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## Histamine regulation of microglia: gene-environment interaction in the regulation of central nervous system inflammation

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### Abstract

Microglia mediate neuroinflammation and regulate brain development and homeostasis. Microglial abnormalities are implicated in a range of neuropsychiatric pathology, including Tourette syndrome (TS) and autism. Histamine (HA) is both a neurotransmitter and an immune modulator. HA deficiency has been implicated as a rare cause of TS and may contribute to other neuropsychiatric conditions. *In vitro* studies suggest that HA can regulate microglia, but this has never been explored *in vivo*. We used immunohistochemistry to examine the effects of HA deficiency in *histidine decarboxylase (Hdc)* knockout mice and of HA receptor stimulation in wild-type animals. We find HA to regulate microglia *in vivo*, via the H4 receptor. Chronic HA deficiency in *Hdc* knockout mice reduces ramifications of microglia in the striatum and (at trend level) in the hypothalamus, but not elsewhere in the brain. Depletion of histaminergic neurons in the hypothalamus has a similar effect. Microglia expressing IGF-1 are particularly reduced. However, the microglial response to challenge with lipopolysaccharide (LPS) is potentiated in *Hdc* knockout mice. Genetic abnormalities in histaminergic signaling may produce a vulnerability to inflammatory challenge, setting the state for pathogenically dysregulated neuroimmune responses.

### Keywords

basal ganglia; H4R receptor; IGF-1; lipopolysaccharide

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### CONFLICT OF INTEREST

The authors have no conflict of interest of relevance to this work to disclose.

## INTRODUCTION

Histamine (HA) is produced both by immune cells and by a group of neurons in the posterior tuberomammillary nucleus of the hypothalamus; in the central nervous system it functions as a neurotransmitter, with diverse functions in different circuitries (1). Histamine receptors are prominent in many brain regions, especially in the cortico-basal ganglia circuitry (1). Dysregulation of brain histamine is increasingly appreciated as a potential contributor to neuropsychiatric disease (2). For example, disruption of histamine biosynthesis has been implicated as a rare genetic cause of Tourette syndrome (TS) (3-6). Abnormalities in histaminergic signaling have also been hypothesized to contribute to Parkinson's disease (7), Huntington's disease (8, 9), Alzheimer disease's (10, 11), narcolepsy (12, 13), and drug addiction (1, 2).

Peripheral HA is an important modulator of immune and allergic responses (e.g. 14, 15). Dysregulated neuroinflammation has been proposed to contribute to TS and a variety of other neuropsychiatric conditions (16-18), although these connections remain unproven in most cases. The possible role of HA as a regulator of neuroinflammatory processes in the brain has received scant attention.

Microglia are the primary inflammatory cells in the brain; inflammatory challenge, such as administration of lipopolysaccharide (LPS), induces them to produce inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). More recently it has been appreciated that microglia are required for normal brain development, homeostasis, synaptic function, and adult neurogenesis, even in the absence of inflammation (19-21). Consistent with this variety of functional roles, microglia can take on a range of phenotypes. Insulin-like growth factor 1 (IGF-1) is an important mediator of homeostatic microglial function. IGF-1 is upregulated in neuroprotective microglia induced by T<sub>H</sub>2 cytokines such as IL-4 (22). Support by IGF-1 expressing neurotrophic microglia is required for survival of cortical neurons during postnatal development (22), for neurogenesis induced by environmental enrichment and exercise in the adult (23, 24), and for neuroprotection after ischemic injury (25, 26). IGF-1-positive microglia also serve a neuroprotective role in animal models of neurodegenerative diseases such as amyotrophic lateral sclerosis (27) and retinitis pigmentosa (28).

Microglial dysregulation is seen in a variety of neuropsychiatric conditions, including TS, although whether such dysregulation is central to pathophysiology remains to be established (18, 29-31). Inflammation-related genes have been found to be upregulated in postmortem brain from individuals with TS (31-33), and microglia in postmortem tissue have morphological hallmarks of activation, though they are normal in number (31). A recent positron emission tomography study similarly suggested increased microglial activation in patients (30). In mice, knockout of the *HoxB8* gene, which is expressed in a subset of microglia, leads to microglial deficits and a marked increase in grooming behavior; similar grooming phenotypes have been described in other animal models of TS (34), obsessive compulsive disorder (OCD) (35-37), and autism (21, 38). Strikingly, these behavioral manifestations of the *HoxB8* knockout have been reported to be rescued by repopulation of the brain with wild-type microglia (39).

These observations led us to investigate the role of histamine in the regulation of microglia. A few *in vitro* studies have attempted to address this question, but much is unclear. In N9 cells, a microglia-like cell line, HA can induce migration, through activation of the H4 histamine receptor (but not the H1 receptor) (40). N9 cells, like brain microglia, produce pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  when stimulated with LPS. When they are stimulated by HA, however, N9 cells do not produce these pro-inflammatory cytokines – in fact, HA blocks LPS-induced N9 cell motility and IL-1 $\beta$  secretion. This leads to the hypothesis that HA might oppose the pro-inflammatory activation of microglia – a role that has also been suggested for other aminergic neurotransmitters (41). Similar findings have recently been reported in microglial cultures (42).

In contrast, other reports using primary microglial cultures from neonatal rat brain suggest that HA can have a pro-inflammatory effect. In one recent study, Dong et al (43) found that HA activated microglia *in vitro* in a dose- and time-dependent manner. Activation was mediated by both the H1 and the H4 receptors and led to the production of the inflammatory cytokines interleukin-6 (IL-6) and TNF- $\alpha$ . Zhu and colleagues reported similar results, also in primary microglial cultures (44). HA has been reported to induce nitric oxide synthase (iNOS), another marker of an inflammatory phenotype, in cultured microglia (45).

These divergent results may derive from the fact that all of these studies have been performed *in vitro*. *In vitro* studies have examined microglia isolated from neonates; even if they accurately recapitulate events *in vivo* in pups, this may not extrapolate to adult animals. Microglia in the adult have a less inflammatory expression profile than those seen in neonates or aging animals (46). In addition, such preparations eliminate the multitudinous cell-cell interactions that, it is increasingly recognized, fine-tune microglial phenotype and function *in vivo*. It remains unclear, therefore, how histamine modulates microglial activation *in vivo*, and whether this varies depending on brain region or animal state. We therefore used genetic and pharmacological tools to examine the regulation of microglia by HA in the intact brain.

## MATERIALS AND METHODS

### Mice

All experiments were conducted under the auspices of the Yale Institutional Animal Care and Use Committee, in accordance with NIH guidelines. Adult male mice aged 2-4 months were used for all experiments. C57Bl/6 wild-types were purchased from Jackson Laboratories ([www.jax.org](http://www.jax.org)). *Hdc*-KO mice have been previously described (3, 47); the mice used in these experiments were backcrossed onto C57Bl/6 for 9 generations. Knockout mice were generated by intercrossing heterozygotes in our vivarium; WT littermates were used as controls. Targeted depletion of histaminergic neurons was performed in *Hdc*-cre transgenic mice (48), which were obtained from Jackson Laboratories ([jaxmice.jax.org/strain/021198](http://jaxmice.jax.org/strain/021198)) and backcrossed with C57Bl/6J mice in our vivarium. HDC-Cre negative littermates were used as negative controls in these experiments.

## Drugs

Histamine dihydrochloride and VUF8430 dihydrobromide were purchased from Sigma Aldrich. 4-methylhistamine dihydrochloride, 2-pyridylethylamine dihydrochloride and JNJ 10191584 maleate were purchased from Tocris Biosciences. Lipopolysaccharide from *Escherichia coli* (055:B5) was purchased from Santa Cruz Biotechnology. Except for JNJ 10191584, which was dissolved in 1% carboxymethylcellulose (Calbiochem), all the drugs were dissolved in sterile 0.9% sodium chloride.

