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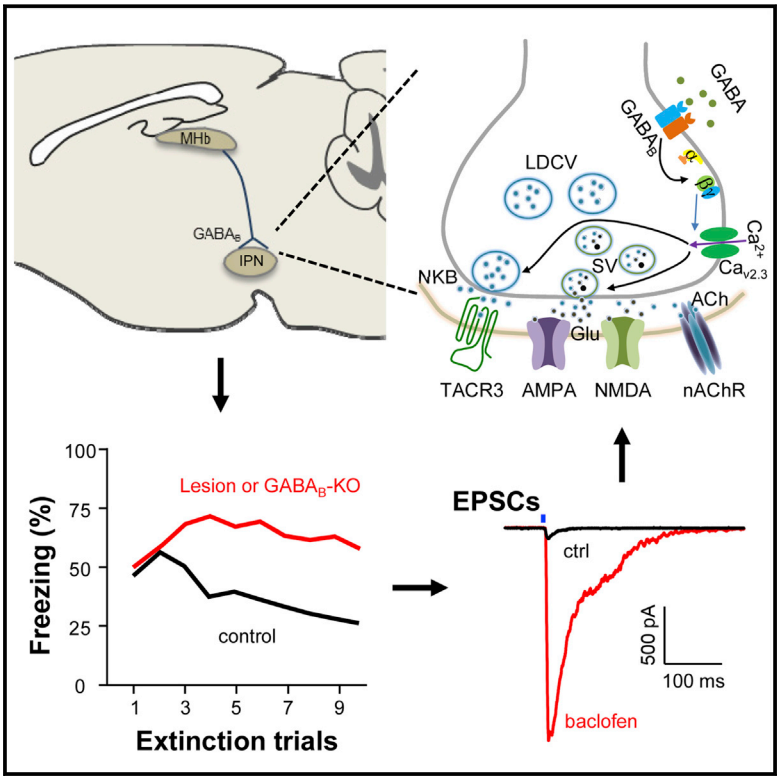
Presynaptic Excitation via $GABA_B$ Receptors in Habenula Cholinergic Neurons Regulates Fear Memory Expression

Graphical Abstract



Highlights

- Habenula cholinergic neurons reduce fear memory expression
- Presynaptic $GABA_B$ activity of habenula neurons facilitates fear extinction
- $GABA_B$ drastically potentiates the corelease of multiple neurotransmitters
- $GABA_B$ amplifies presynaptic Ca^{2+} entry through $Ca_{v2.3}$ channels

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In Brief

Fear behavior in the face of danger relies on presynaptic excitation in a habenular circuit that is unexpectedly mediated by $GABA_B$ receptors, synaptic proteins typically responsible for inhibitory signaling.

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Presynaptic Excitation via $GABA_B$ Receptors in Habenula Cholinergic Neurons Regulates Fear Memory Expression

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SUMMARY

Fear behaviors are regulated by adaptive mechanisms that dampen their expression in the absence of danger. By studying circuits and the molecular mechanisms underlying this adaptive response, we show that cholinergic neurons of the medial habenula reduce fear memory expression through $GABA_B$ presynaptic excitation. Ablating these neurons or inactivating their $GABA_B$ receptors impairs fear extinction in mice, whereas activating the neurons or their axonal $GABA_B$ receptors reduces conditioned fear. Although considered exclusively inhibitory, here, $GABA_B$ mediates excitation by amplifying presynaptic Ca^{2+} entry through $Ca_{v2.3}$ channels and potentiating co-release of glutamate, acetylcholine, and neurokinin B to excite interpeduncular neurons. Activating the receptors for these neurotransmitters or enhancing neurotransmission with a phosphodiesterase inhibitor reduces fear responses of both wild-type and $GABA_B$ mutant mice. We identify the role of an extra-amygdalar circuit and presynaptic $GABA_B$ receptors in fear control, suggesting that boosting neurotransmission in this pathway might ameliorate some fear disorders.

INTRODUCTION

Excessive fear or fear that persists in the absence of threat is dysfunctional, as in phobias and post-traumatic stress disorder (PTSD) (Milad and Quirk, 2012; VanElzakker et al., 2014). The formation and expression of fear memory involves the extended amygdala and its connected brain areas (Janak and Tye, 2015; LeDoux, 2000; Tovote et al., 2015). The circuit between the amygdala and the limbic prefrontal cortex is critical for reducing fear responses during the extinction phase (Herry et al., 2010; Myers and Davis, 2007). However, the functions and molecular mechanisms of extra-amygdalar circuits in fear extinction remain unclear.

The habenulo-interpeduncular pathway may regulate aversive responses (Figures S1A and S1B). In mammals, the medial habenula (MHb) in the epithalamus receives excitatory signals from brain areas that promote fear and anxiety (Yamaguchi et al., 2013). A majority of MHb neurons express cholinergic markers and corelease glutamate and acetylcholine to activate their postsynaptic neurons in the midbrain interpeduncular nucleus (IPN) (Frahm et al., 2015; Hu et al., 2012; Qin and Luo, 2009; Ren et al., 2011). IPN neurons then project caudally to a set of brainstem nuclei, where neurons send their outputs back to the limbic forebrain areas (Goto et al., 2001; Pollak Dorocic et al., 2014) (Figure S1A). The MHb in mammals is involved in behavioral processes related to aversive and/or appetitive stimuli, such as stress, pain, and nicotine addiction (Fowler et al., 2011; Frahm et al., 2011, 2015; Hu et al., 2012; Kobayashi et al., 2013; Soria-Gómez et al., 2015). Disrupting this pathway in fish impacts behavioral responses to aversive stimuli (Agetsuma et al., 2010; Lee et al., 2010). It remains unclear whether the findings from the studies of fish apply to mammals, because the fish MHb homolog receives direct sensory but not limbic inputs (Stephenson-Jones et al., 2012).

Habenula cholinergic neurons express a particularly high level of $GABA_B$ receptors along their axonal projections to the IPN (Margeta-Mitrovic et al., 1999). $GABA_B$ is a key receptor for the major neurotransmitter GABA (gamma aminobutyric acid), and $GABA_B$ has to date been considered to be exclusively inhibitory (Gassmann and Bettler, 2012). Activating $GABA_B$ suppresses transmitter release at presynaptic sites and hyperpolarizes membrane potentials at postsynaptic sites (Dutar and Nicoll, 1988a; Gassmann and Bettler, 2012; Newberry and Nicoll, 1984). Altered $GABA_B$ signaling is associated with anxiety and fear disorders (Cryan and Kaupmann, 2005). The $GABA_B$ agonist baclofen has been used to treat PTSD in clinical trials (Drake et al., 2003; Manteghi et al., 2014), suggesting a potential role in fear control for $GABA_B$ receptors that are expressed in MHb neurons. However, no studies have as yet addressed the behavioral functions, physiological effects, or molecular mechanisms of $GABA_B$ signaling in the habenulo-interpeduncular pathway.

Here, we analyzed the functions of habenula cholinergic neurons and their $GABA_B$ receptors in the acquisition and expression of cued fear memory. We found that both the neuronal

activity and the presynaptic $GABA_B$ signaling of these neurons are critical for extinguishing fear memory. Critically, $GABA_B$ mediates its behavioral effect by strongly potentiating the corelease of multiple neurotransmitters. At the molecular level, $GABA_B$ produces presynaptic excitation by facilitating Ca^{2+} entry through the $Ca_{v2.3}$ channel. Our data demonstrate that $GABA_B$ signaling in a specific synapse of an evolutionarily-conserved pathway controls expression of fear memory, hence suggesting alternative therapeutic approaches to treating fear disorders.

RESULTS

Habenula Cholinergic Neurons Control Fear Memory Expression

We first ablated cholinergic neurons in the MHb to test whether they are required for forming or expressing fear memory. To selectively kill these neurons, we infused the Cre-dependent adeno-associated virus (AAV) vector AAV-flex-taCasp3-TEVp into the bilateral habenula of a *ChAT-Cre* mouse, which expressed the Cre recombinase under the promoter of the gene encoding choline acetyltransferase (*ChAT*) (Figures 1A and 1B). As a control for the accuracy of the *ChAT-Cre* driver line (Gong et al., 2007), infusion of the AAV-DIO-EmGFP vectors led to the expression of enhanced membrane green fluorescent protein (EmGFP) in ChAT-immunopositive neurons within the MHb of these mice (henceforth referred to as *MHb-ChAT-EmGFP* mice for simplicity; Figure 1C). This approach also selectively labeled ChAT-expressing axons from the MHb to the IPN (Figure S1C). Consistent with the effect of taCasp3 on inducing cell apoptosis (Yang et al., 2013), injecting AAV-flex-taCasp3-TEVp into the *ChAT-Cre* (abbreviated as *MHb-ChAT-taCasp3*) mice eliminated cholinergic neurons in the MHb and abolished cholinergic axonal terminals in the IPN (Figures 1C, S1C, and S1D). The ChAT expression pattern remained unchanged in other major cholinergic brain areas (Figures S1E–S1G), indicating that the vector-induced loss of cholinergic neurons was restricted within the MHb and that cholinergic inputs to the IPN arose predominantly from the MHb.

