Brain 5-HT deficiency increases stress vulnerability and impairs antidepressant responses following psychosocial stress

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Brain serotonin (5-HT) deficiency and exposure to psychosocial stress have both been implicated in the etiology of depression and anxiety disorders, but whether 5-HT deficiency influences susceptibility to depression- and anxiety-like phenotypes induced by psychosocial stress has not been formally established. Most clinically effective antidepressants increase the extracellular levels of 5-HT, and thus it has been hypothesized that antidepressant responses result from the reversal of endogenous 5-HT deficiency, but this hypothesis remains highly controversial. Here we evaluated the impact of brain 5-HT deficiency on stress susceptibility and antidepressant-like responses using tryptophan hydroxylase 2 knockin (Tph2KI) mice, which display 60–80% reductions in brain 5-HT. Our results demonstrate that 5-HT deficiency leads to increased susceptibility to social defeat stress (SDS), a model of psychosocial stress, and prevents the fluoxetine (FLX)-induced reversal of SDS-induced social avoidance, suggesting that 5-HT deficiency may impair antidepressant responses. In light of recent clinical and preclinical studies highlighting the potential of inhibiting the lateral habenula (LHb) to achieve antidepressant and antidepressant-like responses, we also examined whether LHb inhibition could achieve antidepressant-like responses in FLX-insensitive Tph2KI mice subjected to SDS. Our data reveal that using designer receptors exclusively activated by designer drugs (DREADDs) to inhibit LHb activity leads to reduced SDS-induced social avoidance behavior in both WT and Tph2KI mice. This observation provides additional preclinical evidence that inhibiting the LHb might represent a promising alternative therapeutic approach under conditions in which selective 5-HT reuptake inhibitors are ineffective.

serotonin | stress | antidepressants | habenula

Stress is thought to play a key role in the development of neuropsychiatric disorders, but only a small percentage of individuals exposed to stress develop mental illnesses. To account for this, diathesis–stress hypotheses of neuropsychiatric disorders postulate that stress only leads to maladaptive behavior and psychiatric disease in individuals who harbor certain vulnerability factors (or diatheses) (1–3). However, the diatheses that lead to increased susceptibility to psychosocial stress remain largely unknown.

Levels of brain serotonin (5-HT) have long been implicated in the development and treatment of neuropsychiatric disorders. Indeed, biomarkers of 5-HT deficiency have been identified in subpopulations of depression patients (4), and mutations within 5-HT–system genes have been associated with affective disorders, anxiety disorders, and alterations in stress sensitivity (3, 5–8). In addition, most antidepressant drugs increase extracellular levels of brain 5-HT. However, whether brain 5-HT deficiency alters stress susceptibility or contributes to the development of aberrant emotional behavior remains largely unresolved. Here we examined the consequences of low levels of brain 5-HT on susceptibility to psychosocial stress using a modified version of the social defeat stress (SDS) paradigm (9). To model brain 5-HT deficiency, we used tryptophan hydroxylase 2 (R439H) knockin (Tph2KI) mice, which display 60–80% reductions in brain 5-HT (3, 10–13). These animals express a rare mutant form of the brain 5-HT synthetic enzyme tryptophan hydroxylase 2, which was first identified in a cohort of patients with major depression (5). Our prior work with these animals has shown that 5-HT deficiency significantly impacts behavioral responses to mild early life stress (13) but not to chronic mild stress (14), suggesting that low levels of 5-HT may alter susceptibility to certain types of stressors or to stressors applied during specific developmental periods.

Prior research has shown that SDS can lead to a long-lasting behavioral avoidance phenotype, which can be reversed by chronic administration of antidepressants, such as fluoxetine (FLX) (15). Our previous work has shown that Tph2KI mice exhibit decreased sensitivity to several of the signature effects of chronic FLX, a selective 5-HT reuptake inhibitor (SSRI), in the absence of stress (12), but whether 5-HT deficiency impacts antidepressant responses following stress has not been reported. Interestingly, several mutations in Tph2 have been associated with poor treatment responses in depression patients (16–18), which could suggest that 5-HT deficiency contributes to SSRI treatment resistance. Here we also sought to investigate the effects of 5-HT deficiency on antidepressant-like responses to chronic FLX following psychosocial stress.

Significance

The biological factors that determine whether an individual develops mental illness, such as depression or posttraumatic stress disorder, or responds adequately to pharmacotherapy remain almost completely unknown. Using genetically modified mice, we demonstrate that low levels of brain serotonin lead to increased vulnerability to psychosocial stress and prevent the antidepressant-like effects of fluoxetine following stress exposure. Our data also show that inhibiting the lateral habenula can reverse stress-induced behavioral avoidance in serotonin-deficient animals, which fail to respond to fluoxetine. Our results provide additional insight into the serotonin deficiency hypothesis of depression and highlight the potential of targeting the lateral habenula to treat depression and anxiety disorders in patients who fail to respond to selective serotonin reuptake inhibitors.

