSCalibur flow cytometer (BD Biosciences, United States) was used to analyze the cells. For analysis of rat ipsilateral hypothalamus tissues, the primary antibodies were rabbit anti-H_1R, anti-H_2R, anti-H_3R, anti-H_4R, anti-PAR2, and anti-TLR4, or normal rabbit IgG, while FITC-conjugated goat anti-rabbit was used as the secondary antibody with PE-conjugated mouse anti-OX-42 antibody or isotype control (1:200). For analysis of mouse tissues, the primary antibody was rabbit anti-Iba1 or normal rabbit IgG, and the secondary antibody was FITC-conjugated goat anti-rabbit.

In vitro Studies

P815 Cell Culture

The P815, mast cells line derived from mouse tumor cells was kindly provided by Fu Ning, PhD, Department of Immunology, Southern Medical University. As previously described (Zhang X. et al., 2016), the cells were incubated with DMEM medium containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2/95% air. For assays, P815 cells (1×10^6 cells) were treated with or without LPS (1 μg/ml) for 12, 24, 48, and 72 h.

Microglia-Enriched Cultures

Mice primary microglia were prepared as previously described with slight modifications (Dong et al., 2014). Briefly, brain tissues of postnatal (P1–P2) BABL/c mice were grinded; the resulting cells were cultured in poly-D-lysine precoated cell culture flasks with DMEM containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. After reaching a confluent monolayer of glial cells (10–14 days), microglia were separated from astrocytes by shaking for 5 h at 100 r.p.m., then replanted on 24-well culture plates at a density of 10^5 cells/cm^2. The enriched microglia were > 98% pure as determined by expression of Iba1.

Co-culture of P815 Cells and Microglia

As previously described (Zhang X. et al., 2016), P815 cells (1×10^6 cells) were treated with cromolyn for 30 min, then cells were stimulated with LPS (1 μg/ml) for 12, 24, 48, and 72 h. Primary microglia (1×10^6 cells) were treated with conditioned medium (CM) from P815 cells with or without LPS treatment for the given time periods. The conditioned cells were further incubated for 6, 12, and 24 h. In addition, we also stimulated microglia with cromolyn (10 μg/ml), LPS (1 μg/ml), or LPS and cromolyn together.

Cytokines Assay

The expression of TNF-α and IL-6 in rat ipsilateral and contralateral hypothalamus or ipsilateral and contralateral cerebral cortex tissue extracts were quantified with a commercial ELISA kit from R&D Systems (Minneapolis, MN, United States). Histamine and mast cells tryptase contents in the supernatant of mast cells were tested with a detection ELISA kit from Fitzgerald (Birmingham, United Kingdom). The levels of selected cytokines (TNF-α, IL-6, IL-1β, INF-γ, RANTES, and IL-10) in the culture media were measured with a Milliplex kit (Merck & Millipore, United States) following the manufacturer's instructions. All samples were run in duplicate.

Western Blotting

Ipsilateral hypothalamus tissue extracts and primary microglia cells were collected and homogenized in lysis buffer. The cell lysate was used to assess protein expression by western blotting as previously described (Dong et al., 2017). The primary antibodies were: rabbit antibodies against JNK, phospho-JNK, p38, phosphop38, ERK, phospho-ERK, AKT, and phosphoAKT (1:1000). And the secondary antibody was goat-anti-rabbit (1:1000). Protein bands were detected with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, United States).

Flow Cytometry Analysis

Microglia were pelleted by centrifugation at 450 g for 10 min, then fixed in 4% paraformaldehyde for 30 min. After washing, the cells were re-suspended in PBS. Cells were incubated overnight

at 4°C with rabbit anti-H_1R, anti-H_2R, anti-H_3R, anti-H_4R, anti-PAR2, and anti-TLR4 antibodies or normal rabbit IgG. Subsequently, they were incubated at 37°C for 1 h, followed by 1 μg/ml of FITC-conjugated goat anti-rabbit secondary antibody or isotype control (1:200). Finally, the cells were re-suspended in PBS. FACSCalibur flow cytometer (BD Biosciences, United States) was used to analyze the cells.

Statistical Analysis

All values are expressed as means ± SD. Significant differences ($P < 0.05$) between treatments and control were determined by one-way ANOVA followed by the *post hoc* least significant difference test.

RESULTS

Cromolyn Alleviated LPS-Induced Mast Cell Activation and Memory Impairment

To evaluate whether brain mast cells are involved in LPS-induced neuroinflammation, we determined the activation of brain mast cells in the hypothalamus at 30 min or 24 h after LPS injection. Brain mast cells were quantified in tissue sections stained with toluidine blue (TB) and mast cell tryptase (**Figure 1A**). As shown in **Figure 1B**, intraperitoneal injection of 1 mg/kg LPS 30 min or 24 h induced a significant increase in the number of activated mast cells in both the ipsilateral and contralateral hypothalamus as compared with that in the control group. Treatment with mast cell stabilizer cromolyn (200 μg) repressed the mast cell activation induced by LPS IP injection.

Rats were pretreatment of cromolyn (200 μg) 30 min before LPS administration to determine the effect of mast cells on LPS-induced memory impairment. Contextual assessment and Y-maze test were used to assess rats' cognitive function after LPS treatment for 1 day. As shown in **Figure 1C**, the rats exposed to LPS alone exhibited a significant reduction in cognitive function compared to those given only saline. Pre-treatment with cromolyn significantly promoted freezing behavior and the number of learning trials, suggesting cromolyn alleviates LPS-induced memory dysfunction. Together, these results indicate that mast cells play a role in memory impairment induced by LPS and cromolyn can limit the adverse cognitive outcomes caused by endotoxemia.

Stabilization of Mast Cell Inhibited LPS-Induced Microglia Activation in Hypothalamus

The effects of brain mast cells on LPS-induced microglia activation were determined through immunostaining for Iba1, a marker for microglia. IP injection of LPS induced a large number of microglia activation in both ipsilateral and contralateral hypothalamus. The cell morphology changes of activated microglia are processes retraction and cell body enlarged. And activated microglia were showed by notable Iba1-IR positive. Pretreatment of Cromolyn (200 μg) significantly suppressed LPS-induced activation of microglia in both ipsilateral and contralateral hypothalamus (**Figure 2A**). Quantification of Iba1 positive cells in the hypothalamus was shown in **Figure 2B**. However, the effect of Cromolyn is partial inhibition, but not completely reverse. These results indicate that stabilizing mast cells partial suppress LPS-induced activation of hypothalamus microglia.

The mast cell-deficient $Kit^{W-sh/W-sh}$ mice were used to further confirm the role of mast cells in LPS induced microglia activation. As shown in **Figures 2C,D**, IP injection of LPS into WT mice induced significant microglia activation in the hypothalamus 24 h later, but had less effect in the $Kit^{W-sh/W-sh}$ mice. Further characterization by flow cytometry (**Figure 2E**) demonstrated that WT mice stimulated with LPS expressed Iba-1 at levels three times higher than the corresponding saline group. However, mast cell-deficient $Kit^{W-sh/W-sh}$ mice treated with LPS had only two times greater Iba-1 expression than their control group, indicating indicating that mast cells contribute to LPS-induced activation of microglia.

Stabilization of Mast Cell Inhibited LPS-Induced Proinflammatory Factors Production and MAPK Activation in Hypothalamus

Excessive proinflammatory cytokines released from activated microglia is involved in microglia-mediated neuroinflammation. The proinflammatory cytokines IL-6 and TNF-α were detected in present study. We found that IP injection of LPS (1 mg/kg) significantly promoted TNF-α release in ipsilateral and contralateral hypothalamus. IP injection of LPS also upregulated IL-6 and TNF-α content in ipsilateral cerebral cortex and contralateral cerebral cortex. Cromolyn (200 μg) was able to alleviate TNF-α and IL-6 levels in the ipsilateral and contralateral hypothalamus induced by LPS injection of 30 min or 24 h (**Figures 3A,B**). Site-directed injection of cromolyn in the hypothalamus also inhibited the production of TNF-α and IL-6 in cerebral cortex at 24 h after LPS injection, but it is not statistically significant on IL-6 and TNF-α levels in cerebral cortex induced by LPS injection of 30 min. These results indicated that stabilizing mast cells prevents LPS-induced proinflammatory cytokines release.

Mitogen-activated protein kinases (MAPK) and NF-κB are the predominant signaling transduction pathways responsible for the synthesis and production of proinflammatory mediators in LPS-induced neuroinflammation and microglia activation (Akundi et al., 2005; Ciallella et al., 2005). Phosphorylation of AKT is a downstream target of PI3K activation and therefore is a proxy for activation of the PI3K pathway (Desai and Thurmond, 2011). We investigated whether cromolyn could affect LPS-induced phosphorylation of MAPK and AKT. As shown in **Figure 3C**, IP injection of LPS for 30 min or 24 h induced MAPK, AKT, and NF-κB p65 phosphorylation. Cromolyn (200 μg) was given 30 min before LPS treatment partially inhibited LPS-induced MAPK, AKT, and NF-κB p65 phosphorylation. These results indicate that stabilization of mast cells suppress LPS-induced MAPK, AKT, and NF-κB signaling pathway activation.

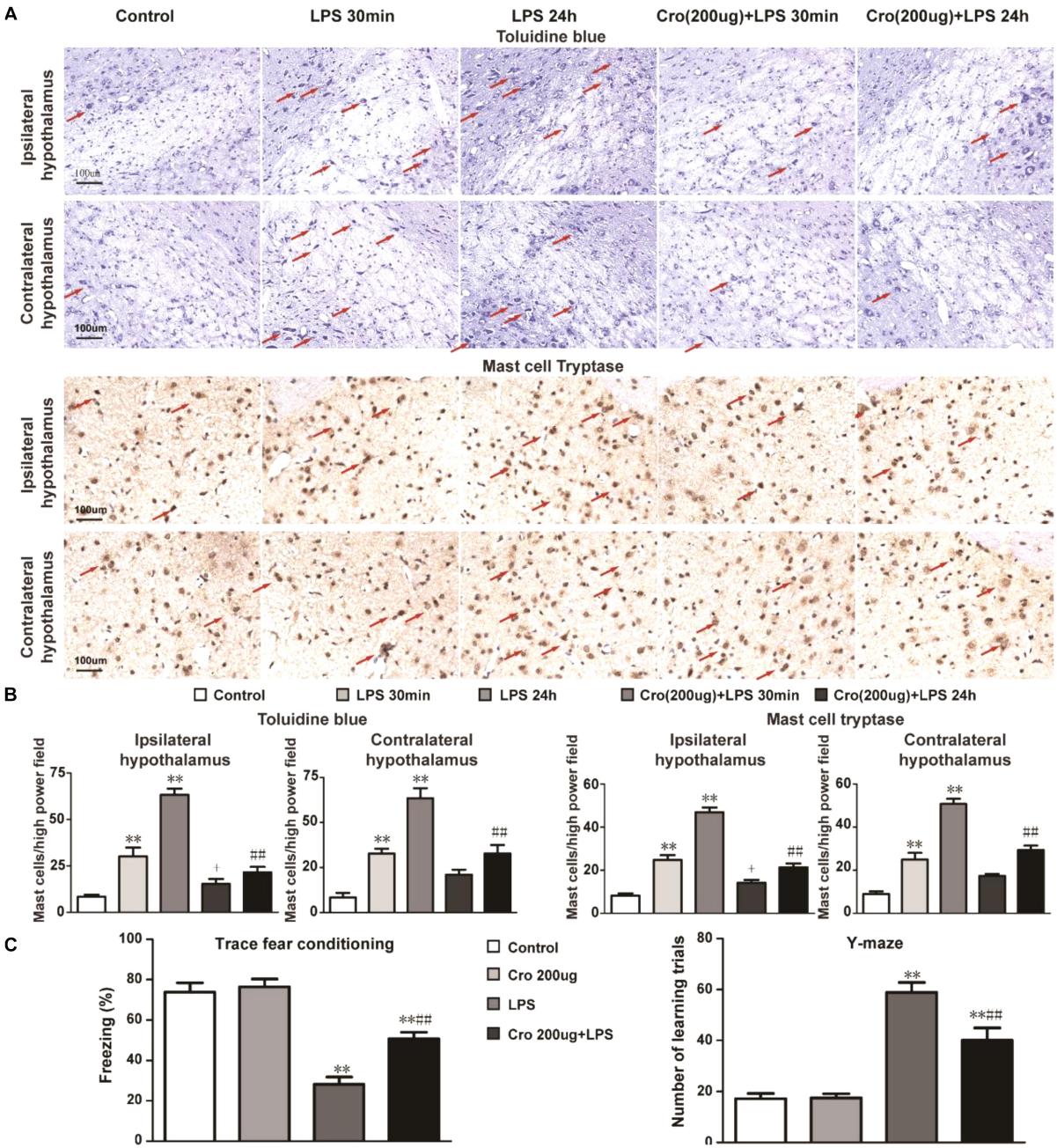

FIGURE 1 | Cromolyn repressed LPS-induced mast cell activation in rat hypothalamus and memory impairment. **(A)** Rat brain mast cells were stained with toluidine blue and Mast Cell Tryptase. Scale bar: 100 μm. **(B)** Quantification of activated mast cells in rat ipsilateral and contralateral hypothalamus. $^{**}P < 0.01$ vs. Control group. $^{+}P < 0.05$ vs. LPS 30 min group, $^{\#\#}P < 0.01$ vs. LPS 24 h group. **(C)** Context fear response, as measured by freezing behavior, was determined in the rats. The Y-maze test was performed after TFC in the rats. $^{**}P < 0.01$ vs. Control group. $^{\#\#}P < 0.01$ vs. LPS 24 h group. The data are presented as the mean ± SD ($n = 6$).

LPS Changed Receptor Expression in Hypothalamus Microglia

Degranulated mast cells can release tryptase and histamine, which induced microglial activation and inflammatory cytokines release (Zhang S. et al., 2012; Dong et al., 2014). Flow cytometry analysis was used to explore whether LPS-induced activated mast cells can change some receptor proteins expression in microglia. As shown in the **Figure 4**, the expressions of H_1R, H_4R, PAR2, and TLR4 were upregulation, but H_2R and H_3R expressions were deregulation in the ipsilateral hypothalamus microglia after IP injection of LPS for 24 h. Pretreatment of cromolyn (200 μg) 30 min before LPS administration inhibited

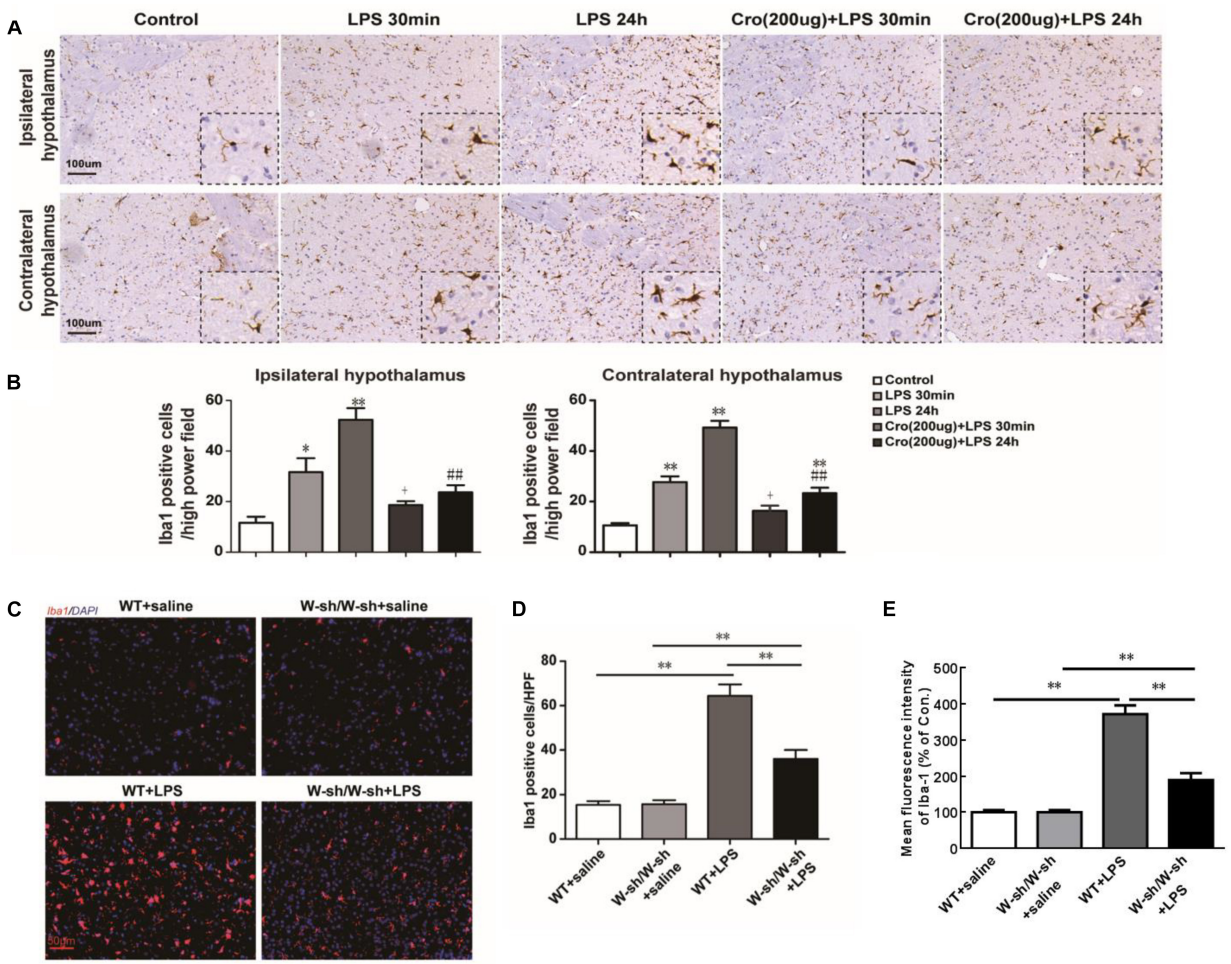

FIGURE 2 | Stabilization of mast cell inhibited LPS-induced microglia activation in hypothalamus. **(A,B)** Immunohistochemical analysis was used to detect Iba1, markers of microglia, expression in rat hypothalamus. The activated microglia cells had larger cell body, poorly ramified short and thick processes. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group. $^{+}P < 0.05$ vs. LPS 30 min group, $^{\#\#}P < 0.01$ vs. LPS 24 h group. **(C)** The effect of LPS on microglia activation was decreased in mast cell-deficient $Kit^{W-sh/W-sh}$ mice. Immunofluorescence was used to detect Iba1 expression in mice hypothalamus. **(D)** Quantification of Iba1 positive cells in the hypothalamus. **(E)** For flow cytometry analysis of the expression of Iba. $^{**}P < 0.01$. Data are presented as the mean ± SD ($n = 6$). HPF, high power field.

LPS-induced TLR4 upregulation. However, cromolyn only had a tendency to inhibit the effect of LPS on other receptor protein expression in microglia, but it has no statistical significance. These results indicate that LPS-induced mast cells activation change some receptor expression in hypothalamus microglia, and mast cell stabilizer cromolyn had a tendency to inhibit the effect of LPS.

Cromolyn Inhibited LPS-Induced P815 Cells Degranulation

To observe the mast cells activation and the effect of LPS on mediator release, we quantified the expression levels of histamine and mast cell tryptase in the supernatant after stimulation with LPS based on previously described method (Zhang X. et al., 2016). As shown in **Figures 5A,B**, LPS (1 μg/ml) stimulation of P815 cells for 12, 24, and 48 h all increased the level of histamine and tryptase, indicating that degranulation was induced. We also quantified the secretion of a number of cytokines (TNF-α, IL-6, IFN-γ, RANTES, IL-1β, GM-CSF, and IL-10). In contrast to histamine and mast cell tryptase released, stimulation of P815 with LPS for 48 h had no effect on the levels of secreted cytokines (**Figures 5C–I**). Treatment with cromolyn (10 μg/ml) alone did not induce histamine and tryptase release from P815, but did inhibit the LPS-induced histamine and tryptase release from P815 cells (**Figures 5J,K**). These results indicate that LPS treatment (12–48 h) can induce P815 cells degranulation, and this process is inhibited by the mast cell stabilizer cromolyn.

Cromolyn Inhibited Activated P815 Cell-Induced Pro-inflammatory Cytokines Production From Microglia

Conditioned medium (CM) from P815 cells given different treatment was used to explore the effects of activated mast cells on the activation of primary microglia. Microglia were

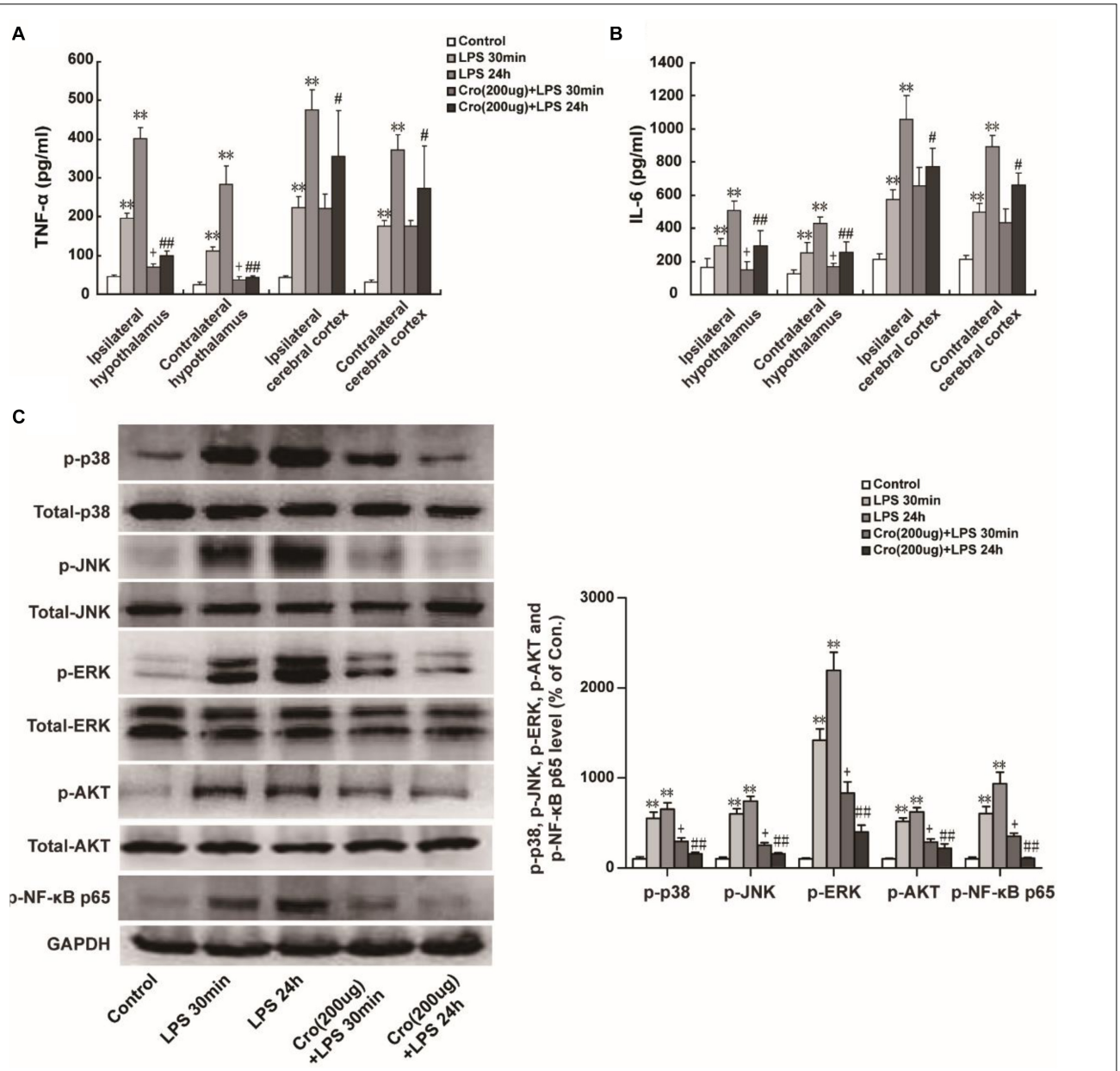

FIGURE 3 | Stabilization of mast cell inhibited LPS-induced proinflammatory factors production and MAPK activation in hypothalamus. IP injection of LPS (1 mg/kg) for 30 min or 24 h significantly increased TNF-α **(A)** and IL-6 **(B)** expression in ipsilateral and contralateral hypothalamus or ipsilateral and contralateral cerebral cortex in rats. Furthermore, Cromolyn (200 μg) could partial inhibit the effect of LPS. $^{**}P < 0.01$ vs. Control group. $^{+}P < 0.05$ vs. LPS 30 min group,$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS 24 h group. **(C)** Protein expressions of p-p38, p-JNK, p-ERK, p-AKT, and p-NF-κB p65 in ipsilateral hypothalamus of rats were examined by Western blotting. $^{**}P < 0.01$ vs. Control group. $^{+}P < 0.05$ vs. LPS 30 min group, $^{\#\#}P < 0.01$ vs. LPS 24 h group. Data are presented as the mean ± SD ($n = 6$).

incubated with CM for an additional 6, 12, and 24 h. Cytokine levels (TNF-α, IL-6, IFN-γ, RANTES, IL-1β, GM-CSF, and IL-10) in the supernatant were quantified to estimate the levels of microglia activation. Compared with control group, CM from P815 cells treated with LPS (1 μg/ml) evoked inflammation-related cytokines TNF-α, IL-6, RANTES, IL-1β, and IL-10 production from microglia (**Figure 6A**).

However, residual LPS in the CM will continue to stimulate microglia; therefore, we cannot conclusively determine the effect of this CM on microglia activation. To address this issue, cytokines levels in the supernatant of microglia stimulated by LPS alone and in the supernatant of microglia stimulated by CM from P815 cells with LPS were compared. As shown in the **Figure 6B**, the levels of pro-inflammatory cytokines TNF-α, RANTES, and IL-1β were significantly upregulated for microglia stimulated by CM from LPS-induced P815 (48 h) than that stimulated by LPS alone at longer timepoints (24 h). This process was inhibited by treatment with the mast cell stabilizer cromolyn (10 μg/ml). We

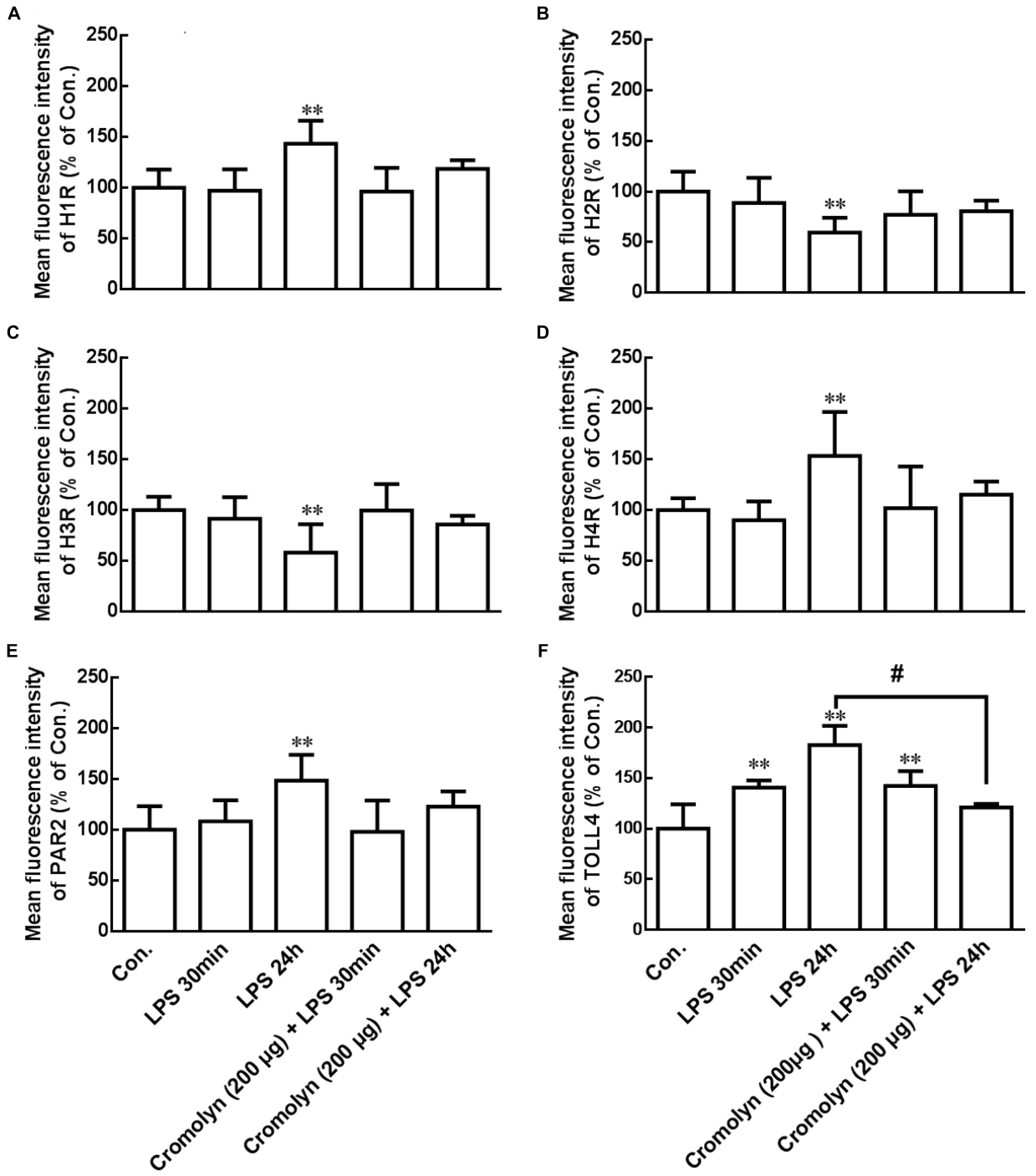

FIGURE 4 | LPS induced receptor change in microglia of hypothalamus. Flow cytometry analysis was used to determine the receptor expression of H_1R, H_2R, H_3R, H_4R, PAR2, and TLR4 in the rat ipsilateral hypothalamus microglia. Mast cell stabilizer cromolyn inhibits LPS-induced TLR4 increase on microglia in rat hypothalamus. $^{**}P < 0.01$ vs. Control group. $^{\#}P < 0.05$ vs. LPS 24 h group. Data are presented as the mean ± SD ($n = 6$).

also found that the level of GM-CSF released from microglia was significantly inhibited by CM from P815 with LPS as compared with that by LPS alone, which effect was partially reversed by cromolyn. However, there was no significant difference observed in between the level of anti-inflammatory cytokine IL-10 in microglia stimulated by CM from P815 with LPS and that stimulated by LPS alone. We also found that cromolyn alone had no effect on cytokine production from microglia with or without LPS. Therefore, the inhibitory effect of cromolyn on microglia activation relies on reducing mast cell activation.

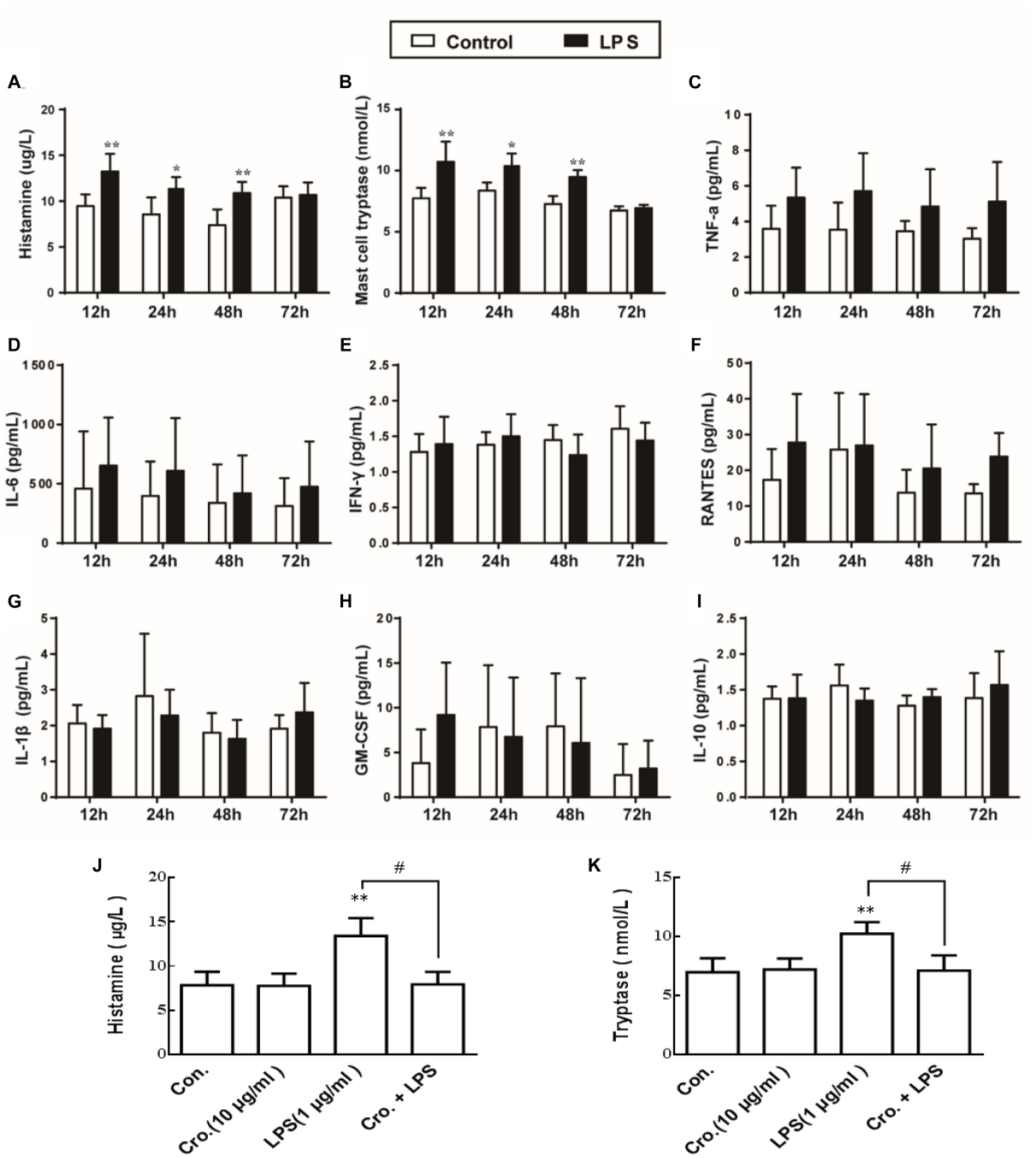

FIGURE 5 | Cromolyn inhibited LPS-induced P815 cells degranulation. P815 cells were treated with LPS for 12, 24, and 48 h. ELISA was used to detect the expression of **(A)** histamine, **(B)** tryptase, **(C)** TNF-α, **(D)** IL-6, **(E)** INF-γ, **(F)** RANTES, **(G)** IL-1β, **(H)** GM-CSF, and **(I)** IL-10 in the medium. Cromolyn inhibited LPS-induced histamine **(J)** and tryptase **(K)** release from P815 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group. $^{\#}P < 0.05$ vs. LPS group. The data are presented as the mean ± SD ($n = 4$).

MAPK Signaling Pathways Were Involved in the Mast Cells-Induced Microglia Activation

A previous *in vivo* study identified the MAPK and AKT signaling pathways as important in mast cells-induced microglia activation; we therefore validated the involvement of these signaling pathways *in vitro*. Treatment of microglia with CM from LPS-stimulated P815 cells resulted in sharp upregulation of phosphorylation of AKT and P38, this result was ameliorated by pretreatment with cromolyn (10 μg/ml) (**Figures 7A,B**).

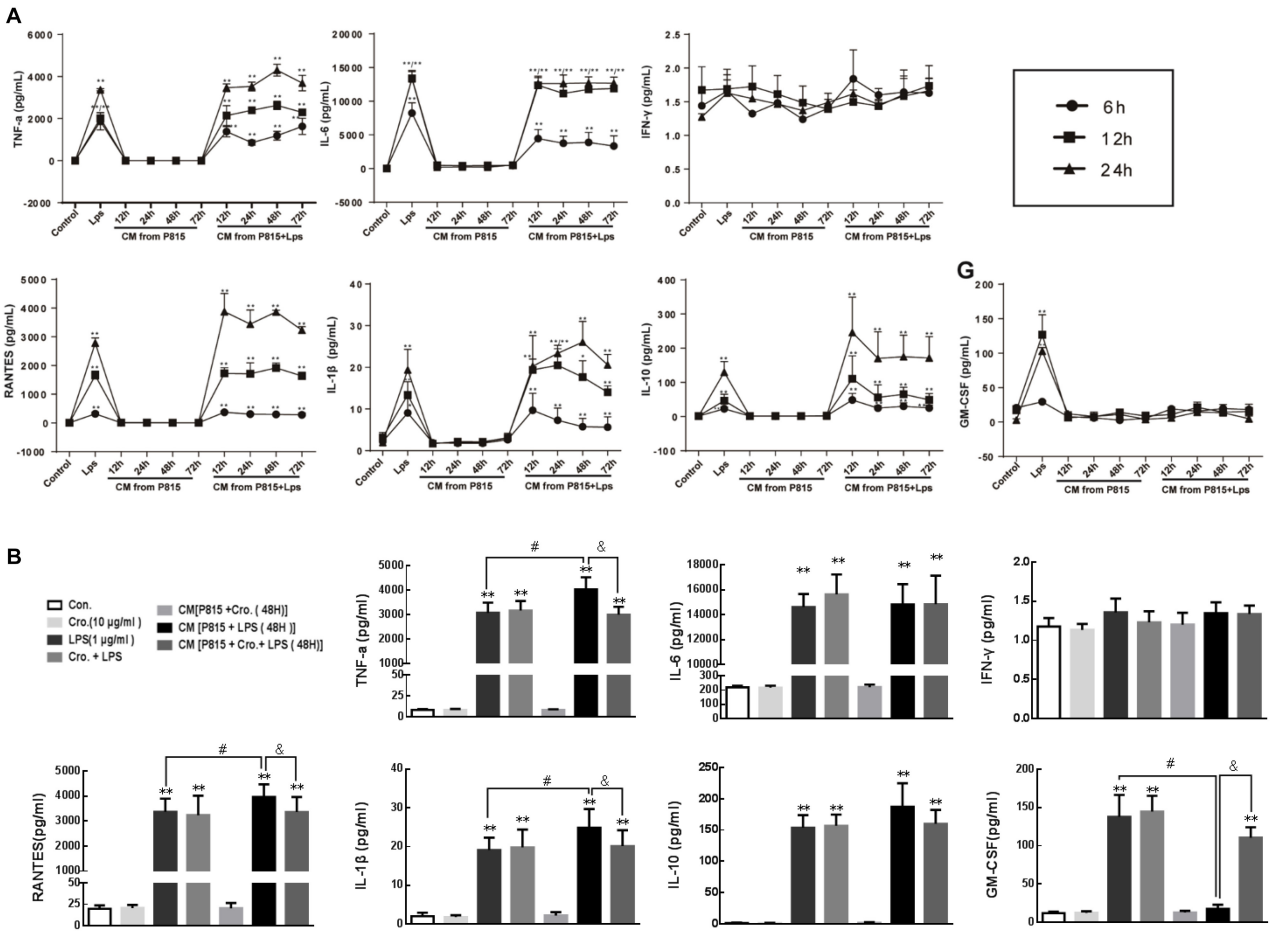

FIGURE 6 | Cromolyn inhibited activated P815 cell-induced pro-inflammatory cytokines production from microglia. **(A)** Primary microglia was treated with CM from P815 cells with or without LPS stimulation for 12, 24, 48, and 72 h for different incubation times (6, 12, and 24 h). ELISA was used to detect the expression of TNF-α, IL-6, INF-γ, RANTES, IL-1β, IL-10, and GM-CSF. **(B)** TNF-α, RANTES, and IL-1β levels in microglia stimulated by CM from P815 with LPS (48 h) were higher than that stimulated by LPS alone at longer time points (24 h), which was inhibited by mast cell stabilizer cromolyn (10 μg/ml). $^{**}P < 0.01$ vs. Control group. $^{\#}P < 0.05$ vs. LPS group. $^{\&}P < 0.05$ vs. CM from P815 with LPS (48 h) group. The data are presented as the mean ± SD ($n = 4$).

These changes were consistent with *in vivo* study, and support that MAPK signaling may be involved in the activation of microglia by mast cells.

Cromolyn Inhibited Activated P815 Cell-Induced H_1R, H_4R, and TLR4 Increase in Microglia

In vivo, we found that LPS induced receptor protein expression change in microglia of the hypothalamus. Mast cell stabilizer cromolyn, however, can inhibit the effect of LPS. To further support this, the effect of LPS on receptor protein expression change in microglia was examined *in vitro* by flow cytometry.

P815 cells were stimulated by treatment with LPS (1 μg/ml) for 24 h. As shown in **Figure 8**, this stimulation downregulated H_2R and H_3R, and upregulated TLR4 in microglia. Treatment with cromolyn (10 μg/ml) alone did not affect receptor protein expression in microglia with or without the stimulation of LPS, but did inhibit the increased expression of H_1R, H_4R, and TLR4 in microglia stimulated by CM from P815 with LPS (48 h) These results suggest that LPS-induced P815 cell activation can stimulate H_1R, H_4R, and TLR4 upregulation in microglia and that inhibitory effect of cromolyn acts only through mast cells.

DISCUSSION

One of the most important revelations in neuroscience research is the realization of communication between the immune system and CNS. Proinflammatory cytokines released by microglia (the brain's resident macrophages), play a key role in this communication, and also have fundamental roles in neurodegenerative diseases. Microglia also responds to mediators released from other immune cells, such as mast cells (Zhang S. et al., 2012; Dong et al., 2014). We have previously found that histamine, tryptase and mast cell degranulation can all induce microglia activation. However, the role of mast cells in LPS-induced microglia activation, neuroinflammation and

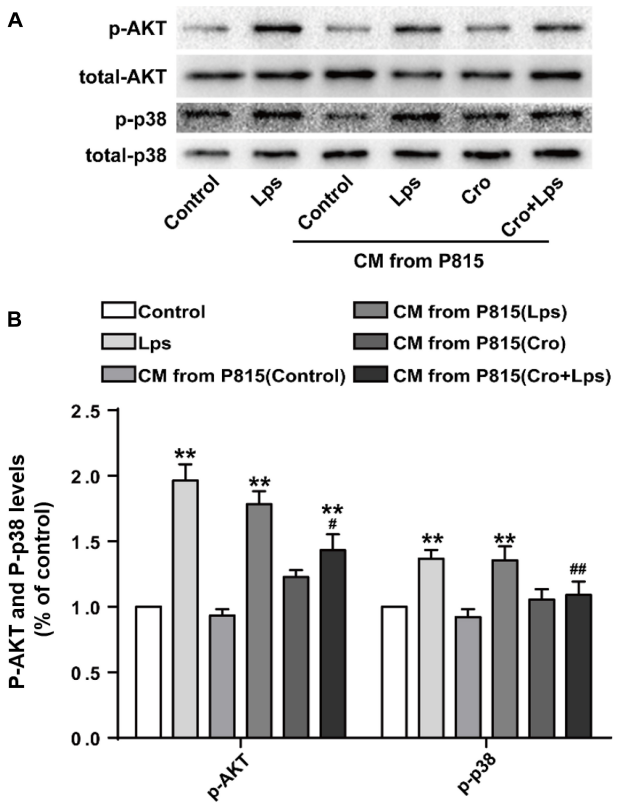

FIGURE 7 | MAPK signaling pathways were involved in the mast cells-induced microglia activation. **(A)** The expression of p-AKT and p-p38 were detected by Western blotting. **(B)** Levels of p-AKT and p-p38 were quantified and normalized to GAPDH levels. Each value was expressed relative to the control, which was set to 1. $^{**}P < 0.01$ vs. Medium group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CM from P815 with LPS (48 h) group. The data are presented as the mean ± SD ($n = 4$).

cognitive impairment has not been clarified. In this paper, we report that the stabilization of mast cells inhibits LPS-induced microglia activation and neuroinflammation and also alleviates LPS-induced cognitive dysfunction.

Neuroinflammation is an underlying pathological component in a wide range of neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and multiple sclerosis (Skaper et al., 2017). In treating neuroinflammation, considerable efforts has been directed to restraining the inflammatory cascade of peripheral phagocyte and neutrophil infiltration and glia activation; less focus has been placed on the ability of resident brain cells, such as mast cells, to initiate an immediate host response in the meninges and cerebral parenchyma (Silver and Curley, 2013).

Microglia are regarded as the tissue-specific macrophages of the brain. Microgliosis is an important element of brain's response to inflammation, which has an increased number of microglia (Ransohoff, 2007). In previous studies, we found that mast cell tryptase and histamine can stimulate microglia activation through the PAR2, H_1R, and H_4R (Zhang S. et al., 2012; Dong et al., 2014), and that mast cell degranulation induced by compound 48/80 can promote microglia activation (Dong et al., 2017). Thus, we hypothesized that mast cells may play a key role in neuroinflammation. In the present study, we found that IP injection of LPS for 30 min or 24 h induced activation of mast cells and microglia in both the ipsilateral and contralateral hypothalamus. Coincident with the change in microglia activation in the hypothalamus, we also observed an increase in the proinflammatory cytokines TNF-α and IL-6 contents of the hypothalamus and cortex, which may have been released from activated microglia. Levels of these cytokines were higher in the cortex, suggesting that LPS induces more production of proinflammatory cytokines in the cortex than in the hypothalamus.

Mast cells are found on the brain side of the BBB. They lie in close proximity to the basal side of blood vessel walls (Khalil et al., 2007), and they act not only as first responders in harmful situations but also as environmental "sensors" to communicate with glia, the extracellular matrix, and even neurons (Lindsberg et al., 2010). Activated brain mast cells can promote BBB breakdown and neutrophil infiltration, resulting in neuroinflammation that contributes to postoperative cognitive dysfunction. Therefore, inhibition of mast cell activation should be neuroprotective. In the present study, inhibiting mast cell degranulation by the mast cell stabilizer cromolyn limited microglia activation and release of TNF-α and IL-6, and alleviated LPS-induced cognitive impairment. Specifically, site-directed injection of cromolyn into the hypothalamus inhibited the production of TNF-α and IL-6 induced by LPS in both hypothalamus and cerebral cortex. However, the inhibitory effect on cortex cytokine production occurred only at 24 h after LPS injection; no significant effect was observed at 30 min after injection. This may be because the number of mast cells in the cerebral cortex is much less than that in the hypothalamus, so the effects of cromolyn are slowed in the cortex.

