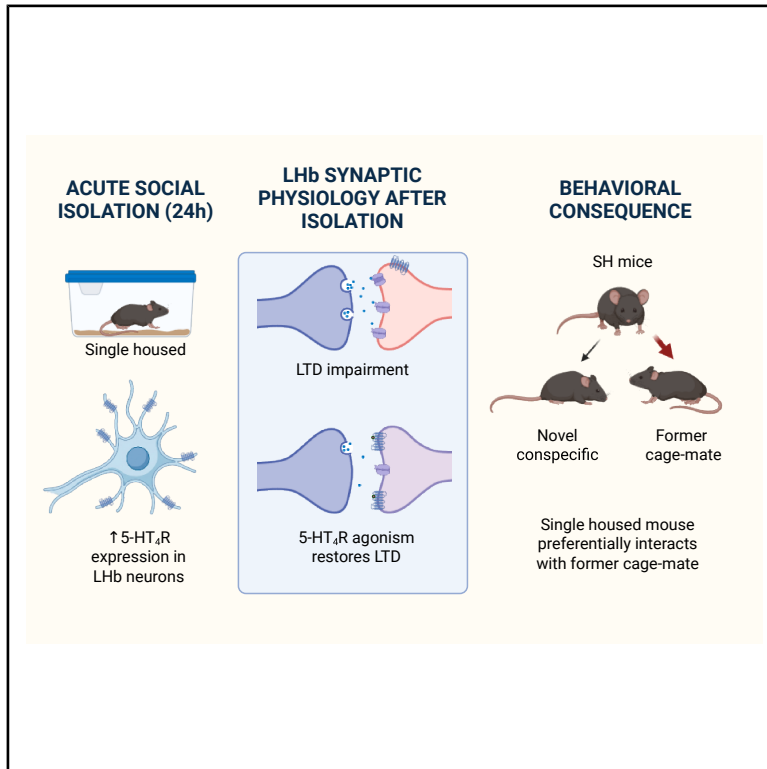


## Lateral habenula 5-HT<sub>4</sub> receptor signaling restores social novelty preference after acute isolation

### Graphical abstract



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### In brief

Kang et al. show that acute social isolation upregulates 5-HT<sub>4</sub>R in the lateral habenula, altering synaptic plasticity. This shift drives a familiarity-biased social preference. Pharmacological activation of 5-HT<sub>4</sub>R restores both synaptic function and social behavior, revealing a mechanism for adaptive social value regulation.

### Highlights

- Acute social isolation induces a neuron-specific upregulation of 5-HT<sub>4</sub>R in the LHb in mice
- Long-term depression (LTD) and long-term potentiation (LTP) in LHb are altered after isolation
- 5-HT<sub>4</sub>R activation restores LTD and normalizes hyperexcitability in isolated mice
- Isolated mice show familiarity-biased social preference that is restored by 5-HT<sub>4</sub>R activation



## Report

# Lateral habenula 5-HT<sub>4</sub> receptor signaling restores social novelty preference after acute isolation

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## SUMMARY

Social homeostasis—the maintenance of stable social contact—is disrupted by acute social stressors such as isolation. While the lateral habenula (LHb) is known for encoding negative emotional salience, it also modulates social behavior. However, how the LHb contributes to restoring social balance after acute stress remains unclear. Here, we demonstrate that acute social isolation upregulates serotonin receptor 4 (5-HT<sub>4</sub>R) expression in the LHb. Electrophysiological analysis shows impaired long-term depression and facilitated long-term potentiation in isolated mice, which is restored by pharmacological activation of 5-HT<sub>4</sub>R. Behaviorally, group-housed mice show the typical social preference for novelty. On the other hand, isolated mice interact more with familiar mice than novel mice, which is restored by LHb-targeted 5-HT<sub>4</sub>R activation. These findings suggest that social isolation shifts social preference through 5-HT<sub>4</sub>R-mediated metaplasticity in the LHb. This study provides mechanistic insight into how acute social stress alters social behavior, with implications for social dysfunction in neuropsychiatric disorders.

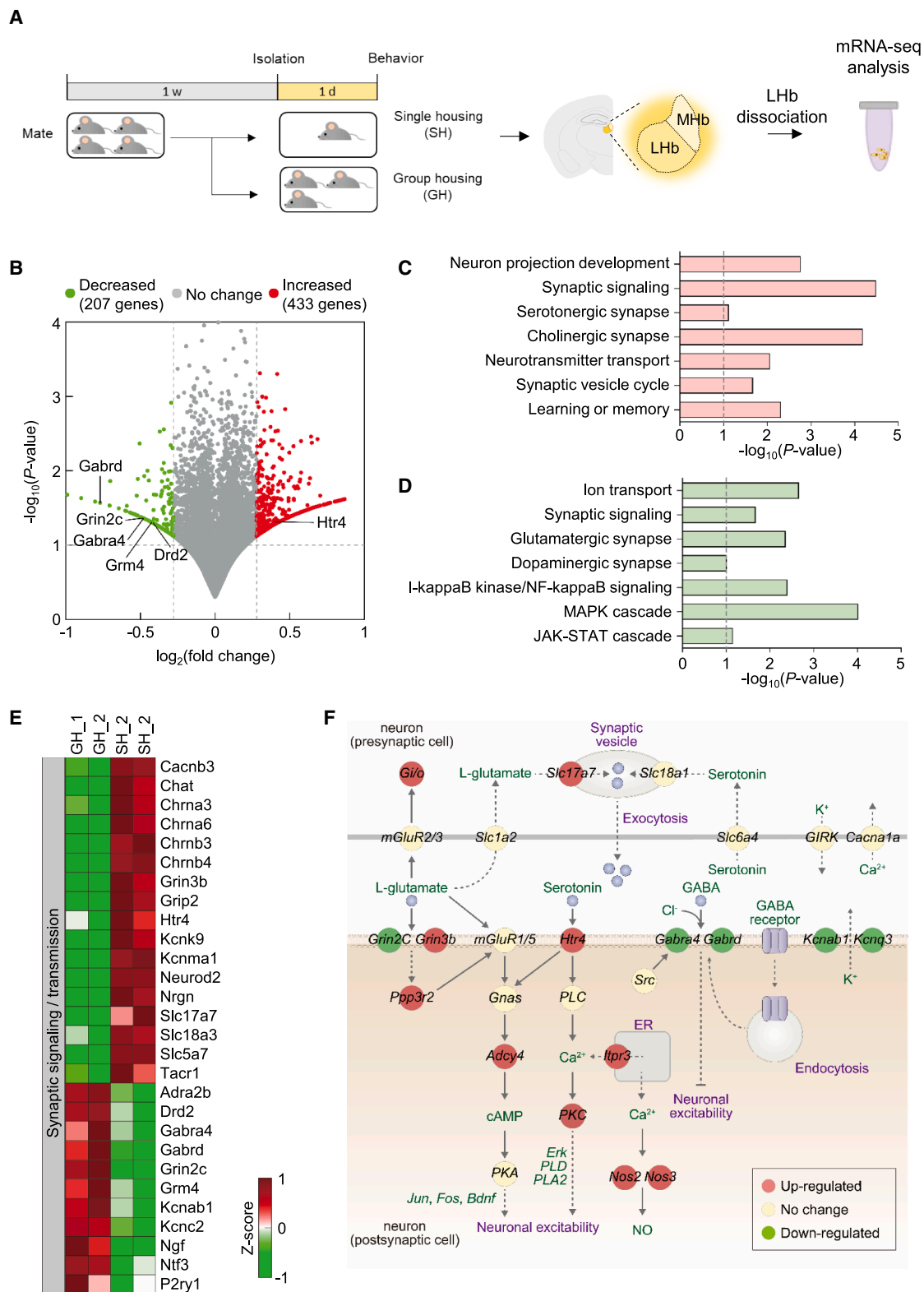
## INTRODUCTION

Appropriate social relationships through social interactions are critical for the survival of social animals, including rodents, primates,<sup>1,2</sup> as well as humans.<sup>2</sup> Chronic social isolation is regarded as a severe psychosocial stressor implicating an etiology of neuropsychiatric disorders such as schizophrenia,<sup>3</sup> depression,<sup>4,5</sup> anxiety,<sup>6</sup> and aggression.<sup>7</sup> Numerous studies have suggested that chronic social isolation results in cognitive dysfunction and social deficits.<sup>8,9</sup> Moreover, acute social isolation induces prosocial behaviors, such as social cravings and increased affiliative behaviors in rodents and humans, by so maintaining a “social homeostasis” or driving compensatory social craving.<sup>10</sup> However, it is not known whether acutely isolated individuals demonstrate general social preference changes or those specific toward familiar or novel conspecifics.

Monoaminergic neurotransmission regulates the prosocial behaviors induced by social isolation.<sup>11</sup> The habenula is an essential hub region of the brain that modulates monoamine systems, such as the dopamine and serotonin systems. The habenula complex containing medial habenula (MHb) and LHb is located

in the epithalamus.<sup>12</sup> The LHb mainly conveys negatively valenced information from the forebrain to monoamine systems through the stria medullaris (SM) and fasciculus retroflexus (FR).<sup>12–14</sup> LHb activity encodes social behaviors. Specifically, inhibitory input from the basal forebrain to the LHb is associated with social aggression and conditioned preference for social reward.<sup>15</sup> Additionally, optogenetic manipulations of the medial prefrontal cortex (mPFC) projecting to the LHb regulate social motivation.<sup>16</sup> The expression of cFos, a marker of neuronal activity, in the LHb, is enhanced by social stressors, such as social defeat<sup>17</sup> or isolation.<sup>18</sup> In addition, acute social isolation induces synaptic potentiation at dopaminergic neurons in the dorsal raphe (DR), receiving input from the LHb.<sup>19</sup> Evidence suggests that the LHb is influenced by social stress and affects animal's social decisions. However, it is unclear whether altered LHb activity and synaptic plasticity induced by acute social isolation influence social preferences or decision-making. Recent work<sup>20</sup> further highlights the LHb's role in regulating social preference, though the mechanisms by which acute social isolation alters social behavior through LHb-mediated pathways remain to be elucidated.





