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Serotonin inputs to the dorsal BNST modulate anxiety in a 5-HT_{1A} receptor dependent manner

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Abstract

Serotonin (5-HT) neurons project from the raphe nuclei throughout the brain where they act to maintain homeostasis. Here, we study 5-HT inputs into the bed nucleus of the stria terminalis (BNST), a major subdivision of the extended amygdala that has been proposed to regulate responses to anxiogenic environments in humans and rodents. While the dorsal part of the BNST (dBNST) receives dense 5-HT innervation, whether and how 5-HT in the dBNST normally modulates anxiety remains unclear. Using optogenetics, we demonstrate that activation of 5-HT terminals in the dBNST reduces anxiety in a highly anxiogenic environment. Further analysis revealed that optogenetic inhibition of 5-HT inputs into the dBNST increases anxiety in a less anxiogenic environment. We found that 5-HT predominantly hyperpolarizes dBNST neurons, reducing their activity in a manner that can be blocked by a 5-HT_{1A} antagonist. Finally, we demonstrate that activation of 5-HT_{1A} receptors in the dBNST is necessary for the anxiolytic effect observed following optogenetic stimulation of 5-HT inputs into the dBNST. These data reveal that 5-HT release in the dBNST modulates anxiety-like behavior via 5-HT_{1A} receptors under naturalistic conditions.

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Conflict of Interest

The authors declare no conflict of interest.

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Introduction

Serotonin (5-HT) cells are located in the raphe nucleus (RPH) and release 5-HT throughout the forebrain to regulate the activity of local networks and behavioral processes, including anxiety-related behaviors[1]. However, the mechanisms by which 5-HT acts in target areas to regulate anxiety have not been fully elucidated. Among the forebrain areas that receive 5-HT innervation, the bed nucleus of the stria terminalis (BNST), one of the two major divisions of the extended amygdala has been classically proposed to be a critical site involved in anxiety-like behaviors[2–4]. In contrast, the central nucleus of the amygdala (CeA), another component of the extended amygdala, has been classically involved in phasic fear responses[4–7]. However, while recent evidence indicates that the extended amygdala is involved in evaluating and responding to threat[4], whether these two divisions individually contribute to anxiety states is not fully understood[4, 8].

The BNST has been proposed to be a center for assessing affectively salient information and consequently signaling for shifting behavioral states[9]. Moreover, it is a heterogeneous structure with a complex microcircuitry that can modulate anxiety bidirectionally[10]. For example, while direct inhibition of the whole dorsal BNST (dBNST) results in anxiolysis, inhibition of distinct dBNST subregions can have opposing effects on anxiety[3, 11]. Thus, it has been suggested that competing patterns of activity within BNST circuits shift the behavioral outcome depending on the context[9, 10].

Within the BNST, the dBNST receives the densest 5-HT innervation[12, 13]. However, few studies have directly examined how 5-HT modulates neural activity within the dBNST. For example, in dBNST slices, bath application of 5-HT results in predominantly hyperpolarizing responses, most likely through 5-HT_{1A} receptors[14], which are inhibitory. Remarkably, while activation of local 5-HT_{1A} receptors results in anxiolysis[14, 15], activation of the excitatory 5-HT_{2C} receptors results in anxiogenesis[16, 17]. These findings suggest a complex role for 5-HT in modulating BNST function that is dependent on the balance of excitatory and inhibitory 5-HT receptors.

Here, using optogenetics, we test how endogenous and induced 5-HT release in the extended amygdala affect anxious behavior. We identify a functional role for the 5-HT in the dBNST, but not the CeA, in modulating anxiety. We also find that 5-HT regulates anxiety responses in the dBNST by activating 5-HT_{1A} receptors.

Material and Methods

Detailed information on experimental procedures is provided in Supplementary Material and Methods.

Mice

For optogenetic stimulation male SJL-Tg (Tph2-COP4*H134R/EYFP) 5Gfng/J mice (C57 background) (Jackson Laboratory, Bar Harbor, ME) were used[18]. In this line TPH2 immunofluorescence strongly co-localizes with anti-GFP (ChR2-EYFP +) antibody labeled neurons (81% for dorsal raphe and 87.1% for medial raphe (DRN and MRN respectively)).

ChR2-EYFP+ neurons were virtually all TPH2+ (100% for DRN and MRN), which together suggests that ChR2-EYFP is selectively expressed in 5-HT neurons with no ectopic expression[18].

For optogenetic inhibition male Pet-Ai35 (Pet-Arch) mice (129SvEv background) were used. Specifically, male Pet-Arch mice were generated by breeding male 129S-Gt (ROSA) 26Sortm35.1 (CAG-aop3/GFP) Hze/J (Ai35(RCL-Arch/GFP)) mice (Stock nb. 012735; Jackson Laboratory, Bar Harbor, ME) to Cg-Tg(Fev-cre)1Esd/J (ePet-Cre) female mice (Stock nb. 012712; Jackson Laboratory, Bar Harbor, ME).

Fiber implantation

Male TPH-ChR2 (+) and (−) mice were implanted bilaterally with fiber optics in the dBNST (x=±0.9–0.95, y=+0.38–0.45, z=−4.1–4.15) or in the CeA into a beveled guide cannula to allow preferential illumination of the CeA and not the basolateral amygdala[19] (x=±2.25, y=−1.06, z=−4.4). Pet-Arch (+) and (−) mice were implanted bilaterally in the dBNST with fiber optics (x=±0.9–0.95, y=+0.38–0.5, z=−4.1–4.2). For figure 5 TPH-ChR2 (+) mice were unilaterally implanted with a cannula that was epoxied to an optical fiber aimed at the dBNST (x=±0.9–0.95, y=+0.38–0.45, z=−4.1–4.15). Locations of implants of mice used for the experiments can be found in supplementary figure 6.