The mast cell deficient $Kit^{W-sh/W-sh}$ mice were used to further demonstrate the role of mast cells in LPS-induced neuroinflammation. We found that LPS IP injection induced significantly microglia activation in WT mice, while LPS had less effect on microglia in $Kit^{W-sh/W-sh}$ mice, indicating that activated mast cells, as "first responders," can evoke, expand and prolong immune responses (Silver and Curley, 2013).

The factors from brain mast cells that responsible for the over activation of microglia have not been illuminated to date. Mast cells are characterized by a rapid release a large number of chemokines via degranulation (Feuser et al., 2012). Mast cell secretory mediators released in the CNS change the functions of T cell (Jutel et al., 2001), vascular elements (Esposito et al., 2002), and neuron (Koszegi et al., 2006). These cytokines further induce microglia activation (Chakraborty et al., 2010; Skuljec et al., 2011). We have found *in vitro* that tryptase released from mast cells induced microglia activation through PAR2-MAPK-NF-κB signaling pathways (Zhang S. et al., 2012), and histamine induced microglia activation via the H_1R and H_4R-MAPK and PI3K/AKT- NF-κB signaling

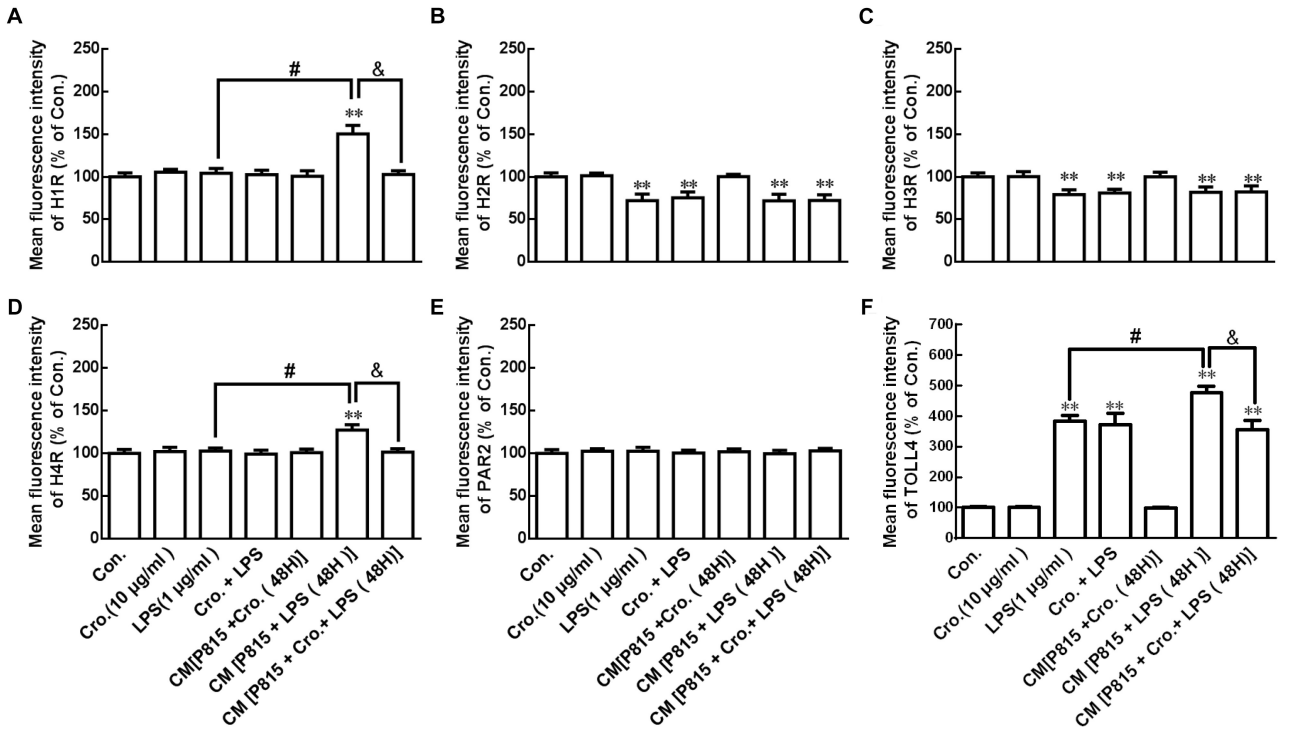

FIGURE 8 | Cromolyn inhibited activated P815 cell -induced H_1R, H_4R, and TLR4 increase in microglia. Flow cytometry analysis was used to determine the receptor expression of H_1R, H_2R, H_3R, H_4R, PAR2, and TLR4 in microglia. $^{**}P < 0.01$ vs. Control group. $^{\#}P < 0.05$ vs. LPS group. $^{\&}P < 0.05$ vs. CM from P815 with LPS (48 h) group. The data are presented as the mean ± SD ($n = 4$).

pathways (Dong et al., 2014). *In vivo*, we found that site-directed injection of the mast cell degranulator Compound 48/80 in the hypothalamus induced microglia activation and increased microglial expression of H_1R, H_4R, PAR2, and TLR4 (Dong et al., 2017). Here, we demonstrate that IP injection of LPS also significantly increased the expression of H_1R, H_4R, PAR2, and TLR4 in microglia after 24 h. After LPS-induced degranulation of mast cell, the released mediators combine with these receptors on microglia to induce microglial activation, followed by activation of the MAPK and AKT-NF-κB signaling pathways. These contribute to exacerbate neuroinflammation-related disease (Skaper et al., 2014). Notably, pretreatment with cromolyn (200 μg) 30 min before LPS administration inhibited LPS-induced TLR4 upregulation. Cromolyn treatment also tended to inhibit the effects of LPS on microglial expression of other receptor proteins, but the results were not statistically significance due to high SD values. Nonetheless, we cannot completely deny the inhibitory effect of cromolyn on LPS-induced receptor protein expression.

In vitro, we found that 1 μg/ml LPS stimulated P815 mast cells to release histamine and tryptase, but had no effect on mast cell production of TNF-α, IL-6, IFN-γ, RANTES, IL-1β, GM-CSF, or IL-10. This suggests that TNF-α is not actually released from mast cells, and therefore mast cells have no role in LPS-induced inflammation mediated by TNF-α. Furthermore, the mast cell stabilizer cromolyn inhibits LPS-induced mast cell degranulation.

We also found that in the absence of mast cells, cromolyn had no effect on the production of cytokines by microglia, with or without LPS stimulation. In contrast, the conditioned media (CM) from LPS-induced P815 mast cells could induce microglia to release TNF-α, IL-6, RANTES, IL-1β, and IL-10. Notably, LPS remains present in the conditioned media in at least trace amounts, and can activate microglia directly. However, TNF-α, RANTES, and IL-1β levels 48 h after stimulation by CM from LPS-induced P815 cells were higher than in microglia stimulated by LPS alone (24 h). Furthermore, CM stimulation of microglia was inhibited by if the mast cells were also treated with the mast cell stabilizer cromolyn (10 μg/ml). We also found that the release of GM-CSF from microglia was significantly inhibited by CM from LPS-induced P815 cells comparing to that induced by direct LPS, and this release was partially inhibited by cromolyn. Histamine and tryptase released from LPS-induced P815 cells may have a role in inhibiting GM-CSF release from microglia; further research is needed to confirm this effect.

While most cytokines released by microglia activated by LPS are pro-inflammatory, LPS also induces release of the anti-inflammatory cytokine IL-10. Notably, we observed no significant difference in IL-10 levels in microglia stimulated by CM from LPS-induced P815 compared to those stimulated by LPS alone, suggesting that IL-10 release was solely induced by LPS. This implies that activated mast cells can only provoke the release of pro-inflammatory cytokines

from microglia. These findings are also consistent with our previous report that released products of activated P815 cells changed microglial phenotypes toward M1/2b (Zhang X. et al., 2016). Therefore, mast cells may have a role in modulating the timing of cytokine release by microglia, thereby prolonging the inflammatory response.

In summary, the present study demonstrates that LPS can induce mast cell degranulation, which stimulates the production of inflammatory factors by activated microglia, and this process can be inhibited by the "mast cell stabilizer" cromolyn. These results imply that mast cell degranulation can aggravate LPS-induced neuroinflammation by evoking microglia activation, and the stabilization of mast cells can inhibit LPS-induced neuroinflammation and memory impairment. Investigating the role of mast cell activation in neuroinflammation is an important emerging research topic that needs to be explored in order to understand and effectively treat neuroinflammation-related diseases.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

SZ and HDi conceived and designed the study. HDo, XianZ, XiaoZ, and YW developed the methodology. HDo, YW, and XiaoZ acquired the data. SZ, HDo, and YQ analyzed and interpreted the data. HDo, SZ, and YW wrote, reviewed, and/or revised the manuscript. SZ supervised the study.

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Brain Histamine Modulates the Antidepressant-Like Effect of the 3-Iodothyroacetic Acid (TA1)

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3-iodothyroacetic acid (TA1), an end metabolite of thyroid hormone, has been shown to produce behavioral effects in mice that are dependent on brain histamine. We now aim to verify whether pharmacologically administered TA1 has brain bioavailability and is able to induce histamine-dependent antidepressant-like behaviors. TA1 brain, liver and plasma levels were measured by LC/MS-MS in male CD1 mice, sacrificed 15 min after receiving a high TA1 dose (330 μgkg^{-1}). The hypothalamic mTOR/AKT/GSK-β cascade activation was evaluated in mice treated with 0.4, 1.32, 4 μgkg^{-1} TA1 by Western-blot. Mast cells were visualized by immuno-histochemistry in brain slices obtained from mice treated with 4 μgkg^{-1} TA1. Histamine release triggered by TA1 (20–1000 nM) was also evaluated in mouse peritoneal mast cells. After receiving TA1 (1.32, 4 or 11 μgkg^{-1}; i.p.) CD1 male mice were subjected to the forced swim (FST) and the tail suspension tests (TST). Spontaneous locomotor and exploratory activities, motor incoordination, and anxiolytic or anxiogenic effects, were evaluated. Parallel behavioral tests were also carried out in mice that, prior to receiving TA1, were pre-treated with pyrilamine (10 $mgkg^{-1}$; PYR) or zolantidine (5 $mgkg^{-1}$; ZOL), histamine type 1 and type 2 receptor antagonists, respectively, or with *p*-chloro-phenylalanine (100 $mgkg^{-1}$; PCPA), an inhibitor of serotonin synthesis. TA1 given i.p. to mice rapidly distributes in the brain, activates the hypothalamic mTOR/AKT and GSK-3β cascade and triggers mast cells degranulation. Furthermore, TA1 induces antidepressant effects and stimulates locomotion with a mechanism that appears to depend on the histaminergic system. TA1 antidepressant effect depends on brain histamine, thus highlighting a relationship between the immune system, brain inflammation and the thyroid.

Keywords: 3-iodothyroacetic acid, thyroid hormone, histamine, mast cells, antidepressant effect

Abbreviations: BBB, blood brain barrier; FST, forced swim test; H1R, type 1 histamine receptor; H2R, type 2 histamine receptor; PCPA, p-chloro-phenylalanine; PYR, pyrilamine; T1AM, 3-iodothyronamine; TA1, 3-iodothyroacetic acid; TST, tail suspension test; ZOL, zolantidine.

INTRODUCTION

Recent evidence indicates that thyroid hormone metabolism may generate compounds endowed of behavioral and metabolic effects.

The TA1 is the last iodinated thyroacetic acid produced by sequential metabolism of thyroid hormone carried out by deiodinases and amine oxidases activities. While little is still known about the TA1 tissue levels and their physiological significance, the pharmacological effects of TA1 have been extensively studied. In this respect, we have described that the systemic administration of low TA1 doses (μgkg^{-1}) induced central and peripheral effects. The central effects that were reported include the activation of neuronal signaling, such as the pro-survival PI3K/AKT cascade (Laurino et al., 2018b), the stimulation of memory and reversion of scopolamine-induced amnesia (Musilli et al., 2014; Laurino et al., 2015a), the stimulation of wakefulness in mice prone to sleep for a high acute dose of ethanol (Laurino et al., 2018a). In addition, recent data indicate that TA1 may behave as a potent anticonvulsant and neuroprotective agent against excitotoxicity (Laurino et al., 2018b). Among peripheral effects, TA1 has been reported to induce itching, along with a reduction of noxious and painful sensitivity (Laurino et al., 2015b). Common determinants of TA1 central and peripheral effects are their rapid onset (within 15 min of administration), the fact that they were described by typical inverted U-shaped dose-effect curves, and their dependence on the activation of the histaminergic system. Such behavioral effects indicate that TA1 may cross the BBB and that histamine might derive from a site where it is "ready to be released." However, direct evidence of brain distribution, as well as the source of histamine involvement in TA1 behavioral effects remain elusive.

In the brain, histamine is present in the histaminergic neurons, that are localized in the hypothalamus from where they project to most of the brain areas, but also in non-neuronal cells including mast cells, that are multifunctional bone marrow-derived tissue-dwelling cells and are considered one of the major sources of histamine in body tissues, including the brain. Mast cells are mainly localized along the blood vessels on the brain side of the BBB (Skaper and Fusco, 2014) and are considered crucial mediators of glial cells-neurons communication, whose main signaling pathway is represented by the activation of the PI3K/AKT cascade (Dong et al., 2017). At the BBB, mast cells represent the first-line of defense against brain invasion by xenobiotics, quickly releasing pre-stored and newly synthesized mediators, including histamine, serotonin and other pro-inflammatory signals (Theoharides and Cochrane, 2004). Despite the low number of mast cells in the healthy brain, it is estimated they can store up to 50% of the brain histamine (Yamatodani et al., 1982). Potentially, mast cells may be a target for TA1. Indeed, recent evidence indicates that histamine, irrespective of its derivation (neuronal and not neuronal), is implicated in the regulation of feeding and sleep-awake cycle (Lin et al., 1986), as well as in the stimulation of locomotion and emotional behaviors, including modulation of anxiety and depression (Lamberti et al., 1998; Wada et al., 1991; Chikahisa et al., 2013).

The aim of the present work is to investigate whether TA1: (i) has brain bioavailability after systemic administration, (ii) can trigger histamine release from mast cells and (iii) is endowed of histamine-dependent antidepressant-like effects in rodents.

MATERIALS AND METHODS

Animals

Male CD1 mice (weight: 20–30 g) purchased from ENVIGO (Italy) were used in the present study. Five mice were housed per cage. Cages were placed in the experimental room 24 h prior to testing to ensure adaptation. Animals were housed at 23 ± 1°C under a 12 h light–dark cycle (lights on at 07:00) and were fed a standard laboratory diet with *ad libitum* access to water. Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). The experimental protocols were approved by the ethical Committee of the Italian Council of Health, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

For this study we received the permission from the Ethical Committee for animal health 176/2017-PR) from the Italian Ministry of Health.

Male CD1 mice (20–30 g) from ENVIGO (Italy) were used. The animals were kept at 22 ± 1°C with a 12 h light–dark cycle (light on at 07:00 h) and were fed a standard laboratory diet with water *ad libitum*. Five mice were housed per cage.

Determination of TA1 Brain, Liver and Plasma Distribution After Systemic Administration

Twenty male mice were divided in two groups of 10 mice each. One group received injection i.p. of a saline solution (control mice), while the other received 330 μgkg^{-1} TA1 dissolved in saline solution (Veh) (Laurino et al., 2018a). This dose which was approximately ten times higher than the doses of TA1 found active on mice behavior, Was chosen to be sure to detect TA1 levels in the analyzed tissues.

Five mice from each group were sacrificed 15 min after TA1 administration, the remaining mice (five from each group) were sacrificed after 60 min. At each time point, the blood, the brain and the liver were removed and quickly frozen until used to assess the TA levels by liquid chromatography tandem mass spectrometry (LC/MS-MS). Due to the small amount of plasma obtained, TA1 determination was carried on a pooled plasma sample from five control or treated mice (at 15 and 60 min after treatment).

Assays were performed on plasma (300 μL), brain and liver samples (130–190 mg). Each sample was placed in a 2 mL Precellys tube, 1 ml of 85:15 (v/v) Acetonitrile/0.1 M

HCl $_{(aq)}$ solution containing 5 nM internal standard (TA1-D4) was added, and the sample was sonicated for 30 minutes and then homogenized using a Precellys 24 beads grinder (2.8 mm ceramic, zirconium oxide, beads). The homogenate of each sample was placed in an ultrasound bath (LBS1 3Lt, Falc Instruments, Treviglio, Italy) for 15 minutes and then centrifuged for 15 min at 1300 × *g* at room temperature. The residual pellet was discarded and the supernatant was placed in a new 12 ml glass centrifuge tube. The solution was subjected to liquid/liquid extraction with hexane (3 mL ×1 mL). The upper phase (hexane) was discarded and the lower phase (acetonitrile) was dried under a gentle stream of nitrogen at 45°C. Samples were then dissolved in 100 μL reconstitution solvent mixture (H_2O:MeOH, 70:30) and analyzed using LC-MS/MS to assess TA1 concentration as described elsewhere (Saba et al., 2010).

In vivo Mast Cells Staining

CD1 mice were treated intraperitoneally with Veh or 4 μgkg^{-1} TA1. After 15 min from injection, animals were sacrificed by CO_2 inhalation and brains were collected and fixed for 24 h in Mota fluid (1% lead acetate in 49.57% Absolute ethanol 49.75% Water and 0.5% Acetic acid), dehydrated in graded ethanol and embedded in paraffin. The presence of mast cells and their content in secretion granules were highlighted by both conventional histological staining and histochemistry. In particular, 5 μm thick histological coronal sections collected at the hippocampus level, were stained with Astra blue (Fluka, Buchs, Switzerland). This cationic dye binds specifically to heparin contained in the mast cell granules (Cinci et al., 2010). Mast cells were histochemically labeled with FITC conjugated avidin (1:400; Sigma Aldrich, Milan, Italy). Avidin is able to electrostatically bind with high sensitivity to mast cell granules (Bacci et al., 2014).

Mice Peritoneal Mast Cells Isolation and Culture: The Effect of TA1 on Histamine Release

Mast cells were isolated from peritoneum of CD1 mice as described in Meurer et al. (Meurer et al., 2016). Briefly, a small incision below the sternum of the animal was performed without puncturing the peritoneum. 10 ml of ice cold sterile PBS were injected in the peritoneal cavity and a soft massage of about 30 s was performed. Cell suspension was centrifuged at 4°C at 300 × *g* for 10 min, re-suspended in 5 ml of PBS and then centrifuged again at 4°C at 300 × *g* for 10 min. Cells were suspended in RPMI medium and incubated at 37°C and 5% CO_2. After 3 days, not adherent cells were removed and fresh culture medium was added. After 6 days, 5 ml of fresh medium were added and at day 10 mast cells (represented by the non-adherent population) were harvested and used for experiments.

Mast cells were plated in 12 well plate (about 25000 cells/well) and treated with vehicle (PBS) alone, 5 mg/ml *Apis mellifera* venom (ENTOMON s.a.s Florence, Italy, gently gifted by Prof. Stefano Turillazzi and prepared in vehicle) or 20 nM, 100 nM, and 1 μM of TA1. Culture media were harvested after 5, 15, 30, 60, 90, and 120 min after treatment. Histamine was measured by the method described in Tiligada et al. (2000) with some modifications. Briefly, 50 μl of each sample was incubated with 12.5 μl of 0.44 M of NaOH+ 0.1% O-phthaldialdehyde (Sigma Aldrich, Milan, Italy) for 10 min at RT. Then the reaction was stopped by adding 12.5 μl of 0.5 M HCl and fluorescence was read by a microplate reader (360 nm excitation and 450 nm emission). Results for each treatment were expressed as baseline-corrected (Veh) fluorescence.

Vehicle or 1 μM TA1 treated cells were placed on a slide after 15 min of treatment and stained with Astra blue to visualize histamine content.

Determination of Signaling Activity of TA1 at Hypothalamus

Twelve male CD1 mice were randomly divided in 4 groups of 3 mice each. One group received i.p. Veh the other mice received 0.4 or 1.32 or 4 μgkg^{-1} TA1. Mice were sacrificed 15 min after administration to remove the hypothalamus. The tissue was frozen at −80°C until used for western-blot analysis.

Western Blot

Proteins (20 μg) isolated from mouse hypothalamus were separated via 4–20% SDS-PAGE and transferred into PVDF membranes (60 min at 398 mA) using standard procedures. Blots were incubated overnight at 4°C with specific antibodies against p- AKT S473, AKT, p-GSK-3β S9, GSK-3β, p-mTOR S2448 and mTOR (Cell Signaling Technology, Denver, MA, United States) and GAPDH (Merk-Millipore, Darmstadt, Germany). Primary antibodies were diluted in PBS containing 1% albumin or 5% non-fat dry milk and 0.05% Tween. The antigen–antibody complexes were visualized using appropriate secondary antibodies (1:10 000, diluted in PBS containing 1% albumin or 5% non-fat dry milk and 0.05% Tween) and incubated for 1 h at room temperature. Blots were then extensively washed with PBS containing 0.1% Tween and developed using an enhanced chemiluminescence detection system (Pierce, Rodano, Italy). Exposition and developing time were standardized for all blots. Densitometric analysis of scanned images was performed on a Macintosh iMac computer using the public domain NIH Image J program. Results are presented as the mean ± SEM of different gels and expressed as arbitrary units (AU), which depict the ratio between levels of target phosphorylated protein and the total protein expression normalized to basal levels.

BEHAVIORAL STUDIES

Treatments

Mice were randomized to receive i.p., administration of saline solution (Veh), or 1.32 or 4 μgkg^{-1} TA1 dissolved in Veh (Laurino et al., 2018a). Experiments were also performed in mice pre-treated i.p. with Veh or with antagonists of type 1 and type 2 histamine receptors, i.e., pyrilamine (10 $mgkg^{-1}$; PYR, prepared in Veh) and zolantidine (5 $mgkg^{-1}$ i.p.; ZOL, prepared in Veh), 20 min before they received 4 μgkg^{-1} TA1 or Veh. When p-chloro-phenylalanine (100 $mgkg^{-}1$; PCPA), the inhibitor of the

tryptophan hydroxylase activity was used 4 μgkg^{-1} TA1 or Veh, were administered to mice which had received a daily injection of PCPA for 4 days. TA1 or Veh were administered 1 h after the last PCPA injection. All drugs were administered at a volume of 10 $mlkg^{-1}$ body weight.

Mouse behavior was observed 15 min after TA1 administration according to the specific methods listed below.

The Hole Board-Platform

The hole-board test was performed according to (Romanelli et al., 2006). The experimental setting consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter), distributed four by four in an equidistant, grid-like manner. Mice were placed in the center of the board one by one and allowed to move about freely for a period of 5 min. Two electric eyes, crossing the plane from midpoint to midpoint of the opposite sides, thus dividing the plane into four equal quadrants, automatically signaled mouse movements (locomotor activity). Miniature photoelectric cells in each of the 16 holes recorded hole exploration. Animal groups consisted of 10 mice and were tested 10 min after the injections.

The Rota-Road Test

The integrity of the animals' motor coordination was assessed using a rota-rod apparatus at a rotating speed of 24 rpm. The numbers of falls from the rod in 30 s, 15 min after drug administration were counted.

The Forced Swimming Test (FST)

The test was conducted as described by Porsolt et al. (1977). Mice (10 animals/group) were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1°C. In the test, the time of immobility was measured during a 6-min period. A decrease in the duration of immobility is indicative of an antidepressant effect.

The Tail Suspension Test (TST)

A piece of tape was adhered to the upper middle of the tail of each animal, creating a flap with the overlap of tape. Mice were suspended from a plastic rod mounted 50 cm above the surface by fastening the tail to the rod with adhesive tape. The duration of the test was 6 min. Immobility was defined as the absence of any limb or body movements, except those caused by respiration.

The Light Dark Box

The light–dark box was made of white and black opaque apparatus (length 50 cm, width 20.5 cm, and height 19 cm) consisted of two equal acrylic compartments, one dark and one white, illuminated by a 60-W bulb lamp and separated by a divider with a 10 cm × 3.2 cm opening at floor level. Each mouse was placed in the middle of the light chamber facing a side away from the door and then released. Mice' behaviors were scored for 300 s and included the latency to the first step into the dark compartment, the duration of time spent in the light chamber, the number of full-body transitions between chambers. These behaviors have previously been measured as a reflection of anxiety in this apparatus (Bourin and Hascoët, 2003). After testing, subjects were removed from the light–dark box and returned to their home cage in colony room. The apparatus was cleaned with 70% ethanol after each use and allowed to dry before the next subject was tested.

Statistical Analysis

Data are expressed as mean ± SEM of independent experiments. Statistical analysis was performed by the One or Two way ANOVA test followed by Tukey or Dunnett or Bonferroni Multiple Comparison Test. The threshold of statistical significance was set at $P < 0.05$. Data analysis was performed using the GraphPad Prism 5.0 statistical program (GraphPad software, San Diego, CA, United States).

RESULTS

Endogenous and Pharmacological Tissue Levels of TA1

3-iodothyroacetic acid endogenous or pharmacological tissue levels were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) in mice sacrificed 15 min after treatment with Veh or with 330 μgkg^{-1} TA1 (Chiellini et al., 2012).

In Veh-treated mice, we were unable to measure TA1 endogenous levels in the brain and in the pooled plasma sample, whereas TA1 was detected in liver (**Table 1**).

In TA1-treated mice, 15 min after pharmacological administration, TA1 was recovered in the pooled plasma sample (19% of the dose administered), in the brain and in the liver of all the mice treated (1.3 and 32% of the dose administered, respectively). In the liver, pharmacological administration produced a 165-fold increase of TA1 level (ng/g) when compared to endogenous levels.

As expected, TA1 tissue levels reduced with time. After 60 min from TA1 administration, plasma and liver levels decreased in parallel resulting approximately ten times lower than those measured at 15 min. In the brain, TA1 levels at 60 min were only four times lower than those measured at 15 min. These results suggest that tissues have different TA1 clearance capacity,

TABLE 1 | 3-iodothyroacetic acid (TA1) levels in mice following intra-peritoneal administration (i.p.) of TA1 (330 μgkg^{-1}).

Treatments	Plasma	Brain (ng/g of tissues)	Liver
Saline	N.D.	N.D.	0.64 ± 0.2
TA1 (15 min)	63.7[a]	4.2 ± 1	106 ± 22
	19%°°	1.3%	32%
TA1 (60 min)	9.4[a]	1.1 ± 0.3	10.47 ± 0.45
	2.8%	0.33%	3.17%

[a]*from pooled blood samples.* °°*The percentage of TA1 recovered in respect of the administered dose (330* μgkg^{-1}*).*

with the brain conserving TA1 levels for longer than plasma or liver (**Table 1**).

TA1 Degranulates Brain Mast Cells

We then investigated whether treatment with 4 μgkg^{-1} TA1 was associated with brain mast cells degranulation.

Our results indicated that, at our staining conditions, i.e., both avidin and Astra Blue, very few mast cells were detectable in the brain. Although the low number of mast cells could be considered as a sign of the extreme specificity of our staining protocols, we could also not exclude that the method of tissue fixation may not allow the retention of mast cells granular content in the central portion of the brain (**Figure 1A**). These technical considerations/limitations notwithstanding, we were still able to highlight differences in mast cell granular content between the two experimental groups (**Figures 1B–E**).

In fact, in Veh-treated mice, the mast cells detected showed substantially intact granular content (**Figures 1B,D**). Instead, in brain slices prepared from mice treated with 4 μgkg^{-1} TA1, the few mast cells that were stained showed reduced granular content (**Figures 1C,E**).

TA1 Releases Histamine From Peritoneal Mast Cells

To confirm the capacity of TA1 to trigger histamine release from mast cells, we performed *in vitro* experiments exposing isolated mouse peritoneal mast cells to increasing concentrations of TA1.

Our results indicated that TA1 effectively degranulated peritoneal mast cells (**Figures 2A,B**). In fact, histamine levels in cell medium significantly increased (compared to Veh) when mast cells were exposed to increasing TA1 concentrations (**Figure 2A**) or to bee venom (BV) and mast cells appear degranulated (**Figure 2B**).

In particular, the Two-way ANOVA test indicated that, at the concentrations tested, TA1 significantly increased histamine release at all the times of cell exposure without a clear concentration dependent effect on the time of exposure. Kinetic data indicated that the releasing capacity of the acid showed a trend to increase from 5 to 15 min of cell exposure and then it remained almost stable up to 30 min (**Figure 2A**) indicating granular histamine depauperation in the absence of a fast re-synthesis.

TA1 Activates the Hypothalamic AKT/mTOR and Reduces the GSK-3β Activity

Since kinetic data indicated the presence of TA1 in the brain of mice 15 min after administration, we investigated whether TA1 was able to activate any signaling activity when administered at the pharmacological doses previously found to induce behavioral effects (Laurino et al., 2018a). For this aim, mice were sacrificed 15 min after treatment with 4 μgkg^{-1} TA1 or vehicle (Veh) and the hypothalamus was isolated.

Our results showed that the treatment with TA1 produced activation of the AKT/p-mTOR/GSK-3β cascade depending on the dose administered. In fact, while p-GSK-3β levels resulted significantly increased in the hypothalamus of mice that received 1.32 and 4 μgkg^{-1} TA1 (**Figures 3A,C**; $^*P < 0.05$ vs. Veh), the levels of p-AKT and p-mTOR were found increased over Veh only in those mice treated with 4 μgkg^{-1} TA1 (**Figures 3A–D**,

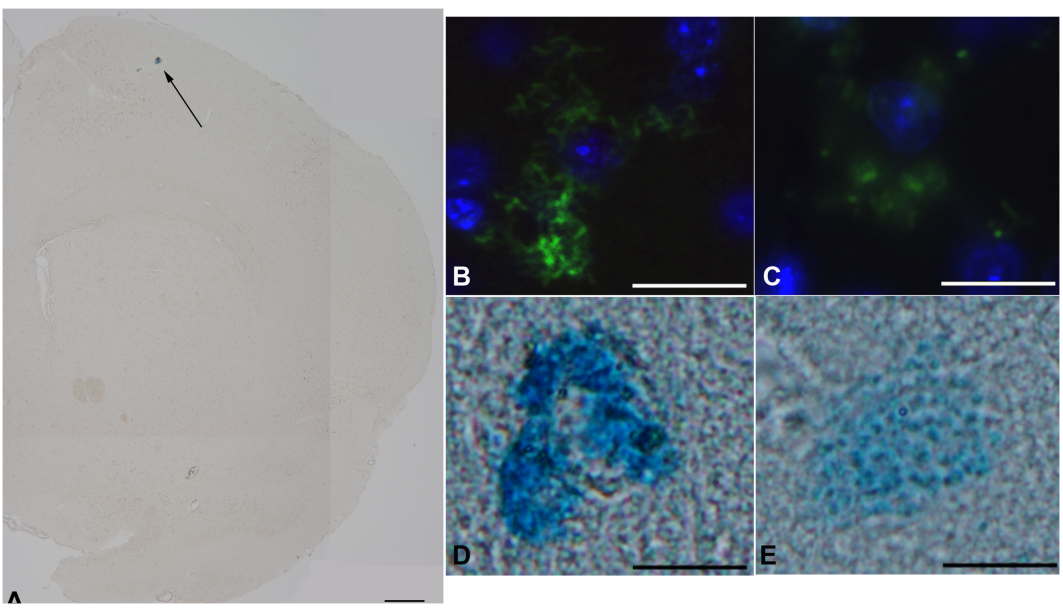

FIGURE 1 | The effect of TA1 on brain mast cell degranulation. Mast cells were stained as described in Methods. **(A)** Astra blue staining, representative image of a hemisphere. Few mast cells are visible in the upper portion (black arrows) 35x magnification; scale bar 600 μm. **(B,C)** Representative images of mast cells exposed to Veh (picture B) or to 4 μgkg^{-1} TA1 **(C)** stained with FITC conjugated avidin (green). **(D,E)** Representative images of Astra blue stained mast cells exposed to Veh **(D)** or to 4 μgkg^{-1} TA1 **(E)**. 1000× magnification; scale bar 100 μm.

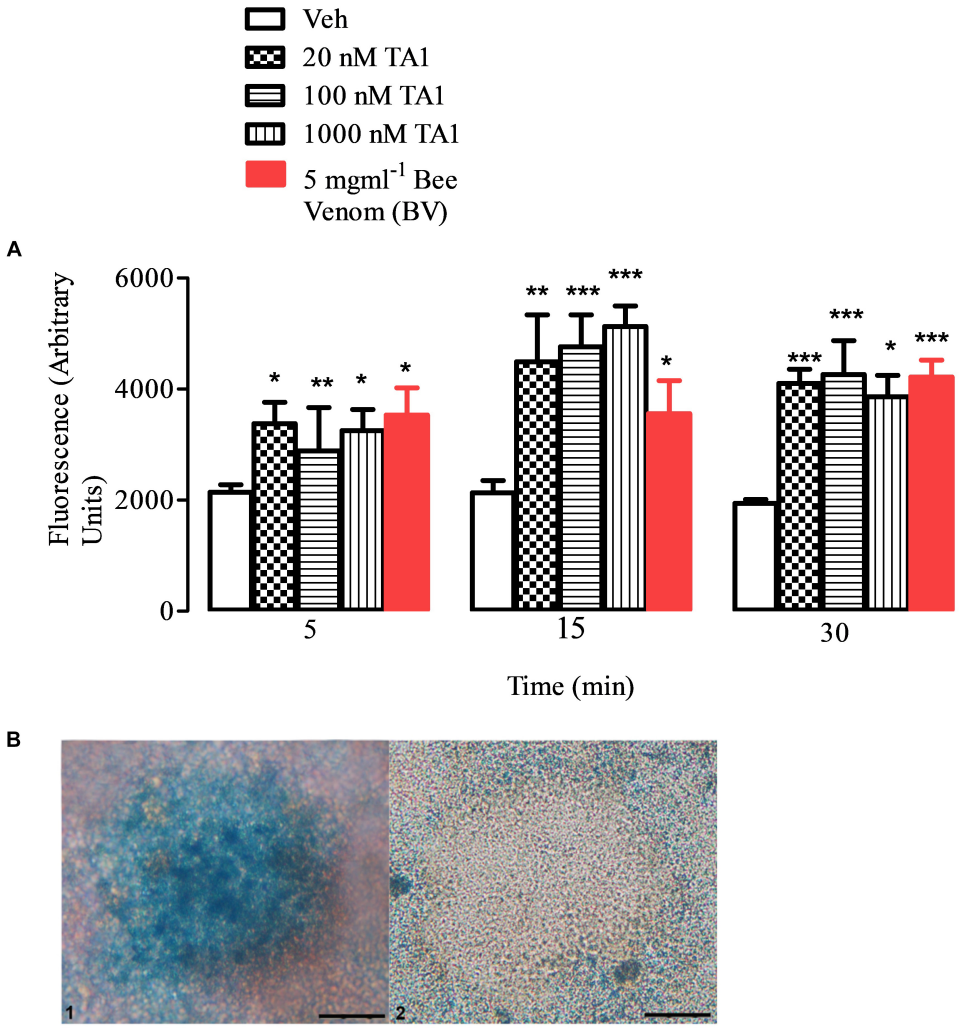

FIGURE 2 | TA1 releases histamine from peritoneal mast cells. Peritoneal mast cells were prepared and exposed to 20, 100, or 1000 nM TA1 as described in Methods. **(A)** Histamine accumulation was evaluated by fluorescence in cell medium after 5, 15, 30 min of cell exposure to TA1 or to *Apis mellifera* venom (BV; 5 mgml^{-1}). Results are expressed as means ± SEM of three different experiments run in duplicate (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. Veh.). **(B)** Picture 1: mast cells stained with Astra Blue following 15 min exposure to Veh. A representative image is showed. Picture 2: representative image of mast cells stained with Astra Blue following 15 min exposure to 1 μM TA1.

One-way ANOVA test followed by Tukey *post hoc* test; *$P < 0.05$ vs. Veh).

TA1 TREATMENT INDUCES ANTIDEPRESSANT-LIKE EFFECTS

TA1 Reduces the Immobility Time in the FST

The FST is a validated methodological tool for pre-clinical assessment of antidepressant drug activity. In this test, the behavioral immobility of the mice represents a condition of "despair," since the mice give up moving after realizing that escape is impossible. Consistently, we performed the FST to investigate the possible antidepressant effects of TA1.

One-way ANOVA analysis of the data obtained from the FST indicated that 4 and 11 μgkg^{-1} TA1 significantly reduced the immobility time (**$P < 0.01$ and ***$P < 0.001$ vs. Veh. respectively and °$P < 0.05$ vs. 1.32 μgkg^{-1} TA1 *post hoc* Tukey Multiple Comparison Test), thus supporting a possible antidepressant effect of TA1, which required to be confirmed by additional tests (**Figure 4A**).

TA1 Reduces the Immobility Time in the TST

The TST is a test based on the assumption that the animal will try to escape the stressful situation (i.e., mice suspended). After a certain time, the animal ceases to struggle and immobility occurs. As for the FST, longer immobility times are sign of depressive behavior.

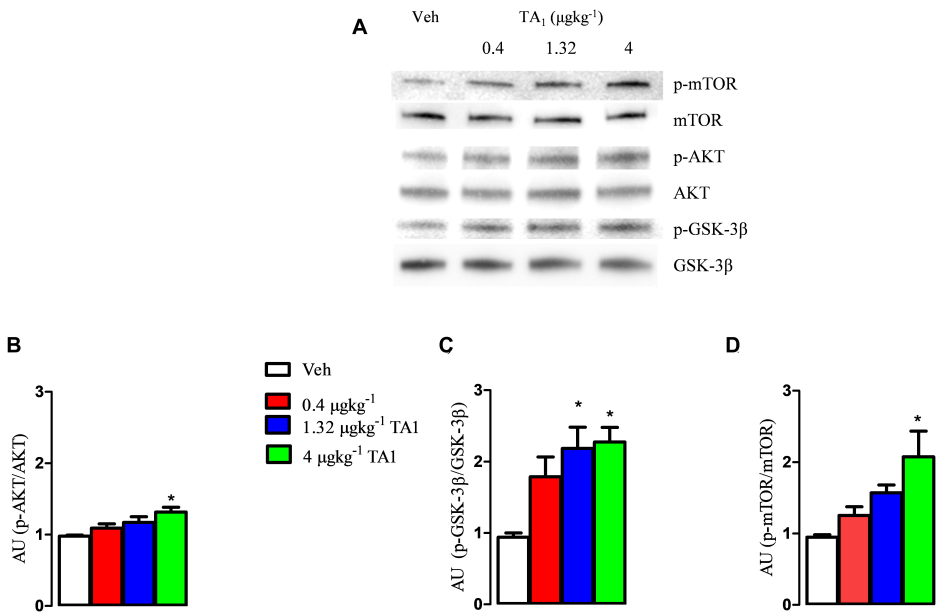

FIGURE 3 | The effects of TA1 on hypothalamic p-PI3K/AKT and p GSK-3 levels. Male CD1 mice received an intraperitoneal injection of either vehicle (Veh) or 0.4, 1.32 and 4 μgkg^{-1} TA1. Hypothalamic levels of p-AKT **(B)**, p-GSK-3β **(C)**, and p-mTOR **(D)** were assessed 15 min after mice treatment. **(A)** A representative experiment is shown. **(B–D)** Densitometric analysis is showed. Results are presented as the mean ± SEM of 3 different experiments; $^{*}P < 0.05$ vs. Veh. (One-way ANOVA test followed by Tukey Multiple Comparison Test).

Mice were subjected to the TST in order to confirm the results obtained in the FST. Results showed that 4 and 11 μgkg^{-1} TA1 treatment significantly reduced the immobility time in the TST too ($^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Veh treated mice; **Figure 4B**).

The Antidepressant Effect of TA1 Includes the Activation of the Histaminergic System

As previously reported, the behavioral effects induced by TA1 resulted to be mediated by the activation of the histaminergic system, since these actions are modulated by anti-histaminergic drugs (Laurino et al., 2015a, 2017a,b). In line with this evidence, we aimed to investigate whether the antidepressant effects of TA1 were also modulated by treatment with anti-histaminergic drugs (i.e., PYR and ZOL).

As a control, the treatment of mice with ZOL and PYR alone did not affect the immobility time of mice in both FST and TST tests in respect of Vehicle (Veh) treated mice (One-way Anova test followed by Tukey Multiple Comparison test **Figures 4C–E**).

In mice pre-treated with 10 $mgkg^{-1}$ PYR, TA1, at all the doses tested, failed to reduce the immobility time of the mice (**Figure 4C**). Instead, in mice pre-treated with 5 $mgkg^{-1}$ ZOL, 4 and 11 μgkg^{-1} TA1 significantly reduced the immobility time in the FST ($^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Veh alone respectively, $^{\#\#\#}P < 0.001$ and $^{\#\#}P < 0.01$ vs. ZOL respectively, Tukey Multiple Comparison Test analysis) (**Figure 4D**).

Consistently, in the TST, in mice pre-treated with 10 $mgkg^{-1}$ PYR, TA1 4 and 11 μgkg^{-1} did not induce any significantly reduction of the immobility time of the mice (**Figure 4E**).

The Antidepressant Effect of TA1 Does Not Include the Serotoninergic System

To investigate the involvement of serotonin in TA1 antidepressant effects, we performed the FST on mice deprived of serotonin after a 4 day treatment with PCPA, an inhibitor of the tryptophan hydroxylase activity.

Our results showed that mice treated for 4 days with 100 $mgkg^{-1}$ PCPA had immobility time similar to Veh-treated mice (**Figure 4F**, One-Way Anova test followed by Tukey Multiple Comparison test). At this condition, the administration of 4 and 11 μgkg^{-1} TA1 still significantly reduced the immobility time vs. Veh-treated mice (**Figure 4F**; $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. Veh, $^{\#\#\#}P < 0.001$ vs. PCPA, $^{\circ\circ\circ}P < 0.01$ vs. 1.32 μgkg^{-1} TA1, Tukey Multiple Comparison Test). These data indicated that the serotoninergic system was not involved in TA1 antidepressant effect.

TA1 Does Not Show Anxiolytic-Like Effects

The antidepressant effect of drugs might derive from their anxiolytic features. To exclude that TA1 was endowed of anxiolytic effects we performed the light-dark box test with mice which received 4 and 11 μgkg^{-1} TA1. The One-way Anova test of results followed by Dunnett's *post hoc* test indicated that mice treated with TA1 and Veh spent a similar amount of time in the light (**Figure 5A**), in the dark compartments (**Figure 5B**) and had a similar number of transitions from light to dark compartments (**Figure 5C**).

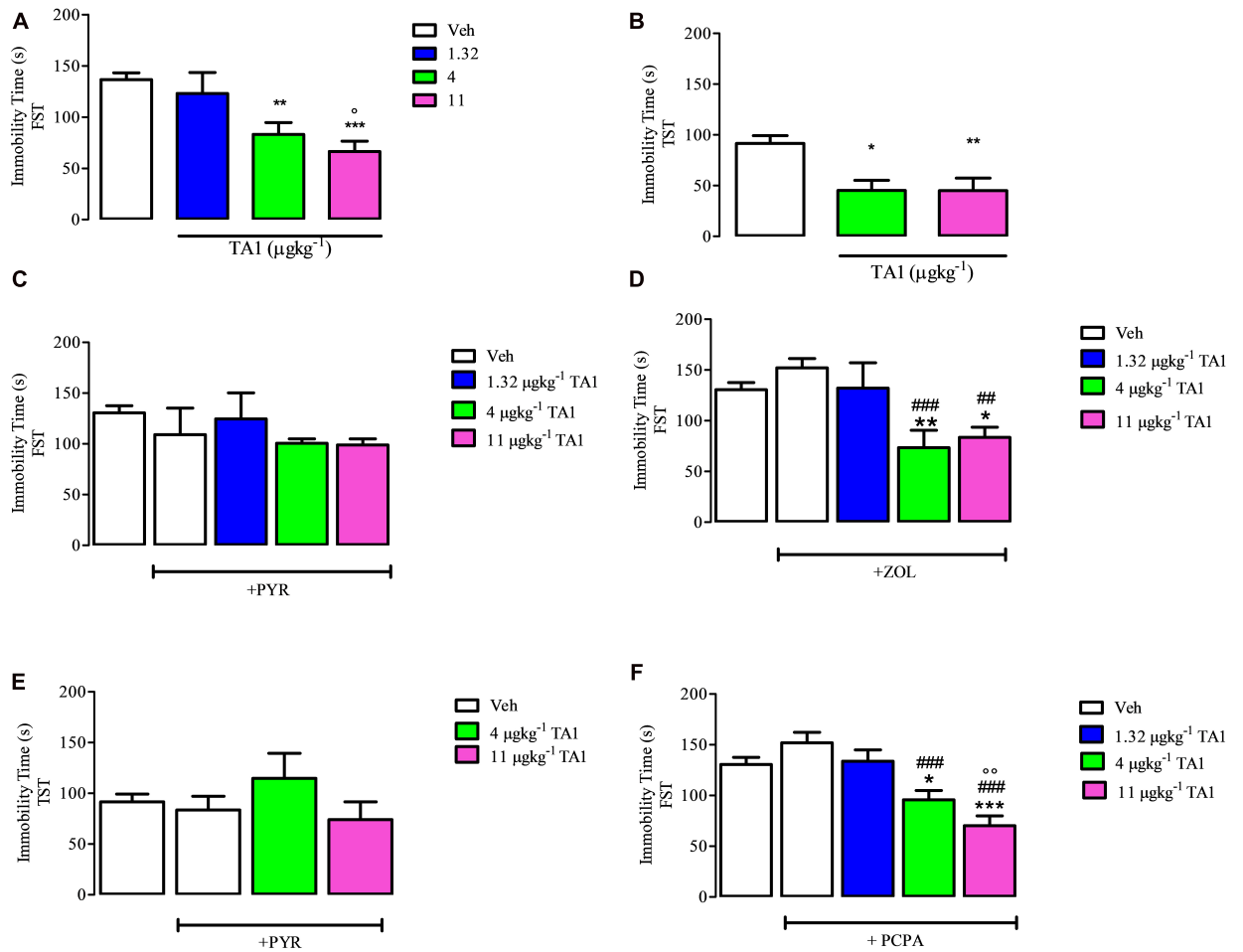

FIGURE 4 | The effect of 3-iodothyroacetic acid (TA1) treatment on the immobility time in the forced swim test (FST) and in the tail suspension test (TST): evidence for the involvement of histamine and not of serotonin. Mice received 1.32, 4 and 11 μgkg^{-1} TA1 and after 15 min they were subjected to the FST **(A)** or to TST **(B)**. The immobility time was measured as described in Methods. **(A)** The immobility time measured in mice subjected to the FST is reported. Results are the means ± SEM of three different experiments were at least ten mice were used (**$P < 0.01$ and ***$P < 0.001$ vs. Veh and °$P < 0.05$ vs. 1.32 μgkg^{-1} TA1; One-way ANOVA test followed by Tukey Multiple Comparison Test). **(B)** Immobility time measured in mice subjected to the TST is reported. Results are the means ± SEM of three different experiments using at least ten mice (*$P < 0.05$ and **$P < 0.01$ vs. Veh; One-way ANOVA test followed by Tukey Multiple Comparison Test). **(C,D)** The FST was then repeated in mice pre-treated with Vehicle (Veh) or with pyrilamine (10 $mgkg^{-1}$; PYR; **C**) or with zolantidine (5 $mgkg^{-1}$, ZOL; **D**) before receiving 4 or 11 μgkg^{-1} TA1 as described in Methods. The Immobility time was measured. Results are the means ± SEM of three different experiments were at least ten mice were used (*$P < 0.05$ and **$P < 0.01$ vs. Veh; ###$P < 0.001$ and ##$P < 0.01$ vs. ZOL, One-way ANOVA test followed by Tukey Multiple Comparison Test); **(E)** The TST was repeated in mice pre-treated with pyrilamine (10 $mgkg^{-1}$; PYR) before receiving 4 and 11 μgkg^{-1} TA1 as described in Methods. The Immobility time was measured. Results are the means ± SEM of three different experiments were at least ten mice were used (One-way ANOVA test followed by Tukey Multiple Comparison Test); **(F)** the FST was repeated in mice pre-treated for 4 days with p-chloro methyltyrosine (100 $mgkg^{-1}$; PCPA) and then with 1.32, 4 and 11 μgkg^{-1} TA1 as described in Methods. The Immobility time was measured. Results are the means ± SEM of three different experiments were at least ten mice were used (*$P < 0.05$ and ***$P < 0.001$ vs. Veh, ###$P < 0.001$ vs. PCPA, °°$P < 0.01$ vs. 1.32 μgkg^{-1}; One-way ANOVA test followed by Tukey Multiple Comparison Test).

