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5-HT2C receptors in the BNST are necessary for the enhancement of fear learning by selective serotonin reuptake inhibitors

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) are widely prescribed to treat anxiety and depression, yet they paradoxically increase anxiety during initial treatment. Acute administration of these drugs prior to learning can also enhance Pavlovian cued fear conditioning. This potentiation has been previously reported to depend upon the bed nucleus of the stria terminalis (BNST). Here, using temporary inactivation, we confirmed that the BNST is not necessary for the acquisition of cued or contextual fear memory. Systemic administration of the SSRI citalopram prior to fear conditioning led to an upregulation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) in the oval nucleus of the BNST, and a majority of these neurons expressed the 5-HT2C receptor. Finally, local infusions of a 5-HT2C receptor antagonist directly into the oval nucleus of the BNST prevented the fear memory-enhancing effects of citalopram. These findings highlight the ability of the BNST circuitry to be recruited into gating fear and anxiety-like behaviors.

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed to treat anxiety disorders and depression (Kent et al., 1998; van der Kolk et al., 1994). However, they paradoxically increase anxiety in humans when they are given acutely (Mir and Taylor, 1997), and can increase the risk of suicidal ideation (Teicher et al., 1990). Rodent models of anxiety including the elevated plus maze, social interaction task and novelty suppressed feeding task reveal a similar anxiogenic effect of acute SSRI administration (Griebel et al., 1999; Bodnoff et al., 1989; Dekeyne et al., 2000).

Previous research has revealed that acute SSRI administration prior to fear conditioning enhances the consolidation of fear memories (Burghardt et al., 2004; Ravinder et al., 2013). One advantage of using fear conditioning to investigate the actions of SSRIs is that it is a

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model of emotional learning for which the underlying neural circuitry has been characterized in great detail (Johansen et al,. 2011; Orsini and Maren, 2012; Pape and Pare, 2010). Fear conditioning engages fear circuits as well as mechanisms involved in learning and memory. Moreover, many anxiety disorders in humans can be characterized as abnormalities in the acquisition or extinction of conditioned fear (Grillon, 2002; Milad et al., 2008).

The enhancing effects of SSRIs on fear conditioning appear to involve neural activity within the bed nucleus of the stria terminalis (BNST), as systemic injections or intra-BNST infusions of the SSRI fluoxetine potentiate fear learning (Ravinder et al., 2013). Systemic administration of SSRIs also lead to upregulation of the immediate early gene Arc (activity-regulated cytoskeleton associated protein) in the oval nucleus of the BNST (BNSTov; Ravinder et al., 2013). The BNSTov, which is a subregion of the anterolateral BNST (BNST-AL), is one of a dozen defined cell groups within the BNST (Alheid 2003). In general, the BNST has been implicated in processing both adaptive and pathological anxiety, with the majority of studies focusing on its contribution to an animal's response to unpredictable stressful events and anxiety (Alheid 2003; Dunn and Williams, 1995).

Lesions of the BNST do not interfere with fear conditioning (LeDoux et al., 1988; Sullivan et al., 2004). Instead, they disrupt the expression of longer "anxiety-like" states (Walker et al., 2003). This has led to the idea that short duration cues (such as a 30 second tone) recruit amygdalar circuits, whereas long-duration cues, including contextual cues, recruit the BNST (Lee and Davis, 1997; Walker e, 2009). However, there is also evidence that BNST activity can modulate fear conditioning even when short duration cues are used. A subset of BNST-AL neurons develops inhibitory responses to a short duration conditioned stimulus (CS), whereas a separate group of neurons in the anteromedial BNST develop positive CS responses (Haufler et al., 2013). As described above, local infusions of SSRIs into the BNST prior to fear conditioning enhance fear memory consolidation (Ravinder et al., 2013).

Systemic injections of SSRIs enhance both the consolidation and the expression of fear responses, and this latter effect is blocked by the co-administration of a 5-HT2C antagonist (Burghardt et al., 2007). Several lines of evidence suggest that 5-HT2C receptors within the BNST might play a role in the fear enhancing effects of SSRIs. Systemic activation of 5-HT2C receptors increases c-fos expression in the BNST as well as anxiety-like behavior (Bagdy et al., 2001; Singewald et al., 2003). Conversely, 5-HT2C knockout mice show decreased anxiety (Heisler et al., 2007). Importantly, 5-HT2C receptor antagonists block the anxogenic effects of different SSRIs, including fluoxetine and citalopram (Bagdy et al., 2001; Dekeyne et al., 2000).

The goal of the present study was to determine if the fear-enhancing effects of SSRI administration depend on 5-HT2C receptors in the BNST. We first confirmed that temporary inactivation of the BNST does not interfere with the acquisition of cued or contextual fear conditioning. We then characterized the neurons in the BNSTov that show upregulation of the immediate-early gene Arc following SSRI administration plus fear conditioning and determined that the majority of these neurons express 5-HT2C receptors. Finally, enhanced fear conditioning by SSRI administration was blocked by local infusions of a 5-HT2C

antagonist into the BNSTov. Together, these experiments support the idea that recruitment of BNST activity can modulate the consolidation of fear memories.

