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Short communication

# The transcription factor Hhex regulates inflammation-related genes in microglia

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ABSTRACT

Microglia have diverse physiological and pathological functions. However, the transcriptional mechanisms remain elusive. Here we sought new transcription factors relevant to microglial functions from the microglial transcriptome of stressed mice and evaluated their roles in primary microglia. TLR2 and TLR4 agonists increased Rel, Atf3, and Cebpb and decreased Hhex in primary microglia as repeated social defeat stress. Although Hhex was not studied in microglia, TLR2 and TLR4 agonists decreased Hhex, and Hhex overexpression attenuated TLR4-increased expression of inflammation-related genes. These findings suggest that Hhex negatively regulates inflammation-related genes in microglia and that TLR2/4 activation reduces Hhex, facilitating TLR4-mediated neuroinflammation.



Microglia are immune cells in the brain.[1] Historically, the roles of microglia have been characterized in the pathology of brain injury and neurological disorders accompanied by neuronal and glial death.[2] In these devastating conditions, microglia are activated by cellular debris, secrete proinflammatory substances, and exacerbate neuronal damages. The discovery of microglial functions in pain and dynamic microglial morphology in the resting state have sparked the scrutiny of their physiological processes.[3,4] Now it is accepted that microglia play physiological roles in negatively and positively regulating synaptic and neuronal activity. Alongside, clinical studies with patient samples and basic studies with rodent models suggested microglia as an essential player for the pathology of mental illnesses such as depression.[5,6]

Despite diverse microglial functions in physiological and pathological processes, how microglia adapt their properties to each of these functions remains elusive. Transcriptome analyses of microglia revealed distinct gene expression profiles of neuropathic pain and neurological disorders.[7,8] Some of these studies identified essential transcription factors in each disease context. However, the current knowledge is still short of explaining the diversity of microglial functions, especially in physiological conditions.

Repeated social defeat stress (R-SDS) that has been used to study depression pathology increases the expression of histological markers for microglial activation in the medial prefrontal cortex (mPFC) and not in the nucleus accumbens (NAc).[6] The innate immune receptors Toll-like receptor (TLR) 2 and TLR4 mediate this microglial activation, and TLR2/4 in mPFC microglia are essential for the development of depression-related behavior. Here we sought new transcription factors relevant to microglial functions from the microglial transcriptome of socially defeated mice and evaluated their roles in primary microglia.

To this aim, we first reanalyzed the previously published DNA microarray data of microglia isolated from the mPFC and NAc of wild-type and TLR2/4-deficient mice with or without R-SDS.[6] We extracted differentially expressed genes (DEGs) with more than a 2-

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fold increase or decrease with R-SDS. We enriched transcription factors with gene ontology terms and identified 17 transcription factors in the DEGs (Fig. 1). Among these, we focused on the genes differentially expressed between wild-type and TLR2/4-deficient mice in the mPFC and/or NAc. Hmbox1 was excluded as its multiple probes yielded inconsistent results. Thus, we chose 11 transcription factors Tfdp2, Klf4, Rel, Atf3, Tsc22d3, Cebpb, Tgif1, Elk3, Zkscan17, Hhex and Batf3 for further analyses. Consistent with mPFC specificity in stress-induced microglial activation in histology, several transcription factors preferentially increased (e.g., Tfbp2, Klf4, Cebpb) or decreased (e.g., Zkscan17, Batf3) in the mPFC. Nonetheless, others increased (e.g., Tsc22d3) or decreased (e.g., Hhex) in both the mPFC and NAc microglia. These findings suggest that TLR2/4 signaling is involved in both mPFC and NAc microglia.

TLR2/4 signaling induces the gene expression changes of these transcription factors within the microglia (i.e., cell-autonomously) or via some other cells (i.e., cell-non-autonomously). We used primary microglia isolated from adult mouse brains to seek the transcription factors whose expression was cell-autonomously regulated by TLR2/4 signaling. TLR4 activates broader intracellular signaling pathways than TLR2.[9] Thus, we first examined the effects of TLR4 stimulation by its ligand lipopolysaccharide (LPS) on the expression of the above transcription factors.