TA1 Increases Mouse Spontaneous Locomotor Activity: Evidence for Histamine Involvement

The hole-board test is a validated model for evaluating the spontaneous locomotor activity and exploratory behavior of animals in a new environment (File, 1973).

Mice were put on the hole board platform 15 min after receiving 1.32, 4 and 11 μgkg^{-1} TA1 or Veh to measure the effect of the treatment on their locomotor and exploratory activities, i.e., curiosity (**Figures 6A,B**).

One-Way ANOVA analysis of the results indicated that only 4 μgkg^{-1} TA1 treatment, and not 1.32 or 11 μgkg^{-1}, induced a significant increase of mice locomotor activity (**$P < 0.01$ vs. Veh treated mice). On the other hand, any of the TA1 doses administered modified the mice exploratory activity (curiosity) (**Figure 6B**).

Mice receiving PYR and ZOL showed locomotor activity similar to Veh-treated mice (**Figures 6C,D**, One-way Anova test followed by Tukey Multiple Comparison Test). We next verified whether TA1 stimulation

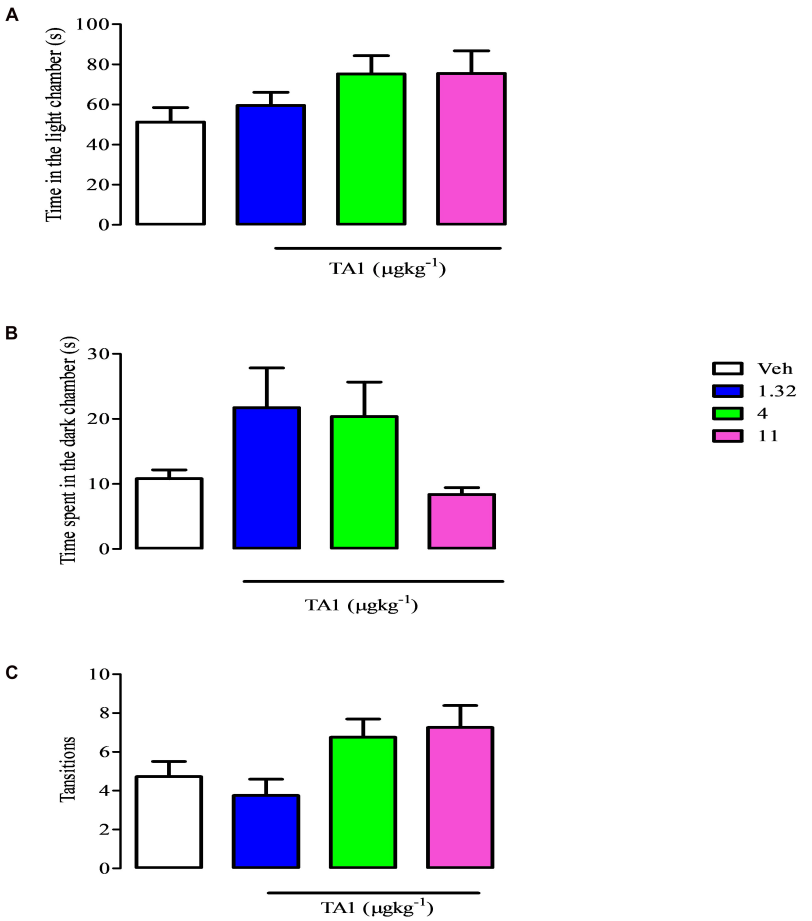

FIGURE 5 | TA1 does not show anxyolitic-like effects. The light dark paradigm was carried on mice received 1.32, 4 and 11 μgkg^{-1} TA1 or vehicle (Veh) as described in Methods. Results are expressed as the means ± SEM of measurements relative to 10 mice. Mice behaviors were scored for 300 sec and included **(A)** the duration of time spent in the light chamber, **(B)** the latency to the first step into the dark compartment, **(C)** the number of full-body transitions between chambers.

of mice locomotion was modified in mice treated with PYR and ZOL.

In mice treated with PYR, 4 μgkg^{-1} TA1 conserved its capacity to stimulate mice locomotor activity ($^{*}P < 0.05$ vs. Veh, $\#p < 0.05$ vs. PYR, Tukey Multiple Comparison Test, **Figure 6C**), while in ZOL pre-treated mice, 4 μgkg^{-1} TA1 significantly reduced mice locomotor activity ($^{**}P < 0.01$ vs. Veh, $\#P < 0.05$ vs. ZOL; Tukey's Multiple Comparison Test, **Figure 6D**).

PYR (10 $mgkg^{-1}$), but not ZOL-treatment, induced *per se* reduction of mice exploratory activity (curiosity) ($^{*}P < 0.05$ vs. Veh, Tukey's Multiple Comparison Test; **Figures 6E,F**). Interestingly, in these mice, the administration of 4 μgkg^{-1} TA1 reverted PYR-induced depression of mice curiosity (**Figure 6E**; $\#P < 0.05$ vs. PYR,). Instead, 4 μgkg^{-1} TA1 did not affect the curiosity of mice pre-treated with ZOL (**Figure 6F**).

TA1 Does Not Produce Motor Incoordination

Mice receiving 4 and 11 μgkg^{-1} TA1 i.p. were then put on the accelerated rota-road as described in "Methods." In these settings, mice treated with TA1 or with Veh showed similar performances with respect to the number of falls (data not shown), thus suggesting that TA1 did not compromise motor coordination.

DISCUSSION

We here demonstrate that TA1 shows brain bioavailability after systemic administration. The presence of TA1 in the brain is associated with the activation of the hypothalamic AKT and GSK-3β signaling pathways and the induction of emotional effects, including antidepressant-like effects. This result is independent on modulation of anxiety but it is associated with a mild stimulation of mice locomotion. Overall, these results demonstrate that TA1 is able to cross the BBB, a site where TA1 could activate mast cell degranulation. Indeed, TA1 systemic administration is associated with mast cell degranulation, and *in vitro* evidence demonstrates that TA1 may trigger histamine release from peritoneal mast cells. Interestingly, TA1 antidepressant-like effects and the stimulation of mice locomotion are modulated by anti-histaminergic drugs, thus confirming the dependence of these effects on the release of histamine. Even though the source of histamine involved in such

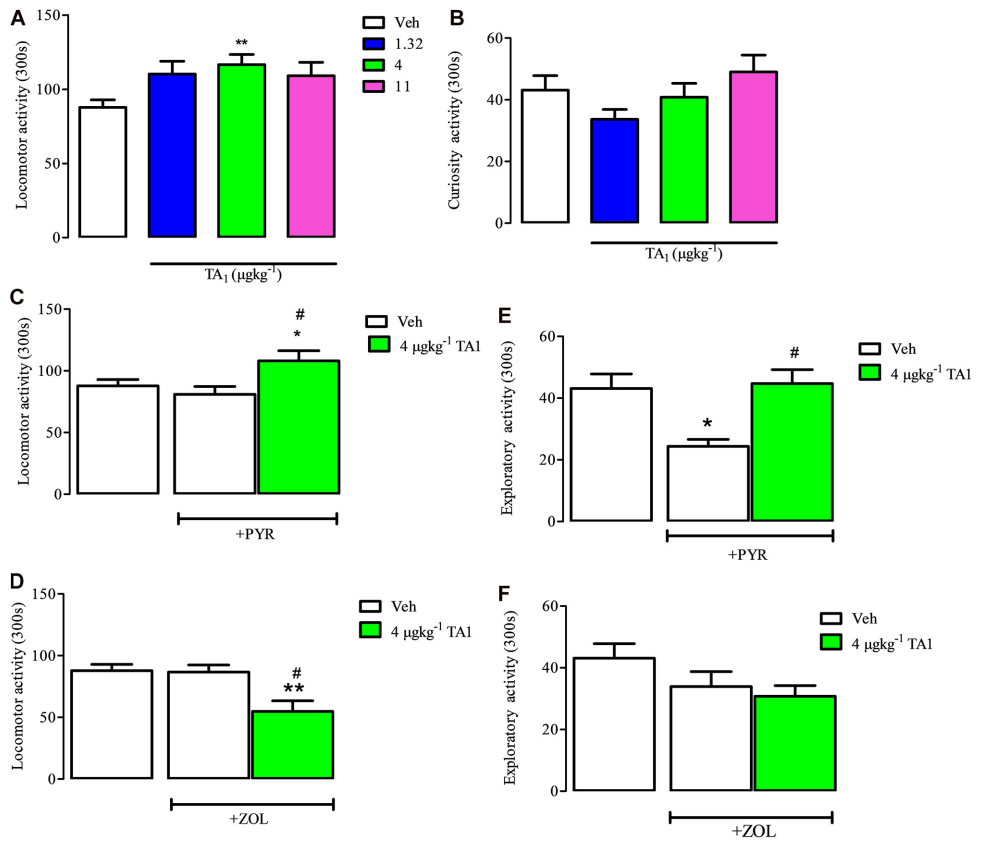

FIGURE 6 | TA1 increases mouse spontaneous locomotor activity: evidence for histamine involvement. Mice were put on the hole board 15 min after 1.32, 4, and 11 μgkg^{-1} TA1 administration to evaluate the effect of the treatments on spontaneous locomotor activity and exploratory activities. Results are expressed as the means ± SEM of measurements relative to 10 mice. **(A,B)** The effect of TA1 treatment on spontaneous mice locomotion **(A)** and exploratory activity (curiosity, **B**) is showed (**P < 0.01 vs. One-way ANOVA test followed by Tukey Multiple Comparison Test). **(C,D)** Mice locomotion was evaluated in mice pre-treated i.p. with Veh or with pyrilamine (10 mgkg^{-1}; PYR; **C**) or with zolantidine (5 mgkg^{-1}; ZOL; **D**),before receiving 4 μgkg^{-1} TA1 or Veh as described in Methods. Results are the means ± SEM of measures relative to 10 mice (*P < 0.05 and **P < 0.01 vs. Veh, #P < 0.05 vs. PYR/ZOL, One-was ANOVA test followed by Tukey Multiple Comparison Test). **(E,F)** Mice curiosity (exploratory activity) was evaluated in mice pre-treated with Veh or with PYR **(E)** or ZOL **(F)** before receiving 4 μgkg^{-1} TA1 or Veh. (*P < 0.05 and #P < 0.05 vs. PYR, One-way ANOVA test followed by Tukey Multiple Comparison Test).

effects (and in general with all the behavioral effects observed after administering TA1) remains elusive, our results provide further insight into the mechanism of TA1 action, suggesting that mast cells are a possible source of the histamine responsible for TA1 central-mediated behavioral effects.

TA1 is considered a by-product of thyroid hormone metabolism, produced by at least two independent synthetic pathways. However, little is known about the pattern of tissue distribution of endogenous and/or pharmacologically administered TA1. This issue has limited the exploration of the possible pathogenic or diagnostic role of this thyroid hormone metabolite in thyroid diseases. Our present results indicate that the endogenous levels of TA1 in mice brain and plasma are undetectable at our settings, whereas endogenous TA1 is highly detectable in liver. As expected, TA1 tissue levels increased following pharmacological administration. In particular, TA1 brain and plasma levels became detectable just 15 min after systemic administration, and, at this time point, a significant increase was also observed in liver as compared to control mice. These data provide evidence that TA1 is able to cross the BBB, and confirm that the liver is a preferential site not only of thyroid hormone but also of thyroid hormone metabolites accumulation (Porsolt et al., 1977; Saba et al., 2010). In line with this, our data also indicate that TA1 liver levels do not derive exclusively from local synthesis. Another interesting aspect of TA1 pharmacokinetic, is the different kinetic of tissue level reduction. Our data show that 60 min after administration, the decrease of TA1 brain levels was less than liver and plasma levels, suggesting that TA1 brain clearance might be lower than that of the liver. Moreover, even though the amount of TA1 recovered in the brain is only a small percentage of the dose administered, the brain may represent a site of "preservation of low levels" of TA1. These data are consistent with our previous observations (Laurino et al., 2015b) and suggest TA1 brain levels may be homeostatically controlled. However, the mechanism used by TA1 to cross the BBB and liver membranes remains to be investigated. Whichever mechanism is adopted, the passage of TA1 across the BBB implies an interaction with the cells

lying on the brain side of the barrier, including mast cells, and the distribution in brain areas, including the hypothalamus where histaminergic neurons are highly concentrated. As a novel finding, our data demonstrate that TA1 has the capacity to degranulate mast cells and to trigger histamine release from peritoneal mast cells, showing a maximum effectiveness at 15 min after cell exposure and a minimum TA1 effective concentration of 20 nM. Notably, this concentration is in the range of the pharmacological doses of TA1 administered to mice, and the time of 15 min is also in line with the activation of the hypothalamic signals and the onset of the behavioral effects here described. Interestingly, the accumulation of histamine in cell medium did not depend linearly on the concentration of TA1. In fact, at 1 μM TA1, the histamine medium content did not increase further with respect to the amount released by 20 and 100 nM TA1, thus indicating a fast release of histamine in the absence of re-synthesis. The absence of a linear concentration-dependent effect might also explain the U-shaped dose-effect curves described for the *in vivo* effects of TA1 reported in literature. To note, mast cells express all the four subtypes of histamine receptors (Csaba et al., 2007) and can store thyroid hormone (Harvima et al., 2014). Overall, the fact that TA1, a thyroid hormone metabolite, may trigger histamine release from mast cells reinforces the hypothesis of a dual relationship between the endocrine, including the thyroid, and the immune system, a relationship which might have interesting clinical implications.

15 min after TA1 administration, an intracellular hypothalamic signaling is found to be activated. In particular, a 4 μgkg^{-1} TA1 dosage results in the activation of the AKT which is among the targets of mast cells-derived histamine (Kim et al., 2018), but it is also the cascade activated in the enhancement of cognition and the neuroprotection offered by type 3 histamine receptor antagonists/inverse agonists, whose effects are mainly due to the disinhibition of neuronal histamine release (Bitner et al., 2011; Bhowmik et al., 2014). At the same doses that are effective on signaling activation, TA1 induces antidepressant-like effects. As already observed in the case of pro-cognitive properties, these effects are prevented by pre-treating mice with an H1R antagonist (Lamberti et al., 1998). In addition, our results demonstrate that the antidepressant effect of TA1 is not a consequence of an anxiolytic effect and it is unrelated to serotonin, the amine co-stored with histamine in mast cells and strongly interplaying with neuronal histamine in the control of mood tone (Munari et al., 2015). Instead, we found that TA1 stimulates mice movements on the plane without giving motor incoordination. As for the antidepressant-like effects, mice locomotor activity results modulated by antihistaminergic drug treatment revealing a main role for the H2R activation. Interestingly, TA1 was also found to stimulate mice curiosity when the H1R is blocked, a condition which depresses, *per se*, mice curiosity. This finding highlights the role of TA1 in stimulating curiosity even when this is particularly depressed, a condition mimicking depression, and reinforces the involvement of the histaminergic system in TA1 behavioral effects. All the effects here described were observed in a the same narrow range of doses previously reported to stimulate memory (Laurino et al., 2015a; Bellusci et al., 2017), wakefulness (Laurino et al., 2018a), to protect neuron from excitotoxicity, to reduce PZT-induced seizures (Laurino et al., 2018b). Overall, these behavioral effects indicate that TA1 is a mild psychostimulant which, interestingly, rapidly increases the level of attention, the mood tone and cognition capacity of the subject without inducing motor incoordination.

In conclusion, our findings indicate that TA1 is a potent antidepressant-like drug displaying a rapid onset of action whose mechanism appears to involve brain histamine. Our data suggest that histamine may derive, at least in part, from brain mast cells. The observation that TA1 triggers histamine release from mast cells opens the way to the existence of a novel and not-yet-explored relationship between thyroid endocrine components, anxiety like behaviors and the immune system (Landucci et al., 2019). In addition, since mast cells are ubiquitous cells, the degranulating capacity of TA1 has to be taken into consideration when the pharmacological effects of TA1 are investigated.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The experimental protocols were approved by the ethical Committee of the Italian Council of Health, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

AUTHOR CONTRIBUTIONS

LR designed the experimental protocol and wrote the manuscript. AL and EL performed the behavioral and biochemical experiments. LB performed the LC/MS-MS analysis. GC designed protocol for the analysis of TA1 in tissues. GD performed the drug administration and monitoring of the experimental settings. MG performed the Western Blot. LC performed the histochemistry experiments.

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frontiers in Cellular Neuroscience

BRIEF RESEARCH REPORT
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Meningeal Mast Cells Contribute to ATP-Induced Nociceptive Firing in Trigeminal Nerve Terminals: Direct and Indirect Purinergic Mechanisms Triggering Migraine Pain

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Peripheral mechanisms of primary headaches such as a migraine remain unclear. Meningeal afferents surrounded by multiple mast cells have been suggested as a major source of migraine pain. Extracellular ATP released during migraine attacks is a likely candidate for activating meningeal afferents via neuronal P2X receptors. Recently, we showed that ATP also increased degranulation of resident meningeal mast cells (Nurkhametova et al., 2019). However, the contribution of ATP-induced mast cell degranulation in aggravating the migraine pain remains unknown. Here we explored the role of meningeal mast cells in the pro-nociceptive effects of extracellular ATP. The impact of mast cells on ATP mediated activation of peripheral branches of trigeminal nerves was measured electrophysiologically in the dura mater of adult wild type (WT) or mast cell deficient mice. We found that a spontaneous spiking activity in the meningeal afferents, at baseline level, did not differ in two groups. However, in WT mice, meningeal application of ATP dramatically (24.6-fold) increased nociceptive firing, peaking at frequencies around 10 Hz. In contrast, in mast cell deficient animals, ATP-induced excitation was significantly weaker (3.5-fold). Application of serotonin to meninges in WT induced strong spiking. Moreover, in WT mice, the 5-HT3 antagonist MDL-7222 inhibited not only serotonin but also the ATP induced nociceptive firing. Our data suggest that extracellular ATP activates nociceptive firing in meningeal trigeminal afferents via amplified degranulation of resident mast cells in addition to direct excitatory action on the nerve terminals. This highlights the importance of mast cell degranulation via extracellular ATP, in aggravating the migraine pain.

Keywords: ATP, 5-HT3, mast cells, pain, migraine

INTRODUCTION

Mast cells are immune cells implicated in various inflammatory diseases. Since several original studies by Theoharides et al. (1995, 2005), the role of meningeal mast cells as triggers of migraine attacks was further explored by others, showing the pro-nociceptive role of mast cell derived pro-inflammatory cytokines/chemokines (Reuter et al., 2001; Levy et al., 2007; Baun et al., 2012; Conti et al., 2019). We recently showed that serotonin appeared to be the most important neurotransmitter released by degranulated dural mast cells to activate peripheral meningeal nerve fibers (Kilinc et al., 2017). Despite several potential candidates, it remains, however, unclear which signal or chemical agent initially triggers the activation of meningeal mast cells.

In the frame of the current Research Topic, we published a recent study showing that extracellular ATP acts through the P2X7 subtype of purinergic receptors on meningeal mast cells, leading to both mast cell activation and degranulation (Nurkhametova et al., 2019). Similar results were found also in human mast cells line (Wareham and Seward, 2016). Based on these findings, we hypothesized that this mast-cell based mechanism can indirectly contribute to ATP-induced activation of meningeal afferents. Notably, it is well established that ATP directly excites trigeminal nerve terminals (Zhao and Levy, 2015; Yegutkin et al., 2016; Zakharov et al., 2016), mainly via P2X3 receptors (Yegutkin et al., 2016). Thus, ATP potentially may have a dual complementary migraine pain promoting effect. Given a plethora of pro-inflammatory and pro-nociceptive substances released from active mast cells (Conti et al., 2019) these data suggest that ATP-driven mechanisms might significantly contribute both to meningeal neuroinflammation and to prolonged pain in migraine.

Here, we set out to differentiate the indirect, mast cell-mediated, and direct actions of ATP on meningeal afferents in isolated mouse hemiskull preparations, in mice deficient of mast cells. Our data highlight the importance of ATP driven mast cell degranulation in the aggravation of nociceptive firing in migraine pain.

MATERIALS AND METHODS

Animals

Experiments were performed on 10–12-week-old male WT C57BL/6J and C57BL/6J-KitW-v/J mice provided by the Animal Facilities of the University of Eastern Finland (UEF). All procedures were approved by the Committee for the Welfare of Laboratory Animals of the University of Eastern Finland and the Provincial Government of Kuopio. Experiments were conducted according to the European Community Council guidelines (Directives 86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Toluidine Blue Staining of Meningeal Mast Cells

Toluidine Blue staining was used to identify mast cells in meningeal tissues as previously described by Shelukhina et al. (2017) and Nurkhametova et al. (2019). In short, the brains were carefully removed from the hemiskulls leaving the meninges intact on bone tissue. The hemiskulls were filled with artificial cerebrospinal fluid (ACSF) (in mM): NaCl 115, KCl 3, CaCl2 2, MgCl2 1, NaH2PO4 1, NaHCO3 25, glucose 11) for 10 min (room temperature) and oxygenated with 95% O2/ 5% CO2. The hemiskulls were then transferred to 4% paraformaldehyde and fixed overnight at 4°C followed by three washes with phosphate buffered saline (PBS). Meningeal tissues were dissected from hemiskulls and placed on glass slides (Polysine® Thermo-Scientific, United States) for staining with Toluidine Blue (Levy et al., 2007; Kilinc et al., 2017). Images were acquired with an Olympus AX-TFSM microscope (Olympus, Japan).

Electrophysiology

Isolated whole-mount mouse hemiskulls were used for spike recordings as previously described (Zakharov et al., 2015; Kilinc et al., 2017; Mikhailov et al., 2019). In short, hemiskulls were cleaned from cranial muscles, keeping the dura mater with meningeal nerves and vessels intact. The main meningeal branch of the trigeminal nerve was cleaned from surrounding tissue, cut and placed inside the glass electrode filled with the ACSF. All recordings of electrical activity from trigeminal nerves were performed from hemiskull preparations continuously perfused by ACSF oxygenated with 95% O2/ 5% CO2. Trigeminal nerve spiking activity was registered using DAM80 amplifier (World Precision Instruments, Sarasota, FL, United States). Electrical signals were digitized using a NI PCI6221 board (National Instruments, United States) stored on a PC for off-line analysis. Signals were visualized by WinEDR v.3.2.7 software (University of Strathclyde, Glasgow, United Kingdom) and analyzed with Matlab-based software (Zakharov et al., 2015). All agonists and the antagonist of 5-HT3 receptors (ATP from Sigma-Aldrich, Germany and serotonin and MDL-7222 from Tocris Bioscience, United Kingdom) were prepared immediately before usage and were applied to the receptive fields in meninges by fast perfusion (7 ml/min). ATP and serotonin were dissolved in water, while MDL-7222 was first dissolved in DMSO (stock concentration 30 mM) and then diluted to a final concentration of 10 μM in the basic solution.

Statistical Analysis

Experimental data were analyzed using Matlab (MathWorks, Inc., United States). Data are presented as mean ± SEM (standard error of mean). The data were analyzed using Student's paired *t*-test and Mann–Whitney *U*-test when appropriate, the differences accepted significant at $p \leq 0.05$.

RESULTS

ATP Induced Activation of Meningeal Afferents Reduced in Mast Cells Deficient Mice

We first verified that the mast cell deficient animals were indeed devoid of mast cells. As demonstrated in **Figures 1A,C**,

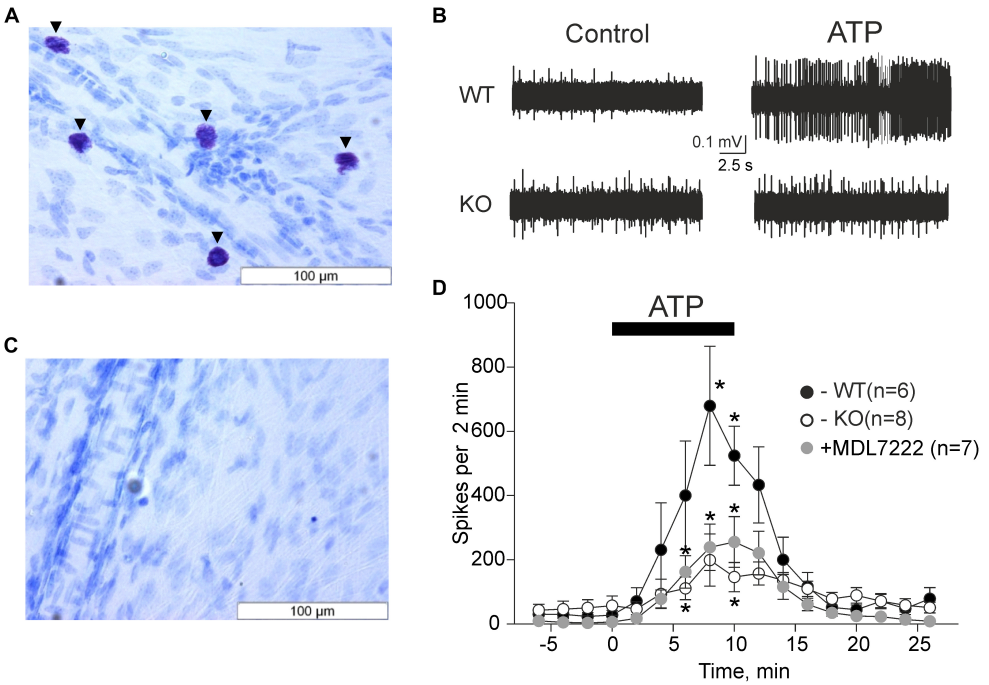

FIGURE 1 | ATP induced meningeal firing was significantly reduced in KO mice compared to WT mice and in the presence of the 5-HT3 antagonist MDL-7222. Toluidine Blue staining of meningeal tissue in the WT **(A)** and KO **(C)** mice. **(A)** Toluidine Blue-stained mast cells are shown along the middle meningeal artery (arrows) in the dura mater. **(C)** KO mice show no Toluidine Blue stained cells. **(B)** Representative traces of nociceptive firing recorded from trigeminal nerve fibers in WT and KO mice, respectively, before and after application of 100 μM ATP. **(D)** The time-course of nociceptive firing during application of 100 μM ATP in the WT (black circles, $n = 6$), KO (white circles, $n = 8$) mice, and 100 μM ATP in the presence of 10 μM MDL-7222 in WT mice (gray circles, $n = 7$). Each point represents a mean spike frequency for 2 min of recording. Mean ± SEM, nerve activation levels compared to the baseline using the Student's paired *t*-test, $^*p < 0.05$.

where WT meninges contained a vast amount of mast cells, there were no mast cells in the meninges of C57BL/6J-KitW-v/J mice (KO mice).

The pro-nociceptive action of ATP on trigeminal meningeal nerve fibers was electrophysiologically recorded in WT and KO mice. The baseline frequency of meningeal spikes (measured during 2 min before ATP application) was not significantly different in the two groups (27.7 ± 14.8 spikes in the WT, $n = 6$ versus 57.0 ± 29.9 spikes in KO mice, $n = 8$, $p = 0.322$). The application of ATP (100 μM) via rapid perfusion produced a pronounced firing in nerve fibers in both groups of mice (**Figure 1B**). In WT mice, the frequency of nociceptive spikes after application of ATP increased from the resting value of 27.7 spikes to 400.2 ± 169.1 spikes 6 min after ATP application ($p = 0.105$ as compared to baseline activity, $n = 6$) and to 679.2 ± 185.1 spikes 8 min after ATP application ($p = 0.024$, $n = 6$). In sharp contrast, in KO animals, ATP increased spiking activity from the resting value of 57.0 spikes only to 111 ± 35.5 spikes ($p = 0.034$, $n = 8$) by 6 min and to 199 ± 81.2 spikes ($p = 0.057$, $n = 8$) by 8 min. The detailed time-course of ATP action in WT and KO mice is shown in **Figure 1D**. Comparative analysis indicated that during the maximal effect (6–8 min of ATP action) the spike frequency in KO mice was significantly lower ($p = 0.02$) compared to the WT mice (**Figure 1D**).

MDL-7222 Inhibits ATP Mediated Nociceptive Firing

We recently showed that ATP efficiently promoted the degranulation of meningeal mast cells (Nurkhametova et al., 2019), a process which is associated with the release of multiple active mediators including serotonin. Endogenous serotonin derived from dural mast cells is a likely candidate to excite nerve fibers as it strongly promotes firing of rat meningeal afferents mainly via neuronal ligand-gated 5-HT3 receptors (Kilinc et al., 2017). Therefore, we next investigated the hypothesis that the part of the pro-nociceptive effect of ATP was mediated by endogenous serotonin via 5-HT3 receptors. To this end, we performed experiments where ATP was applied together with the 5-HT3 receptor antagonist MDL-7222. In the presence of this 5-HT3 blocker, ATP (100 μM) was still able to increase the frequency of meningeal spikes from 6.4 ± 2.8 spikes to 160.3 ± 49.9 ($p = 0.027$, $n = 7$) by 6 min, and to 235.9 ± 71 spikes ($p = 0.023$, $n = 7$) by 8 min. However, this effect was significantly ($p = 0.035$) weaker than the peak frequency induced by ATP alone (679.2 ± 185.1 spikes by 8 min, $p = 0.024$, **Figure 1D**).

Serotonin Induces Nociceptive Firing via 5-HT3 Receptors

In order to confirm that low concentrations of serotonin close to physiological levels of this monoamine (Nagata et al., 2006;

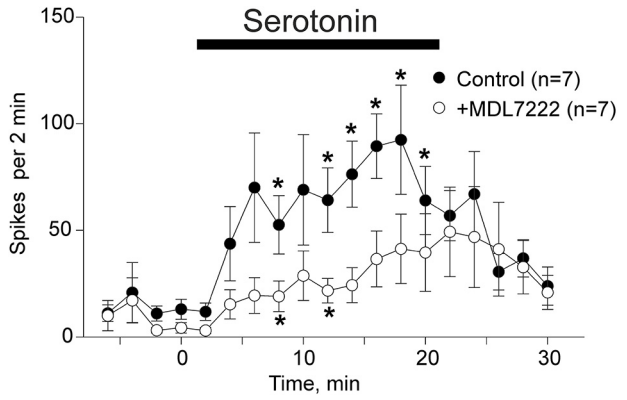

FIGURE 2 | Serotonin induced nociceptive firing and the inhibitory effect of 5-HT3 antagonist MDL-7222 on serotonin-induced activation of meningeal afferents. The time-course of nociceptive firing before and after application of 2 μM serotonin (black circles) and 2 μM serotonin in the presence of 10 μM MDL-7222 (white circles; $n = 7$ in both groups). Each point represents the mean spike frequency of a 2 min recording period. Mean ± SEM, Student's paired t-test, $^*p < 0.05$.

Ćulafic et al., 2007) are active in mice, we applied this monoamine to mouse meninges.

Application of 2 μM of serotonin increased spiking activity of trigeminal nerves in WT mice from 13 ± 4.7 spikes to 89.4 ± 15.1 spikes by 16 min ($p = 0.002$ as compared to baseline activity) and then to 92.4 ± 25.6 spikes by 18 min ($p = 0.015$) after serotonin application ($n = 7$, **Figure 2**). This excitatory action of serotonin was largely prevented in the presence of the 5-HT3 receptor antagonist MDL-7222 (10 μM) down to 36.6 ± 13.1 spikes by 16 min ($p = 0.057$, $n = 7$, **Figure 2**) and 41.3 ± 16.3 spikes by 18 min after serotonin applied together with MDL-7222 ($p = 0.084$, $n = 7$, **Figure 2**).

Comparison of the spike frequency in the period of maximal serotonin-induced activity (14–18 min) showed that the number of spikes was significantly weaker when this agonist was applied together with MDL-7222 ($p = 0.038$, $n = 7$).

Spectral Analysis of the Pro-nociceptive Effect of ATP

To compare the functional sequences of ATP induced signaling in the presence and absence of mast cells, we performed spectral analysis of firing activity in the meningeal nerves, which normally sends this information to the second order brainstem neurons (Andreou et al., 2015).

Figures 3A,B show that the pro-nociceptive effect of ATP in WT mice was characterized by high-frequency discharges. Notably, the spectral analysis revealed that in the WT mice the activity peaked at 10 Hz, which is sufficient for the temporal summation of excitatory signals at the level of secondary nociceptive neurons (Zakharov et al., 2015). In contrast, in KO mice, spectral analysis indicated a prevailing activity at 0.6 Hz (**Figure 3C**). Similar results were obtained also in the presence of MDL-7222 (**Figure 3D**). Thus, in the absence of mast cells, and when the action of serotonin was blocked, ATP-induced high frequency events were significantly reduced.

DISCUSSION

Here, we demonstrate for the first time the potent excitatory action of extracellular ATP on nociceptive firing of mouse meningeal afferents implicated in generation of migraine pain and the key role of mast cells in this phenomenon.

Despite the high prevalence of migraine, the mechanisms of pain generation in this common disorder have not been fully discovered. The trigeminovascular system of the meninges comprising trigeminal nerve fibers densely innervating dura mater blood vessels, is a well-recognized origin site of migraine pain (Messlinger, 2009; Olesen et al., 2009; Noseda and Burstein, 2013; Pietrobon and Moskowitz, 2013; Zakharov et al., 2015).

Recent evidence also suggests an important role for meningeal mast cells in triggering migraine pain. Thus, mast cells are densely present in meningeal tissues, located adjacent to both nerves and vessels (Theoharides et al., 1995, 2005; Levy et al., 2007). The contact between mast cells and nerve endings forms a neuro-immune synapse where active substances released by mast cells can activate neighboring nociceptive fibers and compounds released from active fibers, in turn, can degranulate mast cells (Dimitriadou et al., 1991). There is a long list of active substances, which can take part in the crosstalk between neurons and mast cells. Thus, degranulation of mast cells leads to release of multiple pro-inflammatory substances including enzymes, neurotrophic factors, pro-inflammatory cytokines, histamine and serotonin (Wernersson and Pejler, 2014; Conti et al., 2019). Degranulation of dural mast cells can strongly activate meningeal nerve fibers (Levy et al., 2007; Kilinc et al., 2017). Interestingly, we found that histamine is weak in excitation of meningeal nerve terminals (Kilinc et al., 2017, see also Schwenger et al., 2007). In contrast, serotonin is a powerful inducer of nociceptive firing in meningeal afferents, operating via ligand-gated 5-HT3 receptors (Kilinc et al., 2017).

One of the endogenous substances, which can activate meningeal afferents, is extracellular ATP, a powerful pro-nociceptive and pro-inflammatory agent (Giniatullin and Nistri, 2013; Burnstock et al., 2014). The purinergic hypothesis of migraine, suggesting an important role of ATP in migraine pathophysiology, was first proposed by Burnstock (1981). We previously showed in rats, that ATP induced nociceptive firing in trigeminal nerves, through ATP-gated P2X3 receptors (Yegutkin et al., 2016; Zakharov et al., 2016). The other study showed that dural topical application of ATP activated more than half of A-delta and C-fibers (Zhao and Levy, 2015). In the current study, we also found that ATP produced a huge (24.6-fold) activation of meningeal trigeminal nerve fibers in mice.

Besides this direct excitatory action on nerve terminals, extracellular ATP is also known as a substance triggering mast cell degranulation (Wareham and Seward, 2016; Nurkhametova et al., 2019). Here, we tested the hypothesis that this concomitant action of ATP contributes to activation of trigeminal fibers via degranulation of dural mast cells and the release of additional

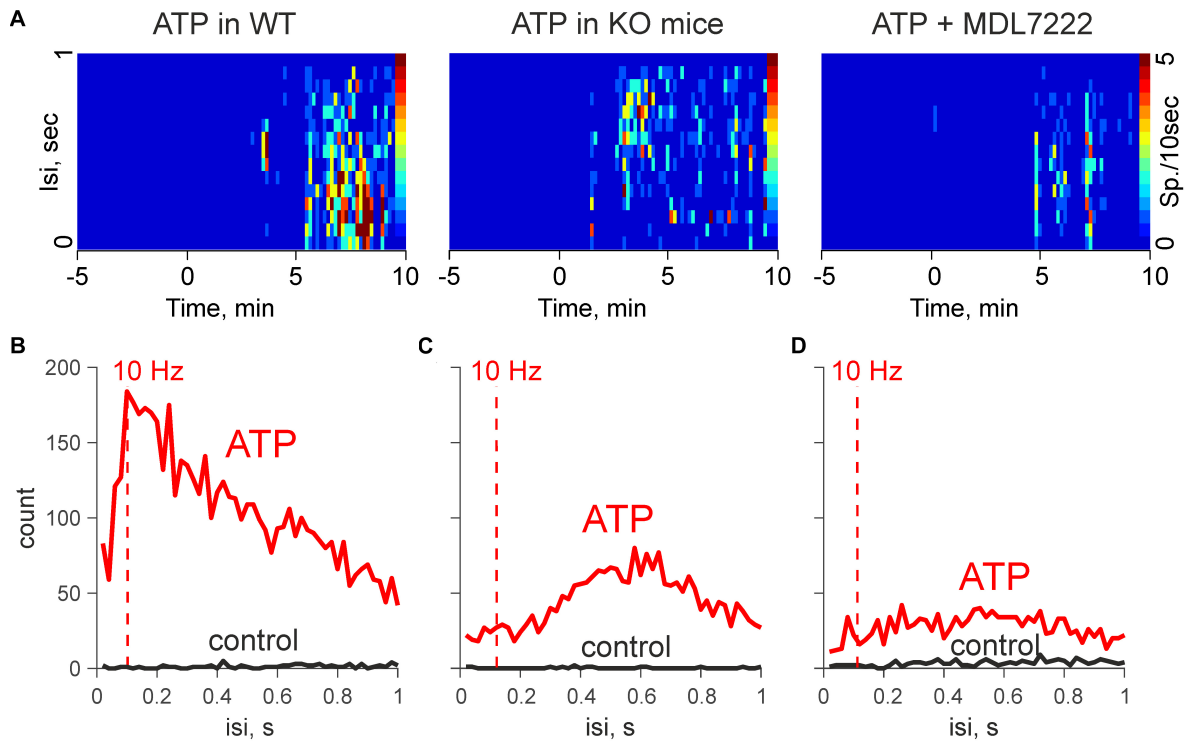

FIGURE 3 | ATP induced high-frequency discharges are reduced in KO mice and in the presence of MDL-7222. **(A)** Heat maps during application of 100 μM ATP in WT (control and in the presence of MDL-7222) and in KO mice. **(B)** Mean interspike intervals during application of 100 μM ATP in WT mice (n = 6). **(C)** Mean interspike intervals in control (black) and after application of 100 μM ATP in KO (n = 8). **(D)** Mean interspike intervals during application of 100 μM ATP in presence of MDL-7222 in WT (n = 7).

excitatory agents, such as serotonin. To test this hypothesis, we used C57BL/6J-KitW-v/J mice deficient in mast cells and found that mast cell deficient mice were significantly less sensitive to the excitatory action of extracellular ATP suggesting that mast cells provided an additional component for the pro-nociceptive action.

As serotonin is a well-known mast cell mediator stored in granules and easily released upon activation (Wernersson and Pejler, 2014), we tested its action on mouse trigeminal afferents. We found that concentrations as low as 2 μM of this biogenic amine are able to excite nerve terminals similar to ATP. Notably, like in rats (Kilinc et al., 2017), this effect of serotonin was antagonized by the specific 5-HT3 antagonist MDL-7222 demonstrating the role of the ligand-gated 5-HT3 receptor as a main target of serotonin.

Moreover, when testing the action of ATP in WT mice, ATP-induced firing was also reduced in the presence of MDL-7222 suggesting that the action of ATP is partially mediated by 5-HT3 receptors. It is worth noting that serotonin can promote release of the migraine mediator CGRP (Kilinc et al., 2017) and contributes to meningeal neuroinflammation (Buzzi and Moskowitz, 2005) which can be a reason for long-lasting pain in migraine. Thus, serotonin can be considered as the endogenous amplifier of purinergic nociception in meninges. On the other hand, at the level of 'postsynaptic' neuronal membrane, there could be the inhibitory interactions between 5-HT3 and P2X channels (Barajas-López et al., 2002), which are most significant at high agonist concentrations. This negative mechanism can limit an excessive excitation of afferents when the high level of ATP and serotonin are co-released. ATP-induced firing discharges around 10 Hz detected in the WT and missing in KO mice and in the presence of MDL-7222 may be important for the nociceptive traffic amplification at the level of second order neurons via temporal summation of input signals in the excitatory synapses in the brainstem (Zakharov et al., 2015).

In summary, we report that extracellular ATP, a powerful pro-nociceptive agent, which can be released during a migraine attack (Karatas et al., 2013), stimulates nociceptive firing in trigeminal afferents via a dual mechanism, including degranulation of resident mast cells and by the direct excitatory action on nerve terminals. ATP can be released from multiple cellular sources including astrocytes, neurons, platelets, and endothelial cells, primarily via exocytosis and/or pannexin/connexin hemichannels (Pankratov et al., 2006; Pangrsic et al., 2007; Lohman et al., 2012). Notably, ATP release could be enhanced in migraine-associated conditions such as shear stress and hypo-osmotic cell swelling (Wei et al., 2011; Burnstock and Knight, 2017) and local inflammation (Dosch et al., 2018). We suggest that ATP-driven mechanisms contribute both to excitation and to meningeal neuroinflammation in the local neuro-immune unit formed by dural mast cells and trigeminal afferent fibers.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. Requests to access the datasets should be directed to Rashid.Giniatullin@uef.fi.

AUTHOR CONTRIBUTIONS

KK, VG, and OG contributed to data collection, analysis, interpretation, and writing the manuscript. RaisaG contributed to data collection and analysis. DN contributed to writing and editing the manuscript. OM and PL provided the KO mouse line and contributed to writing the manuscript. GS contributed to the study design and supervision of the study. TM and RashidG contributed to the study design and supervision, writing the manuscript, and the final editing. All authors approved the final version of the manuscript.

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frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Secretion of Mast Cell Inflammatory Mediators Is Enhanced by CADM1-Dependent Adhesion to Sensory Neurons

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Neuroimmune interactions are important in the pathophysiology of many chronic inflammatory diseases, particularly those associated with alterations in sensory processing and pain. Mast cells and sensory neuron nerve endings are found in areas of the body exposed to the external environment, both are specialized to sense potential damage by injury or pathogens and signal to the immune system and nervous system, respectively, to elicit protective responses. Cell adhesion molecule 1 (CADM1), also known as SynCAM1, has previously been identified as an adhesion molecule which may couple mast cells to sensory neurons however, whether this molecule exerts a functional as well as structural role in neuroimmune cross-talk is unknown. Here we show, using a newly developed *in vitro* co-culture system consisting of murine bone marrow derived mast cells (BMMC) and adult sensory neurons isolated from dorsal root ganglions (DRG), that CADM1 is expressed in mast cells and adult sensory neurons and mediates strong adhesion between the two cell types. Non-neuronal cells in the DRG cultures did not express CADM1, and mast cells did not adhere to them. The interaction of BMMCs with sensory neurons was found to induce mast cell degranulation and IL-6 secretion and to enhance responses to antigen stimulation and activation of FcεRI receptors. Secretion of TNFα in contrast was not affected, nor was secretion evoked by compound 48/80. Co-cultures of BMMCs with HEK 293 cells, which also express CADM1, while also leading to adhesion did not replicate the effects of sensory neurons on mast cells, indicative of a neuron-specific interaction. Application of a CADM1 blocking peptide or knockdown of CADM1 in BMMCs significantly decreased BMMC attachment to sensory neurites and abolished the enhanced secretory responses of mast cells. In conclusion, CADM1 is necessary and sufficient to drive mast cell-sensory neuron adhesion and promote the development of a microenvironment in which neurons enhance mast cell responsiveness to antigen, this interaction could explain why the incidence of painful neuroinflammatory disorders such as irritable bowel syndrome (IBS) are increased in atopic patients.