Methods

Subjects

Adult male Sprague Dawley rats (Charles River Laboratories; 250–325g) were housed individually with *ad libitum* access to food and water and maintained on a 12 hour light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by Columbia University's Animal Care and Use Committee.

Surgery

Rats were anesthetized with a mixture of isoflurane and oxygen and mounted in a stereotaxic apparatus. Betadine was applied to the scalp and a local anesthetic (bupivacaine, s.c.) was injected under the scalp. The scalp was incised and small burr holes were made in the skull to insert cannulas into the BNST (−0.12 AP, +/− 1.5 ML; Paxinos and Watson, 2009). To avoid placing the cannulas within the lateral ventricles, they were inserted at a 10 degree angle. 26-gauge guide cannula (Plastics One) were inserted just dorsal to the BNSTov (−6.0 DV) and cemented, with skull screws, to the skull. Dummy cannulas were inserted to prevent clogging. Rats received an analgesic (carprofen, 5 mg/kg, i.p.) and 5 ml of lactated ringer (s.c.). Animals recovered for one week before behavioral testing.

Behavior

On the first day, rats were habituated to the training context for 20 min. 24 hours later, rats were placed in a rodent conditioning chamber with a metal grid floor (Coulbourn Instruments). In Experiment 1, rats received 5 tone-shock pairings: CS = 5 kHz tone, 80dB, 30 sec; US = 0.5mA shock, 1 sec, tone coterminating with the shock. Five CS-US pairings were given to elicit sufficient contextual fear learning and robust freezing to the CS on testing day. In Experiments 2 and 3, rats received 1 tone-shock pairing such that animals receiving saline injections exhibited 50% freezing to the CS on testing day. This allowed us to measure increased fear learning in citalopram-treated animals, and was consistent with previous research (Ravinder et al., 2013; Burghardt et al., 2004). 24 hours later rats were placed in a different context with a black plexiglass floor washed with peppermint soap, different light placements and with walls made of a different material (either metal or clear plastic). They received 10 or 20 CS tones (30 sec duration; 60–120 sec inter-tone intervals). Animals which were tested for contextual fear conditioning were exposed to the conditioning chamber 24 hours after training for 10 minutes with no CS tones. Behavior was recorded by video camera and analyzed off-line. Time spent freezing to each CS or context (immobility with the exception of breathing) was manually scored for each animal by an observer blind to group assignment. At the end of behavioral experiments, animals were sacrificed by carbon dioxide inhalation, their brains removed and stored in 4% paraformaldehyde in phosphate buffer (PB). Brains were sectioned at a thickness of 100 μm. Nissl staining and light microscopy were used to verify cannula placements within the

amygdala. Animals with cannula placements outside the BNST were excluded from analysis.

Drug administration

Experiment 1: 15 min prior to fear conditioning training, animals received bilateral intra-BNST infusions of vehicle (0.5 μL/side; 0.9% sterile saline) or muscimol (4.4 nmol in 0.5 μL/side). This dose has been used previously to temporarily inactivate the BNST (Fendt et al., 2003). Experiment 2: 1 hour prior to fear conditioning training, animals received systemic injections of either 0.9% saline vehicle or citalopram hydrobromide (Sigma Aldrich) dissolved in vehicle (10mg/kg; i.p). Experiment 3: 1 hour prior to fear conditioning training, animals received systemic injections of either 0.9% saline vehicle or citalopram hydrobromide (Sigma Aldrich) dissolved in vehicle (10mg/kg; i.p). 15 minutes prior to fear conditioning training, animals received bilateral intra-BNST infusions of vehicle (40% DMSO and 60% saline solution, 0.25μL), or the 5-HT2C antagonist, RS-102221, 0.5μg per side in 0.25μl of a 40% DMSO and 60% saline solution. Solutions were infused at a rate of 0.1 μL/min through infusion cannula extending 1mm from the tip of the guide cannula that were attached to 1 μL Hamilton syringes with polyethylene tubing. The cannulae were left in place for 2 min after infusion to ensure the entire drug dose was delivered.