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Serotonin is a monoamine neurotransmitter whose responses are mediated by serotonin receptor (5-HT<sub>R</sub>) subtypes, including 5-HT<sub>1R</sub>–5-HT<sub>7R</sub>.<sup>21</sup> This serotonin-driven signal transduction encodes emotional, motivational, or pathological states such as anxiety, reward, mood disorders, and addiction.<sup>22</sup> Serotonin deficiency mice exhibit susceptibility to social defeat stress. Interestingly, inactivation of the LHb reverses behavioral deficits produced by social stress.<sup>23</sup> Several isoforms of 5-HT<sub>R</sub>s, such as 5-HT<sub>1R</sub>, 5-HT<sub>2R</sub>, 5-HT<sub>3R</sub>, 5-HT<sub>5R</sub>, and 5-HT<sub>7R</sub>, are primarily expressed in the LHb.<sup>24–27</sup> 5-HT<sub>1A</sub>R and 5-HT<sub>1B</sub>R modulate glutamatergic and GABAergic transmissions via presynaptic release probability regulation.<sup>25</sup> In addition, 5-HT<sub>2R</sub> and 5-HT<sub>3R</sub> modulate output firing by regulating postsynaptic responses in the LHb.<sup>26,27</sup> In rodents and humans, 5-HT<sub>4</sub>R has been implicated as a critical modulator of mood and anxiety.<sup>28–30</sup> Pharmacological activation of 5-HT<sub>4</sub>R enhances the antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) in rodents.<sup>31</sup> On the other hand, the genetic deletion of *htr4* showed resistance to physical stress.<sup>30</sup> Moreover, silencing 5-HT<sub>4</sub>R in the hippocampus results in depressive and anxiogenic behaviors as well as reduced neural activity.<sup>28,30,32</sup> Despite these findings, whether LHb 5-HT<sub>4</sub>R contributes to social behavioral adaptation under social stress remains largely unknown.

In this study, we investigated the molecular and neural mechanisms that mediate changes in social decision-making triggered by acute social stress. We developed an acute social isolation model in which a mouse is housed alone for 24 h. We found that the socially isolated mice spent significantly more time investigating a familiar conspecific over an unfamiliar conspecific when both mice were simultaneously available. Then, we performed extracellular field recording to access synaptic responses in the LHb and gene profile screening through mRNA sequencing analysis. Indeed, we found that acute social isolation disrupted synaptic plasticity in the LHb and enhanced *htr4* expression in the LHb. Interestingly, changes in synaptic transmission and social behavior were recovered via pharmacological activation of the 5-HT<sub>4</sub>R, which was found to be upregulated after social isolation. Our findings suggest 5-HT<sub>4</sub>R as a novel molecular mechanism underlying social decision-making following social isolation. Furthermore, this is the first study to show that, although acute social isolation increases prosocial behaviors or social cravings, acutely isolated mice prefer familiar mice to unfamiliar mice in situations where they can decide which social interactions to pursue. This finding suggests that prosocial behaviors induced by acute social isolation are mediated by neural circuit mechanisms distinct from those underlying

normal prosocial behaviors. Therefore, this study provides new insight into the neural consequences of social isolation.

## RESULTS

### Social isolation-induced *htr4* expression in the LHb

The LHb is a key brain region involved in the regulation of social behavior.<sup>16,20</sup> However, transcriptomic changes in the LHb following exposure to acute social isolation stress remain unexplored. To examine the molecular nature associated with social isolation, we performed bulk mRNA sequencing of the LHb tissues from GH and SH mice (Figure 1A). Comparing mRNA expression profiles, we identified 640 differentially expressed genes (DEGs), including 433 upregulated and 207 downregulated genes in SH mice, compared to those in GH mice (Figure 1B; Table S1). Next, we examined cellular processes affected by social isolation through gene set enrichment analysis of the up or downregulated genes using ConsensusPathDB. The upregulated genes were significantly associated with pathways related to synaptic functions in habenular (neuron projection development, synaptic signaling, serotonergic synapse, cholinergic synapse, and learning or memory) (Figure 1C). In contrast, the downregulated genes were mainly associated with pathways related to ion transport, glutamatergic synapse, and dopaminergic synapse, as well as MAPK and JAK-STAT signaling pathways (Figure 1D). Interestingly, several types of neurotransmitter receptors were up or downregulated in the SH mice, including acetylcholine (*Chrna3*, *Chrna6*, and *Chrnb3*), serotonin (*Htr4*) receptors, glutamate (*Grin3b*, *Grin2c*, and *Grm4*), GABA (*Gabbr1*, *Gabra4*, and *Gabrd*), and dopamine (*Drd2*) (Figures 1E and S1E). These data suggest a potential link between acute social isolation and synaptic function in the LHb. To examine the collective actions of up and downregulated signaling pathways, we reconstructed a network model describing interactions among genes involved in synaptic function-related processes. The network also showed up-regulation of cholinergic and serotonergic signaling pathways in the SH mice, compared to those in GH mice (Figure 1F). The most remarkable change among these receptors was the expression of *htr4*.

### Upregulation of *htr4* mRNA in socially isolated LHb region

The 5-HT<sub>4</sub>R receptor remains the least studied serotonin receptor subtype in the LHb, and its baseline expression in this region has not been previously reported. We found that *htr4* mRNA expression in GH mice was faint but significantly upregulated in socially isolated mice (Figure S1). To quantify these changes,

**Figure 1. Gene expression profiling analysis for the acute social stress model**

(A) Workflow for RNA-seq analysis.

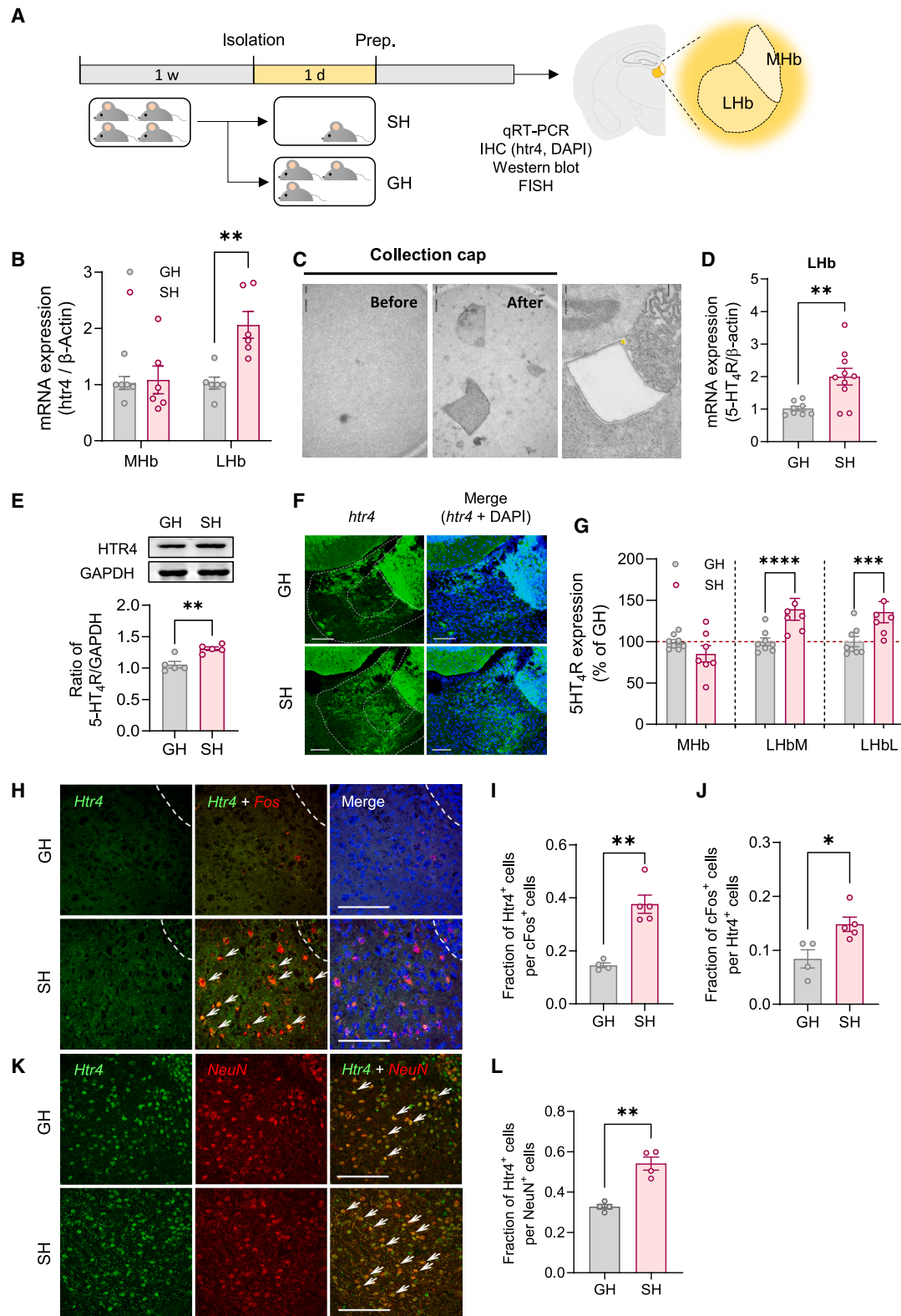
(B) Volcano plot showing differentially expressed genes (DEGs) in SH mice compared to GH mice. Red and green dots represent upregulated and downregulated genes, respectively; gray dots represent genes whose expressions were not significantly different.

(C and D) GO biological processes and pathways are represented by the up (C), and downregulated (D), genes. Statistical significance was displayed as  $-\log_{10}(p)$  value where the enrichment  $p$  value is obtained from ConsensusPathDB. The dotted line indicates the  $p$  value cutoff used.

(E) Heatmap showing the differential expression of the genes involved in the pathways related to synaptic signaling. The color bar represents the gradient of the Z-scores.

(F) A network model describing the interactions among the up and downregulated genes involved in cellular pathways related to synaptic signaling. Nodes were arranged according to KEGG pathway and Wikipathway models.

Arrow and suppressive links, activation, and inhibition reactions, respectively.



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we performed RT-qPCR analysis in an acute social isolation model (Figures 2A and 2B). The habenula complex was divided into MHb and LHb for sample pooling. Consistent with bulk-RNA sequencing analysis results, *htr4* expression in the LHb of SH mice showed a more than 2.5-fold increase compared with GH mice (Figure 2B). To ensure precise regional specificity, we used a laser capture microscope to microdissected the lightly nissl-stained LHb region (Figure 2C), completely excluding the MHb portions. Similar to the results in Figure 2B, the increase in *htr4* expression was observed in the LHb region of the SH mice compared to the GH mice (Figure 2D). Next, to determine the protein expression level of 5-HT<sub>4</sub>R, we conducted a western blot analysis. 5-HT<sub>4</sub>R was also elevated in the LHb region of SH mice (Figure 2E).

To visualize spatial expression changes, we next performed free-floating immunohistochemistry analysis (Figure 2F). We found that 5-HT<sub>4</sub>R expressions were consistently higher in both the LHbM and LHbL of SH mice than in those of GH mice (Figures 2F and 2G). Paradoxically, there were no significant changes in the MHb upon social isolation despite its high basal expression (Figure 2G).