## Surgery

Intracerebroventricular (ICV) drug infusion was performed under anesthesia using standard sterile stereotaxic technique. Animals were anesthetized with ketamine:xylazine 100:10 mg/kg. An infusion syringe was inserted into the lateral ventricle at coordinates  $-0.70$  mm anteroposterior, 2 mm dorsoventral, and 1.4 mm mediolateral (relative to bregma, using the atlas of Paxinos (49)). Drugs were infused in a total volume of 1  $\mu$ l using an UltraMicroPump III (World Precision Instruments Inc) at a rate of 100 nl/min.

For specific depletion of HDC cells in the tuberomammillary nucleus (TMN), we used an AAV5-flex-taCasp3-TEVp virus (50) ( $1 \times 10^{12}$  viral particles, University of North Carolina Vector Core). Viral infusion injections were performed bilaterally in the TMN, at coordinates  $-2$  mm anteroposterior,  $-5.2$  mm dorsoventral,  $\pm 0.6$  mm mediolateral. A volume of 0.5  $\mu$ l was injected in each hemisphere (1  $\mu$ l total) at a flow rate of 100 nl/min (51).

## Perfusion and tissue preparation

For histology, animals were deeply anesthetized using ketamine/xylazine and transcardially perfused with 0.5-1 vol/weight PBS followed by 1-2 vol/weight of 4% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed in 4% PFA overnight, then transferred to sucrose 15% and 30% successively. Brains were sliced using a cryostat; 30  $\mu$ m sections were collected and stored floating in cryoprotectant solution (30% glycerin + 30% ethylene glycol in PBS) at 4°C until use.

## Immunohistochemistry and bright field microscopy

Brain slices were washed thoroughly with PBS. Endogenous peroxidases were inactivated with Bloxall blocking solution (Vector Labs). Nonspecific staining was reduced by incubation of samples with 3% normal goat serum in PBS + 0.1% Triton X100. Sections were then incubated overnight at 4°C with anti-Iba1 primary antibody (Wako, Japan) diluted 1:500 with 3% normal goat serum in PBS + 0.1% Triton X100. Samples were washed and then incubated with biotinylated goat anti-rabbit antibody (Vector Labs) in PBS + 0.1% Triton X100 with 2% normal goat serum for 1 hour at room temperature. Brain slices were washed again and developed using the Vectastain Elite ABC kit and diaminobenzidine (DAB; Vector Labs). Slices were mounted on charged slides, allowed to dry completely, counterstained with Nuclear Fast Red (Vector Labs), dehydrated through graded alcohol washes followed by CitriSolv (CitraSolv), and then mounted and coverslipped using D.P.X. (Sigma Aldrich). All sections compared within each experiment were processed and stained in parallel to ensure comparability.

Mounted sections were visualized using a Leica DM1000 microscope. Images in the middorsal striatum were collected at 200 $\times$ . Several images were collected for each mouse, but only one field was collected for each section, to prevent overlap. For microglial cell counts, cells were manually counted with the Photoshop counter tool in each image by an investigator blind to experimental condition, and averaged across all sections from that mouse. Total optical density analysis was performed using the same images, using an automated procedure in ImageJ software, as illustrated in Supplemental Figure 1.

### Immunofluorescence and confocal microscopy

Free-floating sections were washed with PBS, treated with Bloxall Solution, washed again, incubated in 5% normal donkey serum solution in PBS 0.3% Triton X100 for 1 hour, and then incubated overnight with goat polyclonal anti-IGF-1 1:50 (R&D Systems) and rabbit anti-Iba1 1:500 (Wako, Japan). After 3 washes, slices were incubated with Cy3-conjugated donkey anti-goat antibody and Alexa 488-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch), both at 1:400, for an hour at room temperature. Samples were washed again and mounted in Vectashield HardSet Mounting Medium (Vector Labs) and analyzed by confocal microscopy.

Confocal imaging was performed by sequential scanning of slices using an Olympus Fluoview FV-1000 confocal microscope, using a Kalman filter with an acquisition speed of 4 pixel/sec. 15  $\mu$ m z-stacks were generated by using a fixed step size of 0.5  $\mu$ m.

Quantification of double-positive cells was performed blind to genotype by counting fluorescent cells above background in the striatum bilaterally in multiple views from multiple slices for each animal; all counted slices were averaged for each animal for analysis.

For counting of *Hdc*-positive cells in TMN-depleted animals, coronal slices samples were washed and incubated for 1 hour in 5% normal donkey serum (Jackson ImmunoResearch), and then incubated ON at 4 $^{\circ}$ C with rabbit anti-HDC (1:500, Acris). For secondary detection an Alexafluor555 donkey anti rabbit antibody (1:400, Life sciences) was used. Confocal imaging was performed as described above. Quantification was performed by counting single positive cells above background in the TMN bilaterally without overlap. Four sections per mouse were used per experiment.

### *In situ* hybridization

*In situ* hybridization was performed as described previously (52). Briefly, probes were generated by *in vitro* transcription from templates amplified by PCR from mouse cDNA. See Supplementary Table 1 for primer sequences. PCR products were verified by sequencing. Radiolabeled probes were generated using the MAXIscript kit (Ambion) with T7 polymerase and [ $^{35}$ S]rCTP, purified through a mini Quick Spin RNA Columns (Roche) and hybridized [hybridization buffer: 50% (v/v) formamide, 3 $\times$ SSC, 50 mM NaPO $_4$ , 10 mM dithiothreitol, 1 $\times$ Denhart's, 0.25 g/l tRNA, 10% dextran SO $_4$ ] to post-fixed (4% PFA) coronal sections mounted on glass slides. Following overnight hybridization at 55 $^{\circ}$ C, slides were washed several times with increasingly dilute SSC buffer and then rinsed in dH $_2$ O, dehydrated in 100% ethanol, air dried for several hours and exposed to autoradiographic film

(Amersham Hyperfilm MP, GE Healthcare Life Sciences). Images were captured using a computer-controlled digital camera (Cohu Inc.) and imported into ImageJ for densitometric analysis of the signal of antisense probes. No signal was detected with sense sequence controls in the regions of interest for H1 or H4 genes.

### Real Time rt-PCR

Unfixed whole striatum samples were quickly dissected immediately after euthanasia and frozen in liquid nitrogen. For quantification of cytokines after LPS challenge, mice were injected approximately 4 hours before dissection with 1 mg/kg LPS (53). Tissue was homogenized in 1 ml of Trizol reagent (Invitrogen), and RNA was isolated following the manufacturer's instructions. Poly(A)<sup>+</sup> mRNA was purified from total RNA using the PolyATract mRNA Isolation System III (Promega) following the manufacturer's instructions. These samples were subjected to retrotranscription using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Real Time PCR was carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems) using SybrGreen PCR Core Reagent (Applied Biosystems). See Supplementary Table 1 for primer sequences. Absolute amounts of cDNA present on each sample were determined with respective standard curves, and genes of interest were normalized to actin.

### Statistical analysis

Most data were analyzed using t-tests or ANOVAs Prism 6.0d (GraphPad), as detailed in the Results section and figure legends for each analysis. A mixed model analysis in SPSS 19.0 (IBM) was used to analyze LPS response data to allow for differing number of samples across groups (Figure 5). Alpha was set at 0.05 for all analyses. 2-tailed tests were used in most cases; 1-tailed tests were used as appropriate for confirmatory experiments, where detailed in the text. In immunohistochemical experiments in which multiple slices were examined from each animal, data from all slices was averaged before analysis – that is, the unit of analysis was the animal, not the slice.

