Sex differences in anxiety and fear learning behavior following genetic manipulation of the 5-HT_{1A} receptor in the bed nucleus of the stria terminalis (BNST)

By

Cayce Dorrier

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Chemistry Department

University of North Carolina at Chapel Hill

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Approved:

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Thomas Kash, Thesis Advisor
Brian Hogan, Reader
Kevin Weeks, Reader

Abstract

The *bed nucleus of the stria terminalis (*BNST) is a critical node in the fear and anxiety circuitry of the brain that plays a key role in an organism's ability to respond to stress. Serotonin 1A (5-HT_{1A}) receptor signaling in the BNST has been implicated in anxiety and fear-related behavior using acute pharmacological manipulations that target pre- and post-synaptic receptors (Levita et al., 2004; Gomes et al., 2012). The goal of this project is to elucidate the role of post-synaptic 5-HT_{1A} receptors within the BNST in anxiety, fear, and depressive-like behavior using a genetic deletion approach that selectively targets postsynaptic 5-HT_{1A} receptors. Viral vectors containing the gene for Cre recombinase and a fluorescent tag (AAV5-Cre-GFP) or a control vector (AAV5-GFP) were infused into the BNST of genetically modified male and female 5-HT_{1A} receptor flox mice. One month later, behavior on the elevated plus maze (EPM), open field, forced swim, novelty-suppressed feeding and fear conditioning assays were assessed. The results of this project indicate that the genetic deletion of 5-HT_{1A}Rs in the BNST has an anxiolytic-like effect in the EPM but enhances fear consolidation and contextual fear following a tone-shock protocol in male mice. These seemingly discrepant results may reflect the fact that under low stress conditions (e.g. the EPM), deletion of the 5-HT_{1A} receptor disinhibits BNST output neurons which are anxiolytic (Kim et al., 2013; Jennings et al., 2013). In high stress (e.g. footshock) conditions, local neurons are recruited which are both anxiogenic and fear-enhancing (Levita et al, 2004). Deletion of 5-HT_{1A} receptors from these neurons potentiates their fear enhancing actions, resulting in an increase in fear consolidation and contextual fear. The behavioral phenotypes of the female mice following this genetic manipulation were less pronounced, which could be due to the anatomical differences of the BNST between male and female mice.

Introduction

Anxiety disorders afflict an estimated 18% of adults in the United States and pose a significant public health burden, costing more than $42 billion annually. The bed nucleus of the stria terminalis (BNST) is a critical relay station in the brain that has been implicated in anxiety-like behaviors. As shown in Figure 1, it projects to and receives projections from the amygdala, hippocampus, ventral tegmental area, dorsal raphe, and periaqueductal gray. Of particular interest to this project is the serotonin (5-hydroxytryptamine; 5-HT) projection of serotonin from the dorsal raphe (DR) to the BNST.

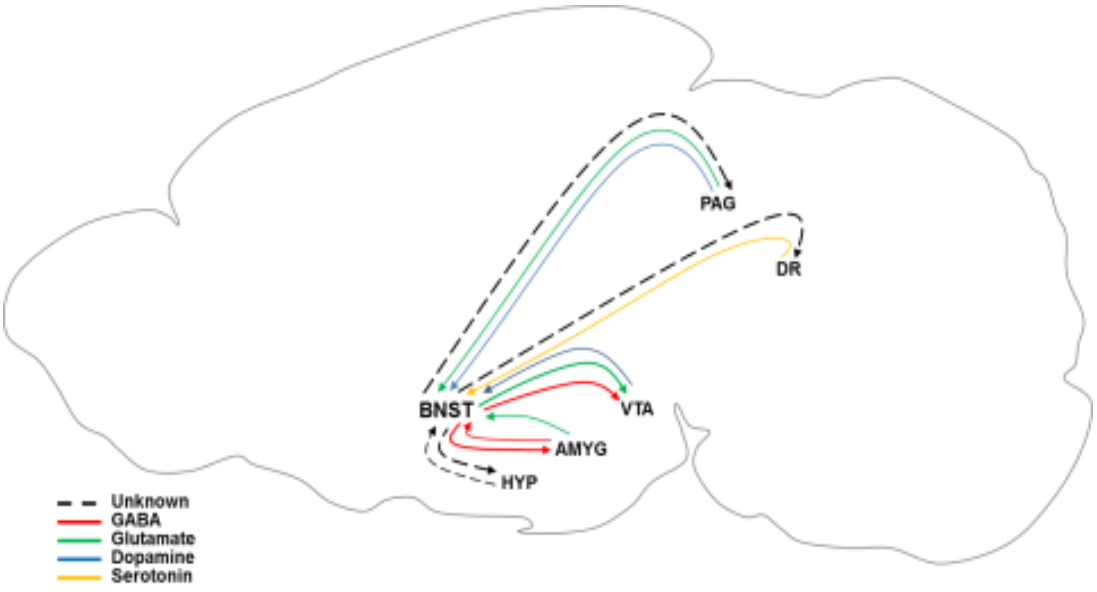


Figure 1. The neuronal inputs and outputs of the BNST. The BNST projects to and receives projections from many other brain regions, acting as a relay station in the brain. Image from Emily Lowery-Gionta, PhD.

Electrical stimulation of the BNST causes behavioral responses similar to that of stressful stimuli (Casada and Dafny, 1991). The goal of the current study is to elucidate the role of 5-HT_{1A} receptor signaling in the BNST in these aversive behavioral responses using a targeted genetic deletion approach.

Many anti-anxiety drugs work by preventing the reuptake of the neurotransmitter 5-HT. The 5-HT receptors are classified into seven families based on sequence homology, pharmacological characteristics and effector coupling (5-HT_{1-7}) and these can further subdivided into subtypes (5-HT_{1A}, 5-HT_{1B}, etc.). Here we will focus on the 5-HT_{1A} receptor subtype, a Gi/o-coupled GPCR that inhibits adenylyl cyclase activity and closes G protein-coupled inwardly-rectifying potassium channels (GIRKs). For this reason, activation of 5-HT_{1A}Rs has a hyperpolarizing effect on cells and is generally inhibitory. It has been implicated as the primary subtype of serotonin responsible for the inhibitory action of serotonin in the BNST (Guo et al., 2009), and activation of the 5-HT_{1A} receptor in the BNST using a 5-HT_{1A}R agonist has been shown to have anxiolytic effects in male rats (Levita et al., 2004).

Additionally, activation of 5-HT_{1A} receptors in the BNST decreased contextual fear learning (Gomes el al., 2012). Together, these studies indicate that 5-HT_{1A} receptor signaling in the BNST attenuates aversive behaviors. In agreement with these acute pharmacological studies, global deletion of the 5-HT_{1A}R using a genetic knock-out approach has been shown to increase anxiety-like and depressive behaviors in both male and female mice (Ramboz et al., 1998; Parks et al., 1998).

