

# Suppression of Brain Mast Cells Degranulation Inhibits Microglial Activation and Central Nervous System Inflammation

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**Abstract** Brain inflammation has a critical role in the pathophysiology of brain diseases. Microglia, the resident immune cells in the brain, play an important role in brain inflammation, while brain mast cells are the “first responder” in the injury rather than microglia. Functional aspects of mast cell-microglia interactions remain poorly understood. Our results demonstrated that site-directed injection of the “mast cell degranulator” compound 48/80 (C48/80) in the hypothalamus induced mast cell degranulation, microglial activation, and inflammatory factor production, which initiated the acute brain inflammatory response. “Mast cell stabilizer” disodium cromoglycate (cromolyn) inhibited this effect, including decrease of inflammatory cytokines, reduced microglial activation, inhibition of MAPK and AKT pathways, and repression of protein expression of histamine receptor 1 (H<sub>1</sub>R), histamine receptor 4 (H<sub>4</sub>R), protease-activated receptor 2 (PAR2), and toll-like receptor 4 (TLR4) in microglia. We also demonstrated that C48/80 had no effect on microglial activation in mast cell-deficient Kit<sup>W-sh/W-sh</sup> mice. These results implicate that activated brain mast cells trigger microglial activation and stabilization of mast cell inhibits microglial activation-induced central nervous system (CNS) inflammation.

Interactions between mast cells and microglia could constitute a new and unique therapeutic target for CNS immune inflammation-related diseases.

**Keywords** Mast cell degranulation · Microglial activation · CNS immune inflammation · Compound 48/80 · Cromolyn

## Introduction

The initiation and propagation of neuroinflammation appear to rely very much on the interaction between glia, immune cells, and neurons, although the glia-immune cell connection remained to be fully explored. Microglia, the resident immune cells in the brain, play a pivotal role in immune surveillance of the central nervous system (CNS). Consequently, these cells are likely to play an important role in either development of protective immune responses or progression of damaging inflammation during CNS disease states. Pathological states within the nervous system, including injury, ischemic stroke [1], and infection [2], can lead to microglial activation and production of a host of factors, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), prostaglandin E2 (PGE2), interleukin-6 (IL-6), nitric oxide (NO), and reactive oxygen species (ROS). Accumulation of these proinflammatory and cytotoxic factors is deleterious directly to neurons and subsequently induces further activation of microglia, resulting in a vicious cycle [3, 4]. Thus, inhibition of microglial activation and subsequent inflammatory process may identify novel therapeutic strategies to eliminate microglia deleterious effects [5].

Besides releasing proinflammatory mediators, microglia also respond to proinflammatory signals released from other nonneuronal cells of immune origin. Mast cells represent a potentially important and underappreciated peripheral immune signaling link to the brain in an inflammatory setting

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[6]. Mast cells, best known for their role in allergic inflammation, are distributed in a variety of anatomical sites, including the CNS, where they are often found adjacent to blood vessels and nerves [7]. Mast cells are located perivascularly close to neurons and microglia and may contribute to brain inflammation through different mechanisms [8]. Mast cells can move through the blood–brain barrier of normal brain [9] but may also traverse the blood–spinal cord barrier and blood–brain barrier when compromised by disease. Theoharides et al. have suggested that perinatal mast cell activation by infectious, stress-related, environmental or allergic triggers can lead to release of proinflammatory and neurotoxic molecules, therefore contributing to brain inflammation and autism spectrum disorder pathogenesis, at least in a subgroup of these patients [10]. These reactions are mediated by degranulation, whereby a number of preformed or newly synthesized mediators and cytokines are secreted by activated mast cells, such as GnRH [11], monoamines [12], specific proteases, cytokines, and histamine [13]. These neurotransmitters and neuromodulators can initiate and modulate several inflammatory pathways implicated in neuroinflammatory diseases affected by stress [14]. Notably, mast cell mediators can recruit and activate T cells as well as permit them to enter the brain by disrupting the BBB [15].

Several molecular mechanisms for potential interactions between mast cells and microglia have been determined *in vitro* [16]. However, the effect of mast cell on microglia is still unclear. Yuan reported that mast cell activation can induce microglia to release neurotrophin [17]. We have previously reported that tryptase and histamine can induce microglial activation and inflammatory mediator release [18, 19], which also suggests the importance of mast cells in induction of inflammation in the CNS. However, the direct effect of mast cell on microglial activation has not been reported. In this study, mast cell degranulator and stabilizer, mast cell-deficient *Kit<sup>W-sh/W-sh</sup>* mice were used to investigate the effect of mast cells on microglial activation and CNS immune inflammation.

## Materials and Methods

### Animals

Male Sprague Dawley (about 250 g) rats, mast cell-deficient *Kit<sup>W-sh/W-sh</sup>* mice (STOCK *Kit<sup>W-sh</sup>/HNihrJaeBsmJNju*, 6 months old), and littermate controls were purchased from Mode Animal Research Center of Nanjing University (Nanjing, China). All animals were housed in groups of five animals per cage under standard laboratory conditions with free access to food and water, constant room temperature of 22 °C, 50–60 % humidity, a 12:12-day–night cycle, etc. All experiments were carried out according to the National

Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and the Guidelines for the Care and Use of Animals in Neuroscience Research by the Society for Neuroscience and approved by Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC).

### Reagents

Compound 48/80 (C48/80), disodium cromoglycate (cromolyn), and toluidine blue were purchased from Sigma (St. Louis, MO, USA). Anti-Mast Cell Tryptase antibody (AA1) and fluoroshield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) were purchased from Abcam (Hong Kong, China). Specific mouse anti-Iba1 antibody was purchased from AbD Serotec (Raleigh, NC, USA). Specific rabbit monoclonal antibodies against p38, Phospho-p38, Jun N-terminal kinase (JNK), Phospho-JNK, extracellular signal-regulated kinases (ERK), Phospho-ERK, AKT, Phospho-AKT, and goat anti-rabbit secondary antibody were obtained from Cell Signaling (Beverly, MA, USA). Specific rabbit monoclonal anti-H<sub>3</sub> receptor antibody was purchased from Abcam (Hong Kong, China). Specific rabbit polyclonal anti-H<sub>1</sub> receptor and rabbit polyclonal anti-H<sub>2</sub> receptor antibodies were purchased from Alomone Labs Ltd. (Israel), and rabbit polyclonal anti-H<sub>4</sub> receptor was purchased from Santa Cruz (CA, USA). Phycoerythrin (PE)-conjugated mouse anti-rat OX-42 monoclonal antibody and isotype control and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody were purchased from BD (BD Biosciences, USA). Rat IL-6 Immunoassay Kit and Rat TNF- $\alpha$  Immunoassay Kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