## RESULTS

### Modulation of microglia by histamine *in vivo*

We evaluated the effect of histamine deficiency on microglial number and activation by examining microglia in the dorsal striatum of *Hdc* knockout mice, which have undetectable levels of histamine in the striatum (3, 47), using anti-Iba1 immunohistochemistry. We focused on the striatum because of our interest in TS, in which striatal abnormalities are well established (54, 55), and because we have previously documented neuronal abnormalities in this structure in these mice (3, 56). Stained microglial processes were less prominent in *Hdc*-KO striatum than in controls (Figure 1A). To quantify this we computed the % area of tissue covered by Iba1-positive cells and processes, using ImageJ (Supplementary Figure 1). This revealed a reduced area covered by Iba1 immunoreactivity in knockout animals. The total number of microglia, on the other hand, was not altered (Figure 1B, C). As the number of microglia cells is not reduced in the *Hdc*-KO mice, and the soma size is qualitatively normal, the decrease of optical density could be attributed to decreased ramifications (Figure 1A). Alterations in Iba1 immunostaining density were not uniform throughout the brain: a trend



towards reduced Iba1 immunostaining density was seen in hypothalamus, where histaminergic neuronal cell bodies are located ( $p = 0.054$ , Figure 1D), but there was no alteration in other regions examined, including motor cortex (Figure 1E) hippocampus, substantia nigra, or globus pallidus (Supplemental Figure 2). This pattern confirms the importance of studying microglial regulation *in vivo*; regional variation in microglial activation would not be apparent in *in vitro* studies.

Much HA in the brain is produced by *Hdc*-expressing histaminergic neurons in the tuberomammillary nucleus (TMN) in the posterior hypothalamus (1). To further confirm the effect of HA deficiency on microglia in the striatum, we selectively depleted histaminergic neurons in the TMN through targeted ablation. A virus that expresses constitutively active Caspase 3 when activated by *cre* recombinase (50) was stereotaxically introduced, bilaterally, into the TMN of *Hdc*-Cre transgenic mice. This produced a ~50% reduction in the number of *Hdc*-expressing cells, relative to wild-type mice injected with the same virus (Figure 1 F, H) (51). Iba1 staining in the striatum showed a normal number of microglia cells but reduced area covered by microglia processes in *Hdc*-ablated mice, compared to controls (Figure 1 G, I, J), paralleling what is seen in *Hdc* knockout mice. This indicates that neurally derived HA, specifically, can regulate microglia.

To test whether increased histamine is able to regulate microglia acutely *in vivo*, we infused HA into wild-type mice, as in a previous study (3). Wild-type adult male C57Bl/6 mice were infused ICV with 20  $\mu\text{g}$  of histamine dihydrochloride in 1  $\mu\text{l}$  sterile saline, or saline alone, under ketamine/xylazine anesthesia. In a pilot experiment, mice were sacrificed 1-14 days later and tissue was stained for Iba1. Histamine increased both microglia number and density of ramifications in the striatum, with an increase apparent as early as 3 days after HA infusion, prominent at 7 days, and persisting at least 14 days (Supplementary Figure 3). We replicated this effect at 7 days and documented a robust increase in both microglial number and Iba1 immunostaining density (Figure 1K, L). The microglial response to exogenous histamine was apparent more broadly than the abnormalities in *Hdc*-KO mice but still showed some regional specificity: increased Iba1 staining was also observed in the hypothalamus, the motor cortex and globus pallidus after HA infusion, whereas substantia nigra and hippocampus showed no response to exogenous HA (Figure 1M, N, and Supplementary Figure 3).

### Microglial regulation by the histamine H4 receptor *in vivo*

HA acts on four receptors (H1-H4), all of which are present in the central nervous system (1). H1 and H4 are expressed on microglia and regulate them in primary culture, but only H4 regulates N9 cells (43, 44). H1 is also present on neurons; H4 is generally believed not to be (1). We quantified the expression of H1 and H4 mRNA in the striatum of *Hdc* knockout mice by *in situ* hybridization. H1 mRNA was not significantly altered in KO mice (Figure 2A, C). H4 mRNA, on the other hand, was significantly decreased in KO mice (Figure 2B, D). These results were confirmed using real time rt-PCR measurement of mRNA in striatal homogenate: H4 mRNA levels were reduced in the *Hdc*-KO mice, but no changes were seen for H1 mRNA (Figure 2E, F).

We next tested the ability of H1 and H4 agonists to recapitulate the regulation of microglia by HA *in vivo* in wild-type mice. H1 and H4 agonists were infused ICV over a range of concentrations in a dose-finding study (Supplementary Figure 4), and then the highest concentration of each was then infused in a larger number of animals in a follow-up experiment. Mice were euthanized 3 days after drug infusion (corresponding to the first marked activation by HA under similar conditions; see Supplementary Figure 3).

The H1 agonist 2-pyridylethylamine has been reported to activate microglia in primary cultures (43) but not in an immortalized microglia-like cell line (40). It produced no effect on either Iba1 staining density or microglial number even at a high dose of 40 ug (Supplementary Figure 4 and Figure 3A).

In contrast, ICV infusion of 40 ug of the H4 agonist VUF 8430 produced an increase in the total Iba1-immunopositive area and in microglia cell number, similarly to HA infusion (Figure 3B; Supplementary Figure 4). Similar results were obtained with another H4 agonist, 4-methylhistamine (4-MH), which produced an increase in total Iba1 staining area (Figure 3B, Supplementary Figure 4). There was a slight increase of Iba1<sup>+</sup> cells after 4-MH, but it did not reach statistical significance (Figure 3B).

We next asked whether H4 antagonism could attenuate the changes produced by HA in microglial cells. Mice were treated systemically with the H4 antagonist JNJ 10191584 (10 mg/kg intraperitoneal) prior to 20 µg ICV HA (as in Figure 1; JNJ 10191584 was given 15 minutes before the induction of surgical anesthesia, approximately 45 minutes before the initiation of the HA infusion). H4 antagonism blocked the effects of HA on microglial cells, as measured by Iba1 staining density (Figure 3C); effects of HA on microglial number were not significant in this experiment.

### HDC-KO mice have reduced IGF1-positive microglia

Microglia can take on a range of phenotypes *in vivo* and can be proinflammatory or neuroprotective. We used rtPCR on acutely dissected striatal homogenate to examine expression of cytokines associated with inflammatory and neuroprotective profiles (Figure 4). IL-1β, TNF-α, IL-6 and iNOS have all been reported to be regulated by HA *in vitro*, though published data have been inconsistent. *In vivo*, between-genotype comparisons did not reveal significant differences in any of these comparisons (Figure 4A). There was a nominal difference suggesting a decrease in the neurotrophin IGF-1, though it did not reach statistical significance (Figure 4B). IGF-1 is an important factor in microglial support for neuronal function and a mediator of microglial neuroprotection in a variety of contexts (22, 23, 27, 57).

IGF-1 is significantly retained in cells and therefore can be examined with cell-type specificity *in vivo* using co-immunofluorescence (Figure 4D). We found the number of IGF1<sup>+</sup>Iba1<sup>+</sup> double-positive cells, as a fraction of all Iba1<sup>+</sup> cells, to be significantly reduced in the *Hdc*-KO mice (Figure 4C, D). This reduction is region-specific: no differences between *Hdc*-KO and WT mice were seen in the motor cortex (Figure 4C).



## Deficiency of histamine results in increased susceptibility to inflammatory challenge

IGF-1 expressing microglia have been proposed to be neuroprotective and to limit inflammatory responses (25, 26). We speculated that the reduction in IGF-1-expressing microglia in *Hdc*-KO mice would lead to a dysregulated response to pro-inflammatory stimulation. Such an effect would be consistent with the inhibitory effect that HA has on the response of N9 cells (40) and neonatal microglial cultures (42) to lipopolysaccharide (LPS) in some studies *in vitro* (though not in all).

We treated HDC-KO and WT mice with LPS at 0.5 and 1.0 mg/kg and evaluated microglial number and activation in the striatum by immunohistochemistry, 7 days later (the peak of HA effects on microglia; see Supplemental Figure 3). Saline-injected KO mice had a reduced density of Iba1, as before (Figure 5A, B). However, microglia in *Hdc*-KO mice responded significantly more robustly to LPS, with a particularly marked effect after 1 mg/kg LPS (Figure 5A, B). This effect was not seen in the effect of LPS on total Iba1-positive cell count (Figure 5C). Overall, these dose-response curves indicate an overexuberant response to inflammatory challenge in mice that lack HA. This differential response of microglia in *Hdc*-KO mice is brain-region specific: the response of microglia to LPS was also exacerbated in the hypothalamus of *Hdc*-KO mice, but not in the motor cortex (Figure 5D).

