

Research report

Region-specific regulation of central histaminergic H3 receptor expression in a mouse model of cow's milk allergy

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HIGHLIGHTS

- Mice with milk allergy exhibited changes in their innate burrowing behavior.
- H3 receptor staining was elevated in select brain regions of mice with milk allergy.
- Expression of H1 and H3 receptors were also elevated in specific regions of the brain.
- H1, H2, and H3 receptors were all elevated in the intestines of allergic mice.
- The central histaminergic system may be affected in various disease conditions.

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ABSTRACT

Central histaminergic H3 receptor (H3R) has been extensively investigated as a potential therapeutic target for various neurological and neurodegenerative disorders. Despite promising results in preclinical rodent models, clinical trials have not provided conclusive evidence for the benefit of H3R antagonists to alleviate cognitive and behavioral symptoms of these disorders. Inconsistent pharmacological efficacies may arise from aberrant changes in H3R over time during disease development. Because H3R is involved in feedback inhibition of histamine synthesis and secretion, the expression of the autoreceptor may also be reciprocally regulated by altered histamine levels in a pathological condition. Thus, we investigated H3R expression in a mouse model of cow's milk allergy, a condition associated with increased histamine levels. Mice were sensitized to bovine whey proteins (WP) over 5 weeks and H3R protein and transcript levels were examined in the brain. Substantially increased H3R immunoreactivity was observed in various brain regions of WP-sensitized mice compared to sham mice. Elevated H3R expression was also found in the thalamic/hypothalamic region. The expression of histaminergic H1, but not H2, receptor subtype was also increased in this and the midbrain regions. Unlike the brain, all three histaminergic receptors were increased in the small intestine. These results indicated that the central histaminergic receptors were altered in WP-sensitized mice in a subtype- and region-specific manner, which likely contributed to behavioral changes we observed in these mice. Our study also suggests that altered levels of H3R could be considered during a pharmacological intervention of a neurological disease.

1. Introduction

The histaminergic H3 receptor (H3R) in the central nervous system (CNS) gained considerable interest in the field as a potential therapeutic target for many neurological and cognitive disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia,

depression, attention deficit hyperactivity disorder (ADHD), and substance abuse (Day et al., 2007; Esbenshade et al., 2006; Kumar et al., 2019; Medhurst et al., 2007; Nuutinen et al., 2016; Pillot et al., 2002b; Sadek et al., 2016). The involvement of H3R in these conditions is not unexpected considering that its intrinsic ligand, histamine, is a crucial modulatory neurotransmitter for various aspects of animal behavior. In

Abbreviations: AD, Alzheimer's disease; ADHD, attention deficit hyperactivity disorder; CMA, cow's milk allergy; CNS, central nervous system; CT, cholera toxin; FC, frontal cortex; HDC, histidine decarboxylase; HPC, hippocampus; H1R, H2R, H3R, histamine H1, H2, H3 receptors; HypTH, hypothalamus; MB, midbrain; PC, parietal cortex; PD, Parkinson's disease; STR, striatum; THAL, thalamus; TMN, tuberomammillary nucleus; WP, whey protein

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the CNS, histamine is produced and released by histaminergic neurons located in the tuberomammillary nucleus (TMN) in the posterior hypothalamus and modulates brain functions concerning arousal, satiety, locomotion, learning, memory, emotion, attention, and cognition through extensive projections (Haas et al., 2008; Mochizuki et al., 1992; Panula et al., 1989; Watanabe et al., 1984; Yoshizawa et al., 2009), also see review by (Hu and Chen, 2017). In addition to the neurons of the TMN, brain resident mast cells are major contributors of central histamine, supplying up to 50% of the histamine found in the brain (Goldschmidt et al., 1985; Grzanna and Shultz, 1982).

The H3R is abundantly expressed in various regions of the brain including the cerebral cortex, basal ganglia, hypothalamus, and hippocampus among others (Lovenberg et al., 1999; Pillot et al., 2002a; Pollard et al., 1993). The $G_{i/o}$ -protein coupled receptor functions as a presynaptic autoreceptor that inhibits the release and synthesis of histamine or as a postsynaptic heteroreceptor that regulates other neurotransmitter systems (Arrang et al., 1995, 1983, 1987; Ligneau et al., 1998; Schlicker et al., 1989; Vanhanen et al., 2016). In particular, the ability of H3R to affect dopaminergic receptor expression and signaling via formation of complexes with dopamine D1 or D2 receptors in striatal medium spiny neurons has been well documented (Moreno et al., 2011, 2014; Rapanelli et al., 2016); also see reviews by (Bolam and Ellender, 2016; Ellenbroek, 2013). The function of H3R is thought to be particularly important for wakefulness, cognition and behavior, and inhibition of H3R activity with specific antagonists or inverse agonists has been considered as a therapeutic approach. Indeed, pitolisant, a H3R inverse agonist/antagonist, has been approved to treat narcolepsy by the European Medicines Agency in 2016 and by the United States Food and Drug Administration in 2019. In preclinical studies, antagonism of H3R has been demonstrated to improve pharmacologically induced cognitive impairment in rodent models of schizophrenia (Bardgett et al., 2010; Brown et al., 2013), attention and hyperactivity in an ADHD rat model (Kim et al., 2018), and cognitive deficiencies and pathology in transgenic AD mice (Bardgett et al., 2011). However, except for the use of pitolisant for narcolepsy and associated cataplexy (Dauvilliers et al., 2019, 2013; Szakacs et al., 2017), clinical trials with H3R inverse agonist/antagonists have not shown substantial success in improving behavior and cognition as observed in these animal models (Grove et al., 2014; Jarskog et al., 2015; Kubo et al., 2015; Weisler et al., 2012). Such inconsistencies between preclinical and clinical results suggest that H3R responses to pharmacological inhibitors may be regulated differently through changes in expression levels, affinity, and/or activity in various diseases, especially in chronic conditions.