Laser Delivery

For TPH-ChR2 mice, stimulation of RPH terminals in the dBNST and CeA involved delivery of an average of 3–5 mW[3] or 7–8 mW[19] of blue light 473 nm light (5-ms pulse, 20 Hz)[18] respectively. Stimulation frequency was set at 20 Hz because 1) ChR2 has been shown to faithfully evoke spikes up to this frequency in 5-HT neurons, 2) is within the range of phasic neuronal firing in the RPH of awake behaving mice and 3) this stimulation frequency has been extensively used in the previous literature[20–24]. For Pet-Arch mice, constant green 532nm light was used (3–5 mW).

C-fos induction

TPH-ChR2 and control mice were placed in a new cage (250–300 lux) and stimulated for 5 min with blue light. Pet-Arch and control mice were placed in a new cage (50–60 lux) for 6 min and received 1 min epoch of green light. 90 min later, mice were perfused with 4% paraformaldehyde, brains were removed and processed for immunohistochemistry. (Detailed information in Supplementary Material and Methods).

Behavioral assays

Mice were tested in behavioral tests in the following order: open-field, elevated-plus maze and then the novelty suppressed feeding test, with at least 2–3 days between trials. Because the 129SvEV strain is known to display “high-anxiety” behavior, while the C57 strain displays “low anxiety” behavior in anxiety paradigms[25–28], light levels were adjusted to ensure the ability to detect changes in anxiety states. For ChR2 experiments (C57), light levels in the behavioral testing room were set at 250–300 lux to enhance the anxiogenic potential of the environment. For Arch manipulations (129SvEv), light levels were maintained at 50–60 lux to maintain a moderately anxiogenic environment. An experimenter

blind to the genotype performed all testing. Behavioral output measures were generated by automated analyses unless specified otherwise. Littermate Chr2 or Arch genotype (+) and (−) animals were used for all experiments. Animals were housed in mixed genotype groups. Animals were assigned to group based on genotype; therefore, no randomization was used for assignment. (Detailed information in Supplementary Material and Methods).

Slice Preparation and Electrophysiology

Whole-cell patch-clamp recordings were made from neurons in the dBNST and terminals expressing ChR2 were activated using a 470 nm LED.

Statistical Analysis

All statistical analyses were performed using Stat View (SAS Institute Inc.). The number of mice in each group was selected based on pilot experiments (*Data not shown*) and on power analyses that assumed a normal distribution, a 20% change in mean and 15% variation, indicating that 9 mice per group would be needed for behavioral paradigms. Mice that lost head stages during behavioral testing or mice where fiber/cannula were misplaced were removed from the analysis. Final group numbers are shown in Results section and in Figure legends. All experiments described in the manuscript have been performed in at least two cohorts of mice with consistent results. Figures 1–3,5 reflect the result of a single cohort per experiment. Figure 4 reflects data combined from two smaller cohorts. Group differences were detected using either one-way ANOVA or two-way repeated-measures ANOVA with Bonferroni or Fisher post hoc tests. For paired comparisons, a paired Student's t-test was used. Unpaired Student's t-tests were used for single-variable comparisons. Significance was set at $p<0.05$. All data are shown as means ± SEM.

Results

Optogenetic stimulation of 5-HT inputs to the dBNST and the CeA decreases novel cage induced c-fos expression

In order to target 5-HT inputs in the dBNST and the CeA, we took advantage of the TpH2-ChR2-EYFP BAC (TPH-ChR2) mouse line (TPH: 5-HT cell marker), which expresses ChR2-EYFP exclusively in 5-HT neurons in the DRN and MRN[18] and can be detected in their axon terminals in the dBNST and the CeA (Supplemental Figure 1A).

Exposure of mice to bright novel environments increases c-fos expression in both the BNST and the CeA presumably as a reflection of increased sustained neuronal activity[29]. To assess whether 5-HT modulated this activity, we investigated the effects of photostimulating 5-HT inputs to the dBNST and CeA. TPH-ChR2 and control mice were bilaterally implanted with fiber optics either over the dBNST (TPH-ChR2$^{RPH\text{-}dBNST}$) or over the CeA (TPH-ChR2$^{RPH\text{-}CeA}$) and exposed to a bright new cage for c-fos induction (5 min, 250–300 lux) while simultaneously undergoing photostimulation (Figure 1A). Photostimulation of TPH-ChR2$^{RPH\text{-}dBNST}$ mice resulted in fewer c-fos positive cells in the dBNST while no change in the ventral BNST (vBNST) was detected (Figure 1B, C) (n=4–5/group), suggesting that the light power used preferentially recruits 5-HT inputs in the dBNST. Photostimulation in TPH-ChR2$^{RPH\text{-}CeA}$ mice resulted in fewer c-fos positive cells in the lateral (CeL) and

medial (CeM) parts of the CeA (Figure 1D, E) (n=4–5/group). Furthermore, we detected no changes in the number of TPH positive cells expressing c-fos in the DRN or MRN in either TPH-ChR2$^{RPH\text{-}dBNST}$ (Supplementary Figure 1B,C) (n=4/group) or TPH-ChR2$^{RPH\text{-}CeA}$ mice when compared to their respective control group (Supplementary Figure 1D,E) (n=3–4/group).