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Recent clinical (19) and preclinical studies (20–22) have highlighted the potential of inhibiting the lateral habenula (LHb) (23) to achieve antidepressant or antidepressant-like effects in individuals or animal models that display reduced sensitivity to standard antidepressant therapies. Here we have used designer receptors exclusively activated by designer drugs (DREADDs) (24) to inhibit the LHb and reverse stress-induced social avoidance in wild-type (WT) and Tph2KI mice.

Results

To evaluate whether brain 5-HT deficiency alters susceptibility to psychosocial stress, we subjected WT and Tph2KI mice to 7 d of SDS, a subthreshold SDS paradigm that prior studies suggested would be insufficient to induce behavioral avoidance in WT animals (25). Our results demonstrate that brain 5-HT deficiency does, in fact, lead to increased susceptibility to psychosocial stress. Specifically, 7 d of SDS led to a significant overall reduction in social preference ratio in mice [significant main effect of stress, $F_{(1,\ 76)} = 7.79$, $P = 0.0066$; Fig. 1*A*]. However, a significant genotype-by-stress interaction was also observed [$F_{(1,\ 76)} = 4.44$, $P = 0.0385$]. Post hoc analyses revealed that SDS only led to a significant reduction in social preference in Tph2KI mice, not in WT animals, and SDS-exposed Tph2KI animals exhibited lower preference ratios than any other group ($P < 0.05$). In contrast, 10 d of SDS induced significant social avoidance in both WT and Tph2KI animals [$F_{(1,\ 44)} = 11.44$, $P = 0.0015$; Fig. 1*B*], and no significant interactions or genotype differences were observed.

One brain region that has been heavily implicated in mediating susceptibility to SDS is the nucleus accumbens (NAc) (15, 26). Although the precise molecular mechanisms leading to increased vulnerability to stress remain unknown, alterations in several signal transduction pathways in the NAc have been associated with susceptibility to SDS. For example, changes in the phosphorylation status of extracellular signal-related kinase (ERK) and glycogen synthase kinase 3 beta (GSK3β) in the NAc have been associated with increased SDS vulnerability in C57BL/6 mice (26, 27), as have altered protein and mRNA levels of disheveled (DVL) isoforms (27). To evaluate whether dysregulation of these signaling pathways is associated with the increased susceptibility of Tph2KI mice to SDS, we performed Western blotting and real-time PCR experiments. Our results indicate that neither GSK3β (Fig. 2*A*) nor ERK1/2 (Fig. 2*B*) phosphorylation is altered in the NAc of SDS-exposed Tph2KI mice compared with SDS-exposed WT animals following 7 d of SDS. However, WT animals exhibit a significant reduction in the levels of nuclear β-catenin in the NAc following 7 d of SDS but Tph2KI mice do not [genotype-by-stress interaction, $F_{(1,\ 16)} = 10.13$, $P = 0.0058$; Fig. 2*C*]. SDS-exposed WT animals have significantly less nuclear β-catenin in the NAc than control WT animals and SDS-exposed

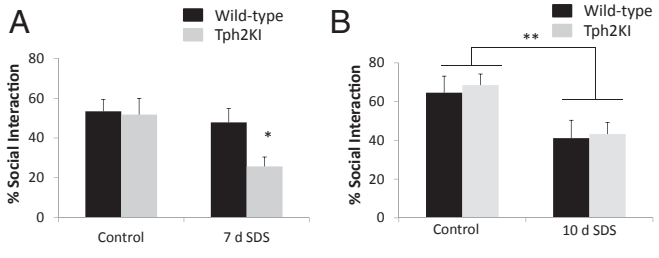

Fig. 1. Susceptibility of WT and Tph2KI mice to social defeat stress. Social preference scores of WT and Tph2KI mice following either 7 d (*A*) or 10 d (*B*) of SDS. *$P < 0.05$ by Tukey's post hoc analysis compared with the Tph2KI control. ** denotes the main effect of SDS by two-way ANOVA, $P < 0.05$. n = 19–21 per group in *A* and n = 10–15 per group for *B*. The error bars represent the SEM.