While an ample number of studies exist examining H3R-mediated regulation of histaminergic and other neurotransmitter systems, the number of studies on the regulation of H3R in the CNS are relatively limited. Nevertheless, altered levels and activities of H3R have been reported in different pathological conditions. In a rat model of PD (Anichtchik et al., 2000; Ryu et al., 1994b), increased levels of H3R were reported in the substantia nigra and ventral striatum ipsilateral to 6-hydroxydopamine lesion, where histamine concentrations were also elevated (Anichtchik et al., 2000; Rinne et al., 2002). In a rodent model of unilateral traumatic brain injury, H3R mRNA expression was decreased in the ipsilateral thalamus in parallel with increased mast cell numbers (Lozada et al., 2005). Interruption of histaminergic input from the hypothalamus resulted in upregulation of postsynaptic H3R (Pollard et al., 1993) while hippocampal H3R expression decreased in transgenic mice lacking histidine decarboxylase (HDC), which catalyzes the conversion of histidine to histamine (Chepkova et al., 2012). In humans, increased H3R binding activity of a tritiated subtype-specific agonist, [^{3} H]-N- α -methylhistamine, was described in the substantia nigra of postmortem brains from PD patients (Anichtchik et al., 2001) and the prefrontal cortex of schizophrenic patients (Jin et al., 2009). In AD and Huntington's disease patients, H3R mRNA was altered in a number of locations throughout the brain (Shan et al., 2012; van Wamelen et al.,

2011). Similarly, a postmortem study of the histaminergic system in autism patients found that gene expression of all three histamine receptor subtypes was altered in the dorsolateral prefrontal cortex compared to healthy controls (Wright et al., 2017). Interestingly, these are neurological disorders that are associated with increased histamine levels (Nuutinen and Panula, 2010; Shan et al., 2015). These findings not only demonstrate that the H3R is differentially regulated depending on the brain regions and disease conditions, but also suggest that its levels may be influenced by altered local and/or systemic histamine concentrations.

In the present study, we therefore assessed the expression of H3R receptor in a mouse model of food allergy, another pathologic condition associated with increased histamine levels from mast cell activation. Previously (Germundson et al., 2018), we induced non-anaphylactic cow's milk allergy (CMA) by sensitizing C57BL/6J mice with bovine whey proteins (WP) in order to assess food-allergy-associated behavioral changes often observed in patients with mood and behavioral disorders such as anxiety, depression, schizophrenia, and autism (Addolorato et al., 1998; Costa-Pinto et al., 2006; Ferro et al., 2016; Garg and Silverberg, 2014; Patten and Williams, 2007; Severance et al., 2012; Shanahan et al., 2014; Tan et al., 2019; Topal et al., 2016; Xu et al., 2018). We demonstrated that sensitization to WP altered innate burrowing behavior in association with increased allergen-specific serum IgE levels and brain and intestinal mast cell numbers in a sex- and age-dependent manner (Germundson et al., 2018). We hypothesized that expression of central H3R is modulated in association with these CMA-induced behavioral and immunological changes and investigated the expression of H3R in different regions of the brain. Because the older male mice (10 months old) exhibited the most robust CMA-associated behavioral changes in our previous study (Germundson et al., 2018), we used this group of sham and WP-sensitized mice. In addition to the brain, we also assessed H3R in the small intestines, the site of more direct allergic insult.

2. Results

2.1. WP sensitization resulted in reduced innate burrowing behavior in male mice

Previously, we demonstrated that WP-sensitized male, but not female, mice exhibited significantly decreased digging behavior compared to sex-matched sham mice when measured by placing the mice in a cage filled with thick bedding (Germundson et al., 2018). This aspect of instinctive burrowing behavior was assessed using another test, the marble-burying test (Deacon, 2006). Male sham mice demonstrated significantly greater levels of activity than treatment-matched females by 3-fold (male sham [$n = 6$]: 6 ± 1 , female sham [$n = 7$]: 2 ± 1 ; $p = 0.01$, two-way ANOVA), indicating that there was a distinct baseline sex difference in this behavior (Fig. 1). WP-sensitized male mice displayed more than 50% decrease in the average marble burying activity (male WP [$n = 6$]: 2.5 ± 0.9 ; $p = 0.02$ compared to sham male mice), the level equivalent to the female sham mice. While the average marble burying activity of WP-sensitized female mice was also lower than their sham counterparts, the difference did not reach statistical significance (female WP [$n = 7$]: 1.1 ± 0.5 ; $p = 0.6$ when compared to female sham mice). Thus, we again demonstrated that WP sensitization elicited observable changes in their innate burrowing behavior of C57BL/6J male mice. Due to lack of a significant behavioral effect of WP sensitization in female mice, all subsequent analyses focused on male mice.

2.2. H3R immunoreactivity was altered in WP-sensitized mice in a region-specific manner

H3R was immunohistochemically detected in the brain sections of sham and WP-sensitized male mice to determine potential effects of

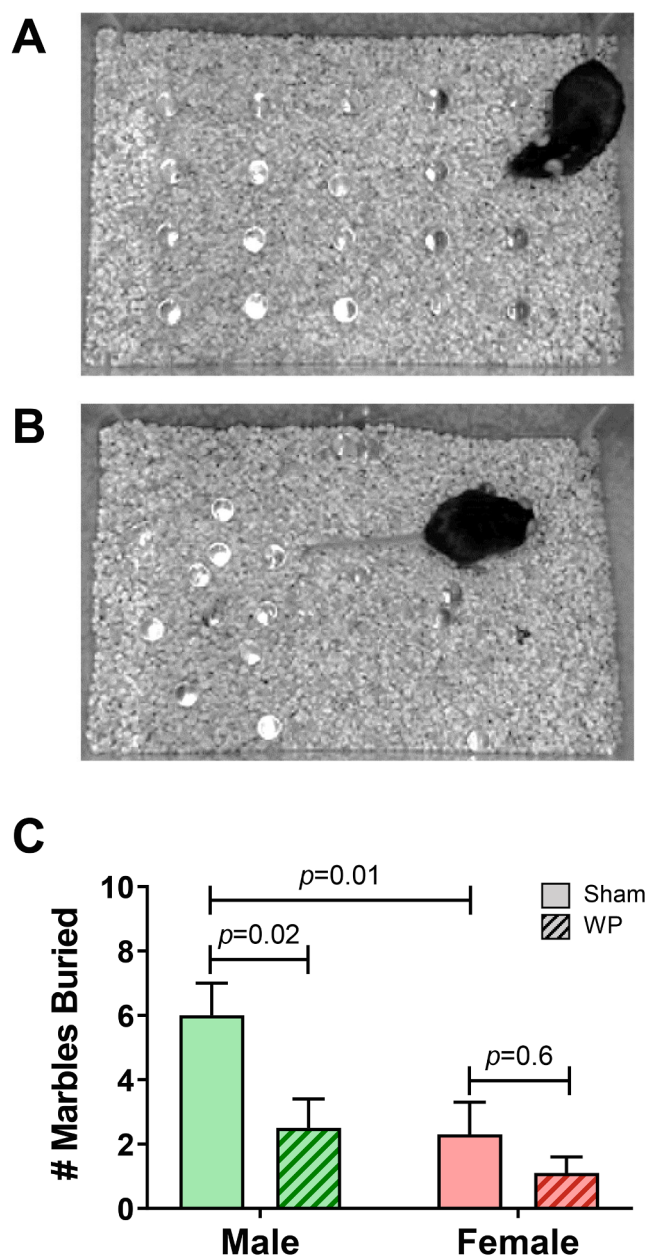


Fig. 1. Marble burying activities in sham and WP-sensitized male and female mice. Mice from each treatment group were individually placed in a cage with 20 evenly-spaced marbles on corncob bedding for 15 min. An example mouse is pictured in the test cage at the beginning (A) and near the end of the test (B). Marbles buried more than 50% by bedding at the end of the testing period were recorded as buried and graphed (C). Data is presented as the average number of buried marbles $\pm$ SEM for the group.