5-HT2C receptor and Arc dual-labeling experiments

Animals received systemic injections of either saline or citalopram as described above. 45 minutes after fear conditioning, animals were given an overdose of sodium pentobarbital (100 mg/kg), and then perfused transcardially with 0.9% saline and 4% paraformaldehyde in 0.1M phosphate buffer (PB). Tissue was post-fixed for 4 hours then transferred to a 20% sucrose solution. Tissue was sliced into 80 μM coronal sections using a Vibratome. Every other slice was collected, so there was no need to correct for double counting. 5 or 6 slices per animal were collected, covering the rostro-caudal extent of the BNSTov. Tissue was washed in 0.1M PB 3 times, then in PB with 1% Triton (PBT) 3 times for 5 min each wash. Slices were blocked in 1% bovine serum albumin (BSA; Sigma Fraction V, A-3059) in PBT for 1 hr and incubated for 48 hours at 4 degrees C in primary antibodies in BSA. Primaries used were a mouse monoclonal anti Arc/Arg3.1 antibody (1:250; Santa Cruz Biotechnology, sc-17839) and a goat polyclonal antibody against the 5HT2C receptor (1:1000; Santa Cruz Biotechnology, sc-15081). Slices were washed in PBT and incubated in secondary antibodies in PBT for 1 hr. Secondary antibodies used were donkey anti-mouse Alexa Fluor-488, and donkey anti-goat Alexa-594 (1:200; Life Technologies). Slices were washed in PBS, mounted on slides and coverslipped.

Microscopy

Double-labeling was assessed by confocal microscopy using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, NY) with LSM 510-META scanning confocal attachments. Stacked images were collected as 1 μm multitract optical sections. LSM 510 software (Carl Zeiss) was used to visualize doubly-labeled fluorescent slices at 25× and to capture images. Manual counting was performed on images of the BNSTov; 2 samples per oval nucleus were counted and summed together.

Specificity of antibodies

We used a goat polyclonal anti-5-HT2C receptor antibody (1:1000, Santa Cruz Biotechnology; sc-15081) that is directed toward a 19 amino acid sequence at the Nterminus of the receptor. This specific antibody does not react with the closely related 5-HT2A receptors, as 5HT2C knockout animals (in which the 5-HT2A receptors are intact) show no immunoreactivity of this antibody while 5-HT2A immunoreactivity is unaltered (Bubar et al., 2005). Primary antibody only and secondary antibody only controls contained a complete absence of specific labeling of cells. Arc protein expression was revealed using the anti-Arc/Arg3.1 antibody (mouse monoclonal; Santa Cruz Biotechnology, sc-17839) which has been used extensively to label Arc protein in the amygdala and BNST (Ploski et al., 2008; Ravinder et al., 2013).

Results

To confirm that the BNST does not normally contribute to the acquisition of either context or cued fear conditioning, rats were prepared with bilateral cannulas aimed at the BNST. After recovery, rats were habituated to the training context. 24 hours later, rats received local infusions of saline vehicle (0.5 μL/side; n=7) or muscimol (4.4 nmol in 0.5 μL/side; n=6). 30 minutes later, rats were presented with 5 CS tones each coterminating with a 1 sec US. A oneway repeated measures ANOVA revealed a main effect of tone ($F(4,44) = 27.1$, $p<0.001$); but no effect of drug, ($F(1,11) = 0.89$; $p=0.77$) or interaction ($F(4,44) = 0.16$; $p=0.96$). 24 hours later, animals were placed in the training context for 10 minutes. An unpaired Student's t-test revealed no significant difference in the amount of freezing to the training context 24 hours after training ($p=0.86$). 48 hours after training, animals were placed in a novel context and presented with 20 CS tones. A one-way repeated measures ANOVA revealed a main effect of tone ($F(19,209) = 5.52$, $p<0.001$); but no effect of drug, ($F(1,11) = 0.15$; $p=0.7$) or interaction ($F(19,209) = 1.36$; $p=0.15$). Thus, in agreement with previous lesion studies (LeDoux et al., 1988; Duvarci et al., 2009), temporary inactivation of the BNST prior to training had no effect on the acquisition of cued or contextual fear memories (Figure 1).

Acute administration of the SSRI fluoxetine enhances fear conditioning and Arc protein expression in the BNST (Burghardt et al., 2004; Ravinder et al., 2013). The SSRI citalopram also inhibits reuptake of serotonin from the synapse where it can interact with serotonin receptors. Thus, we determined whether neurons within the BNSTov showed increased Arc expression following citalopram administration and fear conditioning, and if those neurons also expressed the 5-HT2C receptor. 24 hours after habituation to the training context, animals were administered citalopram (10mg/kg, i.p. in saline) or saline. 1 hour later they received 1 CS-US pairing. 45 min later, they were perfused and their brains collected for immunohistochemistry (Figure 2A). Animals receiving citalopram (n=6) before conditioning had significantly higher levels of Arc protein expression than those receiving saline (n=5; $p<0.001$; Figure 2B). We then determined whether Arc-expressing neurons also expressed 5-HT2C receptors. The percentage of Arc-expressing BNSTov neurons which co-express 5-HT2C receptors in animals receiving citalopram was 74.3 ± 4.3% (Figure 2C–E).

