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Pharmacological depletion of serotonin in the basolateral amygdala complex reduces anxiety and disrupts fear conditioning

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Abstract

The basolateral and lateral amygdala nuclei complex (BLC) is implicated in a number of emotional responses including conditioned fear and social anxiety. Based on previous studies demonstrating that enhanced serotonin release in the BLC leads to increased anxiety and fear responses, we hypothesized that pharmacologically depleting serotonin in the BLC using 5,7-dihydroxytryptamine (5,7-DHT) injections would lead to diminished anxiety and disrupted fear conditioning. To test this hypothesis, 5,7-DHT (a serotonin-depleting agent) was bilaterally injected into the BLC. Desipramine (a norepinephrine reuptake inhibitor) was systemically administered to prevent non-selective effects on norepinephrine. After 5 days, 5-7-DHT-treated rats showed increases in the duration of social interaction (SI) time, suggestive of reduced anxiety-like behavior. We then used a cue-induced fear conditioning protocol with shock as the unconditioned stimulus and tone as the conditioned stimulus for rats pretreated with bilateral 5,7-DHT, or vehicle, injections into the BLC. Compared to vehicle-treated rats, 5,7-DHT rats had reduced acquisition of fear during conditioning (measured by freezing time during tone), also had reduced fear retrieval/recall on subsequent testing days. Ex vivo analyses revealed that 5,7-DHT reduced local 5-HT concentrations in the BLC by ~40% without altering local norepinephrine or dopamine concentrations. These data provide additional support for 5-HT playing a critical role in modulating anxiety-like behavior and fear-associated memories through its actions within the BLC.

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Keywords

Anxiety; Fear; Dorsal raphe; 5-ht; Sert; Amygdala; 5,7-dht

1. Introduction

Serotonin (5-HT) plays a critical role in regulating adaptive stress responses to aversive stimuli and is strongly implicated in stress-related anxiety disorders including post-traumatic stress disorder and panic disorder. Serotoninergic neurotransmission is a major therapeutic target for treating these disorders [see review (Hale et al., 2012)]. Yet, serotonin regulation of anxiety and fear-associated behaviors and associated autonomic and endocrine responses to stressful stimuli is complex in due to functional heterogeneity among subpopulations of serotonergic neurons and the large number of serotonin receptors; in addition, serotonin's effects on physiological and behavioral responses to aversive stimuli appear to depend on the brain region where it is released (Hale et al., 2013).

One area where serotonin plays an important role in modulating anxiety and fear responses is the basolateral amygdala complex (BLC; which includes the basolateral and lateral nuclei). The BLC is highly responsive to stressful stimuli (Brydges et al., 2013; Butler et al., 2011; Henderson et al., 2012; Johnson et al., 2008; Singewald et al., 2003) and plays a critical role in fear conditioning, which is critical for survival [see reviews (Johansen et al., 2011; Johansen et al., 2012)]. Serotonergic neurons located in the brainstem dorsal and median raphe nuclei project to the amygdala, hippocampus, and ventromedial prefrontal cortex (PFC). Within the BLC, extracellular levels of 5-HT increase rapidly during conditioned fear (Zanoveli et al., 2009) and following exposure to inescapable stress (Amat et al., 1998). Following inescapable stress the increase in extracellular 5-HT is prolonged relative to either escapable stress or restraint stress, and remains elevated 100% above escapable stress or restraint stress controls for 24 h. The persistent increases in extracellular 5-HT concentrations within the amygdala following stress may contribute to a net loss of local GABA inhibition and subsequent increase in excitation of glutamatergic projection neurons. In support of this, serotonin acutely increases GABAergic tone in the BLC by exciting local GABAergic interneurons via the postsynaptic 5-HT_{2A} receptors (Jiang et al., 2009; McDonald and Mascagni, 2007; Rainnie, 1999), but stress downregulates the 5-HT_{2A} receptor and reduces serotonin's effects on local GABAergic tone (Jiang et al., 2009). In general, increases in the excitability of amygdala glutamatergic projection neurons lead to enhanced fear conditioned behavior, so, stress-induced downregulation of 5-HT_{2A} receptors, loss of GABAergic tone, and disinhibition of glutamatergic projection neurons should also enhance fear conditioning. This hypothesis is supported by work done by Bosker and Ravinder where a single systemic injection of serotonin reuptake inhibitor in rats increases extracellular 5-HT in the amygdala by ~150% (Bosker et al., 2001) and also enhances acquisition of fear associated freezing responses, and increased fear conditioned freezing responses (Ravinder et al., 2013). In contrast, reduction of 5-HT tone in the amygdala using the serotonin neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) reduces conflict anxiety (Sommer et al., 2001), but little is known about how the depletion of 5-HT affects acquisition of fear conditioning.