We compared the *MHb-ChAT-taCasp3* mice with the *MHb-ChAT-EmGFP* control mice on a test of cued fear memory. In this test, we conditioned mice by presenting a 20-s auditory tone (conditioned stimulus [CS]) that co-terminated with a 1-s footshock for five trials (Figure 1D). As training progressed, both groups became increasingly immobilized (freezing) during the tone. The two groups of mice exhibited similar freezing levels during the conditioning phase (Figure 1E; see Table S1 for statistical analysis), suggesting that habenula cholinergic neurons are dispensable for forming fear memory.

We then examined whether ablating these neurons affected fear memory expression. Following behavioral protocols for testing fear extinction (Milad and Quirk, 2002; Soria-Gómez et al., 2015), we placed a mouse in a test chamber that was distinct from the training chamber and repetitively delivered CS tones in the absence of footshock (ten trials with random intervals) (Figure 1D). The *MHb-ChAT-EmGFP* control mice showed the normal response pattern: freezing responses decreased gradually within a session and became fully extinguished after ~3 days. The freezing levels of *MHb-ChAT-taCasp3* mice were initially similar to those of control mice, but decayed more slowly, resulting in significantly higher freezing ratios, even after 5 days (Figures 1F and 1G; Table S1). In a different experiment, we tested animal freezing responses to a 180-s continuous auditory tone (Soria-Gómez et al., 2015) (Figure 1D). In both test sessions on days 1 and 6, the *MHb-ChAT-taCasp3* mice showed long-lasting freezing responses during the tones, resulting in significantly higher total freezing time than for control mice (Figures 1H and 1I; Table S1). Ablating habenula cholinergic neurons did not affect locomotor activity (Figures S1H and S1I; Table S1), thus ruling out the possibility that a general suppression of locomotor activity caused the freezing increase. Therefore, habenula cholinergic neurons are necessary for reducing fear responses to conditioned stimuli that no longer actively predict threat.

We next examined whether activating habenula cholinergic neurons would be sufficient for reducing freezing responses. In these experiments, we used *ChAT-ChR2-EYFP* mice, in which the light-sensitive cation channel ChannelRhodopsin2 (ChR2) was selectively expressed in the somata and axons of cholinergic neurons (Figures 1J, S1J, and S1K) (Ren et al., 2011). Taking advantage of the fact that the axons of bilateral MHb neurons converge into a single nucleus along the midline, we used one optical fiber to activate the bilateral cholinergic inputs. The optical fiber was implanted into the dorsal IPN with its tip tapered to minimize tissue damage (Figures 1J and S1L). We induced strong fear responses by conditioning *ChAT-ChR2-EYFP* mice with ten tone-footshock pairs (Figure S1M). During the extinction phase in the following days, the mice in the stimulation group received light pulses to activate the cholinergic terminals during auditory tones (Figure 1K). Optogenetic stimulation resulted in significantly lower levels of freezing throughout the test. As a control, delivering light pulses to wild-type littermate mice and omitting light pulses to the control *ChAT-ChR2-EYFP* mice had no effect (Figures 1L and 1M; Table S1). These results therefore suggest that activating habenula cholinergic neurons can effectively substitute for fear extinction.

$GABA_B$ Activity in the Habenulo-Interpeduncular Pathway Controls Fear Extinction

We investigated how $GABA_B$ receptors in habenula cholinergic neurons may affect fear behavior. Both essential subunits, $GABA_{B(1)}$ and $GABA_{B(2)}$, are richly expressed in habenula cholinergic neurons and their axon terminals within the IPN (Figures 2A, 2B, and S2A–S2C). To genetically inactivate the $GABA_{B(1)}$-encoding gene (*Gabbr1*) in cholinergic neurons, we generated $GABA_{B(1)}$ conditional knockout (*ChAT-GABA*$_{B(1)}$-KO; CKO) mice by crossing the *ChAT-Cre* mouse line with the *GABA*$_{B(1)}^{lox511/lox511}$ line. In the *ChAT-GABA*$_{B(1)}$-KO mice, $GABA_{B(1)}$ expression was abolished in the cholinergic neurons in the MHb and in their axonal terminals within the central and rostral IPN (Figures 2C and 2D and S2D–S2F); $GABA_{B(1)}$ expression appeared normal in the lateral IPN, cortex, and hippocampus of these mice (Figures S2G–S2I). This allowed us to investigate the potential role in fear memory expression for the $GABA_B$ activity of cholinergic neurons.

Both *ChAT-GABA*$_{B(1)}$-KO mice and their wild-type littermates showed similar increases in freezing during the conditioning

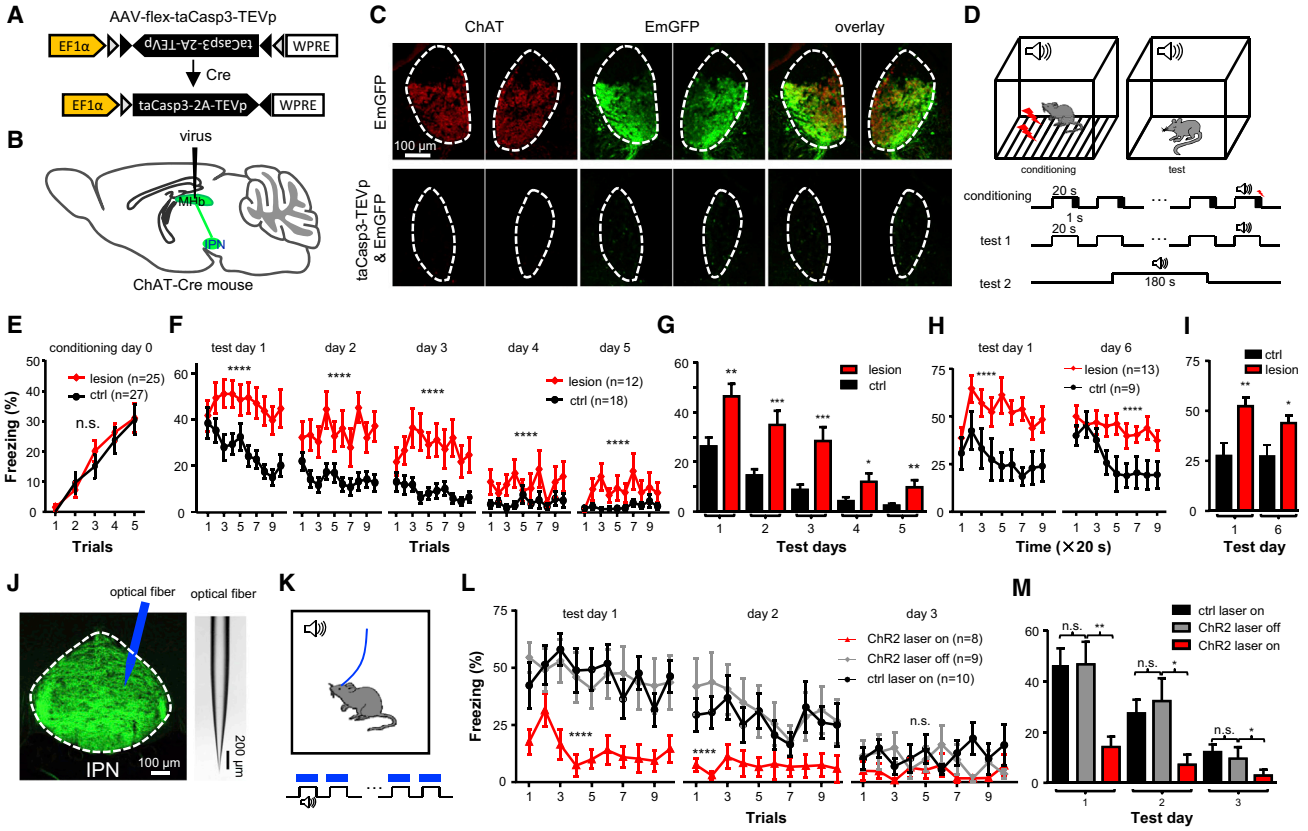

Figure 1. Ablating Habenula Cholinergic Neurons Enhances the Expression of Conditioned Fear, whereas Activating These Cells Reduces Fear Responses

(A and B) We generated a *MHb-ChAT-taCasp3* (lesion) mouse or a *MHb-ChAT-EmGFP* (ctrl) mouse by infusing a mixture of AAV-DIO-EmGFP vectors and AAV-flex-taCasp3-TEVp (A) or AAV-DIO-EmGFP only vectors into the MHb of a *ChAT-Cre* mouse (B).

(C) Coexpression of EmGFP (green) and ChAT (red) in the bilateral MHb of a control mouse (upper) and the lack of such expression and reduction of MHb volume in a lesion mouse (lower).

(D) Methods for cued fear conditioning and tests. In test sessions, mice were exposed to either ten discrete conditioned stimuli (test 1) or one 180-s continuous tone (test 2).