To determine whether 5-HT2C receptors in the BNSTov mediate the enhancing effects of SSRIs on fear conditioning, we implanted bilateral cannulas aimed at the BNSTov. A week later, animals were habituated to the training context. 24 hours later, they were assigned to one of four groups (Figure 3A). Animals received a systemic injection of either saline vehicle or citalopram dissolved in saline (10mg/kg, i.p.) 1 hour prior to fear conditioning. 15 minutes prior to fear conditioning, animals received infusions of either vehicle (0.25μL; 80% saline, 20% DMSO) or the 5-HT2C receptor antagonist RS102221 (1 μg/side dissolved in 0.25μL 80% saline, 20% DMSO) such that four groups of animals were established (Figure 3A). Rats were then conditioned with one CS-US pairing (Figure 3B). A two-way ANOVA found no significant effects of the injection, BNST infusion or interaction on freezing during the 30 seconds before the CS (pre-CS) (Injection: $F(1,36)=0.21$; $p=0.65$; Infusion: $F(1,36) = 0.48$; $p=0.49$; Interaction: $F(1,36) = 0.004$; $p=0.95$) or to freezing to the CS (Injection: $F(1,36) = 0.02$; $p=0.89$; Infusion: $F(1,36)=0.2$; $p=0.66$; Interaction: $F(1,36) = 0.41$; $p = 0.53$). 24 hours later, animals received 10 CS tones (Figure 3C). A two-way repeated measures ANOVA revealed a significant interaction between systemic injections and local infusions ($F(1,36)=4.45$; $p<0.05$). Averaging freezing scores across all 10 tones, a one-way ANOVA across all four groups found a significant effect of group ($F(1,36) = 4.96$; $p<0.01$), with post-hoc tests revealing that the group receiving systemic citalopram and local infusions of saline showed significantly higher freezing levels than all other groups ($p<0.05$ for all comparisons; Bonferroni post-hoc tests). Thus, systemic injections of the SSRI citalopram enhance fear conditioning, which can be blocked by local infusions of 5-HT2C antagonists into the BNST.

Discussion

Consistent with earlier reports (LeDoux et al., 1988; Duvarci et al., 2009), we found that temporary inactivation of the BNST did not affect the acquisition of cued or context fear suggesting that the BNST contributes to the expression rather than the acquisition of contextual fear memories. Systemic injections of the SSRI citalopram did, however, enhance fear learning when given prior to fear conditioning. Citalopram also upregulated the expression of Arc protein within the BNSTov, and a majority of those cells (74%) expressed the 5-HT2C receptor, suggesting a role for this receptor in the behavioral effects of citalopram. Accordingly, intra-BNSTov infusion of a 5-HT2C receptor antagonist eliminated the effects of systemic citalopram injections. Together these results suggest that citalopram acts on a subset of neurons expressing the 5-HT2C receptor within the BNSTov to enhance fear memory consolidation.

Studies using electrolytic or neurotoxic lesions of the BNST have demonstrated that the BNST is not necessary for cued fear conditioning (LeDoux et al., 1988; Gewirtz et al.,1998; Sullivan et al., 2004; Duvarci et al., 2009). The timing of irreversible lesions or reversible inactivation of the BNST can reveal the contribution of this structure to either the acquisition and/or expression of contextual fear. If lesions are made *before* training (Duvarci et al., 2009; LeDoux et al., 1988), and contextual fear conditioning is impaired, it is unclear whether BNST activity was necessary for acquisition or expression of contextual fear. However if lesions or reversible inactivation are performed *after* training (Resstel et al., 2008; Sullivan et al,. 2004), the reduction in the amount of freezing to the conditioning

context suggests that BNST activity is necessary for the expression of fear memory. Here, we inactivated the BNST before training; the animals were tested drug free. Since there was no difference in freezing behavior on testing day, this suggests that BNST activity during training is not necessary for the learning or acquisition of contextual fear memory. However, it should be noted that more recent studies in which specific regions or cell types of the BNST are manipulated reveal that the BNST is functionally heterogeneous (Jennings et al., 2013; Kim et al., 2013). When activity of individual neurons within the BNST-AL is recorded during fear conditioning, a subset of neurons acquire inhibitory responses to a short-duration CS. These responses persist when animals are tested days later, at which time BNST-AM neurons have acquired excitatory responses to the CS (Haufler et al., 2013). Moreover, there are intrinsic BNST networks which could be disrupted by large infusions of muscimol (Turesson et al., 2013). Thus, while the data presented here are in agreement with past lesion and inactivation studies, future work should take into account the functional differences between adjacent regions of the BNST by making more targeted manipulations.