Optogenetic activation of 5-HT inputs to the dBNST, but not to the CeA, results in anxiolysis

Next, we examined the ability of 5-HT inputs to the dBNST (Figure 2A) to modulate anxiety in three well-validated behavioral tasks: the open-field test, the elevated-plus maze and the novelty suppressed feeding test (n=8–10/group). In the open-field, mice were tested using a 9 min session with three 3 min epochs (OFF/ON/OFF). Compared to control mice, TPH-ChR2$^{RPH\text{-}dBNST}$ mice spent more time in the center of the arena during the ON epoch (Figure 2B and Supplementary Figure 2A, B). Importantly, no changes in total distance (Figure 2C) or ambulatory velocity were detected (Supplementary Figure 2C). We then tested behavior in the elevated-plus maze using the same 3 epoch approach. TPH-ChR2$^{RPH\text{-}dBNST}$ mice spent more time in the open arms (Figure 2D and Supplementary Figure 2D, E) and had increased open arm entries (Figure 2E) during the ON epoch but not the OFF epoch compared to controls. Ambulatory velocity and total distance between groups did not differ (Supplementary Figure 2F, G).

We next investigated the effects of our optogenetic manipulations in the novelty suppressed feeding test, which measures the latency of a mouse to consume food placed in brightly lit aversive arena[30]. The task was repeated twice on different days for each mouse, counterbalanced for light stimulation (ON) or no stimulation (OFF). The latency to feed was reduced in the TPH-ChR2$^{RPH\text{-}dBNST}$ light ON group compared to controls (Figure 2F and Supplementary Figure 1H) without changes in home cage consumption (Figure 2G).

Next, we investigated the functional contribution of 5-HT inputs to the CeA (Figure 3A) in the same behavioral paradigms and testing conditions as previously used (n=6–7/group). Despite suppression of c-fos in the CeA with light stimulation, no behavioral effect of light was detected in TPH-ChR2$^{RPH\text{-}CeA}$ mice compared to their controls in in any of the parameters examined (Figure 3B–G and Supplementary Figure 3A–H). Thus, activating the RPH-dBNST pathway, but not RPH-CeA, reduces anxiety.

Optogenetic inhibition of 5-HT inputs to the dBNST increases anxiety-like behaviors

We next tested whether 5-HT inputs modulate dBNST activity under naturalistic conditions by inhibiting local 5-HT terminals. To do so, we used a Pet-Arch-GFP (Pet-Arch) mouse line, which exhibits robust Arch-GFP expression in RPH neurons and their terminals in the BNST (Supplemental Figure 4A).

Pet-Arch and control mice on a 129SvEv background were bilaterally implanted with fiber optics over the dBNST (Pet-Arch$^{RPH\text{-}dBNST}$) (Figure 4A). To ensure the ability to detect anxiogenic responses in a 129SvEv background, mice were exposed to a dimly lit (50–60 lux) new cage, bright enough to ensure c-fos induction, but dim enough to allow detection of additional c-fos activation. Mice were then exposed for 6 min while alternating the light in 1

min epochs (OFF/ON/OFF/ON/OFF/ON). One minute epochs were chosen because sustained activation (>1 min.) of Arch in projections has been reported to result in a paradoxical increase rather than inhibition of spontaneous release[31]. Photostimulation of Pet-Arch$^{RPH\text{-}dBNST}$ mice resulted in an increase in the number of c-fos positive cells in the dBNST along with no changes in the vBNST in a new cage (Figure 4B, C) (n=5/group).

To test whether the changes in c-fos activation correspond to changes in behavior, we tested Pet-Arch$^{RPH\text{-}dBNST}$ and control mice in the open-field and elevated-plus maze tests using a 6 min session under the same conditions as above (n=8–11/group). In the open-field test, we found that Pet-Arch$^{RPH\text{-}dBNST}$ mice spent significantly less time in the center during light ON but not light OFF conditions compared to controls (Figure 4D and Supplementary Figure 4B). Importantly, no changes were detected in total distance traveled (Figure 4E) or in ambulatory velocity (Supplementary Figure 4C). In the elevated-plus maze Pet-Arch$^{RPH\text{-}dBNST}$ mice spent less time in the open arms during ON but not OFF epochs compared to controls (Figure 4F and Supplementary Figure 4D). Similarly, open arm entries were lower during ON but not OFF epochs (Figure 4G). No changes in ambulatory velocity or total distance were detected between groups in either condition (Supplementary Figure 4E, F). Taken together, these data demonstrate that 5-HT inputs to the dBNST modulate anxiety under naturalistic conditions.

5-HT inhibition of dBNST is mediated through 5-HT$_{1A}$ receptors

To identify the receptor mediating the dominant effects of 5-HT in the dBNST, we performed in vitro whole-cell patch-clamp recordings from dBNST neurons (Figure 5A). We observed a delayed hyperpolarizing response in 67% of dBNST cells that was maximal at 45 seconds post illumination, with a mean effect size of −4.69±1.01 mV in TPH-ChR2 mice (Figure 5B, C) (n=7–8 cells/group). This light-evoked hyperpolarization was not found in control mice and was blocked in TPH-ChR2 mice with WAY 100,635, a 5-HT$_{1A}$ receptor antagonist (Figure 5B, C)[32]. Importantly, we saw no evidence of fast depolarizing responses following light stimulation and no net effect of 5-HT was observed in the remaining 33% of cells.