5-HT_{1A} also plays a role in many other behavioral phenotypes such as feeding and motor control. Fluoxetine, a selective serotonin reuptake inhibitor, has been shown to suppress food intake in animal models of feeding, and the combination of fluoxetine and a 5-HT_{1A}R antagonist

intensifies this effect (Dominic et al., 1998). A 5-HT$_{1A}$R antagonist has been shown to decrease locomotor activity, although this effect was most pronounced with microinjections into the hippocampus (Belcheva et al., 1997). Conversely, a non-selective 5-HT$_{1B/A}$ agonist has been shown to increase forward locomotion (Aronsen et al., 2014). These phenotypes in addition to fear and anxiety will be studied using a BNST 5-HT$_{1A}$R knock-out model to determine if they are modulated through the BNST.

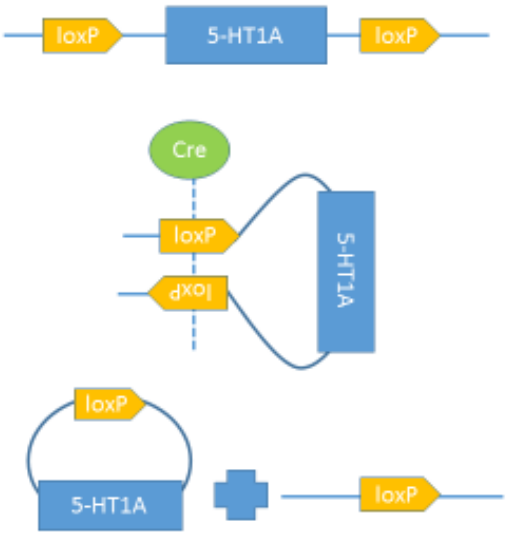


Figure 2. Schematic of the mechanism of action of the AAV5-CRE-GFP virus in the 5-HT1a flox mice.

The receptor will be deleted using genetically modified 5-HT$_{1A}$ flox mice, in which 2 *loxP* segments surround the gene for the 5-HT$_{1A}$ receptor. This 34 base pair sequence is recognized and bound by the enzyme cre recombinase. In the 5-HT$_{1A}$ flox mice, a single recombinase will bind to each *loxP* segment. The two enzymes will then come together, forming a tetramer and bringing the two *loxP* sites together in a recombination event that will excise the segment of the gene corresponding to the 5-HT$_{1A}$ receptor (Nagy et al., 2000). This recombination event essentially knocks out this receptor, as depicted in Figure 2. Injecting a virus containing the gene for cre recombinase into the BNST of these mice will insert this gene into the DNA of BNST neurons. The cell's machinery is then able to produce this enzyme in only BNST neurons, leading to the knock-out of 5-HT$_{1A}$ receptors in this specific brain region.

Behavioral testing will be used to determine if the absence of this receptor leads to an increase an anxiety- and depressive-like behaviors, feeding behaviors, and fear learning in these mice. Specifics tests include elevated plus maze (EPM), open field, forced swim, novelty-suppressed feeding and fear conditioning. The body weight of all mice was recorded after surgery and throughout the cohort of behavioral experiments to look for any evidence of changes in feeding behaviors.

All experiments were conducted on male and female mice in order to look for any sex-specific differences in behavioral outcomes associated with 5-HT_{1A} receptor deletion in the BNST. Without any drug treatment or genetic manipulation, male and female mice respond differently to many behavioral tests and paradigms. For example, female mice appear less anxious than males in the elevated plus maze, show less immobility in the forced swim test and more often fail to adapt to repeated stress procedures than male mice (Blanchard et al., 1991). In response to 5-HT_{1A}, female mice have been shown to be more sensitive to the 5-HT_{1A} agonist 8-OH-DHAP (Blanchard et al., 1991).

While both sexes have been studied in the context of a global 5-HT_{1A} receptor deletion, the studies on 5-HT_{1A} receptors in the BNST have not included female animals. Additionally, while many brain regions such as the hippocampus implicated in the global knockout lack drastic anatomical sex differences, the volume of the medial posterior region of the BNST is significantly larger in male rats than in females (del Abril et al., 1987). This difference has been correlated to a greater number of apoptotic nuclei in the principle nucleus of the BNST in female rats sacrificed on postnatal day 12 (Chung et al., 2000). This anatomical difference could lead to differences in behavior when the BNST is the only targeted brain region. It is important to study how genetic manipulations of 5-HT systems affect both males and females, especially given that

women are approximately twice as likely to be afflicted with anxiety and depression as men and are thus more likely to be prescribed drugs such as SSRIs that target 5-HT systems (Kessler et al., 1993, Kessler et al., 2005).

Based on the previous studies cited above, our hypothesis is that the deletion of the 5-HT_{1A} receptor in the BNST will increase anxiety and depressive-like behavior and fear learning in mice with the potential for sex differences in behavior.

Methods

Mice

All animals were group housed on a 12 hour light cycle with *ad libitum* access to rodent chow and water, unless described otherwise. 5-HT_{1A} flox mice were obtained from Eric Delpire, Vanderbilt University Department of Anesthesiology. Three separate cohorts of these mice were run through the experiments explained below, each containing mice receiving the virus harboring the gene for cre recombinase (AAV5-Cre-GFP, designated as cre) and a control virus (AAV5-GFP, designated as control). Both of the AAV viruses were produced by the Gene Therapy Center Vector Core at the University of North Carolina at Chapel Hill. The first cohort of mice contained 15 male mice, n=8 cre and n=7 control, the second 12 female mice, n=7 cre and n=5 control, and the third 6 male mice, n=3 cre and n=3 control. The behavioral data from the two cohorts of male mice was pooled to create an overall male total of n=11 cre and n=10 control. SERT-cre mice for the immunohistochemistry studies were obtained from Dr. Bryan Roth.

Stereotaxic Surgery

Twenty-four hours prior to surgery, 5-HT_{1A} flox mice were given a solution of acetaminophen in their drinking water (5 ml/200 ml v/v). The mice were then anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). 32 gauge needles connected to a 1 µL Hamilton syringe were used to bilaterally inject 400 nl either AAV5-Cre-GFP or AAV5-GFP into the BNST (coordinates from Bregma: ± 1.0 ML, 0.4 AP, -4.35 DV). Mice were allowed to recover for 6 weeks after surgery.

Body Weight Measurements

The body weight of each mouse was recorded approximately every other day from the day of surgery through the end of the behavioral experiments to look for changes in body weight over time.

