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# Sex-dependent modulation of anxiety and fear by 5-HT$_{1A}$ receptors in the bed nucleus of the stria terminalis

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## Abstract

Serotonin (5-hydroxytryptamine; 5-HT) coordinates behavioral responses to stress through a variety of presynaptic and postsynaptic receptors distributed across functionally diverse neuronal networks in the central nervous system. Efferent 5-HT projections from the dorsal raphe nucleus (DRN) to the bed nucleus of the stria terminalis (BNST) are generally thought to enhance anxiety and aversive learning by activating 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) signaling in the BNST, although an opposing role for postsynaptic 5-HT$_{1A}$ receptors has recently been suggested. In the present study, we sought to delineate a role for postsynaptic 5-HT$_{1A}$ receptors in the BNST in aversive behaviors using a conditional knockdown of the 5-HT$_{1A}$ receptor. Both males and females were tested to dissect out sex-specific effects. We found that male mice have significantly reduced fear memory recall relative to female mice and inactivation of 5-HT$_{1A}$ receptor in the BNST increases contextual fear conditioning in male mice so that they resemble the females. This coincided with an increase in neuronal excitability in males, suggesting that 5-HT$_{1A}$ receptor deletion may enhance contextual fear recall by disinhibiting fear memory circuits in the BNST. Interestingly, 5-HT$_{1A}$ receptor knockdown did not significantly alter anxiety-like behavior in male or female mice, which is in agreement with previous findings that anxiety and fear are modulated by dissociable circuits in the BNST. Overall, these results suggest that BNST 5-HT$_{1A}$ receptors do not

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Author contributions

C.A.M wrote the manuscript with editorial assistance from G.B-DLR and T.L.K. C.A.M and T.L.K designed the studies and C.A.M performed surgeries, behavior, electrophysiology, and data analysis. G.B-DLR and M.M. performed data analysis for behavioral and electrophysiological studies. C.E.D performed surgeries, behavioral experiments, and data analysis. J.F.D performed surgeries, immunohistochemistry, and imaging. D.P. assisted with electrophysiological experiments. C.A.G performed behavioral analysis. O.J.H performed behavior, imaging and analysis. G.T. and Z.A.M performed *in situ* hybridization and imaging. E.D. generated the *htr1a*$^{fl/fl}$ mice.

significantly alter behavior under basal conditions, but can act as a molecular brake that buffer against excessive activation of aversive circuits in more threatening contexts.

**Keywords**

Serotonin (5-HT); 5-$HT_{1A}$ receptor; bed nucleus of the stria terminalis (BNST); anxiety; contextual fear

## 1. INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) neurons originate in a cluster of nuclei in the midbrain and pons known as the raphe nuclei, where they orchestrate emotional responses to stress through a variety of pre- and postsynaptic receptors distributed across functionally diverse neuronal networks in the central nervous system[1–4]. The dorsal raphe nucleus (DRN) has reciprocal connections with the bed nucleus of the stria terminalis (BNST), a part of the extended amygdala that acts as a conduit between forebrain limbic structures and hypothalamic and brainstem regions[5,6]. The BNST is therefore ideally positioned to relay autonomic, sensory and stress-related information to emotional processing centers of the brain. Efferent projections from the DRN to the BNST are generally thought to enhance anxiety and aversive learning by activating 5-$HT_{2C}$ receptor signaling in the BNST[7–10], although an opposing role for 5-HT at 5-$HT_{1A}$ receptors has recently been suggested[11]. Earlier studies also support a model in which 5-$HT_{1A}$ receptor signaling in the BNST is anxiolytic. In 2004, Levita et al. found that direct infusion of a non-selective 5-$HT_1$ receptor agonist (of 5-carboxamidotryptamine or 5-CT) into the BNST attenuated acoustic startle response, while others have shown that cannabidiol reduces anxiety and contextual fear recall via activation of 5-$HT_{1A}$ receptors[12–14]. These diverging behavioral actions of 5-HT seem to mirror the physiological effects of 5-$HT_{2C}$ and 5-$HT_{1A}$ receptor activation on neuronal excitability, with 5-$HT_{2C}$ receptors having a net excitatory and 5-$HT_{1A}$ receptors having a net inhibitory postsynaptic effect[15].

Converging lines of evidence point toward an anxiolytic role for 5-$HT_{1A}$ receptors in the BNST, but the precise contribution of presynaptic and postsynaptic 5-$HT_{1A}$ receptors to these behavioral outcomes is unclear. While activation of postsynaptic 5-$HT_{1A}$ receptors may articulate the anti-aversive effects of 5-HT, there is also the possibility that activation of presynaptic 5-$HT_{1A}$ heteroreceptors may exert an anxiolytic effect by inhibiting glutamate release from presynaptic terminals[16–19], which would tend to reduce postsynaptic 5-$HT_{2C}$ receptor signaling. In the present study, we generated a conditioned knockout of the *htr1a* allele (*htr1a*$^{fl/fl}$) to delineate the role of postsynaptic 5-$HT_{1A}$ receptors in aversive behavior. Site-specific infusion of an adeno-associated viral vector (AAV-cre-GFP) into the BNST allowed for selective targeting of 5-$HT_{1A}$ receptor knockdown to postsynaptic neurons in order to isolate their contribution to aversive behaviors.

The BNST plays a key role in processing threat, but its precise contribution to unconditioned or diffuse threat (“anxiety”) and conditioned or discrete threat (fear) remains controversial[20–23]. In light of previous reports that 5-HT can reduce anxiety via 5-$HT_{1A}$ receptor activation in the BNST[12,13,24], we interrogated the role of postsynaptic 5-$HT_{1A}$

receptor knockdown in multiple behavioral assays that explore conditioned responses threat and unconditioned anxiety. Our overarching hypothesis was that genetic knockdown of 5-$HT_{1A}$ receptors in the BNST would enhance anxiety and conditioned fear memory by increasing neuronal excitability in neural circuits that process anxiety and fear. The BNST is also a sexually dimorphic region that displays differentially responsiveness to stress[25–28], so we compared male and female *htr1a*$^{fl/fl}$ mice in this study. Earlier studies report that inactivation of the BNST can prevent impaired learning after stress in males but not in females[29], suggesting that 5-$HT_{1A}$ receptor knockdown in the BNST may differentially impact aversive behaviors in male and female mice.

## 2. RESULTS AND DISCUSSION

### 2.1 Generation and validation of conditional 5-$HT_{1A}$ receptor knockout mouse line

The 5-$HT_{1A}$ receptor is widely distributed throughout the mammalian brain and is generally thought to have an anxiolytic function based on pharmacological and genetic knockout studies[30–34]. In the search for a neural substrate underpinning the anxiolytic effects of 5-$HT_{1A}$ receptors, many studies have pinpointed the extended amygdala and particularly the BNST as a potential candidate, although to date these studies have used pharmacological manipulations that do not discriminate between pre and postsynaptic effects[12–14]. The general consensus of these earlier studies is that 5-$HT_{1A}$R agonists administered into the BNST are anxiolytic and reduce contextual fear recall, presumably by activating postsynaptic 5-$HT_{1A}$ receptors. We generated a conditional *htr1a*$^{fl/fl}$ line with lox P sites flanking the sites flanking the *htr1a* open reading frame (ORF) to determine whether postsynaptic 5-$HT_{1A}$ receptors were critically involved in its putative anti-aversive actions in the BNST. We used a combination of fluorescence *in situ* hybridization and slice electrophysiology to verify that mice homozygous for the floxed *htr1a* allele exhibit functional deletion of 5-$HT_{1A}$ receptors upon Cre-mediated recombination with AAV-Cre-GFP. Our results indicate that 5-$HT_{1A}$ receptor mRNA was significantly reduced in *htr1a*$^{fl/fl}$:Cre$^{BNST}$ mice relative to controls in the dBNST ($t_{10}$=3.10, p<0.05) and vBNST ($t_{10}$=3.36, p<0.01) (Figure 1A–D). Next, *htr1a*$^{fl/fl}$:Cre$^{BNST}$ and *htr1a*$^{fl/fl}$:GFP$^{BNST}$ mice were injected with red fluorescent retrograde tracer beads into the ventral tegmental area (VTA) to target neuronal populations in the BNST that are known to express 5-$HT_{1A}$ receptors[9]. Recording from these VTA-projecting neurons in the presence of the selective 5-$HT_7$ antagonist SB 269970, we found that bath application of the 5-$HT_1$ agonist 5-CT hyperpolarized 5/11 neurons tested from control mice and 0/10 neurons from *htr1a*$^{fl/fl}$:Cre$^{BNST}$ mice (Fisher's exact test, odds ratio = 17.77, 95% CI (0.8360, 377.7), p<0.05) (Figure 1E–H). Together, these results indicate that *htr1a*$^{fl/fl}$ mice can be used to genetically knockdown 5-$HT_{1A}$ receptors in the BNST.