(E) Freezing response levels across trials during the cued fear conditioning on day 0.

(F) Freezing responses across trials in the extinction sessions on days 1–5, in which we presented ten discrete conditioned stimuli but omitted footshocks.

(G) Overall freezing responses of the lesion mice and control mice to discrete tones.

(H and I) Effects of lesion on the freezing responses (H) and overall freezing ratio (I) to a continuous 180 s tone.

(J) Distribution of ChR2-EYFP-expressing fibers (green) within the IPN and the method of delivering light with a tapered optical fiber.

(K) Method of testing the effect of optogenetic stimulation on freezing responses. Blue bar indicates light stimuli (5 ms light pulses at 50 Hz).

(L and M) Freezing responses across trials (L) and total freezing response levels (M) of the stimulation mice and control mice during extinction sessions. For a control, we gave light stimulation to wild-type littermates or omitted light stimulation to *ChAT-ChR2-EYFP* mice.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., not significant; two-way ANOVA on difference between phenotypes or between stimulation and control (E, F, H, and L) and t tests (G, I, and M). Error bars indicate SEM.

See Table S1 for statistical analyses in detail. See also Figure S1.

phase (Figure 2E; Table S1), indicating that the $GABA_B$ activity of cholinergic neurons is not required for forming cued fear memory. In the extinction sessions using discrete CS, a tone without shock elicited significant higher freezing levels from $GABA_B$ CKO mice, even after extinction training for 5 days (Figures 2F and 2G; Table S1). When tested with a continuous 180-s tone, wild-type mice gradually reduced their freezing, but the $GABA_B$ CKO mice continued to freeze and exhibited significantly higher freezing levels on days 1 and 6 (Figures S2J and S2K; Table S1). *ChAT-GABA*$_{B(1)}$-KO mice did not show abnormality in locomotor activity before the tests, nor in general locomotion in an open field, nor in anxiety levels in heightened or illuminated open spaces, nor in pain sensitivity to footshock (Figures S2L–S2R; Table S1). Apparently $GABA_B$ activity of the cholinergic neurons does not affect various global functions. Rather, it is critical to controlling fear extinction.

We then examined how pharmacological manipulation of $GABA_B$ activity in the IPN of wild-type mice would affect fear responses. C57BL6/N wild-type mice were conditioned with five tone-footshock pairs. Immediately before the extinction sessions, we infused the $GABA_B$ antagonist 2-OH-saclofen (saclofen) directly into the IPN (Figure S3A). Saclofen pretreatment

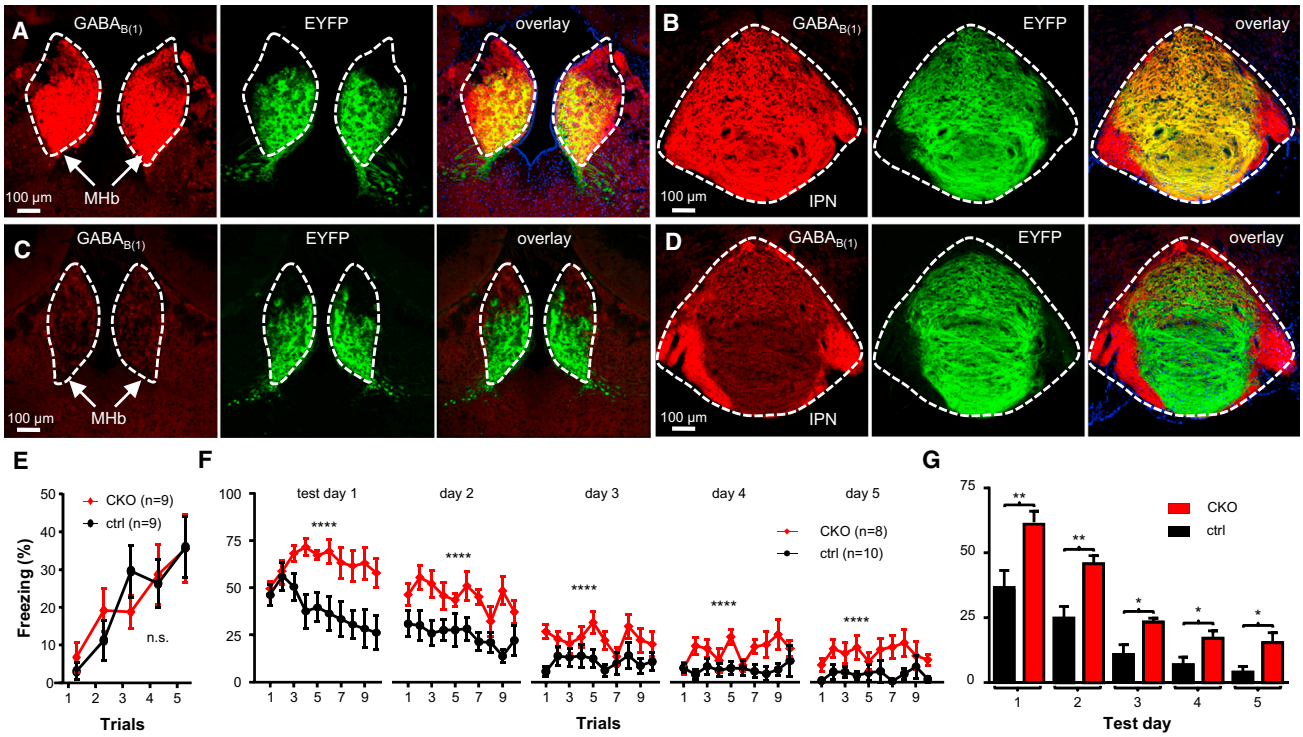

Figure 2. Genetically Inactivating *GABA$_B$* in Cholinergic Neurons Enhances the Expression of Learned Fear

(A and B) Strong GABA$_{B(1)}$ immunoreactivity (red) in habenula cholinergic neurons (green; A) and their axonal terminals in the IPN (green; B) of a *ChAT-ChR2-EYFP* mouse.

(C and D) GABA$_{B(1)}$ immunoreactivity was markedly reduced in the MHb (C) and IPN (D) of a *ChAT-GABA*$_{B(1)}$-KO (CKO) mouse. GABA$_{B(1)}$ expression remained unchanged in the lateral IPN that received non-cholinergic inputs from the dorsal MHb.

(E) Freezing responses of both CKO mice and wild-type littermate controls (ctrl) during fear conditioning.

(F) Freezing responses of CKO mice and control mice during the fear extinction sessions on days 1–5. ****$p < 0.0001$ for difference between phenotypes in all sessions, two-way ANOVA.

(G) Total freezing responses of the mutant and control mice. *$p < 0.05$; **$p < 0.01$; t tests. Error bars indicate SEM.

See Table S1 for statistical analyses in detail. See also Figure S2.

significantly increased the freezing responses to both discrete trials of conditioned stimulus and a 180-s continuous tone (Figures 3A, 3B, S3B, and S3C; Table S1). Saclofen did not reduce locomotor activity prior to the presentation of auditory tone (Figure S3D; Table S1), again indicating that GABA$_B$ activity in the IPN reduces freezing levels but not general locomotion.

If blocking GABA$_B$ in the IPN could increase fear responses, then we reasoned that activating GABA$_B$ might do the opposite, reducing freezing responses during extinction tests. One day after a wild-type mouse was subjected to fear conditioning with ten trials of tone-footshock stimuli, intra-IPN pretreatment of baclofen (3 pmol) accelerated fear extinction for all behavioral sessions that were based on discrete auditory tones (Figures 3C and 3D; Table S1). At the high dose of 30 pmol, baclofen produced a sedative effect, and we needed to increase the waiting time from 5 min to 1.5 hr before starting the tests. Possibly because of the longer waiting time, this baclofen pretreatment significantly reduced freezing on extinction session on day 6, but not on day 1, when we tested with a 180-s continuous tone (Figures S3E–S3G; Table S1). Knocking out GABA$_B$ in cholinergic neurons prevented baclofen from facilitating fear extinction (Figures 3E, 3F, and S3H–S3J; Table S1), demonstrating that the fear-reducing effect of baclofen requires GABA$_B$ receptors within the cholinergic neural processes.

GABA$_B$ Activity Mediates Presynaptic Excitation

To study the synaptic basis for the fear-reducing effect of GABA$_B$ activity, we performed whole-cell patch recording from interpeduncular neurons in brain slices of *ChAT-ChR2-EYFP* mice (Figure 4A). Stimulating the ChR2-EYFP-expressing terminals with a single brief light pulse evoked fast excitatory postsynaptic currents (EPSCs) (Figures 4A and 4B), whereas shining light onto EmGFP-expressing terminals of a control mouse did not produce any current (Figure S4A). In agreement with glutamate corelease by the habenula cholinergic neurons (Hu et al., 2012; Ren et al., 2011), the EPSCs were resistant to nicotinic blockers but were blocked by an AMPA-type glutamate receptor antagonist (Figures 4C and S4B).