Behavior

EPM

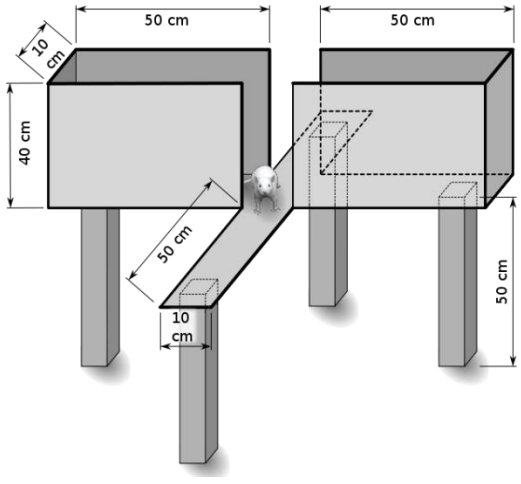


Figure 3. A standard EPM apparatus. A mouse is placed in the center of the maze and allowed to explore the arms for 5 min.

Mice were placed in the center of a standard elevated plus maze, depicted in Figure 3, facing an open arm and allowed to explore the maze for 5 min. The number of open arm entries, probability of entering the open arm, the time spent in the open arm, and the total distance traveled in the maze were scored using EthoVision XT 7. In this test, mice exhibiting anxiety-like behavior avoid the open arms (Pellow et al., 1985).

Open Field

Mice were placed into the corner of a white Plexiglas open field arena (25 x 25 x 25 cm) and were allowed to explore the arena for 30 min. The total distance travelled, time spent in the central 25% of the box, and the latency to enter the center of the box were scored using EthoVision XT 7. Avoidance of the center of the box reflects heightened anxiety (Choleris et al., 2001).

Forced Swim

Mice were placed in a clear, plexiglass container that was filled ¾ full with water at 25°C for 6 min. Immobility during the last 4 minutes were hand-scored using The Observer XT 10. This test has been used to compare the effects of various antidepressants as it puts mice in a situation where they can lose hope of escaping a stressful environment (Can et al., 2012). Mice who exhibit more behavioral despair are more likely to spend a higher percentage of their time in an immobile state (Pellow et al., 1895).

Novelty Suppressed Feeding

Mice were introduced to Froot Loops (Kellogg's) 48 hours before experimentation and food deprived for 24 hours prior to experimentation. 45 min before experimentation mice were placed into new cages without food. The open field box was modified for this experiment by adding bedding and taping colored paper to the walls of the box. A single Froot Loop was placed on a piece of filter paper in the center of the box. The mice were then placed on the box until they began to eat the Froot Loop, and their latency to feed was recorded. They were then immediately transferred to their home cage with a pre-determined weight of Froot Loops (around 1 g.) for 10 min. After this time period the final weight of the food was recorded and the mice were placed

back into their home cages. The latency to feed is a measure of anxiety, with a higher latency to feed reflective of more anxiety- like behaviors, and the food consumption post-test reflects feeding behaviors. (Samuels and Hen, 2001).

Fear Conditioning

Fear recall and extinction was assessed through a 3 day protocol, presented in Figure 4. In Day 1 the mice were placed in a hood and carried straight to the room containing a fear conditioning chamber (Med Associates) that was cleaned with a 1% vanilla/19.5 % ethanol/ 78.5% water solution. After an initial 3 min baseline period, 5 tone/shock pairings occurred in intervals ranging from 60-120 s. After another 2 min period the mice were removed from the box. In Day 2, which tested cued fear learning, the mice were placed in a cabinet and carried around a hallway to the behavior room. The mice were placed in a different fear conditioning chamber (Med Associates) that contained a striped pattern on the walls and was cleaned and scented with a 70% EtOH solution. After an initial 3 min baseline period, a tone lasting 30 s was repeated 10 times with 5 s between each tone. After another 2 min period the mice were moved from the box. Day 3 tested contextual fear recall by placing mice in the same box with the same scent as day 1 for 10 minutes without a tone or shock. Percent freezing, defined as a lack of all movement except for respiration, was used as the behavioral parameter in these experiments with a higher percent freezing indicative of an increase in fear learning (Seidl et al., 2000). Each mice was judged every 5 s as freezing or unfreezing.

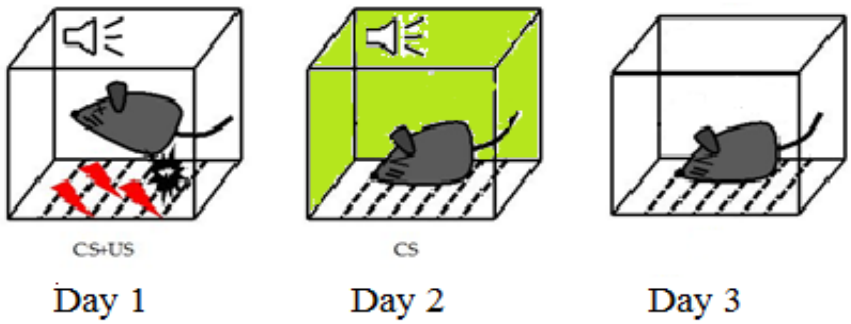


Figure 4. Fear conditioning protocol. A three-day protocol was used to test for fear learning in the mice, where CS represents the conditioned stimulus (a tone) and US represents the unconditioned stimulus (a footshock).

Immunohistochemistry

Immunohistochemistry was performed using anti-green fluorescent protein (anti-GFP) primary antibodies to label BNST cell bodies containing either the cre or the control virus. Slices 45 µm in thickness containing the BNST were collected using a Leica VT1000S vibratome (Leica Microsystems, Nussloch, Germany) and stored in a 50% glycerol solution at −20 °C until immunohistochemistry was performed. Slices were washed in PBS three times for 5 min followed by a 30 min incubation in 50% methanol, a 5 min incubation in 3% H_2O_2 in PBS, three 10 min PBS washes, a 30 min incubation in 0.5% Triton X-100 in PBS, and an additional 10 min PBS wash. The slices were then incubated for 60 min in a blocking solution of 0.1% Triton/10% Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA) in PBS and transferred to a primary solution of the blocking solution containing a 1:500 dilution of anti-Green Fluorescent Protein (Aves Labs, Tigard, Oregon) where they incubated overnight at room temperature.

Slices were then washed with PBS three times for 10 min before incubating for 2 hrs at room temperature in a secondary solution containing a 1:200 dilution of Alexa Fluor 488 Donkey anti-mouse (Jackson ImmunoResearch, West Grove, PA) in PBS. Slices were then washed in PBS

four times for 10 min, mounted on slides and allowed to air dry, and covered with Vecta-Shield Mounting Medium (Vector Laboratories, Burlingame, CA). Images of the BNST were collected on an Olympus FV1000 confocal microscope with FluoView 1000 Software using the 20x objectives and a Zeiss AXIO Zoom V16 microscope.