To generate primary microglia, we transcardially perfused adult C57BL/6N female mice of 8–10 weeks old with phosphate-buffered saline (PBS) under deep anesthesia with sodium pentobarbital (200 mg/kg intraperitoneally). For dissociating cells, the brains with the olfactory bulbs and cerebellum removed were incubated in Hank's balanced salt solution containing papain (Worthington) and DNase I (Worthington) at 37 °C for 30 min. After myelin removal with 30% Percoll discontinuous gradient centrifugation, microglia were incubated with magnetic beads bound to anti-CD11b antibodies (Miltenyi Biotech) and isolated with Auto MACS-Pro. The isolated microglia were cultured in DMEM/F12 containing 10% FBS, 10 ng/ml M-CSF, and 50 ng/ml TGF-$\beta_1$.[10] On the 4th day after the beginning of culture, 100 ng/ml LPS (Sigma–Aldrich) or 10 ng/ml LTA (Invivogen) was added to the medium, and microglia were harvested 6 h later (Fig. 2A). PBS or water was used as a vehicle for LPS or LTA, respectively. Total RNA was purified from harvested microglia and used for cDNA synthesis and real-

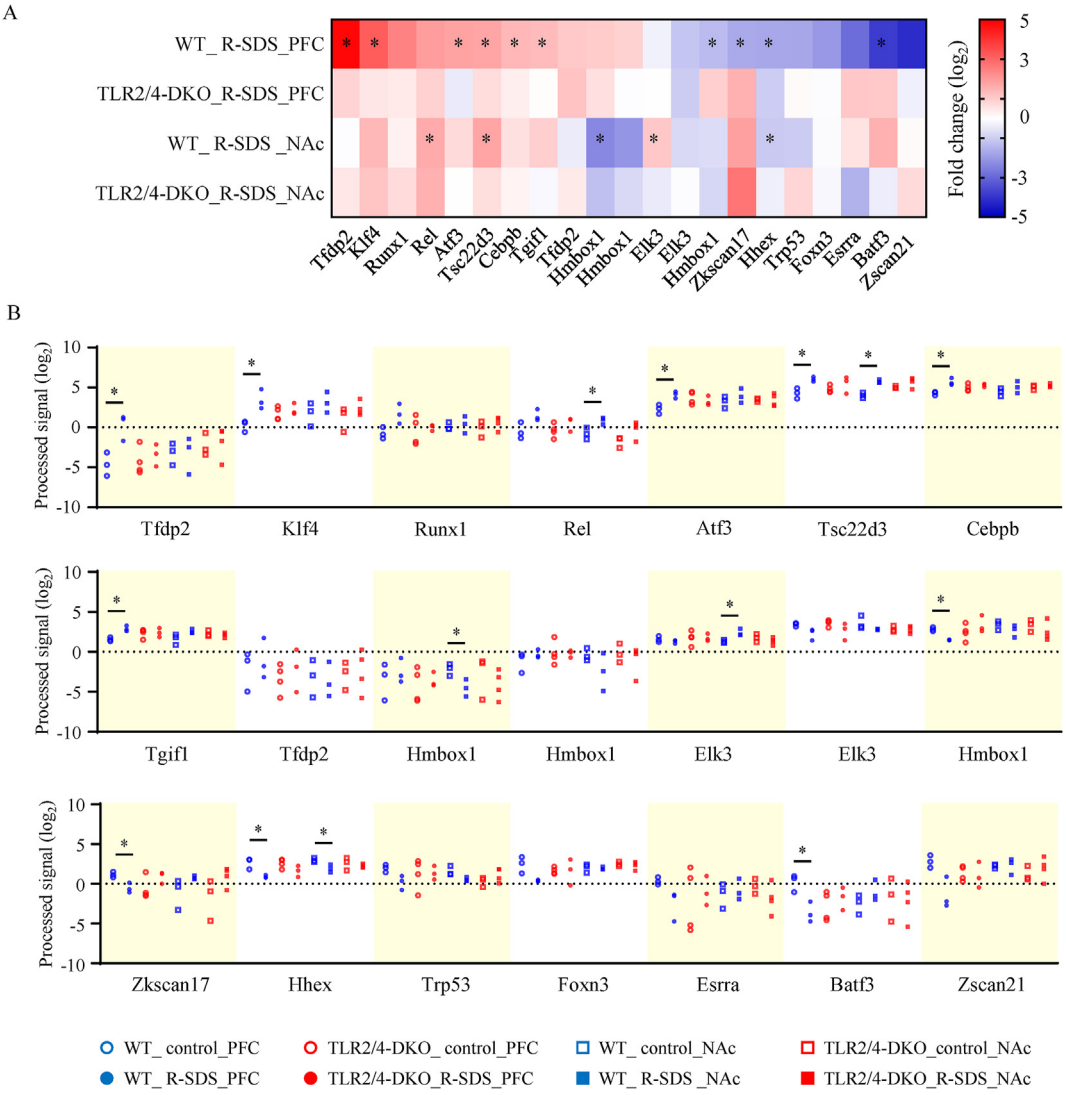


**Fig. 1.** R-SDS alters the expression of transcription factors in microglia. (A) A heat map showing R-SDS-induced changes in the expression of transcription factors in microglia isolated from the mPFC and NAc of wild-type mice (WT_R-SDS) and TLR2/4-deficient mice (TLR2/4-DKO_R-SDS). (B) The expression levels of the transcription factors shown in (A) in microglia isolated from the mPFC or NAc of wild-type mice and TLR2/4-deficient mice with or without R-SDS (R-SDS or control, respectively). *$P < 0.05$ for unpaired *t*-test compared to the respective controls.