CMA in its expression. The specificity of the anti-H3R antibody toward the H3R was validated by western blot analysis using an epitope-adsorbed antibody preparation (Supplementary Fig. 1). Although we did not observe immunostaining in the TMN, where the histaminergic cell bodies reside, H3R staining was observed in various areas of the brain (Fig. 2, A–J). The majority of immunopositive structures showed morphology consistent with neuronal fibers. Greater intensities of staining were noticeable in distinct regions of the WP-sensitized brains, particularly in the cingulate, piriform, motor, and somatosensory cortices, and the hippocampus, the areas important for motor, cognitive, and limbic functions. To validate these qualitative observations between the groups, H3R staining was quantified by densitometry from photomicrographs. The optical density (OD) values of H3R immunoreactivity

were significantly greater in WP-sensitized mice when compared to the sham group by 1.5 fold and 3 fold in the cingulate (Fig. 2, A&B) and piriform cortices (Fig. 2, C&D), respectively (the cingulate cortex: male sham [$n = 11$] 29.7 ± 2.0 , male WP [$n = 11$] 42.1 ± 3.0 ; $p = 0.003$; the piriform cortex: male sham [$n = 11$] 23.7 ± 2.5 , male WP [$n = 11$] 64.3 ± 6.5 ; p less than 0.0001). In comparison to the cingulate and piriform cortices, the motor (Fig. 2, E&F) and somatosensory (Fig. 2, G&H) cortices had lower baseline H3R immunoreactivity. However, The OD values for WP-sensitized mice in these areas were still 1.8-fold and 1.2-fold greater than the sham mouse group, respectively (the motor cortex: male sham [$n = 11$] 3.8 ± 0.5 , male WP [$n = 13$] 7.2 ± 0.8 ; $p = 0.003$; the somatosensory cortex: male sham [$n = 11$] 2.7 ± 0.3 , male WP [$n = 12$] 3.6 ± 0.2 ; $p = 0.03$). In the hippocampus (Fig. 2, I&J), the difference in H3R immunoreactivity between the male sham and WP-sensitized hippocampus was 1.5 fold (male sham [$n = 12$] 8.6 ± 0.8 , male WP [$n = 11$] 12.3 ± 1.1 ; $p = 0.009$). These results demonstrated that the levels of H3R were substantially more abundant in these regions of the brain after WP sensitization.

2.3. WP sensitization elicited differential effects on the mRNA expression of H3 receptor subtype in distinct regions of the brain

In order to determine whether the augmented H3R immunoreactivity in the WP-sensitized male mice was associated with increased gene expression, the levels of H3R transcripts were quantified by RT-qPCR from the areas including the frontal cortex/striatum (Region 1), the parietal cortex/hippocampus (Region 2), the thalamus/hypothalamus (Region 3), and the midbrain (Region 4) (Fig. 3A). H3R expression in Region 3, which included the TMN, was significantly greater in WP-sensitized mice by ~ 3 fold (male sham [$n = 6$]: 1.0 ± 0.4 , male WP [$n = 6$]: 2.7 ± 0.6 ; $p_{adj} = 0.001$), while Regions 1, 2, and 4 were unaffected (Fig. 3B). Although a small rise in the average group value for H3R expression (1.4 fold) was detected in Region 4, the difference did not reach statistical significance when compared to the sham group. These results indicated that H3R mRNA expression was increased after WP-sensitization in the region where the cell bodies of histaminergic neurons reside.

To follow up with this finding, we examined whether other histaminergic receptor subtypes, H1R and H2R, were also regulated after WP sensitization in each of these regions. Similarly to H3R, the expression of H1R in Regions 1 and 2 in WP-sensitized mice were comparable to the sham group (Fig. 3C). Although a decreasing trend in H1R expression was detected in Region 2, the difference did not reach statistical significance (male sham [$n = 6$]: 1.0 ± 0.3 , male WP [$n = 5$]: 0.48 ± 0.08 ; $p = 0.1$). In Region 3 and 4, however, WP sensitization increased the relative expression of H1R by 2.1- and 1.7-fold, respectively (Region 3: sham [$n = 6$]: 1.0 ± 0.2 , WP [$n = 6$]: 2.1 ± 0.4 ; $p = 0.0005$, Region 4: sham [$n = 6$]: 1.0 ± 0.2 , WP [$n = 6$]: 1.7 ± 0.2 ; $p = 0.03$). In contrast, the expression of H2R was not affected by WP sensitization in any of the 4 brain regions (Fig. 3D). These results indicated that the expression of the three histaminergic receptor subtypes were differentially regulated in the brains of WP-sensitized mice in a region-specific manner.

We also investigated whether WP sensitization could affect histamine synthesis by regulating the expression of HDC, the rate-limiting catalytic enzyme of histamine biosynthesis (Fig. 3E). In Region 3 where the histaminergic neurons of the TMN are located, HDC expression was readily detectable, and the relative expression of HDC was approximately 3 fold greater than the sham group (sham [$n = 4$]: 1.0 ± 0.1 , WP [$n = 5$]: 3 ± 1 ; $p = 0.03$). There was a decreasing trend for HDC expression in Region 2, although it was not statistically significant (sham [$n = 6$]: 1.0 ± 0.3 , WP [$n = 6$]: 0.4 ± 0.1 ; $p = 0.4$). HDC expression was not affected in the other regions. These results suggested that the capacity of the histaminergic neurons in the TMN to synthesize histamine as their monoaminergic neurotransmitter was specifically affected in the TMN by WP sensitization.