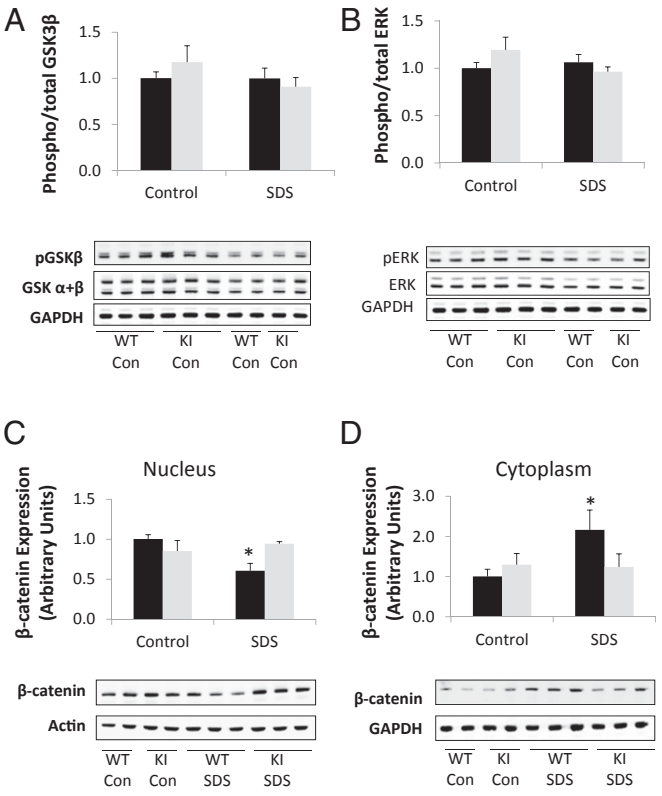

Fig. 2. Signaling consequences of social defeat in the nucleus accumbens of WT and Tph2KI mice. Quantification and representative images of Western blots showing the levels of pGSK3β (*A*) or pERK (*B*) in the nucleus accumbens following 7 d of SDS in WT and Tph2KI mice. Quantification and representative images of Western blots from cell fractionation experiments documenting the levels of β-catenin in the nucleus (*C*) and cytosol (*D*) in the nucleus accumbens of WT and Tph2KI mice following 7 d of SDS. n = 5 per group. *$P < 0.05$ vs. WT control by Tukey's post hoc test. The error bars represent the SEM.

Tph2KI mice ($P < 0.05$ by Tukey's test) after the 7-d SDS paradigm. When the cytoplasmic levels of β-catenin were measured in the NAc, SDS was shown to significantly increase cytoplasmic β-catenin [main effect of SDS, $F_{(1,\ 16)} = 4.56$, $P = 0.049$; Fig. 2*D*]. However, a significant genotype-by-stress interaction was also observed [$F_{(1,\ 16)} = 4.79$, $P = 0.044$; Fig. 2*D*], and Tukey's post hoc tests revealed that SDS only led to a significant increase in cytoplasmic β-catenin in WT mice, not in Tph2KI animals (Fig. 2*D*).

In addition to the NAc, several other brain regions have also been implicated in susceptibility to SDS, including the medial frontal cortex (mFC) (28, 29) and the amygdala (Amyg) (30). Interestingly, Tph2KI mice have previously been reported to exhibit aberrant connectivity between the mFC and the Amyg (31). Thus, we also sought to examine whether molecular alterations within these brain regions are also associated with the increased susceptibility of Tph2KI mice to SDS. Real-time PCR analysis revealed that 7 d of SDS led to a significant overall increase in DVL-1 expression in the mFC [main effect of SDS, $F_{(1,\ 16)} = 16.66$, $P = 0.0009$; Fig. 3*A*]. In addition, 5-HT deficiency led to a significant increase in DVL-1 mRNA in the mFC regardless of stress [main effect of genotype, $F_{(1,\ 16)} = 12.45$, $P = 0.0028$; Fig. 3*A*]. However, a significant genotype-by-stress interaction was also observed [$F_{(1,\ 16)} = 5.66$, $P = 0.03$]. Tukey's post hoc analysis revealed that SDS-exposed Tph2KI mice exhibited higher mRNA levels of DVL-1 in the mFC than any other group (Fig. 3*A*). In contrast, the levels of DVL-2 (Fig. 3*B*)