We then examined how baclofen affects the glutamatergic EPSCs. We had expected that it would reduce the EPSCs, because GABA$_B$ action has to date been considered to be purely inhibitory. Unexpectedly, the drug increased the EPSC amplitude in a cell from ~100 pA to nearly 2,000 pA (Figures 4B and 4C). For the entire population of recorded

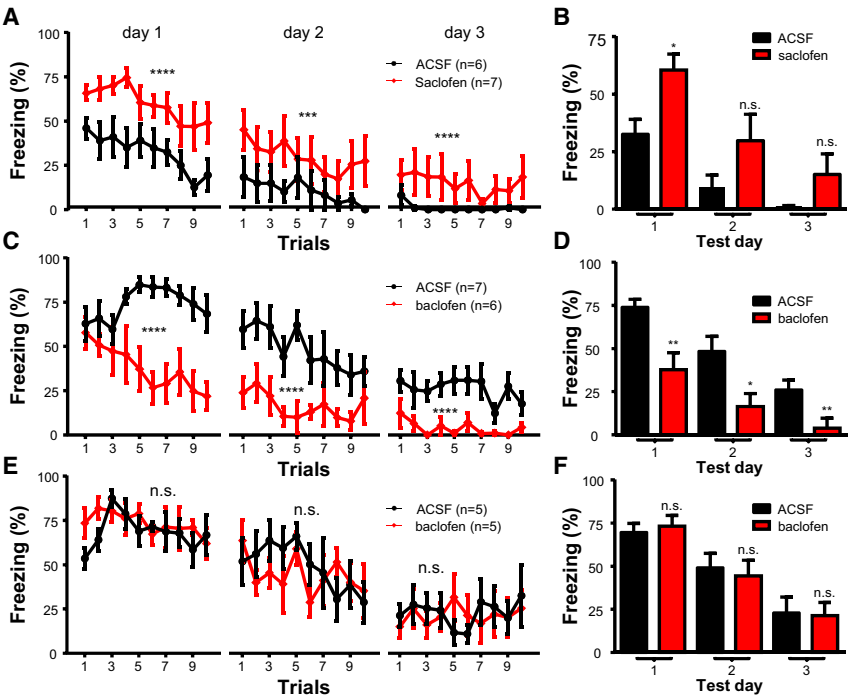

Figure 3. $GABA_B$ Activity in the Interpeduncular Nucleus Facilitates Fear Extinction

(A and B) The effects of pre-test intra-IPN infusion of the $GABA_B$ antagonist 2-OH-saclofen (saclofen; 300 pmol) on freezing responses across trials (A) and total freezing response levels (B) of C57BL6/N wild-type mice during the extinction sessions on days 1–3.

(C and D) The effects of pre-test injection of baclofen (3 pmol) into the IPN on the freezing responses across trials (C) and overall freezing ratio (D) of wild-type mice.

(E and F) Lack of significant effects by intra-IPN pretreatment of baclofen on the freezing responses across trials (E) and overall freezing ratio (F) of *ChAT-$GABA_{B(1)}$*-KO mice. Mice were trained with five conditioning trials in (A) and (B) and ten trials in (C)–(F).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n.s., not significant; two-way ANOVA for difference between drugs and ACSF control in (A), (C), and (E) and t tests in (B), (D), and (F). Error bars indicate SEM. See Table S1 for statistical analyses in detail. See also Figure S3.

neurons, baclofen potentiated the EPSCs by more than 10-fold, increasing the EPSC amplitude from ~100 pA to >1,000 pA (Figure 4D; Table S1). The potentiation was dose-dependent and was saturable, with an EC_{50} of ~1 μM (Figures S4C and S4D); this value is consistent with the binding affinity of baclofen for $GABA_B$ receptors (Kaupmann et al., 1997). Baclofen did not change the EPSC rising time (Figures S4E and S4F), suggesting that its effect is monosynaptic. Baclofen also potently enhanced the EPSCs elicited by electrically stimulating the fasciculus retroflexus (fr), which contains the incoming fiber tract from the MHb (Figures S4G–S4I; Table S1). This rules out the possibility that baclofen acts on ChR2 to enhance EPSCs. After ablating habenula cholinergic neurons, electrical stimulation failed to elicit any EPSCs, even in the presence of baclofen (Figure S4J), indicating that baclofen selectively increases neurotransmitter release from the axonal terminals of these neurons.

We carried out three experiments to test whether presynaptic $GABA_B$ receptors, or a yet-to-be-identified receptor for baclofen, were mediating the unusual excitatory effect. First, knocking out *$GABA_{B(1)}$* in cholinergic neurons prevented baclofen from potentiating EPSCs (Figures 4E and 4F; Table S1), indicating that $GABA_B$ receptors in the presynaptic neurons are essential. Second, antagonizing $GABA_B$ with saclofen reversibly abolished baclofen's potentiatory effect (Figures S4K–S4M; Table S1), demonstrating the necessity of $GABA_B$ activity within the IPN. Third, baclofen did not alter inward currents induced by directly puffing the glutamate agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) onto the interpeduncular neurons of wild-type mice (Figures S4N and S4O; Table S1), excluding the possibility that baclofen directly increased the activity of postsynaptic glutamate receptors. We can thus conclude that baclofen activates presynaptic $GABA_B$ receptors to boost glutamate release.

Baclofen mimics the effect of endogenous GABA. Directly perfusing GABA into the slice preparation significantly potentiated light-evoked EPSCs (Figures 4G–4I; Table S1). Furthermore, endogenously released GABA also enhances EPSCs. To show this, we induced GABA release by optogenetically stimulating the GABAergic neuropil in the IPN of *VGAT-ChR2-EYFP* mice, in which ChR2 expression was under the control of the promoter of the gene encoding the vesicular GABA transporter (*VGAT*) (Zhao et al., 2011) (Figures 4J, S4P, and S4Q). Optically-induced GABA release significantly enhanced the EPSCs evoked by electrical stimulation of the incoming habenular fiber (Figures 4K and 4L; Table S1).

$GABA_B$ Potentiates Neurotransmitter Corelease to Reduce Fear

In light of glutamate and acetylcholine corelease by habenula cholinergic neurons, we asked whether $GABA_B$ activity could also potentiate cholinergic EPSCs. In the presence of the glutamate antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX), a train of light pulses (10 Hz, 5-ms pulses for 1 s) elicited a slow EPSC of <50 pA in an interpeduncular neuron of a *ChAT-ChR2-EYFP* mouse (Figures 5A and 5B). Following baclofen application, the EPSC reached the peak amplitude of ~700 pA and quickly decayed despite continuous light stimulation. In most cells (12/18), these slow EPSCs were fully blocked by nicotinic antagonists, indicating that they were cholinergic in nature (Figures 5A–5C; Table S1). For the 12 fully-blocked cells, baclofen amplified cholinergic EPSCs 9-fold (Figure 5C; Table S1), indicating that $GABA_B$ activity can strongly enhance acetylcholine release.

Baclofen also potentiated EPSCs mediated by the peptide neurokinin B (NKB). This was revealed by our observation that

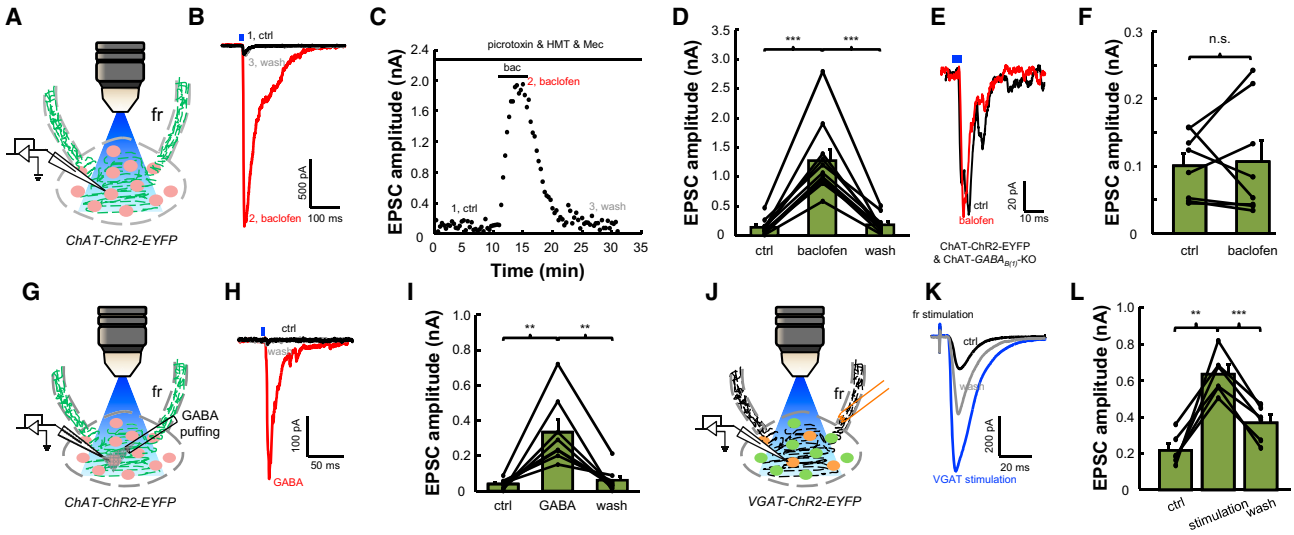

Figure 4. Presynaptic GABA$_B$ Activity Drastically Potentiates Glutamate Release from Habenular Neurons

(A–D) Baclofen (1 μM) produced an over tenfold increase in glutamatergic EPSCs. The cartoon in (A) illustrates the method of whole-cell recordings and optogenetic stimulation of the ChR2-EYFP-expressing axons from the fasciculus retroflexus (fr). Raw traces (B) and a time-series plot of the EPSC amplitude (C) draw data from one representative cell. The blue dot in (B) indicates 5-ms blue light stimulation. Horizontal lines in (C) show the timing of drug applications. Glutamatergic EPSCs were isolated with the GABA$_A$ blocker picrotoxin (50 μM) and a mixture of nicotinic blockers hexamethonium (HMT, 50 μM) and mecamylamine (MEC, 5 μM). Numbers in (B) correspond to time in (C). n = 11 cells in (D).