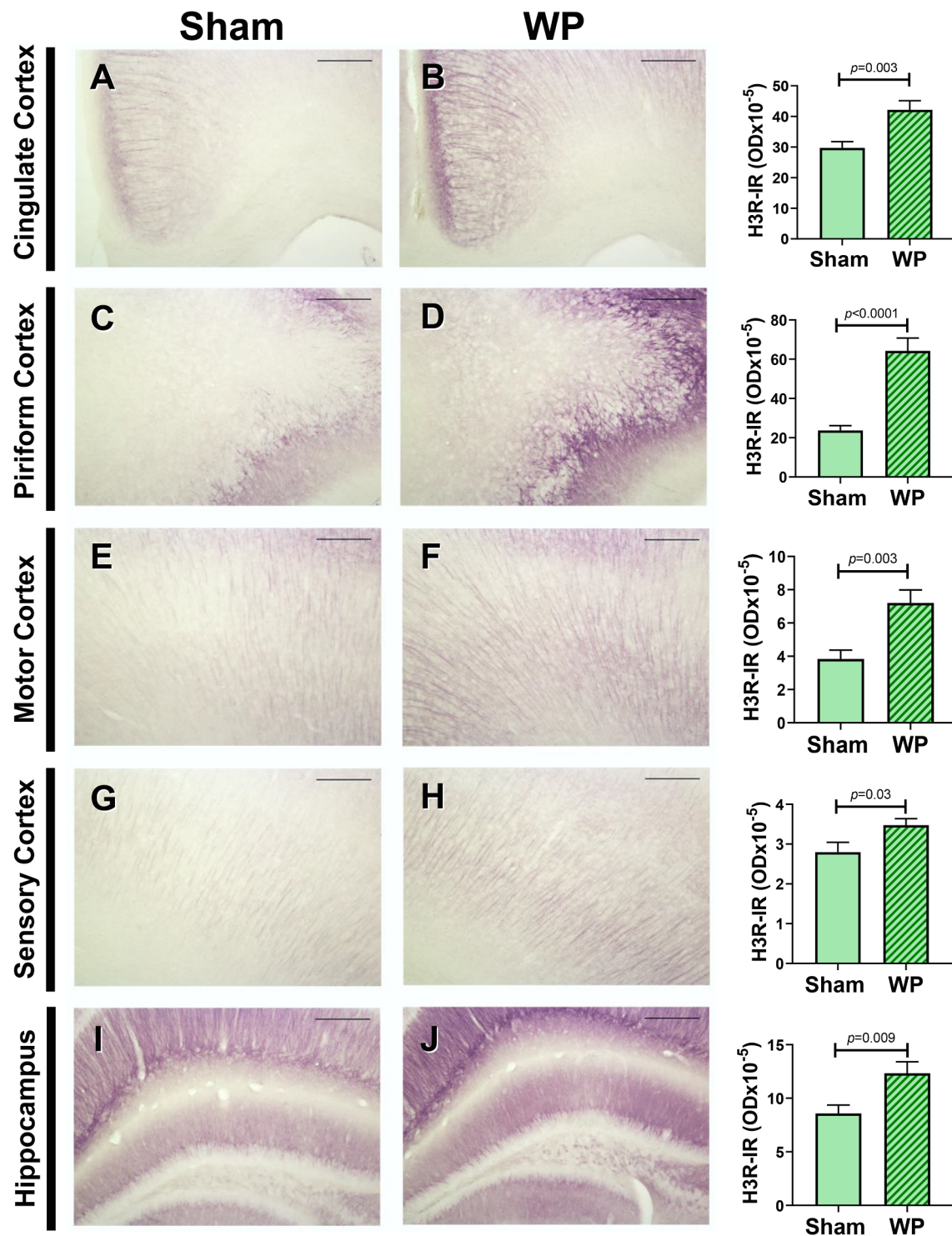


Fig. 2. Comparisons of H3R immunoreactivity between sham and WP-sensitized mice in different regions of the brain. Representative photomicrographs demonstrating the presence and localization of the H3R immunoreactivity in sham and WP-sensitized male mice including the cingulate (A, B), piriform (C, D), somatosensory (E, F), and motor cortices (G, H), and the hippocampus (I, J) (10x objective; scale bar = 200 μ m). Graphs represent the quantification of H3R immunoreactivity for corresponding brain regions presented as the average OD value for each group $\pm$ SEM.

2.4. WP sensitization influenced the levels of the three histaminergic receptor subtypes in the small intestine

Because we observed that WP sensitization differentially regulated the expression of the three histaminergic receptor subtypes in the brain, we next investigated whether these receptors were similarly regulated at the site of allergen insult in the small intestines. When the levels of

H1R, H2R, and H3R were evaluated in the ileum using immunohistochemistry, all receptor subtypes were detectable in the ileum of both sham and WP-sensitized mice (Fig. 4). While the staining for each receptor was particularly prominent in the enterocytes and/or lamina propria, the staining was more intense in the villi of WP-sensitized mice than sham mice. Unlike our observations in the brain where only H1R and H3R were altered in select regions, these results showed

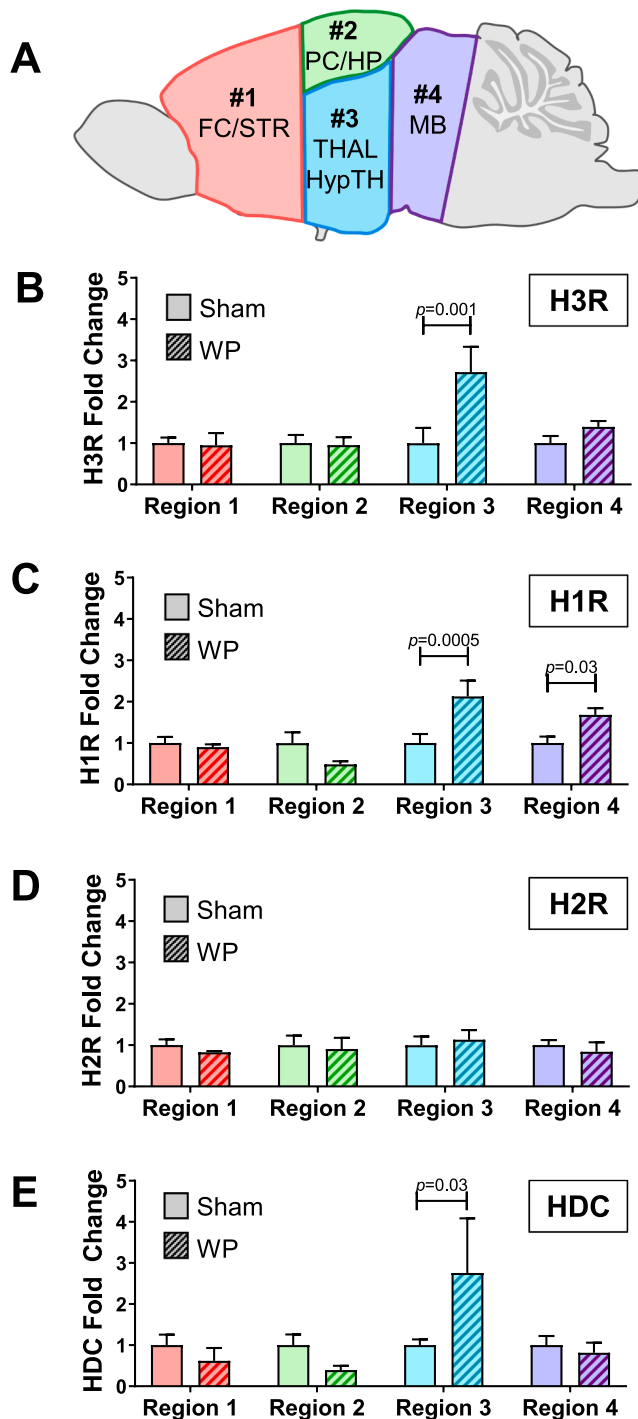


Fig. 3. Comparisons of the gene expression for H1R, H2R, H3R and HDC in various brain regions of sham and WP-sensitized mice. (A) Right mouse brain hemisphere depicting the 4 regions dissected for the transcript analysis using RT-qPCR. FC/STR = frontal cortex/striatum, PC/HP = parietal cortex/hippocampus, THAL/HypTH = thalamus/hypothalamus, MB = midbrain. (B-E) Total RNA was isolated individually from the 4 regions and RT-qPCR was performed to determine the relative expressions of H3R (B), H1R (C), H2R (D), and HDC (E) in WP-sensitized mice (solid bars) compared to sham control mice (striped bars). Ribosomal 18S was used as the reference gene, and the Cq value for each target gene was normalized to the 18S Cq value from the same sample. Data is presented as the average fold change in relative expression for each group $\pm$ SEM.

that the levels of all three histaminergic receptors were induced with WP sensitization.