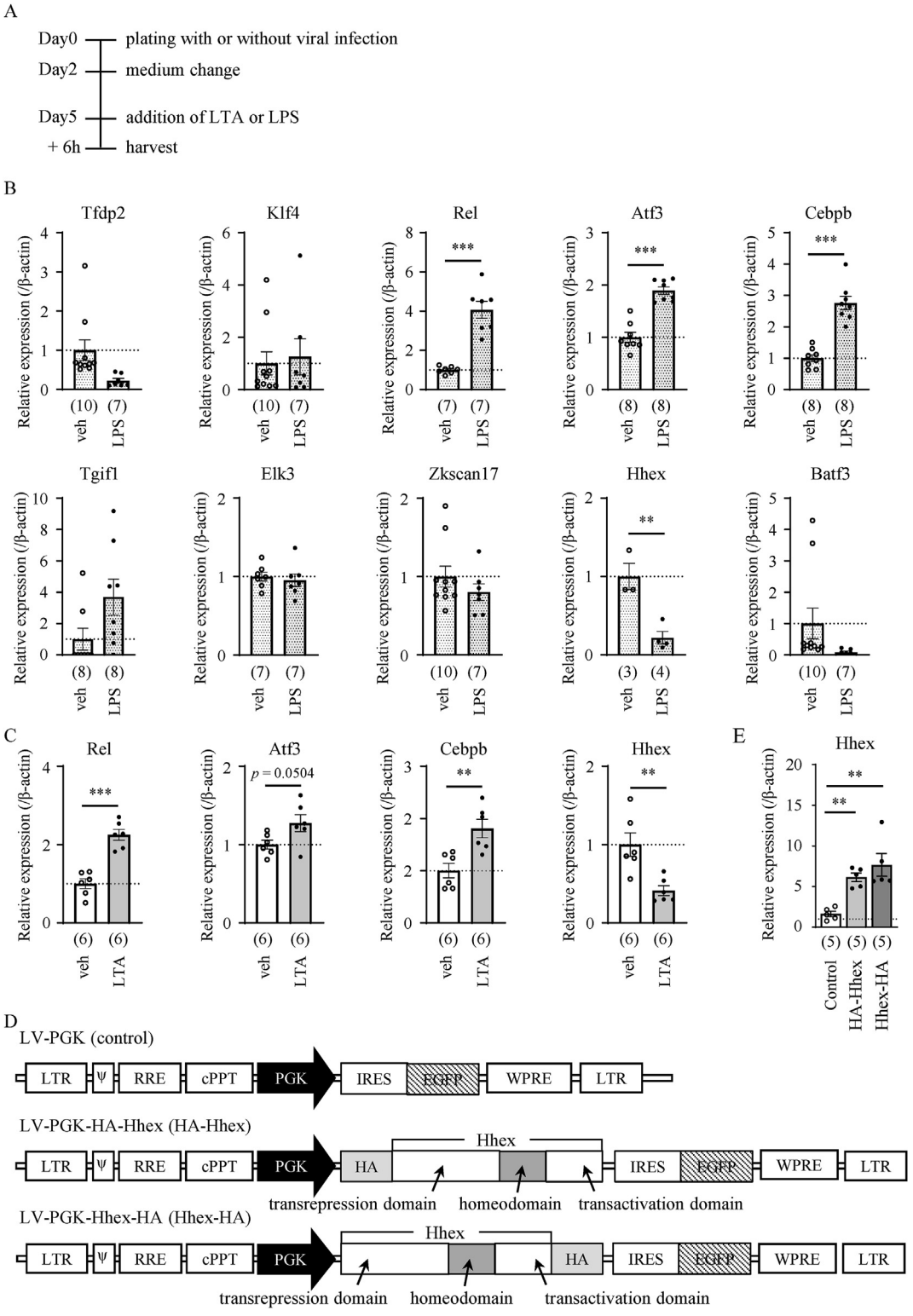


**Fig. 2.** LTA and LPS increase Rel, Atf3, and Cebpb and decrease Hhex in primary microglia. (A) An experimental schedule. (B, C) LPS- and LTA-induced changes in the expression of transcripton factors normalized to that of β-actin in primary microglia. (D) Lentiviral vectors for overexpressing Hhex fused to HA at its N- or C-terminus with EGFP (HA-Hhex and Hhex-HA, respectively) or EGFP alone (control). Note that Hhex is composed of transrepression domain, homeodomain and transactivation domain. (E) The expression levels of Hhex in primary microglia with or without Hhex overexpression. Data are shown as mean ± SEM of at least two independent experiments. The number of samples is shown below each bar. $^{**}P < 0.01$, $^{***}P < 0.001$ for unpaired t-test (B and C). $^{**}P < 0.01$ for Tukey's multiple comparison test (E).