### 2.2. 5-$HT_{1A}$ receptor knockdown differentially modulates fear memory in male and female mice.

In light of previous studies pinpointing a role for 5-$HT_{1A}$ receptor signaling in the effects of cannabidiol on contextual fear memory[14], we analyzed contextual fear recall in male and female mice *htr1a*$^{fl/fl}$:Cre$^{BNST}$ mice. There was no significant interaction between Sex and Cre on percent freezing during fear learning, nor any significant main effects. However,

there was a significant interaction between Sex and Cre on percent freezing during contextual fear recall (2-way ANOVA; $F_{1,33}$=16.99, p<0.001). Bonferroni post-tests revealed that 5-$HT_{1A}$ receptor knockdown increased percent freezing in males ($t_{33}$=4.045 p<0.001) but not in females ($t_{33}$=1.755, ns) (Figure 2A–C)[35]. We also found that contextual freezing was significantly higher in females compared to males in the control groups ($t_{33}$=3.498, p<0.01), suggesting that females may exhibit heightened contextual fear that is relatively insensitive to manipulations of 5-$HT_{1A}$ receptors. This led us to speculate that females may have lower basal levels of expression of 5-$HT_{1A}$ receptors in aversive memory circuits. However, our results indicate that 5-$HT_{1A}$ receptor protein levels in the BNST did not significantly differ by sex ($t_8$=0.2431, ns) (Figure 3A–B). It is interesting to note that the BNST is a sexually dimorphic region in which males display twice as many neurons in the principal nucleus of the BNST as females[36]. It was also found that CRF mRNA is enhanced in male mice with diminished expression of GAD67 relative to female mice[26,28]. These anatomical and neurochemical differences highlight that BNST circuits are differentially organized in males and females, which may account for the divergent behavioral phenotypes observed here.

### 2.3. Neuronal excitability in the BNST enhanced by 5-$HT_{1A}$ receptor knockdown after contextual fear

Previous reports found that cannabidiol attenuated c-fos expression in the BNST after contextual fear recall in a 5-$HT_{1A}$ receptor dependent manner[37], supporting the idea that 5-$HT_{1A}$ receptors can inhibit the activity of aversive memory circuits in the BNST. Together with earlier studies indicating that 5-$HT_{1A}$ receptors have a net inhibitory effect on BNST neurons via activation of $G_i$-signaling pathways[12,15], we predicted that 5-$HT_{1A}$ receptor knockdown would exacerbate the increase in neuronal excitability during contextual fear recall which may account for exaggerated freezing responses in male mice during re-exposure to these aversive contexts. In order to test this hypothesis, *htr1a*$^{fl/fl}$::$Cre^{BNST}$ and *htr1a*$^{fl/fl}$::$GFP^{BNST}$ mice were submitted to fear conditioning followed by contextual fear recall. A second group of *htr1a*$^{fl/fl}$::$Cre^{BNST}$ and *htr1a*$^{fl/fl}$::$GFP^{BNST}$ mice were exposed to the conditioning chamber on the first day without receiving footshocks as a control group. Three-way ANOVAs revealed that there was no significant interaction between Sex, Cre and Conditioning on resting membrane potential (RMP) ($F_{1,65}$=0.342, ns), but there was a significant two-way interaction between Cre and Conditioning ($F_{1,65}$=7.716, p<0.01) and significant main effects of Conditioning ($F_{1,65}$=7.753, p<0.01) and Cre (F1,65=18.973, p<0.001) (Figure 4A, C). This suggests that the effect of Conditioning on RMP differs in Cre and Control mice, but this two-way interaction is not modified by Sex. Simple main effects of Cre reveal differences in both male and female conditioned but not in habituated mice, which was surprising given that activation 5-$HT_{1A}$ receptors has a hyperpolarizing effect on RMP in wild-type mice[15]. This indicates that the presence of 5-$HT_{1A}$ receptors may only exert an appreciable influence over resting membrane potential and neuronal excitability in the presence of an aversive stimulus, when 5-HT is released in the BNST. Given that these receptors are in relatively low abundance in the BNST, it may stand to reason that their absence would not significantly impact neuronal excitability in the resting state. It was also surprising that conditioning alone did not significantly alter excitability in

the male or female control mice. This suggests that the 5-$HT_{1A}$ receptors may have a buffering effect on RMP after fear conditioning that is unmasked after 5-$HT_{1A}$ knockdown.

Next, we looked at two measures of neuronal excitability; rheobase and current induced spiking. Rheobase is defined as the minimum current amplitude required to reach the depolarization threshold for action potential firing and should decrease as the cell becomes more excitable. We found no significant 3-way interaction between Sex, Cre and Conditioning on rheobase ($F_{1,63}$=0.990, ns) nor any significant two-way interactions or main effects. However, looking at simple main effects revealed a significant effect of Cre in conditioned male mice (F=5.546, $p<0.05$), which is consistent with our contextual fear data that 5-$HT_{1A}$ knockdown augments fear in male mice (Figure 4B, D). We then looked at current-induced spiking, which was defined as the number of spikes during a 30 pA current injection. Here we found no significant three-way interaction between Sex, Cre and Conditioning ($F_{1,62}$=0.607, ns) nor any significant two-way interactions. We did observe significant main effects of Cre ($F_{1,62}$ = 4,359, $p<0.05$) and Conditioning ($F_{1,62}$=8.903, $p<0.01$) on the number of action potentials fired at 30 pA. We then looked at simple main effects and found a significant effect of Cre in conditioned males only (F=5.750, $p<0.05$), which is similar to what we observed for rheobase (Figure 4E).

### 2.4 Genetic knockdown of 5-$HT_{1A}$ receptors in the BNST does not alter anxiety-like behavior in the EPM, open field or novelty-suppressed feeding tests.

In order to assess the contribution of postsynaptic 5-$HT_{1A}$ receptors to anxiety-like behavior, male and female *htr1a*$^{fl/fl}$:$Cre^{BNST}$ mice were submitted to the elevated plus maze (EPM), which is based on the tendency of the mouse to explore new environments and their conflicting tendency to avoid open spaces. More time spent in the open arms is taken as an inverse index of anxiety-like behavior[38]. Overall, there was no significant interaction between Sex and Cre on open arm time, probability, latency or distance moved in the EPM. However, there was a significant main effect of Sex on open arm probability in the EPM ($F_{1,78}$=7.386, $p<0.01$). Posthoc comparisons revealed a significant difference between male and female Cre mice, with females exhibiting a decrease in open arm probability which is indicative of increased anxiety-like behavior (t=2.303, $p<0.05$) (Figure 5A–D).

We performed two additional assays to provide converging information on anxiety-like behavior: the open field test and the novelty suppressed feeding test.[39]. These tests both represent conflict assays between the natural tendency of the mouse to avoid open spaces and their conflicting drive to explore new environments (open field) and consume palatable food (novelty-suppressed feeding). Important differences between these two constructs should be considered, such as the fact that mice are food deprived for 24 hours before the novelty suppressed feeding test, which can be a stressor. Both assays also have potential confounds which should be considered, including differences in locomotor activity in the open field and satiety/hunger in the novelty-suppressed feeding test. We controlled for both of these variables by measuring distance moved in the open field and performing a 10-minute home cage feeding assay immediately following the novelty-suppressed feeding test.