3. Discussion

Under the hypothesis that the levels of central H3R become modulated during a prolonged pathologic condition, we immunohistochemically examined the presence of the receptor in the brain sections from WP-sensitized mice. Using these mice, we previously showed that WP-sensitized mice had normal levels of overall mobility but exhibited a significant decrease in their digging activity (Germundson et al., 2018), which is thought to reflect innate burrowing behavior in mice (Deacon, 2006). In the present study, we confirmed this behavior change with the marble burying test. Although this test is sometimes performed for 30–60 min, we found that the shorter test period used in this study reduced the possibility for buried marbles to be uncovered by continued digging, which obscures the final number of buried marbles. Importantly, we observed significant differences between sham and WP-sensitized mice after 15 min (Fig. 1). Thus, our WP-sensitized mouse model serves as a useful tool to detect significant behavioral changes associated with CMA even in the absence of anaphylaxis (Germundson et al., 2018).

As previously described elsewhere (Lovenberg et al., 1999; Pillot et al., 2002a; Pollard et al., 1993), H3R immunoreactivity was detected throughout the brains of our sham and WP-sensitized mice. In association with the behavioral changes in WP-sensitized mice, we found marked increases in H3R immunoreactivity in the cingulate, piriform, somatosensory and motor cortices, and the hippocampus, the regions important for limbic, motor, memory, and cognitive functions (Fig. 2) (Andersson et al., 2010; Liljeholm and O'Doherty, 2012; Royet et al., 2003). Unexpectedly, we did not observe H3R immunoreactivity in the TMN, where the cell bodies of histaminergic neurons are located, even though we were able to detect the H3R mRNA with RT-qPCR and its expression was significantly elevated in this region in WP-sensitized mice (Fig. 3B, Region 3). One possible explanation for this discrepancy between H3R immunoreactivity and mRNA expression in this region may be that mRNA is produced in the cell bodies while translated, functional receptor proteins are distributed and localized in the axons. Alternatively, we might have only detected a particular isoform of H3R with the antibody we used, which was raised against a rat H3R peptide sequence corresponding to amino acids 228–242 (Alomone Labs, Jerusalem, Israel). H3R receptors exist in multiple functionally competent isoforms, and the location of the epitope, the third intracellular loop, is a site of truncation (Drutel et al., 2001; Esbenshade et al., 2008; Morisset et al., 2001). These isoforms are differentially expressed in the brain (Drutel et al., 2001; Morisset et al., 2001); thus, it is possible that the cell bodies of the TMN have one or more of the truncated H3R isoforms that were not detected with the antibody used in this study. This notion, however, needs to be further validated.

The change in H3R availability may be a result of feedback inhibition of histaminergic function. H3R has been shown to inhibit the release and synthesis of histamine from presynaptic neurons in an auto-crine manner (Arrang et al., 1983, 1987; Ellenbroek, 2013), and its upregulation has been observed in pathological conditions that are associated with elevated levels of histamine (Anichtchik et al., 2000; Rinne et al., 2002; Ryu et al., 1994b). It is possible that histamine levels were elevated in WP-sensitized mice after the antigen challenge, and consequently, the expression of H3R was increased to downregulate histamine synthesis and release and thus, its concentration. Alternatively, it is feasible that WP sensitization and challenge had influenced the central histamine production, increasing histamine synthesis by the TMN. This notion is supported by the increased HDC expression observed in Region 3 of WP-sensitized mice (Fig. 3E). Elevated histamine production and release by the TMN might have resulted in subsequent upregulation of H3R expression in this region (Fig. 3B). Despite the source, the amount of histamine in the brain should be examined. In