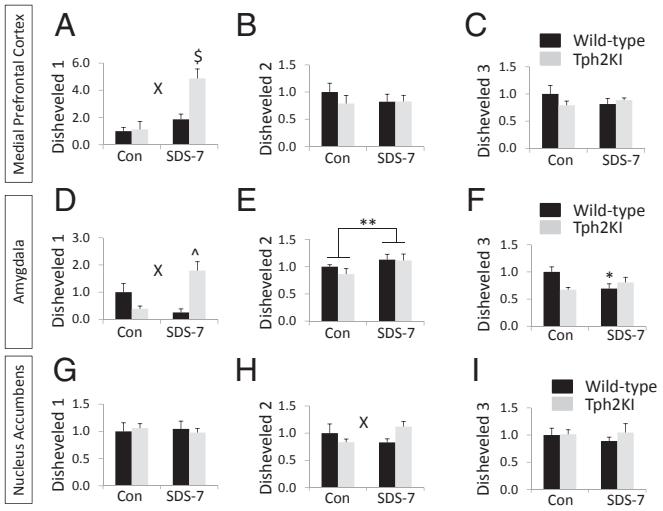

Fig. 3. Effects of 7 d of social defeat on gene expression in the amygdala, frontal cortex, and nucleus accumbens of WT and Tph2KI mice. Quantification of the mRNA levels of DVL-1 (*A*), DVL-2 (*B*), and DVL-3 (*C*) in the medial prefrontal cortex of control WT and Tph2KI mice and in animals exposed to 7 d of SDS (SDS-7). Quantification of the mRNA levels of DVL-1 (*D*), DVL-2 (*E*), and DVL-3 (*F*) in the amygdala of control WT and Tph2KI mice and in animals exposed to 7 d of SDS. Quantification of the mRNA levels of DVL-1 (*G*), DVL-2 (*H*), and DVL-3 (*I*) in the nucleus accumbens of control WT and Tph2KI mice and in animals exposed to 7 d of SDS. $n = 5$ per group. $^{\$}P < 0.05$ vs. all other groups by Tukey's post hoc tests. $^{\wedge}P < 0.05$ vs. WT SDS and Tph2KI control groups by Tukey's post hoc tests. $^{*}P < 0.05$ vs. WT control by Tukey's post hoc test. ** indicates the significant main effect of SDS by two-way ANOVA. X indicates significant genotype-by-stress interaction by two-way ANOVA ($P < 0.05$). The error bars represent the SEM.

and DVL-3 (Fig. 3*C*) were not significantly impacted in the mFC by 5-HT deficiency or stress.

Tph2KI animals also exhibited increased mRNA levels of DVL-1 in the Amyg compared with WT controls [main effect of genotype, $F_{(1, 16)} = 4.65$, $P = 0.047$; Fig. 3*D*]. Although 7 d of SDS did not lead to an overall main effect on DVL-1 expression in the Amyg, a significant genotype-by-stress interaction was observed [$F_{(1, 16)} = 24.84$, $P = 0.0001$; Fig. 3*D*]. SDS-exposed Tph2KI mice had significantly higher mRNA levels of DVL-1 in the Amyg than either control Tph2KI mice or SDS-exposed WT animals (Fig. 3*D*). In addition, 7 d of SDS led to a significant increase in DVL-2 mRNA in the Amyg [main effect of SDS, $F_{(1, 16)} = 5.17$, $P = 0.037$; Fig. 3*E*] but 5-HT deficiency did not significantly impact this result. Although there were no significant main effects of genotype or SDS on the levels of DVL-3, a significant genotype-by-stress interaction was observed [$F_{(1, 16)} = 8.98$, $P = 0.0086$; Fig. 3*F*]. Post hoc analyses revealed that 7 d of SDS led to a significant decrease in DVL-3 expression in the Amyg in WT mice, but no significant effects were observed in Tph2KI animals (Fig. 3*F*).

In contrast to the mFC and Amyg, no significant differences in the mRNA levels of DVL-1 were observed in the NAc of WT vs. 5-HT–deficient mice at baseline or following exposure to 7 d of SDS (Fig. 3*G*). A significant genotype-by-stress interaction was observed for the mRNA levels of DVL-2 [$F_{(1, 16)} = 6.47$, $P = 0.0216$; Fig. 3*H*]. Specifically, 7 d of SDS decreased DVL-2 expression in the NAc of WT animals, whereas it increased DVL-2 mRNA expression in Tph2KI mice. Consequently, Tph2KI mice exposed to 7 d of SDS had higher levels of DVL-2 mRNA in the NAc compared with control Tph2KI mice or WT animals subjected to 7 d of SDS, but these individual group comparisons did not reach statistical significance by Tukey's post hoc tests. No significant effects of genotype or stress were observed on levels of DVL-3 mRNA in the NAc (Fig. 3*I*).

SDS-induced changes in gene expression in each of these brain regions were also investigated following 10 d of SDS. In the mFC, Tph2KI mice were observed to exhibit a significant overall increase in DVL-1 expression compared with WT animals [$F_{(1, 36)} = 6.98$, $P = 0.012$; Fig. 4*A*], which is consistent with the results shown in Fig. 3*A*, but no significant effects of SDS were observed. Similar to the results observed after 7 d, no significant differences in the expression of DVL-2 (Fig. 4*B*) or DVL-3 (Fig. 4*C*) were observed following 10 d of SDS. In the Amyg, 10 d of SDS led to a significant increase in the expression of DVL-1 [$F_{(1, 36)} = 5.68$, $P = 0.023$; Fig. 4*D*], but no significant genotype or interaction effects were observed. Similar trends were observed for the expression of DVL-2 ($P = 0.1$; Fig. 4*E*) and DVL-3 ($P = 0.06$; Fig. 4*F*), but neither of these effects reached statistical significance. In the NAc, we observed no significant alterations in the expression of DVL-1 (Fig. 4*G*), but 10 d of SDS did induce a significant reduction in the levels of DVL-2 [$F_{(1, 36)} = 4.82$, $P = 0.035$; Fig. 4*H*). No significant alterations in the mRNA levels of DVL-3 were observed following 10 d of SDS in WT and Tph2KI mice (Fig. 4*I*).