To corroborate the differential effect of LPS challenge in *Hdc*-KO mice, cytokine mRNA was quantified 4 hr after LPS challenge (1  $\mu$ g/ml). *Hdc*-KO mice showed an exaggerated production of IL-1 $\beta$  in response to LPS compared to WT controls (Figure 5E). A similar trend was seen for TNF- $\alpha$  (Supplementary Figure 5). In contrast, arginase-1, a neuroprotective factor, showed a trend toward reduction after LPS in *Hdc*-KO mice, but not in controls (Supplementary Figure 5).

## DISCUSSION

Microglia, the brain's resident inflammatory cells, have critical roles both in the response to injury or infection and in normal brain development and homeostasis. They are dysregulated in a variety of neuropsychiatric disease states (18), although the causal role of this dysregulation remains to be established in most cases. They are modulated by innumerable neurotransmitters, cytokines, and other factors.

We have examined the modulation of microglia by histamine (HA) *in vivo*, using exogenous HA infusion, chronic histamine deficiency in the *Hdc* knockout mouse, selective removal of the primary source of histamine in the brain (histaminergic neurons in the TMN), and a range of pharmacological manipulations. The potential importance of HA regulation of microglia (45) is highlighted by the fact that both HA and microglial dysregulation are implicated in neuropsychiatric disease states such as Tourette syndrome (TS) and a range of neurodegenerative syndromes. Our finding of microglial abnormalities in the *Hdc* knockout mouse, a validated model of the pathophysiology of TS (Figure 1) (3), further emphasizes the importance of understanding HA's role in regulating microglial function. Previous examination of HA regulation of microglia has been limited to *in vitro* studies of immortalized N9 cells (40) or primary microglial cultures from neonates (42-44). These

studies have found HA to regulate microglia but have produced conflicting data as to the nature of that regulation – specifically, whether HA-stimulated microglia have a pro-inflammatory or neuroprotective phenotype (57) (40). Our study is the first examination of these questions *in vivo*.

Several findings stand out. We confirm that HA can regulate microglia *in vivo*, as manifested by increased total area of Iba1 staining after ICV HA infusion, and decreased Iba1-immunoreactive density in *Hdc*-KO mice. In neonatal cultures, the H1 and H4 receptors have been implicated in microglial activation; the H4 receptor plays a particularly prominent role in immortalized cells (40). Our data indicate that, under these conditions, the H4 receptor is the primary mediator of microglial modulation by histamine: ICV treatment with an H1 agonist, at comparable doses (43), has no significant effect on microglia, and an H4 antagonist blocks the effect produced by ICV histamine (Figure 3).

These *in vivo* effects may result from direct HA action on microglia; alternatively, they may be an indirect result of HA effects on other cell types. The H4 receptor has not been reported to be expressed in striatal neurons or glia, apart from microglia. Most studies have described its expression in the brain as being largely restricted to hematopoietic cells (1); if this is the case, then the effects of H4 agonists (Figure 2) suggest a direct effect on microglia. H4 receptor RNA and protein have been identified in many regions of the brain (58, 59), but cell-type specificity has not been characterized in detail *in vivo*. One study has reported electrophysiological effects of H4 agonists on neurons in somatosensory cortex, suggesting the presence of functional receptors in these cells (58); however, it remains possible that this effect is indirect, via H4 receptors on microglia or other local cell types. There is, to our knowledge, no evidence suggesting the presence of the H4 receptors on astrocytes. H4 expression has, on the other hand, been reported in isolated microglia and in *in vitro* studies of immortalized microglia-like cells (40, 44).

Chronic HA deficiency in the *Hdc* knockout mouse (3) leads to effects opposite to those of exogenous HA administration, across multiple experiments. *Hdc* knockout mice have a normal number of microglia but a reduced density of microglial ramifications, as measured by the total area occupied by Iba1 expression (Figure 1). Interestingly, this effect is also observed in the hypothalamus, but not elsewhere in the brain (Supplementary Figure 2). This pattern correlates with levels of HDC protein, which we have found to be high in hypothalamus (where the histaminergic cell bodies are located) and in striatum, relative to other forebrain structures (60). H4 receptor expression is reduced in *Hdc*-KO mice (Figure 2), suggesting that chronic HA deficiency may also lead to a reduction in the mechanisms of microglial HA responsivity. Selective depletion of *Hdc*-expressing neurons in the TMN induces similar abnormalities, suggesting that neurotransmitter histamine is a key modulator of striatal microglia (as opposed to perivascular mast cells, or autocrine signal from microglia cells themselves).

Our data suggest that HA deficiency makes microglial cells more vulnerable to an inflammatory challenge. HA deficiency in the *Hdc*-KO mouse leads to a reduction in IGF-1 positive microglia (Figure 4). IGF-1-expressing microglia are also induced by T<sub>H</sub>2 cytokines such as IL-4. They have been shown to protect against neural cell death in organotypic

hippocampal cultures (61), to support cortical neurons during development (22), and to promote neurogenesis and preserve hippocampal function in adulthood (23, 24), suggesting a neuroprotective function (57). In pathological contexts, IGF-1 positive microglia protect the brain after ischemic injury and in neurodegenerative conditions (25-28). Their reduction in *Hdc*-KO mice may produce a state of vulnerability.

Consistent with this, the *Hdc*-KO mouse shows an exaggerated microglial response to a pro-inflammatory challenge with LPS (Figure 5). These data suggest an overly exuberant inflammatory response to an immune challenge. Such a model is consistent with *in vitro* findings in an immortalized microglia-like cell line, in which HA was shown to activate N9 cells (by a chemotaxis assay) but to antagonize the activation produced by LPS stimulation (40); similar findings have recently been reported in primary microglial cultures (42). Our *in vivo* findings contrast, however, with other studies in acutely isolated microglia from neonates, which suggest that HA induces a pro-inflammatory phenotype, characterized by production of such cytokines as IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , and oxidative enzymes such as iNOS (43-45).

### Microglial heterogeneity

It is increasingly appreciated that microglia in the brain exhibit remarkable phenotypic heterogeneity (57) and are responsive to a wide range of factors in the milieu (62). Different protocols for microglial isolation, culture, and study may produce markedly different cellular phenotypes and thus divergent molecular and functional results (62); this may explain the divergent reports of the effects of HA on microglia studied *in vitro* (42-44). This reinforces the importance of studying them *in vivo*.

Several other aspects of our findings further emphasize the importance of *in vivo* studies. We find abnormalities in microglia in *Hdc*-KO animals in the striatum and in the hypothalamus, but not elsewhere; such regional variation would be lost in studies of isolated or cultured microglia. Our observation that neuron-derived HA is a key regulator of microglia would, additionally, not be possible *in vitro*. *In vivo* examinations such as we present here are technically challenging, but they are essential to characterize the full panoply of functions of these enigmatic cells in the brain.

We have focused largely on microglia in the adult mouse striatum. HA's regulatory effects may differ in other contexts; the fact that microglial abnormalities are not uniform throughout the brain of *Hdc*-KO mice (Figure 1 and Supplementary Figure 2) supports this. Development may also modulate microglial responses to HA. The ability of HA to produce a pro-inflammatory phenotype in previous studies (43, 44) may be an artifact of *in vitro* analysis in primary cultures, but it might equally well be a result of the fact that those studies were performed on microglia from neonates, not adults. *In vivo* examination of the variation in the ability of HA to regulate microglia across development is an important goal for future work.

One distinction that has garnered substantial recent interest is the distinction between brain-resident microglia, which populate the central nervous system early in development, and macrophage-lineage cells that enter the brain at a later point in ontogeny. We have not

examined this distinction in the current work. The possibility that HA differentially regulates these two populations merits future study.