(E and F) Raw traces from a single cell (E) and group data (F) show that knocking out *GABA*$_{B(1)}$ in cholinergic neurons abolished the potentiatory effect of baclofen (n = 8 cells).

(G–I) GABA puff (1 mM) potentiated glutamatergic EPSCs. Schematic in (G) illustrates the method of recording and GABA puff. Raw traces in (H) shows the responses of a single cell and (I) shows group data (n = 7 cells).

(J–L) Optogenetically stimulating (5 ms; 10 Hz, 15 s) GABAergic neuropils in the IPN of a *VGAT-ChR2-EYFP* mouse potentiated EPSCs that were evoked by electric stimulation of the fiber tract fr. (J) illustrates the method of recording and optogenetic stimulation. (K) shows the raw traces from a single cell, with stimulation artifacts clipped for clarity. (L) shows group data (n = 6 cells).

***p < 0.001; **p < 0.01; n.s., not significant; two-sided paired t tests. Error bars indicate SEM.

See Table S1 for statistical analyses in detail. See also Figure S4.

some cells (6/18 cells) expressed a large inward current that was resistant to nicotinic blockers (Figure 5D). Consistent with the expression of NKB in habenular neurons (Marksteiner et al., 1992), the presence of NKB receptor antagonists abolished this additional current in the six cells tested (Figures 5E and 5F; Table S1). This provides evidence both that NKB serves as a neurotransmitter in the brain and that GABA$_B$ activity gates the release of NKB.

Given that all three coreleased neurotransmitters evoked excitatory currents, we tested whether baclofen could indeed increase the ability of habenular neurons to elicit action potential firing in interpeduncular neurons. We stimulated ChR2-expressing axonal terminals at various frequencies (5-ms pulses at 1–20 Hz for 1 s) and recorded action potential firing from interpeduncular neurons using cell-attached recording. Baclofen significantly increased the number of action potentials evoked by individual light pulses, especially when the stimulation frequency was <10 Hz (Figures 5G, 5H, S4R, and S4S; Table S1). Therefore, presynaptic GABA$_B$ activity facilitates the spread of excitatory signals across the habenulo-interpeduncular synapse.

Next, we examined whether activating the receptors of the coreleased neurotransmitters could reduce fear memory expression. Wild-type C57BL6/N mice were conditioned with ten tone-footshock pairs. Before the tests in extinction sessions, we separately infused the ionotropic glutamate receptor agonists (AMPA and NMDA), acetylcholine, or a NKB receptor agonist (senktide) into the IPN of individual mice. All of these drugs significantly reduced freezing responses (Figures 6A–6F; Table S1). Similarly, intra-IPN pretreatment of these drugs significantly reduced the freezing responses of *ChAT-GABA*$_{B(1)}$-KO mice to levels comparable to those of wild-type mice (Figures S5A–S5E; Table S1). Therefore, activating the receptors for the coreleased neurotransmitters reduces fear memory expression.

The widespread expression of GABA$_B$ receptors in the brain raises the concern that higher doses of baclofen may be sedative. We tested whether fear could be reduced by potentiating the neurotransmission of habenula cholinergic neurons in a GABA$_B$-independent manner. Habenula cholinergic neurons richly express phosphodiesterase 2A (PDE2A), and blocking PEDA2A activity increases presynaptic cyclic adenosine monophosphate (cAMP) levels and enhances neurotransmitter release (Hu et al., 2012). Intra-IPN pretreatment of the selective PDE2A inhibitor (Bay 60-7550) significantly reduced the freezing responses of wild-type mice during the test sessions for cued fear memory (Figures 6G, 6H, and S6F–S6I; Table S1), suggesting an alternative choice of drugs for the control of fear memory expression.

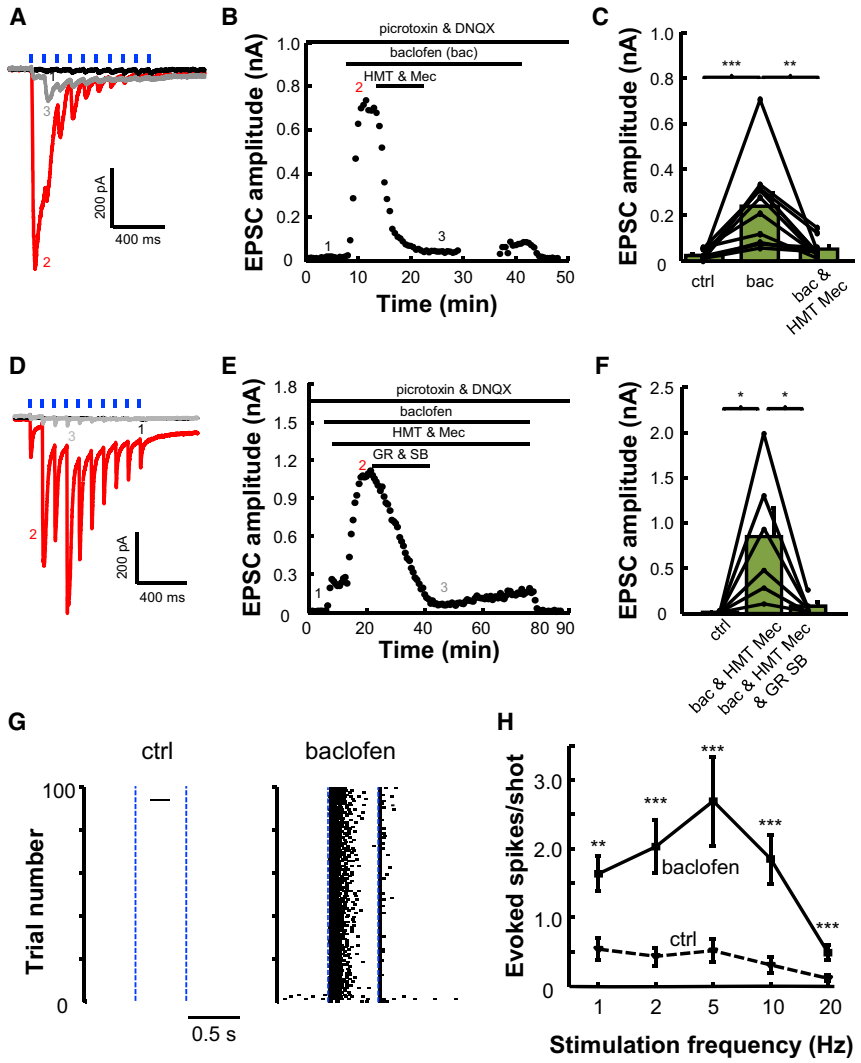

Figure 5. $GABA_B$ Activity Potentiates the Corelease of Acetylcholine and Neurokinin B and Increases the Effectiveness of Propagating Activity from the MHb to the IPN

(A–C) Raw trace (A) and time-series plot of EPSC amplitudes (B) from a representative cell and population data (C) show that baclofen (1 μM) potently increased cholinergic EPSCs, which were isolated by the glutamate receptor blocker DNQX (10 μM) and subsequently eliminated by nicotinic blockers HMT (50 μM) and Mec (5 μM; n = 12 cells).

(D–F) Raw trace (D) and time-series plot of EPSC amplitudes (E) from a single cell and population data (F) show that, in some cells, baclofen potentiated peptidergic EPSCs, which were resistant to nicotinic blockers but were abolished by a mixture of NKB blockers GR159897 (GR; 5 μM) and SB222200 (SB; 20 μM; n = 6 cells).

(G and H) Raster plots of spiking activity of an IPN neuron (G) and mean firing rates of all tested cells (n = 13 cells in H) show the effect of baclofen on light-evoked spiking responses in the IPN of *ChAT-ChR2-EYP* mice. Each row in (G) indicates one trial; dots represents spikes. Blue dashed lines correspond to light pulses.

*p < 0.05; **p < 0.01; ***p < 0.001; two-sided paired t tests. Error bars indicate SEM.

See Table S1 for statistical analyses in detail. See also Figure S4.