Prior work has shown that chronic but not acute antidepressant administration can reverse social avoidance phenotypes induced by SDS (15). Thus, we next examined the ability of the SSRI FLX to reverse stress-induced social avoidance in WT and Tph2KI animals. Chronic administration of FLX for 3 wk following exposure to a 10-d SDS paradigm led to a significant increase in social preference [significant main effect of drug, $F_{(1, 61)} = 6.62$, $P = 0.0125$; Fig. 5*A*]. However, a significant drug-by-genotype interaction was also observed [$F_{(1, 61)} = 6.52$, $P = 0.0132$]. Tukey's post hoc analysis revealed that FLX only led to a significant increase in social preference in WT mice ($P = 0.0041$), not in Tph2KI animals. As a control, FLX did not

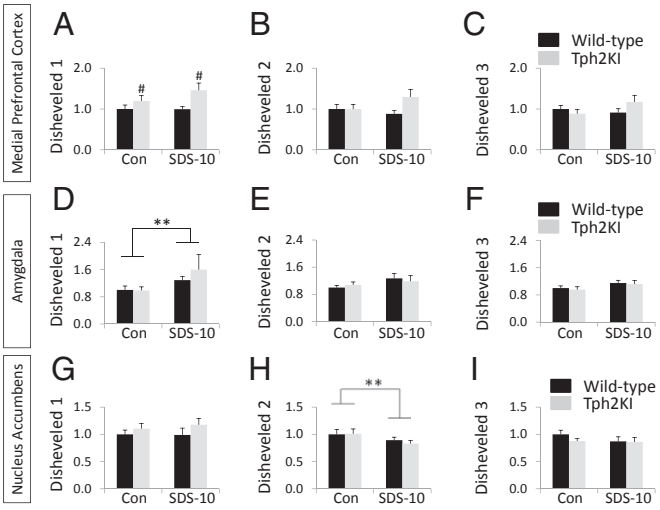

Fig. 4. Effects of 10 d of social defeat on gene expression in the amygdala, frontal cortex, and nucleus accumbens of WT and Tph2KI mice. Quantification of the mRNA levels of DVL-1 (*A*), DVL-2 (*B*), and DVL-3 (*C*) in the medial prefrontal cortex of control WT and Tph2KI mice and in animals exposed to 10 d of SDS (SDS-10). Quantification of the mRNA levels of DVL-1 (*D*), DVL-2 (*E*), and DVL-3 (*F*) in the amygdala of control WT and Tph2KI mice and in animals exposed to 10 d of SDS. Quantification of the mRNA levels of DVL-1 (*G*), DVL-2 (*H*), and DVL-3 (*I*) in the nucleus accumbens of control WT and Tph2KI mice and in animals exposed to 10 d of SDS. $n = 10$ per group. # indicates the significant main effect of genotype by two-way ANOVA ($P < 0.05$). ** indicates the significant main effect of SDS by two-way ANOVA ($P < 0.05$). The error bars represent SEM.

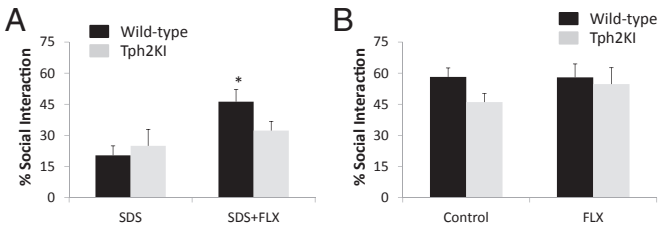

Fig. 5. Antidepressant-like responses of WT and Tph2KI mice to fluoxetine following psychosocial stress. (*A*) Quantification of social preference behavior following chronic fluoxetine treatment in SDS-exposed WT and Tph2KI animals. (*B*) Quantification of social preference behavior following chronic fluoxetine treatment in control WT and Tph2KI animals. n = 13–20 per group in *A* and n = 9–11 per group in *B*. *$P < 0.05$ compared with WT control by Tukey's post hoc test. The error bars represent the SEM.

significantly affect social preference in mice that were not exposed to SDS (Fig. 5*B*).

Due to the fact that chronic FLX failed to reverse stress-induced social avoidance in Tph2KI animals, we next sought to evaluate whether inhibiting the LHb, which has recently been reported to lead to antidepressant-like effects in an animal model of treatment-resistant depression (21), could reverse SDS-induced behavioral avoidance in 5-HT–deficient mice. Administering clozapine-*N*-oxide (CNO), the specific ligand for DREADDs, to WT and Tph2KI animals expressing the inhibitory DREADD [AAV8-hSyn-hM4D(Gi)-mCherry] in the LHb (Fig. 6*A*) led to a significant increase in interaction time in both WT and Tph2KI animals [$F_{(1,\ 32)} = 9.42$, $P = 0.0043$; Fig. 6*B*]. In contrast, mice in which the DREADD virus was targeted incorrectly, which occurred primarily in the hippocampus (Fig. 6*C*), did not exhibit any significant alterations in social avoidance in response to CNO administration [$F_{(1,\ 19)} = 0.0047$, $P = 0.95$; Fig. 6*D*].