Keywords: mast cells, pain, allergy, IGE receptor, CADM1, synCAM1, sensory neurons

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INTRODUCTION

Mast cells are best known for their role in allergic diseases. The symptoms of allergic reactions are instigated by the secretion of a plethora of pro-inflammatory mediators from mast cells following antigen-dependent crosslinking of IgE receptors (FcεRI) (Galli and Tsai, 2012). These mediators include preformed molecules stored in granules such as histamine, serotonin, adenosine 5′-triphosphate (ATP), proteases, tumor necrosis factor-α (TNFα), chemokines, and peptides, as well as de novo synthesized cytokines, growth factors and lipid mediators (Sismanopoulos et al., 2012). Since mast cells are tissue resident cells, the mediators they secrete influence the function of nearby cells expressing cognate receptors. Conversely, mast cells also express a wide variety of other types of receptors whose activation by local mediators may intern amplify antigen-induced responses (Gilfillan et al., 2009), while much is known about mast cell cross-talk with other innate and adaptive immune cells, more recently, their contribution to neuroimmune signaling is also increasingly being recognized (Undem and Taylor-Clark, 2014; Voisin et al., 2017).

Evidence is accumulating that mast cells may contribute to pain experienced in conditions whose pathology involves tissues lying at the interface of the external environment such as intestines, bladder, uterus, airways, skin and meninges (Aich et al., 2015; Gupta and Harvima, 2018). Indeed, mast cells have shown preference to attach to substance P (subP)and calcitonin gene related peptide (CGRP)-positive sensory neurons in human and rat intestine (Stead et al., 1987), respiratory tract (Alving et al., 1991) dura matter (Rozniecki et al., 1999), and other tissues (Spanos et al., 1997; Pavlovic et al., 2008). The number of contacts between mast cells and neurons is increased during infection (Stead et al., 1987), allergic conditions (El-Nour et al., 2005), and inflammatory conditions such as irritable bowel syndrome (Barbara et al., 2004; Theoharides and Conti, 2004) and this correlates to pain (Barbara et al., 2007; Ohman and Simrén, 2010; Di Nardo et al., 2014). Mast cell granule-derived mediators and cytokines, including IL-6 and TNF-α, in turn have been shown to sensitize nociceptors (von Banchet et al., 2005; Barbara et al., 2007; Hensellek et al., 2007) and contribute directly to neurogenic inflammation and pain signaling (Aich et al., 2015; Wouters et al., 2016; Gupta and Harvima, 2018). Knowledge of the adhesion molecules regulating mast-cell sensory neuron contacts may therefore provide new insight into disease mechanisms and strategies for intervention.

Cell adhesion molecule 1 (CADM1, also known as SynCAM 1, Necl-2, SgIgSF, TSLC-1) is reported to contribute to mast cell interactions with neurons (Furuno et al., 2005; Hagiyama et al., 2011), fibroblasts and smooth muscle cells (Moiseeva et al., 2013b). CADM1 is one of four related glycoproteins with a common structure consisting of three extracellular Ig-like domains, a transmembrane region and short conserved cytoplasmic domain that binds adaptor proteins linking it to the cytoskeleton and other intracellular partners (Biederer, 2006). CADM1, 2, 3 can each form weak trans homophilic interactions, while CADM1/2 and CADM3/4 interactions produce strong heterophilic adhesions in neurons (Fogel et al., 2007). Mutations in CADM1 have been implicated in autism spectrum disorder, and its expression is increased in Rett syndrome (Nectoux et al., 2010) which is associated with altered peripheral mechanosensory transduction (Orefice et al., 2016). One study performed to date examining mast cell – sensory neuron adhesions, failed to detect CADM protein expression but did find evidence for nectin-3 mRNA expression in dorsal root ganglia. Neutralizing antibodies aimed at disrupting CADM1/nectin-3 heterophilic interactions reduced mast cell adhesion (Furuno et al., 2012; Moiseeva et al., 2013b). However, since neuronal expression of CADM1 is developmentally regulated (Fogel et al., 2007; Hagiyama et al., 2011; Moiseeva et al., 2013a,b), and the only study performed to date used neurons isolated from newborn mice, whether CADM1 contributes to functional interactions between mature sensory neurons and mast cells remains an open question. It is also unknown how adhesion between sensory neurons and mast cells modulates their responses to allergic activation. To address these questions, we established a co-culture system between adult sensory neurons isolated from dorsal root ganglia (DRG) and functionally mature mast cells generated from haematopoetic stem cells found in bone marrow derived mast cells (BMMCs). Protein expression analysis, showed that distinct variants of CADM1 are expressed in sensory neurons and mast cells. Knockdown of CADM1 in mast cells abolished their adhesion to sensory neurons, conversely blocking CADM1 on sensory neurons with a neutralizing antibody inhibited mast cell adhesion. Functional analysis of mast cells in co-culture furthermore revealed that CADM1-dependent interactions with sensory neurons induced degranulation and IL-6 synthesis, and significantly enhanced FcεRI-activated secretory responses. Separation of the sensory neurons from the mast cells by a porous membrane prevented the effects of co-culture, as did knockdown of CADM1, showing that the functional interaction was adhesion dependent. While the effects of mast cell derived mediators on sensory neurons are well documented, to our knowledge this is the first demonstration of a reciprocal adhesion-dependent effect of sensory neurons on mast cells and their responsiveness to antigen, emphasizing the important role that neuro-immune interactions contribute to allergic diseases.

MATERIALS AND METHODS

All animals were maintained on a 12-h light/dark cycle in a temperature-controlled environment and given food *ad libitum*. All animal procedures were conducted under the Animal (Scientific Procedures) Act 1986, and approved by the UK Home Office.

Bone Marrow-Derived Mast Cells (BMMC) Cell Culture

Bone marrow-derived mast cells were isolated from 8 to 12 week-old C57BL6 wild type mice as described previously (Furuno and Nakanishi, 2011) with modifications. After the mice were sacrificed, bone marrow was collected from the tibia and

the femur by repeated flushing using a 27-G needle syringe filled with calcium- and magnesium- free phosphate buffer solution (PBS, PAN Biotech, Germany). Cells were collected and centrifuged at 340 × *g* for 10 min at 4°C. The pellets obtained were re-suspended with 2-ml lysis buffer [0.83% ammonium chloride, 0.168% Na-carbonate, 1 mM EDTA (pH 7.3)], in which they were incubated for 10 min at room temperature to induce lysis of red blood cells. The lysed cells were centrifuged and resuspended with Iscove's Modified Dulbecco's Media (IMDM, Lonza, United Kingdom). For cell culture, complete medium was supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, United Kingdom), 1% MEM Vitamin (Gibco, United Kingdom), 1% of sodium pyruvate (Gibco, United Kingdom), 100 IU/ml Penicillin, 100 μg/ml streptomycin (PAA Laboratories, United Kingdom), and 0.1 mM non-essential amino acid (Gibco, United Kingdom). In the final step, 10 ng/ml of recombinant mouse stem cell factor SCF (R&D systems, MN, United States) and 5 ng/ml recombinant murine IL-3 (R&D Systems, MN, United States) were added. The cells were cultured in 7.5% CO_2 at 37°C for 4 weeks until they differentiated into BMMCs. Prior to use in experiments, cells from each preparation were analyzed for surface expression of FcεRI and SCF receptor (c-kit), the classic mast cell markers, by flow cytometry. Only cultures in which >95% viable cells stained positive for both c-kit and FcεRI were used.

Dorsal Root Ganglion (DRG) Culture

Dorsal Root Ganglion were isolated and cultured according to previously described procedure (Sleigh et al., 2016). DRGs isolated from adult (8–12 week old) C57BL male mice, were dissociated with 0.06 μg/ml collagenase XI (Sigma) and 0.1 μg/ml Dispase for 1 h at 37°C, followed by gentle trituration. For selective isolation of neurons, gradient centrifuge technique with 15% bovine serum albumin (BSA) in medium was used. Cells were cultured in complete Neurobasal-A medium (NBA, Gibco) containing 2% B-27 supplement (Gibco), 2 mM Glutamax (Gibco), 1% penicillin/streptomycin (Gibco), 10 ng/ml NGF (Sigma) and 1 μM Cytosineβ-D-arabinofuranoside (Ara-C, Sigma) and seeded on 16 mm matrigel (BD) – coated glass coverslips or 96 well flat bottom plates and incubated for 1 day before using in co-culture.

BMMC-DRG Co-culture

After culturing BMMC for 4 weeks, the purity of mast cells was assessed for surface expression of FcεRI and c-Kit by flow cytometry. Only BMMC cultures with >95% $FcεRI^+$ and $c\text{-}Kit^+$ were used for co-culture. 1–3 × 10^5 BMMCs suspended in co-culture medium (50% IMDM and 50% NBA) were added to DRG cultures prepared 24 h previously. Co-cultures were incubated in 37°C with presence of IL-3 (5 ng/ml) for different time points. For some experiments, DRG were preincubated for 30 min prior to co-culture with 1–30 μg/ml of CADM1 blocking peptide (9D2, Medical & Biological Laboratories). For separation experiments, transwells (Costar, Corning) with a 0.4-μm insert were used. DRG were cultured in the lower chamber, while BMMCs were added in the insert.

BMMC Sensitization, Degranulation and Cytokine Secretion Assay

For antigen stimulation experiments, BMMCs (3.5 × 10^5 cells) were sensitized overnight with 0.5 μg/ml anti-dinitrophenyl IgE (anti-DNP IgE, Sigma-Aldrich, United Kingdom). On the following day, IgE-presensitized BMMCs were co-cultured with DRG for various time points and then stimulated by 10–100 ng/ml of dinitrophenyl antigen (DNP) (Sigma-Aldrich, United Kingdom) for 30 min at 37°C. The co-culture supernatant was collected and analyzed for degranulation, IL-6, and TNFα secretion.

Degranulation of mast cells was evaluated by measuring the activity of granule-stored enzyme β-hexosaminidase (β-hex) release (Gilfillan and Tkaczyk, 2006). After BMMC were activated by IgE/Ag cross-linking, supernatants were collected and incubated with the same volume of substrate solution [2 mM *p*-nitrophenyl *N-acetyl* β-D-glucosamine (Sigma-Aldrich, United Kingdom) in 100 mM citrate buffer (pH 4.5)] at 37°C for 2 h. The reaction was stopped by addition 90 μl of Tris–HCL (pH 9). Enzyme activity was evaluated by measuring optical density at 405 nm with a microplate reader (OPTIMA). The total amount of β-hex released was determined by cell lysis with 0.5% Triton X-100. Background absorbance readings (b) were determined from wells containing all buffers except supernatant. The β-Hex activity was calculated using the following formula: degranulation (%) = ((supernatant−b)/(Total−b)) × 100. Fold change of enhancement in degranulation was calculated by dividing the percentage of degranulation in tested condition to the percentage of degranulation in the control condition.

For cytokines production, the experiment was performed as above, but BMMCs were stimulated with DNP for 6 h at 37°C. IL-6 and TNFα were analyzed in supernatants by mouse IL-6 ELISA kit (R&D Systems) and mouse TNFα ELISA kit (R&D Systems), respectively, as per manufacturer's instructions.

Fluorometric Calcein-Adhesion Assay

Adhesion of BMMC was assessed using Calcein Cell Adhesion Assay Kit (Invitrogen, Life Technologies) as previously described (Moiseeva et al., 2013a). Following manufacturer' protocol, 1 × 10^6 BMMCs/ml of serum-free medium were labeled with 5 μM Calcein-AM for 30 min at 37°C. Then, calcein-labeled BMMCs were resuspended with co-culture medium at a density of 1 × 10^5 BMMCs/100 μl/well and co-cultured with DRG for 2 h. Un-attached BMMCs were washed out by spinning the plate upside down at 20 × *g* for 2 min and wells were re-filled with co-culture medium. The fluorescent signal was measured by fluorescence plate reader before and after washing step for total and attached readings, respectively. Calcein excitation wavelength used was 485 nm and emission wavelength was 520 nm. Adhesion was measured as percentages of adherent BMMCs to the total. For comparison control, calcein-labeled BMMCs were seeded on 1-day old Matrigel-coated wells in

same co-culture medium and for the same time like the ones with DRG co-culture.

Flow Cytometry

For BMMC surface protein expression, 1×10^6 BMMCs/sample were used. After washing BMMCs with cold PBS, cells were re-suspended in cold FACS buffer (2 mM EDTA in PBS with 2% FBS). BMMCs were blocked with 1:100 of Fc Block (CD16/32) (eBioscience) for 15 min on ice to prevent non-specific binding of antibodies. After washing with FACS buffer twice, BMMCs were incubated with APC-anti-mouse c-Kit (eBioscience, 1:100) and PE-anti-mouse FcεRI (eBioscience, 1:100), when checking for mast cell differentiation, or rabbit anti-CADM1 (Santa Cruz sc-33198, 1:100) antibodies for 20 min on ice. Alexa Flour®488 anti-rabbit (Invitrogen, 1:1000) was used as secondary antibody. Fluorescence was detected with FACSCalibur (BD Biosciences) at emission wavelengths of 488 nm for Alexa Fluor®488 labeled samples, 660 nm for APC labeled samples or 585 nm for PE labeled samples. For analysis, the viability was first gated based on side scatter and electronic volume from unstained sample. Only viable cells from gated population were included in fluorescence measurements. For CADM1 intracellular expression, BMMCs were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at 4°C. Then, permeablized with 0.1% Triton X-100 for 10 min.

Immunocytochemistry

Cultured cells were washed with ice-cold PBS and fixed with 4% PFA for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Following 1 h incubation with blocking buffer (2% normal donkey serum (Sigma), 0.2% Fish serum gelatine (FSG) Sigma, G7765) and 0.01% Triton X-100) in PBS) at room temperature, cells were stained with primary antibodies and incubated overnight at 4°C. Cells were then washed three times with PBS and stained with Alexa Fluor®anti-mouse or anti-rabbit secondary antibodies (1:1000 Invitrogen) for 2 h in the dark at room temperature. Glass coverslips were mounted onto microscope slides using mounting medium (Vectashield Hard set H1500, Vector) with 4′,6-diamidino-2-phenylindole (DAPI) to stain the nucleus. For negative control, some wells were stained with only secondary antibodies. Images were viewed using a 40× and 60× oil objective (N.A. 1.42) on Nikon A1 confocal microscope. Samples were illuminated at the required wavelength using 405, 488, and 561 nm lasers.

Primary antibodies used for immunocytochemistry were as follows: Rabbit anti-peripherin (Sigma P5117, at 1:1000), Guinea pig anti-Substance P (Abcam ab10353, 1:100), Mouse anti-CGRP (Abcam Ab81887, 1:100), mouse anti-β III Tubulin monoclonal IgG (R&D MAB1195 clone TuJ-1 lot HGQ0113121, 1:1000), Rabbit Anti-CADM1 Polyclonal IgG (H-300) (Santa Cruz sc-33198 lot F0407, 1:300), Alexa Fluor®488 anti-mouse c-Kit (Biolegend (6861), 1:100), Mouse anti-Tryptase (Abcam ab2378, 1:300). For further details please see **Supplementary Table 1**.

Western Blot

A total of 1×10^6 of 4-week old BMMCs or one-day old DRG cultures were lysed in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.3% Triton X-100, pH 8) with 1% of protease inhibitor cocktail III (Fisher Scientific). The insoluble debris was removed by spinning at 14,000 g for 20 min at 4°C. The protein concentration of each lysate was determined using Bradford protein assay (Sigma). For separation of CADM1 protein, 10% SDS resolving gel was used. After running the gel, the protein transfer was performed at 85 V for 90 min at 4°C (protein of interest 110 kDa). Then, the membrane was probed using rabbit anti-mouse CADM1 antibody (1:300, Santa Cruz) and mouse anti-GAPDH (1:5000, Thermo Fisher Scientific) and visualized using Li-cor system, Goat IRDye 800 anti-Rabbit 1:5000 and Goat IRDye 700 anti-Mouse 1:5000.

Amaxa Nucleofection of BMMC

Nucleofector II (Amaxa, Lonza) was used to knockdown CADM1 in BMMC. Basic fibroblasts nucleofector kit (VPI-1002, 90279050) and recommended programs (X-001) was optimized for BMMC transfection. 4-5 $\times 10^6$ BMMCs/reaction were transfected with 2 μg of psi-U6 plasmid (Genecopoeia) expressing CADM1 ShRNA and eGFP or scrambled ShRNA and eGFP. Transfected cells were incubated with complete IMDM for 48 h at 37°C and 7.5% CO_2 before sorted using fluorescence-activated cell sorting (FACS). Only the GFP expressing cells were used for subsequent experiments. Three unique constructs of CADM1 ShRNA plasmids and non-targeting scramble control (Genecopoeia) were tested separately to identify the most efficient construct. CADM1 -ShRNA1 MSH031688-31 (ggacagaatctgtttactaaa), CADM1- ShRNA2 MSH031688-32 (cctccacgtaacttgatgatc), CADM1- ShRNA3 MSH031688-33 (ggagattgaagtcaactgtac), Scramble- ShRNA CSHCTR001.

Statistical Analysis

The results are expressed in the figures as the means ± standard error of the mean (SEM) of at least three independent experiments based on different mouse cultures. Statistical comparisons with the appropriate control data from adhesion assay experiment with blocking peptide and all knockdown experiments were performed using one-way repeated measures (ANOVA) followed by Turkey's post-test. Data from other experiments were analyzed using paired *t*-test. Probability values (p) < 0.05 were considered statistically significant. All data handling, statistical analysis, and graphs were prepared using GraphPad Prism (GraphPad Software, La Jolla, CA, United States)[1].

RESULTS

CADM1 Is Expressed in Mast Cells and Sensory Neurons

As a prelude to understanding the role that cell adhesion plays in regulating mast cell-sensory neuron interactions, we established a co-culture system of C57BL6 mouse BMMCs and primary DRG neurons isolated from adult mice (8–12 weeks). The purity of neurons used in the co-cultures was estimated to be 30%, as quantified by immunohistochemistry of anti-β tubulin positively

[1]www.graphpad.com

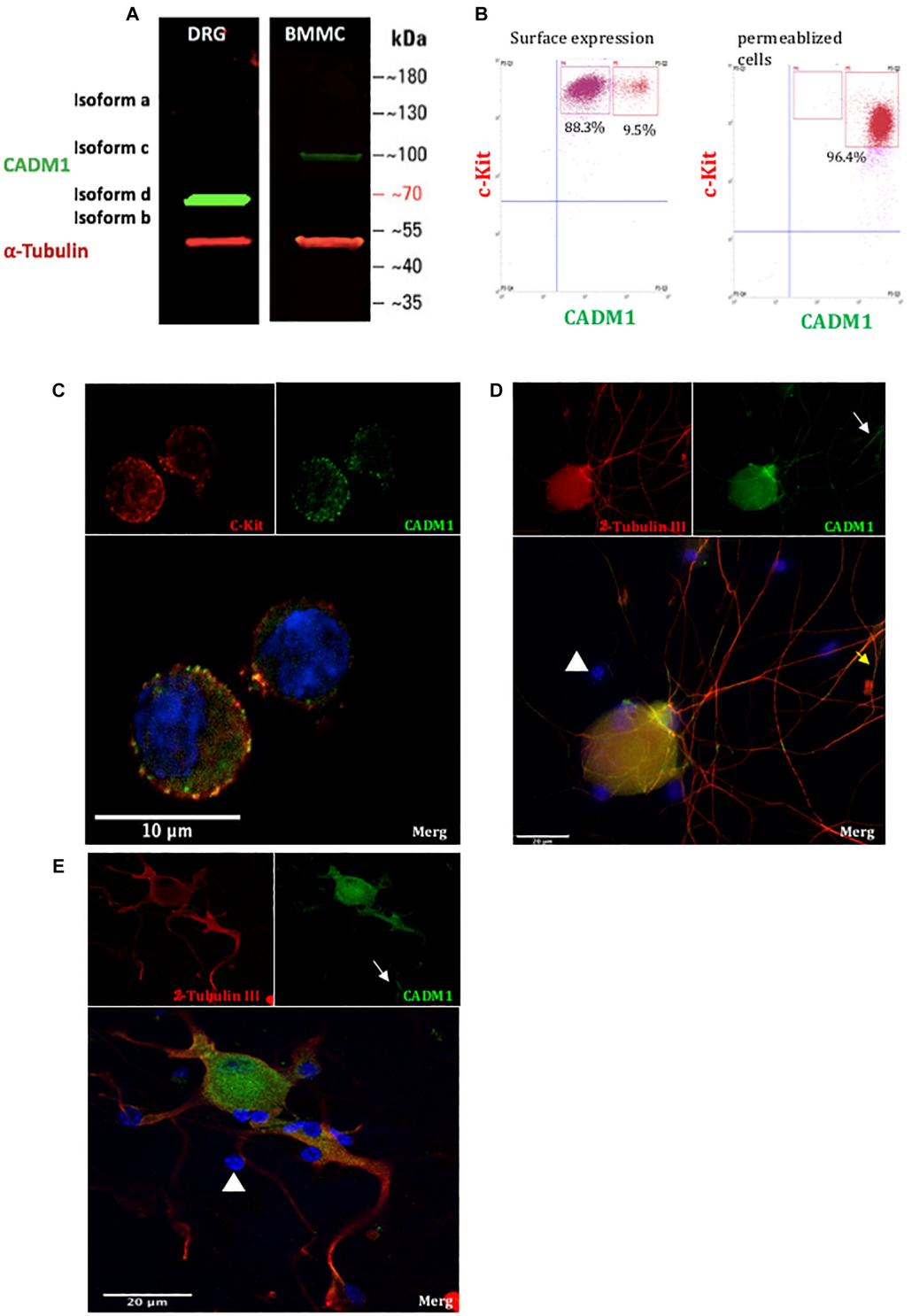

FIGURE 1 | CADM1 is expressed in BMMCs and sensory neurons. **(A)** Immunoblot of lysates prepared from DRG and BMMC mono-cultures probed with anti-CADM1 and anti-α-Tubulin, as a loading control. The m.w. scale is shown to the right of the blot. **(B)** Flow cytometric analysis of surface (left) and total CADM1 expression in Triton X-100-permeablized BMMCs (right). BMMCs were double labeled with conjugated c-Kit and CADM1 Abs. BMMCs were gated as c-kit+ CADM1high cell subset and c-kit+ CADM1medium cell subset. Numbers in plots are the percentages of cells in the indicated gate. **(C)** Confocal immunofluorescence images of c-Kit (red), CADM1 (green), and DAPI (blue) and merged image of mono-cultured BMMCs. Scale bar represents 10 μm. **(D,E)** Confocal immunofluorescence images of β-Tubulin (red), CADM1 (green) and DAPI (blue) in mono-cultured DRGs. White arrows indicate CADM1 immunoreactive neurites. Note that non-neuronal cells, observed as DAPI positive, β-Tubulin negative are also negative for CADM1 (arrowheads). Scale bar represents 20 μm.

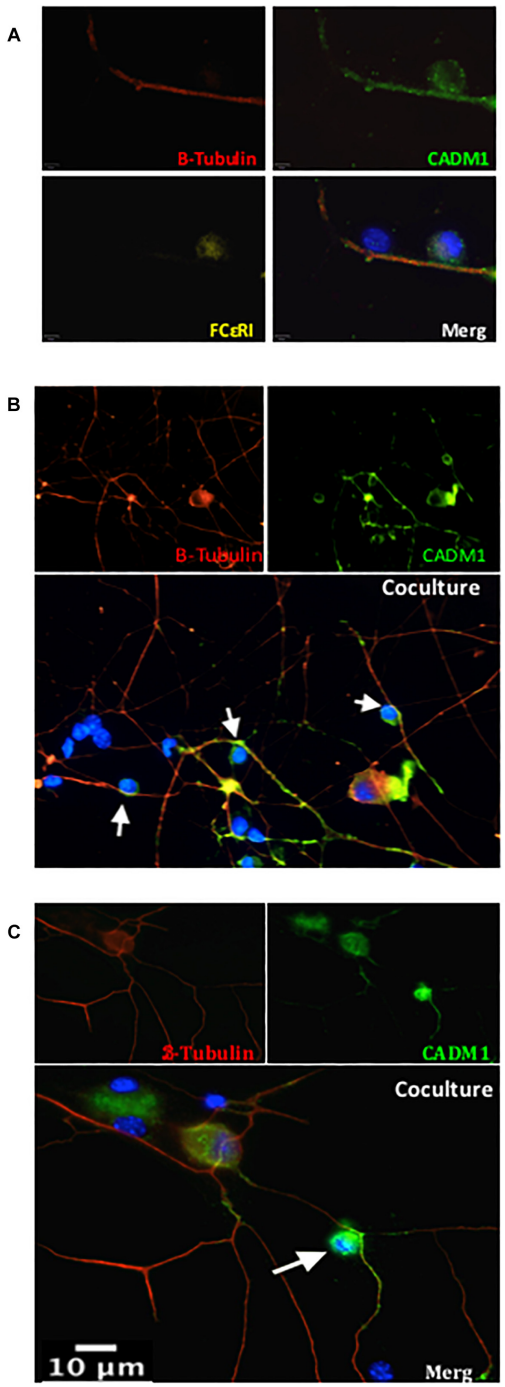

FIGURE 2 | CADM1 is enriched at mast cell-sensory neurite contact sites. Representative immunofluorescent images of BMMCs co-cultured with DRG neurons for 24 h. In panel **(A)** individual and overlaid images are shown from a neurite with two adherent cells. β-Tubulin (red) is used as the neuronal marker, DAPI (blue) nuclear marker labels all cells in the culture, while FcεRI (yellow) specifically labels BMMCs. CADM1 (green) stained positive in the sensory neuron neurite and BMMC, but is absent from the attached non-neuronal, non-mast cell. **(B,C)** Representative individual and overlaid immunofluorescent images of CADM1 (green), β-Tubulin (red) and DAPI (blue) staining of different co-cultures. Increased CADM1 fluorescent intensity at contact sites (white arrows) between BMMCs and neurites (red).

stained cells relative to the total number of DAPI positively stained cells. Prior to co-culture, BMMCs were differentiated for 4 weeks with IL-3 and rm-SCF and assessed for purity and maturity by flow cytometry and their ability to undergo antigen-induced degranulation. Only BMMC cultures in which >99% of cells stained positive for expression of c-Kit and FcεRI, well established markers of mature mast cell, were used in co-culture experiments. The expression and distribution of CADM1 in each cell type before and after 48 h co-culture was then examined. Western blot analysis of lysates prepared from pure BMMCs exhibited a single band for CADM1 with a molecular weight of ∼100 kDa (**Figure 1A**), consistent with previous reports for human and mouse mast cells (Furuno et al., 2005; Moiseeva et al., 2013a). Immunocytochemistry and flow cytometry showed moreover that CADM1 was predominantly located in the plasma membrane of BMMCs (**Figures 1B,C**). CADM1 expression was also observed in lysates prepared from adult DRG cultures, although it had a lower molecular weight (∼70 kDa, **Figure 1A**), consistent with the expression of a distinct splice variant lacking *O*-glycans (Hagiyama et al., 2011). Immunocytochemistry of DRGs maintained in mono-culture for 48 h with the neuronal marker β-tubulin confirmed the expression of CADM1 was specific to neurons and notably absent from non-neuronal glia-like cells which surrounding the neurons, whose presence can be detected from DAPI staining of their nuclei (**Figures 1D,E**). Close inspection of CADM1 staining in the co-cultures showed that it was most intense at the sites of contact between mast cells and neurites (**Figure 2** arrows).

CADM1 Is Necessary for BMMC Adhesion to DRG Neurons

To assess the role of CADM1 in mediating BMMC adhesion to sensory neurons, we developed a fluorimetric adhesion assay based on labeling of BMMCs with calcein, prior to their addition to DRG cultures. Optimization experiments showed that after loading BMMCs with calcein-AM for 30 min, fluorescent labeling is stable for 3 h and subsequently declines over the next 24 h. Isolated adult DRG neurons were cultured for 24 h in a flat-bottomed 96 well tissue culture plate during which time they developed an extensive network of neurites (**Figure 3A**). Calcein-labeled BMMCs were then added to the wells and allowed to adhere for 2 h after which non-adherent cells were removed vigorously by centrifugal spinning the culture plate upside-down at 20 × *g* for 2 min. To control for non-specific adhesion of BMMCs to the matrigel matrix used for supporting the DRG cultures, experiments were done in parallel on wells coated with matrigel but devoid of DRG (**Figure 3B**). Microscopic examination of the wells after spinning, clearly shows the enhancement in BMMC adhesion induced by co-culture with neurons, and moreover that the majority of mast cells attach to neurites rather than cell bodies (**Figure 3A**), consistent with observations made during the immunohistochemistry experiments shown in **Figure 2**. To quantify BMMC adhesion to the neurons, we measured the total fluorescence per well from calcein-labeled BMMCs before (total) and after centrifugation (for adherent). As shown in **Figure 3C**,

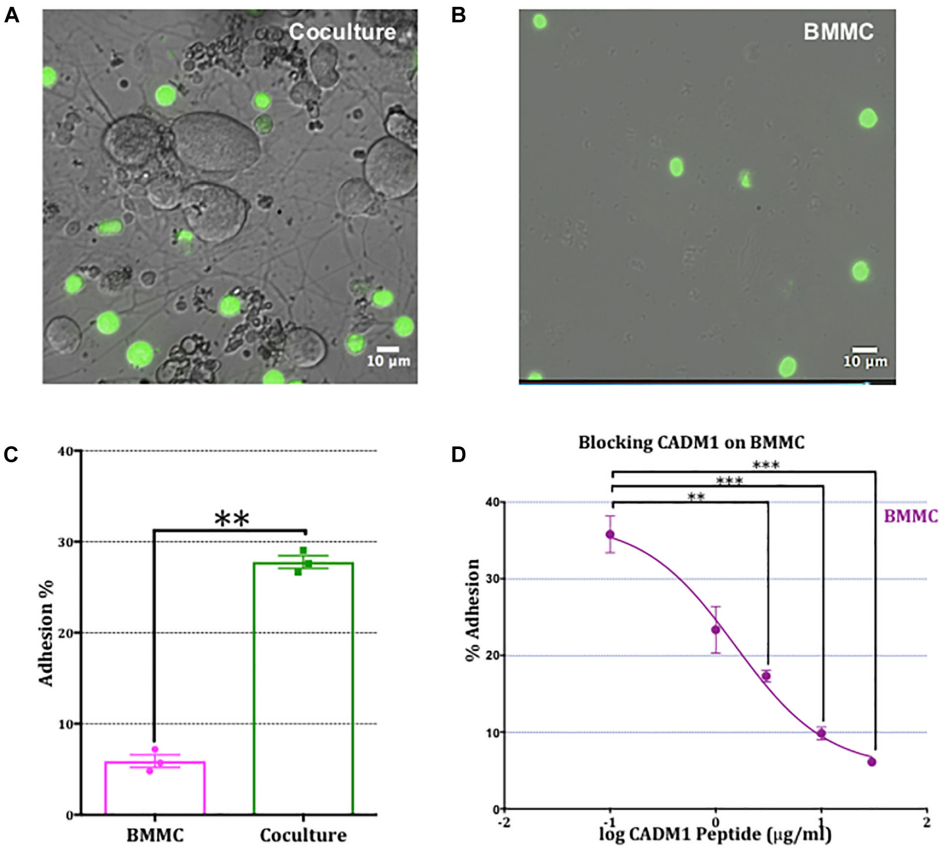

FIGURE 3 | Adhesion of mast cells to sensory neurons is attenuated by a CADM1 blocking peptide. **(A)** Superimposed bright-field and fluorescent image of live calcein-labeled, adherent BMMCs (green) co-cultured with DRG neurons (unlabeled cells) for 2 h. Non-adherent cells have been removed by washing and centrifugation of the plate. **(B)** Image of calcein-labeled BMMCs plated in parallel into matrigel-coated wells devoid of neurons and subjected to the same washing and centrifugation procedure. **(C)** BMMC adhesion quantified from calcein-fluorescence remaining in wells after washing and centrifugation expressed as a percentage of total well fluorescence measured prior to washing procedure. Data shown as mean ± SEM from $N = 3$. Each done in duplicate. Data were analyzed using a two-tailed paired t-test $^{**}p < 0.01$. **(D)** Concentration-dependent inhibition of mast cell adhesion to DRG measured with a CADM1 blocking peptide. Percentage of adherent BMMC was calculated using the calcein adhesion assay. Each condition was done in duplicate, on $N = 3$ cultures. Each point represents the mean ± SEM. One-way ANOVA followed by Turkey's multiple comparison post-test was performed. ** denotes $p < 0.01$ and *** $p < 0.001$ compared to the percentage of BMMC adhesion in the absence of CADM1 blocking peptide.

co-culture with DRG neurons significantly increased the number of adherent BMMCs four-fold. Addition of CADM1 blocking peptide inhibited adhesion of BMMCs to sensory neurons in a concentration-dependent manner (**Figure 3D**) and was almost abolished at the maximum concentration tested (30 $\mu g\ ml^{-1}$), consistent with the hypothesis that CADM1 mediates adhesion between mast cells and sensory neurons.

To validate the results from the CADM1 blocking peptide, we also performed CADM1 knockdown experiments in BMMCs. Three vectors expressing unique CADM1 targeted shRNA or a non-targeting scramble control together with an eGFP reporter gene were transfected into BMMCs. After 48 h, cells were sorted using fluorescence activated sorting and eGFP expressing cells used in subsequent experiments. The efficacy of CADM1 knockdown in sorted eGFP expressing BMMCs as assessed by western blot (**Figure 4A**) showed that all three ShRNA constructs tested were highly effective, with ShRNA2 and ShRNA3 achieving almost complete knockdown. Consistent with the results from the CADM1 blocking peptide experiments, knockdown of CADM1 expression in BMMCs significantly attenuated their adhesion to sensory neurons (**Figure 4B**).

CADM1-Dependent Adhesion to Sensory Neurons Potentiates Antigen-Induced Mast Cell Degranulation and Cytokine Secretion

Having established that BMMCs adhere to sensory neurons via a CADM1-dependent interaction, we next examined potential functional consequences of this interaction. It has been reported that the length of substance P immuno-reactive nerve fibers are increased in airway of allergic conditions such as asthma (Ollerenshaw et al., 1991) and that NGF and TNFα secreted by activated mast cells could enhance neuronal outgrowth (Leon et al., 1994; Kakurai et al., 2006). Immunocytochemical analysis of DRG cultures used for our co-cultures showed they consisted

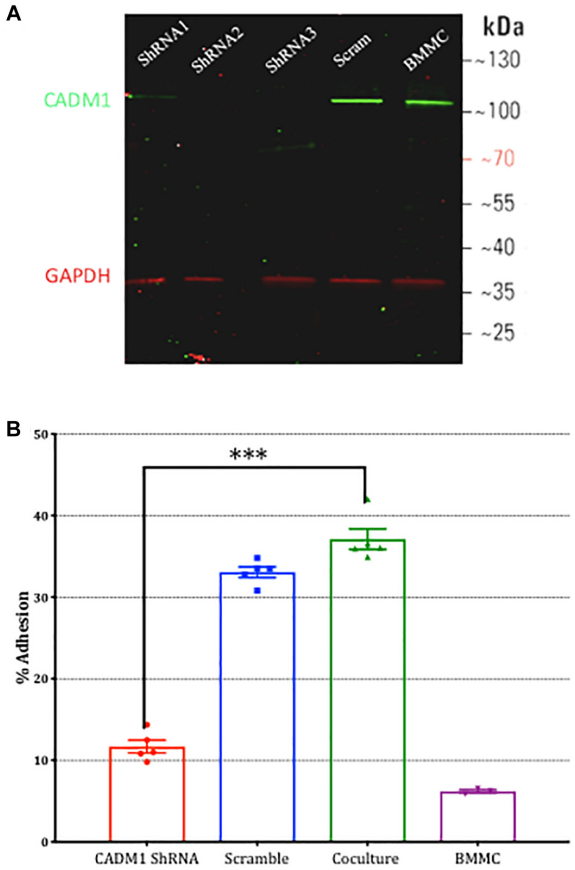

FIGURE 4 | Knockdown of CADM1 in mast cells inhibits their adhesion to sensory neurons. **(A)** Western blot analysis of CADM1 expression in BMMCs transfected with the indicated ShRNA 48 h previously. Expression from un-transfected control cells examined in parallel are also shown (lane labeled BMMC). GAPDH was used as loading control. Data are representative of three independent experiments. **(B)** Adherence of BMMCs transfected with CADM1 ShRNA or scrambled control to neurons tested using the calcein assay. BMMC adhesion in the absence of DRG neurons is shown as a control. CADM1 knockdown significantly reduced BMMC adhesion to sensory neurons ($N = 3$). Each bar represents the mean ± SEM. One-way ANOVA followed by Turkey's multiple comparison post-test was performed. *** denotes $p < 0.001$.

of at least 60% nociceptors, of which ∼50% were peptidergic (**Supplementary Figure 1**). We therefore investigated the effect of co-culturing BMMC on sensory neuron morphology in our *in vitro* system. Two parameters examined were total neurite length and complexity (number of neurite crossing points of a concentric circle set with radii increasing by 20 μm, (Stanko et al., 2015) and comparisons made between DRG monocultures and BMMC-DRG co-cultures. The neurites and BMMCs were detected by immunocytochemistry staining for βIII-tubulin and c-kit, respectively (**Figures 5A–D**). After 48 h of co-culture, no significant change in neurite length nor complexity was detected (**Figures 5E,F**), indicating that at least in the short term (48 h post-axotomy), mast cell adhesion does not alter sensory neurite morphology.

To investigate whether adhesion of mast cells to sensory neurons alters mast cell function and more specifically, antigenic activation of mast cells through FcεRI receptors, we established co-cultures of DRG neurons with anti-DNP IgE-sensitized BMMCs and compared their responses to antigen stimulation with mono-cultures of BMMCs prepared in parallel. Degranulation of mast cells was measured using β-hexosaminidase (β-hex) assays (Kuehn et al., 2010). Remarkably, BMMC basal degranulation and antigen-stimulated degranulation were both significantly potentiated in BMMCs following 6 h co-culture with sensory neurons and continued to increase for up to 24 h, the latest time point tested (**Figure 6**). Indeed after 24 h of co-culture with sensory neurons, mast cell degranulation was approximately double that measured in mono-cultures set up in parallel. In contrast, no such potentiation of antigen-induced degranulation was observed when BMMCs in co-culture were stimulated with compound 48/80 (**Supplementary Figure 2**), indicating that the effect of co-culture was not simply making the mast cells hyper-responsive in a non-specific manner but involved a specific signaling pathway which enhanced their responsiveness to antigen-stimulation in a time-dependent manner.

To examine the possibility that mediators released by DRG neurons in co-culture mediated the enhancement of mast cell degranulation, we performed three different types of experiments. Firstly we examined the impact of stimulating sensory neurons directly with capsaicin on mast cell degranulation. As shown in **Figure 7A**, BMMC degranulation in co-cultures was significantly potentiated by capsaicin showing that chemical communication between sensory neurons and mast cells was functional under co-culture conditions. Next we examined the impact of disrupting contact between the two cell types by (a) incubating BMMCs for 24 h with supernatant from BMMC-DRG co-cultures, and (b) preparing co-cultures of BMMCs and DRGs in which contact between the two cell types was blocked by means of a transwell insert. In either scenario, where direct contact between sensory neurons and mast cells was disrupted, the potentiation of mast cell degranulation by sensory neuron signaling was blocked (**Figures 7B,C**) emphasizing the need for adhesion between the two cell types.

To explore further the specificity of the functional interaction between mast cells and sensory neurons and its dependence on CADM1-mediated adhesion, we also examined the effects of co-culturing BMMCs with HEK cells. Like sensory neurons, HEK cells also express CADM1 and BMMCs adhere to these cells (**Supplementary Figure 3**). However, co-culture of BMMCs with HEK cells did not result in potentiation of mast cell degranulation (**Supplementary Figure 3**), the percentage of basal degranulation measured being 5.8 ± 1.6% in BMMC-HEK co-cultures maintained for 24 h, compared with 6.5 ± 0.7% for BMMC mono-cultures set up in parallel ($N = 3$). Antigen-induced degranulation was similarly unaffected by co-culture with HEK cells (co-culture degranulation 17.9 ± 1.8%, compared with 18.2 ± 1.6% for BMMC mono-cultures set up in parallel, $N = 3$, **Supplementary Figure 3**).

Having established that potentiation of BMMC degranulation was specific to co-culture with DRG and reliant on contact, we

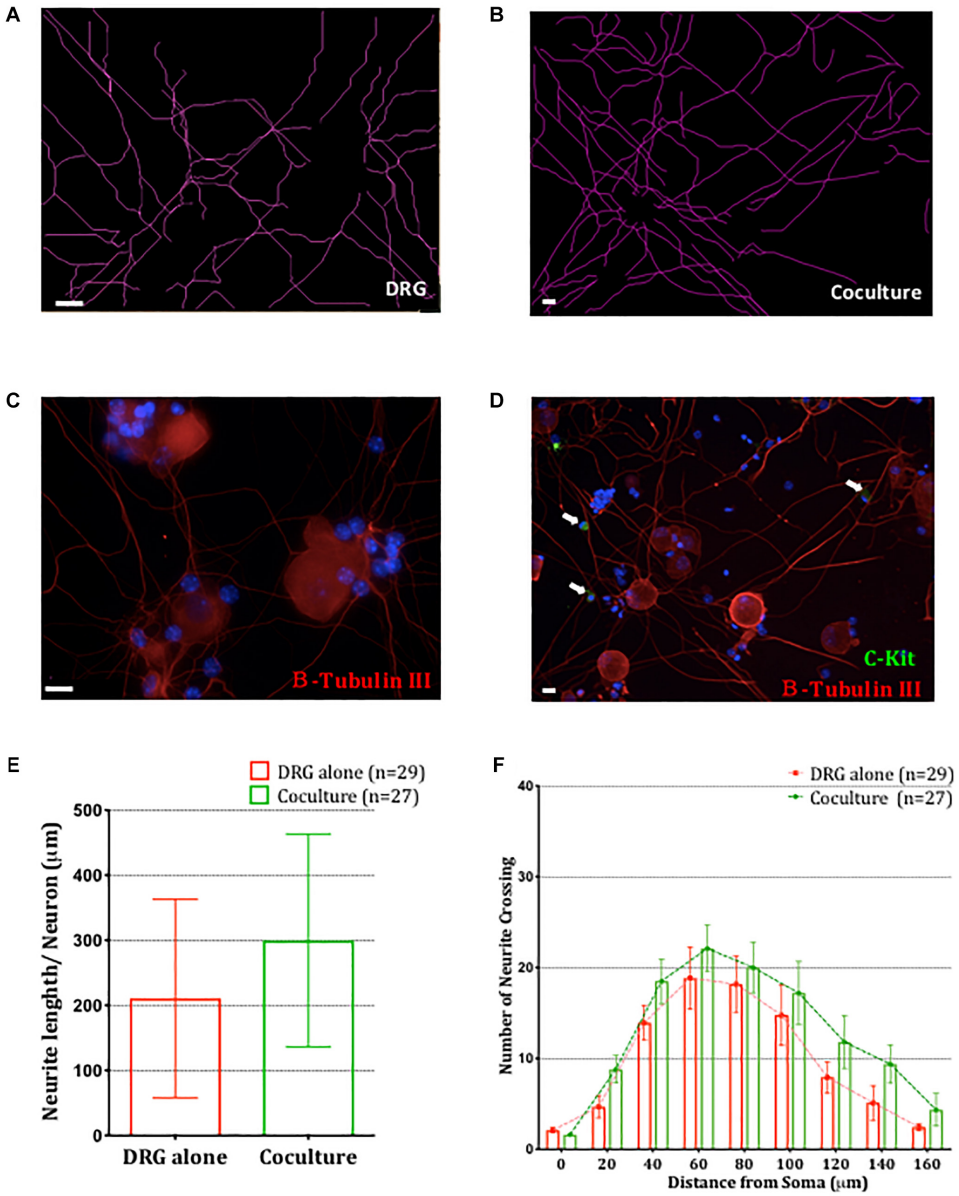

FIGURE 5 | Co-culture of BMMCs with DRG does not impact neuronal morphology. Representative schematic tracings of the neurites from DRG cultured alone **(A)** or in BMMC-DRG co-culture **(B)**. Soma and neurites were visualized with anti-β-III tubulin (red) and BMMCs with anti-c-kit (green, white arrows) in DRG mono-cultures **(C)** and co-cultures **(D)**. DAPI staining of nuclei (blue) show presence of non-neuronal cells in the DRG cultures. **(E)** Quantitative analysis of total neurites length in DRG mono-cultures and co-cultures, compared using unpaired *t*-test. **(F)** Sholl analysis of the neurite complexity. Each bar represents the mean ± SEM of number of crossing neurites found in each given distance from the soma. Statistical analysis using multiple *t*-test. $N = 3$.

next examined the role of CADM1-dependent adhesion in mast cell-sensory neuron crosstalk. Addition of CADM1 blocking peptide to the co-cultures was found to significantly inhibit the potentiation of mast cell degranulation (**Figures 8A,B**), indicating that CADM1-mediated adhesion between the cell types was necessary and sufficient to potentiate mast cell secretion. Consistent with this conclusion, knockdown of CADM1 in BMMCs also significantly attenuated the enhancement of degranulation induced by co-culture with sensory neurons (**Figures 8C,D**).