$GABA_B$ Activity Increases Presynaptic Ca^{2+} Entry through $Ca_{v2.3}$-Containing Channels

What could explain the $GABA_B$ receptor's novel excitatory effect? To address this question, we first tested whether presynaptic $GABA_B$ signals via a G protein. Pertussis toxin inactivates some G proteins and blocks the inhibitory action of $GABA_B$ activity (Dutar and Nicoll, 1988b; Kajikawa et al., 2001). We injected pertussis toxin into the IPN of a *ChAT-ChR2-EYFP* mouse and then recorded EPSCs 2 days after toxin treatment. Pertussis toxin, but not its control vehicle, prevented baclofen from potentiating EPSCs, confirming that presynaptic $GABA_B$ acts through a pertussis toxin-sensitive G protein (Figures S6A–S6F; Table S1). Increasing presynaptic cAMP levels significantly amplified glutamatergic EPSCs (Figures S6G–S6J; Table S1). However, baclofen did not alter cAMP concentration (Figure S6G; Table S1); and when cAMP synthesis was blocked, it could still potentiate EPSCs (Figures S6K and S6L; Table S1). Thus, cAMP cannot be a key downstream messenger for $GABA_B$.

Ca^{2+} is another key second messenger of G protein-coupled receptors, and presynaptic Ca^{2+} is known to be essential for neurotransmitter release (Wheeler et al., 1994). Although $GABA_B$ activity inhibits the activity of Ca^{2+} channels elsewhere (Mintz and Bean, 1993; Wu and Saggau, 1995), we speculated that it may increase presynaptic Ca^{2+} levels to facilitate neurotransmitter release from habenula cholinergic neurons. To analyze baclofen's effect on presynaptic Ca^{2+} levels, we expressed a genetically encoded Ca^{2+} indicator (GCaMP6) in habenular terminals by injecting AAV-DIO-GCaMP6m vectors into the MHb of a *ChAT-Cre* mouse (Figures 7A–7C) (Chen et al., 2013). We then measured GCaMP fluorescence changes within the cholinergic axonal terminals using 2-photon microscopy. Baclofen significantly increased Ca^{2+} transients in habenular terminals evoked by electrical stimulation of their axons at either 1 Hz or 10 Hz (Figures 7D–7G, S6M, and S6N), demonstrating that $GABA_B$ activity amplifies presynaptic Ca^{2+} transients.

We identified the Ca^{2+} channel downstream of $GABA_B$ signaling. Nickel (Ni^{2+}), a blocker of the R-type Ca^{2+} current (Wu et al., 1998), abolished the increase of presynaptic Ca^{2+} influx (Figures 7H and 7I; Table S1). Ni^{2+} also prevented baclofen from potentiating EPSCs, whereas blockers of other types of calcium channel (P/Q-type, T-type, N-type, or L-type) had no effect (Figures 7J–7L and S7A–S7L; Table S1). R-type Ca^{2+} channels contain the $Ca_{v2.3}$ subunit (Sochivko et al., 2002), which is richly expressed in habenula cholinergic neurons and their axonal

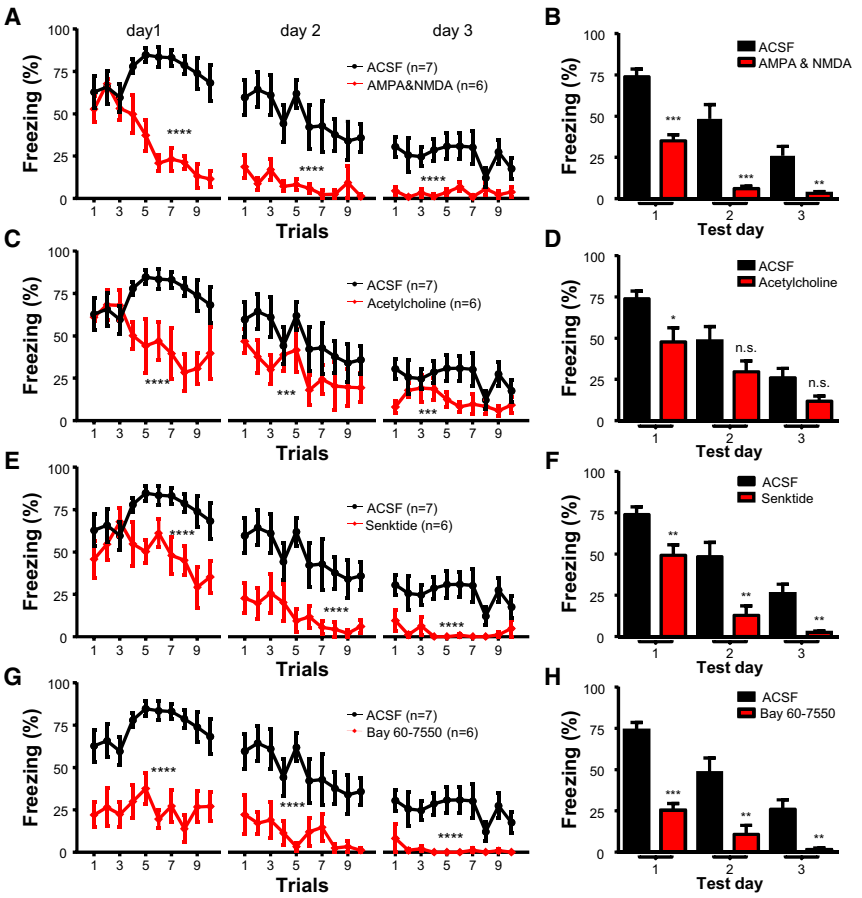

Figure 6. Activating Interpeduncular Neurons or Potentiating Habenular Neurotransmission Reduces Fear Memory Expression.

(A and B) The effect of intra-IPN pretreatment of AMPA (3 pmol) and NMDA (12 pmol) on the freezing responses of wild-type mice across trials (A) and overall freezing ratio (B) of wild-type mice during extinction sessions on days 1–3. Mice were conditioned with ten tone-shock pairs on day 0. For a control, ACSF was infused prior to the extinction sessions.

(C–H) The effect of intra-IPN pretreatment of acetylcholine (0.6 nmol; C and D), the NKB agonist senktide (3 pmol; E and F), and the PDE2A inhibitor Bay 60-7550 (30 pmol; G and H) on the freezing responses of wild-type mice.

*p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant; Two-way ANOVA in (A), (C), (E), and (G) and t tests in (B), (D), (F), and (H). Error bars indicate SEM.

See Table S1 for statistical analyses in detail. See also Figure S5.

terminals in the IPN (Parajuli et al., 2012) (Figures 7M and S7M). Using the CRISPR/Cas9 genome editing tool (Cong et al., 2013), we generated $Ca_{v2.3}$ null mice in the background of *ChAT-ChR2-EYFP* mouse line (Figures 7N, S7N, and S7O). Genetically inactivating the $Ca_{v2.3}$-containing channel abolished baclofen's potentiatory effect (Figures 7O–7Q; Table S1). Therefore, presynaptic $GABA_B$ receptors increase neurotransmitter release by facilitating the opening of the voltage-dependent R-type calcium channels formed with $Ca_{v2.3}$ subunits.

DISCUSSION

Animals and humans adjust their behavioral responses to threats in an experience-dependent manner. Research on fear conditioning and extinction has focused predominantly on the cortico-amygdala circuit (Herry et al., 2010; Milad and Quirk, 2012; Myers and Davis, 2007; Tovote et al., 2015; VanElzakker et al., 2014). Here, we demonstrate that habenula cholinergic neurons and the $GABA_B$ activity in their axonal terminals control fear memory expression through a unique signaling mechanism. We report three major findings. First, ablating habenula cholinergic neurons or selectively inactivating their $GABA_B$ receptors impairs the extinction of cued fear memory, whereas activating these neurons or their $GABA_B$ receptors reduces the expression of conditioned fear. Second, although previously considered purely inhibitory, $GABA_B$ actually mediates the fear-reducing effect by strongly potentiating the corelease of multiple neurotransmitters from habenula cholinergic neurons. Third, $GABA_B$ produces presynaptic excitation by enhancing Ca^{2+} influx through a special Ca^{2+} channel. These conclusions establish the habenulo-interpeduncular pathway as the key site for fear control and substantially expand our understanding of the signaling capacity of GABA and $GABA_B$ receptors. Moreover, our findings provide a circuit and molecular explanation of baclofen's clinical efficacy on fear-related disorders and suggest alternative pharmacotherapeutic solutions.

Habenula Cholinergic Neurons Suppress Fear Memory Expression

Selectively ablating cholinergic neurons in the mouse medial habenula increases freezing responses to a cue that was previously associated with danger but no longer represents a threat. This lesion does not affect fear memory formation or general locomotor activity. Moreover, optogenetically stimulating the axonal terminals of habenula cholinergic neurons reduces freezing responses. The complementary loss- and gain-of-function phenotypes strongly suggest that the activity of habenula cholinergic neurons plays an important role in suppressing the expression of fear memory in mammals.