Because 5-HT elevation has been reported to inhibit the LHb (32) and 5-HT deficiency limits the extent of 5-HT elevation in response to FLX (12), we hypothesized that Tph2KI mice might exhibit persistent increased LHb activity following FLX treatment after SDS exposure, an effect that could potentially underlie their persistent behavioral avoidance. To test this, we evaluated the effects of chronic FLX on the number of cFos$^+$ cells in the LHb in WT and Tph2KI animals 21 d after the completion of a 10-d SDS paradigm. A significant main effect of treatment condition was observed by two-way ANOVA [$F_{(2,\ 81)} = 3.83$, $P = 0.026$; Fig. S1]. Although SDS led to a genotype-independent increase in the number of cFos$^+$ cells in the LHb ($P = 0.0191$), FLX did not significantly reduce the number of cFos$^+$ cells in either genotype. The specificity of the cFos antibody was confirmed using a blocking peptide (Fig. S2).

Discussion

Our results demonstrate that brain 5-HT deficiency can increase vulnerability to psychosocial stress, a finding that supports both the 5-HT deficiency and the diathesis–stress hypotheses of mental illness. Although the molecular mechanisms that confer this increased susceptibility have not been completely elucidated, our data suggest a potential role for DVL/β-catenin signaling in the NAc, Amyg, and mFC. It is possible that the increased DVL-1 expression in the mFC and Amyg observed in SDS-exposed Tph2KI mice compared with SDS-exposed WT mice could underlie the increased susceptibility of Tph2KI animals. It is also possible that the decreased nuclear translocation of β-catenin in WT mice following SDS contributes to the resilience of WT mice to stress and that Tph2KI animals, which do not exhibit decreased nuclear levels of β-catenin following SDS, fail to engage this potential resilience mechanism. A recent study has shown that overexpression of β-catenin in the mouse NAc can promote resilience to SDS, and that patients with major depression exhibit reduced mRNA levels of β-catenin (33). Our results provide further evidence of a potentially important role of β-catenin signaling in mediating resilience to stress.

Our results indicating no significant alterations in the phosphorylation of GSK3β in the NAc argue against a primary role of GSK3β in mediating the increased stress susceptibility of Tph2KI mice following 7 d of SDS. In contrast, two prior studies did report that changes in GSK3β signaling in the NAc were associated with vulnerability vs. resilience to SDS, at least in C57BL/6 mice following 10 d of defeat (26, 27). However, one of these studies reported that increased phosphorylation of GSK3β in the NAc was associated with increased susceptibility to defeat (26), whereas the other reported that susceptible animals exhibited decreased GSK3β phosphorylation in the NAc (27). Although the reasons for the discrepancies between these published studies remain unclear, it appears that the mechanisms through which brain 5-HT deficiency leads to increased stress susceptibility may differ from those governing stress responses in C57BL/6 mice.

Our gene expression profiling results also suggest overlapping but distinct molecular alterations associated with stress susceptibility in WT and Tph2KI mice. For example, Tph2KI mice subjected to 7 or 10 d of SDS exhibit social avoidance behavior and increased DVL-1 expression in the mFC compared with controls. However, WT mice subjected to 10 d of SDS do not display increased DVL-1 expression in the mFC, despite exhibiting social avoidance, a fact that may indicate that increased DVL-1 signaling in the mFC only contributes to stress vulnerability in 5-HT–deficient animals. In contrast, all groups of mice displaying social avoidance (i.e., Tph2KI mice after either 7 or 10 d of SDS, and WT mice after 10 d of stress) exhibited increased DVL-1 in the Amyg compared with controls, a finding that suggests that increased DVL-1 signaling in the Amyg could play a significant role in mediating stress susceptibility regardless of 5-HT status. Future research will be required to

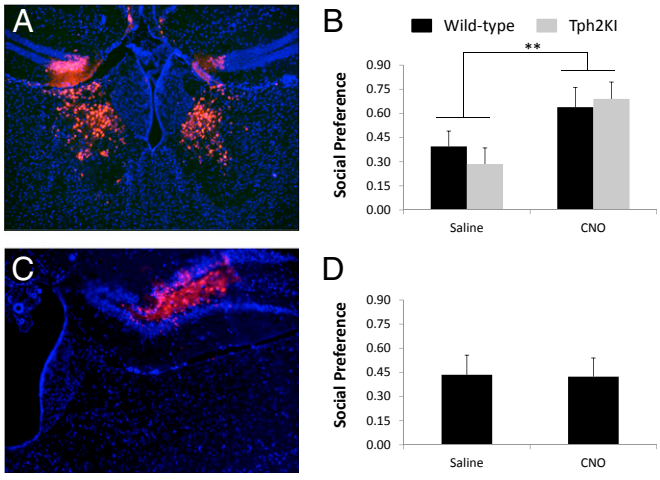

Fig. 6. Antidepressant-like effects of lateral habenula inhibition. (*A*) A representative image showing a stereotactic injection of AAV8-hSyn-M4D (Gi)DREADD-mCherry into the lateral habenula. (*B*) CNO administration to M4D(Gi)DREADD-expressing animals leads to a significant increase in social preference in both WT and Tph2KI mice. (*C*) A representative image showing a missed stereotactic injection, which resulted in M4D(Gi)DREADD expression in the hippocampus, not the lateral habenula. (*D*) CNO administration to improperly localized M4D(Gi)DREADD-expressing animals did not significantly affect social preference. **$P < 0.05$ by two-way ANOVA (significant main effect of CNO). n = 6–11 per group for *B* and n = 13 saline and 8 CNO for *D*. The error bars represent the SEM.

more fully elucidate the cellular and molecular pathways that regulate stress susceptibility.