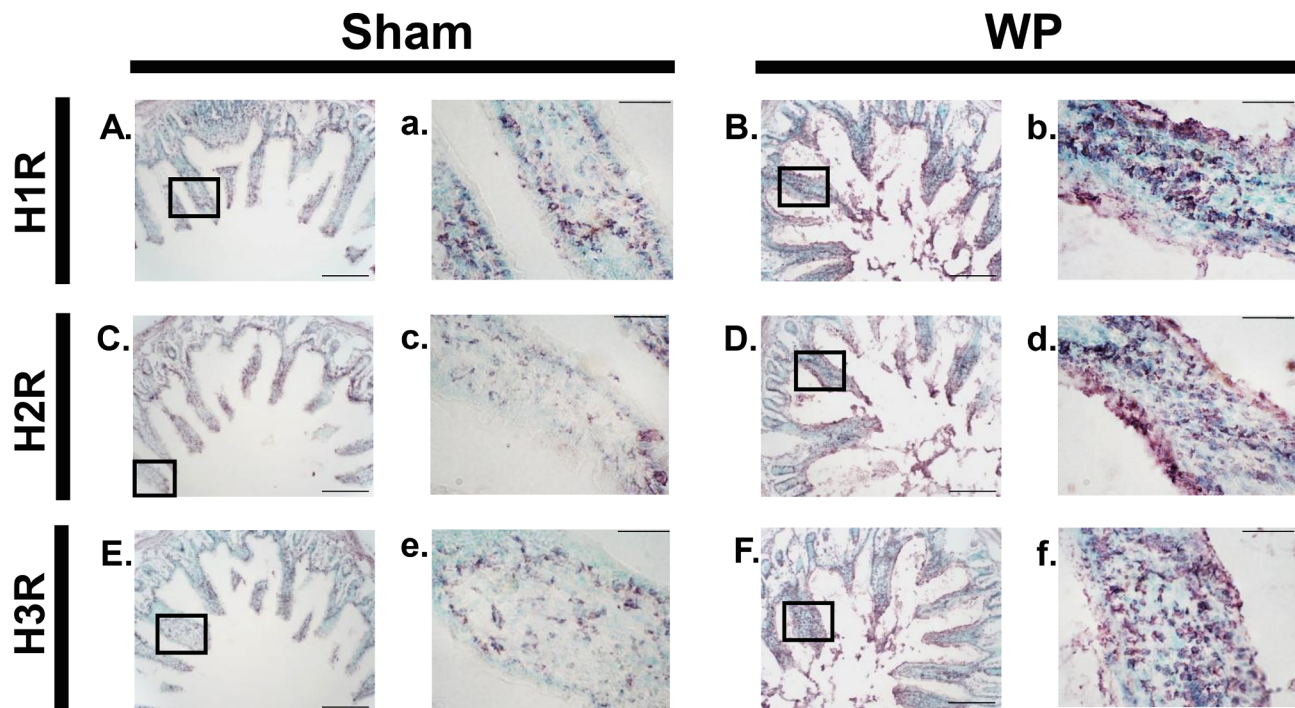


Fig. 4. Immunohistochemical staining of H1R, H2R, and H3R in the small intestines of male sham and WP-sensitized groups. Representative immunohistochemical staining for H1R (A, B), H2R (C, D), and H3R (E, F) in the ileums of sham (A, C, E) and WP-sensitized mice (B, D, F) are shown in photomicrographs taken using a 20x objective (scale bar = 200 μ m). Higher magnification images taken with a 60x objective (scale bar = 50 μ m) particularly highlight the immunoreactivity of WP sensitized males along the enterocytes of villi. Representative photomicrographs at both objectives were taken as a z-stack ($n = 15$, step size of 0.7 μ m) and merged to create a greater depth of focus.

this experiment, we had focused on the behavior, histological changes, and transcription analysis, and therefore we did not process the brain tissue samples to preserve the evanescent aminergic neurotransmitter for quantitation. To validate that the observed changes in H3R expression was indeed due to elevated brain histamine levels, additional studies are warranted.

Other histaminergic receptors in the brain were also examined in this study. In addition to H3R and HDC, the expression of H1R was significantly increased in Region 3 (Fig. 3C). The elevated H1R expression in Region 4, which included the midbrain and rostral brainstem, also indicated that the expression of this receptor subtype was altered in broader areas of the brain by WP sensitization than H3R. In contrast, H2R expression was unchanged in any of the brain regions (Fig. 3D). In addition to its role in wakefulness and arousal (Parmentier et al., 2016), H1R, but not H2R, has been implicated in the anxiogenic function of central histamine (Yuzurihara et al., 2000). Thus, the increased amount of H1R in the brain may also have contributed to the behavioral changes observed in our mouse model.

WP sensitization could have also affected other neurotransmitter systems in the brain. As a presynaptic heteroreceptor, H3R regulates the release of other neurotransmitters, including dopamine, serotonin, glutamate, and gamma-aminobutyric acid (GABA) (Ellenbroek, 2013). Postsynaptic H3R heteroreceptors are mostly found in the striatum, where they heterodimerize with dopamine D1 and D2 receptors to alter downstream signaling (Ryu et al., 1994a). Therefore, upregulation in H3R expression in this brain region may result in modifications of normal striatal activities, influencing motor and limbic functions. Abnormal limbic functions have been described in various neuropsychiatric disorders, such as depression, anxiety disorders, anorexia nervosa, and schizophrenia (Christensen et al., 2015; Liljeholm and O'Doherty, 2012; Lipsman et al., 2015; White et al., 2008).

Because substantial dysregulation in H3R expression was observed in select regions of WP-sensitized brains, we examined whether the changes in peripheral histaminergic receptors had also occurred at the

site of allergen insult. In the small intestines, immunohistochemical staining for H1R, H2R, and H3R was markedly elevated in WP-sensitized mice (Fig. 4). Localized elevation of H1R and H2R expression in the gut has been previously demonstrated in human inflammatory gastrointestinal conditions, including food allergy (Sander et al., 2006). H1R has been shown to influence vasodilation, contractility, transepithelial permeability (Keely et al., 1995; Togias, 2003), and communication with the enteric nervous system (Bell et al., 2015). Furthermore, these histamine receptors have been implicated in immunoregulatory roles by influencing both Th1- and Th2-type helper T lymphocyte responses (Bell et al., 2015; Jutel et al., 2001). Thus, the increases in the expression of these histaminergic receptors may exaggerate and/or prolong the effects of peripheral histamine upon allergen exposure. Since we previously observed an increased number of mast cells in the intestines (Germundson et al., 2018), it is likely that histamine concentration was locally elevated in the WP-sensitized intestines and subsequently altered histamine receptor expression. Dysregulation of the intestinal histaminergic system may also affect brain function via prolonged inflammation, compromised blood brain barrier permeability, nutrient malabsorption, and dysbiosis of intestinal flora.

Inflammation and associated proinflammatory mediators are well known to affect the function of the central nervous system (CNS) and behavior (Skaper et al., 2018). It is therefore feasible that inflammatory responses triggered by food allergy also influence the brain. Indeed, controlled cohort studies have reported increased prevalence of anxiety and depression (Addolorato et al., 1998) or schizophrenia (Severance et al., 2012) in adult food allergy sufferers compared to healthy controls. Similar comorbidities of behavioral disorders, such as autism (Jyonouchi, 2010), ADHD (Miyazaki et al., 2017), and Tourette's syndrome or obsessive-compulsive disorder (Bruun, 1984; Yuce et al., 2014), have also been reported in younger patients. The results from our study suggests that modulation of H3R may be considered a potential therapeutic target for alleviating these allergy-associated mood and behavior symptoms.

In conclusion, we demonstrated dysregulation of the central and intestinal histaminergic systems in association with altered innate burrowing behavior in WP-sensitized mature male mice. In particular, significant increases in H3R was observed in select regions of the brain at both the transcript and protein levels. H1R, H2R, and H3R were also increased in the ileum of sensitized mice, demonstrating that the histaminergic receptor systems were also significantly altered at the peripheral site of allergen insult. The results of this study provide evidence that both central and peripheral histaminergic receptor systems are dysregulated in a pathologic condition, at least in the mouse model of cow's milk allergy. Thus, altered levels of receptors should be taken into account when receptor agonist/antagonists are used to manipulate the receptor activities as a therapeutic strategy.

4. Experimental procedure

4.1. Animals and WP sensitization

In-house-bred 10-month-old male and female C57BL/6J mice were housed in the animal facility at the University of North Dakota under a 12-hour light/dark cycle. The animals were given access to food and water *ad libitum*. Mice were randomly assigned to either sham or WP-sensitization groups ($n = 6$ for males; $n = 8$ for females). Once a week for 5 weeks, mice in the WP-sensitization group were given 200 μ L phosphate-buffered saline (PBS) containing 20 mg WP with an adjuvant of 10 μ g cholera toxin B subunit (CT) (MilliporeSigma, Burlington, MA, United States). The solution was then given via intragastric gavage using flexible, 25-mm feeding needles (Instech Laboratories, Inc., Plymouth Meeting, PA, United States). Sex-matched sham mice were given only the vehicle (10 μ g CT in 200 μ L PBS). On the sixth week, all mice were challenged with 50 mg WP in 200 μ L PBS (Fig. 5). All procedures in this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use was approved by the University of North Dakota Institutional Animal Care and Use Committee (protocol number: 1403–6). Special care was taken to observe the animals after intragastric administration procedures and throughout the experiment for any signs of distress and/or discomfort for prompt remedy or euthanasia to minimize suffering.