Acute SSRI administration before fear conditioning led to an upregulation of Arc protein in the BNSTov. The majority of these neurons express the 5-HT2C receptor. Within the BNST-AL, serotonin elicits complex postsynaptic responses (Guo et al., 2009), suggesting the existence of multiple networks within this structure. Although the primary response to serotonin is inhibitory within the anterior BNST, one specific set of neurons (Type III) containing 5-HT2C receptors depolarizes in response to serotonin (Guo et al., 2009). The majority of neurons activated by SSRIs and fear conditioning co-expressed 5-HT2C receptors, suggesting that SSRIs might preferentially act on this subset of BNSTov neurons. Interestingly, the Type III neurons containing 5-HT2C receptors also express the neuropeptide corticotropin-releasing factor (CRF; Dabrowska et al., 2011). CRF neurons within the BNST pay a major role in an animal's response to stressors (Lee and Davis, 1997; Sahuque et al., 2006; Dabrowska et al., 2013). Evidence suggests that activation of 5-HT2C receptors and CRF receptors in the BNST are both anxiogenic (Gibson et al., 1994; Campbell and Merchant 2003; Lee and Davis, 1997). Thus future work might determine whether manipulations of the BNST CRF system might mimic the effects of citalopram on fear conditioning.

There is evidence that SSRIs alone (in the absence of conditioning) can enhance activation of the BNST (Singewald et al., 2003). This could suggest that citalopram enhances baseline anxiety during conditioning, rather than recruiting the BNST into the fear learning circuit. However, we did not see increases in freezing behavior in these animals during the pre-CS or the CS period during training, nor during the pre-CS period during testing. This suggests that citalopram did not indiscriminately increase animals' baseline anxiety. Nevertheless, future studies should determine the extent to which active neurons within the BNST during acquisition become a permanent part of the memory trace by addressing whether the BNST is also active during memory recall.

It is unclear why citalopram might preferentially act on a specific BNST circuit, if the majority of BNST neurons express 5-HT receptors. However, it should be noted that there is a large body of literature demonstrating that SSRIs interact with specific subtypes of 5-HT receptors as well as non-5-HT receptors and ion channels (for review see Bianchi 2008).

Depending on the type of SSRI administered, the noradrenergic, dopaminergic and opioid systems can be modulated (Bymaster et al., 2002; Goodnick and Goldstein, 1998). These interactions suggest that SSRIs may differentially modulate fear learning and other behavioral paradigms.

Another possibility is that citalopram affects the activity of multiple BNST circuits, but only a subset of those interact with amygdalar circuitry. The central nucleus of the amygdala (CE) and the BNST are reciprocally connected to each other (Dong et al., 2001; Kretteck and Price, 1978). They share almost identical patterns of efferent targets including brainstem areas involved in responses to fear and anxiety, hypothalamic areas involved in autonomic motivation, the parabrachial nucleus and the substantia innominata (Radley et al., 2009; Dong et al., 2001; Petrovich and Swanson, 1999). BNST projections to the CE mostly originate in the BNST-AL and BNST-AM divisions (Sun and Cassell 1993; Dong et al., 2001; Dong and Swanson 2006). The current model of fear conditioning posits that short duration cues recruit CE circuits whereas long-duration cues recruit the BNST which then suppresses the CE (Lee and Davis, 1997; Walker et al., 2009). For there to be a seamless transition from the early to the late components of a sustained threat response, there must be some interaction and coordination between the two structures. The experiments here suggest that modulation of BNST activity by SSRI administration might alter the balance of this interaction. Recruitment of BNST networks during fear conditioning to short-duration CSs could then modulate fear memory consolidation. Overall, our findings suggest that the BNST can modulate fear memory formation and consolidation, specifically during acute SSRI administration. Future studies will be needed to address the precise role of specific subpopulations of BNST neurons in this effect and the connections between the BNST and amygdalar nuclei.

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Highlights

- BNST inactivation does not impair acquisition of cued or context fear conditioning
- SSRI injections enhance cued fear conditioning and Arc expression in the BNST
- The majority of these Arc expressing neurons express 5-HT2C receptors
- BNST infusions of 5-HT2C receptor antagonists block the effects of SSRIs on cued fear