### Surgery and Drug Administration

The rats were randomly allocated to six groups (group A–F) with 12 rats in each group, and investigators were blinded to the experimental treatment. Rats of groups C–F were pretreated with mast cell stabilizer cromolyn (100 or 200  $\mu$ g/ $\mu$ l) for 30 min, simultaneously rats of groups A and B pretreated with sterile saline. After 30 min, rats of groups B–D were injected with C48/80 (1  $\mu$ g/ $\mu$ l) in the hypothalamus and rats of groups A (control rats), E, and F were injected with sterile saline for another 30 min.

Following anesthesia, rats were placed in the stereotaxic apparatus (Stoelting Instruments, USA). Guide cannulas (Plastic One) were inserted into the right hypothalamus as follows: 1.80 mm lateral and 1.90 mm posterior from Bregma, at a 10° angle and 8 mm deep [20]. The animals were allowed to recover in the animal facility for 14 days before use. Animals were handled daily to check the guide cannula and to familiarize them with the investigators. To determine

whether mast cell degranulator C48/80 can activate microglia, we administered C48/80 centrally by an ipsilateral site injection in the hypothalamus. We chose to inject the hypothalamus because mast cells are plentiful in this area. Animals with implanted guide cannulas received 2.5 of 1  $\mu\text{g}/\mu\text{l}$  C48/80 (2.5  $\mu\text{g}$ ) or 2.5  $\mu\text{l}$  0.9 % NaCl directly in the hypothalamus. Animals remained in their cages for 30 min or 24 h and were not restrained. To stabilize mast cells, the same site was pretreated with 1  $\mu\text{l}$  of cromolyn (100 or 200  $\mu\text{g}/\mu\text{l}$ ) 30 min before C48/80 administration. Control animals were pretreated with 1  $\mu\text{l}$  0.9 % NaCl. Rats were killed after drug administration, and their brains, followed by the separation of meninges from which, were used for morphological ( $n=6$ ) and biochemical ( $n=6$ ) analyses.

The mice were divided into four treatment groups. Of the wild-type (WT) mice groups, one group received C48/80 administration ( $n=5$ ) and a control group received saline ( $n=5$ ). Similarly with the Kit<sup>W-sh/W-sh</sup> mice, one group received C48/80 administration ( $n=5$ ) while the other group received saline ( $n=5$ ).

Following anesthesia, mice were mounted in the stereotaxic frame and kept at 37 °C using a heating pad. A burrhole was made to inject into the right hypothalamus as follows: 0.96 mm lateral and 0.82 mm posterior from Bregma, at a 10° angle and 4.82 mm deep [21]. A 33-gauge needle connected to a 10- $\mu\text{l}$  syringe was then lowered to 4.82 mm, and either C48/80 (1  $\mu\text{g}/\mu\text{l}$ , 0.8  $\mu\text{g}$ ) or saline (0.8  $\mu\text{l}$ ) was injected. The needle was then left in place for 5 min before being removed to suture the skin. Mice were killed after drug administration, and their brains, followed by the separation of meninges from which, were used for morphological ( $n=5$ ) analyses.

### Mast Cell Staining and Counting

Slides were stained in 0.05 % toluidine blue for thalamic sections. A 1 % stock solution in 70 % ethanol was dissolved in 0.5 % NaCl solution (pH 2.2–2.3), and the slides were immersed for 30 min in the above concentration. They were then washed twice in distilled water, dehydrated in a series of increasing concentrations of ethanol, followed by placement in butyl acetate ester. Samples were coverslipped using Eukitt® mounting medium and were allowed to dry overnight.

Mast cell counting was always performed in a blinded fashion by an experimenter that was unaware of the sample identity. Criteria for degranulation included loss of purple staining, fuzzy appearance, distorted shape, or multiple granules visible in the vicinity of the cell. For thalamic sections, the entire surface area of the ipsilateral and contralateral thalamus was scanned manually using a light microscope at  $\times 200$  magnification and mast cells were calculated with the help of the Cell D software (Olympus).

### Immunocytochemistry

Rats and mice were anesthetized by chloral hydrate and perfused first with 0.9 % saline and then with cold 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were dissected out and maintained in 4 % paraformaldehyde overnight. Brains were cryopreserved in 30 % sucrose in phosphate-buffered saline (PBS) and then stored at  $-70$  °C until used. Free-floating sections encompassing the entire brain were prepared using a cryostat. Sections were processed for Iba1 and mast cell tryptase immunohistochemistry as follows. After incubation for 1 h in 10 % bovine serum albumin with 0.3 % Triton X-100 in 0.01 M PBS, tissue sections (30  $\mu\text{m}$ ) were incubated with primary antibodies overnight at 4 °C. Primary antibodies used in this study were as follows: rabbit anti-Iba1 (1:200; Wako Chemicals USA, Inc.) and mouse anti-Mast Cell Tryptase (1:100; abcam, USA). Immunostaining was visualized by using 3,3'-diaminobenzidine. Sections were then counterstained with hematoxylin.

### Flow Cytometry Analysis

To determine the receptor expression affected by C48/80 in microglia, the ipsilateral hypothalamus tissues were minced with sterile scissors and digested with 0.25 % Trypsin–EDTA solution for 5 min at 37 °C. Trypsinization was stopped by adding an equal volume of culture medium. The dissociated cells were passed through a 100- $\mu\text{m}$ -pore mesh, pelleted at 200 g for 10 min at 4 °C, and resuspended and fixed in 4 % paraformaldehyde for 30 min. After washing, the cells were incubated with rabbit anti-H<sub>1</sub>R, anti-H<sub>2</sub>R, anti-H<sub>3</sub>R, anti-H<sub>4</sub>R, anti-PAR2, and anti-toll-like receptor 4 (TLR4) antibodies or normal rabbit IgG, respectively, overnight at 4 °C, followed by 1  $\mu\text{g}/\text{ml}$  of FITC-conjugated goat anti-rabbit secondary antibody alone with PE-conjugated mouse anti-rat OX-42 monoclonal antibody or isotype control (1:200) at 37 °C for 1 h. Cells were finally resuspended in PBS and analyzed on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, USA).