To further determine if *htr4* upregulation occurs in LHb neuronal population that is activated by social isolation, we performed RNAscope fluorescent *in situ* hybridization (FISH) targeting *htr4* and the immediate-early gene *cFos* (Figure 2H). Consistent with our RT-qPCR and immunostaining results, *htr4* mRNA was sparsely expressed in the LHb of GH mice but was markedly increased in SH mice. Quantitative colocalization analyses further revealed that both the proportion of *cFos*<sup>+</sup> cells among *htr4*<sup>+</sup> cells and the proportion of *htr4*<sup>+</sup> cells among *cFos*<sup>+</sup> cells were significantly elevated in SH mice (Figures 2H–2J), indicating that *htr4*-expressing neurons are preferentially recruited during acute isolation. To identify

the cellular subtypes contributing to the isolation-induced increase in 5-HT<sub>4</sub>R expression, we performed cell-type-specific analyses using immunohistochemistry analysis, respectively. Quantification of 5-HT<sub>4</sub>R-positive cells revealed a selective elevation of 5-HT<sub>4</sub>R expression in NeuN<sup>+</sup> cells of SH mice, whereas S100β<sup>+</sup> astrocytes showed no detectable change between GH and SH mice (Figures 2K, 2L, and S2). These findings indicate that the up-regulation of 5-HT<sub>4</sub>R following acute social isolation occurs predominantly within LHb neurons, supporting a neuron-specific enhancement of 5-HT<sub>4</sub>R signaling.

Taken together, these findings demonstrate that acute social isolation selectively upregulated 5-HT<sub>4</sub>R expression in the LHb but not in the MHb. 5-HT<sub>4</sub>R has a fast-acting antidepressant effect and is known to modulate mood and anxiety.<sup>27</sup> The LHb receives a massive serotonin input from the DR.<sup>10,22</sup> The increased *htr4* expression may imply a compensatory mechanism responding to a disturbance of serotonin input, which is responsible for the impact of social isolation. To determine whether the serotonin input to the LHb is affected by social isolation, we investigated the serotonin level in the LHb region through 5-HT ELISA analysis. We showed that a significant decrease in intracellular 5-HT level was measured in the microdissected LHb region of SH mice when compared to GH mice (Figures S3A and S3E). Furthermore, acute social isolation reduced the extracellular 5-HT concentration in the LHb region, as assessed by *in vivo* microdialysis (Figures S3A–S3C and S2E). These results show that acute social isolation induces upregulation of *htr4* gene expression in the LHb region to compensate for the reduced serotonin levels. Therefore, we further investigated whether increased 5-HT<sub>4</sub>R expression is involved in the effect of social isolation on synaptic responses and social behavior.

## Figure 2. Upregulation of 5-HT<sub>4</sub>R expression in LHb region for socially isolated mice

(A) Experimental workflow for mRNA and protein analysis in the habenula complex following acute social isolation.

(B) *Htr4* mRNA levels in the medial habenula (MHb) and lateral habenula (LHb). No significant difference was observed in the MHb, while a moderate increase was noted in the LHb of SH mice compared to GH mice (two-way ANOVA test with Sidak's multiple test, MHb; ns,  $p = 0.9750$ ; LHb;  $**p = 0.0019$ ).

(C and D) RT-qPCR analysis of *Htr4* mRNA expression in LCM-isolated LHb tissue. Representative images showing LHb tissue before and after LCM collection using an adhesive cap. Nissl-stained sections of LHb brain tissue are displayed.

Scale bars, 150 μm. *Htr4* mRNA levels were significantly elevated in LHb obtained from SH mice compared with GH group (unpaired *t* test,  $**p = 0.0044$ ). Data are presented as mean ± s.e.m.

(E) Western blot analysis and quantification of 5-HT<sub>4</sub>R protein expression in GH vs. SH mice, with a bar graph indicating relative 5-HT<sub>4</sub>R expression (unpaired *t* test,  $**p = 0.0032$ ;  $n = 5$  sets per group, with 4 mice pooled per set).

(F and G) Immunohistochemistry (IHC) images and quantification showing 5-HT<sub>4</sub>R distribution in GH and SH mice, with increased 5-HT<sub>4</sub>R expression in SH mice. The dashed line indicates the analyzed LHb region.

5-HT<sub>4</sub>R expression is significantly higher in the medial (LHbM) and lateral (LHbL) regions of the SH mouse LHb compared to GH mice (two-way ANOVA, Sidak's multiple comparisons test: GH MHb vs. SH MHb, ns,  $p = 0.5852$ ; SH LHbM vs. GH LHbM,  $**p = 0.0088$ ; SH LHbL vs. GH LHbL,  $*p = 0.0185$ ).

Scale bars, 100 μm.

(H) Determination of *htr4* localization by fluorescence *in situ* hybridization (FISH).

The *htr4* gene was lightly expressed in LHb of GH mice as well. Then, *htr4* was well merged with LHb-activated *Fos*-positive neurons (green color; *Htr4*, red color; *cFos*).

In the SH LHb region, overlapping with *cFos*-positive cell is shown in yellow color. Scale bars, 100 μm.

(I and J) Quantification of the *Htr4*<sup>+</sup> cells among *cFos*-positive cells were significantly increased in the SH mice ( $0.3760 \pm 0.03$ ,  $n = 5$ ) than GH mice ( $0.1455 \pm 0.01$ ,  $n = 4$ ) (unpaired *t* test,  $**p = 0.0018$ ).

Scale bars, 100 μm.

(K) Cell-type specific distribution of 5-HT<sub>4</sub>R in LHb region. Representative confocal images showing neuronal (NeuN) and astrocytic (S100β) markers co-labeled with *Htr4* protein in LHb region of GH and SH mice.

NeuN (red), S100β (red), and *Htr4* (green). Scale bars, 100 μm.

(L) *Htr4*-positive cells were significantly increased in NeuN-positive cells (GH;  $0.3276 \pm 0.01$ , SH;  $0.5415 \pm 0.03$ ) (unpaired *t* test,  $**p = 0.0045$ ). Data is represented as mean ± SEM.

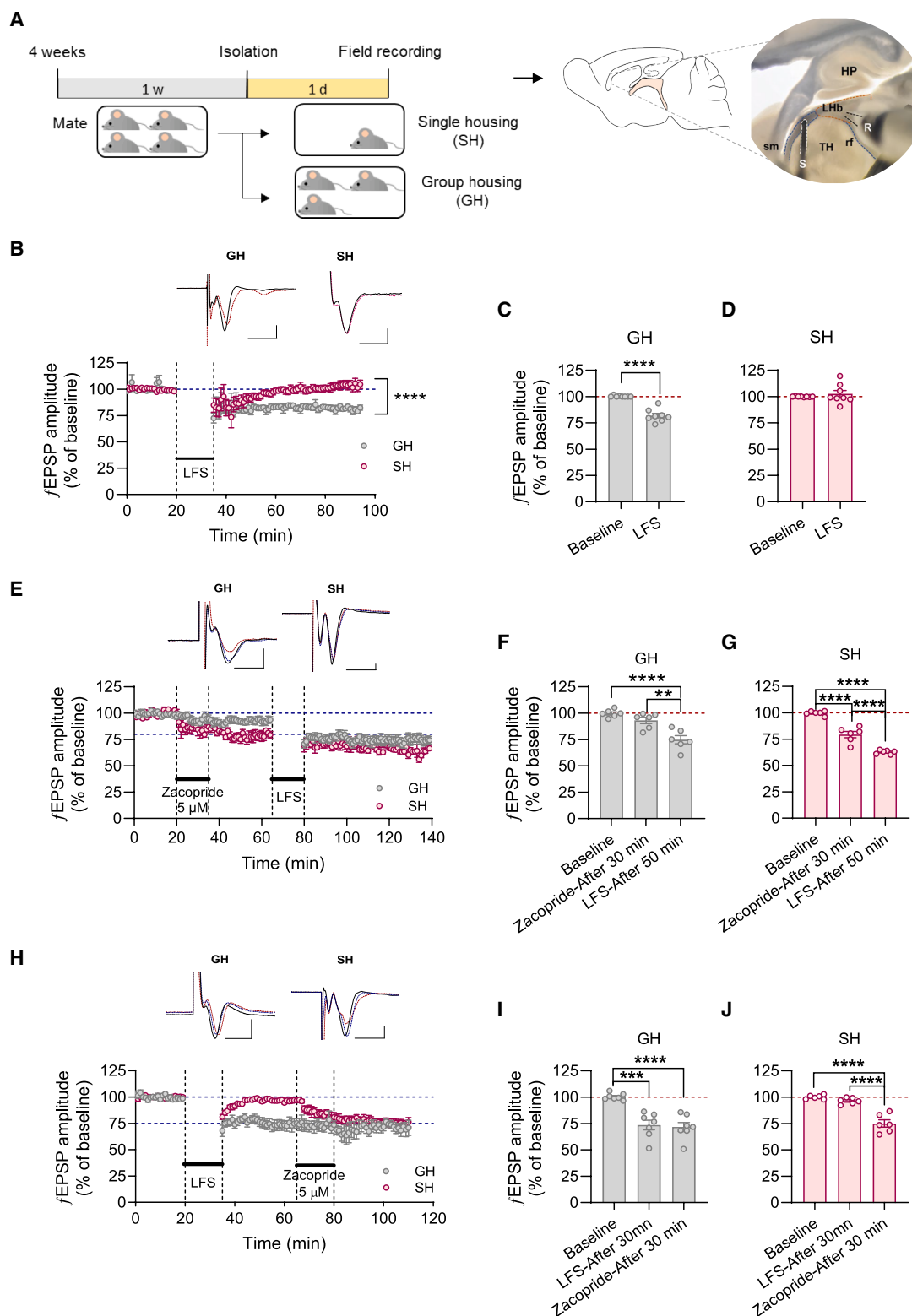


**Figure 3. Activation of 5-HT<sub>4</sub>R tunes synaptic modification by modulating subthreshold**

(A) Experimental flow chart of field extracellular recording after the acute social isolation model (left image).

Graphical illustration of sagittal brain slices containing the LHb (right image). Field excitatory postsynaptic potentials were recorded in the LHb by stimulating the stria medullaris (SM).

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## 5-HT<sub>4</sub>R activation restores altered synaptic plasticity in SH mice by regulating the induction threshold

The Lhb exhibits synaptic potentiation in several pathophysiological states.<sup>33–35</sup> It is well-established that low-frequency stimulation (LFS) (900 pulses of 1 Hz) induces long-lasting depression for AMPA-mediated fEPSP in several brain regions, such as the hippocampus,<sup>36</sup> amygdala,<sup>37</sup> and Lhb.<sup>38,39</sup> We examined whether acute social isolation alters synaptic plasticity in the Lhb. To determine the effect of social isolation on glutamatergic synaptic transmission, we investigated the changes in synaptic responses in the Lhb following acute social isolation for 24 h (Figure 3). To assess field potentials, a stimulus electrode (S) was used to stimulate SM fibers to obtain fEPSPs in sagittal Lhb slices (Figure 3A). LFS-induced LTD was intact in GH mice and completely impaired in SH mice (Figures 3B–3D). Furthermore, social isolation facilitated LTP induced by theta-burst stimulation (100 Hz, 250 ms of intertrain intervals [ITI]) (Figures S5D–S5F).