Finally, we examined whether CADM1-dependent mast-cell sensory neuron cross talk extended to the regulation of pro-inflammatory cytokine secretion. IL-6 and TNFα secretion in mono- and co-cultures were compared in the absence and presence of antigen activation of mast cells. As shown in **Figure 9A**, IL-6 secretion was induced by co-culture with DRG and moreover, antigen-activated secretion significantly increased by 2.5-fold. In contrast, TNFα was not increased, indicating that the signaling pathway enhanced by CADM1 adhesion of mast cells to sensory neurons was specific and selective to the synthesis

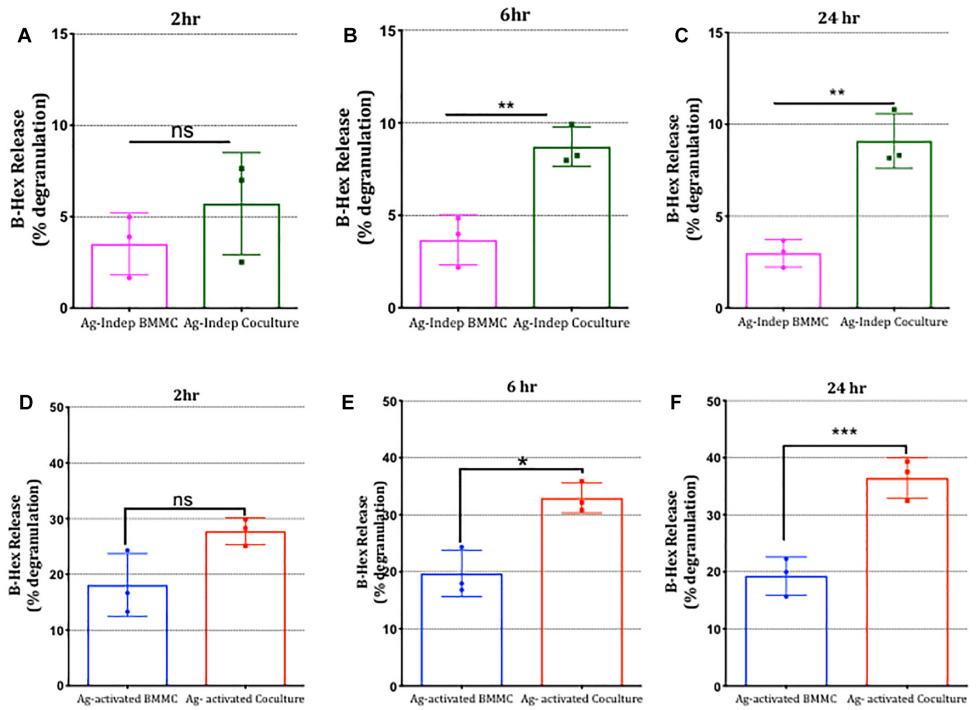

FIGURE 6 | Mast cell degranulation is potentiated following co-culture with sensory neurons. BMMCs pre-sensitized with anti-DNP IGE were cultured alone or with DRG for various times as indicated **(A–F)**. Degranulation measured as secreted β-hexosaminidase (β-hex) in media recovered from the cultures was measured at the times indicated above each graph in the absence **(A–C)** or after 30 min exposure to the antigen (Ag) DNP (10°ng/ml, **D–F**) and expressed as a percentage of total β-hex measured from BMMC lysed with 0.5% Triton X-100. Data shown are mean ± SEM of $N = 3$, each performed in duplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to BMMC alone. Data were analyzed using two-tailed paired *t*-test.

and secretion of specific pro-inflammatory cytokines. Control experiments confirmed that neither cytokine was produced to any significant level in DRG monocultures (**Figures 9A,B**). Knockdown of CADM1 expression in BMMCs significantly attenuated the neuronal induced IL-6 secretion as well as the neuronal enhancement of antigen-activated IL-6 secretion, confirming the critical role played by this adhesion molecule in regulating the signaling pathway controlling cytokine expression and secretion in mast cell-sensory neuron cross talk (**Figure 9C**).

DISCUSSION

Because mast cells mature within tissues when they are in proximity to other cell types (Galli, 2000), one can speculate that mast cell specific interactions with other cells will be regulated by the expression of cognate adhesion receptors on other cells in the tissue and that contact between the cells will promote receptor mediated communication between the cells. Furthermore, anchoring of adhesion molecules at sites of cell contact and consequent stabilization of protein-protein interactions mediated through their cytosolic domains may also influence the effector functions of mast cells in a tissue and cell specific manner. Here we show, that CADM1 mediates adhesion between mast cells and adult sensory neurons, and that this interaction alone is sufficient to induces degranulation and IL-6 secretion from mast cells and also to enhance significantly FcεRI-activated secretion of pro-inflammatory mediators.

At least four isoforms of CADM1 are generated through alternative splicing, with resulting changes in glycosylation impacting on cell specific functions and adhesion strength (Fogel et al., 2007; Hagiyama et al., 2011; Moiseeva et al., 2013a). Our protein expression analysis in BMMCs is consistent with other studies on human (Yang et al., 2006; Moiseeva et al., 2012) and rodent mast cells (Ito et al., 2003) showing expression of a single isoform of CADM1 with a m.w. of ∼100 kDa. This is significantly higher than the predicted molecular weight of any of the four *CADM* gene encoded proteins (m.w. 40–45 kDa), but in agreement with the apparent m.w. of fully glycosylated CADM1c isoform. The specific expression of CADM1c in mast cells may be of significance when it comes to controlling adhesion to sensory neurons. It is known that dimer formation is essential for CADM-mediated adhesion, CADM1c is unique among the four common isoforms in forming heterodimers with CADM1d, thereby significantly increasing the strength of adhesion compared to that formed by homodimers (Hagiyama et al., 2011). BMMCs grown in monocultures notably show very little adhesion to each other. This observation suggests that CADM1c does not mediate significant homotypic adhesion and that trans CADM1 binding is isoform dependent (Hagiyama et al., 2011).

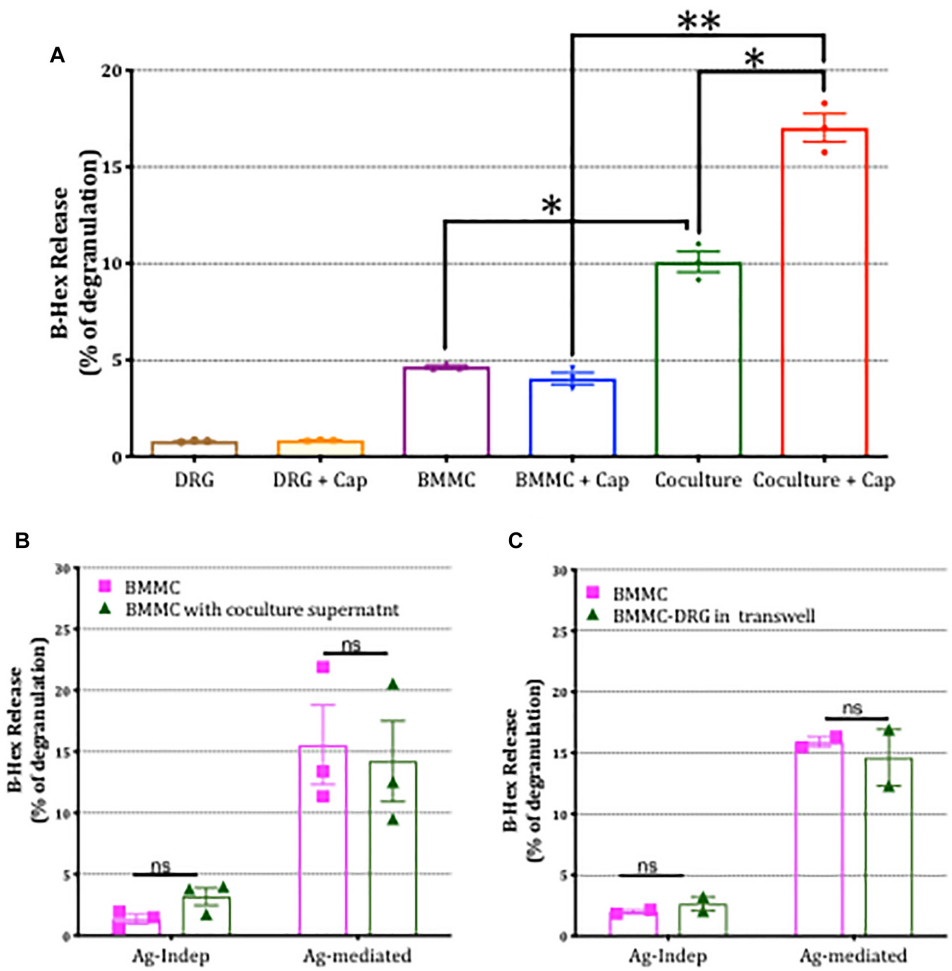

FIGURE 7 | Potentiation of mast cell degranulation by sensory neurons is contact-dependent. **(A)** BMMC degranulation measured as secreted β-hex from the mono-cultures and co-cultures of the indicated cells. Sensory neurons co-cultured with BMMCs for 24 h, were activated with 1 μm capsaicin for 20 min. β-Hex secreted from mono-cultures of DRGs is minimal and shows that the potentiated BMMC degranulation observed in the co-cultures is not simply additive but due to a significant interaction between the two cell types. **(B)** BMMCs pre-sensitized with anti-DNP IGE were cultured alone or with supernatant collected from BMMC-DRG co-cultured for 24 h. Degranulation as measured by secreted β-hex was measured in resting condition (labeled Ag independent) or after 30 min stimulation with the antigen, DNP (10 ng/ml, labeled Ag-mediated), and expressed as a percentage of total β-hex measured from lysed BMMCs. **(C)** Pre-sensitized BMMCs were cultured alone or on the top of DRG cultured using a transwell system for 24 h prior to measuring degranulation. Data shown are mean ± SEM of $N = 3$, each performed in duplicate. Data were analyzed using two-tailed paired *t*-test. * $p < 0.05$, ** $p < 0.01$, n.s is non-significant.

CADM1 expression analysis on isolated adult DRGs showed a thick band of protein with an estimated m.w. of ∼75 kDa, the expected molecular weight of isoform d. In other neurons, CADM1d expression is developmentally regulated, concentrated in neurites and linked to formation of synapses (Fogel et al., 2007; Hagiyama et al., 2011). Our immunocytochemistry analysis of DRG indicated that CADM1 was expressed in the soma of all subtypes of sensory neurons and some neurites. When BMMCs were co-cultured with DRG, BMMCs were observed to have attach to neurites where CADM1 was concentrated. In contrast to our results, a previous study investigating mast cell-sensory neuron interactions failed to identify CADM1 expression in DRG and concluded that nectin3 mediated adhesion with mast cells (Hagiyama et al., 2011; Furuno et al., 2012). Differences between the two studies may arise from the age of mice used to isolate DRG and developmental regulation of CADM1 expression. In agreement with our protein expression analysis in adult DRG, single cell RNA sequence analysis of DRG neurons also indicates expression of CADM1 in all subtypes of sensory neurons and interestingly, also expression of CADM2 in nociceptors (Usoskin et al., 2015). CADM1 and CADM2 protein expression throughout development has been reported in chick DRG and interestingly, in the same report confirmed in mouse (Frei et al., 2014). CADM2 also has a mw ∼76 kDa, and forms strong and specific cis-heterophilic interactions with CADM1 (Frei et al., 2014), and could therefore also be contributing to, or regulating, the formation of trans-heterophilic interactions mediating the adhesion of mast cells to sensory neurites observed in our study.

Blocking CADM1 before co-culture reduced the percentage of BMMCs adhered to sensory neurons. Knockdown CADM1

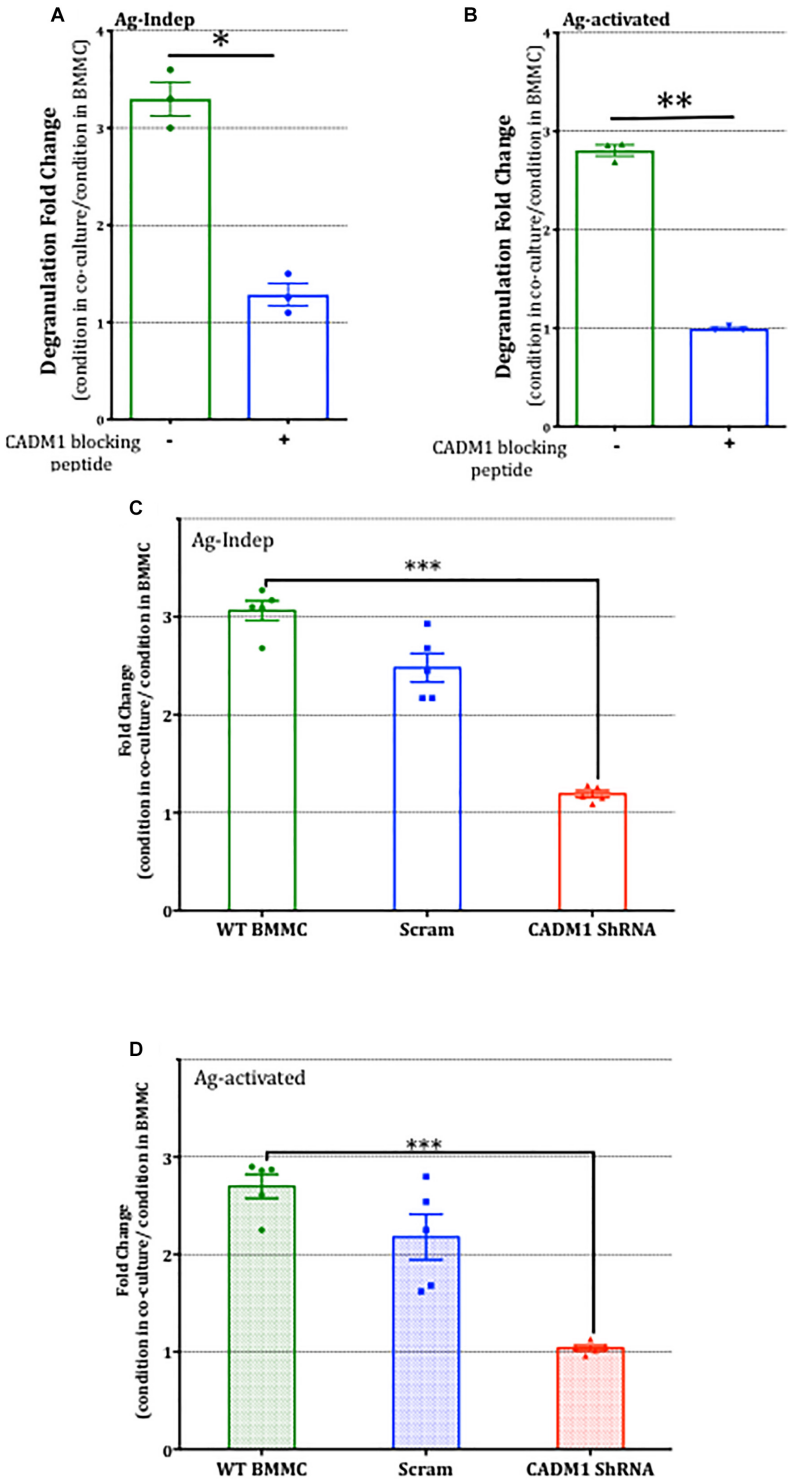

FIGURE 8 | CADM1 is necessary for mast-cell sensor neuron cross-talk. BMMCs pre-sensitized with anti-DNP IGE were co-cultured with DRG for 24 h in the presence or absence of 10 μg/ml CADM1 blocking peptide before degranulation was quantified using the β-Hex assay. Data were normalized to degranulation measurements made from BMMC mono-cultures set up in parallel, and the fold-change in basal Ag-Indep degranulation **(A)** or DNP (10 ng/ml), Ag-activated degranulation **(B)** calculated and compared by two-tailed paired *t*-test, * denotes $p < 0.05$, ** $p < 0.01$. Each bar represents the mean ± SEM ($N = 3$). **(C)** Untransfected (WT BMMC), CADM1-ShRNA or scramble-transfected BMMCs pre-sensitized with anti-DNP IGE were co-cultured with DRG for 24 h before degranulation was quantified using the β-Hex assay. Fold change of Ag-Independent degranulation **(C)** and DNP (10 ng/ml) Ag-activated degranulation **(D)** were calculated and compared by one-way ANOVA followed by Turkey's multiple comparison post-test, ***denotes $p < 0.001$ compared to wt BMMC-DRG co-culture. Each bar represents the mean ± SEM ($N = 3$).

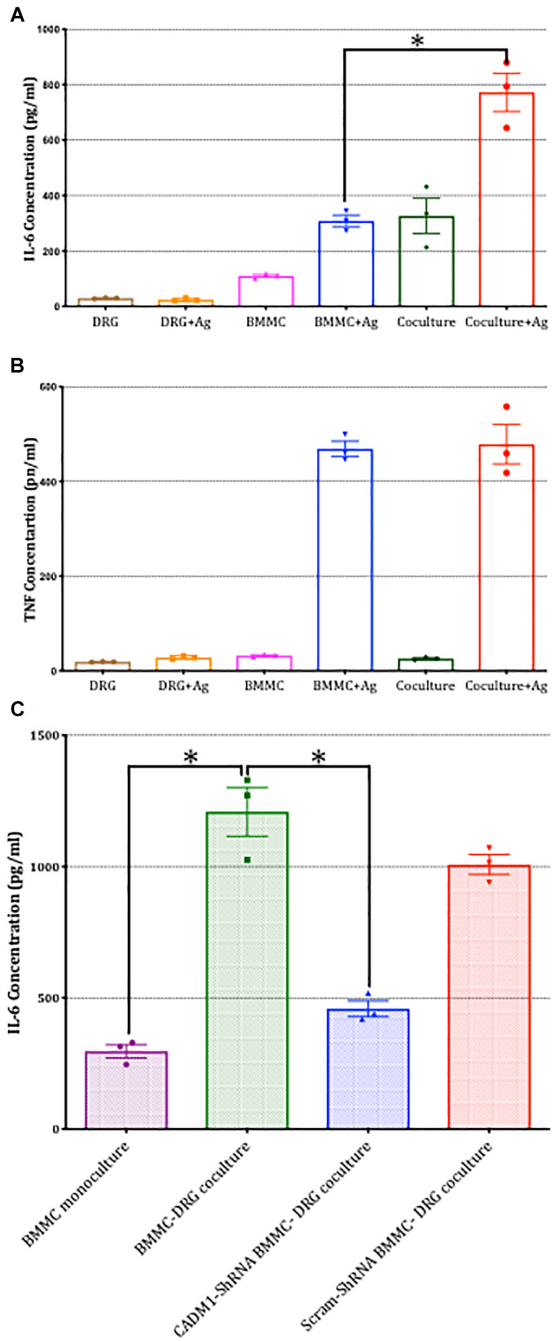

FIGURE 9 | Sensory neuron potentiation of antigen-stimulated cytokine secretion by mast cells is dependent on CADM1. BMMCs pre-sensitized with anti-DNP IGE were cultured alone or with DRG for 24 h. **(A)** Secreted IL-6 and **(B)** TNFα were quantified by ELISA for cells stimulated in parallel with the antigen DNP (10 ng/ml) for 6 h or vehicle control. Data shown are mean ± SEM of $N = 3$, each performed in duplicate. * $p < 0.05$ compared to stimulated BMMC alone. Data were analyzed using two-tailed paired *t*-test. **(C)** BMMC mono-culture and untransfected WT, CADM1-knockdown or scramble-transfected BMMCs were co-cultured with DRG for 24 h, prior to being activated with DNP (10 ng/ml) for 6 h. IL-6 was measured from supernatants and compared by one-way ANOVA followed by Turkey's multiple comparison post-test, *denotes $p < 0.05$ compared to wt BMMC-DRG co-culture. Each bar represents the mean ± SEM ($N = 3$).

expression from BMMCs also significantly reduced their adhesion to neurons. Despite the effectiveness of the ShRNA in ablating expression of CADM1 in BMMCs, the reduction in adhesion achieved was less than that obtained with the blocking peptide. This difference could arise from non-specific inhibition of other structurally-related immunoglobulin superfamily adhesion receptors by the peptide, such as ICAM-1 (Inamura et al., 1998). Another possibility is that CADM1 knockdown leads to compensatory up-regulation of other adhesion molecules such as integrins which are also widely expressed in mast cells and can regulate their signaling (Sperr et al., 1992; Lorentz et al., 2002; Moiseeva et al., 2014). Nonetheless, taken together the results of both types of interference experiments provide strong evidence that CADM1 is primarily responsible for mast cell adhesion to sensory neurons.

Addition of BMMCs to DRG culture for 1 day did not affect either total neurite length or neurite complexity. Although it is reported that activated mast cells produce NGF and TNFα, which enhance neuronal outgrowth and plasticity (Leon et al., 1994; Kakurai et al., 2006), in our co-culture system, the former, at least was not apparent. Key methodological differences that could account for the lack of morphological changes could be the use of adult versus embryonic DRG (Leon et al., 1994; Frei et al., 2014). Embryonic DRG cultures are dependent on NGF for their survival (Melli and Höke, 2009), while adult DRG cultures are not, despite its receptor expression. Indeed, the role of NGF in adult sensory neurons shifts away from the neurotrophic effect to a pro-inflammatory effect that regulates neuronal function and plasticity (Sofroniew et al., 2001). Our functional studies, as discussed below, support the notion that in adult sensory neurons, the role of CADM1 adhesion is shifted from one supporting axonal pathfinding and formation of neural circuits to one regulating neuroimmune crosstalk.

Functional experiments performed on mono-cultures and co-cultures showed for the first time that CADM1-mediated adhesion between mast cells and sensory neurons specifically and selectively induced degranulation and IL-6 secretion from mast cells and moreover significantly enhanced antigen-activated mast cell responses. While adhesion between the two cell types was very rapid and established within 2 h of contact, the enhancement of mast cell secretory functions was relatively delayed, becoming significant after 6 h of co-culture and continuing to increase over the ensuing 24 h, indicating that activation of CADM1-dependent intracellular signaling pathways are necessary. The observation that mast cell degranulation may be modulated by adhesion to other cells, even in the absence of external stimulation, has been reported previously. Co-culture human lung mast cells with airway smooth muscle show increased constitutive histamine release (Hollins et al., 2008) which starts after 16 h of co-culture (Lewis et al., 2016). Activated T cells enhance constitutive histamine release from BMMC also when co-cultured for 16 h (Inamura et al., 1998). Co-culture of LAD2 mast cells with tumor cells, like pancreatic ductal

adenocarcinoma for 24 h also show enhanced constitutive tryptase release (Ma et al., 2013). Here we found that co-culture with HEK cells, which also express CADM1, like sensory neurons, while promoting efficient adhesion, did not however, result in enhanced mast cell secretion implying that additional cell specific interactions facilitated by CADM1-adhesion contribute to the activation and sensitization of mast cells.

Sensory neurons are a potential source of potent mast cell activators, including substance P (Karimi et al., 2000; Okabe et al., 2006), CGRP (Forsythe et al., 2000; Rychter et al., 2011) and ATP (Wareham and Seward, 2016) which may exist as co-transmitters. Exposure to inflammatory mediators drives increased CGRP synthesis and dense core granule biogenesis in peptidergic sensory neurons (Russell et al., 2014). In our co-culture experiments, activation of sensory neurons with capsaicin notably markedly enhanced mast cell degranulation, consistent with the establishment of chemical communication between the cells. Our preliminary experiments with cultures of sensory neurons from trigeminal ganglia, which are enriched in peptidergic neurons, revealed further that co-culture with mast cells was sufficient to significantly increase CGRP secretion (**Supplementary Figure 4**). It would therefore seem likely that the gradual increase in mast cell degranulation we observed over time in our co-cultures reflect the development of an increasing number of functional neuro-immune synapse like structures between mast cells and sensory neurites, potentiating chemical communication between the two cell types which *in vivo* would translate into pain hypersensitivity (Gupta and Harvima, 2018). The ability of CADM1 to promote pre- and post-synaptic specializations and synaptogenesis in the CNS is well established (Frei and Stoeckli, 2017). Crucial to these synapse promoting functions of CADM proteins, are their highly conserved cytosolic domains which recruit scaffold proteins and effector molecules and increased actin polymerization (Cheadle and Biederer, 2012), which is needed for the capture of dense core vesicles (Porat-Shliom et al., 2013; Bharat et al., 2017). Interestingly, CADM1 driven formation of synapses may be activity driven, and the distribution of CADM1 into membrane nanodomains may physically define the contact edges of synapses (Robbins et al., 2010; Perez de Arce et al., 2015; Ribic et al., 2019). We would therefore propose a model in which the formation of trans-heterotypic CADM1 adhesions between mast cells and sensory neurites leads to the formation and stabilization of neuro-immune synapses enriched in peptidergic vesicles, increasing cross-talk between the two cell types and inducing mast cell activation and secretion. Further studies with super-resolution microscopy will be needed to test this model.

In addition to inducing mast cell degranulation and IL-6 secretion, co-culture with sensory neurons and CADM1-dependent adhesion was also found to enhance antigen-induced responses. Activation of mast cells by antigen is mediated by the crosslinking of FcεRI-receptors which triggers a series of tyrosine kinase regulated phosphorylation events, orchestrated through the engagement of multiple adaptor and scaffold proteins, culminating in the activation of signaling that triggers degranulation, lipid mediator synthesis, and transcription of cytokines and chemokines. In the context of the enhanced FcεRI responses observed in our co-cultures, we found that secretion of TNFα was not potentiated. Transcription of TNFα is driven by NFAT and sustained calcium signaling (Klein et al., 2006; Falvo et al., 2010) whereas IL-6 transcription is driven by NF kappaB signaling, therefore the observed enhancement of only one of the two tested cytokines indicates that CADM1-mediated adhesion of mast cells to sensory neurites augments a selective part of the FcεRI signaling. Precedence for selective potentiation of selective cytokines, and excluding TNFα, has been reported previously and shown to be mediated through synergistic activation of specific kinases regulating the activity of transcription factors. Receptor-mediated inhibition of signaling could also be involved in fine tuning the impact of sensory neuron adhesion on mast cell allergen responses. Activation of GPCRs coupled to pertussis toxin sensitive G*i* proteins, lead to synergistic enhancement of degranulation, IL-6 and TNFα secretion, (Kuehn et al., 2008), which is distinct to what we have observed in co-culture. We observed that activation of mast cells with compound 48/80 was not enhanced by co-culture with sensory neurons. Since compound 48/80 function in mast cells is mediated through activation of MrgprB2 and G*i* proteins, a receptor also targeted by substance P (Subramanian et al., 2016), this suggests however, that the enhancement in antigen-evoked responses observed upon adhesion to sensory neurites does not represent a G*i* PCR-mediated amplification system (Gilfillan et al., 2009). CADM1-mediated interactions with the actin cytoskeleton through its cytosolic domains may also prime FcεRIs in a manner similar to that recently described for integrins in mast cells (Shelby et al., 2016; Wakefield et al., 2017; Halova et al., 2018). While further in-depth molecular studies of FcεRI distribution and signaling cascades are needed to understand how CADM1 adhesion potentiates responses to allergens, our data shows unequivocally that CADM1-mediated adhesion to sensory neurons enhances mast cell derived IL-6 secretion and degranulation and would therefore potentiate mast cell regulated inflammatory responses in atopic individuals. *In vivo*, mast cells of IBS patients secrete greater amounts of the IL-6 (Liebregts et al., 2007) in response to neuronal hyperexcitability (O'Malley et al., 2011). Moreover, IL-6 has been found to induce mechanical nociceptive plasticity (Melemedjian et al., 2010; Hughes et al., 2013) that evokes allodynia (Oka et al., 1995; Dina et al., 2008). Blocking IL-6 receptors in neurons reduces inflammation in an antigen-induced arthritis model (Ebbinghaus et al., 2015) and anti-IL-6 receptor antibodies show promising therapeutic potential in controlling pain in rheumatoid arthritis (Nishimoto et al., 2009). Therefore, there is good evidence already that pain-related disorders may involve an increase in IL-6 levels, such as that reproduced in our co-culture system. In conclusion, we show that CADM1 is necessary to drive mast cells-sensory neuron adhesion and contribute to the development of a microenvironment in which neurons enhance mast cell responsiveness to antigen. This interaction

could explain why the incidence of painful neuroinflammatory disorders such as IBS are increased in atopic patients.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Healthy, 8–12 week-old male C57BL wild-type adult mice were used in this study. All animals were maintained on a 12-h light/dark cycle in a temperature-controlled environment and given food *ad libitum*. All animal procedures were conducted under the Animal (Scientific Procedures) Act 1986, and approved by the UK Home Office.

AUTHOR CONTRIBUTIONS

ES, ZD, and RM designed the experiments. ES and RM prepared the manuscript. RM and JM did the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00262/full#supplementary-material

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Mast Cell/Proteinase Activated Receptor 2 (PAR2) Mediated Interactions in the Pathogenesis of Discogenic Back Pain

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Mast cells (MCs) are present in the painful degenerate human intervertebral disc (IVD) and are associated with disease pathogenesis. MCs release granules containing enzymatic and inflammatory factors in response to stimulants or allergens. The serine protease, tryptase, is unique to MCs and its activation of the G-protein coupled receptor, Protease Activated Receptor 2 (PAR2), induces inflammation and degradation in osteoarthritic cartilage. Our previously published work has demonstrated increased levels of MC marker tryptase in IVD samples from discogenic back pain patients compared to healthy control IVD samples including expression of chemotactic agents that may facilitate MC migration into the IVD. To further elucidate MCs' role in the IVD and mechanisms underlying its effects, we investigated whether (1) human IVD cells can promote MC migration, (2) MC tryptase can mediate up-regulation of inflammatory/catabolic process in human IVD cells and tissue, and (3) the potential of PAR2 antagonist to function as a therapeutic drug in *in vitro* human and *ex vivo* bovine pilot models of disease. MC migration was quantitatively assessed using conditioned media from primary human IVD cells and MC migration examined through Matrigel. Exposure to soluble IVD factors significantly enhanced MC migration, suggesting IVD cells can recruit MCs. We also demonstrated significant upregulation of MC chemokine SCF and angiogenic factor VEGFA gene expression in human IVD cells *in vitro* in response to recombinant human tryptase, suggesting tryptase can enhance recruitment of MCs and promotion of angiogenesis into the usually avascular IVD. Furthermore, tryptase can degrade proteoglycans in IVD tissue as demonstrated by significant increases in glycosaminoglycans released into surrounding media. This can create a catabolic microenvironment compromising structural integrity and facilitating vascular migration usually inhibited by the anti-angiogenic IVD matrix. Finally, as a "proof of concept" study, we examined the therapeutic potential of PAR2 antagonist (PAR2A) on human IVD cells and bovine organ culture IVD model. While preliminary data shows

promise and points toward structural restoration of the bovine IVD including down-regulation of VEGFA, effects of PAR2 antagonist on human IVD cells differ between gender and donors suggesting that further validation is required with larger cohorts of human specimens.

Keywords: mast cell, tryptase, PAR2, intervertebral disc, discogenic back pain

INTRODUCTION

Approximately 70–80% of the population will experience chronic low back pain during their lifetime (Andersson, 1999) and large population-based studies have demonstrated that intervertebral disc (IVD) degeneration is a significant cause of chronic low back pain (De Schepper et al., 2010). The huge socioeconomic burden of Discogenic Back Pain (DBP) is not only a result of widespread use of interventions that are costly with limited efficacy, but also the role of DBP in the growing opioid epidemic, as opioids are the most widely prescribed drug used to treat back pain (Balagué et al., 2012; Deyo et al., 2015). Initial DBP therapies are largely conservative and include analgesics, physiotherapy and psychosocial pain management approaches. When these approaches are unsuccessful, highly invasive surgeries (e.g., disc arthroplasty or lumbar fusions) are routinely performed. Yet, these treatments are often short-lived, fail to target the underlying cause of disease and can significantly reduce patient mobility often leading to adjacent segment disc disease (Mannion et al., 2014).

The healthy IVD is characterized by a healthy gelatinous core of proteoglycans and nucleus pulposus (NP) cells of notochordal origin. This NP core is enclosed radially by concentric rings of collagen to form the annulus fibrosus (AF) with fibroblast-like AF cells aligned with the collagen fibrils. The joint is encased caudally and cranially by the cartilage end plate (CEP), hyaline cartilage crucial to bidirectional diffusion of nutrients and metabolites and separates the IVD from the vertebral bodies (Roughley, 2004). Degeneration of the IVD is characterized by significant increases in extracellular matrix (ECM) breakdown, inflammation and nerve/vascular ingrowth (Smith et al., 2011; Lama et al., 2018). Nociceptive neurons and blood vessels expressing nerve growth factor (NGF) have been demonstrated in the painful human IVD (Freemont et al., 1997, 2002), specifically in regions of ECM depletion and injury (Stefanakis et al., 2012; Lama et al., 2018). Our previous work has demonstrated that disc ECM and injury can regulate nerve/vascular growth *in vitro* and *in vivo* (Johnson et al., 2002, 2005; Kim et al., 2011; Purmessur et al., 2015). Additionally, pro-inflammatory cytokines interleukin 1-Beta (IL-1β) and tumor necrosis factor alpha (TNFα), (increased in the degenerate IVD) can up-regulate catabolic enzymes along with neurotrophic and angiogenic factors NGF, vascular endothelial growth factor A (VEGFA), interleukin-6 (IL-6) and substance P in IVD cells (Le Maitre et al., 2005; Séguin et al., 2005; Purmessur et al., 2013a). While the healthy IVD is immune-privileged, increased numbers of innate immune cells have been demonstrated within painful human IVDs compared to healthy IVDs, yet their role within the painful degenerate disc remains unknown (Wiet et al., 2017).

Immune cells play an integral role in tissue remodeling and healing. However, due to the avascular nature of the IVD, repair is commonly described as "frustrated healing" and lacks the native ECM components to restore structure and function (Adams and Roughley, 2006). Innate immune cells, such as mast cells (MCs) and macrophages, have been identified in degenerative human IVD tissue and induce a catabolic and inflammatory phenotype in IVD cells, however, the specific mechanisms underlying these effects remains to be elucidated (Wiet et al., 2017; Nakazawa et al., 2018). MCs function to release preformed granules with enzymatic (tryptase and a disintegrin and metalloproteinase with a thrombospondin motif 5, ADAMTS5) and inflammatory/pain-associated factors (IL-1β, TNFα, VEGFA, NGF and Substance P among others) in response to microenvironmental stimuli or allergens (De Schepper et al., 2010). Furthermore, MCs are active and significantly up-regulated in chronic pain conditions such as migraines, irritable bowel syndrome, rheumatoid arthritis and osteoarthritis (OA) (O'Sullivan et al., 2000; Nigrovic and Lee, 2005; Theoharides et al., 2005; Nakano et al., 2007). In OA, MC-derived mediators enhance mechanical hypersensitivity in nociceptors within the joint space (Sousa-Valente et al., 2018). Macrophages have also been identified in the IVD, however, they do not contain preformed granules but synthesize inflammatory cytokines upon activation [TNFα, IL-1β and prostaglandins (PGE2)] (Gordon and Taylor, 2005; Nakazawa et al., 2018). Similar to MCs, they are present in arthritic diseases and associated with matrix remodeling in disc herniation (Koike et al., 2003; Kobayashi et al., 2009). However, recent studies have demonstrated that macrophages do not directly regulate pain-associated growth factors VEGFA and NGF (Miyagi et al., 2018).

The serine proteinase tryptase is produced by MCs, and its activation of the G-protein coupled receptor, Proteinase Activated Receptor 2 (PAR2; F2RL1), has been shown to induce inflammation and catabolism in human osteoarthritic cartilage (Balagué et al., 2012). PAR2 initiates and enhances painful responses in inflammatory, visceral, and cancer-related pathologies and recent studies demonstrate its activation is sufficient to induce transition to a chronic pain state (Tillu et al., 2015). PAR2 is expressed by multiple cell types including immune cells, nerves and endothelial blood vessel cells, chondrocytes and IVD cells (Klarenbach et al., 2003; Iida et al., 2009; Chen et al., 2011). However, its specific role in DBP remains unknown. PAR2 is also involved in sensitization of neuropeptide receptors (Neurokinin 1) and ion channels (i.e., transient receptor potential cation channel subfamily V member 1; TRPV1) expressed on dorsal root ganglion (DRG) cells, which enhance hyperalgesia and pain (Vergnolle et al., 2001; Amadesi, 2004). Studies by Iida et al. (2009) have demonstrated that PAR2 is expressed in the rat IVD, elevated in painful human IVDs and that its activation

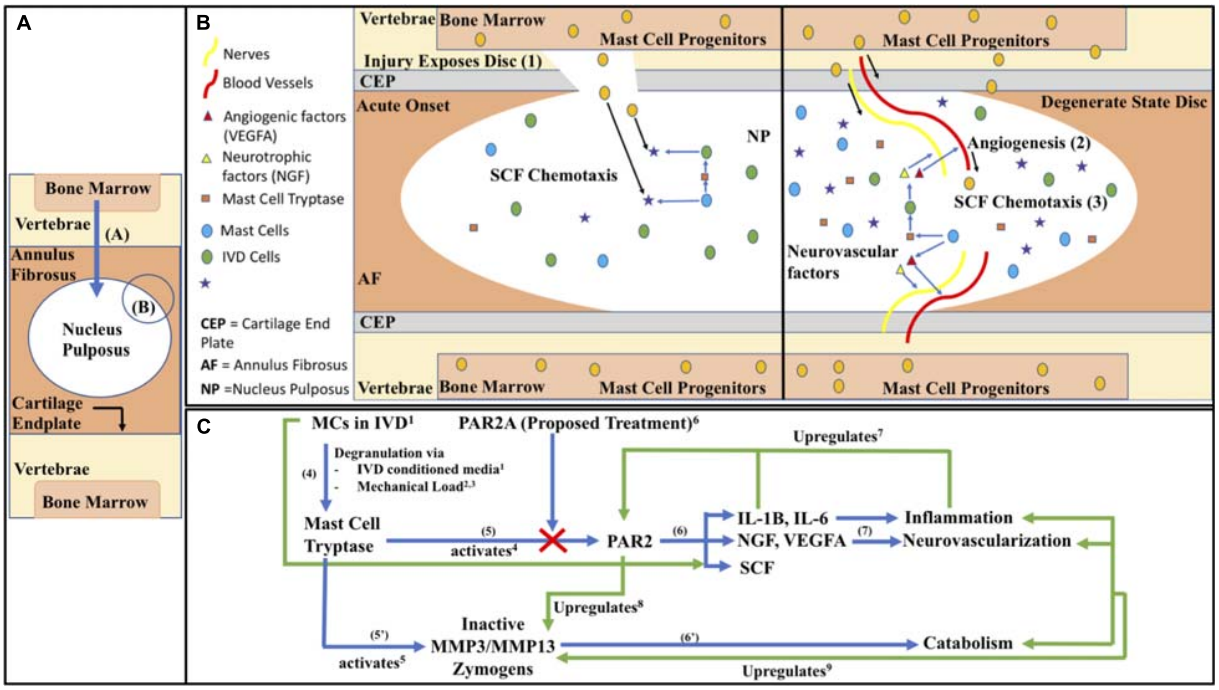

FIGURE 1 | (A) Macroscopic IVD schematic with zoomed in proposed mechanistic models of **(B)** MC invasion into the intradiscal environment in acute onset and chronic state as visualized through (1) Acute injury or matrix perturbation provides physical access of MC progenitors into usually immune-privileged intra-discal microenvironment acutely and (2) in the chronic state access can occur through vascularization, (3) facilitated by the MC chemoattractant SCF (KITLG). **(C)** Biochemical pathways of immune modulation in DBP as follows; (4) MCs in the IVD respond to mechanical load and nutrient deprivation in the harsh microenvironment by releasing tryptase (Wiet et al., 2017[1]; Zhang et al., 2012[2]; Fowlkes et al., 2013[3]). Tryptase can activate (5) PAR2 directly (Molino et al., 1997[4]) as well as (5') matrix enzymes, matrix metalloproteinases 3 and 13 (MMPs) (Magarinos et al., 2013[5]). (6) PAR2 activation by tryptase, abated by PAR2A (Chen et al., 2011[6]) in the present study, leads to downstream factors related to catabolism, (6') such as MMPs (Lee et al., 2010[8]), which can degrade the proteoglycan aggrecan (Magarinos et al., 2013[5]) creating an increasingly permissive environment for inflammation and stimulating increased activation of endogenous pro-MMP (Vo et al., 2013[9]). (7) PAR2's activation also leads to downstream factors related to inflammation, neo-vascularization/neo-innervation, and MC chemotaxis contributing to a synergistic positive feedback loop, whereas PAR2's activation upregulates its own expression through IL-1β, ad general inflammation, perpetuating a chronic degenerate state (Ritchie et al., 2007[7]). Black Arrows represent labeling or directionality, Blue arrows represent forward mechanism steps, Green arrows represent feedback interactions. **(C)** Blunt arrow ends represent effects coming from, pointed ends represent effects going to, and bidirectional pointed ends represent co-regulatory interactions.

upregulates the expression of IL-1β and ADAMTS4 in rat IVD cells. However, whether MC mediators (i.e., tryptase) can activate such inflammatory pathways in human IVD cells is currently unknown and is the focus of the studies described here.

The objectives of this study are based on the following hypothetical model as described in **Figure 1**. Briefly, MC progenitors invade the IVD via injury or matrix perturbation, being specifically recruited to the IVD region from bone marrow, following invasion the IVD microenvironment causes degranulation by the matured MCs and the action of the released mediators either directly on tissue or indirectly via induction of genetic changes on cells, promoting neurovascularization, inflammation, catabolism and ultimately pain. This process is self-perpetuating as elaborated on in **Figure 1**; MC migration is enhanced directly and indirectly through IVD cells, allowing this degenerative process to repeat and progress into a chronic degenerative state, with the eventual compromised state becoming further conducive to the aforementioned markers of a painful IVD. We hypothesize that, due to these intrinsically synergistic mechanisms, inhibition with the PAR2 antagonist oligopeptide FSLLRY-NH_2 (PAR2A) (Chen et al., 2011) possesses therapeutic potential to prevent progression of a chronic inflammatory, catabolic and painful state.

This study aims to determine the potential mechanism by which MCs may invade the IVD joint, investigate tryptase effects on matrix degradation and chemotactic/vascular markers, quantify and validate protein expression of PAR2 in NP, AF and CEP regions of the human IVD via immunohistochemistry and western blot and to determine the therapeutic potential of PAR2A in a bovine *ex vivo* translational model and in human *in vitro* model for proof of concept.

MATERIALS AND METHODS

Tissue Procurement and Histological Processing

Mild to moderately degenerate human cadaveric IVD samples (**Table 1**) were acquired via the Co-operative human tissue

TABLE 1 | Human tissue and cells.

Level/Joint (tissue + cells)	Region (tissue + cells)	Age	Gender
L3-L4; L4-L5	NP, AF, CEP	43	F
L1-L2; L5-S1	NP, AF, CEP	55	M
L3-L4; L4-L5	NP, CEP	45	M
L3-L4; L4-L5	NP, AF, CEP	56	F
L3-L4; L4-L5	NP, AF, CEP	52	M
L2-L3; L3-L4	NP, AF	58	F
L2-L3; L3-L4	NP, AF	46	M
L3-L4	NP, AF	31	F
L4-L5	NP, AF, CEP	30	F
L3-L4	NP, AF	37	F
L4-L5	NP, AF, CEP	59	F
L3-L4	NP	19	F
L3-L4	NP	39	M
Knee	Articular Cartilage	22	F
Knee	Articular Cartilage	20	F
Knee	Articular Cartilage	22	F

network, Midwestern Division (Columbus, Ohio) with institutional IRB exemption. Cadaveric specimens were dissected within 36 h post-mortem and tissue either isolated for cells or histology. For histology, 2 mm (superior to inferior) sagittal sections were collected using a diamond coated band saw. These sections were then fixed in 10% neutral buffered formalin before paraffin embedding for IHC. Human articular cartilage samples (**Table 1**) were acquired from patients with informed consent in accordance with The Ohio State University (Columbus, Ohio) IRB relevant guidelines and regulations under IRB# 2018H0424.

Primary IVD and Articular Cartilage Cells

All primary human IVD and articular cartilage cells (**Table 1**) were obtained from cadaveric samples, and patients undergoing treatment for osteoarthritis of the knee, respectively, sourced as described in Tissue Procurement. Exclusion criteria was applied for IVD samples, specifically, no infectious disease, discs were intact, no discs from spines older than 60 years old and Thompson scale grade not exceeding 3.5 (discs were blinded, graded by authors independently, and grades averaged). Articular cartilage samples were obtained from regions of intact healthy cartilage with microscopic and clinical assessment by Dr. David Flanigan. Cells were isolated using protease from *Streptomyces griseus* (0.05 mg/25 mL) in digestion media (high glucose (4.5 g/mL) DMEM, 1% Penicillin/Streptomycin (P/S), 0.5% Fungizone) for 1 h before 12 h digestion in Collagenase I or II (0.003 g/25 mL) for AF or NP tissue, respectively as cited previously (Purmessur et al., 2011). Following isolation, cells were expanded in Disc Cell Complete media (DCC; high glucose DMEM, 10% FBS, 1% P/S, 50 μg/mL Ascorbic Acid) in standard conditions (37°C, 20% O_2, 5% CO_2) and fed once every 3 days until ∼90% confluent. All cells used for *in vitro* studies were at passage < P4.

Mast Cell Culture

Human Mast Cell line 1 (HMC-1) cells were a generous gift of Dr. J.H. Butterfield (Mayo Clinic). HMC-1 cells were expanded in suspension in Basal HMC-1 media (BMC; Iscove's modified dulbecco's medium, 10% FBS, 1% P/S, 1.2 mM α-thioglycerol) in standard conditions (37°C 20%O_2 5%CO_2) and fed 2–3 times a week until experimental use. The HMC-1 cell line is a well characterized and thoroughly validated human MC line (Nilsson et al., 1994a), however, as this is an immortalized cell line, they contain a c-kit mutation that allows for easier handling and survivability. It has been shown that these cells may have reduced tryptase levels when compared to mature human skin MCs (Guhl et al., 2010).

Generation of 100% MCCM

HMC-1 cells were expanded as described above. To create mast cell conditioned media (MCCM) cells were diluted to 5.0×10^5 cells/mL in BMC and frozen for 24 h at −80°C. Following freezing, the cell suspension was thawed quickly in a 37°C water bath to maximize cell lysis. The resulting cell lysate solution was centrifuged at 1000 × *g* for 5 min to pellet cells and the supernatant collected and filtered using Pall Centrifugal devices (Pall Corp. MAP003C37) at 3000 × *g* for 30 min followed by reconstitution to the original volume with BMC. This final solution represents 100% MCCM including MC related factors such as MC tryptase. This 100% MCCM was then diluted to the desired 50% MCCM with DCC. Viability was assessed via trypan blue uptake before and after lysis. Protein concentration was assessed using a Bradford assay (Bio-Rad protein assay kit) for conditioned media at pre-lysis, post-lysis and post-filtration steps (Bradford, 1976).