### TNF- $\alpha$ and IL-6 Assay

The content of TNF- $\alpha$  and IL-6 in rat brain tissue extracts was measured with a commercial ELISA kit from R&D Systems.

### Western Blotting

Ipsilateral hypothalamus tissue extracts were collected and homogenized in 200 ml of lysis buffer. After incubation for 20 min on ice, cell lysate was centrifuged and protein concentration in the extracts was determined by the Bradford assay. Proteins (50  $\mu\text{g}$ ) in cell extracts were denatured with sodium dodecyl sulfate (SDS) sample buffer and separated by 10 %

SDS–polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes (Millipore) by using a Bio-Rad miniprotein-III wet transfer unit. The membranes were incubated with 5 % BSA dissolved in Tris-buffered saline with Tween 20 (TBST) (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl, and 0.1 % Tween 20) at room temperature for 1 h. This was followed by incubating the membranes with different antibodies overnight at 4 °C. The following primary antibodies were used: rabbit monoclonal anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-ERK, phospho-ERK, anti-AKT, and anti-phospho-AKT (1:1000). After adding the goat-anti-rabbit secondary antibody (1:1000) for 1 h, the protein bands on the membranes were detected with an enhanced chemiluminescence kit.

### Statistical Analysis

All values are means±SEM. The significance of the difference between control and samples treated with various drugs was determined by one-way ANOVA followed by the post hoc least significant difference test. Differences were considered significant at  $P < 0.05$ .

## Results

### Cromolyn Repressed C48/80-Induced Mast Cell Activation in Hypothalamus

At first, we investigated the activated brain mast cells induced by C48/80. Brain mast cells were quantified in toluidine blue (TB) and mast cell tryptase-stained sections (Fig. 1a, b). As shown in Fig. 1c, directed site injection of 2.5 µl of 1 µg/µl C48/80 (2.5 µg), which is a mast cell degranulator, in the right hypothalamus for 30 min significantly induced activated mast cell number which increased in both the ipsilateral and contralateral hypothalamus and which was significantly greater than those of the control group. Mast cell stabilizer cromolyn (100 or 200 µg) repressed the mast cell activation induced by C48/80.

### Stabilization of Mast Cell Inhibited Microglial Activation in Hypothalamus

In order to explore the effects of activated mast cells on microglial activation, immunostaining was used to detect Iba1, marker for microglia. C48/80 could induce notable microglial activation in both the ipsilateral and contralateral hypothalamus, indicated by a large number of Iba1-ir cells, which had larger cell body and poorly ramified short and thick processes. Cromolyn (100 or 200 µg) pretreatment resulted significantly in suppression of microglial activation in both the ipsilateral and contralateral hypothalamus, indicated by

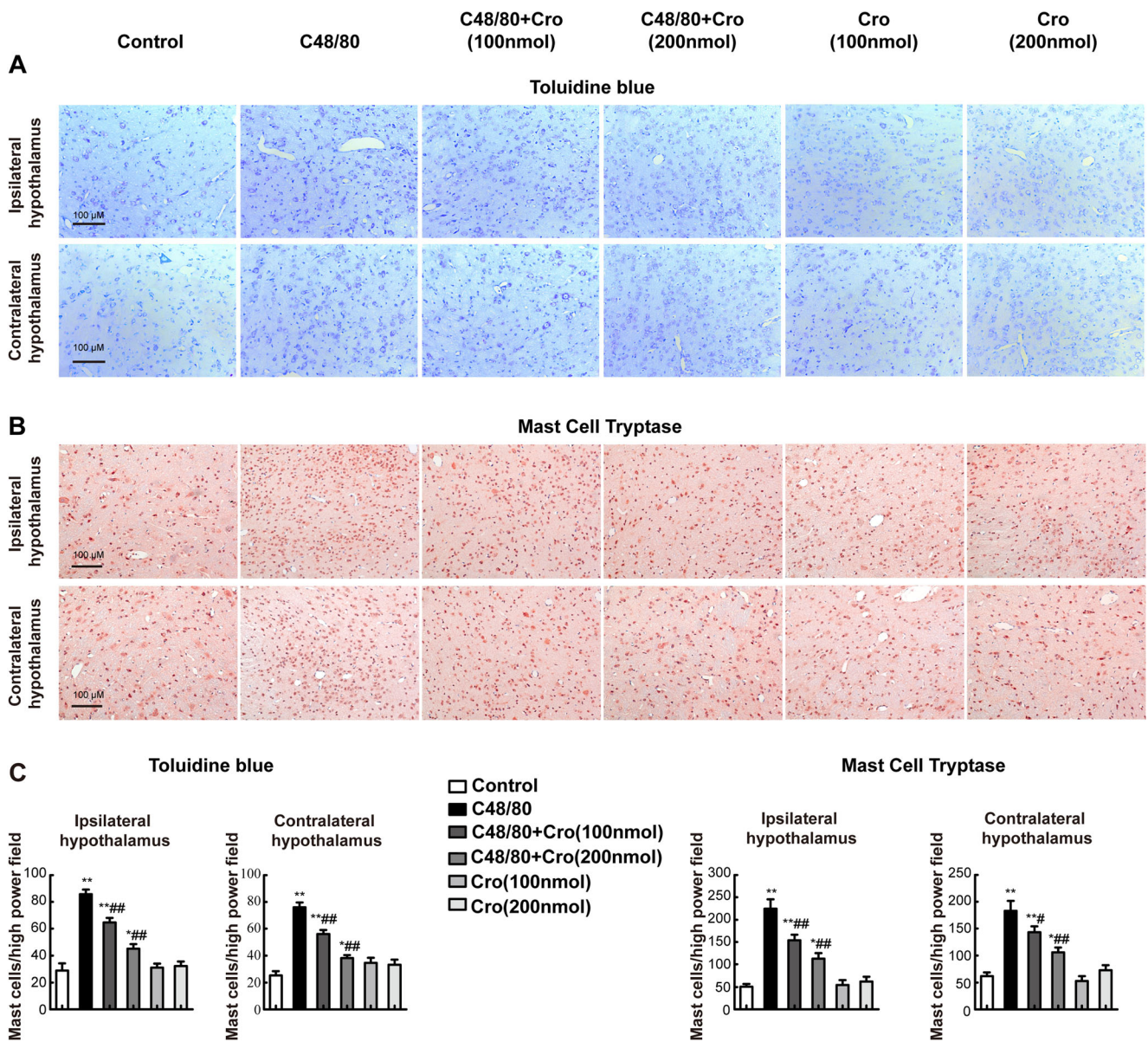
few Iba1-ir cell numbers, small cell body, and ramified and thin processes. Moreover, site injection of cromolyn (100 or 200 µg) alone had no effect on microglial activation in the brain (Fig. 2). However, site injection of C48/80 in the right hypothalamus had no significant effect on the mast cell and microglial activation in both ipsilateral and contralateral cerebral cortices (data not shown). These results suggest that stabilization of mast cells can inhibit microglial activation in the hypothalamus which does not propagate to the cerebral cortex within the analyzed time period.