Our findings also indicate that 5-HT deficiency can negatively impact the ability of FLX to reverse stress-induced social avoidance. These results are consistent with our previous work showing that Tph2KI mice exhibit blunted behavioral, cellular, and molecular responses to chronic FLX administration in the absence of stress (12). Together, these data suggest that 5-HT deficiency could play a role in treatment resistance to SSRIs, a finding that is consistent with the fact that mutations in Tph2 have been reported to play a role in treatment responses in humans (16–18).

A recent case report showed that deep brain stimulation-mediated inhibition of the LHb led to antidepressant effects in a patient with treatment-resistant depression (19). Similarly, deep brain stimulation-mediated inhibition of the LHb in rats has been shown to lead to antidepressant-like effects (22). In addition, DREADD-mediated inhibition of the lateral habenula has been shown to lead to antidepressant-like effects in the forced swim test in rats in the absence of stress (20). Similarly, using stereotactic injections of the GABA agonist muscimol to inhibit LHb activity has also been shown to lead to an antidepressant-like effect in rats bred for high levels of learned helplessness behavior (21). Our current findings support and extend these prior studies by showing that DREADD-mediated LHb inhibition can reverse SDS-induced social avoidance behavior in mice. The fact that LHb inhibition is effective in Tph2KI animals further highlights the potential of inhibiting this structure to achieve antidepressant-like effects in populations that are refractory to SSRIs.

Prior reports have suggested that the LHb plays a key role in animal models of depression and anxiety-like behavior (22, 34–36) and that LHb activity promotes behavioral avoidance (37). Interestingly, the administration of 5-HT and selective 5-HT1B agonists have been shown to inhibit excitatory input to the LHb (32, 38), and acute tryptophan depletion has been reported to increase blood flow in the LHb (39). Based on these published studies, we had hypothesized that the low levels of brain 5-HT in Tph2KI mice would result in increased neuronal activity in the LHb, but our cFos results do not support this hypothesis. However, consistent with the role of the LHb in predicting negative reward and aversive stimuli (40, 41), increased cFos immunoreactivity was observed in the LHb of SDS-exposed animals upon exposure to an unfamiliar male CD1 mouse, but neither 5-HT deficiency nor FLX treatment significantly influenced this effect. Although our cFos data suggest that inhibition of the LHb may not be necessary for FLX to reverse SDS-induced social avoidance, it should be noted that cFos immunoreactivity is not a direct measure of neuronal activity, and very few $cFos^+$ cells in the LHb were observed in any of the experimental conditions. It is possible that other, more sensitive experimental approaches could identify differential LHb activity in WT and Tph2KI animals exposed to SDS and FLX, but future research would be required to test this hypothesis. Nonetheless, our results demonstrate that LHb inhibition is sufficient to achieve antidepressant-like responses in mice subjected to psychosocial stress.

In conclusion, our findings demonstrate that brain 5-HT deficiency can lead to increased susceptibility to psychosocial stress, a fact that could have important implications for our understanding of the etiology of stress-related disorders. In addition, our data show that 5-HT deficiency impairs antidepressant-like responses to FLX but not to LHb inhibition, which provides additional preclinical evidence that LHb inhibition may represent a promising new therapeutic approach to treating treatment-resistant depression.

Materials and Methods

Animals. The tryptophan hydroxylase 2 R439H knockin mouse line used in this study has been described previously (10). Throughout this manuscript, homozygous mice are referred to as WT animals, and homozygous mutant animals are referred to as Tph2KIs. Mice were housed on a 12-h light–dark cycle and provided food and water ad libitum, and only male mice were used. Mice were 8 wk of age at the start of the experiments. All experiments were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee.

Drugs. Fluoxetine was obtained from Spectrum Chemicals and was administered in the drinking water (155 mg/L), which we have previously shown results in a dose of FLX that is ~18–22 $mg \cdot kg^{-1} \cdot d^{-1}$ (12, 42). Clozapine-*N*-oxide was obtained from Enzo Life Sciences and was injected into mice intraperitoneally (1 mg/kg) 12 h and 1 h before social avoidance testing. The AAV8-hSyn-M4D(Gi)-mCherry virus was obtained from the University of North Carolina–Chapel Hill Vector Core.