### **A gene-environment interaction in the regulation of CNS inflammation**

Many aspects of brain function, and most forms of psychopathology, are thought to result from gene-environment interactions, rather than from purely genetic or purely environmental causes. Our finding of differential inflammatory response in *Hdc*-KO mice exemplifies such an interaction. In unchallenged *Hdc*-KO mice, raised with minimal immunological challenge in a virus antigen-free vivarium environment, microglial Iba1 immunoreactivity density is lower than that in controls. However, after immunological challenge, the *Hdc*-KO mice mount an exaggerated response, such that after moderate LPS challenge the Iba1 pattern is reversed, with KO mice showing greater Iba1 expression extent than controls (Figure 5A, B). This is accompanied by increased production of IL-1 $\beta$ , confirming an increased inflammatory response.

This observation is particularly intriguing in the context of the fact that HA deficiency, in the *Hdc*-KO mice and in humans carrying mutations in this and other components of histamine signaling pathways, has been proposed as a potential cause of Tourette syndrome (3-6, 63). TS is well established to be substantially genetic, with recent estimates of heritability centering at around 50% (64). There is also substantial evidence that immunological processes contribute to TS, at least in some cases. Elevated inflammatory microglial markers in the striatum, including Iba1, have been documented in TS, post-mortem (31-33) and in a recent PET imaging study (30). Alterations in peripheral immune markers (65-68) and the response to inflammatory challenge (69, 70) have also been described.

These observations suggest a ‘two-hit’ model of inflammatory dysregulation. A genetic abnormality such as *Hdc* deficiency may produce only subtle deficits in isolation (3) but may create vulnerability for an enhanced or abnormal response to immunologic challenge. A subsequent infectious challenge – rare in mice raised in a virus antigen free vivarium environment but universal in the real world – may lead in such a genetic context to an overly exuberant inflammatory response, such as we see here after LPS challenge (Figure 5). This exaggerated inflammatory response, which resembles that seen in the basal ganglia in TS *post-mortem* (31) and *in vivo* (30), may be an important pathophysiological contributor to disease.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **ACKNOWLEDGEMENTS**

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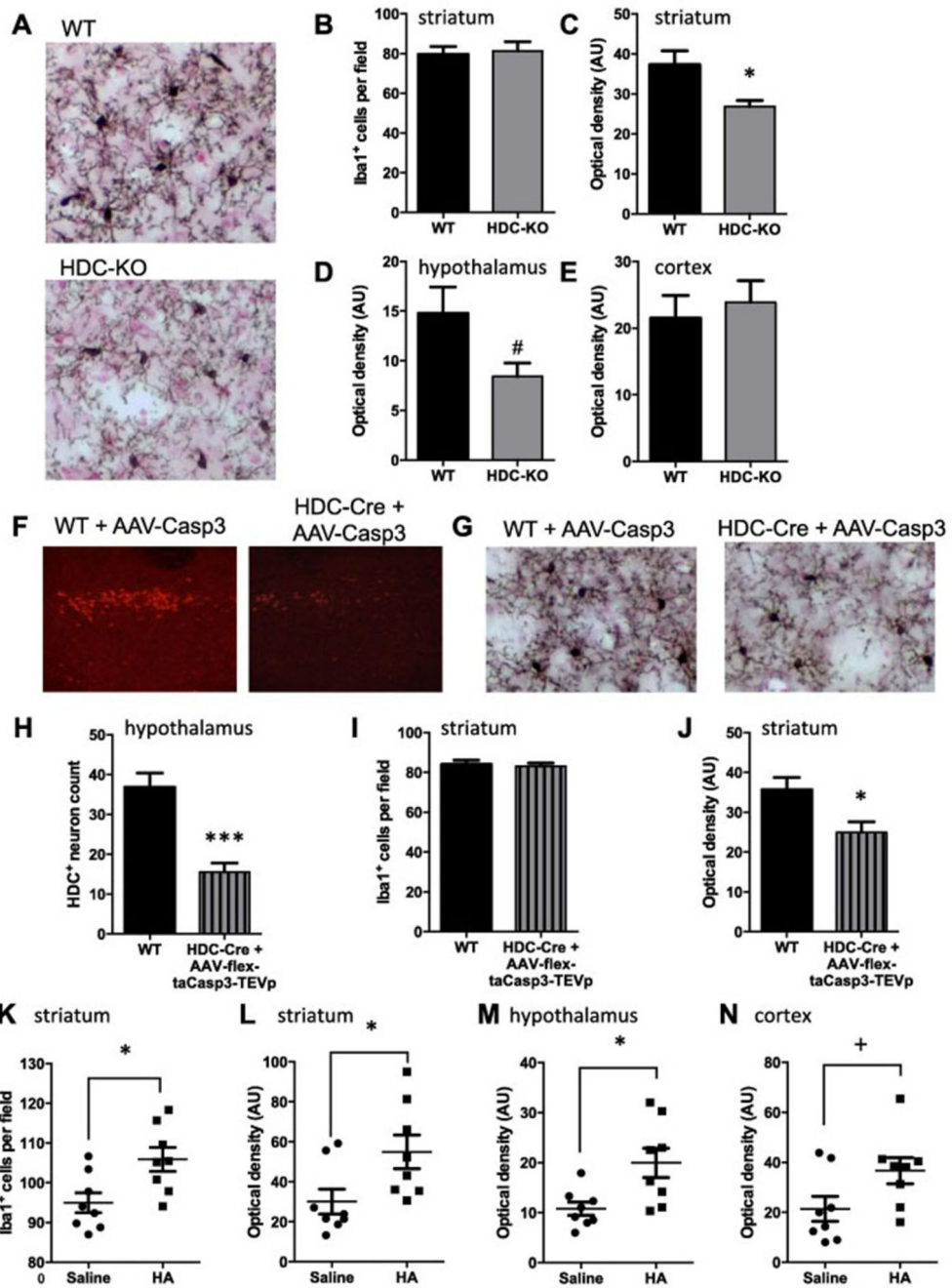


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### HIGHLIGHTS

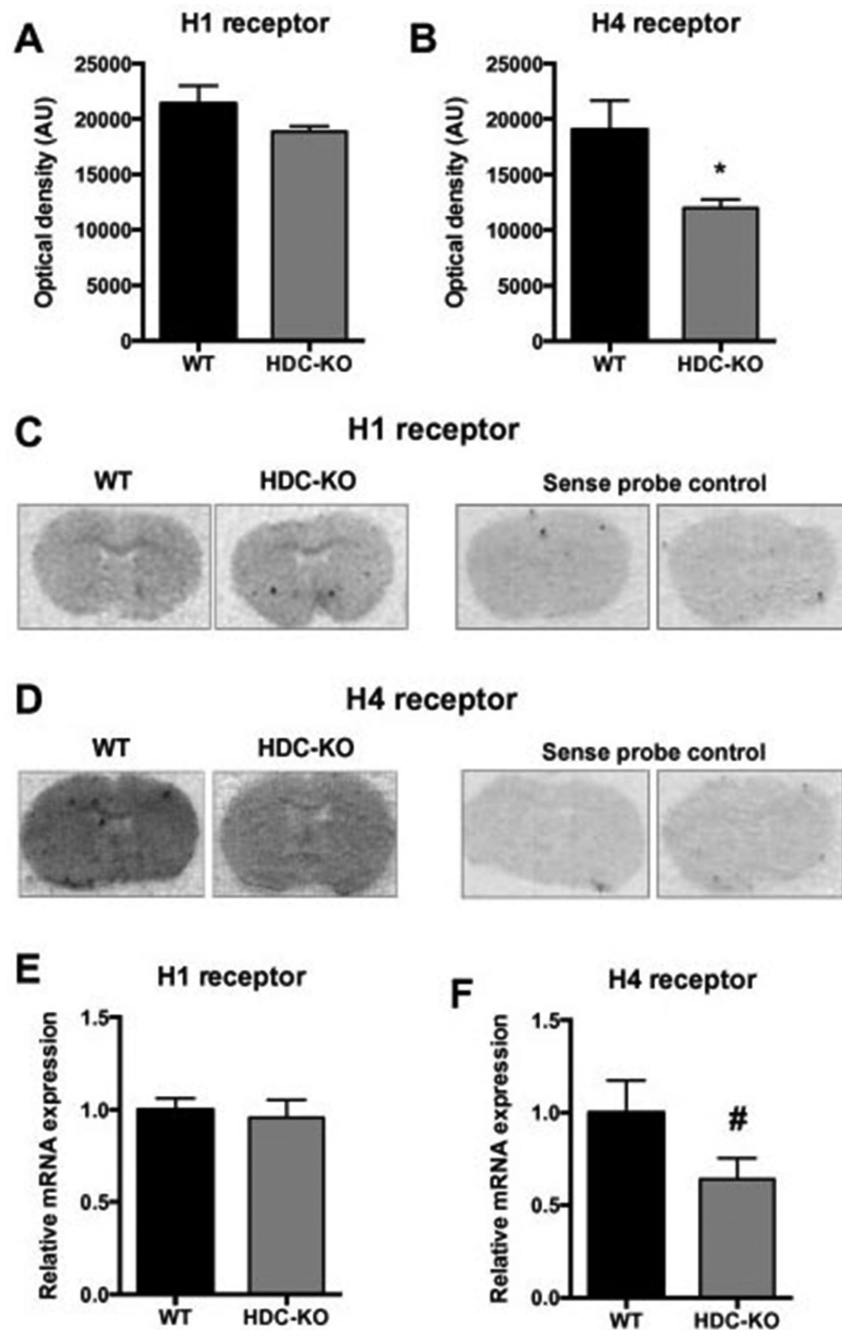
- Histamine regulates microglia *in vivo* in the striatum
- The H4 receptor activates microglia
- Histamine deficiency leads to reduce neuroprotective microglia
- Histamine deficiency leads to a potentiated response to inflammatory challenge.