In the open field test, there was no significant interaction between Sex and Cre on center latency or distance moved. Unlike the EPM, there were no main effects of Sex on any

measure of anxiety-like behavior (Figure 5E–G). Likewise, in the novelty-suppressed feeding test, we saw no significant interaction between Sex and Cre on latency to feed nor any main effects of Sex or Cre. There were also no effects on food consumption in the post-test (Figure 5H–I). These wholly negative results of 5-$HT_{1A}$ receptor knockdown on anxiety-like behaviors were surprising in light of previous studies showing that 5-$HT_{1A}$ receptor agonists in the BNST have an anxiolytic effect[12,13]. Given that 5-$HT_{1A}$ receptor activation has a hyperpolarizing effect on BNST neurons[15], we would expect 5-$HT_{1A}$ receptor knockdown to have an anxiogenic effect by disinhibiting anxiety circuits in the BNST. It is possible that the effects of 5-$HT_{1A}$ knockdown do not manifest under low stress conditions when 5-HT release in the BNST is relatively low. There is also the possibility that postsynaptic 5-$HT_{1A}$ receptors are expressed in both anxiogenic and anxiolytic circuits of the BNST and that their removal has a net neutral effect on anxiety-like behavior. We have previously shown that BNST neurons that project to the VTA and lateral hypothalamus (LH), two anxiolytic outputs of the BNST[40,41], are hyperpolarized by 5-HT in the presence of a 5-$HT_{2C}$ receptor antagonist, which would indicate the presence of 5-$HT_{1A}$ receptors in this population[9,12,15].

There is also evidence in the literature indicating that 5-$HT_{1A}$ receptor signaling can have an antidepressant effect[42,43], so we decided to explore a potential role for 5-$HT_{1A}$ in the BNST in behavioral despair in the forced swim test. We did not observe a significant interaction between Sex and Cre on percent immobility in the forced swim test, but we did see a main effect of Sex suggesting that female mice exhibit elevated depressive-like behavior compared to males ($F_{1,32}=8.172$, $p<0.01$) (Figure 5J). The absence of any effect of 5-$HT_{1A}$ knockdown in the BNST is, however, consistent with previous literature suggesting that the antidepressant effects of 5-$HT_{1A}$ modulation are mediated by prefrontal and hippocampal circuits[44–46]

## 2.5 5-$HT_{1A}$ receptors in the BNST do not significantly modulate anxiety-like behavior after stress

In a previous study, we had found that stimulating 5-HT inputs to the BNST had a robust anxiogenic effect by activating 5-$HT_{2C}$ receptors in corticotropin releasing factor (CRF) neurons[9]. We also found that these neurons were hyperpolarized in the presence of 5-HT with pharmacological blockade of the 5-$HT_{2C}$ receptor, suggesting the presence of 5-$HT_{1A}$ receptors[15]. This led us to speculate whether stimulating 5-HT release in the BNST, which should activate 5-$HT_{2C}$ receptors in CRF neurons, would change the effect of 5-$HT_{1A}$ receptor knockdown from neutral to anxiogenic. Given that restraint stress can stimulate central CRF release, which in turn activates 5-HT neurons in the DRN that release 5-HT release in downstream circuits[47,48], we decided to look at anxiety-like behavior under high stress conditions using two assays: the elevated zero maze (EZM) after restraint stress and the open field test under high light conditions. Previous reports indicate that restraint increases avoidance of the open arms in the elevated zero maze[49], which is indicative of increased anxiety-like behavior. However, our results indicate that there are no discernable differences in anxiety-like behavior between male *htr1a*$^{fl/fl}$:$Cre^{BNST}$ and control mice in the EZM after physical restraint (Figure 6A–D) or the open field under high light conditions (300 lux) (Figure 6E–G). This suggests that 5-$HT_{1A}$ receptors, which are present in low

abundance in the BNST, may not have a discernible effect on anxiety-like behavior even under stressful conditions although they clearly do have a modulatory influence over aversive memory, specifically in males. One possible explanation is that 5-$HT_{1A}$ receptor signaling is upregulated in the BNST in response to footshock in order to buffer the system against excessive stimulation. This neural plasticity may require a consolidation period after the initial stimulus, so the effects of 5-$HT_{1A}$ receptor knockdown are apparent in the contextual fear recall test which occurs 24 hours after the conditioning session.

### 2.6 5-$HT_{1A}$ receptor knockdown in the BNST does not significant alter innate responses to visual threat in the looming disc assay.

The BNST receives retinal inputs and has extensive projections to the midbrain nuclei that process visual stimuli, suggesting that 5-$HT_{1A}$ receptors may contribute innate visual defense responses[50–52]. Another recent study found that innate defensive responses to looming visual stimuli are mediated by parvalbumin-projecting neurons in the superior colliculus of the mouse, which project to neurons in the parabigeminal nucleus and the lateral posterior thalamic nucleus[52], all of which project to the amygdala and may also have direct or indirect connections with the BNST[53–55]. To interrogate a potential role for the BNST in visual threat processing, we examined behavior in the looming disc assay. We found a non-significant trend toward a reduction in maximum escape velocity in male *htr1A*$^{fl/fl}$::$Cre^{BNST}$ mice($t_{13.67}$ (Welch-corrected)=1.85, ns) (Figure 7A–H), but no significant differences in escape latency or freezing time. These data indicate that 5-$HT_{1A}$ knockdown in the BNST does not significantly impair visual function or threat response in male mice.

## 3. CONCLUSIONS

Experimental manipulations that increase neuronal activity in the BNST tend to enhance anxiety[56–58], although there may be differential contributions of specific subnuclei and subpopulations that have led to conflicting reports[40,59]. There is also accumulating evidence from neuroimaging studies that the BNST is involved in the representation of anxiety states in humans with generalized anxiety disorder[60,61]. Given that 5-$HT_{1A}$ receptor activation has been reported to hyperpolarize (inhibit) neurons in the BNST, we hypothesized that 5-$HT_{1A}$ deletion would potentiate anxiety-like behavior. Contrary to our expectations, our results indicate that 5-$HT_{1A}$ receptor knockdown in the BNST does not significantly alter anxiety-like behavior, suggesting that 5-$HT_{1A}$ receptors may not have an appreciable influence over BNST circuits under basal conditions due to their low abundance. On the other hand, 5-$HT_{1A}$ knockdown does enhance contextual fear recall after fear conditioning in males, which is in agreement with previous reports that cannabidiol can reduce contextual fear by activating 5-$HT_{1A}$ receptors in the BNST[14]. This discrepancy between anxiety and fear suggests that high arousal states may induce plasticity in 5-$HT_{1A}$ receptor signaling that enables this receptor to act as a molecular brake that buffers against excessive stimulation. The observation that neuronal excitability is slightly lower in male control mice after fear conditioning but increases in *htr1A*$^{fl/fl}$::$Cre^{BNST}$ mice lends further support to the idea that 5-$HT_{1A}$ acts as a molecular switch that can be turned on under conditions of high arousal.

It is interesting that 5-$HT_{1A}$ knockdown does not appear to have any appreciable effects on contextual fear memory in female mice, especially given that 5-$HT_{1A}$ receptor levels do not significantly differ between males and females. However, it could be argued that the ability to buffer against excessive arousal states may be more adaptive for males from an evolutionary standpoint, especially since males tend to spend more time engaging in high-risk foraging and aggressive behaviors compared to females. This view is supported by the observation that males have significantly reduced contextual fear recall compared to females. Another possibility is that innate differences in fear memory consolidation plays a role in the significant discrepancy between males and females in contextual fear recall. Another possibility is that females have increased sensitivity to the unconditioned stimulus due to their smaller size leading to an exaggerated stress response compared to males, although the fact that males and females exhibit the same level of freezing during conditioning would seem to argue against this. Additional testing at a range of shock intensities to determine whether 5-HT1A knockdown can enhance fear memory in females would be required to rule out this possibility.

## 4. METHODS

### 4.1. Animals

*Htr1a*$^{fl/fl}$ mice were backcrossed for several generations to C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) before using in experiments. All procedures were conducted in accordance with the National Institutes of Health guidelines for animal research and with the approval of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and the Vanderbilt University Medical Center. All animals were group housed on a 12 h light cycle (lights on at 7 a.m.) with *ad libitum* access to rodent chow and water, unless described otherwise.

**Generation of Htr1a$^{fl/fl}$ mice.—***Htr1a*$^{fl/fl}$ mice were generated using homologous recombination in ES cells, the unique exon (exon 1) was targeted to introduce loxP sites around the gene ORF. A loxP site was introduced in the middle of the 5'UTR, while a neomycin resistance gene cassette flanked by Frt and loxP sites was introduced in the 3' UTR. Upon Flp recombination (using Flpe mice), the neomycin-resistance gene cassette was eliminated. Reduction from 3 loxP to 2 loxP was confirmed using PCR. Elimination of exon was also confirmed by crossing the 3 loxP mouse with EIIa-CRE mice and obtaining a 1 loxP mouse.

### 4.2. Viruses

All AAV viruses were produced by the Gene Therapy Center Vector Core at the University of North Carolina at Chapel Hill and had titres of > $10^{12}$ genome copies per ml. Mice were injected with AAV-CMW-Cre-GFP or AAV-CMW-GFP as a control.