5-HT in the dBNST mediates changes in anxiety-like behaviors through 5-HT$_{1A}$ receptors

We next examined whether the observed anxiety changes induced by stimulation of 5-HT terminals could be blocked by a 5-HT$_{1A}$ antagonist infused into the dBNST (n=8/group). TPH-ChR2 mice were unilaterally implanted with a fiber optic attached to a cannula over the dBNST and received either saline or WAY 100,635 (200nM) through the cannula 30 min prior to behavioral testing (Figure 5D). While the unilateral local injections of the 5-HT$_{1A}$ antagonist had no significant effect on their own under highly anxiogenic conditions, they blocked the light-induced anxiolysis in all three anxiety tests (Figure 5E, G, H, I). No changes were detected in either total distance traveled or velocity in the open-field, velocity or total distance in the elevated-plus maze or home cage consumption in the novelty suppressed feeding test (Figure 5F, J and Supplementary Figure 5B, D, E). These data suggest that 5-HT hyperpolarizes dBNST neurons through 5-HT$_{1A}$ receptors resulting in anxiolysis.

Discussion

While it is likely that both the BNST and the CeA contribute to the regulation of responses to potentially threatening situations[4, 8], our results indicate that directly stimulating 5-HT inputs in the dBNST but not in the CeA is anxiolytic. At rest the BNST has low levels of activity[16] and rodents exposure to a bright open-field increases c-fos expression[29]. In this highly anxiogenic context, dBNST activity as measured by c-fos can be inhibited by 5-HT release and results in anxiolysis. These results are consistent with evidence showing that inhibition of the dBNST reduces anxiety[3]. In a less anxiety-provoking environment, we find that inhibition of 5-HT terminals in the dBNST increase dBNST activity resulting in anxiogenesis. These results are the first direct evidence that the RPH-dBNST pathway is involved in anxiety under naturalistic conditions.

Interestingly, using a similar optogenetic approach Marcinkiewcz et al., recently reported that releasing 5-HT in the whole BNST enhances anxiety[24], while we find that enhancing 5-HT release in the dBNST results in anxiolysis. These divergent results are likely due to important methodological differences, which have significant biological implications. For example, we used 3–5 mW of laser power, likely illuminating the oval and anterodorsal divisions of the BNST[3]. Importantly, consistent with our results, direct inhibition of the dBNST and its oval division is anxiolytic[3]. In contrast, Marcinkiewcz et al., used a significantly higher laser power (10 mW) and different positioning of fiber optics[24]. As a result, they likely activate 5-HT inputs in both the dBNST and vBNST[24] and release more 5-HT.

Further, our c-fos results indicate that under bright anxiogenic conditions (250–300 lux) the BNST is active. In contrast, Marcinkiewcz et al., used dim, less anxiogenic conditions (14 lux) that might not engage significant BNST activity[16, 24]. Consequently, our results suggest that when behavioral testing occurs in a high anxiogenic environment (where BNST is active), optogenetically evoked 5-HT release is likely to inhibit oval BNST cells to a degree that results in the observed anxiolysis. Thus taken together, these results highlight the complex role of 5-HT in the BNST reflecting the existence of distinct BNST circuits[9, 10], which can govern opposite behavioral outcomes.

Our results further demonstrate for the first time that 5-HT inputs to the dBNST modulate anxiety under naturalistic conditions. In contrast to our ChR2 manipulations, Pet-Arch (129SvEv) mice were tested under mild anxiogenic conditions that are sufficient to engage BNST function in this strain as our c-fos results indicate. Under these conditions, and in this “high-anxiety” strain, inhibition of 5-HT inputs into the dBNST enhances BNST activity to result in anxiogenesis. Thus taken together, these results suggest that 5-HT inputs into the dBNST regulate anxiety-like behaviors.

We find that optically stimulating 5-HT terminals predominantly results in hyperpolarization of dBNST cells in a 5-HT_{1A} receptor dependent manner[14]. The observed delayed hyperpolarization could be related to the time necessary to achieve sufficient GPCR-mediated activation of GIRK channels to hyperpolarize the membrane. Indeed, in previous 5-HT bath application studies a similar delayed onset of hyperpolarization has been

observed[33]. Alternatively, this delay hyperpolarization may result from polysynaptic activation of neighboring GABAergic cells. However prior work demonstrating direct 5-HT_{1A}-dependent hyperpolarization of dBNST cells via GIRK channel activation[14] makes this second possibility more likely.

Our results also indicate that dBNST 5-HT_{1A} receptors are necessary for the observed light-induced anxiolytic effect as WAY 100,635 blocks the anxiolytic effect of 5-HT. While we did not see anxiogenic effects of the 5-HT_{1A} antagonist alone, this is not entirely surprising, given the unilateral injection and the already highly anxious behavior of the mice treated with saline.