**Figure 1. Microglial modulation by histamine deficiency or stimulation**

**A.** Representative Iba-1 stained striatal fields from WT and *Hdc*-KO mice, with nuclear fast red counterstaining. **B.** Microglia number was quantified as Iba1-positive cells per high-power field in the dorsal striatum, blind to genotype. (See also Supplementary Figure 2.) There was no difference in total microglia in *Hdc*-KO mice ( $N = 7-9$  mice per group; two-tailed t-test:  $t[14] = 0.26$ ,  $p = 0.8$ ). **C.** Optical density of Iba1 immunostaining in dorsal striatum (see Supplementary Figure 1) was reduced in KO mice ( $t[10.7] = 2.75$  [Welch's correction],  $p = 0.019$ ). **D.** Similar reduction in Iba1 staining was observed in the

hypothalamus: two-tailed t-test:  $t[11.7] = 2.1$  [Welch's correction],  $p = 0.054$ ). **E.** There was no alteration in Iba1 staining in motor cortex in *Hdc*-KO mice (two-tailed t-test:  $t[14] = 0.45$ ,  $p = 0.6$ ; see also Supplementary Figure 2). **F.** HDC-expressing neurons in the TMN were selectively ablated using an AAV5-flex-taCasp3-TEVp virus in *HDC-Cre* mice (50); controls were wild-type mice identically infused with virus. **G.** Depletion of HDC neurons in the TMN produced abnormalities in striatal microglia similar to those seen in *Hdc*-KO mice. **H.** HDC neurons were depleted ~ 50% ( $N = 5-6$  mice per group; two-tailed t-test:  $t[9] = 5.3$ ,  $p = 0.0005$ ). **I.** Striatal microglia cell number was not modified by this treatment ( $N = 5-6$  mice per group; two-tailed t-test:  $t[9] = 0.31$ ,  $p = 0.76$ ). **J.** Optical density of Iba1 was reduced in the striatum after HDC neuronal depletion, similarly to what was observed in *Hdc*-KO mice ( $N = 5-6$  mice per group; two-tailed t-test:  $t[9] = 2.633$ ,  $p = 0.027$ ). **K.** Histamine dihydrochloride (20  $\mu\text{g}$ ) was infused into the lateral cerebral ventricle of C57Bl/6 mice (as in ref. 3); microglial activation was examined by Iba1 immunostaining 7 days later (see Supplementary Figure 3 for other time points). Iba1-positive cells were slightly but significantly increased following HA infusion ( $N = 8$  mice per group; two-tailed t-test:  $t[14] = 2.8$ ,  $p = 0.014$ ). **L.** Optical density for Iba1 staining was more markedly increased after HA (two-tailed t-test:  $t[14] = 2.4$ ,  $p = 0.031$ ). **M.** Iba1 optical density was also increased in the hypothalamus after HA infusion ( $N = 8,8$ ; two-tailed t-test:  $t[23] t[14] = 2.847$ ,  $p = 0.013$ ). **N.** Total Iba1 staining was also increased in the motor cortex of wildtype mice compared to vehicle-treated mice ( $N = 8,8$ ; two-tailed t-test:  $t[14] = 2.116$ ,  $p = 0.053$ ). All values are shown as mean  $\pm$  SEM. \* $p < 0.05$ . # $p = 0.054$ . + $p = 0.053$ .



**Figure 2. Differential expression of histamine receptors in HDC-KO mice**

**A.** H1 receptor, visualized using *in situ* hybridization with a specific riboprobe, was unaltered in *Hdc*-KO mice (N = 3-4 mice per group; two-tailed t-test:  $t[5] = 1.3$ ,  $p = 0.26$ ). Quantification was performed relative to a sense control probe, which showed minimal binding. **B.** H4 receptor expression was decreased in *Hdc*-KO mice (two-tailed t-test:  $t[6] = 2.6$ ,  $p = 0.043$ ). **C, D.** Representative images of H1 and H4 receptor autoradiographies respectively; sense control probes showed minimal binding. **E, F.** H1 and H4 expression was confirmed using rtPCR from dissected striatal tissue from *Hdc*-KO and control mice; again,



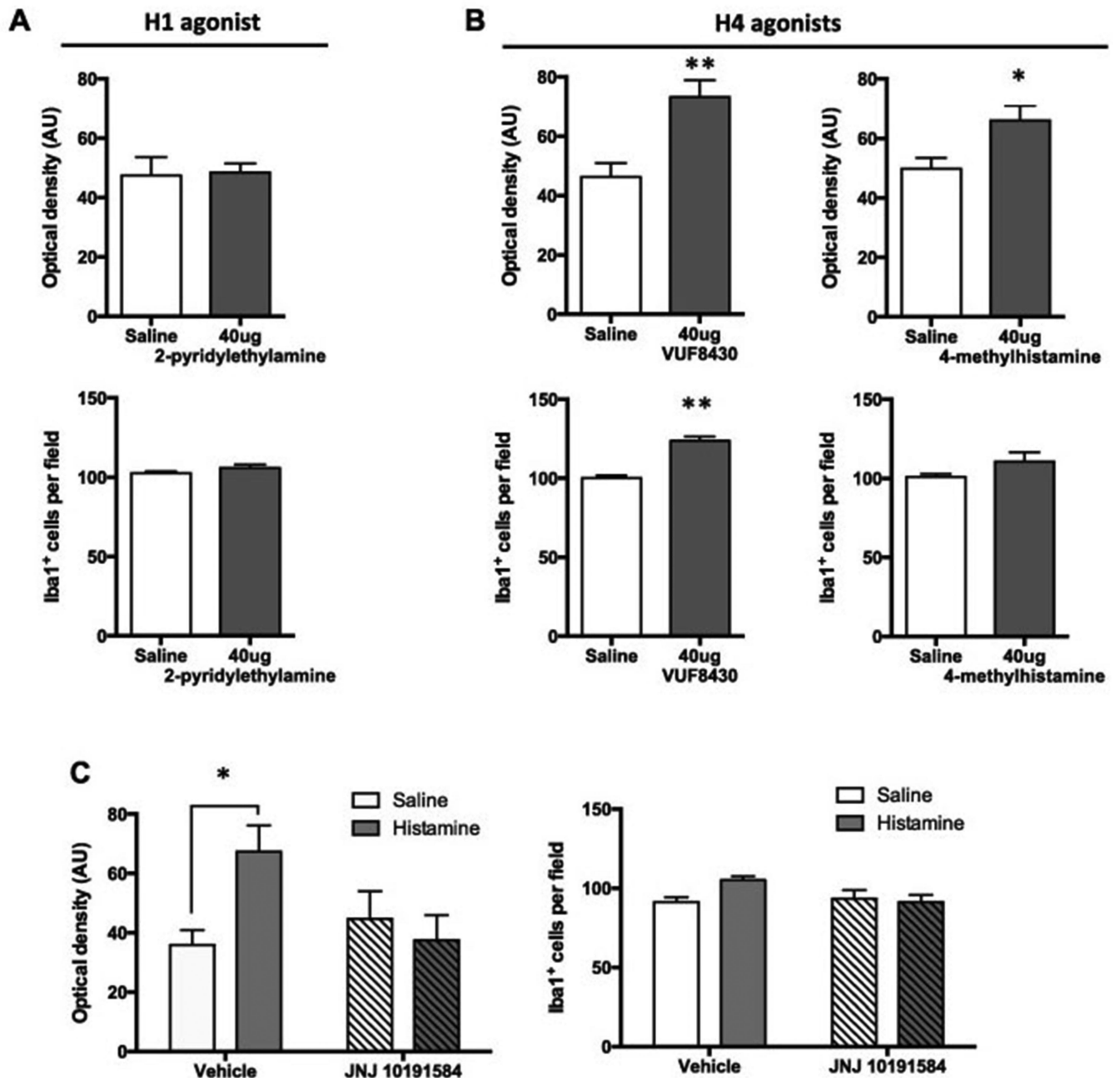
H1 mRNA did not differ between genotypes (N = 10-11 mice per group; two-tailed t-test:  $t[19] = 0.37$ ,  $p = 0.7$ ), but H4 expression was reduced, confirming *in situ* hybridization results (N = 16 mice per group; one-tailed t-test:  $t[30]=1.733$ ,  $p = 0.0467$ ). All values are shown as mean  $\pm$  SEM. \* $p<0.05$  by 2-tailed t-test; #  $p<0.05$  by 1-tailed t-test.