### 4.3. Drugs

5-Carboxamidotryptamine maleate (5-CT), a 5-$HT_1$ agonist, was kept in stock solution in 10 mM at −20°C. The day of use it was diluted in (aCSF) to a working solution of 10 μM. SB

269970, a potent and selective 5-HT7 antagonist, was first dissolved in DMSO at 100 mM and stored at −20°C, then diluted in aCSF to a working concentration of 1 μM.

### 4.4. Stereotaxic surgery

All surgeries were conducted using aseptic technique. Adult *htr1AR*$^{fl/fl}$ mice were used in this study. Before the surgery, mice were deeply anesthetized with isoflurane and then placed into a stereotactic frame (Kopf instruments). An incision was made down the midline of the scalp and a craniotomy was performed above the target region. Mice were injected with 400 nl of AAV-CMW-Cre-GFP or AAV-CMW-GFP into the BNST (± 1.00, 0.30, − 4.35 in mm, midline, Bregma, dorsal surface). Red IX retrograde tracer beads (Lumafluor) were microinjected into the VTA (− 0.30, − 2.90, − 4.60) to fluorescently label VTA-projecting BNST neurons during *ex vivo* slice electrophysiology recordings. Viruses and fluorescent tracers were microinjected using a Neuros Hamilton syringe at a rate of 100 nl min$^{-1}$. Following surgery, all mice were returned to group housing. Mice were allowed to recover for at least 3 weeks before being used for behavioral studies. After all experiments, each mouse was perfused and validated for injection efficiency and viral expression.

### 4.5. Behavioral assays

All behavioral assays were conducted in accordance with previously published procedures from using conditions that were demonstrated to detect differences in anxiety-like and affective behaviors[9,43,50,52]. The order in which the tests were conducted was as follows: 1) EPM, 2) open field, 3) NSF, 5) forced swim, 6) fear conditioning. Animals were given at least 48 hours to recover after the EPM and open field tests and at least 7 days after the NSF and forced swim tests before further behavioral testing was conducted. A subset of animals was tested in the following order: 1) EPM, 2) looming disc, 3) restraint plus EZM and 4) fear conditioning.

**Elevated plus maze.—**This test was conducted according to previously published results which were found to detect anxiogenic effects of fluoxetine in mice[9]. Mice were placed in the center of an elevated plus maze, which consisted of one open arm (35 cm × 5 cm) crossed at a right angle by an enclosed arms (35 cm × 5 cm) with walls that are 15 cm in height. The entire arena is raised at a height of 60 cm from the floor. The mice were allowed to freely explore during a 5 min session. Light levels in the open arms were at ~ 25 lux. Time spent in the open arm, latency to the open arm, open arm probability, and total distance moved were measured.

**Open field (Low and High-Light).—**Mice were placed into the corner of a white Plexiglas open field arena (50 × 50 × 50 cm) and allowed to freely explore for 30 min. Time spent in the center, latency to the center, the number of center entries, and the total distance moved were measured. The center of the open field was defined as the central 25% of the arena. The open field test took place either under high light conditions (300 lux) or low light (25 lux) conditions.

**Novelty-suppressed feeding.—**Forty-eight hours before testing, mice were provided with access to a single piece of Froot Loops cereal (Kellogg's) in their home cage. Twenty-

four hours before testing, home cage chow was removed. Mice were weighed both before food deprivation and before testing to assess body weight loss. Water remained available *ad libitum*. Beginning at least one hour before testing, mice were transferred to a new clean cage so that they were singly housed for the testing session. During the testing session mice were placed into a brightly lit arena (50 × 50 × 50 cm) that contained a single Froot Loop on top of a piece of circular filter paper. Mice were monitored by a live observer and the latency for the mouse to begin eating the pellet was measured. Following the initiation of feeding, mice were removed from the arena and placed back into their home cages. Mice were then provided with 10 min of access to a pre-weighed amount of Froot Loops for a post-test feeding session. After this 10 min post-test, the remaining Froot Loops were weighed, and mice were returned to *ad libitum* home cage chow. Mice were returned to group housing at the end of this session.

**Restraint + Elevated zero maze.—**Mice were placed inside of a 50 ml conical tube with air holes throughout the body of the tube which was taped down to a lab bench for 30 minutes. Immediately afterwards, they were placed in the elevated zero maze (EZM) for behavioral testing. The EZM has a diameter of 60 cm with a 5 cm wide circular corridor that has 16 cm high walls on opposite sides. Time spent in the open areas of the maze is taken as an inverse measure of anxiety-like behavior.

**Forced swim test.—**Forced swim procedures were carried out as described previously[43]. Briefly, mice were placed in a clear, plexiglass container that was filled with 24°C to a height of 15 cm for 6 min. Immobility during the last 4 minutes was hand-scored using Ethovision XT 10 and used as a measure of behavioral despair. Animals exhibiting behavioral despair will spend a higher percentage of their time in an immobile state[62].

**Contextual Fear conditioning.—**A two-day protocol was used to assess contextual fear recall. On the first day, mice were placed into a fear conditioning chamber (Med Associates) containing a grid floor. Prior to the session, the chamber was cleaned with a scented paper towel (19.5% ethanol, 79.5% H2O, 1% vanilla). After a 3 min baseline period during which mice were allowed to explore freely, mice were exposed to a 30 s tone (3 kHz, 80 dB) that co-terminated with a 2 s scrambled foot shock (0.6 mA). A total of 5 tone-shock pairings were delivered with a random inter-tone interval (ITI) of 60–120 s. Following delivery of the last foot shock, mice remained in the conditioning chamber for a 2-min consolidation period. The following day, mice were returned to the same training chamber for 10 min and allowed to freely explore. Freezing behavior was scored at every 5 second interval and averaged for the first 3 minutes. For each session, cameras were mounted overhead for recording freezing behavior, which was later hand-scored every 5s by a trained observer blinded to experimental treatment. Freezing was defined as a lack of movement except as required for respiration.

**Looming disc.—**The looming disc assay was performed as described previously.[50,52] Mice were placed inside of an open top plexiglass box with white matte flooring (40 × 40 × 30 cm) and a dark shelter nest in far left corner. The nest was in the shape of a triangular prism (20 × 12 cm). An LED monitor with a gray screen was placed on top of the arena,

which would display an expanding black disc when triggered. The floor and three walls were covered with a matte coating to prevent reflections of the stimulus. Infrared LEDs were placed around the outer edges of the area for video recording. *Htr1a*$^{fl/fl}$ mice aged 8–12 weeks were group-housed and maintained on a regular light/dark cycle. On the day of looming disc test, each mouse was allowed to freely explore the looming box for 10 min. At the end of the habituation phase, an experimenter would trigger the looming disc when the mouse was at the center of the arena. Flight latency (ms) and velocity to escape into the nest were measured by trained observer blinded to the experimental conditions.

### 4.6. Whole-cell patch clamp electrophysiology

Mice were decapitated under isoflurane anesthesia and brains were rapidly removed and placed in an ice-cold solution of sucrose-artificial cerebrospinal fluid (aCSF) [(in mM): 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 $NaH_2PO_4$, 10 glucose, and 26 $NaHCO_3$ saturated with 95% O 2/5% $CO_2$. Brains were sectioned at 0.07 (mm/s) on a Leica 1200S vibratome to obtain 300 μm coronal slices of the BNST, which were incubated in a heated holding chamber containing normal, oxygenated aCSF [(in mM):124 NaCl, 4.4 KCl, 2 $CaCl_2$,1.2 $MgSO_4$,1 $NaH_2PO_4$,10.0 glucose, and 26.0 $NaHCO_3$] maintained at 30 ± 1°C for at least 1 h before recording. Slices were transferred to a recording chamber (Warner Instruments) submerged in normal, oxygenated aCSF maintained at 28–30°C delivered at a flow rate of 2 ml/min. Neurons of the BNST were visualized using infrared-differential interference contrast (DIC) video-enhanced microscopy (Olympus). Slices were bathed in normal aCSF for at least 30 min before performing whole-cell patch clamp experiments. Borosilicate electrodes pulled with a Flaming-Brown micropipette puller (Sutter Instruments) with a pipette resistance between 3 and 6 MΩ filled with internal solution [(in mM): 135 KCl-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 ATP, and 0.4 GTP, pH 7.35, 290 mOsmol] were used to patch cells in the BNST. Signals were acquired using a Multiclamp 700B amplifier and analyzed with Clampfit 10.3 software (Molecular Devices, Sunnyvale, CA, USA). All experiments were conducted in current clamp mode with the holding current set to zero. For excitability experiments, a current ramp protocol was used to determine the current required to induce firing (rheobase) and the voltage at first spike. The number of spikes generated by discrete current steps was also determined using a 10 pA current step protocol from 0 to 150 pA (VeI plot). In a subset of experiments, the 5HT1a-R agonist 5-CT was bath applied for 10 min before rheobase and VI plots were performed. The 5-HT7 antagonist SB267970 was included in the bath to isolate the contribution of 5-$HT_{1A}$ receptors.