In contrast to our results, direct or 5-HT-mediated activation of 5-HT_{2C} receptors in the whole BNST is anxiogenic[16, 24]. The net effect of 5-HT could thus vary between distinct BNST subregions, depending on their 5-HT receptor composition as high affinity 5-HT_{1A} receptors and lower affinity 5-HT_{2C} receptors are differentially distributed[34]. Indeed, evidence suggests that stress promotes anxiety by enhancing $5HT_{2C}$ receptor signaling in the vBNST[17]. Our results suggest that there is another discrete subset of neurons in the dBNST that are inhibited by 5-HT through 5-HT_{1A} receptors and are anxiolytic under naturalistic conditions that elicit anxious behavior. Future studies might examine how the behavioral context shifts responses to 5-HT in the BNST to ultimately influence behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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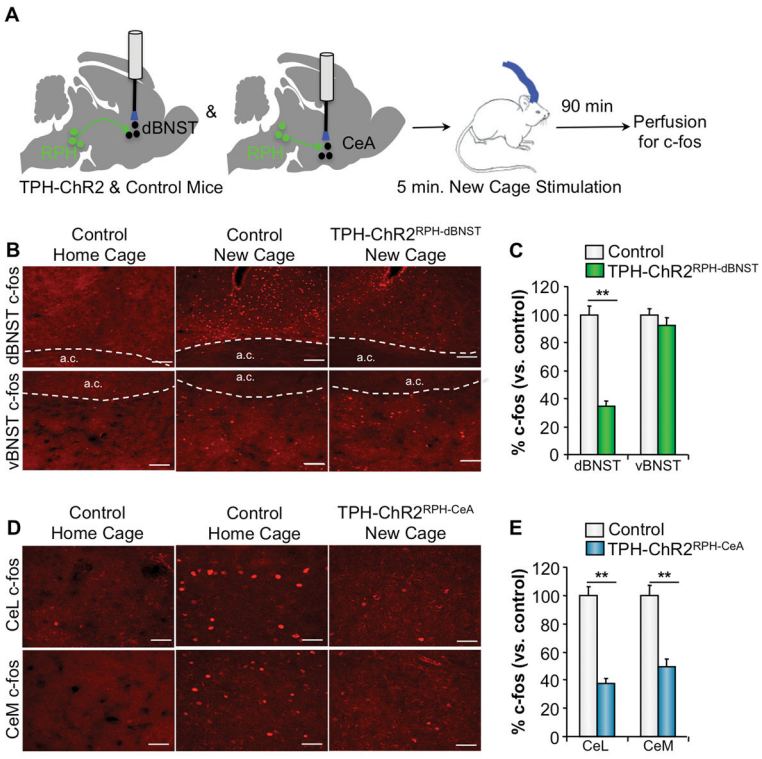

Figure 1. Photostimulation of 5-HT terminals in the dBNST and CeA decreases new cage induced c-fos

(A) Diagram for new cage c-fos induction paradigm. **(B)** Representative images of c-fos staining in the dBNST (Top) and vBNST (Bottom) of control mice in home cage (Left) and in new cage (Center), and TPH-Chr2$^{RPH\text{-}dBNST}$ mice in new cage (Right). Scale bar=100 μm. **(C)** Photostimulation in TPH-Chr2$^{RPH\text{-}dBNST}$ mice reduces the number of c-fos positive cells in the dBNST but not in the vBNST in a new cage (one-way ANOVA dBNST: $F_{1,6}$=35.877, p<0.01; vBNST: $F_{1,6}$=1.257, p=0.3051) (n= 4–5/group). **(D)** Representative images of c-fos staining in the CeL (Top) and CeM (Bottom) of control mice in home cage (Left) and in new cage (Center), and TPH-Chr2$^{RPH\text{-}CeA}$ mice in new cage (Right). Scale bar=50 μm **(E)** Photostimulation in TPH-ChR2$^{PHN\text{-}CeA}$ mice results in fewer c-fos positive cells in the CeL and CeM in a new cage (one-way ANOVA CeL: $F_{1,6}$=82.778, p<0.01; CeM: $F_{1,6}$=34.508, p<0.01) (n= 4–5/group). Means are represented as ±SEM. (*p<0.05; **p<0.01). See also Supplementary Figure 1 and 6A, B.