Habenula cholinergic neurons receive inputs from the septal areas that promote anxiety (Yamaguchi et al., 2013). This at first seems contradictory to our findings on the role of these neurons in reducing fear. However, it is known that the MHb receives inputs from regions other than the septal areas (Qin and Luo, 2009). By focusing on the output of habenula cholinergic neurons rather than a subset of their inputs, this study reveals that the activity of these neurons accelerates the decrease of freezing

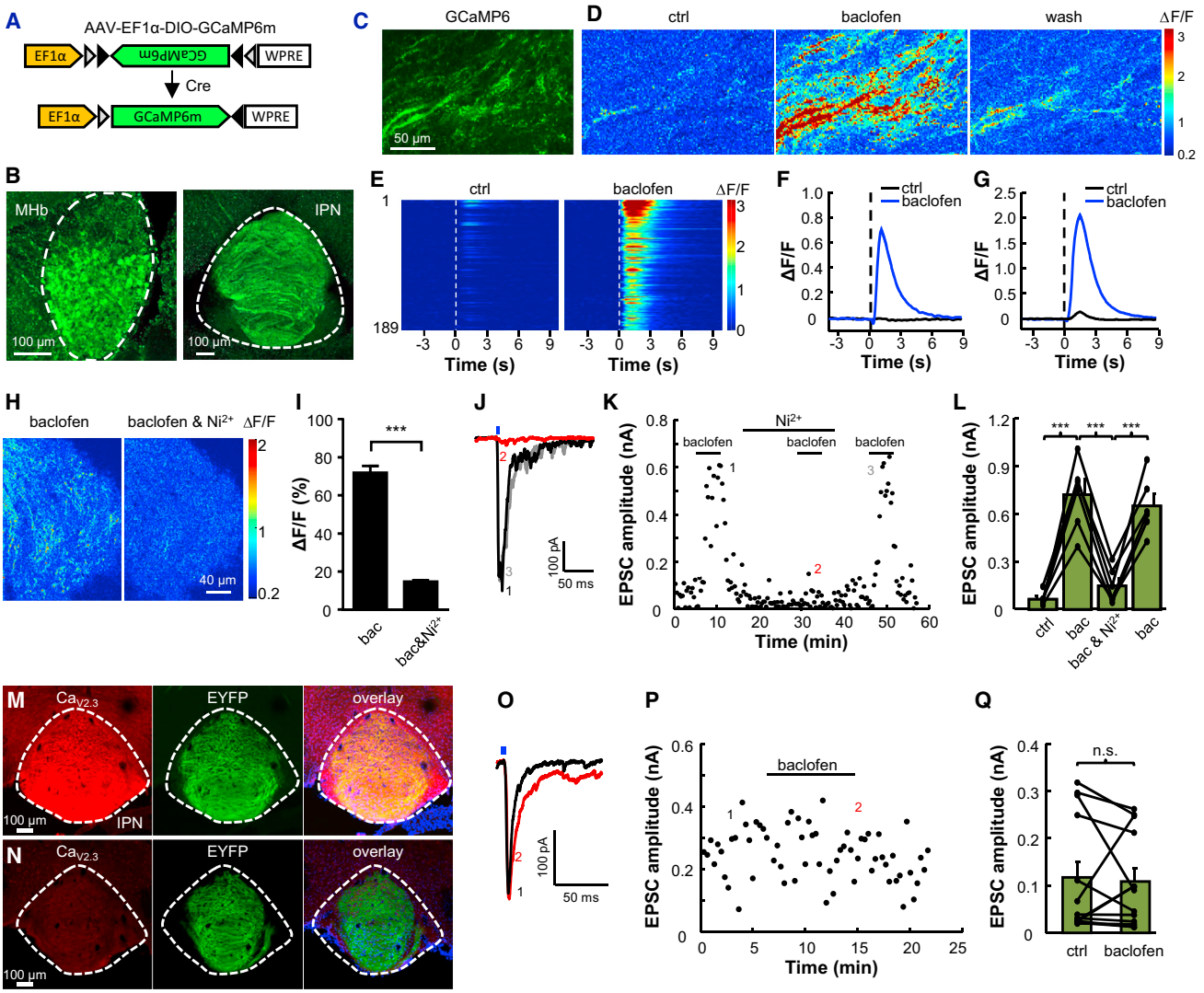

Figure 7. $GABA_B$ Mediates Excitation by Increasing Presynaptic Ca^{2+} Entry through $Ca_{v2.3}$-Containing Channels

(A and B) Injecting AAV-DIO-GCaMP6m vectors (A) into the MHb of a *ChAT-Cre* mouse resulted in GCaMP6 expression in MHb cholinergic neurons and their axonal terminals in the IPN (B).

(C–G) Baclofen (1 μM) increased GCaMP signals in the IPN. (C) Zoom-in view of GCaMP6 expression in the central IPN. (D) Pseudo-color representation of GCaMP fluorescence change within the same area of (C) following electrical fiber stimulation (ten pulses at 10 Hz). (E) Raster plots of GCaMP6 signals for 189 terminal areas from three mice. (F and G) Average GCaMP signals in response to the single-shot (F) and trains (ten pulses at 10 Hz; G) of electrical stimulation. Dashed lines indicate the timing of stimulation. Line width indicates SEM.

(H and I) Ni^{2+} blocked baclofen's potentiatory effect on the evoked Ca^{2+} transients in the axonal terminals of habenula cholinergic neurons. (H) shows the pseudocolor representation of GCaMP fluorescence changes within the IPN. (I) shows average amplitudes of Ca^{2+} transients evoked by single-shot electrical stimulation.

(J–L) Data from a single cell (J and K) and the entire test group (L) show that Ni^{2+} (50 μM) reversibly blocked EPSC potentiation by baclofen (n = 6 cells).

(M and N) $Ca_{v2.3}$ immunoreactivity was observed in ChR2-EYFP+ terminals within the IPN of a *ChAT-ChR2-EYFP* $Ca_{2.3}^{+/+}$ mouse (M), but not a $Ca_{v2.3}$ null mouse (N).

(O–Q) Data from a cell (O and P) and the entire test group (Q) demonstrate that mutating $Ca_{v2.3}$ prevented baclofen from potentiating EPSCs (n = 13 cells).

***p < 0.001; n.s., not significant; two-sided paired t tests in (I, L, and Q). Error bars indicate SEM.

See Table S1 for statistical analyses in detail. See also Figures S6 and S7.

responses to a previous danger-predicting cue. Our observations are consistent with the finding that inactivating or ablating the fish homolog of the MHb causes "helpless" behaviors (Agetsuma et al., 2010; Lee et al., 2010), although it had been unclear whether the fish effect could be extrapolated to mammals, as the habenular structure in fish and mammals receive distinct inputs (Stephenson-Jones et al., 2012). Our findings thus indicate that, despite the difference in inputs across taxa, the habenulo-interpeduncular pathway is evolutionarily conserved to promote active coping to aversive stimuli.

Because neither the MHb nor the IPN connects directly with the amygdala, the current study raises the intriguing question of how habenula neurons contribute to fear control. The habenulo-interpeduncular pathway connects the limbic forebrain areas related to fear processing to several brainstem modulatory centers, including the raphe nuclei (Figure S1A). Serotonergic neurons in the dorsal raphe encode reward signals and selective serotonin reuptake inhibitors alleviate the clinical symptoms of PTSD (Li et al., 2016; Liu et al., 2014; Stein et al., 2000). Possibly, habenula cholinergic neurons regulate the expression of fear memory by controlling the activity of brainstem neurons, including serotonergic neurons, thus modulating fear-related forebrain areas, including the medial prefrontal cortex and amygdala (Herry et al., 2010; Myers and Davis, 2007).

$GABA_B$ Activity in Habenula Cholinergic Neurons Facilitates Fear Extinction

A key finding of the present study is that $GABA_B$ activity on the axonal terminals of habenula cholinergic neurons reduces the expression of cued fear memory. Genetically inactivating $GABA_B$ receptors in these neurons slows the decay of freezing response levels during the extinction phase, but does not disrupt fear memory formation or general locomotion, general anxiety, or pain. Neurochemistry experiments further determine the IPN as the key anatomical site for the fear-reducing effects of $GABA_B$ activity. Blocking $GABA_B$ activity in the IPN immediately before the extinction tests similarly increases freezing, whereas activating $GABA_B$ in the IPN reduces freezing in wild-type mice but not in the $GABA_B$ conditional knockout mice. Tests using discrete conditioned stimuli or 180-s continuous tone revealed similar behavioral phenotypes, suggesting that the change in fear responses is associated with memory expression rather than sensory habituation.