Immunohistochemistry was also performed using anti-GFP and anti-tryptophan hydroxylase (anti-TPH) primary antibodies in SERT-cre mice to verify the serotonin projection from the DR to the BNST. This immunohistochemistry followed the same protocol as above with the addition of a 1:500 dilution of anti-TPH (Sigma-Aldrich, St. Louis, Mo) at the primary step and a 1:200 dilution of Alexa Fluor 647 donkey anti-mouse (Jackson ImmunoResearch, West Grove, PA) at the secondary step.

Statistical Analysis

Statistical differences between cre and control mice were examined separately in males and females using a Student's t-tests with the α level set to 0.05. A two-way ANOVA was also performed with the male and female data pooled together, resulting in a data set with two independent variables. Bonferroni posttests were used to make between group comparisons. All data were analyzed with GraphPad Prism software.

Results

Anatomical verification of 5-HT projection from the DR to the BNST

Transgenic mice expressing Cre recombinase under the control of a serotonin transporter (SERT) promotor (SERT-cre) were injected with a Cre-inducible light activated Channelrhodopsin2

(ChR2)-eYFP virus into the DR. GFP immunohistochemistry was performed to amplify the YFP signal which enabled the visualization of 5-HT neurons and their axon fiber tracts (Narbox-Neme et al., 2008). The presence of axon fibers in the BNST in Figure 5(A) verifies this 5-HT DR→BNST projection.

In the DR, a GFP, tryptophan hydroxylase (TPH) double label was performed to verify that cell bodies expressing ChR2-eYFP were in fact serotonergic. TPH converts the amino acid tryptophan into 5-hydroxytryptophan, a reaction constituting the rate-limiting step in the production of serotonin, and is present only in serotonergic neurons. The overlap of green (GFP) and red (TPH) cell bodies in Figure 5(B) verify that ChR2-eYFP expression is restricted to 5-HT neurons in the DR. The presence of cell bodies containing GFP in the DR also serves to confirm that the injection of ChR2 was a hit.

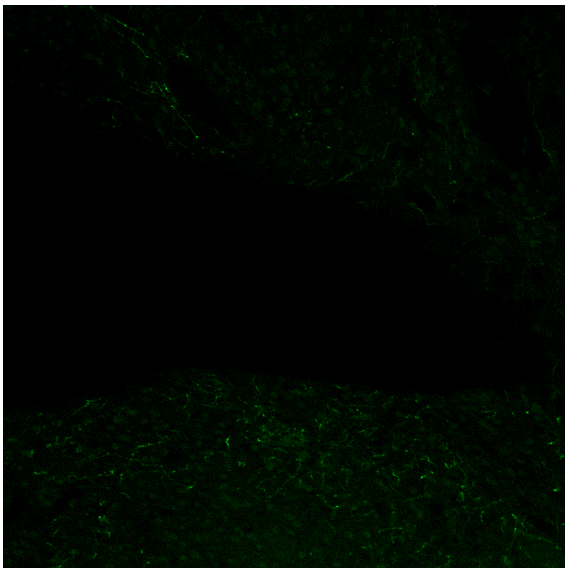
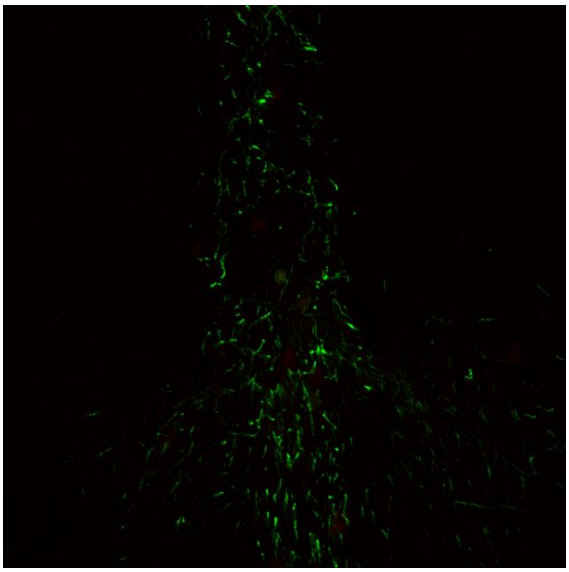
A.  B. 

Figure 5. DR to BNST serotonin projection verification. Immunohistochemistry of the BNST and DR following treatment of SERT-cre mice with ChR2 in the DR. **(A)** GFP labeling in the BNST verifies the projection of serotonergic neurons in the DR to the BNST. **(B)** GFP/TPH colabeling in the DR proves the accuracy of the virus injection and the co-localization of serotonergic neurons in the DR with TPH producing neurons. Images were collected using an Olympus FV1000 confocal microscope with FluoView 1000 Software using the 20x objectives.

Body weight

For this and all further experiments, the mice were separated into two groups, those that received the AAV5-Cre-GFP virus (cre) or those that received the AAV5-GFP virus (control). The body weight of neither the male nor female cre mice varied significantly from the control mice throughout surgery and the course of the behavioral experiments, suggesting that the 5-HT DR→BNST projection is not involved in feeding behavior. The drops in body weight of both the cre and control mice for both sexes occur during the first two weeks post-surgery and the day of the novelty-suppressed feeding test, in which the mice had been food deprived for 24 hours. The average body weight of representative cages of both male and female cre and control mice are presented in Figure 6.