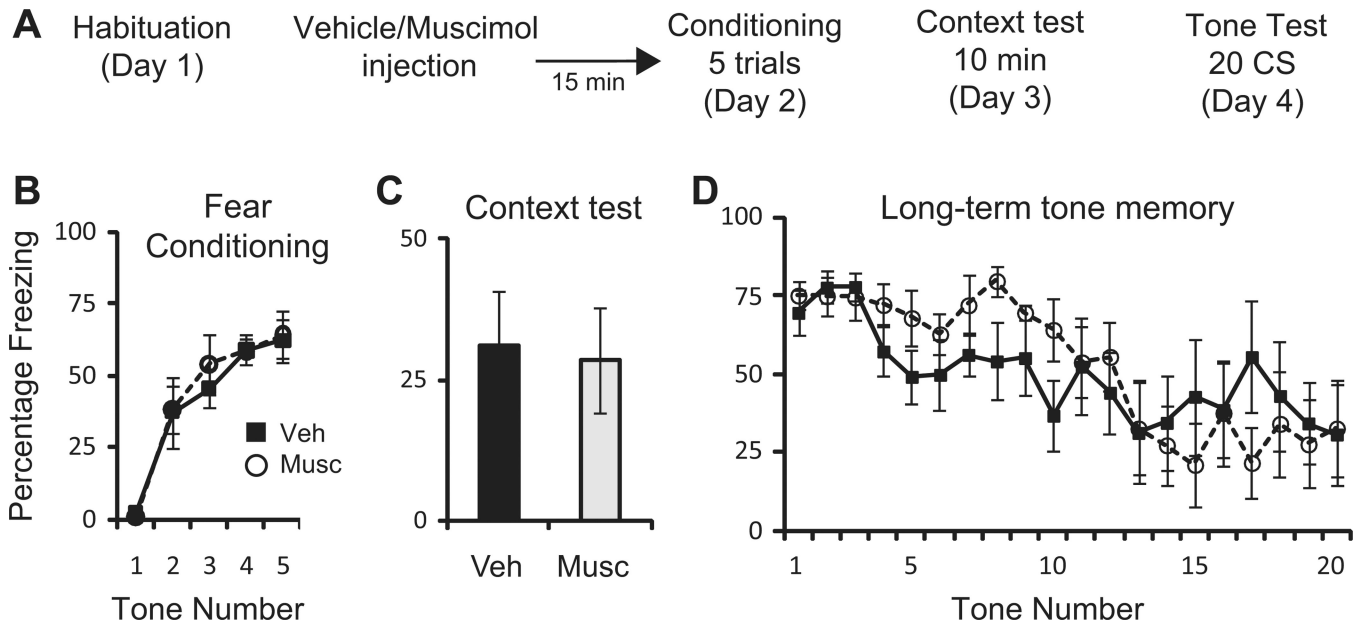

Figure 1.

Temporary inactivation of the BNST does not impair fear conditioning. A: Schematic of behavioral protocol. B: Mean ± SE percent freezing to 5 CS tones during fear conditioning in rats receiving saline vehicle (0.5 μL; n=7) or muscimol (4.4 nmol in 0.5 μL; n=6) 15 min before fear conditioning. C: Mean ± SE percent freezing to the training context for 10 min, 24 hours later. D: Mean ± SE percent freezing to 20 CS tones 48 hours after training.