Our results thus indicate that $GABA_B$ receptors in habenula cholinergic neurons, particularly those in the axonal terminals, are critically important for controlling responses to conditioned stimuli that no longer predict threat. Pharmacological activation of $GABA_B$ or the receptors for the coreleased neurotransmitters in the IPN speeds up the decay, further supporting the assertion that presynaptic $GABA_B$ activity facilitates fear extinction. On the other hand, optogenetic stimulation of habenula cholinergic neurons or infusion of a PDE2A inhibitor into the IPN reduces the overall freezing response levels. Intra-IPN application of saclofen increases the overall freezing response levels. Unlike conditionally knocking out $GABA_{B(1)}$, infusing saclofen also inactivates $GABA_B$ receptors that are expressed by non-cholinergic neurons within the IPN. Thus, the habenulo-interpeduncular pathway can regulate both fear extinction and the overall fear response intensity.

$GABA_B$ Mediates Presynaptic Excitation

Because $GABA_B$ activity had been thought previously to produce only inhibitory responses (Chalifoux and Carter, 2011; Dutar and Nicoll, 1988a, 1988b; Gassmann and Bettler, 2012; Newberry and Nicoll, 1984), it at first appears counterintuitive that both the activity of habenula cholinergic neurons and the activity of $GABA_B$ receptors in their axonal terminals play similar behavioral roles. Our finding that $GABA_B$ mediates strong presynaptic excitation resolves this paradox. Activating $GABA_B$ receptors with baclofen strikingly potentiates glutamatergic EPSCs, whereas knocking out $GABA_B$ in cholinergic neurons completely abolishes the potentiatory effect. Our study provides the first demonstration that $GABA_B$ excites neurons. Baclofen also dramatically potentiates the evoked cholinergic EPSCs, thus revealing that a presynaptic receptor controls neurotransmitter corelease. $GABA_B$ activity further gates the release of NKB to excite interpeduncular neurons, demonstrating both that NKB is exocytotically released as an excitatory neurotransmitter, and a presynaptic receptor gates neuropeptide transmission. GABAergic neurons are densely distributed in the IPN (Hsu et al., 2013), suggesting that GABA may function as a retrograde messenger from interpeduncular neurons to amplify the excitatory effect of habenular outputs.

Thus, baclofen may reduce fear memory expression by potentiating neurotransmitter release from habenula cholinergic neurons to interpeduncular neurons. In support of this possibility, pharmacological activation of receptors for glutamate, acetylcholine, and NKB within the IPN all facilitated fear extinction. The fear-reducing effect of intra-IPN acetylcholine infusion is consistent with the recent finding that inactivating CB1 cannabinoid receptors within habenula neurons increases cholinergic release and reduces behavioral responses to aversive stimuli (Soria-Gómez et al., 2015).

Our data provide plausible circuit- and molecular-level explanations for how baclofen alleviates the symptoms of PTSD patients (Cryan and Kaupmann, 2005; Drake et al., 2003; Manteghi et al., 2014), suggesting that enhancing neurotransmission from habenula cholinergic neurons may prove effective in treating PTSD. One strategy is to target phosphodiesterase 2A, which constitutively inhibits neurotransmission of habenula cholinergic neurons by negatively coupling with the cAMP pathway (Hu et al., 2012). Indeed, locally infusing a selective PDE2A inhibitor into the IPN resulted in decreased freezing responses, suggesting that PDE2A inhibitors may be of use for treating anxiety disorders such as PTSD with the benefit of avoiding baclofen's potentially sedative effect.

$GABA_B$ Activity Increases Presynaptic Ca^{2+} Influx through $Ca_{v2.3}$

Finally, our study reveals the presence of novel signaling cascades for $GABA_B$ receptors (Figure S7P). Our results indicate that, upon ligand binding, presynaptic $GABA_B$ receptors in habenula cholinergic axons dissociate the pertussis toxin-sensitive G protein $G\alpha_{i/o}$-$\beta\gamma$, which in turn facilitates the opening of voltage-dependent calcium channels to increase Ca^{2+} influx. This effect is surprising, because the effect of presynaptic G protein-coupled receptors on Ca^{2+} levels were until now thought to only be inhibitory. Using both pharmacological and genetic approaches, we identify the $Ca_{V2.3}$-constructing Ca^{2+} channel as the key downstream mediator of $GABA_B$ signals. It remains to be further dissected at the molecular level precisely how dissociated G protein subunits couple to $Ca_{v2.3}$. The IPN is the only brain region where $Ca_{V2.3}$ expression is predominantly presynaptic (Parajuli et al., 2012), suggesting that the observed excitatory action requires the compartmentalization of $GABA_B$ receptors, $G\alpha_{i/o}$-$\beta\gamma$, and

$Ca_{V2.3}$ channels into precise microdomains in axonal terminals. The habenulo-interpeduncular pathway may serve as a valuable model for dissecting the detailed molecular mechanism underlying the rich signaling capacity of $GABA_B$ receptors.

Taken together, this study reveals that $GABA_B$ receptors in the habenula cholinergic neurons facilitate fear extinction by potentiating corelease of multiple neurotransmitters. Malfunctions in the habenulo-interpeduncular pathway and in $GABA_B$ signaling have been implicated in psychiatric disorders associated with fear and anxiety (Cryan and Kaupmann, 2005; Hikosaka, 2010; Lecourtier and Kelly, 2007). Our results suggest that potentiating the habenulo-interpeduncular pathway, as by activating habenular $GABA_B$ or inhibiting PDE2A, presents a potentially effective therapeutic approach for treating such disorders.

EXPERIMENTAL PROCEDURES

Detailed materials and methods are available in the Supplemental Experimental Procedures.

Mice

Adult mice of either sex were used. We used simplified genotypes of mouse strains for clarity. Mouse strains include *ChAT-ChR2-EYFP*, *VGAT-ChR2-EYFP*, *ChAT-Cre*, $GABA_{B(1)}^{lox511/lox511}$, $Ca_{v2.3}$ null, and wild-type C57BL6/N mice. All procedures were conducted with the approval of the Animal Care and Use Committee of the National Institute of Biological Sciences, Beijing in accordance with governmental regulations of China.

AAV Vectors and Injection

pAAV-flex-taCasp3-TEVp, pAAV-EF1α-DIO-GCaMP6m, and pAAV-EF1α-DIO-EmGFP were packaged into AAV serotype 2/9 vectors. AAV vectors were injected with pressure into the bilateral MHb of anaesthetized mice.

Behavior

Fear conditioning and extinction were performed essentially as described previously (Milad and Quirk, 2002; Soria-Gómez et al., 2015). For fear conditioning, auditory tones (20 s, 7.5 kHz, 85–90 dB) were coupled to co-terminating footshocks (1 s, 0.7 mA) for five or ten trials. For fear extinction, a mouse was presented with conditioned auditory tones that were no longer coupled to footshocks. We used two slightly different test paradigms to apply tones. First, we repetitively applied the conditioned auditory tone (20 s) for ten discrete trials during the daily extinction phases for 3–5 consecutive days. Alternatively, we applied a continuous auditory tone (7.5 kHz, 180 s) during the extinction sessions on days 1 and 6. Two trained observers scored the mouse behavior off-line in a double-blind manner. Mice were also tested for pain threshold, open field, light/dark box, and elevated plus-maze by following protocols described in the Supplemental Experimental Procedures.

Optogenetic stimulation was carried out essentially as described elsewhere (Liu et al., 2014). Light was delivered through a tapered optical fiber (5-ms pulse duration, 50 Hz frequency, 30 mW output power; fiber outside diameter [OD] = 200 μm and numerical aperture [NA] = 0.39). For the intra-IPN drug injections, a guide cannula was implanted with its tip targeting the dorsal IPN. Immediately prior to extinction tests, drugs (saclofen, baclofen, AMPA and NMDA, senktide, acetylcholine, Bay 60-7550) or artificial cerebrospinal fluid (300 nl) was slowly infused to the IPN via an internal cannula.

Slice Recording and 2-Photon Imaging

Slice preparation and whole-cell recordings were performed as described elsewhere (Ren et al., 2011). GCaMP6m fluorescent signals were imaged with a 20× water immersion objective on a 2-photon microscope (3 frames/s). For photostimulation, blue light pulses (470 nm) were generated with an LED and delivered via a 40× water immersion lens (5 ms, 14 mW). For electrical stimulation, the fasciculus retroflexus was stimulated with bipolar stainless steel microelectrodes (0.2 ms, 100–150 μA). The drugs were delivered either through perfusion or by local ejection.

Measurement of cAMP Levels

Measurement of cAMP levels with ELISA kits were performed as described elsewhere (Hu et al., 2012).

Histology and Immunohistochemistry

History, immunostaining, and fluorescent microscopy were performed essentially as described previously (Ren et al., 2011). For details on procedures and antibodies used, see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.06.026.

AUTHOR CONTRIBUTIONS

J. Zhang and M.L. designed the experiments. J. Zhang, J.L., Y.R., L.T., J. Zhou, C.W., and T.Y. performed behavioral assays. J. Zhang performed physiological recordings. J. Zhang, R.L., J.R., and F.H. performed calcium imaging. L.T., Y.Z., F.W., and J. Zhang generated $Ca_{v2.3}$ mutant mice. Q.F., R.L., and L.T. prepared AAV vectors. B.B. provided $GABA_{B(1)}^{lox511/lox511}$ mice. J. Zhang and M.L. analyzed the data and wrote the paper.

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