To investigate whether up-regulated 5-HT<sub>4</sub>R following acute social isolation influences glutamatergic transmission in the Lhb, we tested a pharmacological activation of 5-HT<sub>4</sub>R with zacopride, a selective 5-HT<sub>4</sub>R agonist (Figure 3E). In GH mice, bath application of zacopride had no significant effect on basal transmission (Figures 3E, 3F, S4A, and S4B). On the other hand, zacopride induced robust depression in SH mice, suggesting activation of upregulated 5-HT<sub>4</sub>R following social isolation induced a chemical LTD in the Lhb (Figures 3E–3G and S4A–S4C). Interestingly, further application of LFS induced LTD in the SH mice (Figure 3E, after 60 min; and G). The results suggest that upregulation of 5-HT<sub>4</sub>R in the Lhb following an acute isolation induced metaplastic changes, thereby establishing a 5-HT<sub>4</sub>R dependency of LFS-induced LTD.

To further investigate the physiological function of upregulated 5-HT<sub>4</sub>R in the SH mice, we applied LFS before the activation of 5-HT<sub>4</sub>R (Figure 3H). Unlike that 5-HT<sub>4</sub>R activation before LFS induced a suppression of basal transmission in GH mice (Figures 3E and 3F), 5-HT<sub>4</sub>R activation did not lead to further chemical LTD, suggesting that 5-HT<sub>4</sub>R-induced LTD shares a common mechanism with LFS-induced LTD in GH mice. On the other hand, SH mice shows impaired LFS-induced LTD, but activating the 5-HT<sub>4</sub>R receptor with zacopride resulted in a long-lasting depression (Figures 3H and 3J). We also found that activating 5-HT<sub>4</sub>R along with LFS or TBS induction rescued the alteration in synaptic plasticity induced by social isolation, restored synaptic responses to control levels (Figures S5G–S5I). Based on these findings, we predict that 5-HT<sub>4</sub>R activity may be involved in lowering the induction threshold for synaptic plasticity by causing a downregulation of basal transmission. These results indicate that 5-HT<sub>4</sub>R activation tunes the basal transmission and synaptic plasticity affected by social stress,

such as acute social isolation, and implies that 5-HT<sub>4</sub>R activation mediates metaplastic Lhb neuron regulation.

To determine the functional impact of 5-HT<sub>4</sub>R modulation on Lhb neuronal activity, we performed whole-cell current-clamp recordings in acute brain slices. We measured resting membrane potential (RMP), maximum firing rates (in response to series of stepped current from 100 to 200 pA), and rheobase currents before and after the bath application of the selective 5-HT<sub>4</sub>R agonist, BIMU-8 (Figures S6A and S6B). Baseline recordings revealed that social isolation significantly altered intrinsic excitability. Neurons from SH mice exhibited a more depolarized RMP compared to GH controls (GH:  $-59.13 \pm 2.23$  mV vs. SH:  $-53.41 \pm 1.90$  mV;  $p = 0.0442$ ; Figure S6C). While BIMU-8 treatment did not significantly alter the RMP in either group (interaction:  $F(1,34) = 0.7382$ ,  $p = 0.3963$ ), it had a profound effect on firing properties. SH neurons displayed a significantly higher maximum firing rate at baseline compared to GH neurons (SH:  $71.14 \pm 9.72$  Hz vs. GH:  $47.75 \pm 5.93$  Hz;  $p = 0.0434$ ). Importantly, BIMU-8 application significantly reduced the firing rate in SH neurons (to  $52.63 \pm 9.25$  Hz;  $p < 0.0001$ ), essentially normalizing it to GH levels, while having no significant effect on GH neurons (interaction:  $F(1,34) = 6.281$ ,  $p = 0.0172$ ; Figure S6D). Consistent with these firing rate changes, the rheobase current was significantly lower in SH neurons at baseline (SH:  $8.17 \pm 0.84$  pA vs. GH:  $14.80 \pm 2.69$  pA), indicating hyperexcitability. BIMU-8 treatment significantly increased the rheobase in SH neurons (to  $14.38 \pm 1.93$  pA;  $p = 0.0182$ ), restoring it to control levels, without affecting GH neurons (interaction:  $F(1,34) = 4.585$ ,  $p = 0.0395$ ; Figure S6E). Consistent with these findings, bath application of the 5-HT<sub>4</sub>R agonist BIMU-8 completely restored LFS-induced LTD in the Lhb from single-housed mice, demonstrating that enhancing 5-HT<sub>4</sub>R signaling is sufficient to reverse the synaptic plasticity deficit (Figures S7A–S7C). Furthermore, the *in vivo* administration of BIMU-8 into the Lhb continued to exhibit restored LFS-LTD in *ex vivo* brain slice (Figures S8A–S8D).

Collectively, these data indicate that SH induces intrinsic hyperexcitability in Lhb neurons, and activation of 5-HT<sub>4</sub>R selectively dampens this aberrant activity, validating the receptor's potential as a therapeutic target for normalizing Lhb function.

## Modulation of social engagement via 5-HT<sub>4</sub>R activation in the Lhb

We assessed whether social isolation for 24 h influences social decision-making behaviors. The mice were housed in a home cage for 1 week before social isolation (Figure 4A). One mouse was randomly chosen and isolated from the home cage for 24 h. We referred to these mice as SH mice. Three mice left in the home cage were used as group-housed (GH) mice. We evaluated whether the impact of isolation on social motivation was

Fr, fasciculus retroflexus fiber; HP, hippocampus; TH, thalamus.

(B–D) Low-frequency stimulation (LFS) (100  $\mu$ s pulse duration, 1 Hz x 900 pulses)-induced LTD was impaired in SH mice and intact in GH mice. Comparisons of fEPSP amplitude between baseline and after LFS in the Lhb from GH and SH mice.

(E–G) Effect of 5-HT<sub>4</sub>R on LFS-induced synaptic depression between GH and SH mice. Impairment of LFS-LTD recovered by downregulating synaptic transmission through activation of 5-HT<sub>4</sub>R in the SH mice (ordinary one-way ANOVA test with Turkey's multiple comparison test, \*\*\* $p = 0.0002$ , \*\*\*\* $p < 0.0001$ ).

(H–J) The effect of 5-HT<sub>4</sub>R post-activation after the LFS-induced LTD in both GH and SH mice (ordinary one-way ANOVA test with Turkey's multiple comparison test, \*\*\* $p = 0.0005$ , \*\*\*\* $p < 0.0001$ ). All data were represented as mean  $\pm$  s.e.m.

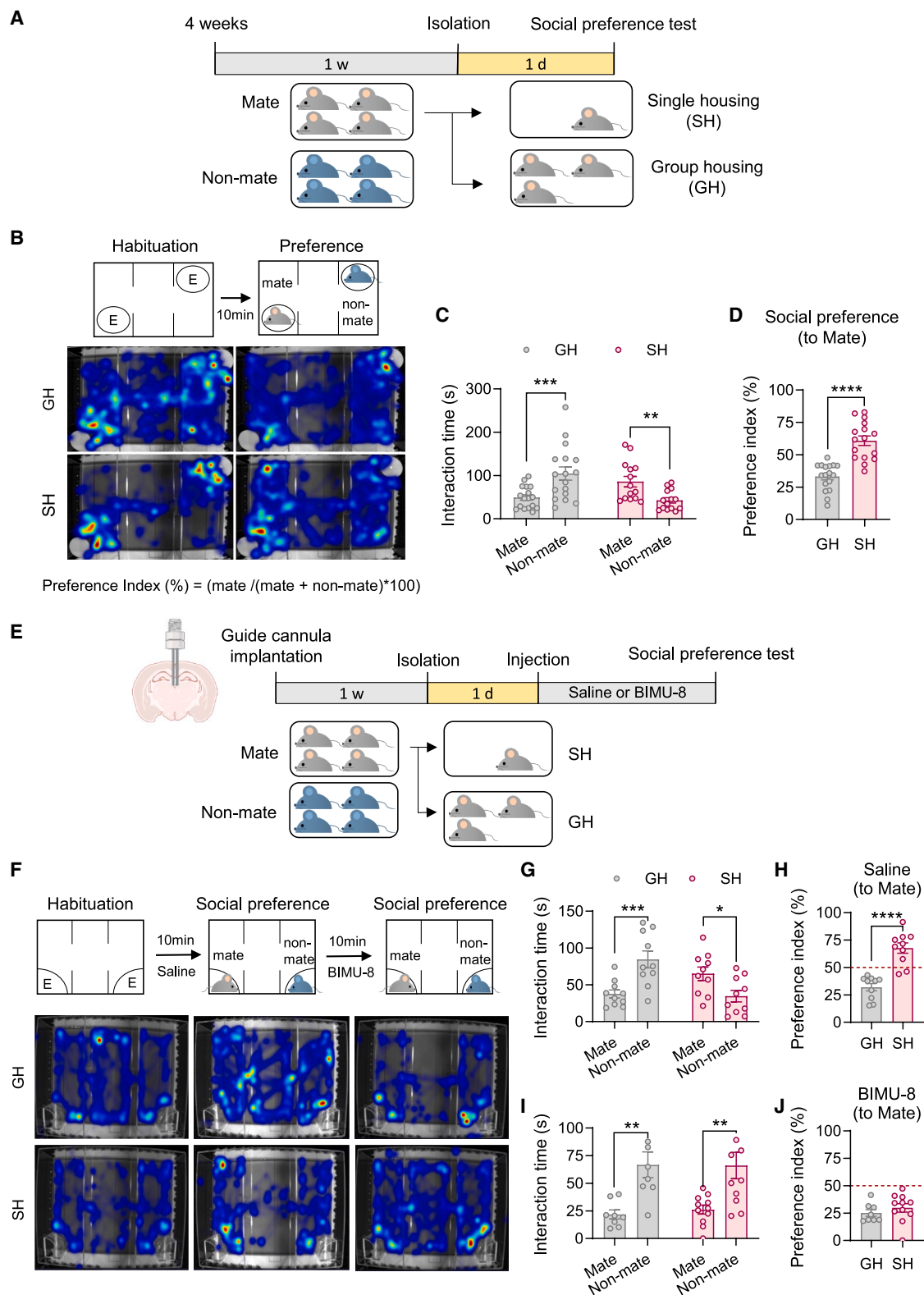


**Figure 4. Modulation of social engagement via 5-HT<sub>2</sub>R activation in the LHb**

(A) Experimental scheme for social preference test with social isolation model. After social isolation, a three-chamber social interaction test was performed (left image).

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distinguishable when the subject mice were exposed to former cage mates and non-cage mate mice (never met with subject mice). Therefore, we modified the conventional three-chamber social interaction task to observe subject mouse social decisions when exposed to cagemate and non-cagemate mice after social isolation (Figure 4A). In the social preference test session, GH mice interacted longer with non-cagemate mice than with cagemate mice, whereas SH mice spent more time with cagemate mice than non-cagemate mice (Figures 4B, 4C and 4D).