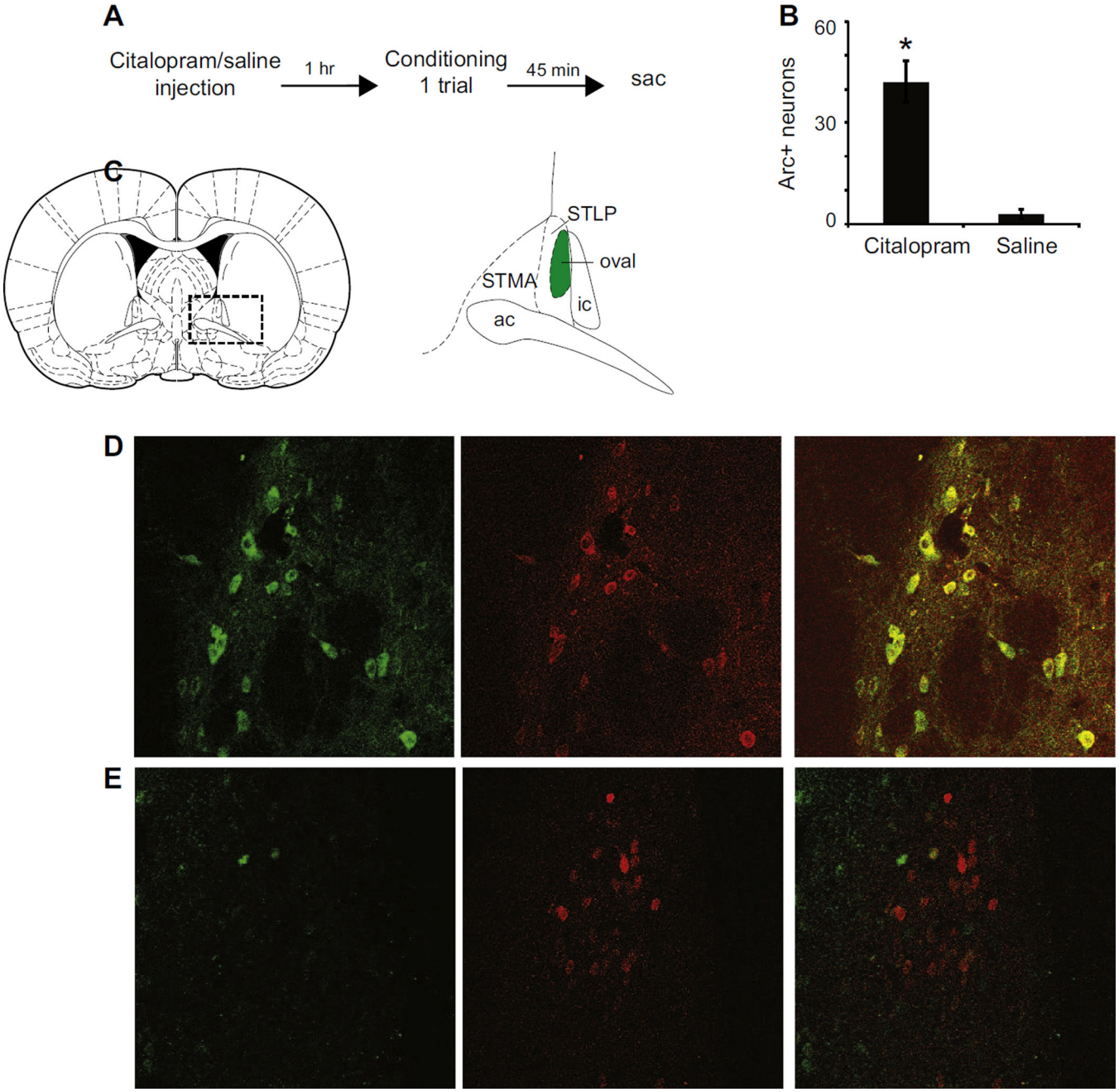

Figure 2.

Systemic citalopram injections increase Arc expression in 5-HT2C containing neurons of the BNSTov. A: Schematic of behavioral protocol. B: Quantification of Arc-expressing cells in the BNSTov following injection of citalopram (n=6) or saline (n=5) followed by fear conditioning, *P<0.001. C: Left, the dotted box shows the location of the BNST at 0.00 Bregma (left, Paxinos and Watson, 2009). Schematic of the principal anterior dorsal BNST subregions (right). ac: anterior commissure, ic: internal capsule, STMA: BNST medial-anterior, STLP: BNST lateral-posterior. All images and cell counts were taken from the shaded oval nucleus. D: Colocalization of Arc-expressing cells (green) and 5-HT2C

receptor-expressing cells (red). Merging of the green and red channels reveals overlap: 74.3 ± 4.3% of Arc-positive cells expressed 5-HT2C receptors. E: Animals receiving saline injections have few Arc-expressing cells, but many 5-HT2C receptor-expressing cells (red) and little overlap.