### C48/80 Had No Effect on Microglial Activation in Hypothalamus of Kit<sup>W-sh/W-sh</sup> Mice

To further confirm whether activated mast cells can induce microglial activation, we used the mast cell-deficient Kit<sup>W-sh/W-sh</sup> mice. In Fig. 3a, TB- and mast cell tryptase-stained cells in hypothalamus were found in WT mice, but not in Kit<sup>W-sh/W-sh</sup> mice, indicating that TB and tryptase staining were specific to mast cells. As shown in Fig. 3b, c, directed site injection of 0.8 µl of 1 µg/µl C48/80 (0.8 µg) in the hypothalamus for 30 min induced notable microglial activation in both the ipsilateral and contralateral hypothalamus of the WT mice but had no effect on the microglial activation in Kit<sup>W-sh/W-sh</sup> mice. These results suggest that C48/80 induces microglial activation through activating mast cells.

### Stabilization of Mast Cell Inhibited C48/80-Induced TNF-α and IL-6 Production

Since microglia-mediated neuroinflammation is mainly due to the excessive proinflammatory factors from activated microglia and their downstream signaling cascades, the levels of proinflammatory factors TNF-α and IL-6 were detected. The results showed that injection of C48/80 (2.5 µg) in hypothalamus for 30 min or 24 h significantly increased TNF-α content in ipsilateral hypothalamus (756 and 847 pg/ml) and contralateral hypothalamus (372 and 554 pg/ml). However, injection of C48/80 in hypothalamus had no effect on the TNF-α content in the ipsilateral and contralateral cerebral cortices. Furthermore, cromolyn (100 or 200 µg) could attenuate the content of TNF-α in the ipsilateral and contralateral hypothalamus (Fig. 4a). The same as the TNF-α, cromolyn also inhibited C48/80-induced IL-6 production in the ipsilateral and contralateral hypothalamus but had no effect on the IL-6 content in the ipsilateral and contralateral cerebral cortices (Fig. 4b). However, site injection of cromolyn (100 or 200 µg) in hypothalamus for 30 min or 24 h alone had no significant effect on the content of TNF-α or IL-6 in the hypothalamus and cerebral cortex. These results suggest



**Fig. 1** Cromolyn repressed C48/80-induced mast cell activation in both the ipsilateral and contralateral hypothalamus. **a** Brain mast cells were stained with toluidine blue (TB). *Scale bar*: 100  $\mu$ m. **b** Immunostaining was used to detect mast cell tryptase. *Scale bar*: 100  $\mu$ m. **c** Quantification

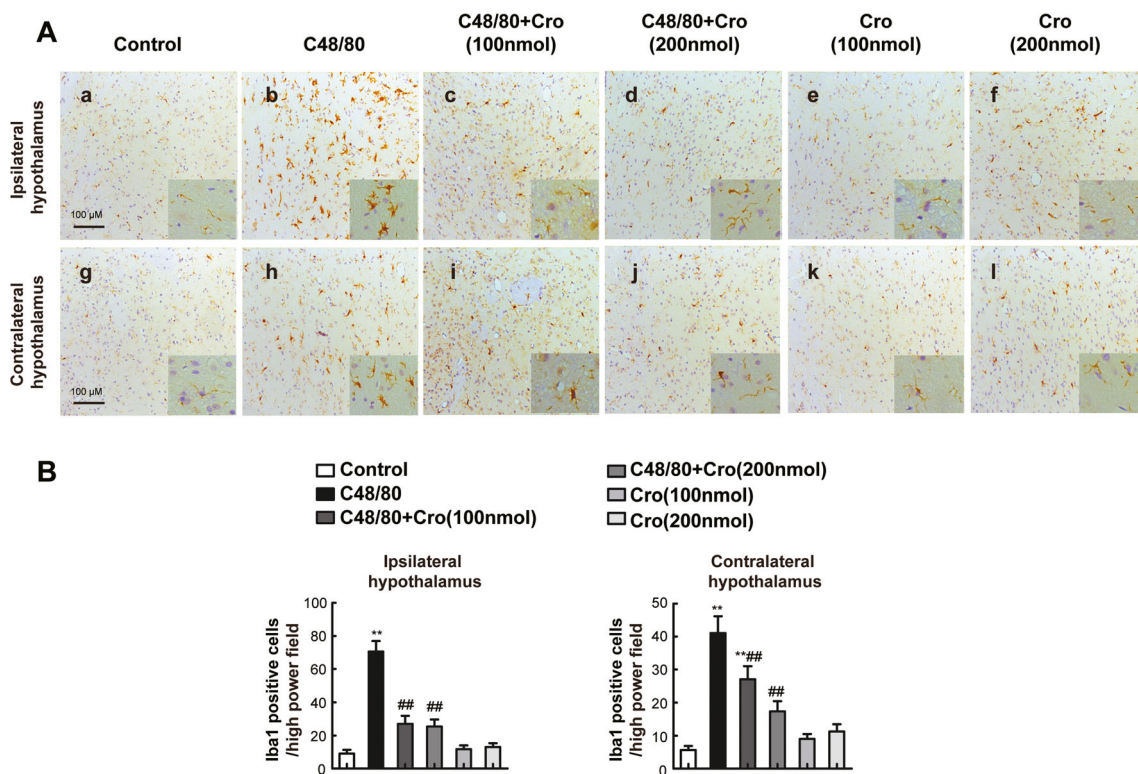
of activated mast cells in the ipsilateral and contralateral hypothalamus. \* $P < 0.05$ , \*\* $P < 0.01$  versus control group. # $P < 0.05$ , ### $P < 0.01$  versus C48/80 group. Data are presented as the mean  $\pm$  SEM ( $n = 6$ )

that stabilization of mast cells can inhibit the production of proinflammatory factors.