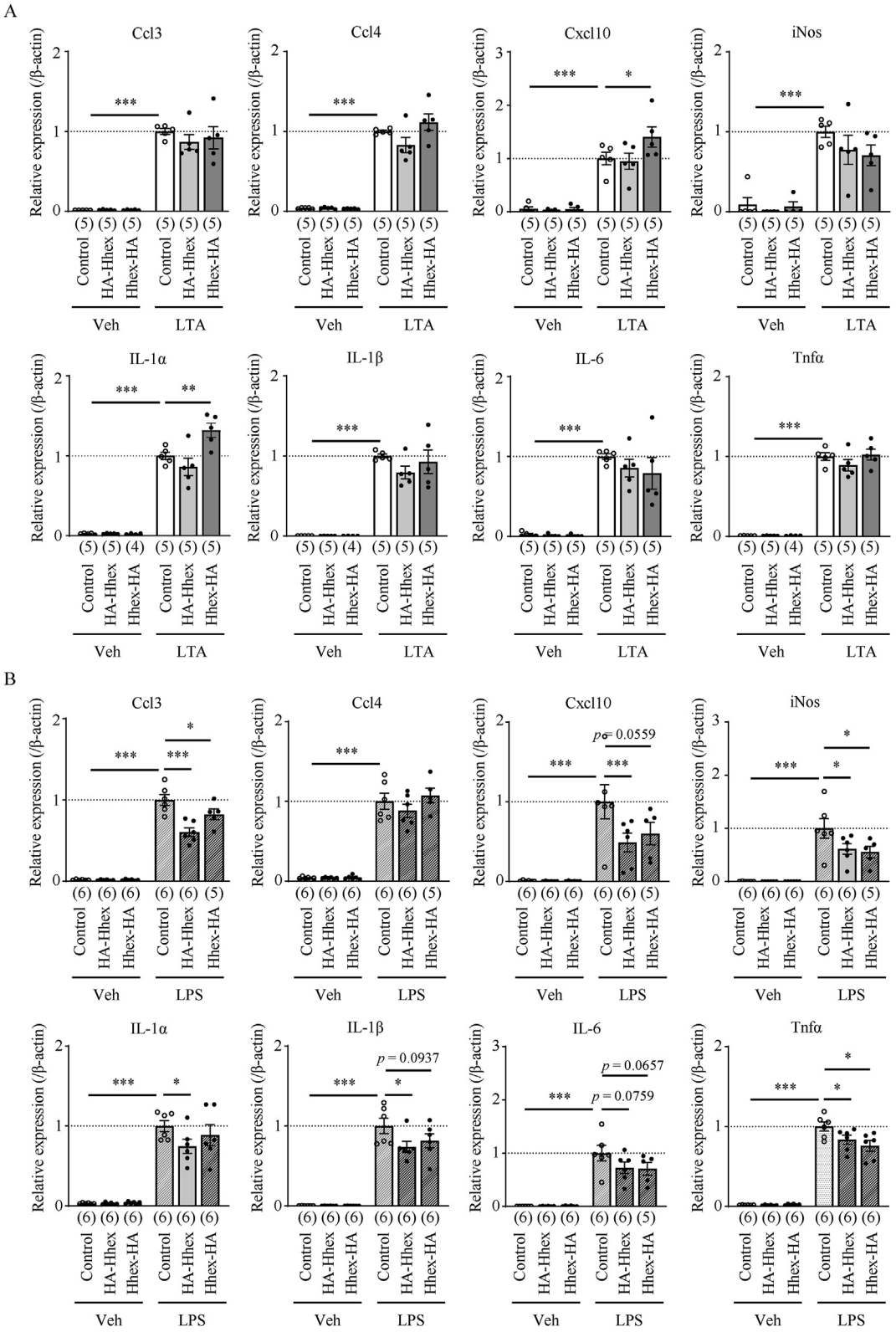


**Fig. 3.** Hhex overexpression attenuates LPS-induced increase in TNFα, IL-1β, IL-6, Ccl3, Cxcl10, and iNos in primary microglia. LTA- or LPS-induced changes (A and B, respectively) in inflammation-related genes with (HA-Hhex and Hhex-HA) or without (control) Hhex overexpression are shown. Data are shown as mean ± SEM of at least two independent experiments. The number of samples is shown below each bar. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for Tukey's multiple comparison test.

time PCR analysis with conventional methods, as previously described.[6] The sequences of primers used in this study are listed in Supplementary material 1. The expression level of each gene was normalized to that of β-actin.

Among the 11 transcription factors chosen above, we could not detect Tsc22d3. Among the 10 that remained, LPS increased Rel, Atf3, and Cebpb and decreased Hhex with statistical significance (Fig. 2B). These changes were in the same direction as those induced by R-SDS (Fig. 1). Then we examined the effects of TLR2 activation by its ligand lipoteichoic acid (LTA) on the expression of these 4 transcription factors in primary microglia. LTA induced the same changes as well (Fig. 2C).

Studies have shown that Rel, Atf3, and Cebpb are crucial for microglial responses.[11] By contrast, Hhex is a Homeobox transcription factor essential for the development of the forebrain, liver, and thyroid gland[12] and regulating hematopoiesis and vasculogenesis.[13,14] However, Hhex mRNA is abundant in the mPFC and NAc microglia of the adult brain, compared with conventional microglial genes such as Aif1 (i.e., Iba-1), P2ry12, and Rel (i.e., NF-κB p65) (Supplementary Fig. 1A). Hhex expression is highly enriched in microglia and endothelial cells (Supplementary Fig. 1B and C). To examine its role