Mast Cell Invasion/Migration in Response to IVD Related Factors

HMC-1 cells were expanded as described in MC culture. The cell invasion/migration assay was performed as cited previously (Brekhman and Neufeld, 2012). Briefly, Matrigel was diluted to 800 μg/mL in Iscove's modified Dulbecco's medium and used to coat the migration insert. HMC-1 cells were diluted to 9.0×10^5 in 0% FBS BMC immediately prior to use. Intervertebral disc conditioned media (IVDCM) ($N = 3$) and articular cartilage conditioned media (ACCM) ($N = 3$) was generated by culturing primary cells from cadaveric human IVD NP or articular cartilage cell samples at 1.0×10^6 cells/mL in 0% FBS DCC for 48 h before collecting this media for use. HMC-1 samples were tested in triplicate for migration with experimental conditions of 0% FBS BMC (negative control), 0% FBS IVDCM, 0% FBS ACCM (Cell Control), or 10% FBS BMC (positive control) added to the bottom of the well. Migrated live MCs were quantified via staining the bottom surface of the membrane with fluorescent dye Calcein (4 μM for 15 min at 37°C), staining all live cells green, and imaged by Nikon-Eclipse inverted microscope. Images were captured at 4× magnification and blinded prior to quantification. The number of migrated MCs were quantified automated via Nikon Analysis Software for each group and normalized to the 10% FBS BMC group

(positive control) as a percent migration, whereas 10% FBS BMC represents 100% migration.

MCCM/IVD Interactions and the PAR2 Pathway

Tryptase Effects on IVD

Cells

Primary human AF and NP cells from cadaveric human tissue (N = 4; **Table 1**) were seeded in 2% agarose gels using a silicone mold at 4.0 × 10^6 cells/mL (1.6 × 10^6 cells/construct) for 24 h in DCC+10%FBS media at 37°C 20%O_2 5%CO_2, followed by 24 h in DCC+2.5% FBS at 37°C 5%CO_2 5%O_2 with 0.00, 0.01, or 0.10 μg/mL (Masuko et al., 2007) of purified recombinant human tryptase from lung MCs (rhTryptase; Promega G5631). One quadrant of each construct was used to assessed viability (4 μM Calcein + 2 μM ethidium for 15 min at 37°C). The remaining portion of each construct was homogenized in TRIzol and mixed with 0.2 mL chloroform/mL TRIzol. This mixture was centrifuged at 12,000 × g for 15 min at 4°C to collect the aqueous upper phase and diluted 1:1 with molecular grade 70% EtOH before mRNA purification using the PuraLink RNA mini kit following manufacturer instructions (Life Technologies) for downstream cDNA synthesis with Maximus H Minus (Thermo Fisher Scientific M1662) and gene expression (qRT-PCR) for MC related chemoattractant SCF and angiogenic marker VEGFA (**Table 2**). Gene expression was quantified using the comparative ΔΔCt method (Livak and Schmittgen, 2001) with levels normalized to the housekeeping gene 18s (**Table 2**).

Tissue

Human cadaveric IVD samples (N = 8; **Table 1**) were freeze-thawed and 8 mm biopsy samples of NP and AF tissue were collected, wet-weight measured, and pre-incubated for 24 h in basal DMEM at 37°C, 20%O_2, 5%CO_2 to account for endogenous aggrecan loss. Non-live tissue (by means of freeze thaw) was utilized to examine the isolated effects of tryptase on proteoglycan accumulation in the absence of any cellular effects. Samples were then incubated with fresh basal media supplemented with 0.0, 1.0, or 5.0 μg/mL rhTryptase (adapted from Magarinos et al., 2013) for 48 h at 37°C 20%O_2 5%CO_2. Information on genes pertaining to our results can be found in **Supplementary Table S1**. Endogenous proteoglycan degradation was measured by sulfated glycosaminoglycan (GAG) release into media indicated by the colorimetric dimethylmethylene blue assay (DMMB; Sigma-Aldrich 341088) with absorbance read at 530 nm. A standard curve using shark chondroitin sulfate from shark cartilage (Sigma-Aldrich C4384) in DMEM with a linear regression analysis R^2 value > 0.95 was used to calculate concentration from absorbance value.

TABLE 2 | Gene assays.

Gene	Assay ID
PAR2 (F2RL1)	Hs00608346_m1
SCF (KITLG)	Hs00241497_m1
VEGFA	Hs00900055_m1; Bt03213282
NGF	Hs00171458_m1
IL-1B	Hs01555410_m1
IL-6	Hs00174131_m1
MMP3	Hs00968305_m1
MMP13	Hs00942584_m1

PAR2 Expression in Human IVD Tissue

Immunohistochemistry

Human cadaveric IVD samples (**Table 1**) of AF (N = 6), NP (N = 9) and CEP (N = 7) were assessed via IHC using SAM11, a primary antibody for PAR2 (1:50; Invitrogen 35-2300). Briefly, tissue slides were deparaffinized, rehydrated, blocked for endogenous peroxidase activity (0.3% H_2O_2 in MeOH), and antigens retrieved using a citrate buffer (90°C, pH 6.0) for 20 min. Blocking for non-specific binding was performed with 5% goat-serum (1% BSA-PBS, 5% goat serum, 0.05% Tween, and 0.05% sodium azide). Primary antibodies were incubated for 2.5 h in background reducing antibody diluent (Dako S3022) followed by incubation with secondary antibody: biotinylated goat anti-mouse (1:200, VectorLabs BA9200). Finally, tissue sections were incubated with streptavidin-horse radish peroxidase and developed with 3,3-diaminobenzidine (DAB) for 90 s (VectorLabs SK-4100). Tissue was counterstained with Gills No. 2 Hematoxylin (Electron Microscopy Sciences 26030-20) and slides dehydrated and mounted. Normal human lung tissue was used for positive and negative controls, with omission of primary antibody for the latter. All antibody concentrations and DAB times were kept consistent across experimental and control samples. Each tissue sample was quantified as described prior (Wiet et al., 2017).

Western Blot

To confirm protein expression of PAR2 in IVD cells western blot was performed as described previously (Walter et al., 2016) for cadaveric IVD cell lysate samples from NP, AF, and CEP cells (N = 2). PAR2 specific antibody SAM11 (1:250; Invitrogen 35-2300) was used with secondary antibody biotinylated goat-anti mouse (1:1000; VectorLabs BA-9200) and run with positive control for β-actin (ACTB; 1:5000; abcam ab8227).

PAR2A Therapeutic Proof of Concept Pilot *in vitro* Human and *ex vivo* Bovine Studies

Bovine *ex vivo* Therapeutic Model

A proof of concept study was performed to evaluate the potential effects of PAR2A on matrix degradation and angiogenesis. Skeletally mature bovine coccygeal motion segments (N = 3) were cleaned of soft tissue and IVD isolation was performed using a diamond coated band saw leaving 1–3 mm of CEP/Vertebral body caudally and cranially for 3 IVDs per bovine sample. Isolated samples were washed in 70% EtOH and PBS before being treated with isolation (Prime Growth 319-511-EL) and neutralizing solution (Prime Growth 319-512-CL) and then culture in Wisent Prime Growth Media (Prime Growth 319-510-CL) + 1% P/S for 24 h at 37°C 5% CO_2 20% O_2 according to Wisent protocol as outlined prior (Grant et al., 2016). Injury was

induced for relevant samples via an "X" cut through the AF region and partial NP removal (Illien-Jünger et al., 2014). After 10 days, samples were split into the following groups: (1) control with no injury or treatment, (2) injury+MCCM, or (3) injury+MCCM+100 nM PAR2A. 10 μL of 100 nM PAR2A (FSLLRY-NH_2) in PBS was injected with a high-precision 25G syringe (Hamilton; Reno, NV, United States) into the relevant bovine IVDs (Illien-Jünger et al., 2014). IVDs were cultured for 96 h and then tissue isolated for histology, cell viability and qRT-PCR. Briefly, the IVD was cut into 3 × 2 mm in width sagittal sections with a ceramic band. Of the three sections the medial segment, which included the site of injury, was assessed for histology with the lateral portions used for viability and gene expression. For gene expression, AF and NP regions were isolated before being flash-frozen in liquid nitrogen and homogenized via crushing with metal rods, washed in 70% EtOH, RNase Zap (Thermo Fisher Scientific AM9780) and cooled in liquid nitrogen. Tissue samples were dissolved, RNA purified, and qRT-PCR performed as described previously for VEGFA to assess the ability of PAR2A for reduction of MC related angiogenic effects (**Table 2**). To assess explant viability, tissue was incubated in MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide; Tokyo Chemical Industry D0801) as described prior (Walter et al., 2016). Each specimen was treated in MTT solution (20 mg/10 mL) for 4 h at 37°C 5% CO_2 20% O_2. The specimens were then fixed in 10% neutral buffered formalin for 48 h before being frozen and cryosectioned (10 μm). Slides were then counterstained with Hoechst 33258 (Sigma-Aldrich 94403) solution (1 μg/mL) for 10 min before being mounted and imaged at 40× magnification. For histology tissue was fixed in 10% neutral buffered formalin before being decalcified in EDTA solution for 7 days, then paraffin embedded, sectioned (10 μm), and stained using alcian blue and picrosirius red solutions (Electron Microscopy Sciences 10350; 26357-02). Stitched images of 16 frames at 4× magnification were taken.

Human *in vitro* Monolayer Therapeutic Model

To assess the clinical relevance of PAR2A on human cells *in vitro*, human NP cells from cadaveric tissue (N = 6; **Table 1**) were seeded in monolayer in duplicate at 1.5 × 10^5 cells/mL and incubated in DCC for 24 h at 37°C 5% CO_2. After 24 h, culture media was removed and replaced with Basal control media, 50% MCCM or 50% MCCM+100 nM PAR2A (Chen et al., 2015) and incubated for 48 h at 37°C 5%CO_2. One well was assessed for viability (4 μM Calcein + 2 μM ethidium for 10 min at 37°C) and one well was used to assess gene expression and lysed using 300 μL 1% 2-mercaptoethanol in lysis buffer and diluted 1:1 in 70% molecular grade EtOH before RNA was purified using PuraLink RNA mini kit as per manufacturer instructions. Downstream cDNA synthesis and gene expression quantification (qRT-PCR) for inflammatory, catabolic, and migratory factors SCF, VEGFA, NGF, IL-1β, and IL-6 (**Table 2**) was completed as described previously.

Statistical Analysis

All tests run with an $\alpha = 0.05$.

MC Migration (N = 3 Biological N = 6 Experimental)

Non-parametric, unpaired, one-tailed T-tests were performed between groups comparing experimental and biological replicates with all data normalized to the 10% FBS positive control group expressed as 100%.

Human *in vitro* 3D Model Gene Expression (N = 4)

Non-parametric, paired, two-tailed T-tests per gene were performed for paired biological replicates comparing fold changes in ΔΔCt gene expression between treatment groups.

Matrix Degradation (N = 8)

Non-parametric, paired, one-tailed, T-tests were performed for biological replicates comparing the GAG released into the media as a measure of proteoglycan degradation.

PAR2 Protein Expression (N = 6–9)

Non-parametric, unpaired, one-way ANOVA for comparing unpaired biological replicates for the percentage of positively stained cells as a percentage of the total cells.

Bovine *ex vivo* Gene Expression (N = 3)

Non-parametric, unpaired, two-tailed T-tests were performed for biological replicates comparing fold changes in ΔΔCt gene expression of *ex vivo* angiogenic growth factor.

Preliminary Human PAR2A Therapeutic Monolayer *in vitro* Model and Demographic Differences (N = 6)

Non-parametric, unpaired, two-tailed T-tests were performed for biological replicates comparing fold changes in ΔΔCT gene expression of inflammatory and pain-associated markers.

RESULTS

MC Migration in Response to IVD Related Factors

To assess the ability of IVD cell related factors to recruit MCs, a migration assay exposing MCs to IVDCM was performed with ACCM, 0% FBS, and 10% FBS controls. Conditioned media from human NP cells induced migration of MCs through the Matrigel 3D matrix and this migration was significantly greater than that of 0% FBS BMC (negative control) medium, or 0% FBS ACCM (cell control) medium alone ($p \leq 0.05$) with no significant difference between 0% FBS ACCM and 0% FBS BMC (**Figure 2**). Data is presented normalized to 10% FBS BMC (positive control) as percent migration with 10% FBS BMC equaling 100%, with an average percentage cell count of 23.3 ± 7.3% for 0% FBS BMC, 30.6 ± 3.6% for 0% FBS ACCM, and 77.1 ± 5.0% for 0% FBS IVDCM as visualized by calcein fluorescent staining of the migrated cells. This data suggests that MCs can migrate in response to IVDCM and more specifically in response to IVD related factors, as the healthy ACCM cell control had little effect compared to negative control.

Tryptase Effects on IVD Cells (NP and AF)

In order to evaluate the metabolic effects of MC Tryptase on IVD cells *in vitro*, NP and AF cells were treated with rhTryptase in 3D

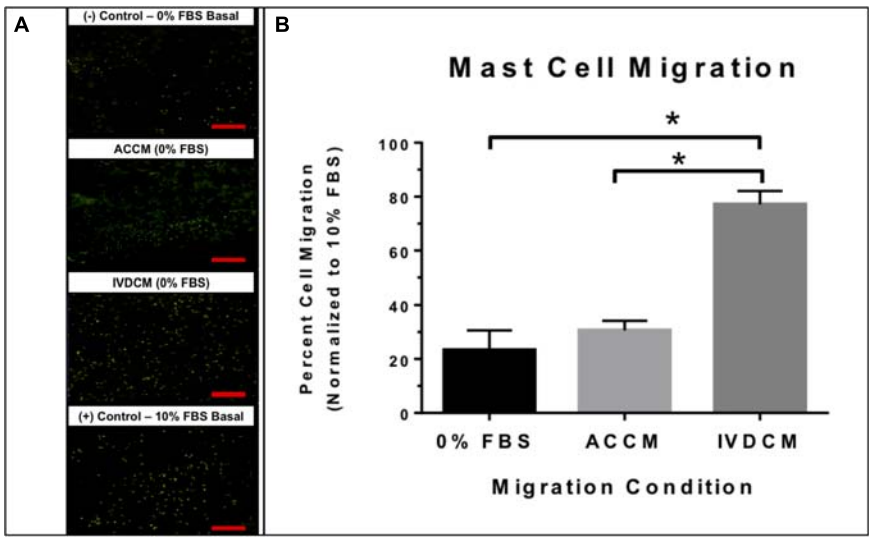

FIGURE 2 | (A) Representative images of (calcein) stained fluorescent MCs migrating in response to 0% FBS BMC (negative control), 0% FBS ACCM (cell control), 0% FBS IVDCM, and 10% FBS BMC (positive control). **(B)** Mast cell migration in response to IVDCM ($N = 3$), ACCM ($N = 3$) and 0% FBS (negative control) ($N = 6$) normalized to 10% (positive control = 100% migrated cells) ($N = 6$; $^*p \leq 0.05$). Red scale bar = 500 μm (4×).

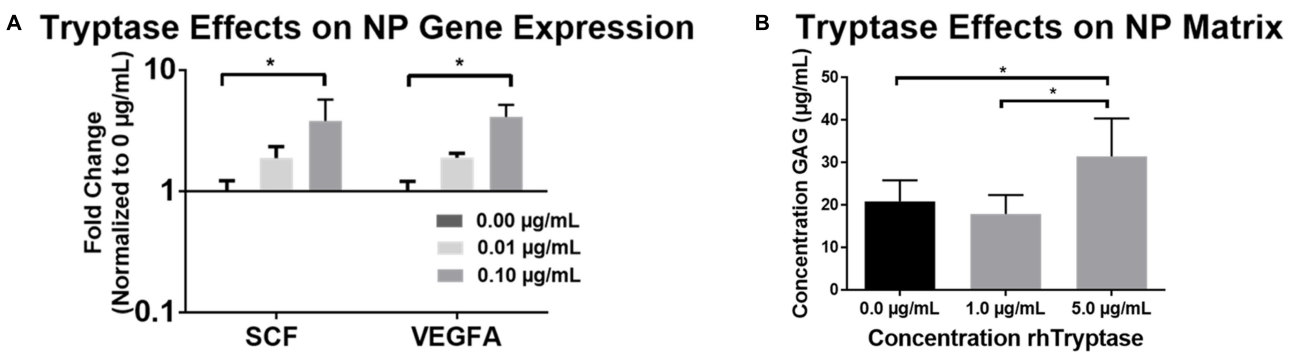

FIGURE 3 | (A) Average fold changes in qRT-PCR gene expression in human NP cells ($N = 4$) in 3D agarose constructs treated with rhTryptase and normalized to untreated controls ($^*p < 0.05$). **(B)** GAG release into media from freeze-thawed human NP explants from cadaveric (autopsy) IVD specimens ($N = 8$; $^*p < 0.05$).

culture and relevant angiogenic (VEGFA) and chemotactic (SCF) factors investigated. rhTryptase at 0.1 μg/mL showed significant upregulation of SCF (3.80-Fold) and VEGFA (4.09-Fold) gene expression relative to untreated control ($p < 0.05$) (**Figure 3A**). No significant differences in SCF or VEGFA were detected for AF cells ($p > 0.05$) (data not shown). No significant differences in viability were detected in any group (NP or AF). This data is indicative of increased angiogenic/chemotactic potential in IVD cells in response to tryptase.

Tryptase Effects on IVD Tissue (NP and AF)

To assess the enzymatically degradative effects (direct and or indirect) of tryptase on IVD tissue, tissue explants were treated with rhTryptase. The addition of 5.0 μg/mL rhTryptase significantly increased the GAG released into media from human NP tissue compared to 1.0 and 0 μg/mL rhTryptase ($p < 0.05$) (**Figure 3B**). No significant differences in GAG release into media were observed in any group from human AF tissue (**Supplementary Figure S1**). The results suggest that tryptase plays a role in enzymatical degradation of the proteoglycan which may affect structure/function of the IVD tissue.

PAR2 Expression in Human IVD Tissue

To evaluate a potential role for PAR2 in mediating the downstream effects of MCs and tryptase, we first performed IHC for PAR2 on cadaveric human IVD tissue. Protein expression was assessed via IHC for PAR2 in human tissue (% of positive cells) in NP (20.5% ± 9.16), AF (13.6% ± 16.48) and CEP (19.6% ± 14.32) cells with no significant differences detected between regions (**Figure 4**). PAR2 protein expression was confirmed in the NP, AF and CEP regions of the human IVD via Western blot (**Figure 4B** and **Supplementary Figure S2**). This expression validates previously reported PAR2 expression in human IVDs

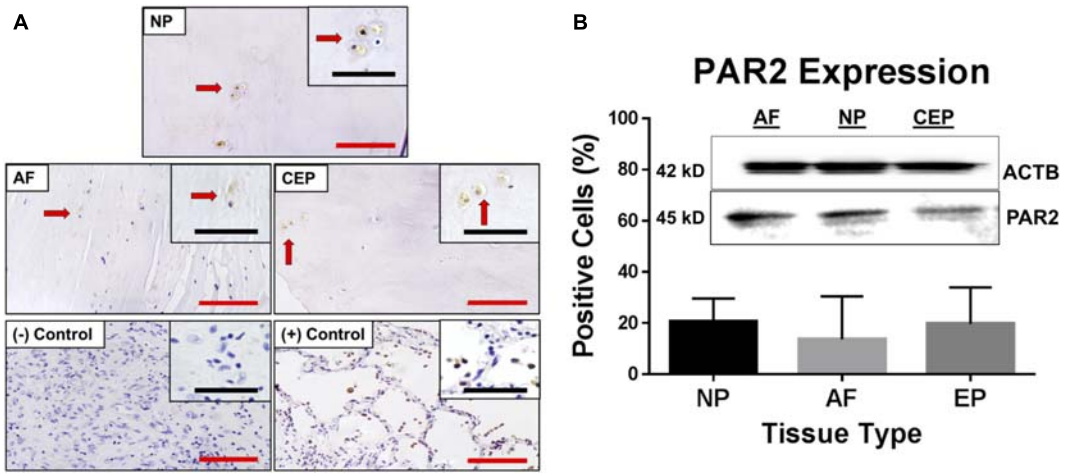

FIGURE 4 | (A) Representative images of immunohistochemical (IHC) PAR2 staining in IVD tissue from all regions; NP, AF and CEP including relevant positive and negative lung tissue controls. **(B)** PAR2 expression presented as % of cells expressing PAR2 as assessed by IHC and confirmed with western blot using the same PAR2 primary antibodies. Red scale bar = 100 μm, Black scale bar = 50 μm. Arrows point to same cells at both magnifications.

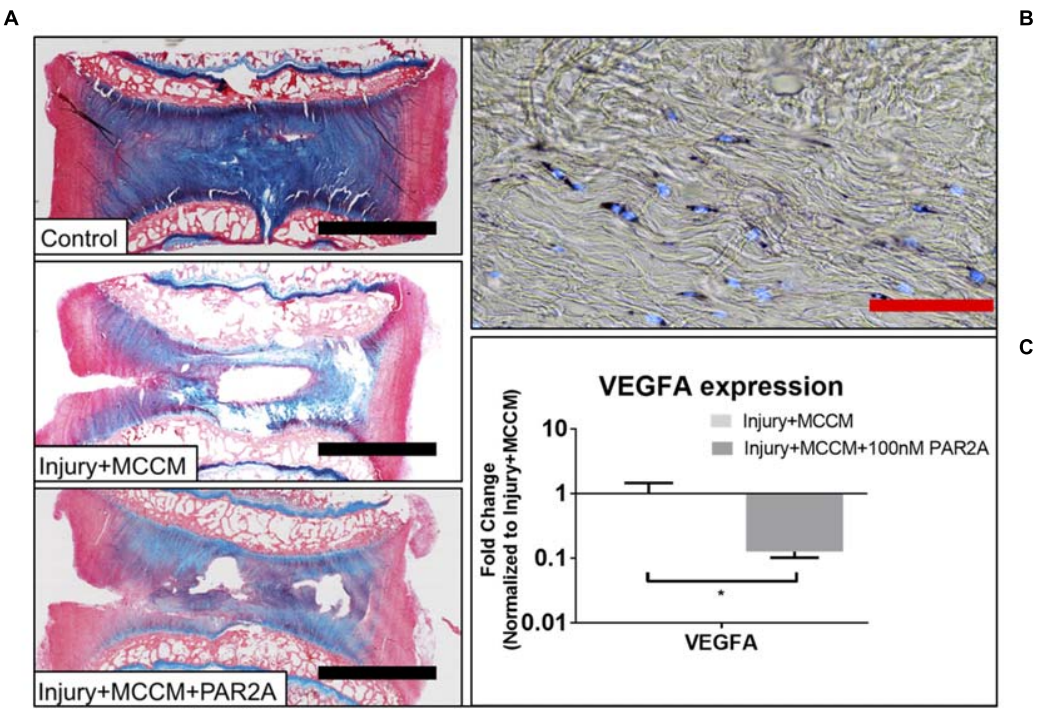

FIGURE 5 | (A) Representative Alcian blue (aggrecan) and picrosirius red (collagen) staining of bovine caudal IVDs after 96 h of culture. Discs were either left intact (control) or injured and cultured with MCCM with or with a PAR2A. **(B)** Representative cell viability image for Injury groups treated with MCCM+PAR2A. **(C)** Average fold change in qRT-PCR gene expression in AF region of bovine *ex vivo* samples (*$p < 0.05$). Red scale bar = 1000 μm, Black scale bar = 50 μm.

(Iida et al., 2009) and points toward a potential pathway mediating MC effects in the human IVD.

Bovine *ex vivo* Therapeutic Model

As a preliminary proof of concept study to assess the potential therapeutic effects of a PAR2 antagonist on MC-induced IVD degeneration, we chose to use a bovine *ex vivo* organ model which has been utilized previously as a model of IVD degeneration and to screen therapeutics (Purmessur et al., 2013b; Illien-Jünger et al., 2014; Grant et al., 2016). Treatment of IVD organ culture samples with PAR2A had no detectable impact on viability for AF as demonstrated by MTT staining co-localized with DNA stain Hoechst (**Figure 5B**). Due to technical limitations, the NP could not be assessed; However, RNA levels and histology suggest

adequate viability due to matrix generation. Treatment with 100 nM PAR2A significantly down-regulated the gene expression of VEGFA (7.87-Fold) in the AF region of MCCM treated injured disc samples relative to MCCM injury controls ($p < 0.05$) (**Figure 5C**). No significant changes in VEGFA expression were observed in the NP (data not shown). Treatment with PAR2A was also correlated with visibly enhanced tissue regeneration as shown by increased histological staining of proteoglycan (blue) in the central regions of the PAR2A IVDs relative to MCCM injury and uninjured controls (**Figure 5A**). These results suggest that PAR2A does not have any detrimental effects on viability but decreases the expression of angiogenic factors while promoting matrix accumulation.

Human *in vitro* Monolayer Therapeutic Model

To further assess the therapeutic potential and clinical translation of PAR2A we evaluated the effects of PAR2A on human cells *in vitro*. PAR2A consistently down-regulated chemokine (SCF), inflammatory (IL-1β; IL-6), neurotrophic (NGF) and angiogenic (VEGFA) markers in human NP IVD cells treated with MCCM compared to human NP cells treated with MCCM alone although this was not statistically significant (**Figure 6A**). When presenting the data as individual human samples, biological differences were observed when assessing gender, where at least 2/3 male NP cells treated with MCCM appeared to respond to PAR2A with down-regulation of the majority of inflammatory and neurovascular factors assessed. When assessing female NP samples, only 1/4 demonstrated a notable response to treatment with PAR2A across a majority of the genes assessed (**Figure 6B**), suggesting gender differences may influence the therapeutic potential of the biologics targeted in the degenerate IVD, however, more validation is needed before any specific conclusions can be drawn.

DISCUSSION

Immune cells, more specifically MCs, have been well documented as a key contributor to the pathology of many musculoskeletal disorders related to joint degradation such as rheumatoid arthritis (Woolley, 2003). Additionally, these cells are known to play a role in several chronic pain conditions such as migraines, inflammatory arthritis and irritable bowel syndrome (O'Sullivan et al., 2000; Nigrovic and Lee, 2005; Levy et al., 2007). Our previously published data has shown significant evidence to support a role for MCs in the pathogenesis of painful IVD degeneration, with increased numbers of MCs in diseased IVD tissue from patients with DBP relative to healthy controls (Wiet et al., 2017). Furthermore, we demonstrated that IVD cells express MC chemoattractants such as SCF and MCs interact with healthy bovine IVD cells to up-regulate catabolic and inflammatory markers such ADAMTS5 and IL-6. Interestingly, soluble factors from bovine IVD cells were able to induce MC degranulation and up-regulation of VEGFA in MCs (Wiet et al., 2017). However, a number of questions still remain with respect to the role of MCs in human DBP and the potential to therapeutically target these pathological effects. Specifically, do IVD cells promote migration of MCs, how do MC mediators such as tryptase interact with human IVD cells and tissue, at the gene and matrix level, and what are the potential pathways down-stream of these effects and can they be therapeutically modulated. The answers to these questions form the basis of the work described here.

To understand the role of MCs in DBP, it is important to investigate how these cells may infiltrate the IVD microenvironment, an environment that is typically immune-privileged. As such, we investigated the ability of soluble factors from human IVD cells to recruit HMC-1 MCs using an *in vitro* migration assay system. Derivatives of the hematopoietic precursor lineage, MCs are unique in their ligand-based maturation at a tissue target site rather than in the stem cell microenvironment, and as such chemoattractant ligands, such as SCF, are crucial in their recruitment. The HMC-1 cell line in particular has been shown to adequately express the C-kit receptor for SCF as well as migrate in a dose dependent manner to SCF. Importantly, they were shown to migrate non-randomly toward SCF rather than random chemokinesis, making it an adequate model to study this mechanism of migration (Nilsson et al., 1994a,b). Our study demonstrates that when MCs are exposed to IVDCM, migration of MCs is significantly upregulated compared to BMC (negative control) groups. Furthermore, this effect was not seen when comparing AC cell control media, ACCM, suggesting this effect is likely specific. We have previously demonstrated that SCF is expressed by human IVD cells from all regions of the IVD in health and disease and is a likely candidate for mediating these effects, however, further work is needed to validate this effect and the role SCF may play (Wiet et al., 2017). Indeed, SCF is a major chemoattractant for MCs and their progenitors *in vivo* (Okayama and Kawakami, 2006) and to our knowledge our study is the first to demonstrate that soluble factors from IVD cells can induce MC migration *in vitro*.

Our previous studies have demonstrated that MCs release soluble factors that can modulate IVD cell behavior and phenotype and one candidate mediator is tryptase, yet the specific effect of tryptase and potential down-stream pathways have not been elucidated. Our study demonstrates that healthy human NP cells, when treated with rhTryptase, exhibit significant upregulation of SCF, and this effect appears to be dose dependent. This suggests that tryptase may help facilitate IVD-induced MC migration into the IVD creating a positive feed-back loop and promoting a chronic inflammatory response whereby more MCs migrate into the diseased IVD and degranulate, releasing their catabolic and inflammatory mediators further enhancing this pathogenic cycle of disease. This mechanistic model is supported by our previous finding of more pronounced SCF expression in the NP region at the protein level (IHC) (Wiet et al., 2017). In this study we also observed significant up-regulation of pro-angiogenic factor VEGFA in NP cells in response to treatment with rhTryptase. This finding is complementary to our previous studies showing significantly increased VEGFA secretion by MCs when exposed to IVDCM from degenerate NP cells relative to basal control, pointing toward MC/IVD interactions mediating pro-angiogenic effects in the painful IVD (Wiet et al., 2017).

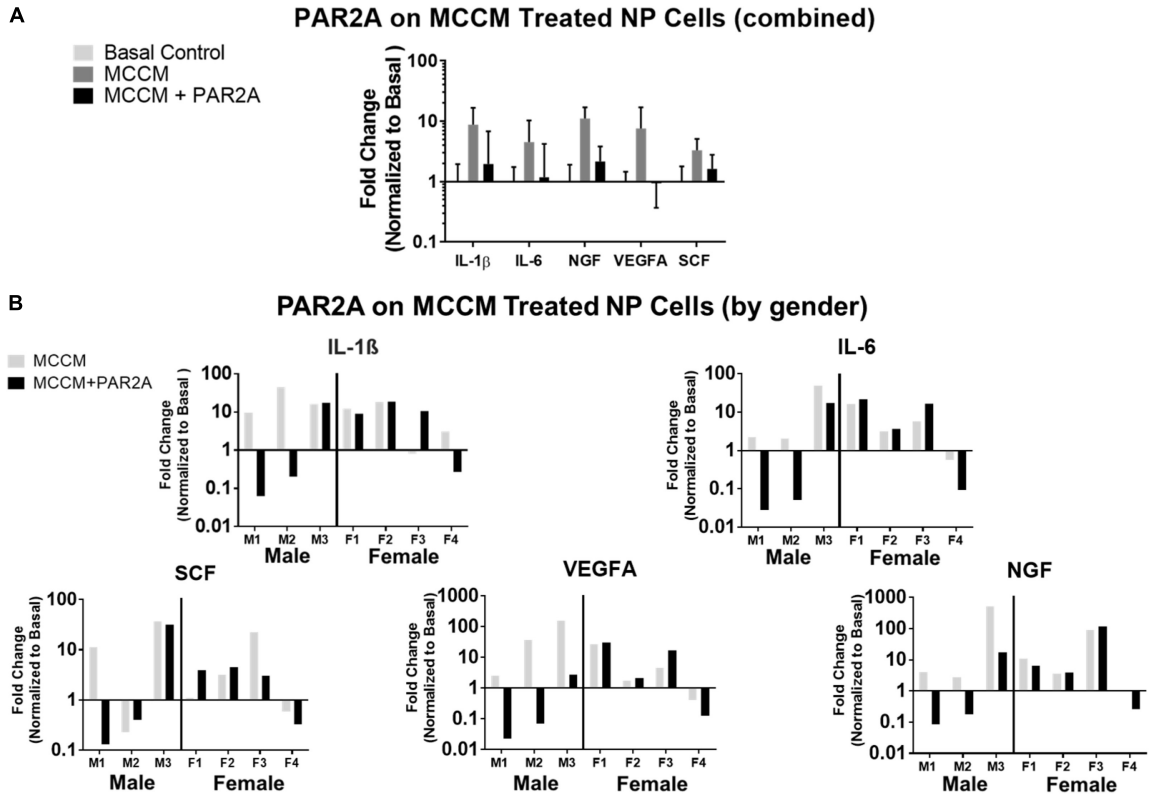

FIGURE 6 | (A) Histogram of average fold change in qRT-PCR gene expression in human NP cells ($N = 7$) treated with MCCM and MCCM+PAR2A normalized to basal untreated controls. **(B)** Histograms of fold changes in qRT-PCR gene expression in human NP cells for individual donors normalized to basal control where left (M1, M2, M3) are males and right (F1, F2, F3, F4) are females.

While the possibility of native MC contamination of IVD cells cannot be definitively excluded given the presence of MCs in cadaveric disc tissue as cited in our previous work (Wiet et al., 2017) MCs are non-adherent cells therefore monolayer culture, used for initial IVD expansion, would very likely exclude MCs from culture. The painful degenerate IVD is characterized by neo-vascular invasion (Freemont et al., 2002; Lee et al., 2011; Stefanakis et al., 2012; Binch et al., 2015; Lama et al., 2018) and given how MCs are associated with angiogenic processes in multiple tissues and pathologies (Kneilling et al., 2007; De Palma et al., 2017; Longo et al., 2018) suggests that tryptase may be associated and enhance this process in the diseased IVD.

In assessing the full spectrum of potential effects that tryptase may have on the IVD there are many considerations beyond the chemokines SCF and VEGFA alone. Tryptase has been shown to activate the zymogen form of MMP3 and MMP13, and these activated proteases can directly degrade aggrecan in femoral cartilage explants (Magarinos et al., 2013). Aggrecan represents the principle structural proteoglycan of the NP, suggesting that tryptase may have similar effects with respect to the ECM of the IVD. Our current study shows that when treated with rhTryptase, isolated NP explants from healthy human IVD specimens released significantly more sulfated glycosaminoglycan (GAG) into the medium, suggesting that MCs can induce degradation of the ECM in the NP via tryptase. This effect was not observed for the AF. These results suggest that, as in the studies by Magarinos et al. (2013), tryptase can enhance degradation of proteoglycan which is relevant to degradation of the IVD. Healthy NP tissue is aggrecan rich, and previous studies have demonstrated that aggrecan is inhibitory to both nerve ingrowth and endothelial cell migration and adhesion (Johnson et al., 2002, 2005). Degradation of proteoglycans, such as aggrecan, by MC enzymes, such as tryptase, could facilitate neurovascular ingrowth into the painful IVD. Specifically, it has been well documented that neo-vascularization and the ingrowth of nociceptive neurons is a marker of DBP progression, and this effect is related to sites of matrix degradation (Smith et al., 2011; Stefanakis et al., 2012; Lama et al., 2018).

To determine potential therapeutic strategies in the context of tryptase, it was crucial to identify the downstream mechanism by which it can affect the IVD cells themselves. Causes of cellular changes related to tryptase however, have not been well characterized. Tryptase has been well documented as a potent activator of PAR2 (Molino et al., 1997) and PAR2 activation has been cited in many catabolic, inflammatory, and pain inducing pathways. Notable among these was the finding that PAR2 activation is sufficient to induce matrix degradation in osteoarthritic cartilage, significantly stimulating catabolic and inflammatory factors such as MMP13 and IL-1β, respectively. Further, this process was also seen to be self-promoting following

onset, with findings that PAR2 is upregulated in diseased tissue and by IL-1β (Boileau et al., 2007). In order to validate the potential of tryptase/PAR2 interactions in IVD pathology, it was important to confirm and quantify the expression of PAR2 in all regions of the human IVD. Our findings validated the work of Iida et al. (2009) that demonstrated clear and levels of expression of PAR2 for non-diseased IVD cells in the NP and AF regions. However, to our knowledge we also show for the first time PAR2 expression in the CEP region with levels that are comparable to that of the NP. Our IHC data, confirmed by western blot, in healthy samples supports a potential role for tryptase/PAR2 interactions in the IVD pathology.

Functional PAR2 activation involves proteolytic cleavage of the amino terminus and uncovering of a tethered ligand interacting with exterior facial loops of the membrane bound protein, leading to a crystalline activation state (Nystedt et al., 1994). The present study sought to investigate this interaction as a therapeutic target using a PAR2 antagonist (PAR2A), the small molecule oligopeptide inhibitor FSLLRY-NH_2. This antagonist functions by blocking this terminal cleavage and has been cited to abate PAR2 pathological effects in several studies such as Chen et al. (2015) and their treatment of neuropathic pain. The skeletally mature bovine IVD is an excellent tool and model for investigating degeneration of the IVD *ex vivo* and for therapeutic screening of targets related to DBP (Purmessur et al., 2013b; Illien-Jünger et al., 2014; Grant et al., 2016) and as such to elucidate the tryptase/PAR2 interaction in disease we developed an *ex vivo* bovine organ culture model representing injury and exposure to MC related soluble factors. Our findings demonstrated that PAR2A had no effect on native cell viability and also demonstrated enhanced matrix regeneration following injury as shown by increased histological blue staining of aggrecan and accumulation of tissue following culture of injured samples in MCCM, when compared to injured controls. Furthermore, in AF tissue the gene expression of VEGFA was significantly downregulated when comparing the same groups. As previously discussed, VEGFA is crucial to the neo-vascularization process and has been shown to promote neurovascular ingrowth in degraded AF tissue (Stefanakis et al., 2012). As such, down-regulation of VEGFA and increased matrix synthesis with treatment of PAR2A highlights its therapeutic potential for restoring structure/function to the IVD while reducing neo-angiogenic processes. However, this "proof of concept" study requires further validation with a larger cohort of IVDs and for a longer time-frame to determine whether effects are sustained.

To further investigate the therapeutic potential of PAR2A on IVD cells we conducted a preliminary *in vitro* human study utilizing PAR2A to screen IVD cells considered most physiologically and clinically relevant to DBP. It is important to note that in order to better elaborate on the microenvironment of a MC modulated disease state in the native IVD, MCCM in its entirety was utilized. This MCCM includes a variety of mediators derived from the granules such as catabolic enzyme ADAMTS5 and inflammatory/pain-associated factors IL-1β, TNFα, VEGFA, NGF and Substance P among others (De Schepper et al., 2010), and tryptase content has been previously validated in these MCCM samples (Wiet et al., 2017) as well as in the present study design. Our study demonstrated that MCCM induced a consistent inflammatory and pathological response in human NP cells. Treatment with PAR2A seemed to abate these effects in a fairly consistent manner, however, when all donors were combined, these results were not statistically significant. Use of human IVD samples for drug screening is highly clinically relevant, yet due to the innate nature of human variability, effects are often dismissed as there exists a pool of non-responders versus responders (Madian et al., 2012). Indeed, DBP in clinical practice has been shown to be enigmatic in its presentation with underlying risk factors not always evident and fully elaborated on in the field. One study in particular found that even with MRI imaging cross-sectional analysis of asymptomatic persons aged 60 or older, 36% had disc herniations, 21% had spinal stenosis and more than 90% had degenerated and or bulging discs (Boden et al., 1990) demonstrating that in many ways our cross-referential understanding of the manifestation of DBP is still emerging. As such, we sought to understand how sample demographics could potentially influence the response to PAR2A treatment. Interestingly, when we examined the effects of PAR2A as a function of male vs. female derived samples we found that in the majority of the assessed genetic markers, at least 2/3 male derived samples responded to PAR2A with down-regulation of the MCCM induced effects. This trend was not nearly as pronounced for female samples, with only 1/4 of these samples demonstrating a consistent response to PAR2A in the assessed genes. It has been shown that gender differences are observed for DBP clinically such as Bartynski et al. (2013) finding that in men, injury related DBP represented 83% of all injury related cases examined with DBP while 65% of progressive onset associated DBP cases being female patients. Additionally, it was found that in cases of progressive onset, significantly more severely degenerative discs in females were reported (65%) when compared to male patients (27%) even though across the entire sample, cases of severely degenerative discs were nearly identical to full-thickness radial fissures (47.3% vs. 52.7%), a type of IVD internal derangement and typical clinical indicator of back pain. It was the conclusion of this group that gender differences may be relevant to the consideration of back pain origin, treatment, and response to treatment. These findings seem to be corroborated by more recent studies such as that Mosley et al. (2019) examining gender differences in a rat AF puncture degenerative model. The study found that in this degenerative model, female rats responded to puncture with decreased collagen organization and density, fibril diameter, and increased molecular damage when compared to males. Furthermore, it was suggested that differences in females are likely to contribute to difficulty in healing, and more pronounced pain in female clinical DBP cases. The application of these findings among others in the context of the present study's findings opens the possibility that demographic considerations are of potential importance in analysis of therapeutic options. While these considerations are potentially promising in the progress toward developing non-surgical intervention therapies for DBP, it is important

to note that significantly more human samples are needed to fully validate and elaborate on these differing outcomes across demographics seen within the present study before any definitive conclusions can be drawn. As such, we report only the potential of PAR2A as a treatment option, and one that should be assessed with consideration of gender among other demographic considerations. Further investigation of these factors is needed to expand upon our preliminary investigation and hopefully elucidate any practical applications there may be to the benefit of the very diverse affected DBP patient population. As such, further work by our group includes longitudinal investigation, expansion of sample size, assessment of age, gender, race and species effects among other factors, to verify the validity of PAR2A as a potential therapeutic strategy for the treatment of DBP.

SIGNIFICANCE

This study highlights chemotactic effects of IVD cells on MCs, evidence toward mast cell/PAR2 interactions regulating inflammatory and angiogenic markers of discogenic back pain, and PAR2A as a potential therapeutic strategy specifically targeting mast cell/PAR2 interactions in IVD pathophysiology.

ETHICS STATEMENT

IRB: 2018H0424 for patient articular cartilage tissue. Autopsy samples from the Cooperative Human Tissue Network are IRB exempted.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study, or acquisition of data, or analysis and interpretation of data, drafted the article or revised it critically for important intellectual content, and approved the final version to be submitted. DP took responsibility for the integrity of the work as a whole, from inception to finished article.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Mast Cells in Gut and Brain and Their Potential Role as an Emerging Therapeutic Target for Neural Diseases

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The mast cells (MCs) are the *leader* cells of inflammation. They are well known for their involvement on allergic reactions through degranulation and release of vasoactive, inflammatory, and nociceptive mediators. Upon encountering potential danger signal, MCs are true sensors of the environment, the first to respond in rapid and selective manner. The MC activates the algic response and modulates the evolution of nociceptive pain, typical of acute inflammation, to neuropathic pain, typical not only of chronic inflammation but also of the dysregulation of the pain system. Yet, MC may contribute to modulate intensity of the associated depressive and anxiogenic component on the neuronal and microglial biological front. Chronic inflammation is a common mediator of these co-morbidities. In parallel to the removal of the etiological factors of tissue damage, the modulation of MC hyperactivity and the reduction of the release of inflammatory factors may constitute a new frontier of pharmacological intervention aimed at preventing the chronicity of inflammation, the evolution of pain, and also the worsening of the depression and anxiogenic state associated with it. So, identifying specific molecules able to modify MC activity may be an important therapeutic tool. Various preclinical evidences suggest that the intestinal microbiota contributes substantially to mood and behavioral disorders. In humans, conditions of the microbiota have been linked to stress, anxiety, depression, and pain. MC is likely the crucial neuroimmune connecting between these components. In this review, the involvement of MCs in pain, stress, and depression is reviewed. We focus on the MC as target that may be mediating stress and mood disorders via microbiota–gut–brain axis.

Keywords: mast cell, neuroinflammation, pain, stress, depression, gut–brain axis, probiotics

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INTRODUCTION

A *basal* inflammation is a protective condition. In physiological levels, both pro- and anti-inflammatory mediators may result essential for induction and maintenance in neuroplasticity phenomena (Bezzi et al., 1998; Avital et al., 2003), whereas in high doses they can cause an acute inflammation that, in turn, can produce a state of chronic inflammation and achieving neural diseases (Dong et al., 2017; Kempuraj et al., 2017). An inflammation state is underlying pain, stress, and depression (Hendriksen et al., 2017; Gupta and Harvima, 2018).

The International Association for the Study of Pain (IASP) defined the pain as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage,*

or described in terms of such damage" (Iasp, 1979). According to this definition, it emerges that pain, originating from peripheral damage, is progressively enriched by neuropsychological and emotional components, which born in the brain and modulate their perceptive and purely subjective components (Mannion and Woolf, 2000). A chronic pain is frequently the first determinant of psychological and mood disorders (Humo et al., 2019).

Stress is a complex dynamic condition in which homeostasis is altered or threatened (Rea et al., 2016). Stress and inflammation represent the main pathogenic factors in multiple diseases that are often comorbid including fibromyalgia syndrome, migraine, as well as irritable bowel syndrome (IBS) (Bennett et al., 1998; Tak et al., 2011). Stress, particularly in early stages of life, is one of the main predictors of the onset of major depression disorder and chronic pain, and it may affect the perception of the pain and exacerbate it (Liu and Chen, 2014; Burke et al., 2017; Humo et al., 2019). Studies point out that major depression is prevalent in patients affected by chronic infections and suggest that a chronic inflammation can increase its incidence (Hauser et al., 2011). Elevations in pro-inflammatory cytokines have been reported in patients suffering from depression and chronic pain (Martinez et al., 2012).

Major depression, emotional, and chronic stress lead to the activation and alteration in limbic regulation of the hypothalamic–pituitary–adrenal (HPA) axis, whose altered regulation is usually associated with centralized pain syndromes (Menke, 2019). Under normal conditions, an acute stress induces the hypothalamus to release the corticotrophin-releasing factor (CRF) that induces the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which causes the adrenal cortex to release glucocorticoids that play metabolic roles (Herman et al., 2003). A negative feedback loop turn off the HPA axis activation (Kageyama and Suda, 2009). Subjects with pain syndromes present altered signaling from HPA axis but also mood disorders, including depression and anxiety (Bao and Swaab, 2019). The crucial link connecting these disorders is the inflammation mediated and modulated by cells, whose *leaders* are the mast cells (MCs). MCs are versatile cells that serve important functions in both innate and adaptive immunity surveillance, and the first responders to insults. They are equipped with extraordinary functional peculiarities and respond strongly to HPA axis activation (Heron and Dubayle, 2013; Skaper, 2016; Theoharides, 2017; Gupta and Harvima, 2018).