### Stabilization of Mast Cell Depressed MAPK and PI3K/AKT Activation in Hypothalamus

Mitogen-activated protein kinases (MAPK) are the predominant signaling transduction pathways responsible for the synthesis and production of proinflammatory mediators from microglia [22, 23]. Phosphorylation of AKT is a downstream target of PI3K activation and therefore is a proxy for activation of the PI3K pathway [24]. So, we

investigated whether C48/80 could affect MAPK and AKT phosphorylation, which might in turn regulate the production of proinflammatory mediators. As shown in Fig. 5, site injection of C48/80 (2.5  $\mu$ g) in hypothalamus for 30 min evoked the phosphorylation of p38, JNK, ERK, and AKT, indicative of p38, JNK, ERK, and AKT activation. However, pretreatment of cromolyn (100 or 200  $\mu$ g) for 30 min before C48/80 administration partially suppressed the effect of C48/80 on the phosphorylation of MAPK and AKT. These results suggest that cromolyn can inhibit mast cell degranulator C48/80-induced MAPK and AKT signaling pathway activation.



**Fig. 2** Stabilization of mast cell inhibited microglial activation in hypothalamus. **a** Immunostaining was used to detect Iba1, markers for microglia. Rats treated with vehicle showed minimal microglial activation in both the ipsilateral and contralateral hypothalamus (**a, g**) indicated by few Iba1-ir cell number and small cell body with ramified and thin processes. In C48/80 (2.5  $\mu$ g)-treated rats, there were numerous Iba1-ir cells in both the ipsilateral and contralateral hypothalamus (**b, h**),

which had larger cell body and poorly ramified short and thick processes. Rats pretreated with cromolyn (100 or 200  $\mu$ g) reduced the number of C48/80-induced Iba1-positive cells. *Scale bar*: 100  $\mu$ m. **b** Quantification of Iba1-positive cells in the ipsilateral and contralateral hypothalamus. \* $P < 0.05$ , \*\* $P < 0.01$  versus control group. # $P < 0.05$ , ## $P < 0.01$  versus C48/80 group. Data are presented as the mean  $\pm$  SEM ( $n = 6$ )

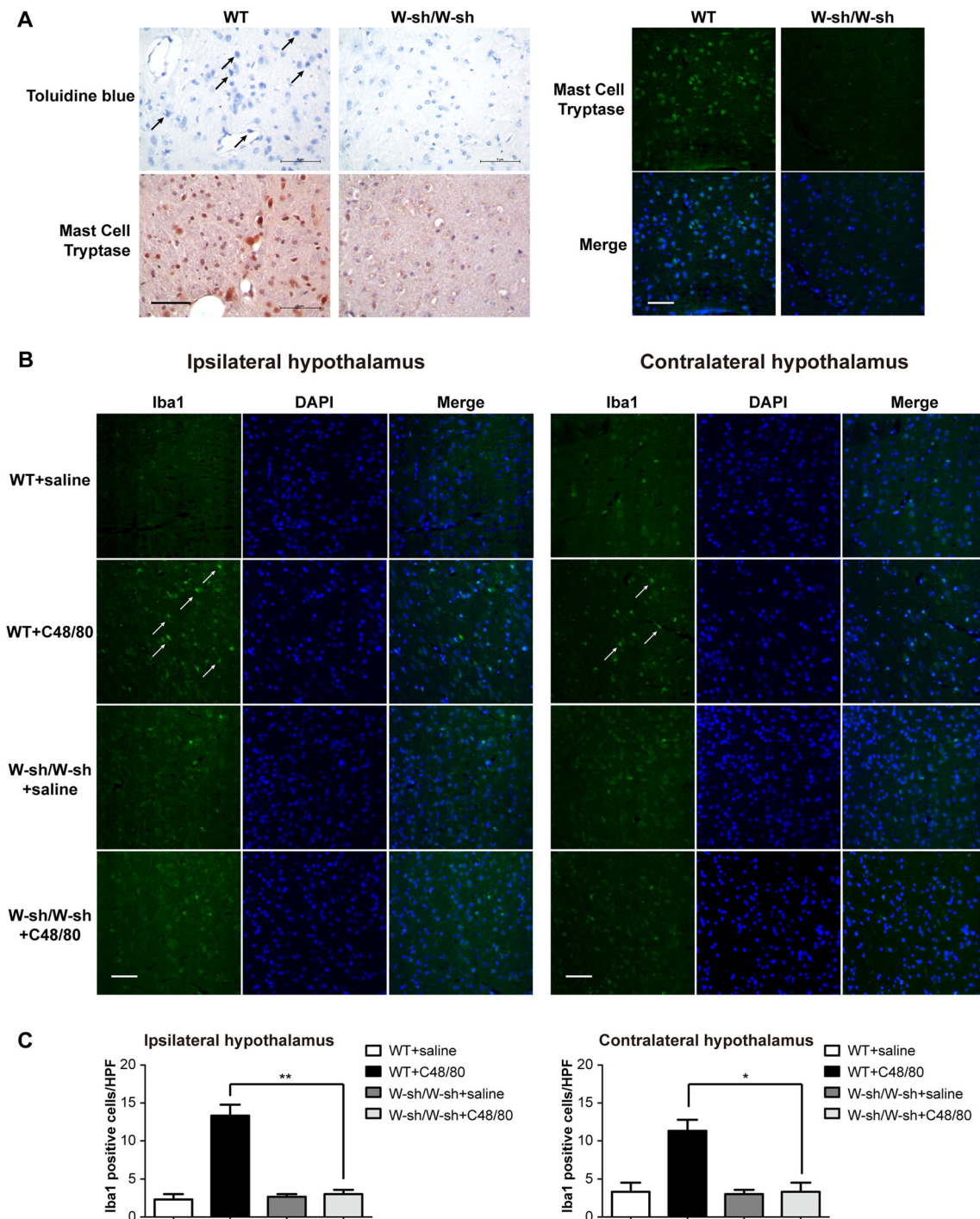
### Cromolyn Inhibited Activated Mast Cells Which Induced Receptor Change on Microglia in Hypothalamus