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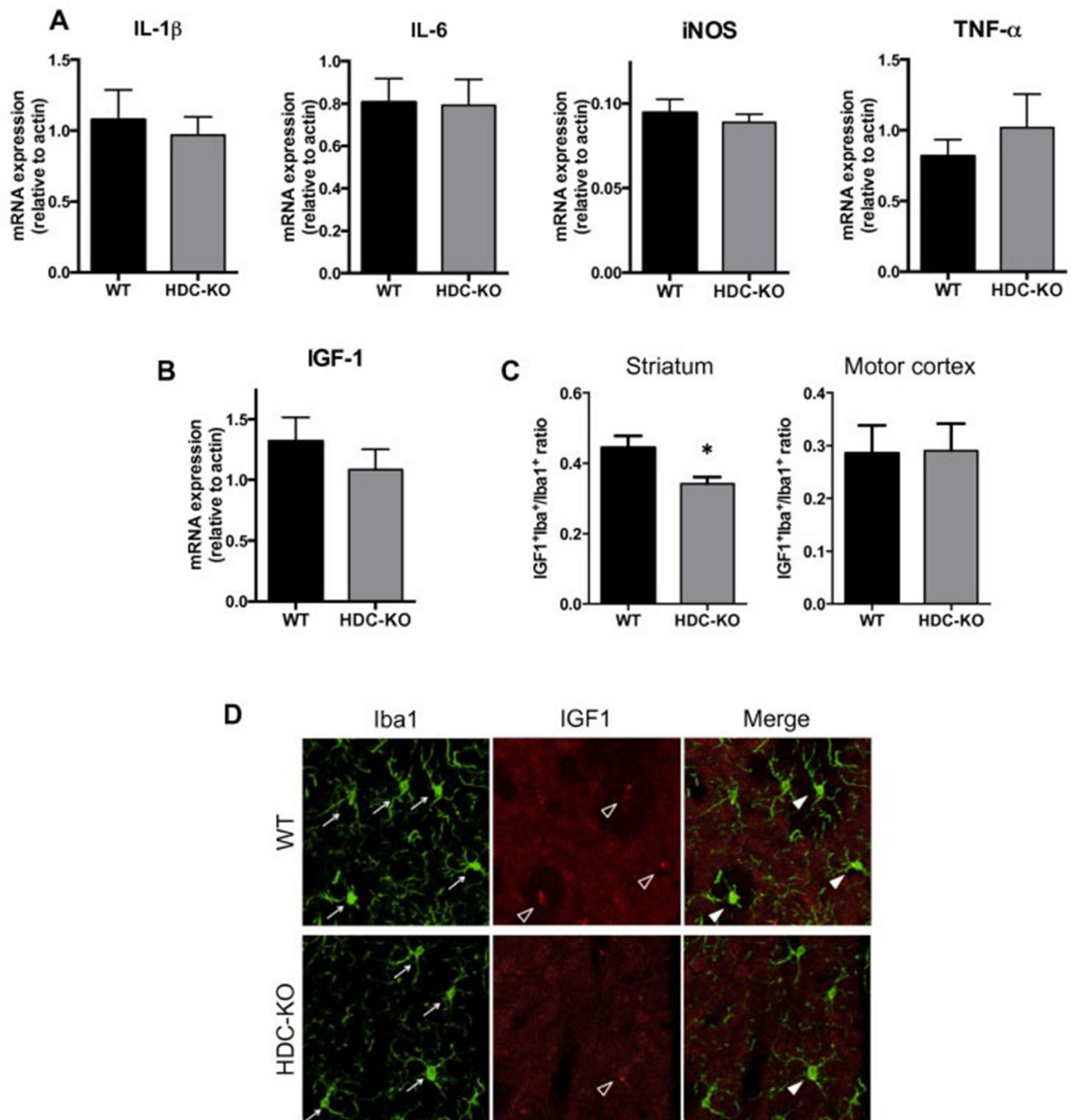
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**Figure 3. Microglial activation *in vivo* is mediated by the H4 receptor**

**A.** The H1 agonist 2-pyridylethylamine, administered ICV, produced no detectable alteration in dorsal striatal microglia 3 days later ( $n = 8,8$ , two-tailed t-test for total density:  $t[14] = 0.15$ ,  $p = 0.89$ ; Iba1<sup>+</sup> cells:  $t[14] = 1.5$ ,  $p = 0.15$ ). **B.** The H4 receptor agonist VUF8430, administered ICV, produced dose-dependent changes in microglial number and activation 3 days later. ( $n = 8-9$  mice per group, two-tailed t-test for total density:  $t[15] = 3.66$ ,  $p = 0.0023$ ; Iba1<sup>+</sup> cells:  $t[15] = 8.02$ ,  $p < 0.001$ ). The H4 agonist 4-methylhistamine showed similar effects on striatal microglia ( $n = 8-9$  mice per group, two-tailed t-test for total density:  $t[15] = 2.702$ ,  $p = 0.016$ ; two-tailed t-test with Welch's correction for Iba1<sup>+</sup> cells:

t[8.408] = 1.6,  $p = 0.15$ ). **C.** H4 antagonism mitigated the HA-induced increase in Iba1 immunostaining [g523]N = 6-7 mice per group; 2-way ANOVA: main effect of histamine,  $F[1,22] = 2.3$ ,  $p = 0.14$ ; main effect of JNJ 10191584,  $F[1,22] = 1.7$ ,  $p = 0.20$ ; interaction:  $F[1,22] = 5.8$ ,  $p = 0.025$ ). H4 antagonism appeared to also mitigate the small HA-induced increase in microglial number, though this interactive effect did not quite reach statistical significance (2-way ANOVA: main effect of histamine,  $F[1,22] = 2.2$ ,  $p = 0.15$ ; main effect of JNJ 10191584,  $F[1,22] = 2.26$ ,  $p = 0.15$ ; interaction:  $F[1,22] = 4.0$ ,  $p = 0.056$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ , main effect of dose.