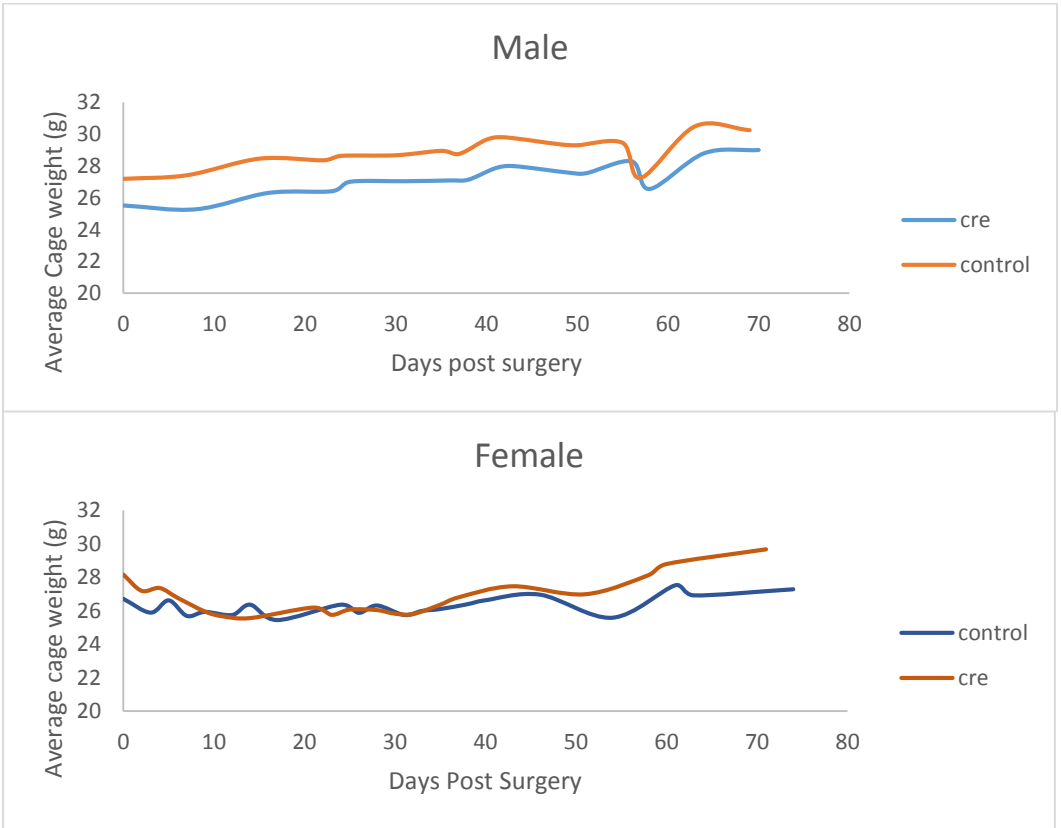


Figure 6. Body Weight. The body weights of a representative 6 male mice (n=3 cre, n=3 control) and 6 female mice (n=3 cre, n=3 control) post-surgery and throughout the behavioral experiments did not vary between the cre and the control groups.

Behavioral Experiments

In the EPM test, the cre male mice spent a significantly greater amount of time in the open arms and were more likely to enter them than the control mice, which suggests that the 5-HT_{1A}R BNST deletion led to a decrease in anxiety-like behavior. There was no significant difference between the cre and control mice in locomotor activity in the EPM for the duration of the test. The female cre mice did not exhibit any significant anxiolytic phenotypes. In another test of anxiety, the open field test, neither male nor female cre and control mice exhibited any significant differences in anxiety-like behaviors or locomotor activity. The results for both of these tests are presented in Figures 7 and 8.

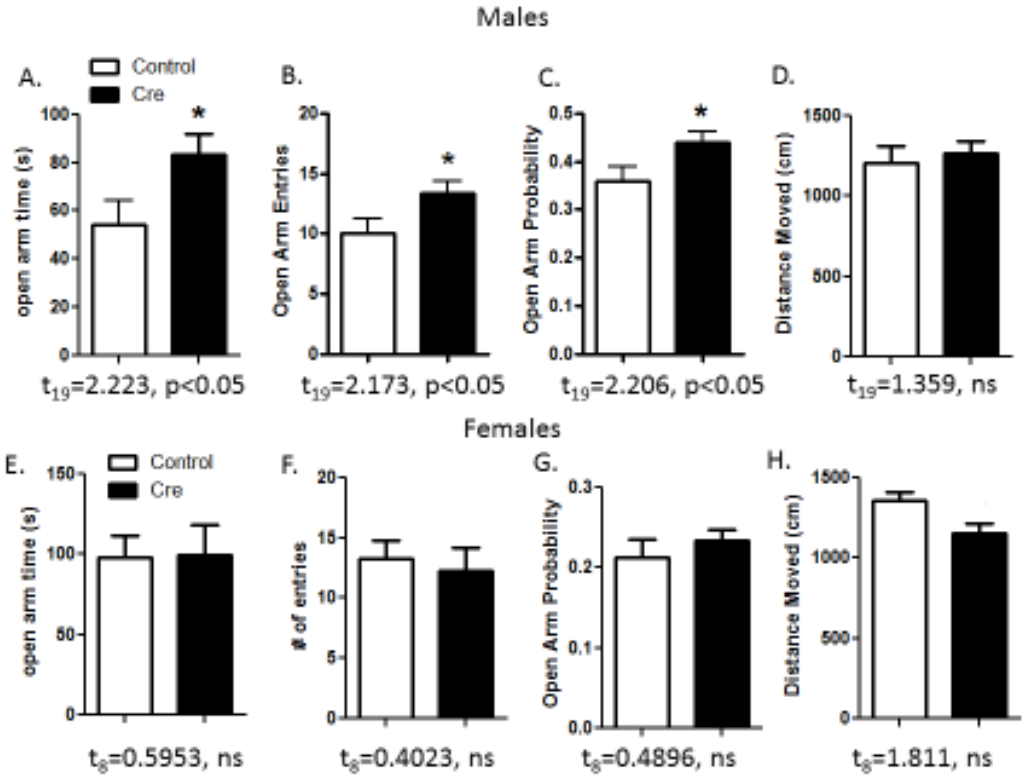


Figure 7. Elevated Plus Maze. (A, B, C) The male cre mice spent a significantly longer amount of time in the open arms, entered them a greater number of times and were more likely to enter them than control mice. **(E, F, G)** The females did not exhibit any significant differences between the cre and the control mice. **(D, H)** Neither male nor female cre and control mice showed any differences in locomotor activity.

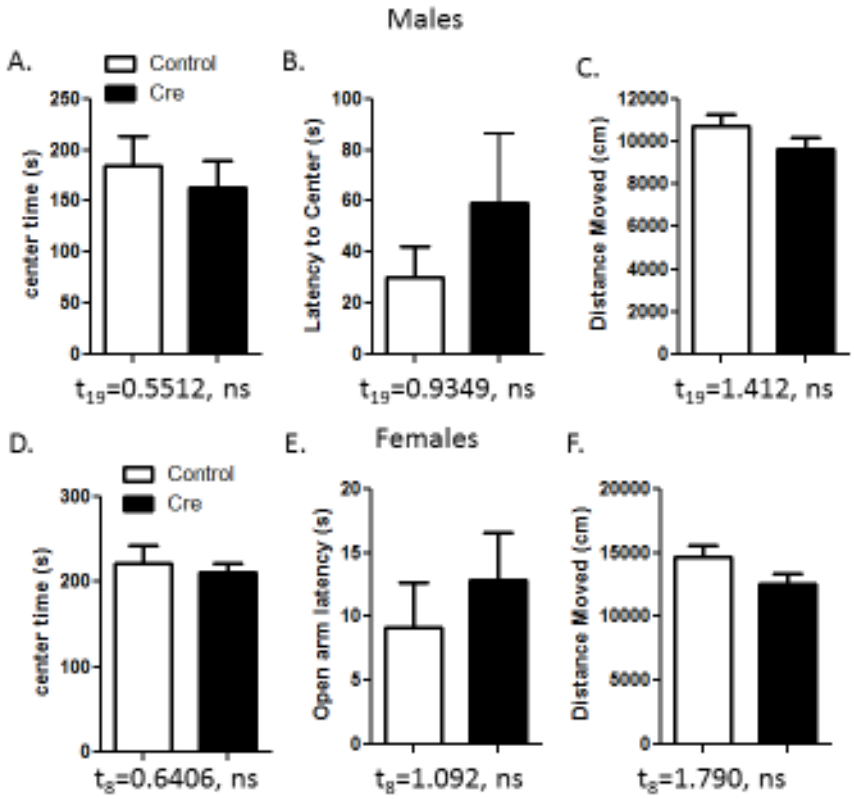


Figure 8. Open Field. (A-F) There were no significant differences between the cre and the control mice in both sexes in the amount of time spent in the center of the box, the latency to the center of the box, and the total distance moved in the box.