We have previously reported that tryptase and histamine, which are the mediators released from degranulated mast cells, were able to induce microglia activation and inflammatory mediator release [18, 19]. In order to determine if degranulated mast cells can modulate expression of some receptor proteins in microglia, flow cytometry analysis was employed in the present study. The results showed that site injection of C48/80 (2.5  $\mu$ g) in hypothalamus for 24 h selectively provoked upregulation of expressions of H<sub>1</sub>R, H<sub>4</sub>R, PAR2, and TLR4 (to 141, 142, 136, and 144 % of control, respectively), but deregulation of H<sub>2</sub>R and H<sub>3</sub>R expressions in the ipsilateral hypothalamus microglia. Site injection of cromolyn (100 or 200  $\mu$ g) in hypothalamus for 24 h alone had no significant effect on the expression of these receptor proteins. However, pretreatment of cromolyn (100 or 200  $\mu$ g) for 30 min before C48/80 administration could partially inhibit the effect of C48/80 on receptor protein expression in microglia (Fig. 6). These results suggest that activated mast cells can modulate receptor protein expression in microglia, which can be inhibited by a mast cell stabilizer.

### Discussion

Signaling mechanisms that regulate neuroinflammation and that target regulators of neuroinflammation may prove to be a useful therapeutic strategy to nervous system disorders. It also raises the question of whether we are missing important therapeutic avenues by studying glia and mast cells in isolation from each other [25]. Emerging evidence suggests the possibility of mast cell–microglia communication. However, the direct evidence that whether mast cells could affect microglial activation is deficiency. In this study, we found that activated brain mast cell was able to activate microglia and aggravate neuroinflammation.

Considerable effort has been directed to inhibiting the inflammatory cascade of blood-borne neutrophil and phagocyte infiltration in neuroinflammation, but few studies have focused on resident brain cell types capable of mounting immediate host responses in the brain and meninges, namely mast cells. Through release of their proinflammatory mediators, mast cells actively participate in the pathogenesis of inflammation [26, 27]. In the present study, we found that directed site injection of mast cell degranulator C48/80 in the right hypothalamus for 30 min induced mast cell and microglial

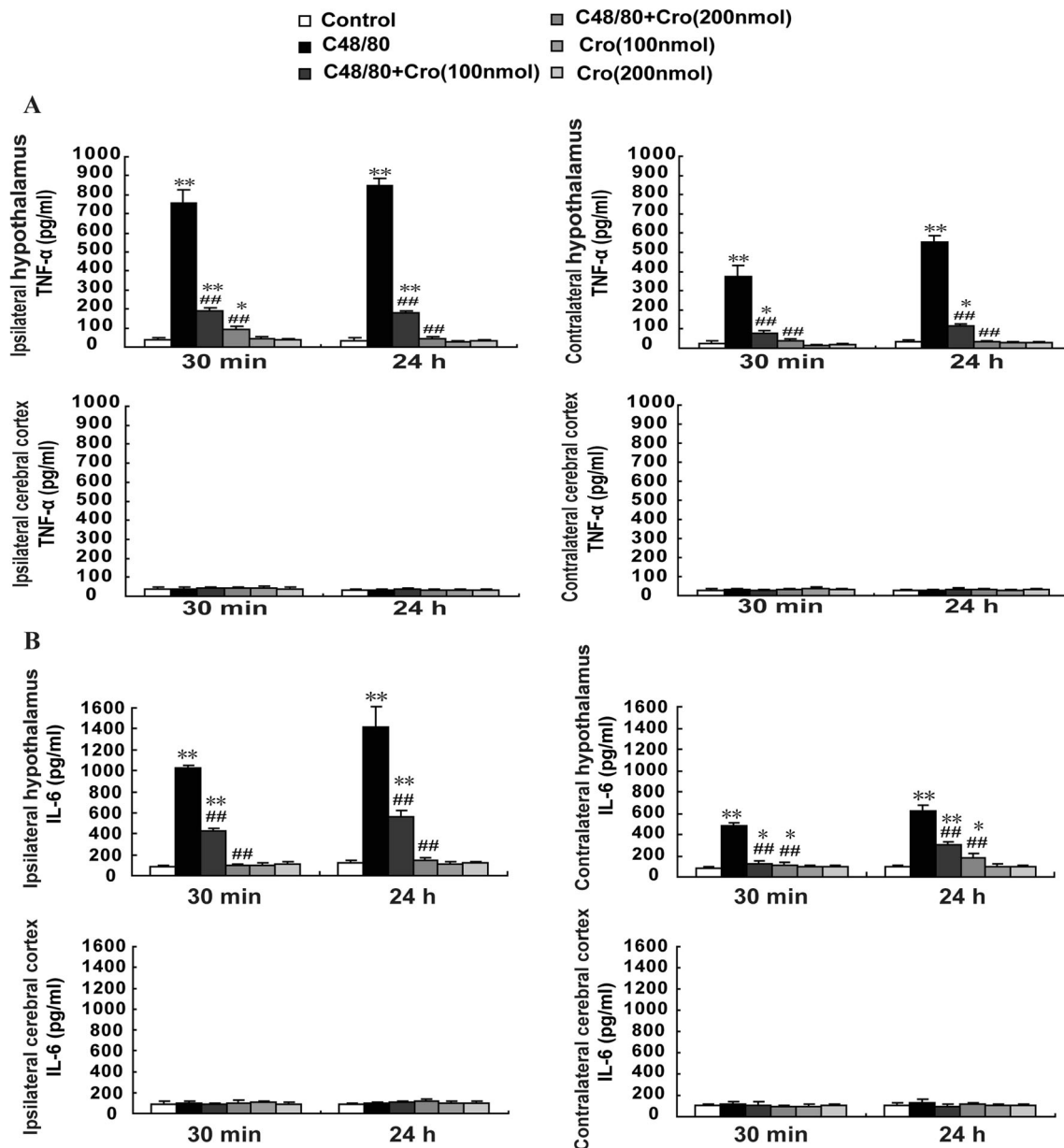


**Fig. 3** C48/80 had no effect on microglial activation in hypothalamus of  $Kit^{W-sh/W-sh}$  mice. **a** Toluidine blue and mast cell tryptase staining of mast cells in the hypothalamus of WT and  $Kit^{W-sh/W-sh}$  mice. **b** Immunostaining was used to detect Iba1, markers for microglia. **c** Quantification of Iba1-