To exclude the possibility that social recognition memory impairment is responsible for the increased preference for cagemate mice, we conducted a conventional three-chamber test using non-cagemate mice as strangers or familiar social stimuli. Both GH and SH mice did not show any bias to either chamber (Figure S9). In the sociability test session, both GH and SH subject mice explored the S1 mice more than the empty wire cage (Figures S9B–S9D). After 10 min, the S1 mouse remained in the wire cage, and the S2 mouse was introduced within the empty cage; we assessed the social interaction of the subject mouse for 10 min. Both GH and SH mice preferentially interacted with the novel mouse (S2) than with the familiar mouse (S1) (Figures S9B, S9E, and S9F). These results suggest that acute social isolation reduced natural social interests in non-cagemate mice (Figures 4C and 4D) without the impairment of social recognition (Figure S9) or object recognition memory (Figure S10).

To further assess how social isolation alters the relative value of social versus non-social stimuli, we performed a social value test in which mice were given a choice between a social target and a novel object (Figure S11). Both GH and SH mice showed a clear preference for the novel mouse over the object, with no significant difference between groups, indicating preserved social novelty preference (Figure S11B–S11D). However, while GH mice did not show a preference between the familiar mouse and the novel object, SH mice exhibited a significantly increased preference for the familiar mouse over the novel object, suggesting that acute social isolation induces a familiarity bias in social value assignment (Figures S11B, S11E, and S11F). These findings indicate that although social recognition and novelty preference remain intact following acute social isolation, the relative value assigned to social stimuli is selectively shifted toward familiar stimuli, reflecting a familiarity bias rather than a deficit in social memory.

We next assessed whether 5-HT<sub>4</sub>R activity in the LHB contributes to the altered social preference induced by acute isolation. Bilateral microinfusion of the 5-HT<sub>4</sub>R agonist BIMU-8 into the LHB (Figure 4E) normalized the familiarity-biased preference of

SH mice: whereas saline-injected SH mice preferentially interacted with cage mate (Figures 4F and 4G, and 4H), BIMU-8 treatment redirected their preference toward non-cage mate mice (Figures 4F, 4I, and 4J). In contrast, BIMU-8 did not affect sociability or social novelty preference in either GH or SH mice (Figure S9G–S9K). GH mice maintained their typical preference for unfamiliar conspecifics regardless of treatment. Together, these results indicate that 5-HT<sub>4</sub>R activation specifically corrects the isolation-induced shift in social value without altering baseline social interaction or social recognition.

To determine whether endogenous 5-HT<sub>4</sub>R signaling is required for normal sociability, we inhibited 5-HT<sub>4</sub>R *in vivo*. Antagonist-treated GH mice exhibited reduced sociability, phenocopying aspects of the behavioral alterations observed following acute isolation (Figures S12A–S12E). *Ex vivo* electrophysiological analyses further revealed that LFS-induced LTD in the LHB was diminished in antagonist-treated mice, paralleling the LTD impairment associated with isolation (Figures S12F and S12G). Taken together, 5-HT<sub>4</sub>R activation and inhibition reveal reciprocal modulation of both social behavior and LHB synaptic plasticity, establishing 5-HT<sub>4</sub>R signaling as a bidirectional regulator that links synaptic adaptations to socially relevant behavioral output.

## DISCUSSION

In this study, we demonstrate that acute social isolation rapidly reshapes social decision-making and synaptic plasticity in the LHB through upregulation of 5-HT<sub>4</sub>R signaling. Acutely isolated mice exhibited a robust preference for familiar cage-mates over non-cage mate conspecifics, despite intact sociability and social recognition memory. At the synaptic level, isolation impaired LFS-induced LTD in the LHB, while pharmacological activation of 5-HT<sub>4</sub>R restored both synaptic plasticity and social preference, indicating that 5-HT<sub>4</sub>R signaling is a key regulator of adaptive social behavior following isolation.

These findings raise an important question regarding why acute isolation selectively alters familiarity-driven social valuation while leaving sociability and social recognition intact. Consistent with previous studies,<sup>40,41</sup> both GH and SH mice exhibited a clear preference for a novel conspecific over a novel object, indicating that baseline social approach and novelty-seeking behavior remain intact following isolation. Importantly, this effect was not driven by a general reduction in novelty preference, but rather by a selective enhancement of familiar social value. While GH mice showed little preference between familiar conspecifics and novel objects,

Experimental illustration of social preference task between cage mate and non-cage mate (right image).

(B) Heatmap visualization image for social interaction task to familiar conspecifics.

(C) Summary histograms for the social interaction time and social preference index between cage mate and non-cage mate (two-way ANOVA test with Sidak's multiple comparison test, \*\*\* $p < 0.0001$ , \*\* $p = 0.0044$ ).

(D) Bar graph showing the social preference index to cage-mate (unpaired  $t$  test, \*\*\*\* $p < 0.0001$ ).

(E) Workflow chart for social preference test via intra-LHB region with saline and BIMU-8.

(F) Experimental illustration of social preference task in saline- and BIMU-8-injected mice (top image). Heatmap visualization image for social interaction task between cage-mate and non-cage-mate (down image).

(G and H) Summary histogram of social interaction time and interaction index in saline-injected mice (two-way ANOVA test with Sidak's multiple comparisons test, \*\*\* $p = 0.0004$ , \* $p = 0.0141$ , unpaired  $t$  test, \*\*\*\* $p < 0.0001$ ).

(I and J) Summary histogram of social interaction time and interaction index in BIMU-8-injected mice (two-way ANOVA test with Sidak's multiple comparisons test, \*\* $p = 0.0016$ , \* $p = 0.0011$ , unpaired  $t$  test, ns,  $p = 0.3882$ ).

SH mice exhibited a robust preference for familiar cage-mates rather than novel objects, revealing a pronounced familiarity bias induced by acute isolation. This distinction becomes clearer when considering the distinct cognitive demands and neural circuits engaged by the behavioral paradigms used in this study.

The social preference task (Figure 4) probes long-term affiliative social memory and the motivational value associated with previously bonded partners, processes tightly linked to LHB-serotonergic pathways that encode social valence.<sup>19,42,43</sup> Our data suggests that acute isolation selectively amplifies LHB-dependent valuation signals for familiar conspecifics. By contrast, the three-chamber social interaction task (Figure S9) primarily assesses discrimination between unfamiliar individuals, a function supported by hippocampal CA2 and ventral CA1 circuits specialized for social novelty processing.<sup>44–46</sup> Consistent with this dissociation, acute isolation altered social value assignment without disrupting hippocampus-dependent social recognition or general sociability.<sup>44,47</sup> Together, these findings support a circuit-level framework in which LHB-centered pathways regulate familiarity-related social valuation, while CA2/vCA1 circuits mediate novelty recognition.

Mechanistically, our results identify 5-HT<sub>4</sub>R signaling as a key modulatory node linking LHB synaptic plasticity to social behavioral adaptation. Acute isolation increased *Htr4* expression in LHB neurons and disrupted LTD induction, suggesting a shift in synaptic state that biases behavioral output. Given that the LHB exerts inhibitory control over serotonergic nuclei, increased LHB activity following acute isolation may reduce serotonergic tone in downstream circuits. In this context, the observed upregulation of *Htr4* may represent a compensatory adaptation to reduced serotonin availability, enhancing receptor sensitivity and signaling efficacy. Accordingly, pharmacological activation of 5-HT<sub>4</sub>R may restore synaptic and behavioral function by re-engaging serotonin-dependent modulation in the LHB. Activation of 5-HT<sub>4</sub>R restored LTD and normalized social preference, redirecting behavior toward novel conspecifics without affecting baseline sociability or social novelty. These findings indicate that 5-HT<sub>4</sub>R-dependent metaplasticity dynamically regulates the motivational value of social stimuli in response to recent social experience. Within this framework, acute isolation may bias LHB output toward encoding familiarity-related positive valence, thereby promoting preferential engagement with familiar partners under socially deprived conditions.

Consistent with this, previous studies have demonstrated that p11 (S100A10) regulates dopaminergic signaling in the LHB through interactions with D2 receptors, thereby influencing affective behaviors.<sup>48</sup> In addition, p11 has been identified as a key regulator of serotonin receptor trafficking, including 5-HT<sub>1B</sub> and 5-HT<sub>4</sub> receptors, by promoting their surface expression and functional signaling.<sup>49,50</sup> Together, these findings suggest that p11 functions as a critical molecular modulator of neuromodulator receptor signaling within LHB circuits. Given this role, p11-dependent mechanisms may contribute to the regulation of serotonergic receptor function in the LHB under social stress conditions.

Activation of 5-HT<sub>4</sub>R reinstated LTD and corrected isolation-induced behavioral biases, whereas pharmacological inhi-

bition produced complementary disruptions even under non-stressed conditions. These mirrored effects, together with prior evidence implicating LHB-serotonergic pathways in social valence and social homeostasis,<sup>10,19,42,43,51</sup> support a model in which 5-HT<sub>4</sub>R-dependent plasticity dynamically tunes the motivational value of social partners according to recent social experience. Within this framework, acute isolation may shift social preference by altering 5-HT<sub>4</sub>R-driven synaptic states in the LHB, enabling rapid recalibration of social engagement as animals move between socially deprived and socially enriched environments.

Histological and molecular analyses revealed low baseline *htr4* expression in the LHB and LHB of GH mice, consistent with prior reports of high *htr4* transcript levels in the MHb. Importantly, Imaris-based quantification demonstrated that isolation-induced *Htr4* upregulation occurred predominantly in NeuN-positive LHB neurons, whereas S100 $\beta$ -positive glial cells showed no significant changes. This cell-type-specific enhancement indicates that acute social stress selectively recruits neuronal 5-HT<sub>4</sub>R expression rather than inducing a global increase across cell populations. Moreover, in our social stress model, *Htr4* upregulation was observed in the LHB but not in the MHb. 5-HT<sub>4</sub>R activation did not alter basal transmission or plasticity in GH mice but successfully rescued LTD deficits in SH mice. As 5-HT<sub>4</sub>R are Gs-coupled receptors that activate the cAMP-PKA signaling cascade, their engagement may suppress the heightened LHB activity induced by social isolation. While other serotonin receptors such as 5-HT<sub>1R</sub>, 5-HT<sub>2R</sub>, and 5-HT<sub>3R</sub> modulate LHB excitability via pre- or postsynaptic mechanisms,<sup>25–27</sup> the physiological role of 5-HT<sub>4</sub>R in the LHB has remained largely unexplored.<sup>52</sup> The LHB sends glutamatergic projections to the VTA, RN, and RMTg, forming inhibitory control pathways that regulate monoaminergic tone.<sup>33,34</sup> Social isolation-induced increases in LHB neuronal activity may suppress serotonergic output through these projection pathways, thereby triggering compensatory upregulation of 5-HT<sub>4</sub>R in LHB neurons (Figure S3D). Activation of 5-HT<sub>4</sub>R may restore serotonin-dependent neuromodulation and promote LTD recovery under social stress.

In conclusion, this study reveals that acute social isolation disrupts synaptic depression in the LHB and alters natural social preference for unfamiliar conspecifics by selectively enhancing the motivational value of familiar individuals. These alterations are accompanied by neuronal 5-HT<sub>4</sub>R upregulation and neurotransmission changes that contribute to plasticity deficits. Our findings provide insight into LHB synaptic mechanisms linking serotonin dynamics to social behavioral adaptation. The complex interactions within the mPFC-LHB-RN axis likely constitute a mechanistic pathway through which isolation shapes social decision-making. Future studies employing cell-type-specific optogenetic or chemogenetic manipulations will be essential for delineating how defined LHB circuits encode and update social motivational states.