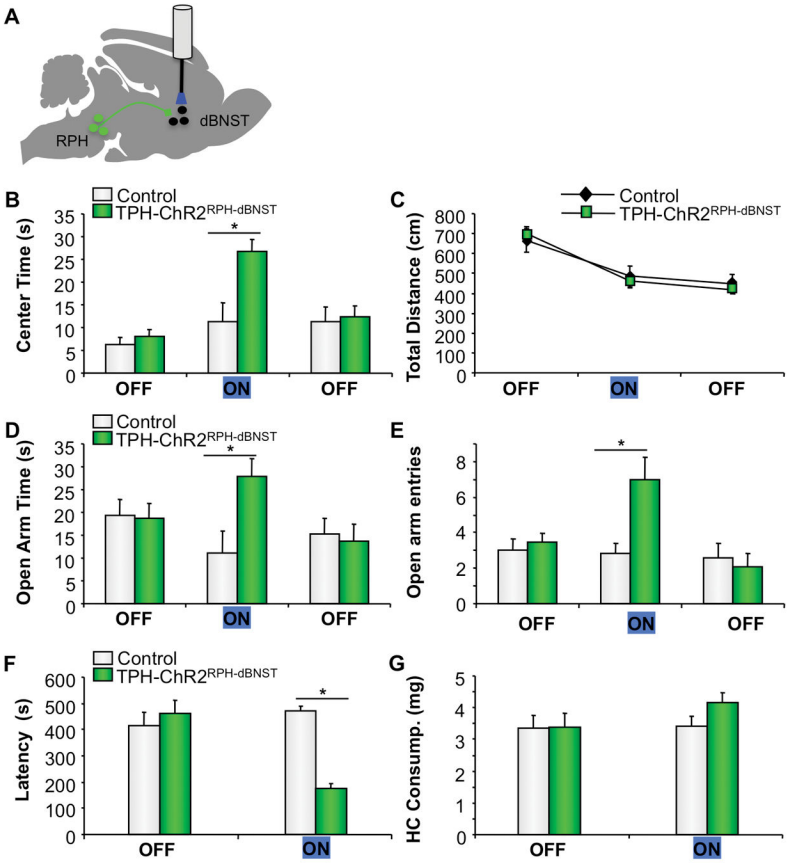

Figure 2. Activation of 5-HT inputs into the dBNST results in anxiolysis

(A) Cartoon of TPH-ChR2$^{RPH\text{-}dBNST}$ mice with fiber optics implanted over dBNST. **(B)** TPH-ChR2$^{RPH\text{-}dBNST}$ mice spent more time in the center in the ON epoch compared to controls (two-way repeated measures ANOVA group x epoch interaction, $F_{2,32}$=7.332, p=0.0024; post hoc Bonferroni corrected t-test, p<0.05). **(C)** No effect on total distance traveled in the open-field was observed (two-way repeated measures ANOVA group x epoch interaction, $F_{2,32}$=0.614, p=0.5475). **(D)** TPH-ChR2$^{RPH\text{-}dBNST}$ mice spent more time in the open arms (two-way repeated measures ANOVA group x epoch interaction, $F_{2,32}$=5.057, p=0.0124; post hoc Bonferroni corrected t-test, p<0.05) and **(E)** showed more open arm entries during photostimulation in the elevated-plus maze (two-way repeated measures ANOVA group x epoch interaction, $F_{2,32}$=4.551, p=0.0182; post hoc Bonferroni corrected t-test, p<0.05). **(F)** Photostimulation of dBNST 5-HT inputs in TPH-ChR2$^{RPH\text{-}dBNST}$ mice decreased the latency to feed (two-way repeated measures ANOVA group x epoch interaction, $F_{1,16}$=41.783, p<0.01; post hoc Bonferroni corrected t-test, p<0.05). **(G)** No changes in home cage (HC) consumption was observed (two-way repeated measures ANOVA group x epoch interaction, $F_{1,16}$=0.540, p=0.4730). (n=8–10/group). Means are represented as ±SEM. (*p<0.05). See also Supplementary Figure 2 and 6A.

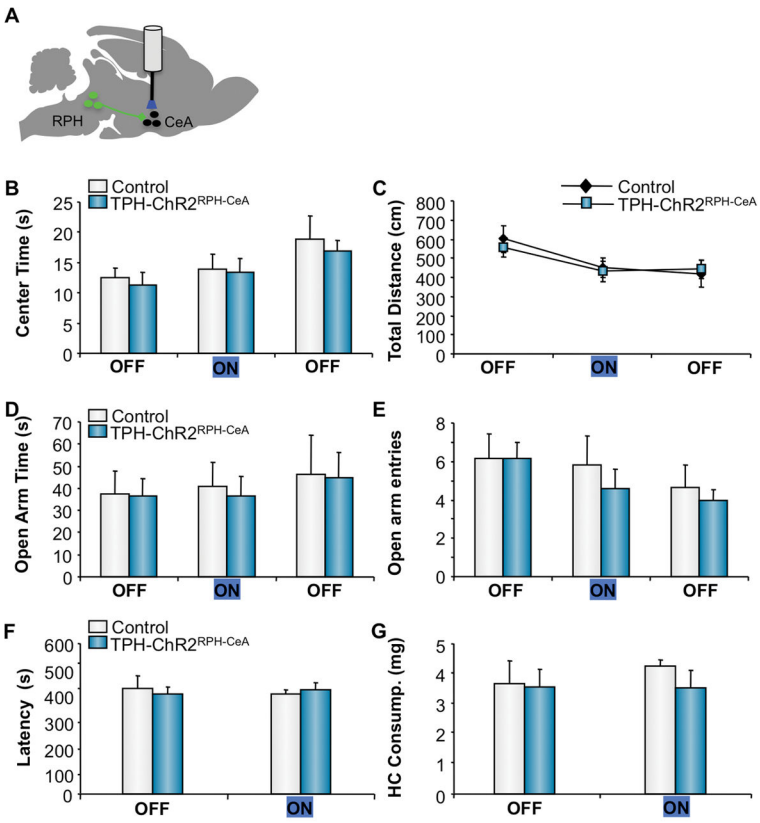

Figure 3. Activation of 5-HT terminals in the CeA has no effect on anxiety

(A) Diagram illustrating TPH-ChR2$^{RPH-CeA}$ mice. **(B, C)** No effect on center time (two-way repeated measures ANOVA group x epoch interaction, $F_{2,22}$=0.043, p=0.9581) or total distance traveled in the open-field was observed (two-way ANOVA group x epoch interaction, $F_{2,22}$=1.127, p=0.3420). **(D, E)** No differences were detected between TPH-ChR2$^{RPH-CeA}$ and control mice in time on the open arms (two-way repeated measures ANOVA group x epoch interaction, $F_{2,22}$=0.026, p=0.9743) or open arm entries in the elevated-plus maze (two-way repeated measures ANOVA group x epoch interaction, $F_{2,22}$=0.303, p=0.7417). **(F)** No effect was detected on latency to eat (two-way repeated measures ANOVA group x epoch interaction, $F_{2,22}$=0.367, p=0.557) or **(G)** in home cage (HC) consumption (two-way repeated measures ANOVA group x epoch interaction, $F_{2,22}$=0.287, p=0.6026). (n=6–7/group). Means are represented as ±SEM. See also Supplementary Figure 3 and 6B.