in microglia, we overexpressed Hhex in primary microglia with lentiviral vectors and counteracted LTA/LPS-induced Hhex reduction. Since Hhex has transrepression and transactivation domains at its N- and C-termini, respectively (Fig. 2D), we generated the lentiviral vectors to express Hhex fused to the HA tag at either terminus. Mouse Hhex cDNA was subcloned into the plasmid pLV-PGK-GFP to obtain pLV-PGK-Hhex-HA and pLV-PGK-HA-Hhex (Fig. 2D). These plasmids were used to generate lentiviral vectors, as previously reported.[6] Three x $10^4$ microglia were plated per well on a 96-well plate and infected with the lentiviral vectors at the MOI of 100. The lentiviral vectors were removed from the culture medium one day later, and microglia were incubated for three more days before we added LPS or LTA or their vehicles. Both lentiviruses expressing Hhex successfully increased Hhex in primary microgia (Fig. 2E).

We previously reported that R-SDS increases many proinflammatory genes in mPFC microglia.[6] We examined the effect of LTA and LPS on the expression of several of these genes. LTA and LPS consistently increased TNFα, IL-1α, IL-1β, IL-6, Ccl3, Ccl4, Cxcl10, and inducible nitric oxide synthase (iNOS). Hhex overexpression attenuated LPS-induced increase in most of these genes, such as TNFα, IL-1β, IL-6, Ccl3, Cxcl10, and iNos, regardless of HA fused to the N- or C-terminus of Hhex (Fig. 3). Notably, Hhex overexpression caused different effects upon LTA stimulation. The effects were marginal, and Cxcl10 and IL-1α expression was even stimulated by Hhex fused to HA at its C-terminus, although this effect was absent with HA at its N-terminus. These findings suggest that Hhex negatively regulates inflammation-related genes in microglia and that TLR2/4 activation reduces Hhex expression, facilitating TLR4-mediated neuroinflammation.