#### Limitations of the study

Although our findings shed light on the role of 5-HT<sub>4</sub>R in modulating LHB synaptic plasticity and social decision-making

following acute social isolation, several limitations should be noted. All experiments were conducted exclusively in adolescent male mice. Because the serotonin system undergoes significant developmental changes during adolescence, the generalizability of our findings to adult animals remains to be determined. While the use of male mice minimized variability associated with the estrous cycle, it limits the generalizability of our findings across sexes. Given well-established sex differences in stress responsiveness, serotonergic signaling, and social behavior, the role of LHB 5-HT<sub>4</sub>R signaling may differ in females. Future studies incorporating female cohorts and accounting for estrous cycle stages will be important to determine the broader applicability of our findings.

In addition, although our data suggest potential subdivision-specific differences within the LHB (e.g., medial vs. lateral subregions), we did not directly test their distinct contributions to social behavior. Furthermore, while we observed reduced serotonin levels, we did not identify the upstream signals or intracellular mechanisms responsible for 5-HT<sub>4</sub>R upregulation in the LHB. Future studies using prolonged isolation paradigms, advanced circuit-mapping approaches, and targeted manipulations of 5-HT<sub>4</sub>R signaling will be important to elucidate how acute social stress reshapes LHB function at both molecular and circuit levels.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeongyeon Kim ([jkim@kbri.re.kr](mailto:jkim@kbri.re.kr)).

### Materials availability

This study did not generate new, unique reagents. Established mouse lines and other reagents are available upon request from the lead contact.

### Data and code availability

- The raw bulk RNA-seq data generated in this study have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE200082 and are publicly available. The accession number is also listed in the [key resources table](#).
- This study did not generate or use custom code.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

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## AUTHOR CONTRIBUTIONS

Conceptualization, J.K. and M.K.; methodology, M.K., K.K., S.U., and B.K.; investigation, M.K., K.K., and B.K.; writing – original draft, M.K., S.C., and

J.K.; writing – review and editing, M.K., S.S., S.C., and J.K.; funding acquisition, J.K., S.C., and M.K.; resources, J.K.; supervision, S.C. and J.K.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## Supplemental information

Supplementary data related to this article can be found online at <https://doi.org/10.1016/j.celrep.2026.117458>.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                                | SOURCE                               | IDENTIFIER                                                                              |
|--------------------------------------------------------------------|--------------------------------------|-----------------------------------------------------------------------------------------|
| <b>Experimental models: Organisms/strains</b>                      |                                      |                                                                                         |
| Mouse: wild type C57BL/6 J                                         | The Jackson Laboratory               | Stock# 000664; RRID:IMSR_JAX:000664                                                     |
| <b>Antibodies</b>                                                  |                                      |                                                                                         |
| Anti-5HT <sub>4</sub> Receptor antibody                            | Abcam                                | CAT# ab60359 (Lot #: GR#222815-7); RRID:AB_2122438                                      |
| Anti-5-HT <sub>4</sub> Receptor (extracellular) antibody           | Almone Lab                           | CAT# ARS-036; RRID:AB_2756796                                                           |
| cFos (9F6) Rabbit mAb                                              | Cell Signaling Technology            | CAT# 2250S; RRID:AB_2247211                                                             |
| Anti-NeuN Antibody, clone A60                                      | Merk Millipore                       | CAT# MAB377; RRID:AB_2298772                                                            |
| Anti-s100 $\beta$ Antibody                                         | Abcam                                | CAT# ab52642; RRID: AB_882426                                                           |
| Rabbit Anti-GAPDH Polyclonal Antibody, Unconjugated                | Bioss antibodies                     | CAT# BS-0459R; RRID:AB_10856088                                                         |
| Goat anti-Mouse IgG2b Antibody HRP Conjugated                      | BETHYL                               | CAT# A90-109P; RRID:AB_67160                                                            |
| Goat anti-Rabbit IgG-heavy and light chain Antibody HRP Conjugated | BETHYL                               | CAT# A120-101P; RRID:AB_67264                                                           |
| Goat anti-Rabbit IgG Alexa Fluor 488                               | Thermo Fisher Scientific             | CAT# A-11034; RRID:AB_2576217                                                           |
| Goat anti-Rabbit IgG Alexa Fluor 594                               | Thermo Fisher Scientific             | CAT# A-11005; RRID:AB_2534073                                                           |
| Mm-Fos-C1 probe                                                    | Advanced Cell Diagnostics            | CAT# 316921-C1                                                                          |
| Mm-Htr4-C3 probe                                                   | Advanced Cell Diagnostics            | CAT# 408241-C3                                                                          |
| <b>Chemicals, peptides, and recombinant proteins</b>               |                                      |                                                                                         |
| 2,2,2-tribromoethanol                                              | Sigma-Aldrich                        | CAT# T48402-100G                                                                        |
| VECTASHIELD HardSet Antifade Mounting Medium with DAPI             | Vector Laboratories                  | CAT# H-1200-10                                                                          |
| SuperSignal West Pico PLUS Chemiluminescent substrate              | Thermo Fischer                       | CAT# 345777                                                                             |
| Normal Goat Serum                                                  | Jackson ImmunoResearch Lab           | CAT# 005-000-121                                                                        |
| Albumin Bovine Fraction V                                          | Thermo-Scientific                    | CAT# J10857-22                                                                          |
| Triton X-100                                                       | Sigma-Aldrich                        | CAT# T8787-250ML                                                                        |
| Reverse Transcription 5 $\times$ Premix (random hexamer)           | ELPIS-Biotech                        | CAT# EBT-1514                                                                           |
| Real-Time qPCR 2 $\times$ Master Mix (SYBR Green and ROX dye)      | ELPIS-Biotech                        | CAT# EBT-1802                                                                           |
| BIMU-8                                                             | Tocris Bioscience                    | CAT# 0903                                                                               |
| Zacopride                                                          | Tocris Bioscience                    | CAT# 1075                                                                               |
| RS39604 hydrochloride                                              | Tocris Bioscience                    | CAT# 0991                                                                               |
| <b>Critical commercial assays</b>                                  |                                      |                                                                                         |
| High-sensitive serotonin ELISA kit                                 | Labor Diagnostika Nord GmbH & Co.KG, | CAT# BAE-5900 R                                                                         |
| RNeasy Micro Kit,                                                  | Qiagen Inc., Basel, Switzerland,     | CAT# 74004                                                                              |
| RNAscope Multiplex Fluorescent Reagent Kit v2 with TSA Vivid dyes  | Advanced Cell Diagnostics            | CAT# 323280                                                                             |
| <b>Software and algorithms</b>                                     |                                      |                                                                                         |
| EthoVision XT                                                      | Nodus                                | <a href="https://www.noldus.com/ethovision-xt">https://www.noldus.com/ethovision-xt</a> |
| GraphPad Prism 10.0                                                | GraphPad software                    | <a href="https://www.graphpad.com/">https://www.graphpad.com/</a> ; RRID:SCR_002798     |
| WinLTP 2.20 b                                                      | WinLTP                               | <a href="https://winltp.co.uk/">https://winltp.co.uk/</a> ; RRID:SCR_008590             |

(Continued on next page)

**Continued**

| REAGENT or RESOURCE | SOURCE            | IDENTIFIER                                                                                                                                                          |
|---------------------|-------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Clampit 10.6        | Molecular Devices | <a href="https://www.moleculardevices.com/products/axon-patch-clamp-system">https://www.moleculardevices.com/products/axon-patch-clamp-system</a> ; RRID:SCR_011323 |
| Clampex 10.6        | Molecular Devices | <a href="https://www.moleculardevices.com/products/axon-patch-clamp-system">https://www.moleculardevices.com/products/axon-patch-clamp-system</a> ; RRID:SCR_011323 |
| IMARIS 9.9          | OXFORD Instrument | <a href="https://imaris.oxinst.com">https://imaris.oxinst.com</a> ; RRID:SCR_007370                                                                                 |

**Deposited data**

|                         |            |                |
|-------------------------|------------|----------------|
| Mouse bulk RNA-seq data | This paper | GEO: GSE200082 |
|-------------------------|------------|----------------|

**Other**

|                                                       |                      |                              |
|-------------------------------------------------------|----------------------|------------------------------|
| CMA 7 6 kDa Microdialysis Probe, 1 mm membrane length | CMA, Sweden          | CAT# CMAP000082              |
| Refrigerated fraction collector                       | CMA470/Microdialysis | CAT# 8002770                 |
| Real-Time PCR 7500 Fast system                        | Applied Biosystems   | CAT# A30299; RRID:SCR_018051 |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

### Animal

Male C57BL/6J mice were purchased from Orient Bio Inc. (Seongnam, South Korea). Mice were used during adolescence (postnatal day 28–35; 20–30 g), a developmental period characterized by heightened sensitivity to social experience and commonly used to study the effects of social isolation on social behavior. All experiments were conducted in adolescent male mice. Female mice were not included in the present study to minimize variability associated with estrous cycle-dependent hormonal modulation of serotonergic signaling and stress responsivity.<sup>53–59</sup> Mice were maintained on a 12 h light–dark cycle in a specific-pathogen-free facility under controlled temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity (60%), with *ad libitum* access to food and water. All procedures were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Brain Research Institute. The mice were maintained on a 12 h light–dark cycle in a specific-pathogen-free facility with controlled temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity (60%) and had free access to food and water. All animal experiments were conducted following the guidelines of the Institutional Animal Care and Use Committee of the Korea Brain Research Institute.

### Acute social isolation

Four C57BL/6J mice were housed in a home cage for more than 1 week. For acute social isolation, a mouse randomly selected was isolated for 24 h in a distinct cage as social isolation (SH) mice. One of the three mice remaining in the home cage was used as a group-housed (GH) mice, and the rest two mice were used as mates in the social behavior test. The social interaction and ultrasound vocalization between SH and GH mice were minimized by allowing sufficient space between the housing cages.

## METHOD DETAILS

### Social preference test

Social preference was assessed based on the social interaction time between cage mates and non-cage mate mice among the GH and SH mice. Before the test, all mice were allowed to adapt for 10 min (habituation session) in an empty three-chamber apparatus ( $60 \times 40 \times 30$  cm) consisting of left, center, and right zones with gates. The cage mate mouse was placed in the left wire cage, and the non-cage mate mouse was placed in another wire cage within the right zone. A test mouse was placed in the center chamber and allowed to interact socially with the cage-mate and non-cage mate mice for 10 min.

### Three-chamber social interaction task

Before the sociability test, all the mice were allowed to adapt for 10 min in a three-chamber apparatus under the same conditions. For the sociability test, we assessed the social interaction between the empty chamber and a stranger mouse (S1) for 10 min. After 10-min intervals, the S1 mouse (familiar) was allowed to stay in the wire cage, and we assessed the social interaction of the mice by placing a novel stranger mouse (S2) in the empty cage for 10 min. All three-chamber tests were conducted using an automatic video-tracking system (EthoVision XT 14 software, Nodus Information Technology Inc., Leesburg, VA, USA).