**Figure 4. Regulation of IGF-1, a putative marker of a neuroprotective microglial phenotype, by HA**

**A-B.** mRNA of inflammatory markers in the striatum of *Hdc*-KO mice, measured from whole tissue by rtPCR. There were no statistically significant differences between groups, but a trend in IGF-1 motivated further examination.  $n = 7,7$ . 2-tailed t-test: IL-1beta,  $t[12] = 0.44$ ,  $p = 0.67$ ; IL-6,  $t[12] = 0.09$ ,  $p = 0.93$ ; TNF-alpha,  $t[12] = 0.76$ ,  $p = 0.46$ ; iNOS,  $t[11] = 0.63$ ,  $p = 0.54$ ; IGF-1,  $t[12] = 0.9$ ,  $p = 0.38$ . **C-D.** IGF-1 expression in microglial cells was detected by double immunofluorescence for Iba1 and IGF-1, followed by confocal

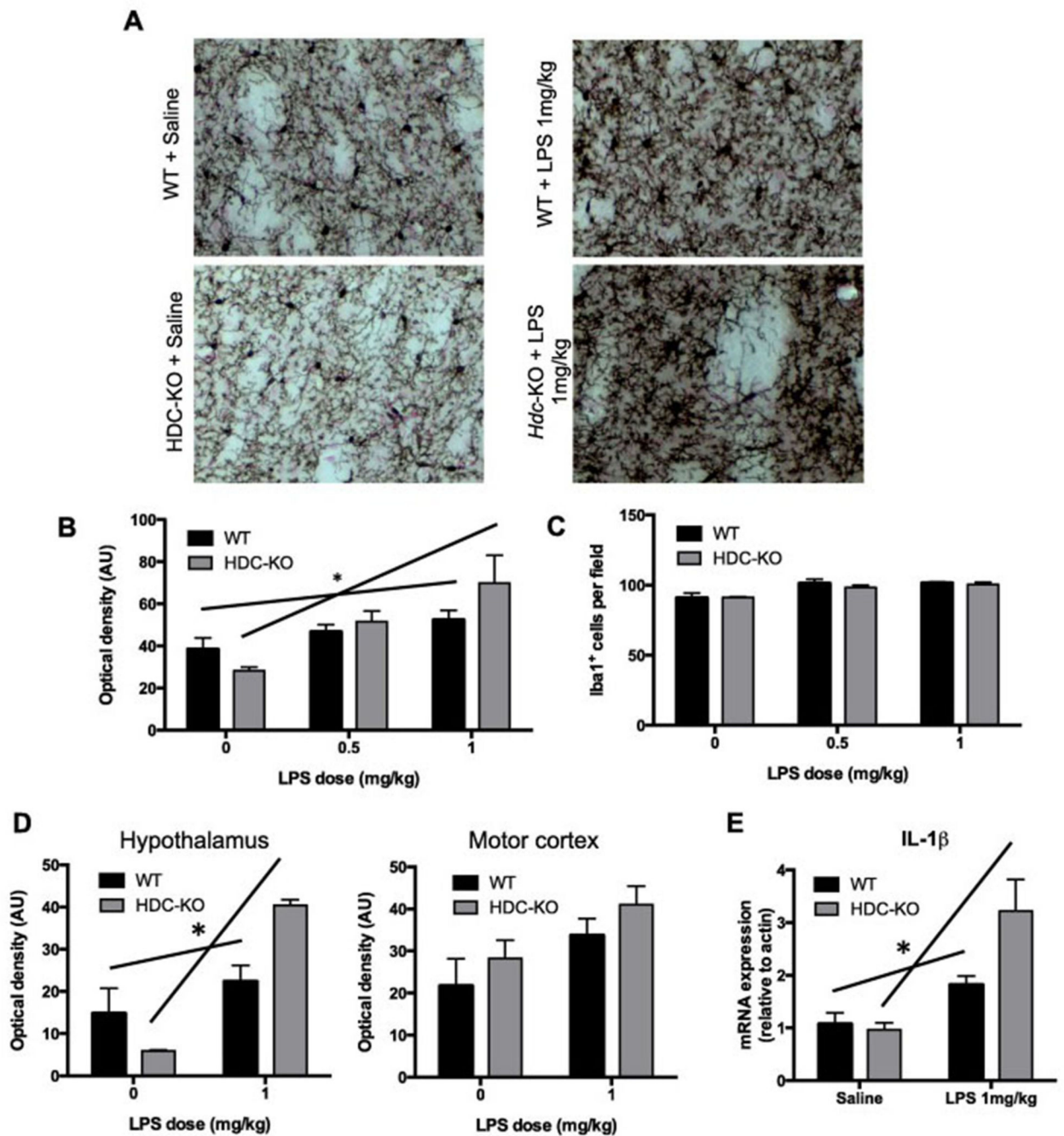
microscopy and quantification of IGF-1 expressing microglia as a subset of total Iba1 positive cells. IGF-1<sup>+</sup>Iba1<sup>+</sup> cells, as a proportion of total Iba1<sup>+</sup> cells, were decreased in the dorsal striatum of *Hdc*-KO mice relative to WT sibling controls (N = 5 WT, 4 KO;  $t[7] = 2.5$ ,  $p = 0.043$ ). No difference was found in the motor cortex ( $t[7] = 0.064$ ,  $p = 0.95$ ). All values shown as mean  $\pm$  SEM. \* $p < 0.05$ .

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**Figure 5. Increased susceptibility of *Hdc*-KO mice to LPS challenge**

**A.** *Hdc*-KO mice and wild-type littermate controls were injected IP with saline or various doses of LPS (0.5 to 1 mg/kg); microglial activation in the dorsal striatum was examined by Iba1 immunostaining 7 days later. **B.** The density of Iba1 immunostaining was disproportionately increased by LPS in a dose-dependent fashion in *Hdc*-KO mice (N = 3-4 mice per group; mixed model: main effect of LPS,  $F[1,18] = 18.9$ ,  $p < 0.0001$ ; main effect of genotype,  $F[1,18] = 1.4$ ,  $p = 0.25$ ; genotype  $\times$  LPS interaction,  $F[1,18] = 4.7$ ,  $p = 0.043$ ). The relationship between LPS dose and Iba1 staining density was significant for WT mice ( $r$



= 0.612,  $p = 0.045$ ) but much more significant for KO animals ( $r = 0.776$ ,  $p = 0.005$ ). **C.** The density of microglial cells was increased in a dose-dependent fashion, with no difference between genotypes (mixed model: main effect of LPS:  $F[1,18] = 22.972$ ,  $p < 0.001$ ; main effect of genotype:  $F[1,18] = 0.037$ ,  $p = 0.85$ ; genotype  $\times$  LPS interaction:  $F[1,18] = 0.09$ ,  $p = 0.76$ ). **D.** Microglial response to LPS was also enhanced in the hypothalamus of *Hdc*-KO mice ( $N = 4,4$ ; 2-way ANOVA: main effect of LPS,  $F[1,12] = 35.27$ ,  $p < 0.0001$ ; main effect of genotype,  $F[1,12] = 1.615$ ,  $p = 0.2278$ ; genotype  $\times$  LPS interaction:  $F[1,12] = 14.38$ ,  $p = 0.0026$ ), but not in the motor cortex ( $N = 4,4$ ; 2-way ANOVA: main effect of LPS,  $F[1,12] = 6.519$ ,  $p = 0.025$ ; main effect of genotype,  $F[1,12] = 2.03$ ,  $p = 0.18$ ; genotype  $\times$  LPS interaction:  $F[1,12] = 0.007$ ,  $p > 0.9$ ). **E.** IL-1 $\beta$  mRNA showed a greater response in *Hdc*-KO mice after 1 mg/kg LPS challenge, corroborating the differential microglial response ( $n = 7,7$ ; 2-way ANOVA: main effect of LPS,  $F[1,24] = 20.20$ ,  $p = 0.0002$ ; main effect of genotype,  $F[1,24] = 3.717$ ,  $p = 0.066$ ; genotype  $\times$  LPS interaction:  $F[1,24] = 5.080$ ,  $p = 0.034$ ). See also Supplementary Figure 5. \*  $p < 0.05$  genotype  $\times$  dose interaction.