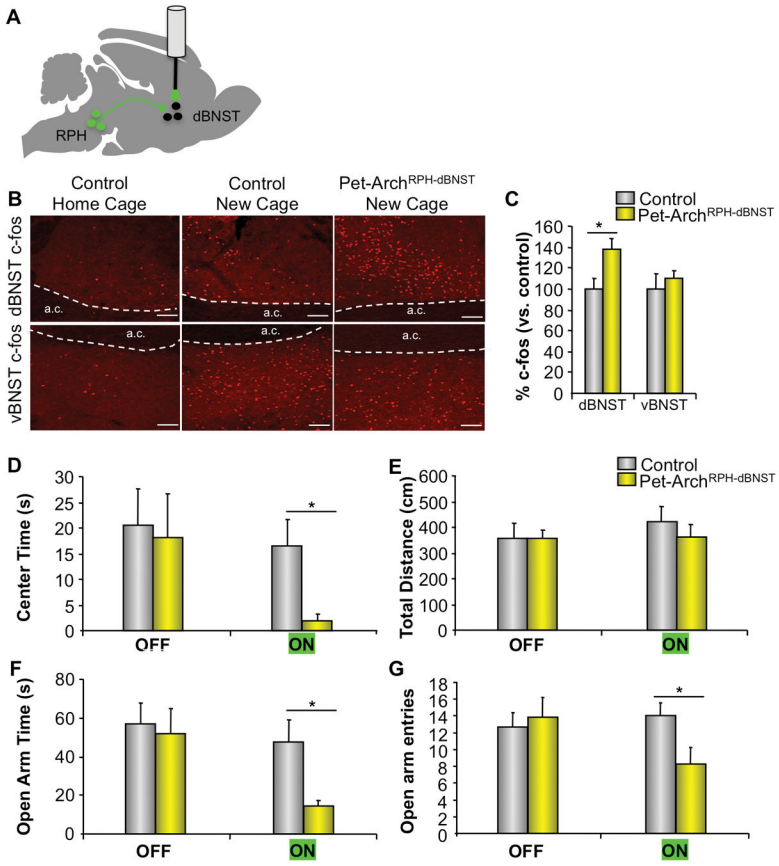

Figure 4. Inhibition of 5-HT inputs in the dBNST increases anxiety-like behaviors

(A) Diagram illustrating Pet-Arch$^{RPH\text{-}dBNST}$ mice. **(B)** Representative images of c-fos staining in the dBNST (Top) and vBNST (Bottom) of control mice in home cage (Left) and in new cage (Center), and Pet-Arch$^{RPH\text{-}dBNST}$ mice in new cage (Right). Scale bar=100 μm. **(C)** Green light illumination of Pet-Arch$^{RPH\text{-}dBNST}$ mice increases c-fos+ cells in the dBNST but not in the vBNST in a new cage (one-way ANOVA dBNST: $F_{1,8}$=6.574, p<0.05; vBNST: $F_{1,8}$=0.450, p=0.5214). (n=5/group) **(D)** During ON epochs, Pet-Arch$^{RPH\text{-}dBNST}$ mice spent significantly less time in the center (unpaired Student's t test, OFF: DF=17, t=0.184, p=0.8656; ON: DF=17, t=2.416, p<0.05). **(E)** Group or light had no effect on total distance traveled in the open-field (unpaired Student's t test, OFF: DF=17, t=0.013, p=0.9896; ON: DF=17, t=0.774, p=0.4670)**. (F)** Pet-Arch$^{RPH\text{-}dBNST}$ mice spent less time in the open arms (unpaired Student's t test, OFF: DF=17, t=0.290, p=0.7754; ON: DF=17, t=2.447, p<0.05) and **(G)** showed a lower number of entries into the open arms during the illumination epoch (unpaired Student's t test, OFF: DF=17, t=0.421, p=0.6794; ON: DF=17, t=2.313, p<0.05). (n=8–11/group). Means are represented as ±SEM. (*p<0.05). See also Supplementary Figure 4 and 6C.