In this study, we explored a novel strategy for identifying new transcription factors by looking into the microglial transcriptome of stressed mice and evaluating their functions in primary microglia, leading to identifying the anti-inflammatory transcription factor Hhex in microglia. Hhex is constitutively expressed in microglia and reduced upon TLR2/4 stimulation to promote inflammatory responses. These properties could make Hhex uniquely serve as a homeostatic inflammation regulator. However, this proposed Hhex function remains tested with its microglia-specific knockout mice. In addition, the mechanism of Hhex's effects is unclear. Generally, multiple transcription factors form a macromolecular complex for transcriptional activation or repression. Rel, Atf3, and Cebpb increased in both TLR2/4-stimulated primary microglia and TLR2/4-activated microglia in the brain of stressed mice. These transcription factors reportedly mediate or augment inflammatory responses to TLR2/4 stimulation. Hhex could suppress their actions, possibly via direct interactions. Also, the Groucho/TLE corepressor could be involved in the Hhex functions as it is recruited to the N-terminal domain of Hhex for transcriptional repression.[15] Whereas TLR2 and TLR4 stimulation increased overlapping proinflammatory genes, Hhex overexpression only reduced it with TLR4 stimulation. Thus, Hhex could act on these TLR4-specific, TRIF-dependent signaling pathways. By contrast, Hhex could amplify TLR2-stimulated Cxcl10 and IL-1α expression, when its C-terminal transactivation domain was altered conformationally, for example, with adjacent HA. To elucidate the roles and actions of Hhex, genome-wide analyses with chromatin-immunoprecipitation sequencing in various neuroinflammatory conditions, including chronic stress, are necessary.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2022.04.006.

## References

1. Rivest S. Regulation of innate immune responses in the brain. *Nat Rev Immunol*. 2009;9(6):429–439.
2. Colonna M, Butovsky O. Microglia function in the central nervous system during health and neurodegeneration. *Annu Rev Immunol*. 2017;35:441–468.
3. Inoue K, Tsuda M. Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nat Rev Neurosci*. 2018;19(3):138–152.
4. Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J Neurosci*. 2009;29(13):3974–3980.
5. Setiawan E, Wilson AA, Mizrahi R, et al. Role of translocator protein density, a marker of neuroinflammation, in the brain during major depressive episodes. *JAMA Psychiatr*. 2015;72(3):268–275.
6. Nie X, Kitaoka S, Tanaka K, et al. The innate immune receptors TLR2/4 mediate repeated social defeat stress-induced social avoidance through prefrontal microglial activation. *Neuron*. 2018;99(3):464–479. e467.
7. Gosselin D, Skola D, Coufal NG, et al. An environment-dependent transcriptional network specifies human microglia identity. *Science*. 2017;356(6344).
8. Tansley S, Uttam S, Ureña Guzmán A, et al. Single-cell RNA sequencing reveals time- and sex-specific responses of mouse spinal cord microglia to peripheral nerve injury and links ApoE to chronic pain. *Nat Commun*. 2022;13(1):843.
9. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34(5):637–650.
10. Bohlen CJ, Bennett FC, Tucker AF, Collins HY, Mulinyawe SB, Barres BA. Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures. *Neuron*. 2017;94(4):759–773. e758.
11. Holtman IR, Skola D, Glass CK. Transcriptional control of microglia phenotypes in health and disease. *J Clin Invest*. 2017;127(9):3220–3229.

12. Martinez Barbera JP, Clements M, Thomas P, et al. The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development*. 2000;127(11):2433–2445.
13. Bedford FK, Ashworth A, Enver T, Wiedemann LM. HEX: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. *Nucleic Acids Res*. 1993;21(5):1245–1249.
14. Soufi A, Jayaraman PS. PRH/Hex: an oligomeric transcription factor and multifunctional regulator of cell fate. *Biochem J*. 2008;412(3):399–413.
15. Desjobert C, Noy P, Swingler T, Williams H, Gaston K, Jayaraman PS. The PRH/Hex repressor protein causes nuclear retention of Groucho/TLE co-repressors. *Biochem J*. 2009;417(1):121–132.