Social Defeat Stress. Age-matched experimental mice were submitted to social defeat stress for either 7 or 10 consecutive days. Every day, each experimental mouse was introduced into the home cage of an unfamiliar male CD1 retired breeder for 5 min, during which time the experimental animal was physically defeated. Resident CD1 aggressors were preselected for aggressive behavior, and only animals that displayed attack latencies reliably shorter than 30 s upon three consecutive screening tests were used as aggressors. Immediately following the first 5-min defeat episode, each experimental mouse was singly housed in a novel cage, where it was housed for the remainder of the social defeat experiment. Control animals were group-housed throughout the experiment and had no exposure to CD1 animals until social avoidance testing.

Social Avoidance Testing. Testing was conducted in a large open field (60 cm by 40 cm) under dim lighting (~30 lx). Two cylindrical wire containers were placed on opposite sides of the open field. One of the containers contained an unfamiliar CD1 retired breeder, and the other container remained empty. Experimental mice were placed in the center of the open field and allowed to explore the arena freely for 5 min. The total interaction time within a 20-cm-diameter interaction zone surrounding each of the two targets was recorded using EthoVision software (Noldus Information Technology) as described previously (43).

Stereotactic Surgery. Before surgery, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A glass micropipette needle attached to a 25-μL Hamilton syringe was inserted into the LHab (stereotactic coordinates: –1.7 mm posterior to bregma; ±0.4 mm lateral of the midline; –2.6 mm ventral of the top of the skull). A volume of 0.2 μL M4D virus was injected into each hemisphere over the course of 1 min using a microinjector pump. Mice were allowed to recover for 3 wk before being subjected to SDS.

Fluorescence Microscopy. The accuracy of stereotactic injections was determined using fluorescence microscopy. Brain sections were obtained from WT and Tph2KI mice injected with M4D-mCherry virus (20-μm-thick) and were coverslipped with SlowFade Gold antifade reagent with DAPI (Life Technologies). Animals in which no mCherry was observed in the lateral habenula or in which the majority of mCherry expression was not within the lateral habenula were considered "misses" and were analyzed separately. A total of 57 surgeries were performed, 36 of which were considered "hits."

Immunohistochemistry. Immunohistochemistry was performed essentially as described previously (14). Briefly, animals were anesthetized with ketamine/xylazine and perfused transcardially with 10% (wt/vol) neutral buffered formalin (NBF). Brains were removed and postfixed in 10% NBF overnight and then cryopreserved in 30% sucrose in PBS for 48 h. Brain sections (25-μm) were obtained using a cryostat and mounted directly onto glass slides. Sections were blocked in 5% BSA in PBS (with 0.1% Triton) for 30 min, washed three times in PBS, and then incubated overnight at 4 °C with primary antibody (cFos; 1:500; Santa Cruz Biotechnology) diluted in 1% BSA in phosphate-buffered saline with 0.1% Triton X-100 (PBS-t). The cFos blocking peptide (Santa Cruz Biotechnology) was diluted 1:500 in 1% BSA in PBS-t and incubated overnight with primary antibody. After three washes in PBS, secondary antibody was applied for 2 h at room temperature. After three more PBS washes, sections were coverslipped with SlowFade Gold antifade reagent with DAPI (Life Technologies), and pictures were taken on a Zeiss Axiovert microscope.

Western Blotting. Animals were killed by cervical dislocation, and brains were rapidly dissected on ice. Bilateral punches (1 mm in diameter, 1 mm in thickness) were obtained from the NAc and snap-frozen in liquid nitrogen. Crude nuclear and cytoplasmic samples were obtained by homogenizing each sample in a solution of 0.32 M sucrose, 20 mM Hepes (pH 7.4), 1 mM EDTA, 1× protease inhibitor mixture, and 1× phosphatase inhibitor mixture. The homogenate was centrifuged for 10 min at 700 × *g* at 4 °C. The supernatant was centrifuged at 16,000 × *g* for 10 min, and the resulting supernatant was used as the crude cytosolic fraction. The initial pellet (from the 2,800-rpm spin) was resuspended and sonicated in protein lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, and protease and phosphatase inhibitors) and used as the nuclear fraction. Western blotting was performed as described previously (13, 44). The protein levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin were measured as loading controls.

Real-Time PCR. Real-time PCR was performed as described previously (12). Primers used were DVL-1 forward: 5′-AGT GGA GCC TCA GAT CAG GA-3′; DVL-1 reverse: 5′-GGT CCT GGG TAC TGG TAG GG-3′; DVL-2 forward: 5′-TGA CAA TGA CGG TTC CAG TG-3′; DVL-2 reverse: 5′-GCG CTG GAT ACT GGT AGG AG-3′; DVL-3 forward: 5′-CTA CAC GCA GCA GTC TGA GG-3′; DVL-3 reverse: 5′-CAT AGC TTG GGT GTG TGT GG-3′; GAPDH forward: 5′-CAT GTT CCA GTA TGA CTC CAC TC-3′; GAPDH reverse: 5′-GGC CTC ACC CCA TTT GAT GT-3′.

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