Increasing evidence has pointed to the relationship between intestinal microbiota and brain, showing that the gut inflammatory *milieu* may play a crucial role in the induction of several nervous conditions including stress, anxiety, and depression as well as in neuroinflammation (Sherwin et al., 2016; Cussotto et al., 2018).

From various studies it emerges that the MC is particularly responsive to microbiota conditions and its stabilization through appropriate combinations of probiotics could represent a new potential therapeutic tool to control neural disorders that underlie its activation (Wouters et al., 2016). Therefore, the goal is to stabilize the MC, and do it starting from the intestine.

In this review, we focus on the MC as potential target that may mediate neural diseases via microbiota–gut–brain axis.

OVERVIEW ON MAST CELLS

Mast cells are heterogeneous and ubiquitous cells of the vascularized tissues where they work as immune gatekeepers at host/environment interfaces, like the skin, airways, gastrointestinal, and urogenital tracts to respond to different allergens, pathogens, parasites, and other danger agents that can be ingested, inhaled, or encountered after breakdown of the epithelial barrier. In addition, MCs organize the inflammatory response, modulating the quality of tissue repair and remodeling at the same time (Traina, 2017; Forsythe, 2019).

Mast cells originate from hematopoietic-derived immune CD34+ multipotential stem cells in the bone marrow and circulate in the blood in low numbers as immature precursors. They migrate to locate in mucosal and connective tissues completing their differentiation in mature MCs on the influence of local residing microenvironment, which defines their phenotype and, consequently, their function (Beaven, 2009; De Zuani et al., 2018). In particular, MCs represent about 2–3% of the immune cellular pool of the lamina propria, and in the muscular and serous layers (3,000–25,000 MCs/mm^3), located in strategic position in proximity of blood, lymphatic vessels, and nerves (Irmak et al., 2019). Stem cell factor (SCF) binds to c-kit tyrosine kinase receptor of MCs and it is a necessary component for their survival, proliferation, and differentiation (Okayama and Kawakami, 2006).

In small quantity, MCs are also present in the brain. Here, they are located in area postrema, parenchyma of thalamus and hypothalamus, leptomeninges, pineal organ, infundibulum, choroid plexus, and in *dura mater* of the spinal cord. Their interaction with meningeal afferents and dural vasculature may have a crucial role in migraine headache (Rozniecki et al., 1999; Xu and Chen, 2015; Arac et al., 2019). In the brain MCs are located on the abluminal side of blood vessels, where they interact with neurons, glia, and endothelial cells (Hendriksen et al., 2017). The total number of MCs present in the central nervous system (CNS) is limited and it is difficult to calculate it because subject to changes related to age, sex, and animal species and also in response to outside environmental conditions (Silver and Curley, 2013). In human healthy brain, in meninges and perivascular area <5 MCs in 5 μm thick tissue sections were found during autopsy (Maslinska et al., 2005). During infection MC numbers increase to 11–20 in meninges and 5–10 in perivascular area. In mice brain, MC numbers are increasing from 150 to 500/50 μm sections thick during development (Nautiyal et al., 2012). But they are very powerful cells and even few MCs are able to release a sufficient amount of inflammatory mediators that can affect the blood–brain barrier (BBB) integrity and, in turn, activate glia and neurons (Hendriksen et al., 2017). All the unique features of MCs allow them to start, amplify, and prolong the inflammatory response. MCs are armed with a vast repertoire of receptors to ligands. MCs possess high affinity receptors, FcεRI for immunoglobulin E (IgE) binding protein on the cell surface and cytoplasmic proteases-containing granules under the influence of molecules, such as SCF, interleukin (IL)-3, and IL-9, as well as bacterial-derived molecules [lipopolysaccharide (LPS) and peptidoglycan]

(Bischoff and Kramer, 2007; Frossi et al., 2017). In this manner, MCs can be activated by pathogens, antigen-bounded Ig, and also by soluble or physic factors, drugs, temperature, pressure via transient-receptor-potential channel 2 (TRPV2). Their receptors include also complements receptors, Toll-like (TLRs, 1–7, 9), nucleotide-binding and oligomerization domain (NOD)-like as well as receptors for cytokines and microbe associated molecular pattern receptors (MAMPs) (Zhang et al., 2012; Traina, 2017).

Interestingly, MCs have the receptors for CRF, strengthening link between stress and MC (Traina, 2017; Kempuraj et al., 2019). In particular, MCs possess both CRF1 and CRF2 receptors, indicating their central role in visceral hyper-sensibility, in sensitizing sensory nerve terminations and, possibly, in lowering pain thresholds (Eller-Smith et al., 2018). In addition, they contain vast CRF stores (Theoharides and Konstantinidou, 2007). A recent study showed that CRF2 represents a negative modulator of MC activation, suggesting a crucial homeostatic role of MC in the CRF system and in the disorders associated with his (D'Costa et al., 2019).

Mast cells own a great number of co-stimulatory molecules. These mediators include members of the tumor necrosis factor TNF/TNFR families, which allow them to interact with different cells populations and with bacteria and fungi through the expression of different pattern-recognition receptors (PRRs) (Frossi et al., 2017; De Zuani et al., 2018). MCs harbor a large amount of granules that contain preformed and *de novo* synthesized molecules. Preformed mediators include histamine, serotonin (5-HT), proteases, heparin, and growth factors including TNF-α, proteoglycans, initiating the early recruitment of immune cells at the infection site. Newly synthesized mediators consist of lipid-derived mediators, such as prostaglandins and leukotrienes and cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-16, and IL-18) that affect the physiology of neighboring cells (Hendriksen et al., 2017; Traina, 2017). Activation of MCs via one of the pathways can release a plethora of pro-nociceptive mediators. MCs are able to respond to activation of the principal stress system, HPA axis and, in turn, pro-inflammatory cytokines are potent stimulators of the HPA axis (Theoharides, 2017). Their aberrant activity may also give to neurodegenerative and mood disorders (Hendriksen et al., 2017). In addition, mediators released by MCs may affect epithelial integrity and viability (Albert-Bayo et al., 2019). MCs represent the connecting link between brain and immune system, because they respond and release neurotransmitters and immune molecules (Forsythe, 2019; **Figure 1**). Finally, in the brain MCs does not possess IgE receptors (FcεRI) (Theoharides and Konstantinidou, 2007). So, the brain does not manifest allergic reactions (Forsythe, 2019).

MAST CELLS AND PAIN

Both peripheral and CNS participate in pain. CNS is involved not only in spino–thalamic–cortical pain system, but also in the limbic areas, which modulate the component emotional-affective of pain, as well as in the cognitive areas, which modulate adaptive, motivational, and relational aspects (Mannion and Woolf, 2000). Headache is one of the main symptoms associated with these conditions (Irmak et al., 2019). Evidence shows the involvement of MCs on pain and stress (Eller-Smith et al., 2018). In stress condition, activation of HPA axis induces an increase of CRF release that could result in MC activation and in sensitization of nerve terminals increasing pain signaling (Eller-Smith et al., 2018). MCs and nerves communicate bi-directionally. Calcitonin gene-related peptide (CGRP) released from meningeal nociceptors may degranulate MCs that release histamine that, in turn, activates mechanosensitive C-fibers that release CGRP and substance P (SP) (Julius and Basbaum, 2001; Irmak et al., 2019). Actually, CGRP co-localizes with SP and both neurotransmitters were found adjacent to mucosal MCs. MCs sustain peripheral neurogenic inflammation through the further release of SP and CGRP, that perpetuate inflammatory molecule release (Julius and Basbaum, 2001). Afferent fibers express the receptors involved in nociception, such as transient receptor potential vanilloid 1 (TRPV1), transient receptor potential ankyrin 1 (TRPA1), and proteinase-activated receptor 2 (PAR2) (Kim et al., 2010). The activation of PAR2 starts downstream sensitization of TRPV1 and TRPA1 involved for the generation of visceral hyper-sensibility (Amadesi et al., 2006; Irmak et al., 2019). PAR-2 is expressed by dorsal root ganglia that co-express TRPV1, TRPV4, TRPA1, and SP and CGRP, is activated by tryptase and SP, and its activation can result in neurogenic inflammation (Amadesi et al., 2006). TRPV1 is an intriguing target for pain control in MC-dependent disorders (Eller-Smith et al., 2018). A possible role of histamine in TRPV1 activation has been suggested (Kajihara et al., 2010; Eller-Smith et al., 2018). SP induces CRF receptor expression on MCs (Asadi et al., 2012). MCs also synthesize nerve growth factor (NGF) that in autocrine mode stimulates the MCs to release pro-nociceptive mediators (Eller-Smith et al., 2018). NGF binds to its receptor TrkA evoking pain hypersensitivity (Eskander et al., 2015).

Evidences suggest that MCs may upgrade a cascade of inflammatory events that result in trigeminal activation (Levy et al., 2006; Irmak et al., 2019). MC releases nitric oxide (NO), crucial mediator of persistent neuronal damage triggering neurogenic inflammation (Ramachandran et al., 2014; Ramachandran, 2018). CGRP and mediators released from MCs induce meningeal vasodilatation and activation of sensory nerve fibers (Irmak et al., 2019). In addition, the nucleotide adenosine triphosphate (ATP) directly excites trigeminal nerve terminals and degranulates MCs, suggesting that ATP may contribute both to excitation and to meningeal neuroinflammation in the unit of dural MCs and trigeminal afferent fibers (Koroleva et al., 2019). A fast inhibitory interaction between respective receptors could revert MC-derived neuroinflammation in sensory nerve endings and influence their time course activation.

MAST CELLS AND STRESS

The exposure to chronic stress may induce irreversible modifications in the brain regions responsible for perception of pain (Fasick et al., 2015).

Specific areas, including hypothalamus, amygdala, prefrontal cortex, and hippocampus, and their interaction with limbic

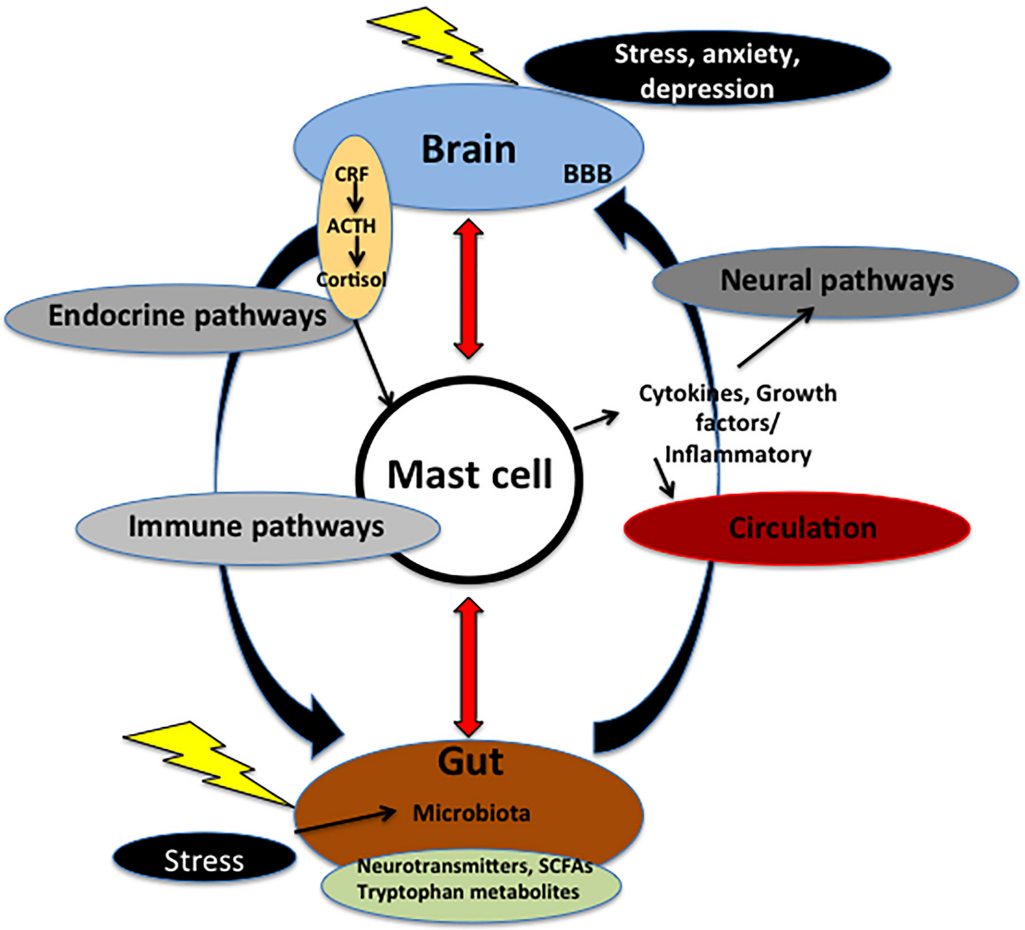

FIGURE 1 | Microbiota–gut–brain axis. The axis is fundamental for the regulation between the brain and the gut. Communication within this axis involves the coordination of different factors and systems. It includes CNS, circulation, and endocrine and immune pathways. Mast cell (MC), emblem of the neuroimmune network, acts at the crossroad of intestinal mucosa, microbiota, and nervous system. Microbiota may control events both in peripheral and CNS via nerve activation, cytokine production, neurotransmitter and SCFAs release, and through systemic circulation. These signals may pass through BBB inside the brain, and, in turn, activate MCs and microglia involved in immune surveillance. In addition, in the brain these molecules control different functions, such as the regulation of HPA axis. This axis regulates various activities, including MC activation. Such activation causes the release of cytokines and growth factors that, in turn, can act both centrally and peripherally. Chronic stress and depression lead to alteration of the HPA axis as well as dysfunctions in the microbial gut environment and pain visceral signaling. On the other hand, in the gut mucosal MCs are influenced by microbiota *milieu* and by visceral conditions. ACTH, adrenocorticotropic hormone; BBB, brain–blood barrier; CRF, corticotrophin-releasing factor; SCFAs, short chain fatty acids.

system are involved in response to stress (Lupien et al., 2009). A crosstalk between MCs and microglia in these areas could explain stress-induced inflammation (Hendriksen et al., 2017; Traina, 2017). Stressful conditions can also activate peripheral MCs, and increase glial activation (Dong et al., 2017). The integration of these centers results in the activation of HPA and in autonomic nervous system (ANS) that modulates also enteric nervous system (ENS), inducing an exacerbation of inflammatory conditions (Rea et al., 2016). Prolonged neuroinflammation has deleterious effects that involve changes in brain parenchyma, BBB alterations, neuronal hyper-excitability, and neuronal death (Hendriksen et al., 2017). The microglia and MCs become hyper-activated, realizing IL-1β, IL-6, and TNF-α (Skaper, 2016; Kempuraj et al., 2017; Traina, 2017). Variety of adhesion molecules, cytokines, chemokines, and metalloproteases contribute in the development of inflammatory response in brain through the degradation of extracellular matrix and tissue remodeling (Hendriksen et al., 2017). Acute stress condition increases BBB permeability resulting in ion unbalance, entry of immune molecules, and instable CNS environment (Hendriksen et al., 2017). The penetration of reactive T cells into the CNS is under the influence of MCs (Silverman et al., 2000). In stress conditions, increased peripheral CRF release consequent to dysregulation of HPA axis activation results in sensitization of nerve terminals (Eller-Smith et al., 2018). A prolonged increase of glucocorticoid levels is associated with a reduction of hippocampal volume and impairments in memory, perception, and attention (Bremner, 2006). Hippocampal volume reduction may be due to high levels of glucocorticoids that damage mature neurons or rather to high levels of cortisol

that suppress neurogenesis (Bremner, 2006). Also a recurrent depression causes a volume reduction of the hippocampus (Bremner et al., 2008).

MAST CELLS AND DEPRESSION

Studies indicate that major depression is prevalent in patients affected by chronic infections suggesting that a chronic inflammation condition can increase depression incidence (Hauser et al., 2011). Major depression disorder attend changes in monoaminergic neurotransmission, imbalance of excitatory/inhibitory signaling, hyperactivity of the HPA axis, abnormalities in neurogenesis (Milenkovic et al., 2019). The disorder was accompanied to increase of circulating IL-1β, IL-6, and TNF-α (Marini et al., 2016). Administration of inflammatory cytokines leads a major depressive disorder condition like to the one induced by stressor agents (Anisman and Hayley, 2012).

Proinflammatory cytokines can induce the activation of indoleamine 2,3-dioxygenase (IDO), enzyme involved in the catabolism of tryptophan to kynurenine and higher levels of kynurenine are linked with a depression condition (Gabbay et al., 2012). Some studies reported that the prevalence of major depression and other neurological and psychiatric symptoms such as anxiety, sleep disorders, and headaches are detected in patients suffering of mastocytosis, a disease characterized by MCs accumulation and activation (Moura et al., 2011; Hendriksen et al., 2017). Such subjects present lower levels of tryptophan and 5-HT, and high IDO1 activity and kynurenine acid. It is know that MCs may be activated by kynurenine metabolites (Hermine et al., 2008; Kawasaki et al., 2014), whereas MCs mediators can affect the IDO pathway bringing to an imbalance between kynurenine and 5-HT ratio. Finally, pro-inflammatory cytokines increase monoamine reuptake by reducing 5-HT levels (Theoharides et al., 2015; Hendriksen et al., 2017). A different pattern of HPA axis activity has been described for atypical depression (Juruena et al., 2018).

MICROBIOTA–GUT–BRAIN AXIS

In the gut, the functional unit established by MC–nerve interaction is a crucial component in the interplay in paracrine signaling (Albert-Bayo et al., 2019). Enteric neurons and vagal and spinal afferents express receptors for molecules released by MCs. These molecules stimulate nerve terminals, thereby modulating the firing threshold. Similarly, neuropeptides and neurotransmitters released by neurons stimulate MC secretion of mediators, which further activate neuronal receptors, supporting the maintenance of this neuro-immune interplay (Forsythe et al., 2012; Albert-Bayo et al., 2019). The intestinal microbiota is composed of trillions of microorganisms, 10 times more numerous than the eukaryotic cells that make up the body (Rea et al., 2016).

Many studies support the relationship between complexity of the dynamic ecosystem of microorganisms that harbor the gut and health status (Eckburg et al., 2005; Cryan and Dinan, 2015). Under physiological and homeostatic conditions the microbiota helps to maintain a multitude of functions such as intestinal peristalsis, control of several metabolic functions, epithelial barrier integrity, pH balance, and immune priming and protection versus invading microorganisms (Kelly et al., 2017). Microbiota controls maturation and function of microglia (Nayak et al., 2014). The microbiota can influence emotional behavior through mechanisms that include microbe-derived bioactive molecules, immune and endocrine cell activation, and vagal nerve stimulation (Dinan and Cryan, 2013). The microbiota plays a crucial role in stress, anxiety, learning and memory, addiction, sexual behavior, social interaction, and depression as well as in neuroinflammation and neurodegeneration (Wang et al., 2016; Cussotto et al., 2018).

The *microbiota–gut–brain axis* is an integrate system that consists of a dynamic matrix of tissues and organs (brain, ANS, glands, gut, immune cells, and microbiota) communicating through a complex multidirectional manner via neural, endocrine, circulatory pathways in order to preserve homeostasis condition and resist to any perturbation to the system (Dinan and Cryan, 2013; Bermúdez-Humarán et al., 2019). Signals from the brain may influence the motor, sensory, and secretory functions of the gut and viceversa, visceral messages from the gut may influence brain function (**Figure 1**). Germ-free (GF) animals (without microbiota, born and maintained in sterile condition) present various immune disorders, including defective microglia (Erny et al., 2015).

Microbial metabolites and products of bacterial fermentation, such as short chain fatty acids (SCFAs), specifically acetate, propionate, and butyrate stimulate enteroendocrine cells to produce various neuropeptides, including neuropeptide Y and SP, that gain access to the circulation and/or receptor affecting ENS neurons or vagal innervation (Cani and Knauf, 2016; **Figure 1**). SCFAs together with other metabolites, such as polyamine, influence the immunity response and may restore physiological conditions in GF animals (Borre et al., 2014; Erny et al., 2015; Rook and Garrett, 2016).

Microbiota promotes tryptophan hydroxylase expression. In the gut, the amino acid tryptophan contributes to synthesis of bioactive molecules, including 5-HT (Agus et al., 2018). SCFAs and tryptophan transmit signals through interaction with enteroendocrine and enterochromaffin cells, and the mucosal immune system, cross the intestinal barrier, go in the systemic circulation, and may cross the BBB (Yano et al., 2015; Kelly et al., 2017; Martin et al., 2018). So, SCFAs and microbial regulation of tryptophan metabolism act as a link between microbiota and brain (Liu et al., 2018; Martin et al., 2018; **Figure 1**). Yet, microbiota produces a variety of other neuroactive molecules, including γ-aminobutyric acid (GABA), catecholamines, and acetylcholine, and may affect the HPA axis (Kelly et al., 2017; Wiley et al., 2017). 5-HT is a crucial substrate in the pathogenesis of mood disorders and intestinal microbiota may be a potential therapeutic target for 5-HT-related brain–gut axis diseases. Various species of bacteria are able of producing 5-HT, including *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Lactobacillus* (Kelly et al., 2017).

Illness, stress, pain, or injury conditions can alter the microbial environment and induce to a large spectrum of effects including alteration of gut motility, loss of intestinal epithelial barrier integrity, antigen penetration, release of LPS into the bloodstream, mucosal MC activation, inflammatory mediator release, visceral hypersensitivity, and nociceptive sensitization, ranging from inflammatory bowel disease (IBD) and IBS to major depressive disorder (Bennett et al., 1998; He, 2004; Brzozowski et al., 2016; Rea et al., 2016). Subjects with IBD present an enhanced risk of anxiety and depression (Maunder, 2005; Goodhand et al., 2012). On the other hand, IBS is a complex disorder in which the inflammation is involved through gut–brain axis, resulting in altered neuroendocrine pathways. IBS is characterized by pain, visceral hyper-sensibility, intestinal microbiota imbalance, gut–brain axis dysfunction, and psychological disorders (Quigley and Craig, 2012). Microbial signals also modulate visceral pain anxiety- and depression-like behavior. In humans, significant changes in the microbiota have been noticed in a variety of amygdala-related clinical disorders, including depression and chronic visceral hypersensitivity. IBS patients present hyperactivity in the amygdala and closed brain regions in response to visceral stimulation (Barbara et al., 2007, 2011; Han et al., 2012; Cowan et al., 2018). Stress condition induces significant modifications in the composition of the microbiota. The mechanism of influence of stress on gut involves action of IL-6, IL-10, IL-1β, and TNF-α (Lew et al., 2018). It has been seen that norepinephrine enhances the virulence of some bacteria (Cogan et al., 2007). Tryptophan-regulating bacteria can function as antidepressant drugs (Dinan and Cryan, 2015). In conclusion, a dysregulation of the microbiota composition (*dysbiosis*) can start or exacerbate intestinal disorders as well as can influence emotional condition (Cryan and Dinan, 2015; Kelly et al., 2017).

MAST CELLS IN THE GUT

Mast cells are present in all layers within the gastrointestinal tract (Albert-Bayo et al., 2019). The close proximity of MCs and nerves is the emblem of the neuro-immune network and has indicated the existence of a bidirectional crosstalk between MCs and nerves acting in tandem with other neural and immune cells (Albert-Bayo et al., 2019). This dialog is crucial in the maintenance of intestinal homeostasis and it is responsible for diseases and in pain visceral perception (Quigley and Craig, 2012).

It has been observed that MC mediators are released in large quantity in gut of IBS subjects and leading to hypersensitivity of afferent neurons (Barbara et al., 2007). There is a growing literature to support the hypothesis that MCs perform a fundamental role in host–microbiota communication, by modulating the influence between them through changes in their activation (Wouters et al., 2016).

A brain–MC interaction is one conceivable mechanism linking stress responses and gastrointestinal symptoms with the involvement of vagal nerve pathway (Dong et al., 2017).

Chronic stress may lead to MC activation and modulate paracellular and transcellular permeability. In IBS an intestinal barrier dysfunction is implicated and the expression of tight junction (TJ) proteins is reduced in correlation with MC activation. Tryptase can activate PAR-2 on epithelial cells by increasing permeability through TJs (McDermott et al., 2003). Epithelial barrier breakdown is associated with an increase in pro-inflammatory cytokines, including IL-4, Il-13, IFN-γ, and TNF-α (Kim et al., 2018). Further molecules released by MCs, including chymase and prostaglandin D2, modulate epithelial chloride and water secretion and intestinal permeability (Wouters et al., 2016; Dong et al., 2017).

Microorganisms, such as bacteria and fungi, can induce MCs activation (De Zuani et al., 2018). Albeit some microorganisms can evoke a pro-inflammatory response, other microorganisms result able to reduce their activation, contributing to maintain their stability. This allows to limit or revert inflammation and to promote homeostatic conditions (Forsythe, 2016; Johnzon et al., 2016).

It has also been shown that MCs may phagocyte bacteria promising a scenario in which MCs may act as intermediate players between the microbiota and the adaptive immune system (Malaviya and Abraham, 2001).

In the gut, MCs are differentially functional in the different regions on the basis of local bacterial charge; in colon MCs have a greater abundance of TLR4 than the MCs present in the small intestine (Frossi et al., 2018). Bacterial challenge induces MC degranulation and release of mediators (Wesolowski and Paumet, 2011). An increase in histamine and tryptase secretion has been reported in biopsies from the gut of IBD patients (De Winter et al., 2012; Wilcz-Villega et al., 2013). These subjects show a lower bacterial diversity in the intestinal microbiota, an increase of the Proteobacteria phylum, and a decrease of Firmicutes (Halfvarson et al., 2017).

Mast cells interact with both the microbiota and the nervous system relating to enteric neurons through release of 5-HT, while are affected by SP or noradrenaline (Buhner and Schermann, 2012).

All these evidences support that MCs may substantially contribute to the balance in gut homeostasis, and their activation is linked to modifications and motor abnormalities and barrier dysfunctions (Barbara et al., 2007; De Zuani et al., 2018). In addition to well-established pharmacotherapy comprised of anti-inflammatories, antibiotics, and proton-pump inhibitors, valid treatment strategies on the microbiota may contain other options including probiotics, prebiotics, and food supplements with anti-inflammatory properties. The beneficial effects of probiotics have been recognized as therapeutic supplement in various disorders (Sarkar et al., 2018).

PROBIOTIC CHALLENGE

Probiotics are defined as "*living microorganisms which, when administered in adequate amounts, confer a health benefit to the host*" (Hill et al., 2014). Probiotic supplementation is particularly useful for developing an understanding of the mechanism of action of selected bacterial strains, and a crucial factor in predicting the favorable health outcomes of nutritional

intervention (Dinan and Cryan, 2013). Various studies reported that specific probiotic strains are able to counteract inflammatory conditions and exert considerable effects on immune cells and inflammation (Dominici et al., 2011; Bellavia et al., 2014; Persichetti et al., 2014; Tomasello et al., 2015a,b; Traina et al., 2016; De Marco et al., 2018; Sichetti et al., 2018). Gut microbiota composition may be effectively affected by dietary ingestion of probiotics and prebiotic, these last ones defined as non-digestible organic substances, capable of selectively stimulating the growth and/or activity of beneficial bacteria (Saulnier et al., 2013). Probiotics decrease plasma cytokine levels and reduce mitogen-stimulated cytokine in healthy subjects (Groeger et al., 2013).

Probiotics are recognized by TLRs in intestinal epithelial cells and immune cells. Probiotics can influence CNS function and modulate the HPA axis attenuating it, and altering the levels of corticosteroid and/or ACTH (Ait-Belgnaoui et al., 2018). HPA axis response to acute stress was attenuated by *Lactobacillus farciminis* (Ait-Belgnaoui et al., 2018). *Lactobacillus rhamnosus* and *Lactobacillus helveticus* influence brain-derived neurotrophic factor (BDNF) levels in basolateral nucleus of amygdala exerting an anxiolytic effect (Peng et al., 2019). Hippocampal *c-Fos* expression is modulated by *L. rhamnosus* and *L. helveticus* (Smith et al., 2014). *L. rhamnosus* reduces hippocampal expression of GABA receptor gene, suggesting a modulation of the balance of inhibition/excitation to control responses to stress, anxiety, and depression (Wiley et al., 2017). Lactobacilli and bifidobacteria are able to metabolize glutamate and produce GABA (Foster and McVey Neufeld, 2013; Wiley et al., 2017). Probiotics can modulate 5-HT, and dopamine, thus affecting both mood and behavior (Cowan et al., 2018; Hoban et al., 2018; Baj et al., 2019). The vagus and enteric nerves are significantly affected by specific probiotics (Dinan and Cryan, 2013). Intestinal microbiota may change the perception of pain and selective probiotic strains may inhibit the hypersensitivity and intestinal permeability induced by the stress (Gareau et al., 2007; Wiley et al., 2017). Probiotic bacteria manipulate intestinal microbiota, enhancing variety and beneficial composition of the bacteria (Karimi and Pena, 2003). An improvement in microbiota metabolites, such as SCFAs and tryptophan, indirectly improves CNS function (Butler et al., 2019). Studies demonstrated that gut microbiota condition regulates BBB permeability (Braniste et al., 2014). However, the mechanisms underlying these beneficial effects are not well understood yet and, currently, very few human studies are present. Probiotic role may include exclusion of pathogenic microorganisms and immune system modulation (Lavasani et al., 2010; Kwon et al., 2013).

And, most likely, the main proponents of this connecting link are the MCs and their products. The ability of specific strains of bacteria to influence MC function and their activation has been studied, sometimes even with conflicting results (De Zuani et al., 2018).

Lactobacillus rhamnosus and *B*ifidobacterium *infantis* probiotic strains reduce depressive-like behavior down-regulating HPA axis (Bravo et al., 2011).

Moreover, it has been reported that *L. rhamnosus* GG and some other probiotic strains show a decreasing effect on the MC numbers in several studies in rodent models (Cassard et al., 2016). Some commensal bacteria can limit MC activation. Pathogenic bacteria, including *Yersinia pestis* and *Salmonella typhimurium*, prevent MCs degranulation in rodents and in humans. This pathogen eludes host innate immunity, involving the MC inactivation (Choi et al., 2013). Both pathogens secrete a tyrosine phosphatase responsible for the suppression of MC activation, leading to a reduced bacterial clearance (Choi et al., 2013; De Zuani et al., 2018). Different commensal bacteria such as *Enterococcus faecalis*, *Lactobacillus paracasei*, and non-pathogenic *Escherichia coli* can delete MC degranulation IgE/Ag-induced in mice (Choi et al., 2016). It is noteworthy that this suppression mechanism is based on impairment of intracellular signaling including an inhibition of the maintenance of elevated intracellular Ca^{2+} levels required for MC degranulation and not down-regulation of FcεRI (Kasakura et al., 2014; Cassard et al., 2016). In addition, different strains of Lactobacilli may suppress MC degranulation but not TNF-α release nor IL-13 (Harata et al., 2016). The role of commensal microorganisms in controlling MC activation was evinced following oral treatment of *E. faecalis* that reduced MC infiltration in a murine model (Choi et al., 2016; De Zuani et al., 2018).

The role of bacteria and MCs can also be linked to the beneficial stabilizer agents. It has been shown that the histamine H1 receptor blocker diphenhydramine, known as Benadryl®, prevents the increase of cytokines from MCs stimulated by bacteria. Some broad-spectrum antibacterial agents inhibit MC activation and its degranulation. In particular, some probiotic strains are able to stabilize MCs, especially *L. rhamnosus GG* (Oksaharju et al., 2011). Oral administration with *L. rhamnosus JB-1* induces inhibition of peritoneal MC degranulation (Forsythe et al., 2012). The effect is mediated by an inhibition of calcium-dependent potassium current (KCa 3.1). This inhibition prevents Ca^{2+} entry required for MC degranulation. In MC regulation of calcium-dependent potassium current is operated through β2-adrenoceptors and adenosine and prostaglandin receptors (Duffy et al., 2007, 2008). Some Lactobacillus strains are able to inhibit IgE-mediated degranulation in MCs through TLR-2 pathway (Kawahara, 2011). A recent study reports that *L. rhamnosus JB-1* reduced stress-induced behavioral deficits in mice, including modifications in sociability and anxiety. This probiotic prevented immunoregulatory alterations related to the stress phenotype suggesting a direct modulation on gut–brain signaling (Bharwani et al., 2017; McVey Neufeld et al., 2018). Administration of specific bacteria induced systemic expansion of Treg, an immune population that produces anti-inflammatory cytokine, such as IL-10 (Kim et al., 2019).

Another study reports that *L. rhamnosus* GG in combination with prebiotics reduces the effects of early-life maternal separation on anxiety-like behavior and hippocampal-dependent learning with modulation of mRNA expression of genes related to stress circuitry, anxiety, and learning in a rodent model (McVey Neufeld et al., 2017).

A recent study reports that probiotic VSL#3 suppress visceral hyper-sensibility through MC-PAR2-TRPV1 pathway in a rodent model (Li et al., 2018).

Kim et al. (2016) report that treatment with *Bifidobacterium longum* KACC 91563 can control the number of MCs in the gut lamina propria. A modality of intercellular communication is the release of membrane vesicles. Microbiota secrete complex extracellular vesicles (EVs), containing protein, DNA, and components of cellular wall within little spherical lipid bilayer acting as messengers. Bacterial EVs may influence the physiology of neighboring cells, inducing intracellular signaling via receptors and giving new features after the acquisition of receptors, enzymes, or genetic material from the vesicles. Bacterial EVs deliver elements at host immune cells in concentrated, preserved, and targeted manner. In particular, MCs internalize EVs specifically. The authors suggest a model of action according to which EVs of *B. longum* enter through intestinal epithelial cells inducing the apoptosis of MCs (Kim et al., 2016).

Bifidobacterium longum, *B. breve*, *L. rhamnosus*, and *L. helveticus* have been used and all exhibited antidepressant effects (Desbonnet et al., 2008). *B. longum* and *L. helveticus* suppress stress-related visceral sensibility via HPA axis control (Ait-Belgnaoui et al., 2018). Beneficial effects of a combination of *Bacillus subtilis* and *Enterococcus faecium* (LCBE) could inhibit the degranulation of MCs, confirming the role of probiotics in the regulation of MCs. In addition, the levels of histamine, which constitute an aggravating mediator that accelerates the development of main lesion of organ, were decreased after administration of LCBE (Guo et al., 2019).

It has been shown that an increase in MCs was countered by inclusion of *B. licheniformis* in the diet of a bird animal model. The strain administration partially alleviated stress condition, suggesting that probiotics can benefit intestinal integrity (Deng et al., 2012).

Probiotics influence mucosal MCs, but also could affect MCs in the brain through microbiota–gut–brain axis. *Lactobacillus reuteri* and Bifidobacteria have positive therapeutic effects on cognitive and emotional impairments in fibromyalgia (Roman et al., 2018). Recently, probiotic supplement on inflammatory markers, and episodic and chronic migraine has been reported (Martami et al., 2019).

Short chain fatty acids also have an effect on MC activation (Diakos et al., 2006). Sodium butyrate reduced the percentage of degranulated MCs and their inflammatory mediator content in weaned pigs. In addition, sodium butyrate reduces the expression of MC-specific tryptase, TNF-α, and IL-6 mRNA. A butyrate-producing probiotic (*Clostridium butyricum*) restores the intestinal epithelial barrier integrity through regulation of TWIK-related potassium channel-1 (Trek-1) (Huang et al., 2016).

Also vitamin D is known to play an immunoregulatory role on MCs. In fact, vitamin D is required to maintain stability of MCs (Liu et al., 2017). A deficiency of vitamin D results in MC activation.

In addition, MCs express 25-hydroxyvitamin D-1α-hydroxylase which enables them to convert inactive 25-hydroxyvitamin D3 (25OHD3) to biologically active 1α,25(OH)2D3 (Yip et al., 2014). 1α,25(OH)2D3 favors apoptosis and inhibits maturation of bone marrow-derived MC precursors in mice (Baroni et al., 2007). Vitamin D regulates NO, which is believed to be an anti-bacterial molecule, produced by MCs. Finally, factors that reduce CRF stabilize activation of MCs (Larauche, 2012).

CONCLUSION

Mast cells are old cells, perhaps, long forgotten, constitutively and strategically located within the mucosal districts, and armed with a wide variety of receptors that allow them the ability to interact strongly with other cells and elements of the microenvironment. The mutual interactions that regulate the MC–microbiota crosstalk show how MCs act at the crossroad of immune system, intestinal mucosa, commensal microbiota, and nervous system.

The evidence supported the idea that there is likely a bi-directional relationship between MC and intestinal microbiota and that MC activity goes well beyond the simple host defense role of regulating microbial composition during pathology condition.

Most of the data obtained so far are derived from *in vitro* or *in vivo* animal studies because human studies are still very limited.

In this review, we refer some interactions that control MC activation and functions, suggesting that various factors and mechanisms can influence the MC–microbiota–gut–brain crosstalk and regulate their output.

A classical approach detected to prevent improper MC activation has been the use of MC stabilizing agents, including Cromolyn, Ketotifene, and Tranilast, although it is not clear to what extent these drugs act and their exact action mechanism (De Zuani et al., 2018). An increase in intracellular Ca^{2+} concentration as a result of Ca^{2+} entry from the extracellular medium is essential for MC degranulation and FcεRI-mediated MC activation (Baba et al., 2008) as well as calcium is required for activation of transcription factors of cytokine genes including IL-4, TNF-α, and IL-13 (Heatley et al., 1975; Chu et al., 2016; Zhang et al., 2016). In addition, the validity of MC stabilizers in the treatment of MC-related disorders is as yet uncertain (He, 2004; Wouters et al., 2016).

Dietary ingestion of probiotics and prebiotics affects gut microbiota composition, underlying the key role played by specific metabolites not only in the gut microbiota composition but also in the brain health state. Some probiotic effects and mechanisms can be similar to those achieved by drugs and may provide suggestion to future interventions.

Indicating strategies preventing MC activation are very more interesting because, once activate, MCs lead perpetuate inflammation state through onset positive feedback loops.

AUTHOR CONTRIBUTIONS

GT researched and summarized the information and wrote the review.

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Mast Cells, Neuroinflammation and Pain in Fibromyalgia Syndrome

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Fibromyalgia Syndrome (FMS) is a disorder of chronic, generalized muscular pain, accompanied by sleep disturbances, fatigue and cognitive dysfunction. There is no definitive pathogenesis except for altered central pain pathways. We previously reported increased serum levels of the neuropeptides substance P (SP) and its structural analogue hemokinin-1 (HK-1) together with the pro-inflammatory cytokines IL-6 and TNF in FMS patients as compared to sedentary controls. We hypothesize that thalamic mast cells contribute to inflammation and pain, by releasing neuro-sensitizing molecules that include histamine, IL-1β, IL-6 and TNF, as well as calcitonin-gene related peptide (CGRP), HK-1 and SP. These molecules could either stimulate thalamic nociceptive neurons directly, or via stimulation of microglia in the diencephalon. As a result, inhibiting mast cell stimulation could be used as a novel approach for reducing pain and the symptoms of FMS.

Keywords: mast cells, pain, neuroinflammation, fibromyalgia syndrome, proinflammatory cytokines (TNF-alpha, IL-1 beta, IL-6)

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INTRODUCTION

Fibromyalgia Syndrome is a disorder of chronic generalized muscular pain, stiffness, generalized fatigue, sleep abnormalities, (Clauw et al., 2011; Schmidt-Wilcke and Clauw, 2011; Clauw, 2014) and cognitive problems (Theoharides et al., 2015b; Hauser et al., 2019) assessed by the FSQ (Ferrari and Russell, 2013), which has about 93% sensitivity and 92% specificity (Clauw, 2014). FMS affects about 5% of adults, primarily women 20–60 years of age (Branco et al., 2010) and belongs to a family of overlapping painful conditions (**Table 1**) known as CSS (Yunus, 2007; Theoharides, 2013). Central sensitization is recognized as the main mechanism involved (Woodman, 2013) and is characterized by allodynia, pain from an otherwise non-painful stimulus, (Russell and Larson, 2009) and hyperalgesia (Staud et al., 2001) due to an exaggerated response to a painful stimulus

Abbreviations: ACR, American College of Rheumatology; ASD, autism spectrum disorder; CGRP, calcitonin-gene related peptide; CIRS, chronic inflammatory response syndrome; CRH, corticotropin-releasing hormone; CSF, cerebrospinal fluid; CSS, central sensitivity syndromes; CXCL8, IL-8; FcεRI, high affinity surface receptors; FMS, fibromyalgia syndrome; FSQ, fibromyalgia survey questionnaire; IBS, irritable bowel syndrome; IC/BPS, interstitial cystitis/bladder pain syndrome; IgE, immunoglobulin E; MCP-1/CCL2, monocyte chemoattractant protein-1; ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome; MRGPRX2, mas-related G-protein coupled receptor member X2; (mt)DNA, mitochondrial; NGF, nerve growth factor; PAF, platelet activating factor; PTSD, post-traumatic stress disorder; SCF, stem cell factor; SP, substance P; TLRs, toll-like receptors; TMD, myogenic temporomandibular disorder.

(Woolf, 2011). The pathogenesis of FMS remains unknown and with no objective diagnostic criteria (McBeth and Mulvey, 2012; Wolfe and Walitt, 2013). FMS patients have reduced tolerance to pain, especially extremes of heat and cold (Desmeules et al., 2003). There is considerable evidence of altered circuity of pain networks and (Jensen et al., 2012; Flodin et al., 2014) abnormal pain processing in FMS (Staud, 2011).

The PubMed database was searched between 1960 and 2018 using the terms fatigue, fibromyalgia, hypothalamus, inflammation, mast cells, pain and stress. Only articles in English were included.

Here we discuss how brain mast cell release of neurosensitizing mediators in the thalamus leads to focal inflammation and contribute to the pathogenesis of FMS.

Neurohormonal Triggers of Mast Cells Contribute to Focal Inflammation in the Diencephalon

It was recently proposed that FMS may involve localized inflammation in the hypothalamus (Theoharides et al., 2015c). Elevations in pro-inflammatory chemokines/cytokines could negatively impact symptoms (Bazzichi et al., 2007; Carvalho et al., 2008; Nugraha et al., 2013) leading to sensitization of peripheral and central nociceptors (Uceyler et al., 2011; Behm et al., 2012; Hornig et al., 2015). Increased levels of the pro-inflammatory chemokine IL-8 (CXCL8) have been reported in the serum and CSF in patients with FMS (Ross et al., 2010; Kadetoff et al., 2012; Rodriguez-Pinto et al., 2014). Chemokines facilitate nociception by directly acting on chemokine receptors present along the pain pathway (Abbadie, 2005; Charo and Ransohoff, 2006).

The cytokines TNF and IL-17 greatly contribute to the inflammatory response (Romero-Sanchez et al., 2011; Griffin et al., 2012). Plasma levels of IL-17 were increased and correlated with levels of TNF in patients with FMS (Pernambuco et al., 2013). CSF and serum IL-17 also positively correlated with pain (Meng et al., 2013) and anxiety (Liu et al., 2012). Mast cells, themselves, can secrete IL-17; moreover, IL-6 and TGFβ from mast cells contribute to the development of Th-17 cells (Kenna and Brown, 2013).

TABLE 1 | Pain Syndromes Comorbid with FMS.

- Chronic inflammatory response syndrome (CIRS)
- Functional dyspepsia
- Gulf War Illness (GWI)
- Interstitial cystitis/bladder pain syndrome (IC/BPS)
- Irritable bowel syndrome (IBS)
- Mast cell mediator disorder (MCMD)
- Mastocytosis
- Migraines
- Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)
- Myogenic temporomandibular disorder (TMD)
- Myofacial pain syndrome
- Post-traumatic stress disorder (PTSD)
- Restless leg syndrome
- Temporomandibular pain syndrome (TMS)
- Tension headache

Fibromyalgia syndrome worsen by stress, (Geenen et al., 2002) which augments pain responses (Bote et al., 2012, 2013). Plasma concentrations of cortisol are increased in the evening, suggesting disruption of the circadian rhythm (Crofford et al., 2004). Serum levels of CRH, which is secreted under stress, were increased in patients with FMS (Tsilioni et al., 2016). CRH was also increased in the CSF of such patients and correlated with severity of pain (McLean et al., 2006). Physiological stress was reported to be the most common trigger in patients with systemic mastocytosis (SM) (Jennings et al., 2014) who also commonly experience FMS (Theoharides et al., 2015d, 2019). We reported increased levels of CRH in the serum of one patient with indolent systemic mastocytosis (Theoharides et al., 2014). CRH can trigger human mast cells to release VEGF without histamine or tryptase (Cao et al., 2005). CRH also has synergistic action with NT stimulating VEGF release. As a result, there is increased vascular permeability in the skin and the blood-brain barrier (BBB) (Esposito et al., 2002; Donelan et al., 2006; Theoharides and Konstantinidou, 2007). Stress also disrupts the gut-blood barrier (Theoharides et al., 1999; Wallon et al., 2008) allowing for gut microbiome-associated molecules, such as propionate (Minerbi et al., 2019) to enter the brain and contribute to focal inflammation. These results have led to the conclusion that mast cells may serve as "immune gate to the brain" (Theoharides, 1990; Ribatti, 2015).

Levels of the neuropeptide SP (Russell, 1998) and NGF (Giovengo et al., 1999) are elevated in the CSF of FMS patients. NGF has been reported to increase nociception and hyperalgesia (Maren, 2017). The SP receptor NK-1 has been involved in the pathophysiology of pain (Greenwood-Van et al., 2014). We reported increased serum levels of SP, its structural analogue Hemokinin-1 (HK-1) and TNF in patients with FMS (Tsilioni et al., 2016). SP (Theoharides et al., 2010a,b) and NGF (Levi-Montalcini, 1987) can stimulate mast cells. Moreover, SP induced mast cell expression of CRHR-1 (Scholzen et al., 2001). Cerebrovascular mast cells were stimulated by CGRP, (Reynier-Rebuffel et al., 1994; Ottosson and Edvinsson, 1997) which is now well established to participate in the pathophysiology of headaches (Edvinsson, 2018). In addition to neuropeptides, sex hormones can also affect mast cell reactivity. For instance, estradiol augments immune (Kovats, 2015) and allergic (Hox et al., 2015) processes. In particular, we had reported expression of estrogen receptors on rodent mast cells (Pang et al., 1995). We also reported that 17β-estradiol further increased stimulation of mast cells by SP (Theoharides et al., 1993). Such findings may help explain why FMS is more common in women.