4.2. Behavior analysis

Behavioral changes in sham and WP-sensitized mice were evaluated by their innate burrowing activity 48 h after the WP challenge. Plastic cages (38.7-cm wide $\times$ 24.8-cm deep $\times$ 29.2-cm tall) were filled with

5-cm-deep corncob bedding and placed in opaque boxes, and 20 glass marbles 14-mm in diameter were placed on the surface of the bedding in an evenly-spaced grid pattern (rows of 5 and columns of 4). Mice were individually placed in the cages and allowed to explore for 15 min (see Fig. 1 A&B). After the test period, the mice were removed from the test cages and the number of marbles that were buried more than 50% in the bedding was recorded. Each cage was cleaned by removing the surface bedding along with visible feces and disinfecting the cage walls and marbles before placing another mouse. The entire bedding was replaced, and cages were washed thoroughly between male and female testing groups.

4.3. Tissue collection

Animals were sacrificed by CO₂-asphyxiation followed by cardiac puncture 3 days post-WP challenge. Atrial blood was collected for the analysis of the immunoglobulin content in the serum, and the remaining blood was cleared by intracardiac perfusion with phosphate-buffered saline (PBS, pH7.4). The brain was carefully removed and hemisected, and the left hemisphere was immersion fixed in 4% paraformaldehyde in PBS for 2 days at 4 °C. The right hemisphere was immediately placed in Allprotect solution (Qiagen Inc., Valencia, CA, United States) and stored at –20 °C until use. The ileum, located in the caudal third of the small intestine, was collected and fixed in 4% paraformaldehyde in PBS for 2 days at 4 °C.

4.4. Antibodies

Primary antibodies used for immunohistochemistry include the following: Rabbit polyclonal anti-H1R (#AHR-001), anti-H2R (#AHR-002), and anti-H3R (#AHR-003) from Alomone Labs (Jerusalem, Israel) diluted 1:1,000 in blocking buffer for brain and 1:100 for ileum. Biotinylated goat anti-rabbit IgG diluted 1:2,000 was used from Vector Laboratories Vectastain Elite ABC-HRP Kit as a secondary antibody (#LS-J1010, Vector Laboratories, Burlingame, CA, United States).

4.5. Immunohistochemical staining of brain sections

The fixed left brain hemispheres were cryoprotected and frozen-sectioned at 40 μ m as previously described (Nagamoto-Combs et al., 2016) and stored in cryoprotective 0.1 M phosphate buffer (pH 7.4) containing 30% (w/v) sucrose, 30% (v/v) ethylene glycol at –20 °C. Following a 10-min treatment with 0.3% (v/v) H₂O₂ and a 1-hour blocking procedure with PBS containing 0.5% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100, and 10% normal goat serum (Thermo

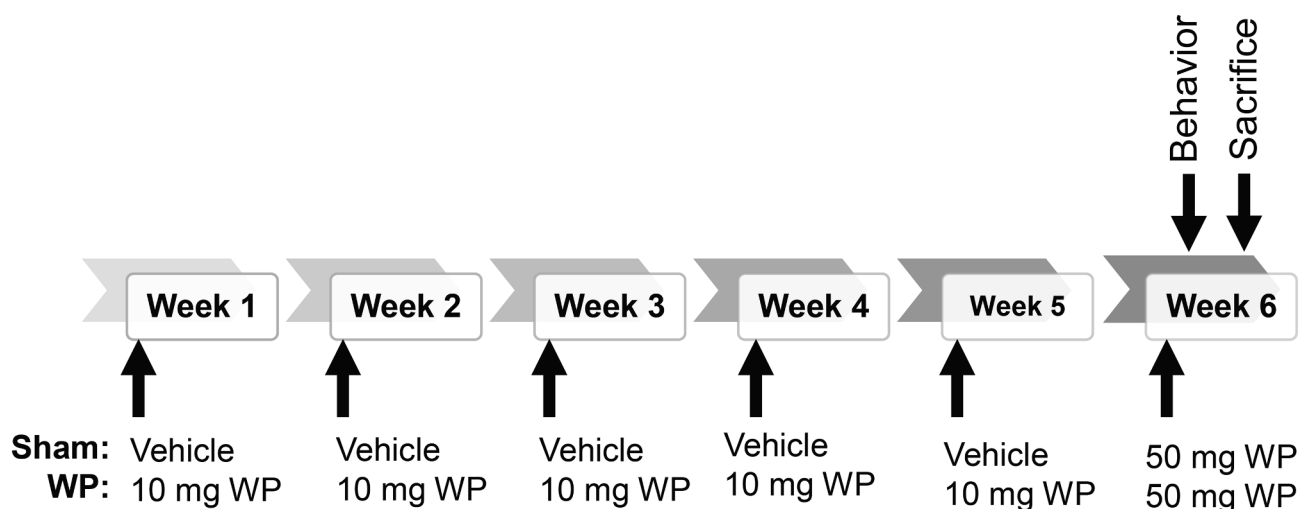


Fig. 5. Schematic diagram of sensitization timeline. The 5-week sensitization period and challenge week are depicted.

Fisher Scientific, Waltham, MA, United States) at room temperature, brain sections were incubated overnight at 4 °C with anti-H3R. Immunoreactivity was visualized using the Vectastain Elite ABC-HRP Kit and Vector VIP Peroxidase Substrate Kit (Vector Laboratories). Brain sections were then mounted on gelatin-subbed glass slides and coverslipped with Permount mounting medium (Fisher Scientific, Hampton, NH, United States) after dehydration through ethanol gradients and clearing with Histo-Clear II (National Diagnostics, Atlanta, GA, United States).

4.6. Immunohistochemical staining of ileal sections

PFA-fixed ileum tissue samples were embedded in O.C.T compound (Sakura Finetek Inc, Torrance, CA, United States) and sectioned at 10 µm on a cryostat. Sections were directly mounted on subbed glass slides and stored at −80 °C until use. For histamine receptor staining, ileal tissues were incubated in 1.0 M HCl for 1 h at 38 °C for antigen retrieval. Tissue sections were allowed to cool to room temperature and rinsed in PBS before treatment in 0.3% H₂O₂ and blocking buffer at room temperature as described above. Tissues were then incubated in anti-H1R, -H2R, or -H3R primary antibody overnight at 4 °C. Immunoreactivity was visualized using the Vectastain Elite ABC-HRP Kit and Vector VIP Peroxidase Substrate Kit (Vector Laboratories). Sections were counterstained with methyl green (MilliporeSigma) for 5 min at 60 °C. Sections were then dehydrated, cleared, and coverslipped.