There were also no significant differences in food consumption or latency to feed between the cre and control mice for both males and females in the novelty-suppressed feeding test. In the forced swim test there were no significant differences between percent immobility between the cre and control mice for both males and females (Figure 9).

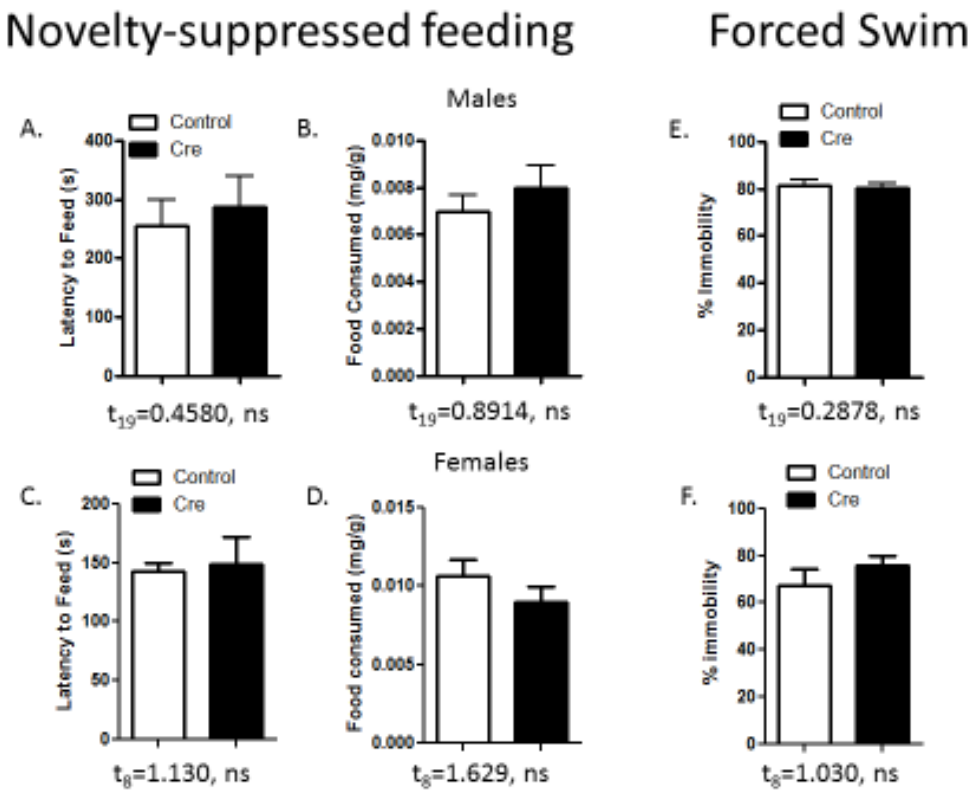


Figure 9. Novelty-suppressed feeding and forced swim. (A, B, C, D) The 5-HT_{1A}R BNST deletion did not have any effect in the latency to feed and food consumption for both males and females in the novelty-suppressed feeding test. **(E, F)** The forced swim test also failed to differentiate between the cre and control mice of both sexes.

As shown in Figure 10, there were no differences between male cre and control mice in fear learning or cued fear. Male cre mice showed a significant increase in cued fear consolidation but not contextual fear learning, although the p value was close to significant at 0.0591. The significant interaction between treatment (cre) and tone in female mice on day 1 suggests that cre females exhibited delayed fear learning. Cre female mice appeared to have an opposite, but not significant, effect in cued fear consolidation than the male cre mice when compared to control mice and no effect in contextual fear recall.

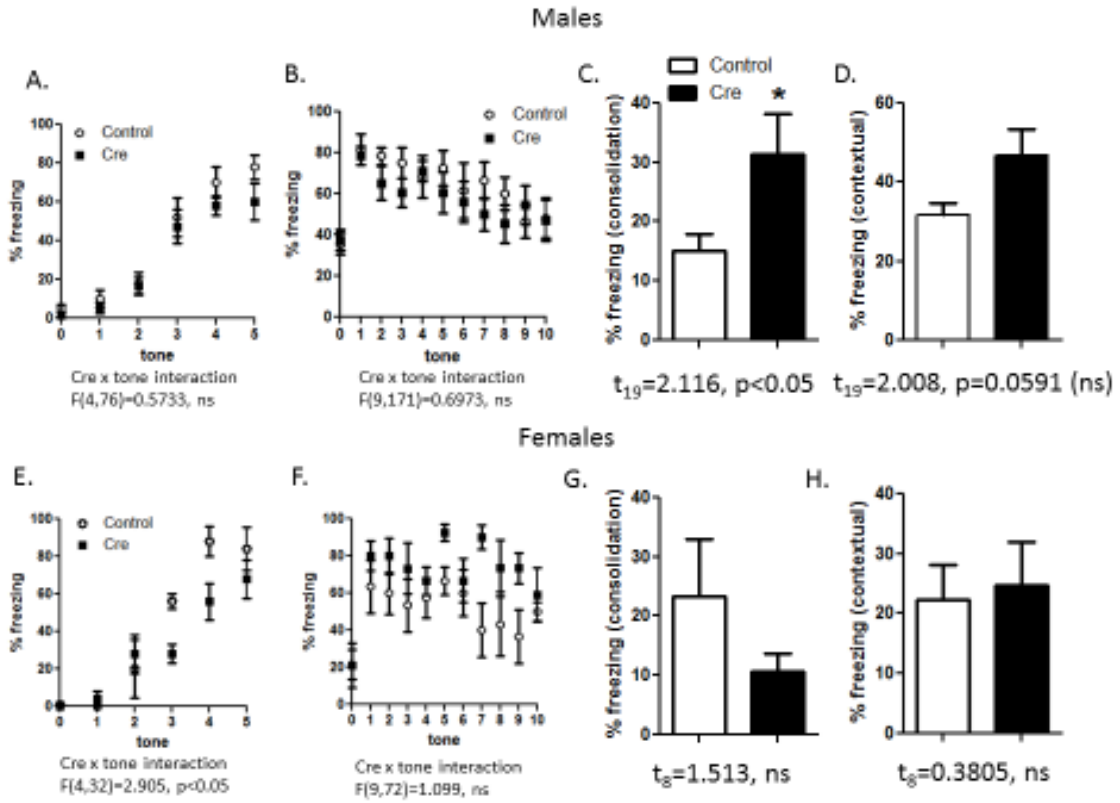
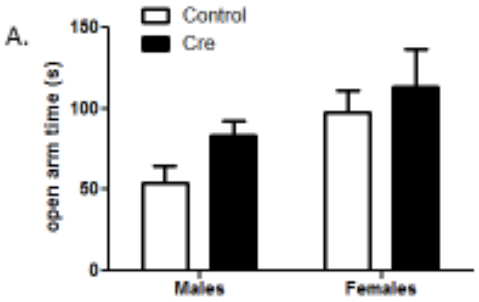