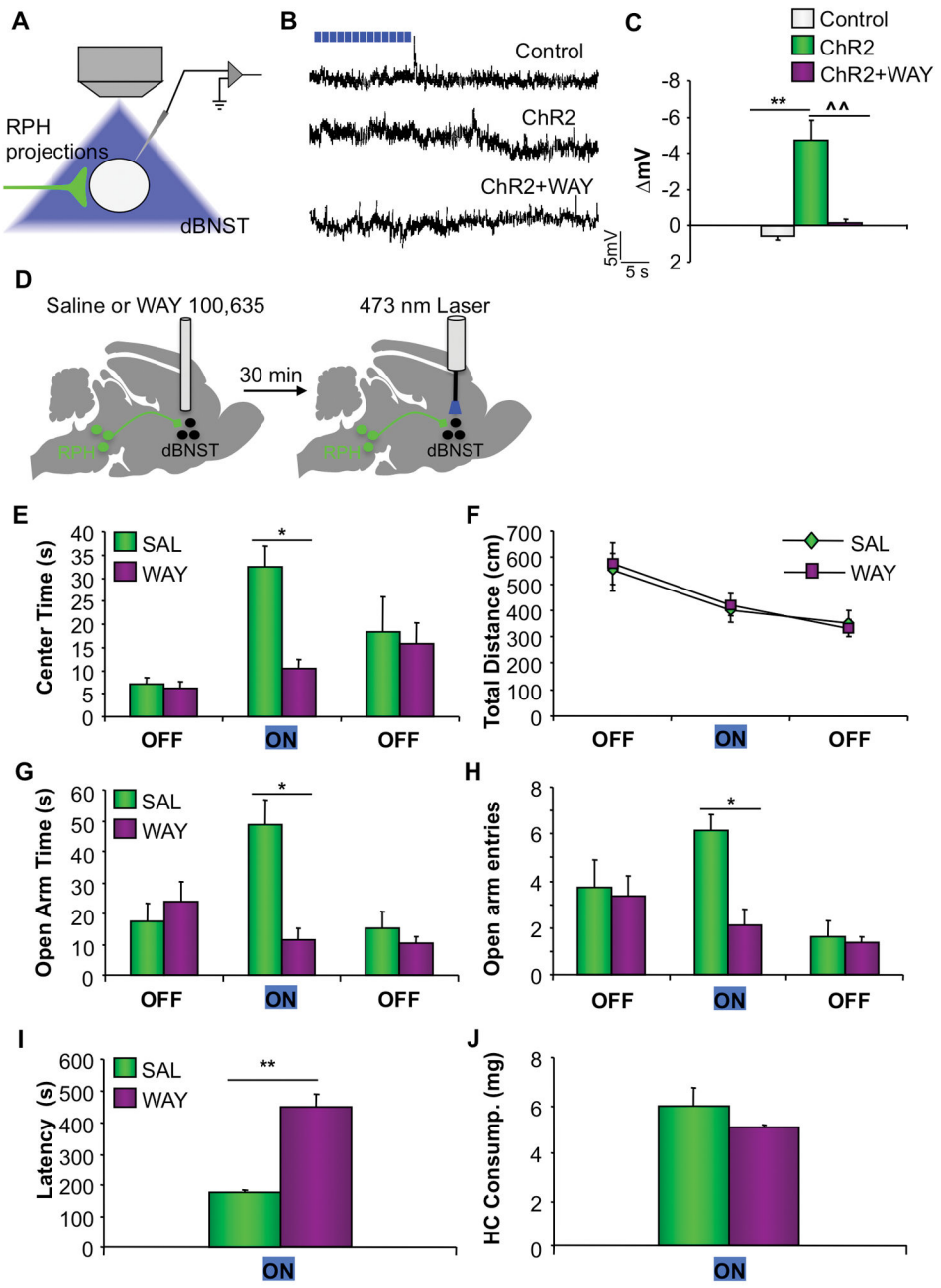

Figure 5. The behavioral effects of 5–HT release in the dBNST are mediated by activation of 5–HT_{1A} receptors

(A) Model depiction of electrophysiological recordings. **(B)** Representative traces from electrophysiological recordings showing a delayed response to photostimulation of 5-HT inputs (20 Hz, 5 ms pulses for 15s) to dBNST neurons in TPH-CHR2 mice but not in controls or TPH-ChR2 mice pretreated with WAY 100,635. **(C)** Average magnitude of hyperpolarization in the dBNST neurons after photostimulation of 5-HT inputs (one-way ANOVA $F_{2,19}$=21.152, p<0.01; Fisher post hoc TPH-ChR2 and control: p<0.01; TPH-ChR2 and TPH-ChR2+WAY: p<0.01) (n=7–8 cells/group). **(D)** Saline or the 5-HT_{1A} receptor

antagonist WAY 100,635 were locally infused into the dBNST of TPH-Chr2$^{RPH\text{-}dBNST}$ mice using a guide cannula 30 min before behavior. **(E)** TPH-Chr2$^{RPH\text{-}dBNST}$ mice pre-treated with WAY spend less time in the center of the open field during the ON epoch than the saline treated mice (two-way repeated measures ANOVA treatment x epoch interaction, $F_{2.28}$=4.933, p=0.0146; post hoc Bonferroni corrected t-test, p<0.05). **(F)** No changes were detected in total distance traveled (two-way repeated measures ANOVA treatment x epoch interaction, $F_{2.28}$=0.236, p=0.7193). (**G)** TPH-Chr2$^{RPH\text{-}dBNST}$ mice treated with WAY spend less time in the open arms (two-way repeated measures ANOVA treatment x epoch interaction, $F_{2.28}$=11.326, p=0.0002; post hoc Bonferroni corrected t-test, p<0.05) and **(H)** show less open arm entries in the elevated-plus maze during the ON epoch when compared to saline treated mice (two-way repeated measures ANOVA treatment x epoch interaction, $F_{2.28}$=4.100, p=0.0274; post hoc Bonferroni corrected t-test, p<0.05). **(I)** 5-HT_{1A} antagonist injection into the dBNST attenuated the light-induced decrease in latency as seen after saline treatment (paired Student's t test, DF=7, t=6.903, p=0.0002) with **(J)** no changes in home cage (HC) consumption (paired Student's t test, DF=7, t=1.102, p=0.3451). (n=8/group). Means are represented as ±SEM. (*p<0.05; **p<0.01 vs. control or saline; ^^p<0.01 vs. WAY in recordings). See also Supplementary Figure 5 and 6D, E.