### Social value preference test

To assess the relative preference for social versus non-social stimuli, mice were tested in a modified three-chamber apparatus (60 × 40 × 30 cm). Mice were first allowed to freely explore the empty apparatus for 10 min for habituation. For the test session, mice were then allowed to freely explore the apparatus for an additional 10 min with a social stimulus placed in one side chamber and a novel object in the opposite chamber, each enclosed in identical wire cages. The social stimulus was either a non-cage mate (novel social session) or a cage mate (familiar social session). The novel object was a plastic object of comparable size to the stimulus mouse. Stimulus locations were counterbalanced across animals, and all apparatus surfaces were cleaned with 70% ethanol between trials. Behavior was recorded and analyzed offline. Preference was evaluated by comparing the time spent investigating the social stimulus versus the novel object, as well as time spent in each chamber when appropriate.

### Cannula implantation

For guide cannula surgery, animals were deeply anesthetized with tribromoethanol (intraperitoneal injection, 250 mg/kg) and mounted on a stereotaxic apparatus. Thereafter, 26-G guide cannulas (Plastics One Inc., #315G, Roanoke, Virginia, USA) were bilaterally implanted into the LHb (Anterior-Posterior (AP): −1.65 mm; Medial-Lateral (ML): ±0.5 mm; Dorsal-Ventral (DV): −2.9 mm, from bregma). Three anchoring screws with implanted cannulas were affixed to the skull with dental cement. The guide cannula was closed using a 32-G dummy cannula (Plastics One Inc., #315G, Roanoke, Virginia, USA) to prevent clogging. After surgery, all mice were allowed to recover for 5 days. Before the social preference test, 0.1 μL of saline or BIMU-8 (Tocris, Cat #4374, 3 μg/mouse) was administered bilaterally at a rate of 0.1 μL/min bilaterally using a Hamilton syringe with a syringe pump. The interval between the drug administration and test session was 10 min. Histological confirmations of whether the tips of the cannula were targeted in the LHb were made after each experiment.

### mRNA sequencing and data analysis

mRNA sequencing was performed using two biological replicates for the GH and SH conditions. For each sample in the two conditions, LHb tissues were collected from four mice (four LHb tissues per sample) and pooled into the tube to minimize the effect of inter-individual variability in mice. Total RNA was then obtained from the LHb tissues. The integrity of the total RNA in each sample was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity values for all the samples were greater than 8.5. Poly(A) mRNA was isolated from the total RNA, and subsequent fragmentation was performed using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. The adaptor-ligated libraries were sequenced using Illumina NovaSeq 6000 (Macrogen, Inc., Seoul, South Korea). From the resulting read sequences for each sample, adapter sequences (TruSeq universal and indexed adapters) were removed using cutadapt software (version 2.7).<sup>60</sup> The remaining reads were then aligned to the *Mus musculus* reference genome (GRCm38) using TopHat2 software (version 2.1.1; <https://ccb.jhu.edu/software/tophat/index.shtml>) with default parameters.<sup>61</sup> After the alignment, we counted the number of reads mapped to the gene features (GTF file of GRCm38.91) using HTSeq.<sup>62</sup> Subsequently, read counts for the samples in each condition were normalized using the trimmed mean of M-value normalization of the edgeR package.<sup>63</sup> The raw data were deposited into the Gene Expression Omnibus database with accession ID GSE200082.

### Identification of differentially expressed genes (DEGs)

The number of reads for the gene features counted by HTSeq was converted to log2-values after adding one (pseudo count) to the read counts. To identify the DEGs, hypothesis testing was performed.<sup>62</sup> Briefly, for each gene, a *t*-statistic value was calculated using Student's *t* test for the comparison of social isolation versus grouped housing. An empirical distribution of the *T*-statistic value for the null hypothesis (i.e., the genes are not differentially expressed) was then estimated by performing all possible combinations of random permutations of the samples. The *p*-values from the Student's *t* test for each gene were computed using a two-tailed test with an empirical null distribution. The DEGs were identified as genes whose analyses showed *p*-values <0.1 and log2-fold changes >0.278 (1.21-fold; the mean of the 0.5th and 99.5th percentiles of the null distribution of log2-fold changes).

### Functional enrichment and network analyses

Gene set enrichment analysis of the DEGs was performed using ConsensusPathDB software (version 35).<sup>64</sup> GO biological processes and pathways enriched by the DEGs were identified as the ones with *p* < 0.1.<sup>65</sup> The network model was built using Cytoscape (version 3.8.2),<sup>66</sup> and the nodes in the network model were arranged based on their localizations and relationships in their associated pathways with the edges indicating activation/inhibition information from the KEGG pathway database.

### The laser capture microdissection microscope (LCM) system

The slides were stained with 1% cresyl violet in 75% ethanol (w/v) for 45 s, dehydrated in a graded ethanol series (50, 75, 95, 100% for 30 s each), in 100% ethanol for 5 min and air-dried. Laser microdissection was performed using a P.A.L.M. MicroBeam (CARL ZEISS). The LHb was collected into AdhesiveCap tubes (Zeiss). Collected LHb tissue was lysed in 10 μL lysis buffer. Lysis buffer was prepared by adding 10 μL beta-mercaptoethanol (β-ME) per mL of RLT solution. Total RNA was immediately isolated using the RNeasy Micro Kit (Qiagen Inc., Basel, Switzerland, Cat# 74004). Quality and quantity of the total RNA samples were determined by measuring the A260/280 and A260/230 ratio using the NanoDrop (Thermo Scientific, Waltham, MA, USA).

### RNA extraction and quantitative real-time PCR (qRT-PCR)

We analyzed the *htr4* gene expression levels using qRT-PCR. The MHb and LHb tissue samples of the four mice were separated with an ice-cold artificial cerebrospinal fluid (aCSF) solution using schel knife (Fine Science Tools, Cat# 10073-14). To determine according to the manufacturer's protocol, total RNA from the pooled samples was extracted using carrier RNA for several samples (RNeasy Micro Kit, Qiagen Inc., Basel, Switzerland, Cat# 74004). The cDNA was synthesized using the Reverse Transcription 5× Premix (random hexamer, ELPIS-Biotech, EBT-1514, Daejeon, Korea) for 60 min at 42°C, followed by enzyme inactivation. We performed qRT-PCR using a Real-Time PCR 7500 Fast system (Applied Biosystems, Foster City, CA, USA) and Real-Time qPCR 2× Master Mix with SYBR Green and ROX dye (ELPIS-Biotech, EBT-1802, Daejeon, Korea). The PCR cycling parameters were 3 min at 94°C, followed by 40 cycles of 10 s at 94°C, 10 s at 55°C, and 30 s at 72°C.  $\beta$ -actin was used to normalize the quantitation gene expression and the relative gene expression was calculated. The following oligonucleotide pairs were used: *htr4* forward primer sequence (5' to 3') GAT GCT AAT GTG AGT TCC AAC GA, *htr4* reverse primer sequence (3' to 5') GCA GCA GAT GGC GTA ATA CCT,  $\beta$ -actin forward primer sequence (5' to 3') GAG CTA TGA GCT GCC TGA CGG, and  $\beta$ -actin reverse primer sequence (3' to 5') CAG CTC AGT AAC AGT CCG CCT. All oligonucleotides were synthesized by BIONICS (Seoul, South Korea).

### Serotonin enzyme-linked immunoassay

According to the manufacturer's protocols, serum levels in the LHb tissues were determined using a serotonin ELISA kit (Labor Diagnostika Nord GmbH & Co. KG, BAE-5900 R, Nordhorn, Germany). Briefly, 100  $\mu$ L of diluted standard, diluted control, and samples were added to the well in the acylation plate. Acylation buffer (25  $\mu$ L) was added to each tube and incubated at room temperature for 30 min. Furthermore, 100  $\mu$ L of acylated samples, standard solution, and control solution were loaded into pre-coated serotonin/5-HIAA microtiter strips with 25  $\mu$ L of serotonin antiserum. The plates were incubated for 20 h in a 4°C cold room. All the contents of the strips were reacted with 100  $\mu$ L of enzyme conjugate and incubated at room temperature for 30 min. After washing, the substrate was loaded into each well and incubated at room temperature for 30 min. The reaction was stopped by adding a stop solution (100  $\mu$ L). The absorbance was read at 450 nm in a multi-microplate reader (Spectramax iD5 multi-mode microplate reader, Molecular Devices, San Jose, USA). Serotonin concentrations were calculated from a standard curve using the GraphPad software (GraphPad Prism 8.0, La Jolla, CA, USA).

### Extracellular field recording

C57BL/6J mice were anesthetized via isoflurane aspiration. Sagittal sections of the LHb slices (300- $\mu$ m thick) were cut in ice-cold sucrose solution containing (in mM): sucrose, 175; NaHCO<sub>3</sub>, 26; glucose, 11; KCl, 3.5; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; MgCl<sub>2</sub>, 1.3; and CaCl<sub>2</sub>, 2 (saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>). Thereafter, the LHb slices were transferred to a holding chamber filled with aCSF, containing (in mM): NaCl, 120; NaHCO<sub>3</sub>, 26; glucose, 11; MgCl<sub>2</sub>, 1.3; NaH<sub>2</sub>PO<sub>4</sub>, 1.25; KCl, 3.5; and CaCl<sub>2</sub>, 2. Freshly cut slices were placed in an incubation chamber containing aCSF and recovered at room temperature (21°C–23°C) for 1 h. For recording, each slice was transferred to a recording chamber and continuously superfused with oxygenated aCSF at a flow rate of 2.5 mL/min at room temperature.

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the LHb with aCSF-filled 3.5–4 M $\Omega$  glass pipettes using WinLTP software ([winltp.com](http://winltp.com); The University of Bristol, UK) and National Instruments Data Acquisition (NI DAQ, BNC-2110, USA). Current stimuli were delivered to a constant stimulus isolation unit (100- $\mu$ s pulse duration) (World Precision Instruments, Inc., Sarasota, FL, USA) using WinLTP 2.31 software. The stimulation rate was 0.067 Hz for all fEPSP recording experiments. After obtaining a stable baseline for 20 min, LTD was induced by applying 900 pulses at 1 Hz (low-frequency stimulation, LFS) through a concentric bipolar electrode (FHC, Bowdoin, Me, USA; 125  $\mu$ m/Rnd/25  $\mu$ m Pt-Ir) with the same stimulating intensity that was used during baseline. The amplitude of the response was used to assess the changes in synaptic strength.