### 4.7. Double Fluorescence In situ hybridization (FISH)

Mice were anesthetized using isoflurane, rapidly decapitated, and brains were rapidly extracted. Immediately after removal, the brains were placed on a square of aluminum foil on dry ice to freeze. Brains were then placed in a − 80°C freezer for no more than 1 week before slicing. Slices of the BNST (12 μm) were made on a Leica CM3050S cryostat (Germany) and placed directly on coverslips. FISH was performed using the Affymetrix ViewRNA 2-Plex Tissue Assay Kit with custom probes for 5-$HT_{1A}$ and Cre designed by Affymetrix (Santa Clara, CA). Slides were coverslipped with Southern Biotech DAPI Fluoromount-G (Birmingham, AL). 3 × 5 tiled *z* stack (15 optical sections comprising 14 μ

m total) images of the entire 12 μ m slice were obtained on a Zeiss 780 confocal microscope for assessment of 5-$HT_{1A}$ and Cre expression. All images were preprocessed with stitching and maximum intensity projection. An image of the BNST from 3 mice in each condition was hand counted for each study using the cell counter plugin in FIJI (ImageJ).

### 4.8 Immunohistochemistry and microscopy

All mice used for behavioral and anatomical tracing experiments were stained for GFP to verify that viral spread of AAV-Cre-GFP was confined to the BNST. Mice were anesthetized with Avertin and transcardially perfused with 30 ml of 0.01 M PBS followed by 30 ml of 4% paraformaldehyde (PFA) in PBS. Brains were extracted and stored in 4% PFA for 24 h at 4 °C before being rinsed twice with and stored in PBS. 45 μm slices were obtained on a Leica VT100S and stored in 50/50 PBS/Glycerol at 4 °C. For the immunohistochemistry, slices were first washed in PBS, then incubated in 0.5% Triton/PBS. Slices were washed in PBS again followed by a 1 hour blocking step in 1% BSA, 10% Normal Donkey Serum in 0.1% Triton-X-100 followed by a 24 h incubation at 4 °C in chicken anti-GFP (1:500; Santa-Cruz) in blocking buffer. The following day, slices were washed in PBS and incubated in Alexafluor 488 Donkey anti-chicken (1:500; Jackson ImmunoResearch), then washed again in PBS. Slices were then mounted on glass slides, coverslipped with Vectashield with Dapi, and imaged using a Zeiss AXIO Zoom V16 microscope with ZEN pro 2012 software.

### 4.9 Western blots

Twenty male and twenty female C57BL6/J were euthanized with isoflurane and immediately decapitated. The brain was extracted and sliced into 1mm sections. Punches from four mice were taken from the BNST and pooled in a tube on dry ice, then stored at −80°C. Tissue was lysed using approximately 20ul of RIPA lysis buffer (150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl pH 8.0) per milligram of tissue that included a 1:100 dilution of a protease inhibitor (Sigma Aldrich P8340) and a phosphatase inhibitor (Sigma Aldrich P5726). After adding the lysis buffer, the samples were sonicated and incubated on ice for 30 min. Protein concentrations were determined using the BioRad DC protein assay kit (Cat #500–0116).4X Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue) was heated and added to each sample. Precision Plus Protein Dual Color Standard (Bio-Rad #1610374) and 20ug of each sample were added to 12% Bio-Rad Mini-PROTEAN TGX Stain-Free gels (Cat #456–8046) and ran in a vertical electrophoresis chamber. The gels were transferred onto low fluorescent PVDF membranes (Bio-Rad #162–0260) using the Bio-Rad Transblot Turbo semidry transfer system. After transfer, the membranes were blocked with 5% non-fat dry milk in TBST for 1 hr at room temperature with agitation. After 5 × 5 minute washes with TBST, the membranes were incubated in 5% BSA/TBST with 1:500 of N-Cadherin produced in rabbit (GeneTex GTX127345) and 1:500 of HTR1A produced in rabbit (ThermoFisher Scientific PA5–28090) for 48 hours at 4°C with agitation. The membranes were washed 5 × 5 min with TBST then incubated in 5% milk/TBST with 1:1000 IRDye 800CW donkey anti-rabbit (LI-COR 925–32213) for 2 hrs at room temperature with agitation. The membranes were washed 5 more times with TBST then imaged on the Azure BioSystems Sapphire Biomolecular Imager. Band quantification was done using AzureSpot 2.0 analysis software.

### 4.10. Data and Statistical Analysis.

Data are presented as mean ± SEM. A two-way analysis of variance (ANOVA) or a Student's t-test was used to analyze behavioral and immunohistochemical data. Some of the sample groups were too small to detect normality (< 8 samples) but parametric tests were used because nonparametric tests lack sufficient power to detect differences in small samples (Graphpad Statistics Guide – http://www.graphpad.com). The standard error of the mean is indicated by error bars for each group of data. Differences were considered significant at $P$ values below 0.05. All data were analyzed with GraphPad Prism software.

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## Abbreviations:

| | |
|---|---|
| **BNST** | Bed nucleus of the stria terminalis |
| **DRN** | dorsal raphe nucleus |
| **EPM** | elevated plus maz |
| **EZM** | elevated zero maze |
| **OF** | open field |
| **NSF** | novelty-suppressed feeding test |

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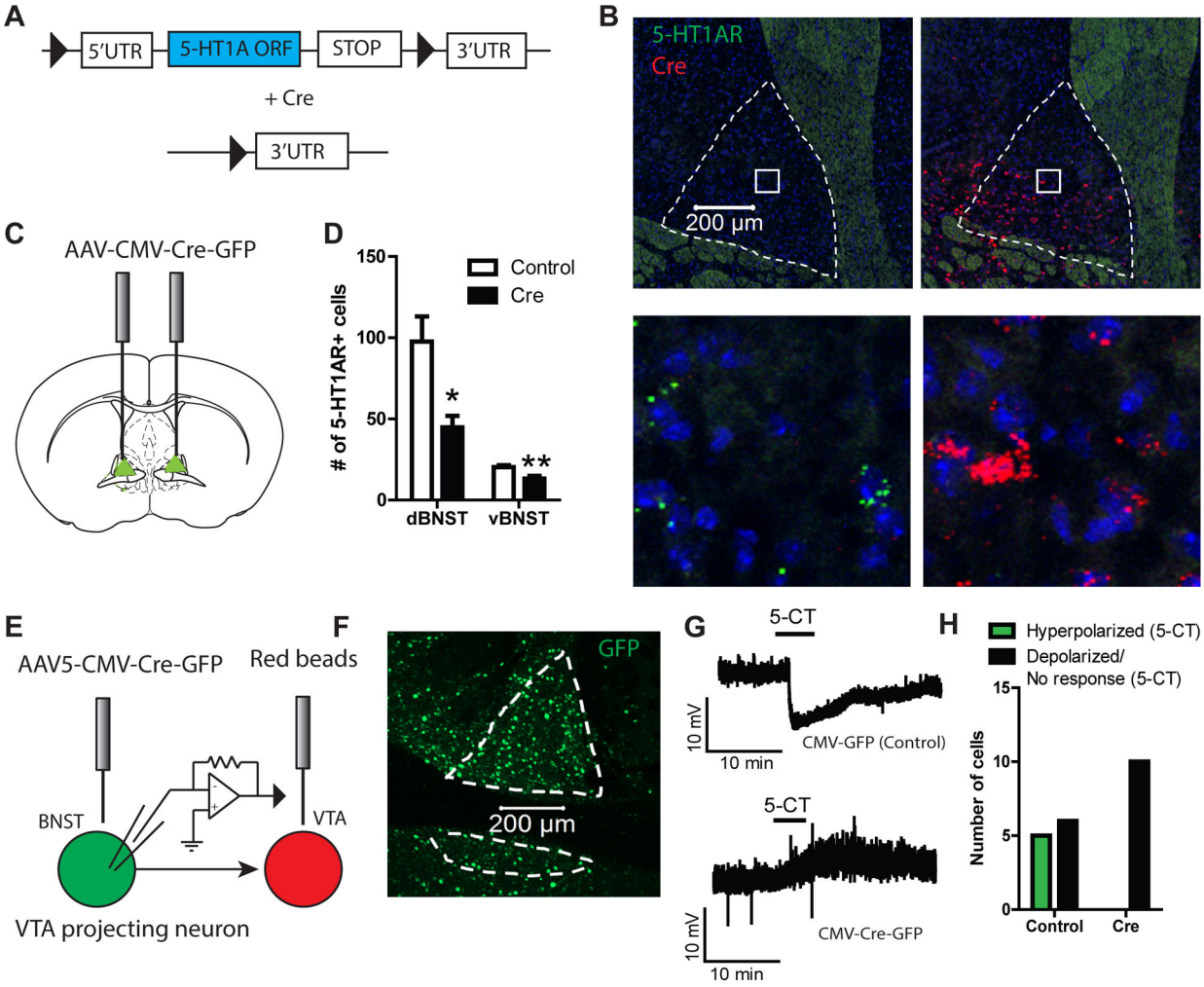