4.7. Densitometric analysis of H3R immunostaining

Immunoreactivity in brain sections was quantified as previously described (Germundson et al., 2018). Briefly, three H3R-immunostained brain sections (0.7 mm apart) from each animal (n = 3–4) across the areas of interest were digitally photographed using an Olympus BX60 microscope Olympus America Inc., Center Valley, PA, United States) and a SPOT RT Slider camera (Diagnostic Instruments, Inc., Sterling Heights, MI, United States). The captured digital photographs were converted to gray scale images, and each area to be quantified was defined using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, United States). The average gray value in each defined area was divide by the total area as the optical density (OD).

4.8. RNA isolation

The right brain hemispheres stored in Allprotect were dissected into the following 4 regions: 1) the frontal cortex and the underlying striatum; 2) the parietal cortex, including the hippocampus; 3) thalamic and hypothalamic areas; and 4) the midbrain (Fig. 3A). Each brain region was homogenized in 500 µL of TRIzol reagent (Thermo Fisher) using 400 mg of zirconium oxide beads and a Bullet Blender tissue homogenizer (Next Advance, New York, NY, United States). Total RNA extracted from each sample was resuspended in 30–50 µL RNase-free H₂O and quantified using a NanoDrop One spectrophotometer (Thermo Fisher). RNA samples were stored at −80 °C until use.

4.9. Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Reverse transcriptase (RT) reactions were carried out from the extracted RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, United States) per the manufacturer's protocol. qPCR reactions were performed using primers obtained from Bio-Rad for H1R (qMmuCED0050826), H2R (qMmuCED0039779), H3R (qMmuCID0007235), and HDC (qMmuCID0011200), with ribosomal 18S rRNA (qMmuCED0005077) as the reference gene. PrimePCR™ SYBR® Green Assay (Bio-Rad) was used for the amplification and fluorescent labeling of gene transcripts. The amplification reactions

were run at 95 °C for 30 sec, 40 cycles of 95 °C for 5 sec, and 60 °C for 30 sec on a CFX96 Touch™ Real-Time PCR Detection System thermal cycler (Bio-Rad). Quantification cycle (Cq) values generated for the target genes were normalized to the 18S Cq values from corresponding RNA samples. Relative gene expression was calculated as $2^{-\Delta Cq}$, where $\Delta Cq = Cq_{\text{target}} - Cq_{18S}$. The fold change of relative expression for a given target gene was determined by dividing the average Cq_{target} value for WP-sensitized mice over the value for sham for each brain region.

4.10. Statistical analysis

Differences in marble burying behavior between sexes and sensitization groups were statistically compared by two-way ANOVA with Bonferroni's multiple comparison test. Differences in densitometric analysis and relative gene expression between sham and WP-sensitized groups were compared by unpaired parametric Student's *t*-test (GraphPad Prism 8). A *p* value less than 0.05 was considered statistically significant. Differences in H3R mRNA expression between the 4 brain regions and sensitization groups were compared using Bonferroni's multiple comparison test correcting for 4 comparisons, considering adjusted *p* values less than 0.0125 significant. For the follow-up comparisons of H1R, H2R, and HDC expression in each brain region, Fisher's LSD multiple comparison was used and a *p* value less than 0.05 was considered significant. All statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, United States).

CRedit authorship contribution statement

Danielle L. Germundson: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Lane P. Vendsel:** Data curation, Formal analysis, Investigation, Writing - original draft. **Kumi Nagamoto-Combs:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

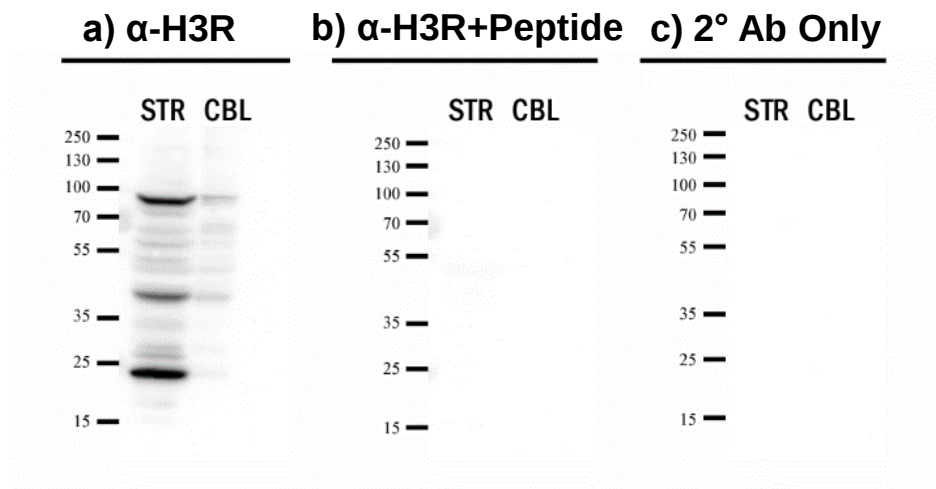
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.brainres.2020.147148>.

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Supplementary Fig 1. Validation of H3R antibody specificity. Western blotting was carried out as described previously¹. Mouse brain tissues from the striatum (STR) and cerebellum (CBL) were homogenized in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄ 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) to extract proteins. Three sets of three lanes, 1) molecular marker, 2) 10 µg STR proteins, and 3) 10 µg CBL proteins, were run on a 10% SDS-polyacrylamide gel. After transferring the resolved proteins on PVDF membrane, the membrane was cut into three sections, each containing the three lanes of molecular markers, STR, and CBL. The membranes were blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 for 1 hour and incubated at 4 °C overnight in (a) 1:1000 dilution of anti-H3R antibody (Alomone AHR-003), (b) 1:1000 dilution of the H3R antibody pre-adsorbed with H3 epitope peptide (Alomone #BLP-HR003) at 1 µg/1 µg antibody for 30 min, or (c) anti-rabbit secondary antibody alone at 1:5000. The signals were detected using chemiluminescent detection system (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Pittsburgh, PA, USA) on a gel documentation system (Aplegen Omega Lum G Gel Documentation System, Gel Company, Inc., San Francisco, CA, USA).

1. Smith N.A., Germundson, D. L., Combs, C. K., Vendsel, L. P., Nagamoto-Combs, K (2019) Astroglia associated with behavioral abnormality in a non-anaphylactic mouse model of cow's milk allergy. *Front. Cell. Neurosci.*, 13(320). <https://doi.org/10.3389/fncel.2019.00320>