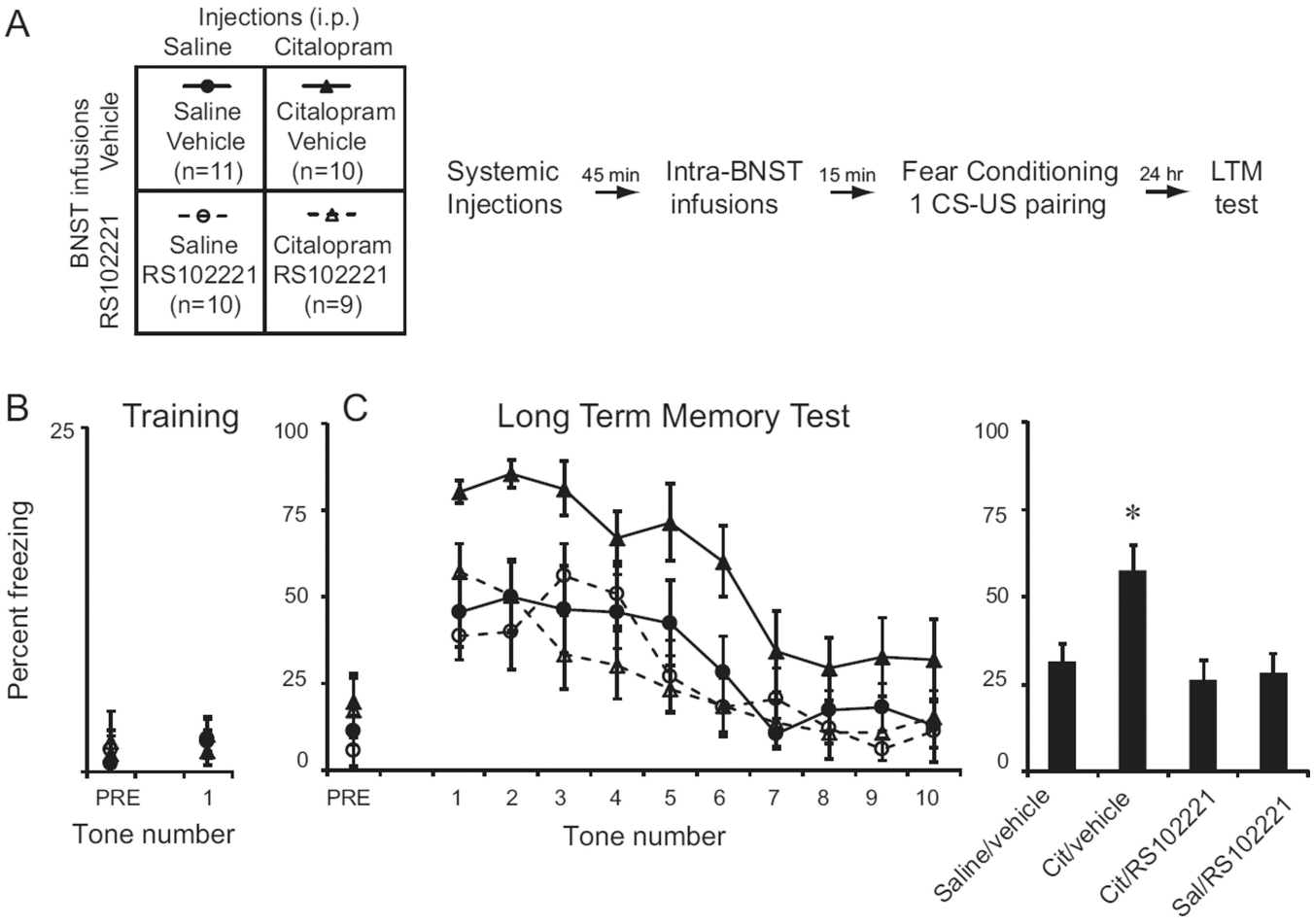

Figure 3.

Intra-BNST infusions of the 5-HT2C receptor antagonist RS-102221 reduce the enhancement of fear learning produced by systemic injections of the SSRI citalopram. A: Schematic of behavioral protocol. Animals received i.p. injections of either 0.9% saline or 10mg/kg citalopram 1 hr prior to fear conditioning and then intra-BNST infusions of vehicle or RS-102221 (1 μg/side) 15 min prior to fear conditioning. 24 hr later they were tested for long term memory (LTM). B: Mean ± SE percent freezing to the pre CS period (30 sec; PRE) and to the CS during fear conditioning. C: Mean ± SE percent freezing to the pre-CS and 10 CS tones 24 hr after training (left). Mean ± SE percent freezing of each group averaged across all 10 tones (right), *P<0.05.

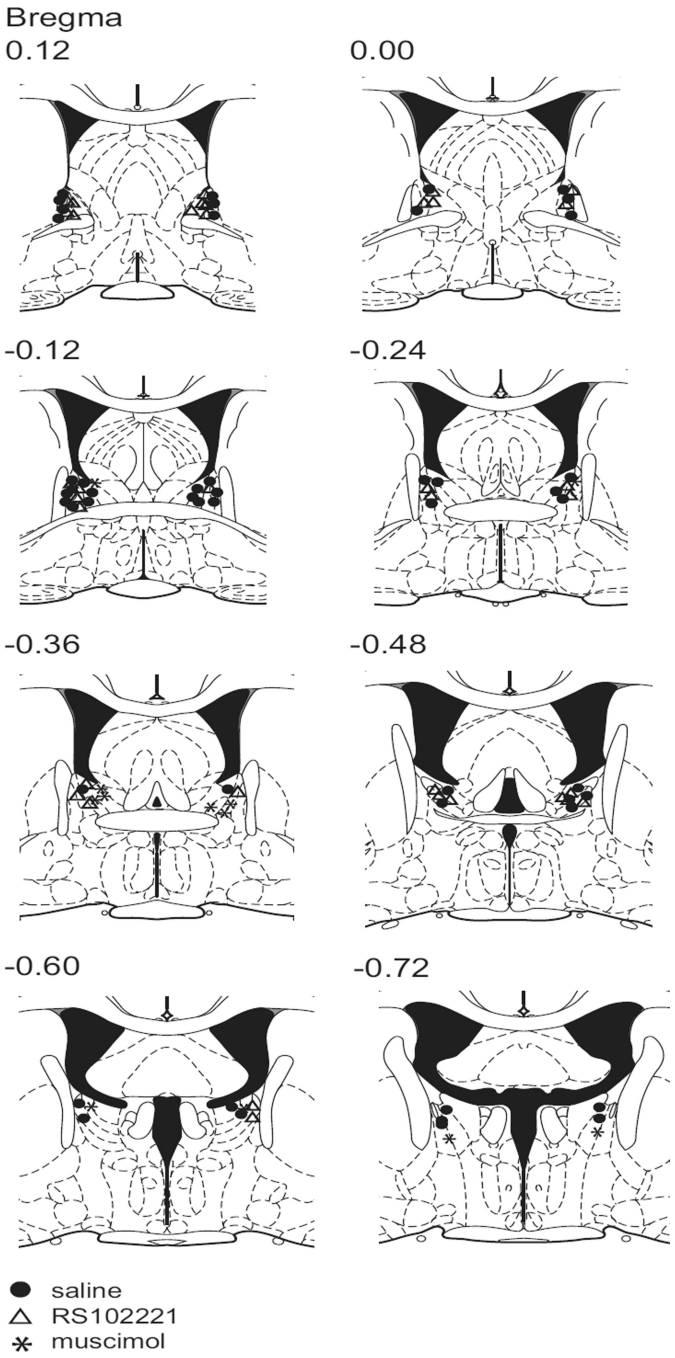

Figure 4.
Histological verification of cannula placements.