**Figure 1: Generation and validation of *htr1a*$^{fl/fl}$ mice.**

(A) Schematic of genetic targeting strategy. (B). Confocal images from *htr1a*$^{fl/fl}$::Cre$^{BNST}$ mice using fluorescence *in situ* hybridization against the 5-HT$_{1A}$ receptor and Cre showing genetic ablation of 5-HT$_{1A}$ receptor. (C) Experimental configuration for microinjection of AAV-CMV-Cre-GFP into the BNST. (D) Histogram showing reduction in 5-HT$_{1A}$ expression in the BNST of *htr1a*$^{fl/fl}$::Cre$^{BNST}$ mice (n=6 Control, n=6 Cre). (E) Experimental configuration of electrophysiological recordings in the BNST from VTA projecting neurons. (F) Representative confocal image of GFP stained BNST tissue from a *htr1a*$^{fl/fl}$::Cre$^{BNST}$ mouse. (G) Representative traces showing the effect of 5-CT on resting membrane potential in VTA-projecting BNST neurons in brain slices from *htr1a*$^{fl/fl}$::Cre$^{BNST}$ and *htr1a*$^{fl/fl}$::GFP$^{BNST}$ mice. (H) Histograms depicting the number of cells (n=11 Control, n=10 Cre) that responded to 5-HT with a depolarization (>2 mV), hyperpolarization (<−2 mV) or no response.

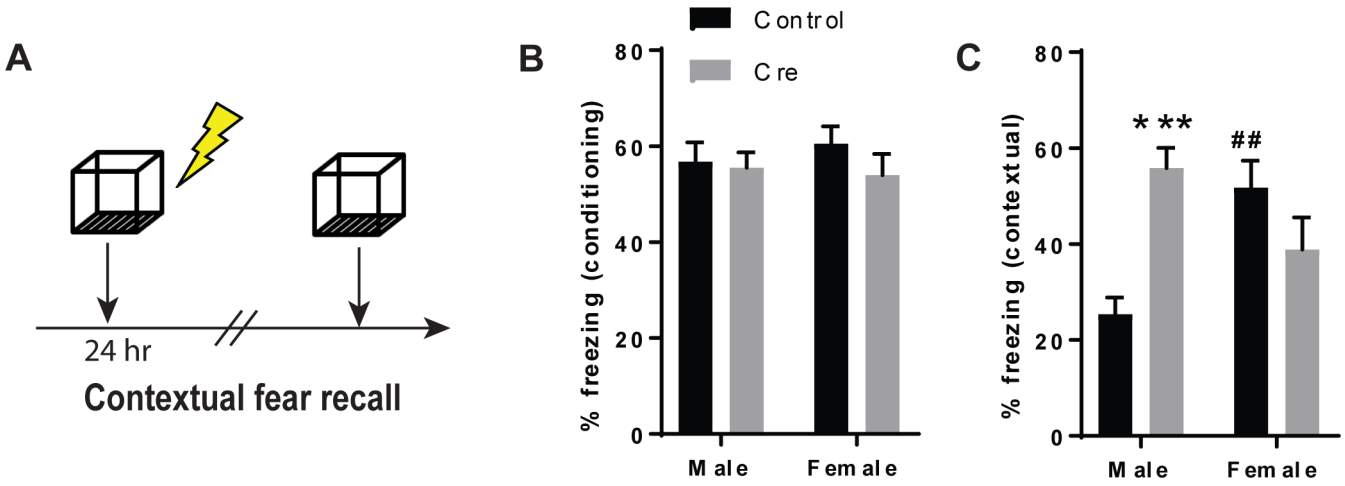


**Figure 2: 5-HT$_{1A}$ receptor knockdown in the BNST enhances contextual fear memory in a sex-dependent manner.**

(A) Schematic of the experimental design for contextual fear conditioning.(B) Histogram depicting percent time freezing during fear conditioning and (C) contextual fear recall. N=9 Control males, N=9 Cre males, N=9 Control females and N=10 Cre females. *** denotes $p<0.001$ for Cre males relative to Control males and ## denotes $p<0.01$ for Control females relative to Control males.

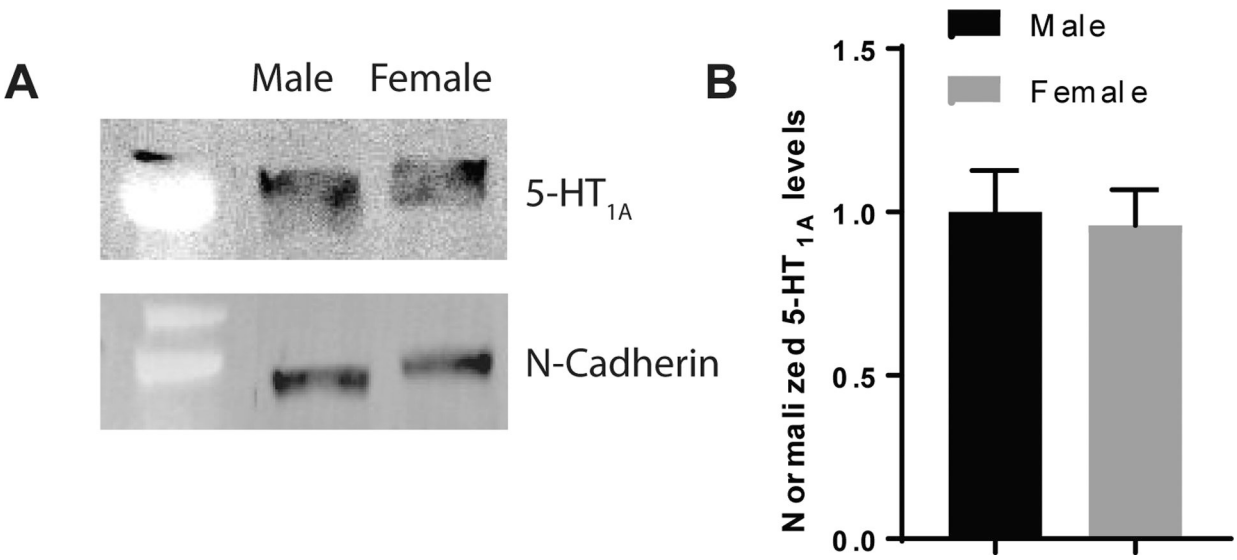


**Figure 3: 5-HT$_{1A}$ receptor protein levels in the BNST do not significantly differ between males and females.**

(A) 5-HT1A and N-Cadherin immunoblots from male and female BNST lysates. (B) Histograms showing normalized 5-HT$_{1A}$ protein levels in male and female mice indicating that 5-HT$_{1A}$ receptor levels in the BNST do not significantly differ by sex. N=5 Male and N=5 female lysate samples (each lysate sample was pooled from 4 mice).