positive cells in the ipsilateral and contralateral hypothalamus. Scale bar: 50  $\mu$ m. \* $P < 0.05$ , \*\* $P < 0.01$ . Data are presented as the mean  $\pm$  SEM ( $n = 5$ )

activation in both the ipsilateral and contralateral hypothalamus but had no effect on microglial activation in mast cell-deficient  $Kit^{W-sh/W-sh}$  mice. Mast cell activation is the “first responder” in this injury and not microglia [28]. Although other resident cells in the CNS produce  $TNF-\alpha$ , most notably

microglia/macrophages [29, 30] and endothelial cells [31], the presence and release of  $TNF-\alpha$  from mast cells preceded its detection in other cells. In our study, we found that injection of mast cell degranulator C48/80 in hypothalamus for 30 min or 24 h significantly increased  $TNF-\alpha$  and IL-6 contents in



**Fig. 4** Stabilization of mast cell inhibited C48/80-induced TNF- $\alpha$  and IL-6 production. Site injection of C48/80 (2.5  $\mu$ g) in hypothalamus for 30 min or 24 h significantly increased TNF- $\alpha$  (a) and IL-6 (b) content in ipsilateral and contralateral hypothalamus. Furthermore, cromolyn (100

or 200  $\mu$ g) could partially inhibit the effect of C48/80. \* $P < 0.05$ , \*\* $P < 0.01$  versus control group. \*\*\* $P < 0.01$  versus C48/80 group. Data are presented as the mean  $\pm$  SEM ( $n = 6$ )

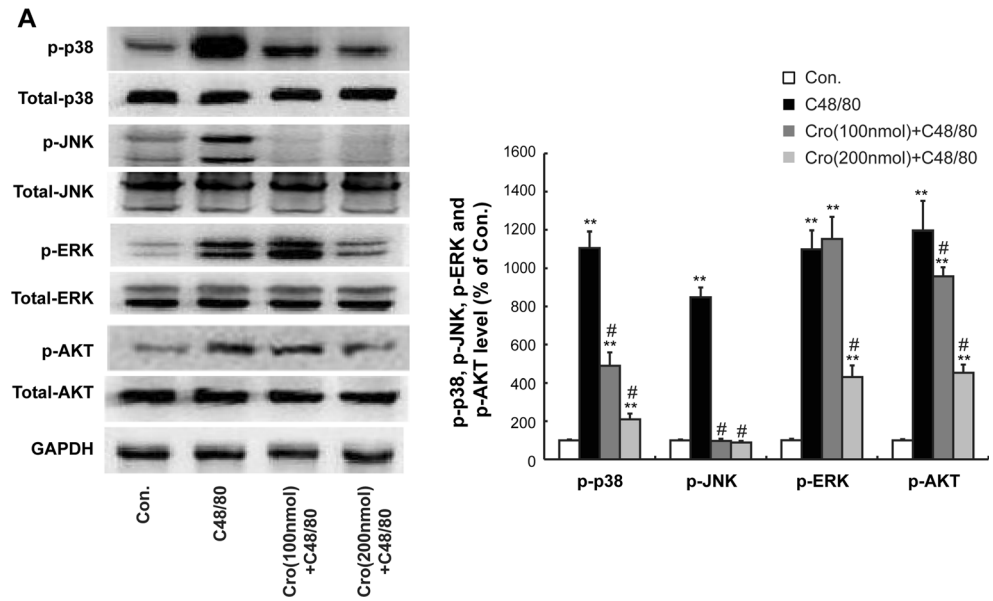
ipsilateral and contralateral hypothalamus. These results further confirm that only the activated mast cells can induce microglial activation and proinflammatory factor release.

Neuroinflammatory disorders are conditions in which components of the nervous system are damaged by inflammatory effectors derived from the innate and adaptive immune systems, as well as glia within the CNS. Microglia, in particular, acts as sensor cells for disturbed brain tissue homeostasis and accumulates locally in response to neuron injury [32]. Microglial activation has been demonstrated to be an early sign that often