In addition to allergic reactions, mast cells contribute to innate immunity, (Galli et al., 2011) autoimmunity (Rottem and Mekori, 2005) and inflammation (Theoharides et al., 2010a).

Thalamic Mast Cells Secrete Neurosensitizing Mediators

Increasing evidence supports the involvement of mast cells in FMS (Lucas et al., 2006; Pollack, 2014) and comorbid disorders (Theoharides, 2013) as well as other inflammatory (Galli et al., 2008; Theoharides et al., 2010a) and painful conditions, (Heron and Dubayle, 2013; Chatterjea and Martinov, 2014) as well

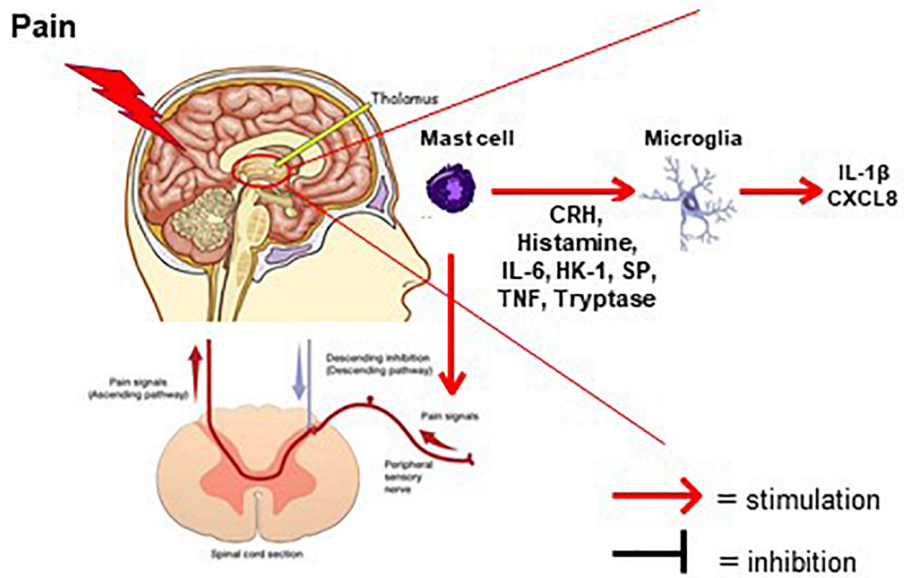

FIGURE 1 | Diagram depicting the involvement of mast cells in the generation of pain in FMS. Mast cells (violet color) in the thalamus secrete pro-inflammatory and neuro-sensitizing mediators (CRH, histamine, IL-6, HK-1, SP, TNF, Tryptase). These mediators can then activate either microglia in thalamic nuclei or ascending nociceptive tracks creating the sensation of pain. Possible natural molecules to inhibit stimulated mast cells and/or microglia are flavonoids such as luteolin or tetramethoxyluteolin (Methlut).

as neuroimmune interactions (Skaper et al., 2017) (**Figure 1**). Chronic urticaria, which involves stimulation of skin mast cells is more common in FMS (Torresani et al., 2009). Moreover, mast cells are significantly increased in the papillary dermis of FMS patients (Blanco et al., 2010). The chemokines monocyte chemoattractant protein-1 (MCP-1/CCL2) and eotaxin (CCL-11) are elevated in plasma of FMS patients (Zhang et al., 2008). MCP-1 is a strong mast cell chemoattractant (Conti et al., 1998) and also triggers mast cells in rodents (Conti and Theoharides, 1994). MCP-1 induced prolonged muscle hyperalgesia in rats via activation of its high-affinity receptor, CC Chemokine receptor 2 (CCR2), on the peripheral nerve terminals (Alvarez et al., 2014). Myoblasts treated with MCP-1 secreted significant amounts of the key pro-inflammatory cytokine IL-1β (Zhang et al., 2008). C-reactive protein (CRP) is now considered a marker of chronic inflammation. CRP may be useful in the diagnostic of FMS (and depression/anxiety that often accompany FMS), even though there is no direct correlation reported (De Berardis et al., 2006, 2017; Orsolini et al., 2018).

Mast cells derive from the bone marrow and mature in response to SCF, which acts via the cell surface tyrosine kinase KIT receptor (Galli et al., 2011). Mast cell progenitors then migrate in all tissues. As a result, mast cell mediators can affect all organs and lead to multiple symptoms. Mast cells are found adjacent to blood vessels and nerve endings; in the brain, mast cells are located in the thalamus, hypothalamus and median eminence (Edvinsson et al., 1976; Lambracht-Hall et al., 1990; Theoharides et al., 2015d).

Mast cells are known to be stimulated by IgE, via activation of its unique surface receptors (FcεRI) (Rivera et al., 2008). Mast cells can also be stimulated via TLRs, (Abraham and St John, 2010; Zhang et al., 2010). Stimulated mast cells secrete multiple vasoactive, pro-inflammatory and neuro-sensitizing molecules (Galli and Tsai, 2008; Theoharides et al., 2010a). Stimulation of mast cells can be augmented by the cytokine IL-33, (Fux et al., 2014) which synergizes with SP to induce release of impressive amounts of VEGF, (Theoharides et al., 2010b) TNF (Taracanova et al., 2017) or IL-1β (Taracanova et al., 2018). As a result, mast cells can serve as "sensors of cell danger" (Theoharides, 1996; Enoksson et al., 2011; Theoharides et al., 2015a).

Mast cell secretory granules store many preformed pro-inflammatory and neuro-sensitizing mediators including bradykinin, histamine, TNF and tryptase (Nakae et al., 2005; Olszewski et al., 2007). Mast cells also release *de novo* synthesized molecules: (a) lipid mediators (leukotrienes, prostaglandins, and PAF), (b) cytokines (IL-6, IL-13, IL-33, TNF) and (c) chemokines (CXCL8, CCL2, CCL5), (Theoharides et al., 2015d; Mukai et al., 2018). Mast cell could often release mediators selectively without histamine or tryptase (Theoharides et al., 2007). Mast cells also release IL-31, which is important in the sensation of itching and pain, in response to IgE and SP, IL-33 and specifically their combination (Petra et al., 2018). We reported that mast cells can release mtDNA, which is mistaken as a pathogen and stimulates inflammatory responses (Zhang B. et al., 2012).

Finally, mast cells can release extracellular vesicles (exosomes) (Skokos et al., 2002, 2003) that could deliver regulatory molecules, including mtDNA and microRNAs (Kawikova and Askenase, 2014). Such microvesicles have been implicated in brain disorders (Tsilioni et al., 2014;

Kawikova and Askenase, 2014) and pain disorders (Rafiee et al., 2018; Silva-Freire et al., 2019). We recently reported that extracellular vesicles are increased in the serum of children with ASD, contained mtDNA and stimulated cultured human microglia to secrete the pro-inflammatory molecules IL-1β and CXCL8 (Tsilioni and Theoharides, 2018).

Mast Cell Interactions With Microglia

Mast cells communicate with microglia (Skaper et al., 2012, 2014b). Mediators secreted from mast cells, (Zhang et al., 2016) such as histamine (Dong et al., 2014) and tryptase, (Zhang S. et al., 2012) can activate microglia leading to secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNF. Microglia can also be activated by CRH secreted from mast cells (Wang et al., 2002; Kempuraj et al., 2004). Stimulation of brain mast cells in mice led to activation of microglia, which was decreased by administration of a mast cell inhibitor (Dong et al., 2017).

Microglia are involved in synapse plasticity, (Shemer et al., 2015; Wu et al., 2015; Reu et al., 2017) but are responsible for innate immunity of the brain (Ransohoff and Brown, 2012; Aguzzi et al., 2013). Microglia contribute to brain inflammation (Hagberg et al., 2012; Aguzzi et al., 2013; Nakagawa and Chiba, 2016) and the pathogenesis of different brain disorders, (Takeda et al., 2014; Reus et al., 2015; Faden et al., 2016; Garden and Campbell, 2016; Groh and Martini, 2017; Koutsouras et al., 2017; Pennisi et al., 2017; Jiang et al., 2018; Thonhoff et al., 2018) especially ASD (Vargas et al., 2005; Morgan et al., 2010; Suzuki et al., 2013; Edmonson et al., 2014; Gupta et al., 2014). Microglia in the thalamus have been discussed in the context of pain, especially maintaining the pain sensation even after the original painful stimulus is not present (Banati, 2002; Hansson, 2010; Saghaei et al., 2013; Blaszczyk et al., 2018).

CONCLUSION

Mast cells have been implicated in headaches (Theoharides, 1983; Theoharides et al., 2005) and pain (Xanthos et al., 2011; Aich et al., 2015; Gupta and Harvima, 2018). Activation of the mast cell-specific receptor, MRGPRX2, (McNeil et al., 2015) and its mouse analogue, Mrgprb2, mediated inflammatory mechanical and thermal hyperalgesia (Green et al., 2019). Hence, mast cells are key players of neuroendocrine (Theoharides, 2017) and painful disorders (Theoharides et al., 2019).

In this context, inhibitors of mast cells (Harvima et al., 2014) would be useful in the treatment of FMS. Natural molecules could include the flavonoids, luteolin (Kempuraj et al., 2008; Theoharides et al., 2015c; Ashaari et al., 2018) and tetramethoxyluteolin, (Theoharides et al., 2017; Theoharides and Tsilioni, 2018) alone or in combination with other substances selected to reduce stress (Theoharides and Kavalioti, 2018). Other natural molecules could include palmitoylethanolamide, (Schweiger et al., 2019) which apparently inhibits neuro-inflammation (Skaper et al., 2013, 2015) and reduces pain (Skaper et al., 2014a; Impellizzeri et al., 2016).

FUTURE DIRECTIONS

Research should focus on identifying in serum of patients with FMS novel molecules that are involved in pain transmission such as bradykinin, CGRP and IL-31. Extracellular vesicles should also be isolated from the serum and CSF of FMS patients, their content identified, and their effect investigated on cultured human mast cells and microglia. Such possible interactions would serve as useful *in vitro* assays for the screening of potential novel treatment agents. Recent reports have also stressed the possible use of the cytokine IL-37, (Mastrangelo et al., 2018) which is known to have anti-inflammatory actions (Cavalli and Dinarello, 2018). It would be important to explore the possible use of IL-37 isoforms in the treatment of FMS.

AUTHOR CONTRIBUTIONS

TT, IT, and MB participated in searching the literature. TT and IT wrote or contributed to the writing of the manuscript. IT prepared the figure.

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REVIEW
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Mast Cells and Sensory Nerves Contribute to Neurogenic Inflammation and Pruritus in Chronic Skin Inflammation

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The intimate interaction between mast cells and sensory nerves can be illustrated by the wheal and surrounding flare in an urticarial reaction in human skin. This reaction is typically associated with an intense itch at the reaction site. Upon activation, cutaneous mast cells release powerful mediators, such as histamine, tryptase, cytokines, and growth factors that can directly stimulate corresponding receptors on itch-mediating sensory nerves. These include, e.g., H1- and H4-receptors, protease-activated receptor-2, IL-31 receptor, and the high-affinity receptor of nerve growth factor (TrkA). On the other hand, sensory nerves can release neuropeptides, including substance P and vasoactive intestinal peptide, that are able to stimulate mast cells to release mediators leading to potentiation of the reciprocal interaction, inflammation, and itch. Even though mast cells are well recognized for their role in allergic skin whealing and urticaria, increasing evidence supports the reciprocal function between mast cells and sensory nerves in neurogenic inflammation in chronic skin diseases, such as psoriasis and atopic dermatitis, which are often characterized by distressing itch, and exacerbated by psychological stress. Increased morphological contacts between mast cells and sensory nerves in the lesional skin in psoriasis and atopic dermatitis as well as experimental models in mice and rats support the essential role for mast cell-sensory nerve communication in consequent pruritus. Therefore, we summarize here the present literature pointing to a close association between mast cells and sensory nerves in pruritic skin diseases as well as review the essential supporting findings on pruritic models in mice and rats.

Keywords: mast cell, sensory neuron, itch, skin, pruritus

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INTRODUCTION

Itching is a very unpleasant sensation that may provoke scratching or the desire to scratch (Savin, 1998). Chronic itch has clinically been classified into 3 groups according to skin changes: (1) pruritus on primary diseased, inflamed skin; (2) pruritus on primary non-diseased, non-inflamed skin; and (3) pruritus with chronic secondary scratch lesions. Pruritus has also been

categorized based on underlying diseases as following: (1) dermatological diseases; (2) systemic diseases including diseases of pregnancy and drug-induced pruritus; (3) neurological diseases; (4) psychiatric/psychosomatic diseases; (5) mixed; and (6) others (Ständer et al., 2007b). Based on its duration, itch can be divided into acute (less than 6 weeks) and chronic (more than 6 weeks) (Azimi et al., 2016).

In the skin, the perception of itch originates from the combined action of nervous, cutaneous and inflammatory cells and substances released by them in the microenvironment. In humans, the nerves responsible for the perception of itch also react to stimuli of pain. There are two types of pruriceptors, specialized nerve fibers sensing itch: fastly conducting A-fibers and non-myelinated, slowly conducting C-fibers, which can be further subdivided based on their responsiveness to different chemical or physical stimuli [reviewed in detail in (LaMotte et al., 2014)].

The itch-specific peripheral sensory neurons may be a subgroup of nociceptive neurons. Subsets of mechano-insensitive nociceptive C-fibers can respond to histamine resulting in the release neuropeptides, including substance P and calcitonin gene -related peptide (CGRP). Instead, the mechano/heat-sensitive nociceptors are not reactive to histamine, but show a response to a cowhage plant protease, leading to stinging itch, which is possibly mediated via protease-activated receptor-2 or -4 (PAR-2 or -4). This type of stinging itch resembles that in skin inflammatory diseases. Nevertheless, cutaneous nerves contain a range of different receptors and ion channels. Therefore, different combinations of receptors may produce a certain type of itch and/or pain sensation. In the spinal dorsal horn, gastrin-releasing peptide-expressing neurons participate in the transmission of itch (Albisetti et al., 2019). The sensing system is further complexed by excitatory interneurons, spinothalamic tract neurons and the central nervous system where these sensations are recognized (Baraniuk, 2012; LaMotte et al., 2014; Talagas et al., 2018). Furthermore, it is noteworthy that chronic inflammation in skin causes additional changes to the neural and proinflammatory cell network.

Free nerve endings in the epidermis are important for sensing itch. It has been proposed that there is a reciprocal synaptic-like interaction between nerves and keratinocytes (Talagas et al., 2018). One possible mediator in this interaction is ß-endorphin, as keratinocytes expressing this mediator have been found around unmyelinated fibers that can be activated via μ-opioid receptor (Bigliardi-Qi et al., 2004). In fact, the whole complex of nerve fibers and the epidermal cells have been proposed to form the "itch receptor" (Greaves, 2010). The skin cells themselves express a wide array of mediators and their receptors involved in the perception of itch (Greaves, 2010). In addition to nerve fibers and epidermal cells, the cells of the immune system constitute yet another part in pruritus. In regard to cellular events in itching skin, mast cells are important players that are involved in neurogenic inflammation and its associated itch. In this review, the fundamental role of mast cells and sensory nerves in itch is discussed.

ITCH-RELATED PROPERTIES OF MAST CELLS

Mast cells are important effector cells in allergic reactions and immunity [reviewed in Conti et al. (2017)], but they also contribute to carcinogenesis (Biswas et al., 2014; Hu et al., 2018; Saadalla et al., 2018). Mast cells are abundant in body-environment interfaces in the skin and the gastrointestinal tract (Gurish and Austen, 2001) as well as present in the meninges of the central nervous system (Bo et al., 1992; Theoharides et al., 2005), and in the lung (Bradding et al., 2006). In the skin, mast cell numbers are highest in the upper dermis and their numbers are not affected by age or sex (Weber et al., 2003), but increase in response to various environmental stimuli as shown after ultraviolet radiation in human skin (Grimbaldeston et al., 2003; Kim et al., 2009), and after exposure to topical sensitizing agents in mice (Kitagaki et al., 1995; Tomimori et al., 2002). It is thus obvious that mast cells constitute an inherent component of itch.

Mast cells are fully loaded with preformed mediators or they produce newly-synthesized mediators, including proteases, histamine, lipid-derived mediators, cytokines, and chemokines which they release upon activation through a variety of mechanisms leading to degranulation, piecemeal degranulation, and/or mediator secretion without degranulation [reviewed in da Silva et al. (2014) and in Gupta and Harvima (2018)]. In type I hypersensitivity, mast cell degranulation is a fundamental event and involves crosslinking of an antigen with two IgE molecules bound to FcεRI receptors (Owen et al., 2013). Mast cell activation has been studied especially in the context of urticaria. It is known that several factors such as infections, stress, certain foods, pseudoallergens, hormones, neuropeptides, and Th2 inflammation may prime mast cells [reviewed in Church et al. (2018)]. Also autoimmune mechanisms may lead to mast cell activation. In these cases, IgE recognizes dermis-derived autoantigens, of which more than 200 are present in chronic spontaneous urticaria (Schmetzer et al., 2018). Another mechanism is the presence of IgG or IgM class antibodies against IgE (Gruber et al., 1988) or against its FcεRI receptor (Hide et al., 1993). These autoantibodies may also target the eosinophils, which release mast-cell activating factors (Puccetti et al., 2005).

ITCH-RELATED FACTORS RELEASED BY MAST CELLS

Mast cells release the contents of their secretory granules to their surroundings upon degranulation. Many of these granule mediators or mediators synthesized *de novo* (Harvima et al., 2014) participate in the development of itch.

Histamine and Its H1 and H4 Receptors

Histamine is the most important pruritogenic mediator of mast cells. Histamine has four receptors, namely H1–H4, of which H1 and H4 are important in pruritus. The function of these receptors in itch has been mainly studied in mouse models, and it has been shown that skin sensory neurons express H1,

H3 and H4 (Rossbach et al., 2011). In mouse models, H1-antagonists have been effective in decreasing itch, which has been known already for decades (Sugimoto et al., 1998), although H4-antagonists (Dunford et al., 2007; Yamaura et al., 2009) have proved to be more potent. Histamine acts also on Transient receptor potential vanilloid receptor-1 (TRPV-1) in sensory neurons (Shim et al., 2007). In keratinocytes, also TRPV-4 is a histaminergic pruriceptor (Chen et al., 2016).

Tryptase and PAR-2

Tryptase, one of the main proteinases secreted by mast cells, can induce pruritus in mice and its effects are inhibited by PAR-2 antibody or PAR-2 antagonist, showing that PAR-2 is involved in tryptase-induced pruritus (Ui et al., 2006). Involvement of tryptase and PAR-2 in itch has also been reported in a mouse model of atopic dermatitis (Zhu et al., 2015). In line with these data, non-lesional and lesional skin biopsies from patients with atopic dermatitis show PAR-2 in sensory nerves with closely located mast cells (Steinhoff et al., 2003).

IL-31 and Its Receptor IL-31RA

Interleukin-31 (IL-31) is important in the pruritus of atopic dermatitis (Sonkoly et al., 2006) and it also participates in the itch of cutaneous lymphoma (Nattkemper et al., 2016). IL-31 has been shown to increase the growth and sprouting of cutaneous sensory nerves (Feld et al., 2016), which express its receptor, IL-31RA (Cevikbas et al., 2014).

Interleukin-31 has been demonstrated to induce mild itch that appears slowly starting at 143 min after skin prick test, which is associated with a long-lasting erythema. By comparison, histamine induces immediate itch that starts within 5 min after skin prick test (Hawro et al., 2014). Human mast cells (Niyonsaba et al., 2010; Petra et al., 2018) and T-cells (Dillon et al., 2004) are sources of IL-31 in skin, thus participating in the development of itch. Moreover, mast cell-derived histamine in addition to IL-31 increase the secretion of brain-derived natriuretic peptide, which in turn affects dendritic cells and keratinocytes to produce cytokines and other mediators, leading to inflammation, and increased itch signaling (Meng et al., 2018).

Leukotrienes and Prostaglandins

Leukotrienes and prostaglandins are also involved in itch, but by different mechanism. When administered intradermally, leukotriene B4 induces itch while prostaglandin E2 does not (Andoh and Kuraishi, 1998). Leukotriene B4 is released from keratinocytes in response to PAR-2 receptor activation (Zhu et al., 2009) and it is involved in the itch-causing cascades of substance P (Andoh et al., 2001) and IL-31 (Andoh et al., 2017a). PAR-2 activation and leukotriene B4 release participate also in dermatophyte-induced itch (Andoh et al., 2014). In addition to producing leukotriene B4 by themselves (Satpathy et al., 2015), human, and murine mast cells also express leukotriene B4 receptors BLT1 and BLT2 (Lundeen et al., 2006). On the contrary, prostaglandin D2, also produced by mast cells themselves (Murakami et al., 1995), decreases histamine release from mast cells and inhibits scratching in a mouse model (Hashimoto et al., 2005). Thus, it seems that mast cells release many mediators that also control their own function.

Neuropeptides and Mast Cell Activation

There are several neuropeptides released by the sensory neurons in the skin, which then activate mast cells. Mast cells degranulate in response to nerve growth factor (NGF) and this signaling acts through TrkA tyrosine receptor (Horigome et al., 1993). Interestingly, mast cells can secrete NGF also by themselves suggesting an autocrine or paracrine mechanism (Nilsson et al., 1997).

The potency of substance P in degranulating mast cells and causing itchy wheals was found already decades ago (Hägermark et al., 1978). The effects of substance P on mast cells are mediated either through neurokinin-1 receptor (NK-1R) or Mas-related G protein coupled receptor-X2 (MRGPRX2) (Kulka et al., 2008; Subramanian et al., 2016). In the initial phases of topical therapy with calcineurin inhibitors pimecrolimus and tacrolimus, pruritus and burning is often present. These compounds have been shown to release substance P and CGRP from primary afferent nerve endings in murine skin, leading to mast cell degranulation and thus release of pruritogenic histamine, and tryptase (Ständer et al., 2007a). Mast cells degranulate rapidly in response to substance P resulting in wheal reaction in the skin (Huttunen et al., 1996; Yamaoka and Kawana, 2007). The ability of substance P to induce histamine release seems to appear only at high concentrations (Weidner et al., 2000). In line with this, substance P decreases mast cell recruitment and degranulation when used as a topical treatment in a murine model of atopic dermatitis (Choi et al., 2018), pointing to a concentration-dependent dual role of substance P.

Interestingly, stimulation of human mast cells with substance P and an IL-1 family member, IL-33, increase the secretion of proinflammatory TNF and IL-1β and these responses are inhibited by a natural flavonoid, methoxyluteolin (Taracanova et al., 2017, 2018). A pure luteolin with Ashwagandha has been proposed as a relief to patients suffering from stress and inflammation-associated diseases (Theoharides and Kavalioti, 2018). This release of cytokines is potentially inhibited also by IL-37, another IL-1 family cytokine [reviewed in Caraffa et al. (2018), Tettamanti et al. (2018), Gallenga et al. (2019)]. The effect of substance P to release proinflammatory cytokines from mast cells points to a mechanism how mast cells participate in the neurogenic inflammation in psoriasis and atopic dermatitis as discussed below in more detail. Recently, Nakamura et al. (2019) reported increased levels of IL-33 in the stratum corneum of patients with atopic dermatitis. All these data emphasize the role of these cytokines in the context of neuroinflammation and itch, in which mast cells obviously participate.

Vasoactive intestinal peptide (VIP) is another potent neuropeptide to degranulate mast cells (Fjellner and Hägermark, 1981; Huttunen et al., 1996), a reaction mediated through VPAC2, and/or MRGPRX2 receptors on mast cells (Kulka et al., 2008; Subramanian et al., 2016). In addition, substance P and VIP have been found to stimulate human mast cells *in vitro*

conditions to release cytokines and chemokines, including TNF-α, GM-CSF, IL-3, CCL2, CCL5, CXCL8, CXCL9, and CXCL10 (Kulka et al., 2008).

MAST CELLS, SKIN DISEASES, AND ITCH

In a retrospective study conducted by Sommer et al. (2007), dermatological disease was the probable cause of the itch in 41,8 % of patients, while almost a similar number of cases (44,8 %) showed no apparent origin of the symptoms. The role of mast cells in the development of itch has been studied mostly in psoriasis, atopic dermatitis and urticaria. It is likely that the increased contacts between nerve fibers and mast cells often seen in these dermatoses constitute the morphological basis for itch chronicization during chronic skin inflammation. The current understanding of the role of mast cells and sensory nerves in itch in these and selected other pruritic dermatoses is discussed here next.

Mast Cells, Sensory Nerves, and Itch in Psoriasis

Psoriasis, a common chronic inflammatory and scaly skin disease, is characterized by pruritus that affects 60–90% of the patients and can appear in different forms, such as stinging, pinching, tickling, crawling, burning or pain sensations (Szepietowski and Reich, 2016). On the other hand, psychosocial stress can exacerbate psoriasis in 40–80% of the patients (Basavaraj et al., 2011). Thus, pruritus and stress may be reciprocally interconnected factors in psoriasis.

Emotional stress is associated with the activation of a variety of neuro-immune-endocrine systems. For example, the hypothalamic-pituitary-adrenal (HPA) axis is activated and stress hormones are released, including corticotroping-releasing hormone (CRH), adrenocorticotropic hormone, and glucocorticoids. Interestingly, human skin has its own functional peripheral equivalent of the HPA axis. Furthermore, numerous other factors are activated in stress, such as α-MSH, neuropeptides, neurotrophins and the sympathetic nervous system (Arck et al., 2006).

Several studies have previously shown that sensory nerve fibers and neuropeptides, including substance P, neurokinin A and VIP, are increased in the psoriatic lesion (Naukkarinen et al., 1989; Eedy et al., 1991; Amatya et al., 2011). Furthermore, the morphologic contacts between neurofilament$^+$ nerves and tryptase$^+$ mast cells are more frequent in psoriatic lesions than in non-lesional psoriatic skin or normal skin (Naukkarinen et al., 1991, 1993). Even the morphologic contacts between substance P$^+$ and CGRP$^+$ fibers and tryptase$^+$ mast cells, but not the contacts between VIP$^+$ fibers and tryptase$^+$ mast cells, have been found to be increased in the psoriatic lesion. Therefore, there is a morphologic basis for mast cell-neural interaction as well as neurogenic inflammation in psoriasis.

The increase in mast cell numbers, especially the MC_{TC}-type (tryptase$^+$ and chymase$^+$) of mast cell, in the psoriatic lesion has been known for decades. However, in contrast to the resistant tryptase, chymase is sensitive to the action of serum protease inhibitors, which may explain the finding that the enzyme activity of chymase is decreased in the psoriatic lesion (Harvima et al., 2008). The net outcome of the partial inactivation of chymase may be an uncontrolled and enhanced substance P-mediated neurogenic inflammation, as chymase degrades substance P and VIP, but tryptase degrades VIP and CGRP (Caughey et al., 1988; Franconi et al., 1989; Walls et al., 1992). Tryptase has the capability to cleave and activate the PAR-2 receptor. Therefore, the serine proteinase may not only activate the receptor on nerves and numerous proinflammatory cells but it may activate PAR-2 on mast cells themselves in a para- or autocrine fashion, as the percentage of tryptase$^+$ mast cells containing PAR-2 immunoreactivity is increased in the psoriatic lesion (Carvalho et al., 2010). Furthermore, the activation of PAR-2 sensitizes TRPV-1 leading to increased substance P and CGRP release (Amadesi et al., 2004) and TRPV-1 is expressed in substance P$^+$ fibers as well as in mast cells in human skin (Ständer et al., 2004).

In addition to neuropeptides, the emotional stress in psoriasis may transmit its signals to the skin through CRH and CRH-R1 receptor on mast cells as suggested previously (Harvima and Nilsson, 2012). This hypothesis is supported by a report that the immunostaining of CRH is increased in the epidermis, sweat glands, and hair follicles in the psoriatic lesion (Kim et al., 2007). In addition, we have analyzed the expression of CRH-R1 immunoreactivity in mast cells in psoriasis and found that the percentage of tryptase$^+$ mast cells expressing CRH-R1 is higher in the lesional than non-lesional skin of 8 psoriatic patients (Haimakainen S et al., unpublished results).

There are several studies that have investigated molecular differences in skin between pruritic and non-pruritic-type of psoriasis. For example, the pruritic-type of psoriasis is characterized by increased levels of substance P and nerve fibers; decreased levels substance P-degrading neutral endopeptidase; increased levels of NGF and/or its receptor TrkA (Nakamura et al., 2003; Chang et al., 2007; Amatya et al., 2011), decreased expression of semaphorin-3A (an axon-guidance molecule) (Taneda et al., 2011; Kou et al., 2012), and increased numbers of total and degranulated mast cells (Nakamura et al., 2003).

Mast Cells, Sensory Nerves, and Itch in Atopic Dermatitis

Atopic dermatitis (AD) is a well-known chronic eczematous skin disease characterized by distressing pruritus that can be exacerbated by inflammatory mediators, sensory nerves, skin dryness, heat, sweat, and emotional stress (Suarez et al., 2012; Murota and Katayama, 2017).

Like in the case of psoriasis, mast cells and sensory nerves have been suggested to play a role in neurogenic inflammation and itch in AD. Regarding mast cells, there are similarities between these diseases. In the lesional atopic dermatitis skin, the number of tryptase$^+$ mast cells is increased slightly, but the activity of chymase is decreased (Järvikallio et al., 1997; Ilves and Harvima, 2015). Like in psoriasis, the possible explanation for the partial inactivation of chymase in AD is the presence of chymase inhibitors in mast cells (Saarinen et al., 2001). The

reduced chymase activity means that the enzyme cannot degrade and control efficiently a variety of proinflammatory peptides and proteins, including IL-6, IL-13, TNF-α, IL-4, IL-5, substance P, and VIP (Franconi et al., 1989; Tunon de Lara et al., 1994; Zhao et al., 2005).

This is relevant for the inflammation, as the percentage of mast cells expressing TNF-α, IL-4, IL-6, and CD30 ligand is increased in the lesional AD skin (Horsmanheimo et al., 1994; Ackermann and Harvima, 1998; Fischer et al., 2006; Ilves and Harvima, 2015). In addition, the severity of itching and tryptase$^+$ and IL-6$^+$ mast cells correlate inversely with the (pro)filaggrin immunostaining in the epidermis of AD skin (Ilves et al., 2015).

Like in the case of psoriasis, there is an increased sensory nerve density in the epidermis and dermis in AD lesions, which suggests an enhanced neurogenic inflammation (Tobin et al., 1992; Urashima and Mihara, 1998; Järvikallio et al., 2003; Tominaga et al., 2009; Kubanov et al., 2015). Previously, it has been demonstrated that there is an increased expression of NGF and amphiregulin in the epidermis, but decreased expression of nerve repulsion factors, semaphorin-3A and anosmin-1, in AD lesions, which can explain the increased nerve density in AD (Tominaga et al., 2009; Tominaga and Takamori, 2014; Kubanov et al., 2015). In addition, another explanation for the increased nerve density is the finding that the expression of NGF is elevated in mast cells in AD lesions, and mast cells can express the receptor of NGF, i.e., TrkA (Nilsson et al., 1997; Xiang and Nilsson, 2000; Groneberg et al., 2005). There are also other regulatory mechanisms between NGF and tryptase, as NGF treatment of mast cells *in vitro* can increase the expression of TrkA as well as the contents of tryptase and histamine (Groneberg et al., 2005), and tryptase can generate mature NGF from pro-NGF (Spinnler et al., 2011). These molecular interactions can explain, at least in part, the finding that the morphologic contacts between tryptase$^+$ mast cells and neurofilament$^+$ fibers are increased in AD lesions (Järvikallio et al., 2003).

Acute psychologic stress can result in marked changes in nerves, neuropeptides and mast cells. For example, the exposure of AD patients to acute Trier social stress test (TSST) associated with decreased NGF$^+$ and PGP9.5$^+$ fibers and decreased contacts between PGP9.5$^+$ fibers and tryptase$^+$ mast cells in AD lesions. However, in the non-lesional AD skin, these parameters increased, rather than decreased. Moreover, a positive correlation was observed between itch and mast cell-nerve fiber contacts in the non-lesional AD skin after TSST or lesional AD skin before TSST (Peters et al., 2014).

The classic histaminergic itch is mediated via H1-receptors on cutaneous sensory nerves, though H4-receptors on nerves can also be involved in pruritus (Ohsawa and Hirasawa, 2014). However, antihistamines usually are not effective in relieving distressing pruritus in chronic skin inflammation. The reason is the non-histaminergic itch that can be mediated through other distinct pathways. The PAR-2 activation on sensory fibers is one of them, as PAR-2$^+$ fibers are increased in AD lesions. Therefore, tryptase released from mast cells can activate PAR-2 on nerve fibers with subsequent release of substance P and CGRP and potentiation of neurogenic inflammation (Steinhoff et al., 2003; Kempkes et al., 2014). Interestingly, lichenified lesions of atopic dermatitis showed no staining for substance P or VIP nor degranulated mast cells, suggesting that other factors may contribute to pruritus in chronic, lichenified lesions (Urashima and Mihara, 1998).

Interleukin-31 can represent another pathway for non-histaminergic itch in AD. This cytokine is derived from Th2 cells and mast cells. In the psoriatic and AD lesions, mast cells show increased levels of IL-31 immunoreactivity compared to normal skin (Niyonsaba et al., 2010). The expressions of the mRNA of IL-31 and its receptor IL-31RA are increased in AD, but not psoriatic, lesions. Furthermore, the receptor is located in small-diameter neurons of human dorsal root ganglia (Sonkoly et al., 2006; Cevikbas et al., 2014).

Interleukin-31 may not only induce slowly developing itch (Hawro et al., 2014), but it may also increase the elongation, branching and density of nerves in AD lesions (Feld et al., 2016).

Mast Cells, Sensory Nerves, and Itch in Urticaria

Urticaria is a fundamentally mast cell-driven disease, in which pruritus is a key symptom in addition to wheals and/or angioedema (Zuberbier et al., 2018). In urticaria, it is the degranulation of mast cells and release of their mediators that cause the symptoms by activating sensory nerves and causing vasodilatation and plasma extravasation (Zuberbier et al., 2018). Although the details of mast cell activation in urticaria are not known yet, mast cells may be activated in urticaria by allergens/autoallergens or IgG antibodies against IgE or its receptor. In patients with chronic urticaria, more than 200 autoantigens reacting with IgE have been detected and of these, IL-24 is a common and functional autoantigen recognized by IgE antibodies (Schmetzer et al., 2018).

Interestingly, also a plethora of other agents are known to prime mast cells for further activation [reviewed in Church et al. (2018)]. Recently, a mouse model showed that Mrgprb2, the ortholog of the human MRGPRX2, and might be a mast cell-specific key receptor in pseudoallergic reactions (McNeil et al., 2015). This receptor is upregulated in the skin of patients with severe chronic urticaria (Fujisawa et al., 2014).

Mast Cells, Sensory Nerves, and Itch in Selected Other Dermatoses

Prurigo nodularis (PN) is a typical example of an itchy dermatosis. The close contact of mast cells and nerve fibers in PN lesions was reported already 20 years ago by Liang et al. (1998). They also noticed that there are increased mast cell numbers in the lesions. There are several other pruritic skin diseases, in which the changes in mast cell numbers have been investigated and they definitely participate in the pathogenesis, although the exact details of their role in pruritus in these dermatoses is not clear yet. Mast cell numbers are increased already in the perilesional skin in patients with hidradenitis suppurativa (HS) and the numbers are further elevated in early and chronic lesions (van der Zee et al., 2012). In line with this finding, about 60 % of patients with HS report moderate or severe pruritus (Matusiak et al., 2018). Similarly, more than one third of patients

with basal cell carcinoma or squamous cell carcinoma report to have pruritus (Mills et al., 2012; Yosipovitch et al., 2014) and elevated mast cell numbers in both tumors have been reported (Diaconu et al., 2007; Haimakainen et al., 2017). The connection between mast cell numbers and itch is not so clear in melanoma, in which less patients report itch (Yosipovitch et al., 2014) and both increased (Toth-Jakatics et al., 2000; Ribatti et al., 2003a,b), and decreased (Biswas et al., 2014; Siiskonen et al., 2015; Rajabi et al., 2017) mast cell numbers have been reported. However, since the exact functions of mast cells in skin cancers are not known at the moment, also their role in itch remains unclear.

Animal Models in Research on Mast Cells and Itch

The growing research performed in experimental animals has revealed that the molecular mechanisms of pruritus as well as the ligands and receptors involved in itch induction are more complex than thought, and it is beyond the scope if this review to describe them all.

Animal models to study pruritus have been mainly developed for AD. Subcutaneous capsaicin injections to neonatal rats causes long-lasting, pruritic skin changes with histopathological findings resembling AD, including increased mast cell number (Back et al., 2012). The Kyoto Fancy Rat Stock 4 is another rat model of AD showing spontaneous itchy dermatitis with increased transepidermal water loss and mast cell numbers (Kuramoto et al., 2015). The bile duct ligation rat model of liver disease has provided a good insight to neuroinflammation and itch. In these rats, PAR-2 receptors are activated and this potentiates the TRPV-1 channels (Belghiti et al., 2013). As discussed above, mast cell tryptase may activate PAR-2, pointing to a role for mast cells also in hepatic pruritus. Elevated tryptase and PAR-2 levels are also found in a mouse model with ovalbumin-induced AD-like dermatitis (Zhu et al., 2015). Mast cells and sensory nerves can be found in apparent morphologic contact in the skin. For example, in a mouse model of hapten-induced AD, mast cells in the lesional skin express high levels of cell adhesion molecule-1 (CADM-1), a molecule that was found to enhance adhesion and communication between sensory nerves and mast cells *in vitro* (Hagiyama et al., 2013). Human mast cells have also been found to express CADM-1 that probably interacts with nectin-3 on nerves (Furuno et al., 2012; Moiseeva et al., 2013).

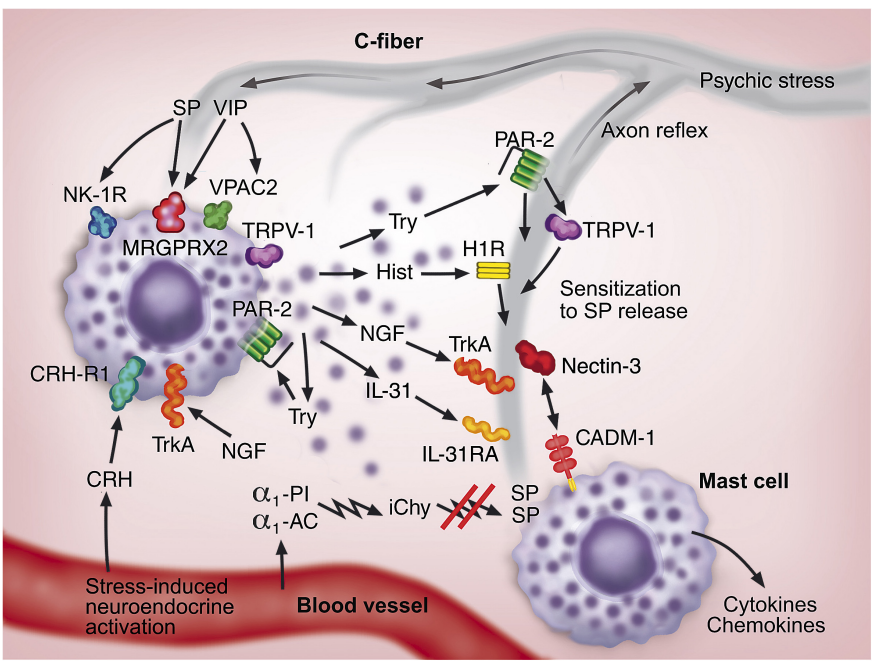

FIGURE 1 | A hypothetical model for the communication between mast cells (two mast cells shown in purple) and sensory nerves (shown in gray) in neurogenic inflammation in the skin. The neuroendocrine and neural systems are activated as a consequence of psychosocial stress. The signals traveling through C-fibers lead to the release of neuropeptides, substance P (SP) and vasoactive intestinal peptide (VIP), from C-fiber endings. The increased cutaneous blood flow conveys corticotropin-releasing hormone (CRH) to the developing inflammation. These neuroendocrine factors activate mast cells through the receptors NK-1R, VPAC2, MRGPRX2, and CRH-R1. Tryptase (Try), histamine (Hist), NGF, IL-31 released from mast cells activate their corresponding receptors PAR-2, H1R, TrkA, and IL-31RA, respectively, on C-fibers. Furthermore, mast cells themselves are activated through PAR-2 and TrkA in an auto- or paracrine fashion. Mast cell-derived mediators activate C-fibers leading to the spread of signal, which can also take place through an axon reflex-related mechanism. NGF and IL-31 support the growth of C-fibers. Chymase released from mast cells is susceptible to the inactivation by serum protease inhibitors, α_1-proteinase inhibitor (α_1-PI) and α_1-antichymotrypsin (α_1-AC), resulting in inactivated chymase (iChy) that cannot degrade and inactivate SP. PAR-2 can sensitize the capsaicin receptor (TRPV-1) in C-fibers enhancing SP and CGRP release. The intimate functional and morphologic communications between mast cells and C-fibers are further strengthened by the cell adhesion molecule-1 (CADM-1) on mast cells and nectin-3 on C-fibers. As a consequence of these multiple interactions, a feedforward loop is developed, which leads to increase in mast cells and C-fibers, development of vicious circle, and potentiation of neurogenic inflammation and itch.

The interaction between nerve fibers and mast cells can be affected by molecules leading to decreased itch and inflammation. In the NC/Nga mouse model of AD, dorsal skin lesions develop spontaneously. The treatment of these lesions with intracutaneous injections of semaphorin-3A, a nerve repulsion factor, for 5 days followed by biopsing on day 11 (Yamaguchi et al., 2008) revealed a significant reduction in the clinical skin score and scratching behavior, decrease in PGP9.5$^+$ nerve fibers in the epidermis, decrease in mast cell numbers, infiltrating CD4$^+$ T cells, IL-4 production, and epidermal thickness (Yamaguchi et al., 2008). In addition, oral administration of chymase inhibitor has been shown to ameliorate dermatitis (Watanabe et al., 2007) as well as scratching behavior in these NC/Nga mice (Terakawa et al., 2008).

In pruritus, the neuroendocrine signals may travel from the central nervous system to periphery and vice versa. MacQueen et al. (1989) showed in 80's that rats exposed to both audiovisual cue and antigen injection were conditioned to mast cell protease II release after reexposure to the audiovisual cue only. A sonic stress for 24 h in mice can induce degranulation of mast cells, changes in endothelium, increase in substance P$^+$ fibers and their contacts with mast cells, and increase in the expression of NGF in skin mast cells (Peters et al., 2005, 2011; Pavlovic et al., 2008). Furthermore, skin mast cells in rats can be activated by stress induced by immobilization (Singh et al., 1999). In summary, there is compelling *in vivo*-evidence that show activation of mast cells induced by stress.

In an AD model induced by IL-13 in mice, the scratching behavior evoked by itch associated with increase in PGP9.5$^+$, CGRP$^+$, and TRPA-1$^+$ (transient receptor potential ankyrin (1) nerves, mast cells, and particularly in TRPA-1$^+$ mast cells. However, there was no increase in TRPV-1 in inflamed skin. In line with this, TRPA-1$^+$ mast cells and nerves and the contacts between them were increased in the lesional skin of patients with AD (Oh et al., 2013). Therefore, this study emphasizes the role of TRPA-1 in itch associated with skin inflammation. In contrast to this, itch evoked by intradermal injections of β2-microglobulin in mouse skin is related, at least in part, to TRPV-1$^+$ primary sensory nerves (Andoh et al., 2017b). Furthermore, itch evoked by IL-31 injections in mice is related to both TRPV-1 and TRPA-1 using knockout mouse models (Cevikbas et al., 2014). In addition, itch induced in mice by lysophosphatidic acid-injections is related to both cation channels (Kittaka et al., 2017).

CONCLUSION AND FUTURE CHALLENGES

Despite thorough and extensive research on mast cells during the past decades, these cells still remain to be an intriguing, complex cell type present in our body-environment interfaces. Although mast cells are physiologically meant to stay alert and to react to potentially noxious agents and conditions, their reactions may turn harmful. In several disorders, their functions seem to increase itch, which is not always beneficial to the host.

The complex functional and morphologic interaction between mast cells and sensory nerves is summarized and illustrated in **Figure 1**. Psychic stress can aggravate itch in several skin diseases. Although the mechanism is not clear in detail, the activation of the systemic HPA axis and/or its equivalent in the skin as well as the activation of neural response can play a role in activating the neurogenic inflammation in the skin. As a consequence, several neuroendocrine mediators, including CRH, substance P and NGF, are released to the circulation and/or are produced locally in the skin. These mediators lead to activation of mast cells and release of their proinflammatory mediators that modify the inflammation of the skin disorder, often increasing itch. The mechanisms are complex owing to the intimate reciprocal communication between mast cells and sensory nerves resulting in increase in mast cell numbers and nerve fibers, development of a vicious circle, and exacerbation of neurogenic inflammation and pruritus.

Future research should aim at further elucidating the details of mast cell biology in different physiological and pathological skin conditions in humans. Here, it will be important to focus on the association of psychic stress with mast cell-neural functions in patients with psoriasis or atopic dermatitis under standardized stress conditions. We should also aim at more in-depth understanding of mast cell heterogeneity and recruitment to the skin and how these may affect mast cell-neural responses in chronic pruritic disorders. In addition, the inhibitors of mast cell mediators or drugs preventing mast cell activation (Harvima et al., 2014) may provide new therapeutic options to treat distressing itch and thereby improve the quality of life of patients.

AUTHOR CONTRIBUTIONS

Both authors wrote and edited the text.

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