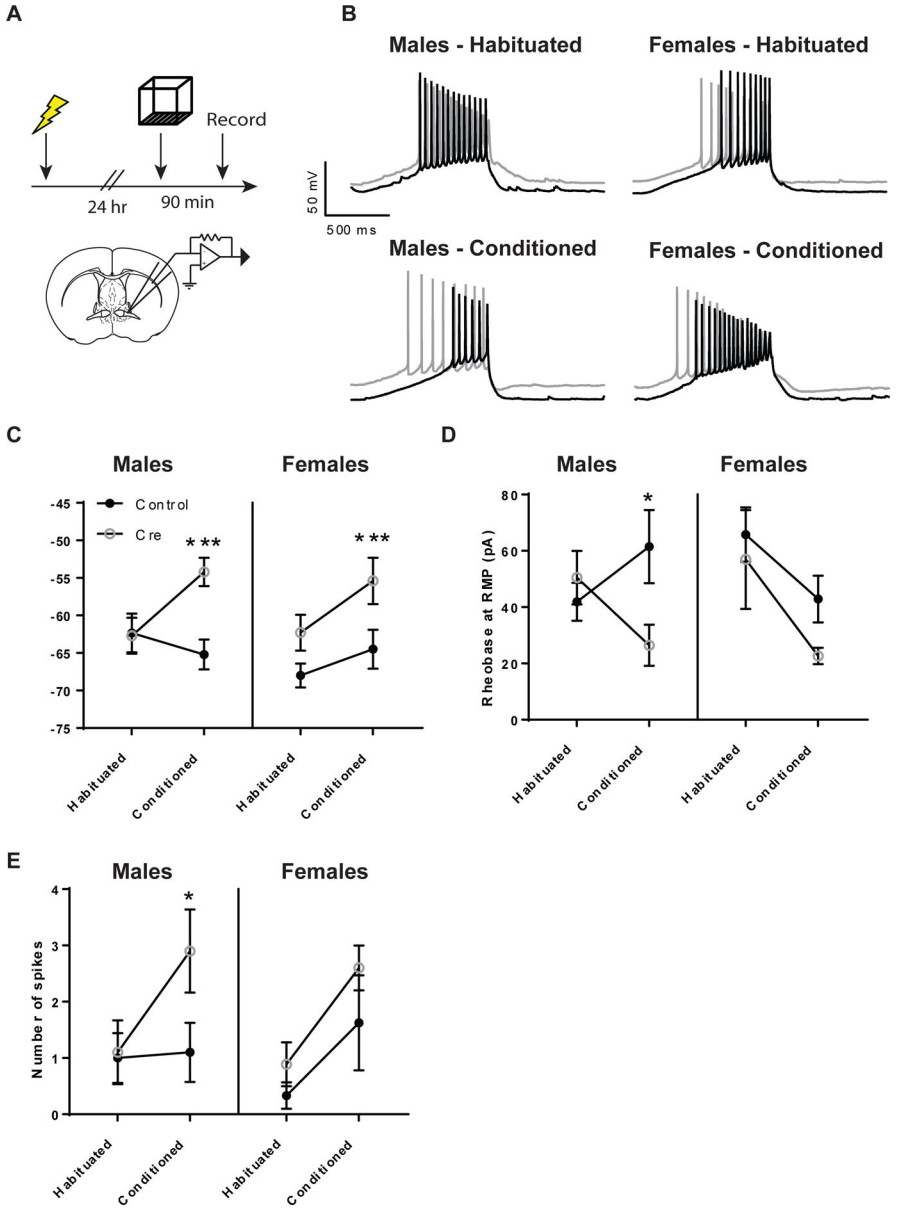


**Figure 4: 5-HT$_{1A}$ receptor knockdown in the BNST enhances neuronal excitability in male mice after contextual fear recall.**

(A) Experimental configuration for contextual fear memory and BNST recordings. (B) Representative traces of rheobase recordings in the BNST (C) Histogram of resting membrane potential (D) Rheobase and (E) Current-induced spiking from habituated and conditioned mice. N= 9 cells from Habituated Control Males, N=10 cells from Habituated Cre Males, N=10 cells from Conditioned Control males, N=10 cells from Conditioned Cre Males, N=9 cells from Habituated Control females, N=10 cells from Habituated Cre females, N=8 cells from Conditioned Control females, N=5 cells from Conditioned Cre females. (*) denotes $p<0.05$ and (***) denotes $p<0.001$.

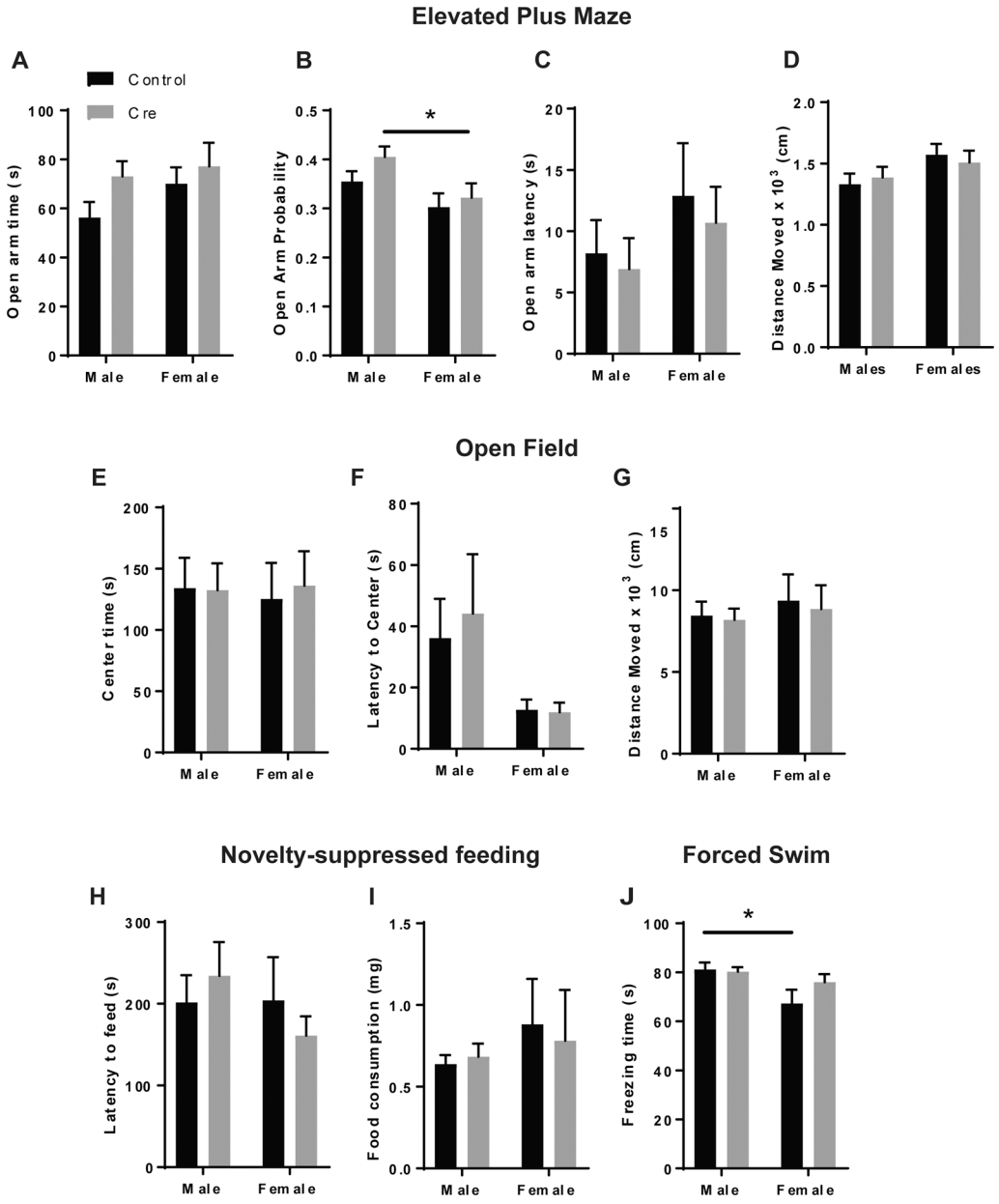


**Figure 5: 5-HT$_{1A}$ receptor knockdown in the BNST does not significantly alter anxiety-like or depressive-like behavior behavior in male and female mice.**

(A) Open arm time, (B) Probability, (C) Latency and (D) Distance moved in the EPM , (F) Latency to center and (G) Distance moved in the open field test. (H) Latency to feed and (I) post-test food consumption in the novelty-suppressed feeding test. (J) Percent immobility time in the forced swim test. For EPM: N=23 control males, N=21 Cre males, N=20 Control females, N=18 Cre females. For open field and novelty-suppressed feeding: N=16 Control males, N=16 Cre males, N=11 Control females, N=9 Cre females. For forced swim: N=10 Control males, N=12 Cre males, N=6 Control females and N=8 Cre females. (*) denotes $p<0.05$