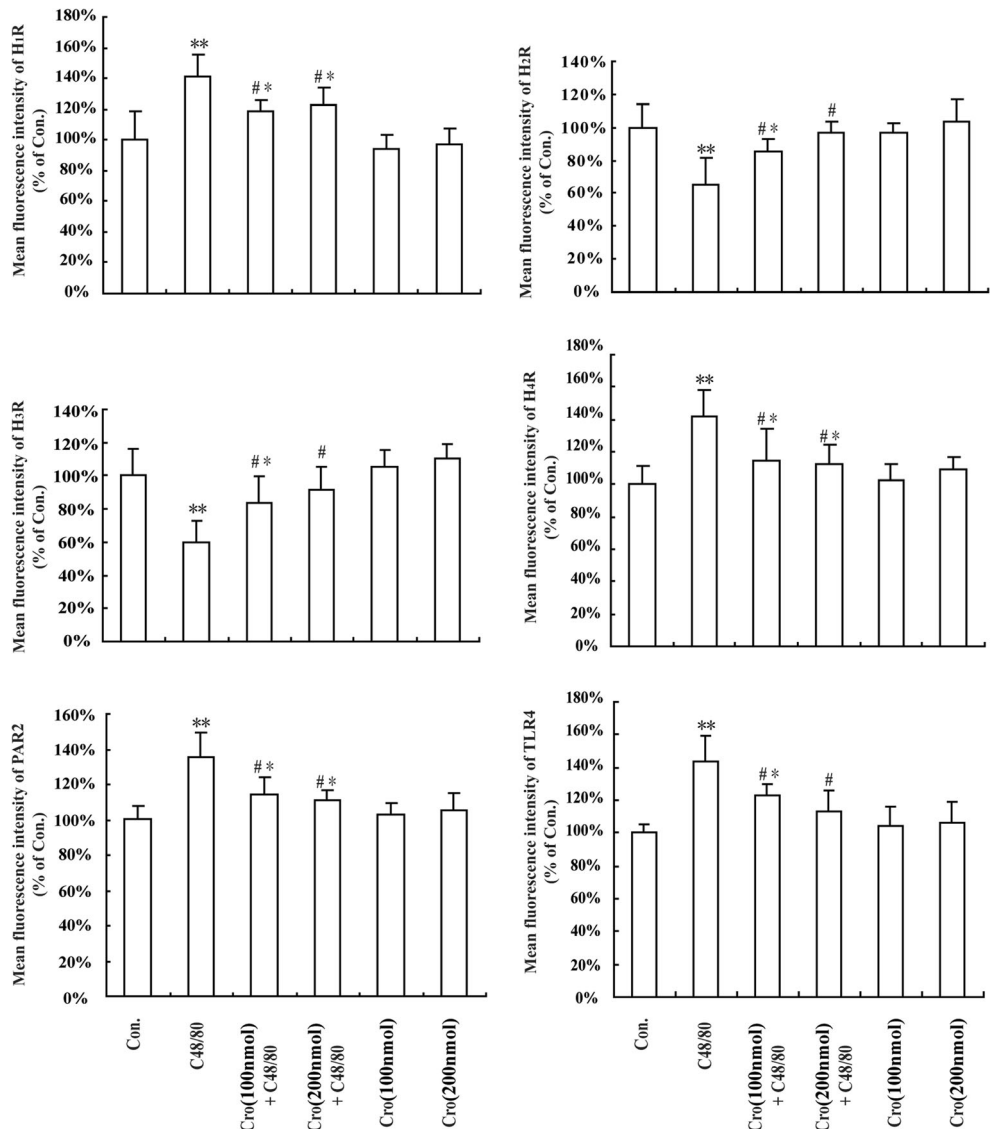
precedes and triggers neuronal death in chronic neurodegenerative diseases [5, 33]. Few studies until now have focused on resident cell types capable of mounting immediate host responses in the brain. Mast cells are effector cells of the innate immune system and represent the “first responders” to injury rather than microglia [28]. If the immediate/early mast cell activation is necessary for the initiation of the inflammatory cascade and ultimate tissue damage, inhibition of this response should be neuroprotective. We found that inhibition of immediate mast cell activation by mast cell stabilizer



**Fig. 5** Stabilization of mast cell depressed MAPK and PI3K/AKT activation in hypothalamus. Western blot checked protein expression of p-p38, p-JNK, p-ERK, and p-AKT in hypothalamus. \* $P < 0.05$ , \*\* $P < 0.01$  versus control group. # $P < 0.05$  versus C48/80 group. Data are presented as the mean  $\pm$  SEM ( $n = 6$ )



**Fig. 6** Cromolyn inhibited activated mast cells which induced receptor change on microglia in hypothalamus. Flow cytometry-assisted expression of H<sub>1</sub>R, H<sub>2</sub>R, H<sub>3</sub>R, H<sub>4</sub>R, PAR2, and TLR4 in the ipsilateral hypothalamus microglia. Site injection of C48/80 (2.5  $\mu$ g) in hypothalamus for 24 h selectively provoked upregulation of expressions of H<sub>1</sub>R, H<sub>4</sub>R, PAR2, and TLR4, but deregulation of H<sub>2</sub>R and H<sub>3</sub>R expressions in the ipsilateral hypothalamus microglia. Pretreatment of cromolyn (100 or 200  $\mu$ g) for 30 min before C48/80 administration could partially inhibit the effect of C48/80 on receptor proteins expression in microglia. \* $P < 0.05$ , \*\* $P < 0.01$  versus control group. # $P < 0.05$  versus C48/80 group. Data are presented as the mean  $\pm$  SEM ( $n = 6$ )



cromolyn limited microglial activation, and C48/80 had no effect on microglial activation in Kit<sup>W-sh/W-sh</sup> mice. Furthermore, pretreatment with cromolyn (100 or 200 µg) 30 min before C48/80 administration could attenuate the content of TNF-α and IL-6 increase induced by C48/80 in the ipsilateral and contralateral hypothalamus. These results indicate that stabilization of mast cell can inhibit microglial activation and production of proinflammatory factors.

The factors responsible for the overactivation of microglia are largely undefined. It is well established that the CNS contains mast cells that are located on the brain side of the blood–brain barrier [34]. They could mediate alterations in blood flow, neurotransmission, and local immune responses in the brain as well [35]. Mast cells are characterized by a large complement of secretory granules which store a wide variety of mediators, including biogenic amines, neuropeptides, cytokines, sulfated proteoglycans, and neutral proteases. Mast cell degranulation is a very rapid process [36], which results in the upregulation of numerous chemokines [37, 38]. When released in the CNS, mast cell secretory products can alter the function of both neural [39], T cell [15], and vascular elements [40]. These cytokines also induce a proinflammatory profile in microglia [41, 42]. We have previously reported that mast cell tryptase can induce microglial activation via the PAR2-MAPK-NF-κB signaling pathway [18] and histamine can evoke microglial activation via the H1R and H4R-MAPK and PI3K/AKT-NF-κB signaling pathways [19], which also suggests the importance of mast cells in induction of inflammation in the CNS. Here, we demonstrate that injection of mast cell degranulator C48/80 in the hypothalamus for 24 h significantly increased the expression of H1R, H4R, PAR2, and TLR4 in microglia. Hence, once mast cells degranulated, the released mediators combine with these receptors on microglia to induce its activation, following with MAPK and AKT signaling pathways, which contributes to neuroinflammation and the exacerbation of CNS immune inflammation-related disease [25].

In summary, the present study identifies that site-directed injection of the “mast cell degranulator” C48/80 in the hypothalamus induces microglial activation, inflammatory factors production, phosphorylation of MAPK and AKT pathways, and increase of protein expressions of H1R, H4R, PAR2, and TLR4 receptors in microglia, which was inhibited by the “mast cell stabilizer” cromolyn. However, C48/80 has no effect on microglial activation in mast cell-deficient Kit<sup>W-sh/W-sh</sup> mice. These results implicate that only activated brain mast cells can trigger microglial activation, and stabilization of mast cell inhibits microglial activation-induced CNS inflammation. Interactions between mast cells and

microglia could constitute a new and unique therapeutic target for CNS immune inflammation-related diseases.

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#### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that there is no conflict of interests regarding the publication of this paper.

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