### Whole cell recordings

Whole cell recordings were obtained using an Axopatch 700 B. The glass capillary pipette was filled with potassium-based internal solution containing 120 mM K-gluconate, 4 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 0.2 mM Na-GTP, 2mM Mg-ATP, and 1mM MgCl<sub>2</sub>. The internal solutions' pH was adjusted to 7.2 with KOH or CsOH and with osmolarity adjusted to approximately 290 mmol/kg by sucrose. Recordings were made under IR-DIC, and cells that were three to four cell layers below the surface of 300  $\mu$ m thick slices were recorded at a bath temperature of 32.0°C  $\pm$  1.0°C. Cells were voltage-clamped at –70 mV to measure access resistance before and at the end of the recording session. Bath solutions were delivered to slices via superfusion driven by a peristaltic pump at a 1.7 mL/min flow rate. The data were discarded if the series resistance changed by over 20%. Membrane potential traces were digitized to 10 kHz, and stored on a microcomputer (Clampex 10 software; Molecular Devices). All the recordings were completed within 6 h of slice preparation.

### Fluorescent of in situ hybridization (FISH) analysis

#### Sample pretreatment

Fixed frozen brain slices (coronal section, 15  $\mu$ m) were prepared with super adhesion glass (SuperFrost Plus slides, Eprelia, Cat. #J1800AMNZ). All slides were removed from the 100% ethanol and baked at 60°C dry oven for 30 min. This step improves the sample attachment to the slides and avoids tissue removal from the slides during subsequent washing steps. After baking the slides to create

a hydrophobic barrier around the sample, an Immedge hydrophobic barrier pen (Vector Laboratories) was used to avoid touching or disrupting the tissue section. Utilizing the multiplex fluorescence V2 RNAscope kit (RNAscope® Intro Pack for Multiplex Fluorescent Reagent Kit v2 Cat. # ACD\_323280), the hydrogen peroxide and Protease III were applied according to the manufacturer's instructions. Because it was observed that a 30-min Protease III treatment caused over-digestion of the leaf sections, the treatment was reduced to 15 min.

### **Probing FISH**

The RNAscope assay was conducted by multiple hybridization steps (probe and AMP binding) and signal development (HRP channel binding) as described in the manufacturer protocol (RNAscope® Intro Pack for Multiplex Fluorescent Reagent Kit v2 with TSA Vivid Dyes, Cat. # 323280, Advanced Cell Diagnostics). Briefly, all probes were customized in Advanced Cell Diagnostics. cFos and Htr4 probe were customized in the C1 and C3 channel (Mm-Fos-C1, Cat. # 316921-C1; Mm-htr4-cust-C3, Cat. # 408241-C3). Single probes and multiplexed assays were used to validate the efficiency of the assays. The hybridizations were performed and analyzed using slides containing multiple independent tissue sections. For probe hybridization, the samples were incubated in the HybEz™ II humidifying system (HybEz Oven, #321721) at 40°C. In our experience, extended probe hybridization times of up to 4 h with the fixed frozen samples improve the probe hybridization and subsequent visualization. The following day, slides were washed in 1 × wash buffer, AMP signal amplification, and HRP labeled probe hybridization steps were carried out according to the manufacturer protocol (Multiplex fluorescence V2 kit, ACD Bio). We have used TSA Vivid fluorophore 520 (green) and fluorophore 570 (orange), which were diluted at 1:1500 to visualize the Mm-htr4-C3 signal in green, Mm-Fos-C1 signal in orange. Developed sample slides after the AMP staining were treated with nuclear stain DAPI. DAPI was removed by flicking the slides within 30 s, and a coverslip was placed utilizing the Everbright hard-set mounting media. These slides were stored overnight in the dark before visualization for complete drying up of hard-set mounting media (ProLong™ Gold Antifade Mount, Invitrogen™, #P36930).

### **Immunohistochemistry (IHC)**

Mice were anesthetized with 2,2,2-tribromoethanol, commercially referred to as Avertin (Sigma Aldrich, St Louis, MO, USA, T48402-100G) (250 mg/kg, intraperitoneal injection). The brain was fixed with ice-cold 4% paraformaldehyde (PFA) followed by saline through cardiac perfusion and removed from the skull. Whole-brain consecutively incubated in 4% PFA solution (overnight in a 4°C cold room) and 30% sucrose (Sigma Aldrich, St Louis, MO, USA, 84097-500G) solution (dissolved in phosphate-buffered saline [PBS] for three days in a 4°C cold room). For cFos analysis, tissue was collected immediately after the 24h isolation period without behavioral challenge. Individual tissue blocks were embedded in Optimal cutting temperature (OCT) cryoprotectants (SAKURA 4583). For immunolabel staining within the MHb and LHb, 35 µm thick cryoprotected coronal sections were serially obtained using a cryotome (CM1860, Leica, Mannheim, Germany).

Immunohistochemistry was performed using the standard free-floating method. Brain slices were washed twice in 10 min with 1 × PBS solution (10 × PBS; Biosesang, Korea, PR4007-100-00). Brain slices were permeabilized using a blocking solution for 90 min at room temperature (22°C–24°C). The blocking solution contained 4% normal goat serum (005-000-121, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), 0.5% bovine serum albumin (Albumin, Bovine Fraction V, Thermo-Scientific, J10857-22), and 0.2% Triton X-100 (Sigma Aldrich, T8787-250ML) in PBS. Primary antibodies were incubated overnight in a 4°C cold room for immunolabel staining. The primary antibodies used were anti-htr4 (Abcam, ab60359, Lot #: GR#222815-7; Alomone labs, ASR-036, dilution rate: 1:500), anti-NeuN (Millipore, MAB377, dilution rate: 1:1000) and anti-s100β (Abcam, Cat. # ab52642, 1:500). The secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, #A-11034, dilution rate: 1:500), goat anti-mouse IgG Alexa Fluor 594 (Invitrogen, #A-11005, dilution rate: 1:500), and goat anti-mouse IgG Alexa Fluor 647 (Invitrogen, #A-28181, dilution rate: 1:500) for 90 min at room temperature. After three washes with PBS (for 5 min × 3), the brain slices were mounted with a DAPI-containing mounting medium (VECTASHIELD HardSet Antifade Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA, USA). Images of the stained tissues were acquired using a confocal microscope (Nikon Eclipse Ti-RCP, Tokyo, Japan). Quantification of immunohistochemical signals was performed using Fiji (ImageJ). Regions of interest (ROIs) corresponding to the LHb were defined based on anatomical landmarks, and identical ROIs were applied across sections. Fluorescence intensity was measured after applying a consistent threshold across all images to exclude background signals. For cell-based analysis, positively labeled cells were counted using the same threshold criteria. Signal intensity values were normalized to the mean intensity of the control group (GH) within each experiment. All analyses were conducted blind to experimental conditions.

### **Western blot analysis**

For Western blot analysis, lateral habenula lysates were prepared in lysis buffer solution containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 0.2% NP-40, and protease inhibitors. Equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose (NC) membranes (Bio-Rad #1620117, 0.45 µm), and incubated with primary antibodies overnight at 4°C. After three washes, we incubated membranes with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h 30 min at room temperature (23°C). Signals were visualized with a SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Fischer, Cat. # 345777). We used the following antibodies: Rb pAb to 5-HT<sub>4</sub> receptor (Abcam, Cat. # Ab60359) at 1:1,000, GAPDH (Bioss, Cat. # bs-0459 R) at 1:10,000, and goat anti-Rabbit HRP conjugated (BETHYL, Cat. # A120-101 P) or goat anti-Mouse HRP conjugated (BETHYL, A90-109 P) at 1:1000. To quantify the intensity of the western blot bands, the area of each band was quantified using the ImageJ program. Band quantitation was obtained from four independent experiments. All statistical data were calculated and graphed using GraphPad Prism 10 (GraphPad, Inc., La Jolla, CA, USA).

### Imaging analysis with IMARIS

Fluorescent *in situ* hybridization images acquired from confocal microscopy were analyzed using IMARIS software (version 9.9; Oxford Instruments). For quantification of *htr4* puncta and cFos expression, the “Spots” function was used to detect discrete RNA puncta and nuclear cFos signals based on intensity thresholds automatically estimated by the software and manually refined to minimize background detection. Regions of interest (ROIs) were defined to encompass the borders of the LHb using anatomical landmarks from the Paxinos mouse brain atlas. For colocalization analysis between *htr4* puncta and cFos<sup>+</sup> cells, the “Colocalize Spots” module was used to identify puncta located within a defined radial distance (0.3–0.5  $\mu$ m) from cFos-labeled nuclei. The proportion of cFos<sup>+</sup> cells containing  $\geq 1$  *htr4* punctum and the proportion of *htr4*<sup>+</sup> puncta localized within cFos<sup>+</sup> cells were automatically computed using the Imaris statistics export function. Cell-type specific quantification of *htr4* expression was performed by generating surfaces for NeuN<sup>+</sup> neurons and S100b<sup>+</sup> astrocytes using the “Surface” function with smoothing parameters (0.2–0.4  $\mu$ m) adjusted to accurately capture cell boundaries. *Htr4*-positive puncta within each cell type were identified through the “Find Spots Close to Surface” tool, using a maximum distance criterion of 0.5  $\mu$ m to assign puncta to their corresponding cellular compartments. All Imaris-generated statistical outputs were exported to GraphPad Prism for visualization and statistical analysis.

### In vivo microdialysis

Briefly, mice were placed in a stereotaxic frame after being anesthetized by isoflurane. Guide cannulas were stereotaxically implanted into the brain, specifically the LHb (AP:  $-1.65$  mm, ML:  $0.4$  mm, DV:  $2.2$  mm). This placement allowed for a CMA7 microdialysis probe (CMA, Sweden) with a  $1$  mm membrane length. The guide cannula was secured to the skull with polycarboxylate cement. After the cannula implantation, animals were given a recovery period of one week before serotonin levels were measured via dialysis. Following the recovery period, a microdialysis probe was inserted through the guide into freely moving mice. The probe was then perfused with CSF (NaCl  $147$  mM, KCl  $2.7$  mM, CaCl<sub>2</sub>  $1.2$  mM, MgCl<sub>2</sub>  $0.85$  mM) at a  $10$   $\mu$ L/min flow rate before sample collection began. After a  $1$ -h equilibration period, samples were collected every  $30$  min at a  $1.5$   $\mu$ L/min perfusion rate into a microcentrifuge tube. Sample collection was done using a refrigerated fraction collector (CMA470/Microdialysis). At each flow rate, the first sample was discarded to allow for equilibration at the new flow rate, and an average of the remaining three sample volumes and serotonin concentrations was taken. The remaining dialysate samples were collected on ice and stored at  $-80^{\circ}\text{C}$  to analyze the basal extracellular levels of serotonin. Extracellular serotonin level was analyzed using the high sensitive serotonin ELISA kit (Labor Diagnostika Nord GmbH & Co.KG, BAE-5900 R, Nordhorn, Germany).

### QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., CA, USA), and values are presented as mean  $\pm$  standard error of the mean (SEM). Comparisons between two experimental groups were performed using Student’s *t* test (Unpaired, two-tailed), and three or more groups were compared using a two-way analysis of variance (ANOVA) with Sidak’s *post-hoc* tests for multiple comparisons.