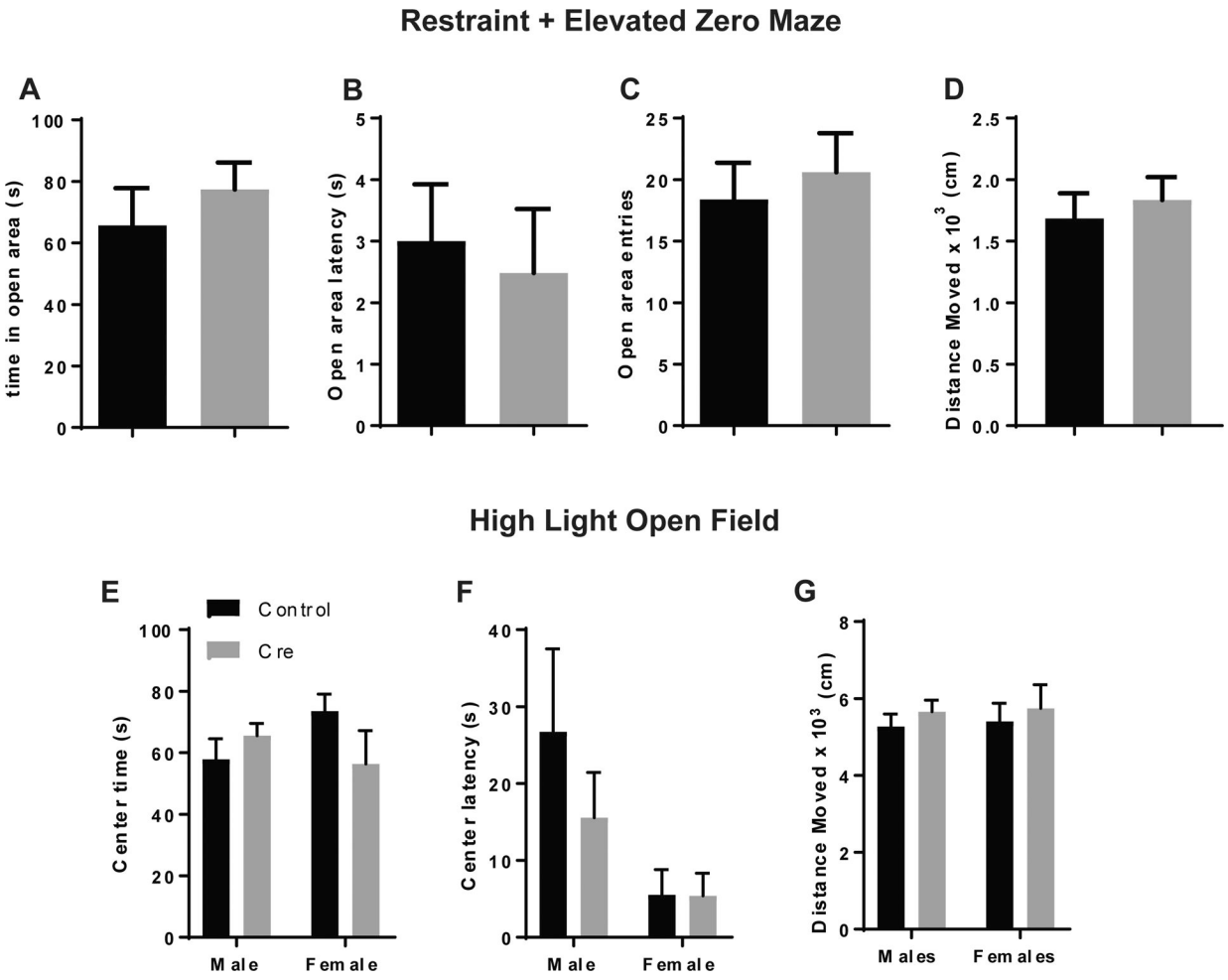


**Figure 6: 5-HT$_{1A}$ receptor knockdown in the BNST does not alter anxiety-behavior in the the EZM after restraint stress or the open field in high light conditions.**

(A) Open area time, (B) Latency, (C) Entries and (D) Distance moved in the EZM after restraint stress. (E) Center time, (F) Center latency and (G) Distance moved in the high light (300 lux) open field. For restraint + EZM: N=5 Control males and N=5 Cre males. For high light open field: N=16 Control males, N=15 Cre males, N=8 Control females, N=7 Cre females.

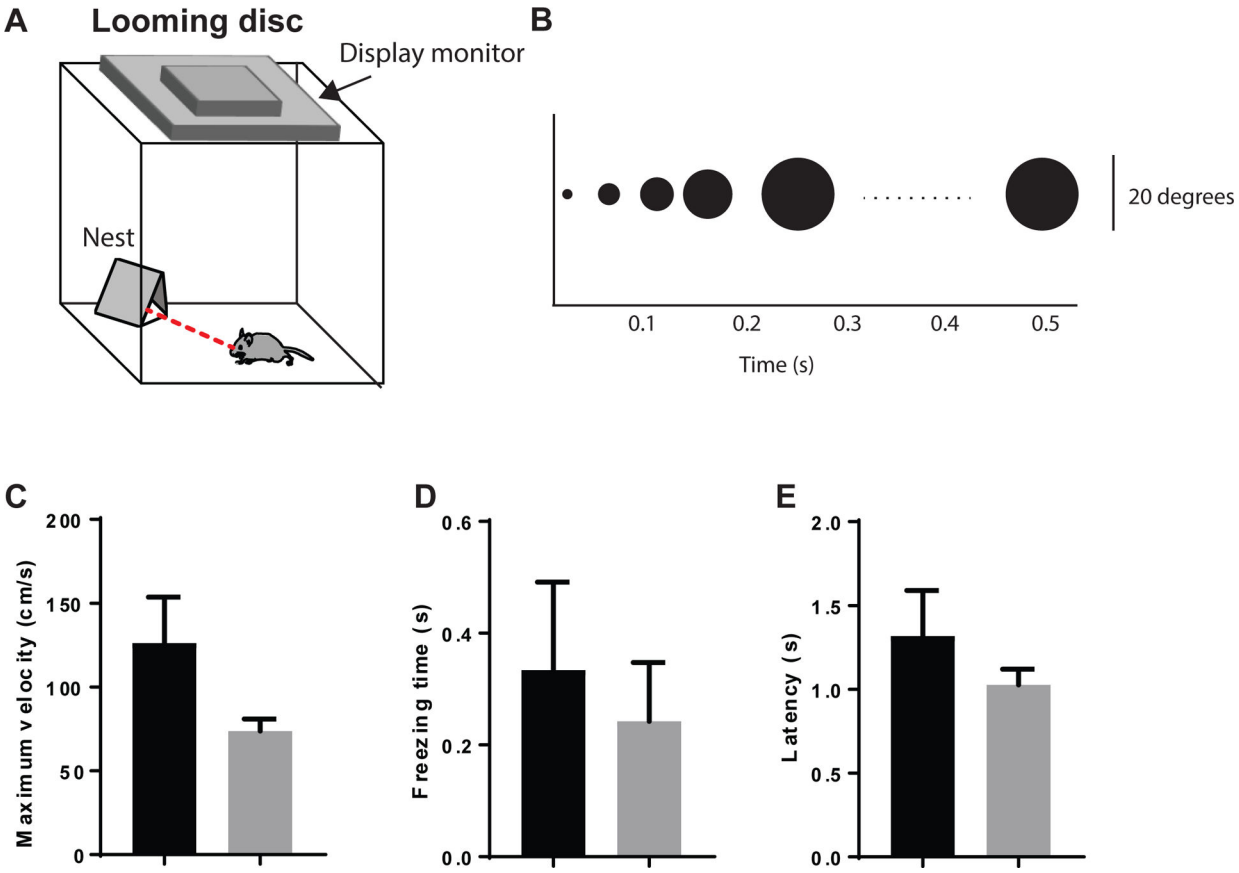


**Figure 7: 5-HT$_{1A}$ receptor knockdown in the BNST does not alter visual threat processing in the looming disc assay.**

(A) Pictorial representation of the looming disc assay. (B) Time course of expanding disc on the display monitor above the looming disc arena. (C) Histogram of maximum velocity, (D) Freezing time, and (E) Escape latency after the looming disc was triggered. N=13 Control males and N=12 Cre males.