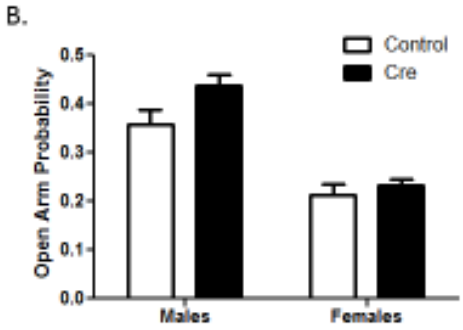
Figure 10. Fear Conditioning. **(A,B)** The deletion of 5-HT_{1A}R in the BNST did not lead to differences in fear learning or cued fear in male mice. **(C,D)** This deletion did lead to a significant increase in cued fear consolidation in male mice but not in contextual fear recall. **(E,F)** Female cre mice exhibited a delay in fear leaning but no significant effects in cued fear. **(G,H)** The deletion led to an opposite, but not significant, effect in cued fear consolidation in female mice than male mice and no differences in contextual fear recall.

The data from the two-way ANOVA tests are shown in Figure 11 (EPM) and Figure 12 (contextual fear). This data indicates that significant sex differences were present in the open arm time and probability of entering the open arms for the EPM test and contextual fear learning. Female mice spent more time in the open arms but were less likely to enter them than male mice. Male mice had a significantly higher percentage of freezing on the third day of the fear conditioning protocol, which measured contextual fear learning.



Source of Variation	P value summary	Significant?
Interaction	ns	No
cre	ns	No
sex	**	Yes

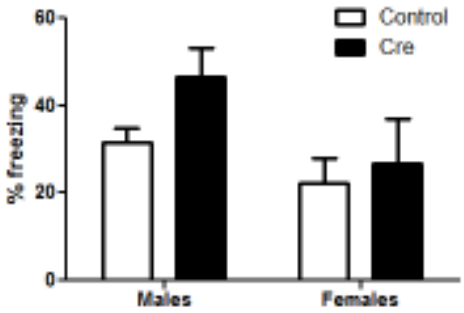
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	322.4	322.4	0.2759
cre	1	3468	3468	2.969
sex	1	9198	9198	7.872
Residual	27	31547	1168	



Source of Variation	P value summary	Significant?
Interaction	ns	No
cre	ns	No
sex	***	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.007802	0.007802	1.413
cre	1	0.01506	0.01506	2.727
sex	1	0.2181	0.2181	39.49
Residual	27	0.1491	0.005522	

Figure 11. Two-way ANOVA data for EPM. (A) There was a significant difference between male and female mice but not cre and control mice in the amount of time spent in the open arms of the EMP apparatus. **(B)** This effect was also reflected in the probability of the mice entering the open arms.



Source of Variation	P value summary	Significant?
Interaction	ns	No
cre	ns	No
sex	*	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	193.4	193.4	0.6284
cre	1	643.7	643.7	2.092
sex	1	1423	1423	4.623
Residual	27	8309	307.8	

Figure 12. Two-way ANOVA for contextual fear. There was a significant difference between the percent immobility on the third day of the fear protocol between male and female mice but not cre and control mice.

Virus Placement Verification

GFP immunohistochemistry was then used to verify the placement of the virus and the ablation of the 5-HT_{1A} receptor in the BNST. Mice that did not have adequate expression of the virus in this brain region were removed from the data set. Representative pictures of a mouse that remained at the data set and a mouse that was removed are presented in Figure 13. Overall, only 2 female cre mice were removed from the data set, making the female cohort n=5 cre and n=5 control.

A.

B.

