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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.

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed to treat anxiety disorders and depression (Kent, Coplan, & Gorman, 1998; van der Kolk et al., 1994). However, they paradoxically increase anxiety in humans when they are given acutely (Mir & Taylor, 1997), and can increase the risk of suicidal ideation (Teicher, Glod, & Cole, 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 (Bodnoff, Suranyi-Cadotte, Quirion, & Meaney, 1989; Dekeyne, Denorme, Monneyron, & Millan, 2000; Griebel, Cohen, Perrault, & Sanger, 1999).

Previous research has revealed that acute SSRI administration prior to fear conditioning enhances the consolidation of fear memories (Burghardt, Sullivan, McEwen, Gorman, & LeDoux, 2004; Ravinder, Burghardt, Brodsky, Bauer, & Chattarji, 2013). One advantage of using fear conditioning to investigate the actions of SSRIs is that it is a model of emotional learning for which the underlying neural circuitry has been characterized in great detail (Johansen, Cain, Ostroff, & LeDoux, 2011; Orsini & Maren, 2012; Pape & 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 & Williams, 1995).

Lesions of the BNST do not interfere with fear conditioning (LeDoux, Iwata, Cicchetti, & Reis, 1988; Sullivan et al., 2004). Instead, they disrupt the expression of longer "anxiety-like" states (Walker, Toufexis, & Davis, 2003). This has led to the idea that short duration cues (such as a 30 s tone) recruit amygdalar circuits, whereas long-duration cues, including contextual cues, recruit the BNST (Lee & Davis, 1997; Walker, Miles, & Davis, 2009). However, there is also evidence that BNST activity can modulate fear

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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, Nagy, & Pare, 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, Bush, McEwen, & LeDoux, 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, Graf, Anheuer, Modos, & Kantor, 2001; Singewald, Salchner, & Sharp, 2003). Conversely, 5-HT2C knockout mice show decreased anxiety (Heisler, Zhou, Bajwa, Hsu, & Tecott, 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.

2. Methods

2.1. Subjects

Adult male Sprague Dawley rats (Charles River Laboratories; 250–325 g) were housed individually with *ad libitum* access to food and water and maintained on a 12 h 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.

2.2. 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 & 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.

2.3. Behavior

On the first day, rats were habituated to the training context for 20 min. 24 h 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, 80 dB, 30 s; US = 0.5 mA shock, 1 s, 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 (Burghardt et al., 2004; Ravinder et al., 2013). 24 h 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 s duration; 60–120 s intertone intervals). Animals which were tested for contextual fear conditioning were exposed to the conditioning chamber 24 h after training for 10 min 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.

2.4. 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, Endres, & Apfelbach, 2003). Experiment 2: 1 h prior to fear conditioning training, animals received systemic injections of either 0.9% saline vehicle or citalopram hydrobromide (Sigma Aldrich) dissolved in vehicle (10 mg/kg; i.p). Experiment 3: 1 h prior to fear conditioning training, animals received systemic injections of either 0.9% saline vehicle or citalopram hydrobromide (Sigma Aldrich) dissolved in vehicle (10 mg/kg; i.p). 15 min prior to fear conditioning training, animals received bilateral intra-BNST infusions of vehicle (40% DMSO and 60% saline solution, 0.25 μL), or the 5-HT2C antagonist, RS-102221, 0.5 μg per side in 0.25 μL of a 40% DMSO and 60% saline solution. Solutions were infused at a rate of 0.1 μL/min through infusion cannula extending 1 mm 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.

2.5. 5-HT2C receptor and Arc dual-labeling experiments

Animals received systemic injections of either saline or citalopram as described above. 45 min 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 0.1 M phosphate buffer (PB). Tissue was post-fixed for 4 h 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 0.1 M 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 h and incubated for 48 h at 4 °C in