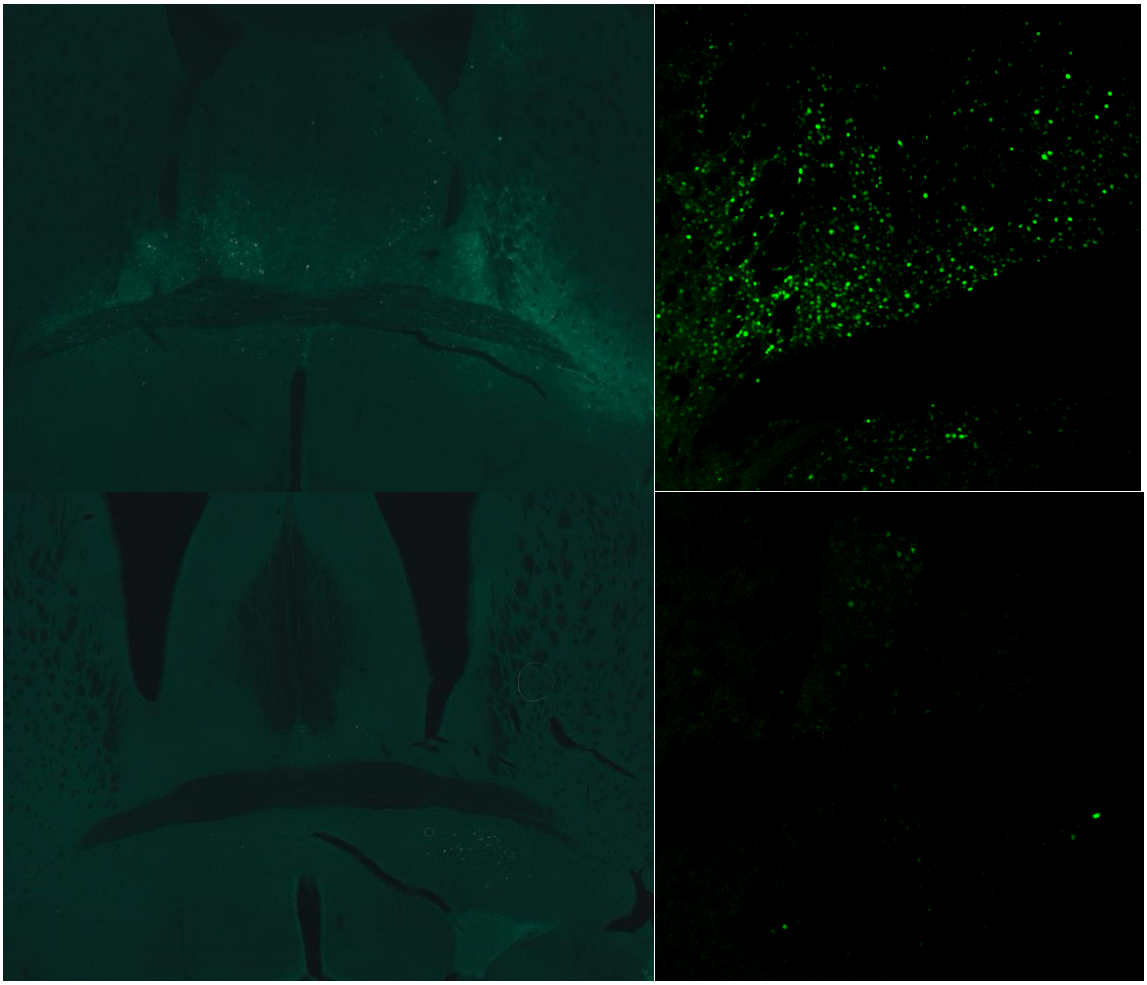


Figure 13. BNST virus placement verification. Images of the BNST following GFP immunohistochemistry. **(A)** Images of a mouse that remained in the data set. **(B)** Images of a mouse that was taken out of the data set. The images on the left were taken with a Zeiss AXIO Zoom V16 microscope and the images on the right were taken with an Olympus FV1000 confocal microscope with FluoView 1000 Software using the 20x objectives.

Discussion

Deletion of 5-HT$_{1A}$R in the BNST leads to a decrease of anxiety-like behavior in male mice

The data above indicates several behavioral phenotypes differ between mice lacking the 5-HT$_{1A}$ receptor in the BNST and control mice. The EPM data suggests that for male mice this deletion leads to a decrease in anxiety-like behavior, indicating that this receptor may be involved in anxiogenic pathways. This is the opposite effect reported in previous studies with a global 5-HT$_{1A}$ deletion. Given that 5-HT$_{1A}$Rs are primarily inhibitory, deletion of this receptor would tend to disinhibit neurons in which they are expressed. This suggests to us that 5-HT$_{1A}$Rs in the BNST are primarily expressed in a subset of neurons that mediate anxiolysis, such as BNST output neurons to the ventral tegmental area (VTA) and lateral hypothalamus (LH) (Kim et al., 2013; Jennings et al., 2013). Deletion of the 5-HT$_{1A}$ receptor in these BNST outputs would result in the disinhibition of these neurons and hence anxiolysis, which is congruent with our results. While these effects differ from those reported in the Levita paper, the only behavioral experiment presented in that paper is the acoustic startle test, which presents mice with a stressor (a loud tone) before testing for any anxiety-related phenotypes. The EPM test does not introduce any stressors and therefore only tests basal anxiety levels. We hypothesize that the presence of a stressor may recruit a different subset of local BNST neurons that promote anxiety-like behavior and enhance fear recall.

The lack of this effect in females could be attributed to the lesser number of overall neurons in the BNST of female mice. Having a smaller BNST volume could be indicative that changes to this brain region would lead to less drastic behavioral phenotypes.

Lack of feeding or depressive effects in both sexes

The lack of significant affects in both the body weight measurements and food consumption in the novelty-suppressed feeding test indicate that this receptor in the BNST does not play a role in feeding behavior. This lack of effect is not surprising as serotonin's feeding-related phenotypes have been attributed to projections to the hypothalamus. (Magalhães et al., 2010). The forced swim results indicate that this receptor also does not play a role in depressive-like behaviors. While BNST lesions have been shown to increase immobility in the forced swim test for both male and female rats, this effect has not been attributed to serotonin and the data presented here indicate that 5-HT_{1A} is not responsible for the effect (Pezuk et al., 2008).

Increase in fear consolidation for male mice

While there were no significant effects of 5-HT_{1A}R deletion on fear learning or cued fear for male mice, there was a significant elevation in freezing for male cre mice in the 2-min period after the tones were stopped on day 2 (i.e. consolidation). The BNST was previously shown to play a critical role in fluoxetine enhancement of cued fear in rats (Ravinder et al., 2012), but this is likely mediated by 5-HT_{2C} rather than 5-HT_{1A} receptors (Burghardt et al., 2013). We hypothesize that this increase in freezing could be due to the cre mice retaining their fear in the context of Day 2. This is consistent with the trend that contextual fear recall (Day 3 of the protocol) was higher for the cre mice than the control mice of both sexes, although not significant. This trend aligns with the finding that the introduction of a 5-HT_{1A} agonist into the BNST decreases contextual fear recall (Gomes et al., 2012) and our hypothesis that the presence of a stressor recruits local BNST neurons which are both anxiogenic and fear enhancing, and that 5-HT_{1A} inhibits these neurons.

Female cre mice exhibited a delay in fear learning for the intermediate tones on Day 1 that was extinguished by the last tone, indicating that both the cre and control mice were able to form the aversion to a shock that was paired with a tone. Effects on cued fear and consolidation were insignificant in females. Increasing the n of both groups could lead to significant effects in this type of learning.

Sex differences in EPM and Contextual Fear

The data presented in the two-way ANOVA analysis showed significant differences between male and female mice in manifestations of anxiety-like behavior and fear learning, but not between cre and control mice. This could be due to the BNST size differences between males and females.

Any significant data between the cre and control mice using a simple T-test was no longer significant using this method, as the threshold for significance was higher due to the increase in independent variables and the low n of the female mice.

Conclusion

Serotonin modulates behaviors including fear, anxiety, depression, and feeding, and treatments preventing the reuptake of serotonin are used every day to treat major psychiatric disorders. Serotonin projections from the DR reach many brain regions, one of them the BNST, a critical relay station in the brain. The experiments above study the 5-HT_{1A} receptor, a receptor for a subtype of serotonin, in the BNST and suggests that the activation of 5-HT_{1A} signaling in the BNST of male mice leads to an increase in anxiety under basal stress levels and a decrease in anxiety and fear learning under high-stress conditions. In female mice, no significant anxiety

phenotypes were reported, which could indicate that the BNST 5-HT_{1A} outputs differ in males and females. Repeating the above experiments with more female mice could lead to significant values for the tests in that gender.

While current treatments in humans cannot target a specific brain region such as the BNST, understanding the specific pathways that lead to certain behaviors could be important for future therapeutics. The data above indicates that serotonin in the BNST is a potential target for treating anxiety and fear disorders.

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