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In the present article, we hypothesized that chronic reduction in serotonergic tone within the BLC region would severely disrupt both the acquisition of conditioned fear, as well as extinction and extinction recall responses. In order to reduce serotonin tone within the BLC, we used 5,7-DHT, which, although the mechanism is not entirely clear, reproducibly depletes local 5-HT by up to 90% in forebrain structures such as the amygdala (Bjorklund et al., 1975; File et al., 1979; Sommer et al., 2001; Tran et al., 2013). To test this hypothesis, we bilaterally injected 5,7-DHT into the BLC to chronically reduce local serotonergic neurotransmission, then assessed anxiety-like behavior and conditioned fear responses, and validated depletion of local 5-HT ex vivo. Since 5,7-DHT has been shown to also reduce local norepinephrine levels at higher doses, a norepinephrine reuptake inhibitor (desipramine) was administered systemically since it has been shown to block this effect (Bjorklund et al., 1975), and norepinephrine, and dopamine were also assessed to confirm that the depletion was specific to 5-HT.

2. Methods and materials

2.1. Animals

All experiments were conducted on adult male Wistar rats (300–325 g), which were purchased from Harlan Laboratories and were housed individually in plastic cages under standard environmental conditions (22 °C; 12/12 light/dark cycle; lights on at 7:00 A.M.) for 7–10 days prior to the surgical manipulations. Food and water were provided *ad libitum*. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, 2011) and the guidelines of the IUPUI Institutional Animal Care and Use Committee.

2.2. Microinjection of 5,7-DHT or vehicle into the BLC

Rats were anesthetized by placing them in a closed Plexiglas® box that was connected to an isoflurane system (MGX Research Machine; Vetamic, Rossville IN, USA) and then with a nose cone connected to the same system during the stereotaxic surgery and during intra-BLC injections of 5,7-DHT or vehicle. Rats were placed into a stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA) with the incisor bar set at − 3.3 mm and nose cone connected to the same system during the surgery. A 33 gauge injector (Plastics One) was lowered into position of the BLC using the following coordinates relative to bregma: anterior, −2.1 mm; lateral, ±5.0 mm; ventral, −8.5 mm, according to a standard stereotaxic atlas of the adult rat brain (Paxinos and Watson, 1986).

The serotonergic toxin 5,7-DHT was used to deplete serotonin within the BLC region. Thirty minutes prior to the injections of 5,7-DHT or vehicle into the BLC, all animals were systemically (i.p.) pretreated with 25 mg/kg of the norepinephrine reuptake inhibitor desipramine (Sigma-Aldrich, St. Louis, MO, USA, dissolved in 0.9% saline). Rats then either received bilateral injections of 5 μg/μl of 5,7-DHT (Sigma-Aldrich; 100 nl per side) or a saline vehicle with 0.1% ascorbic acid. The open-field and social interaction tests were performed 6 days post-BLC injections, and the conditioned fear protocol started on day 7.

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2.3. Open-field behavior test

The open-field arena covered an area of 90 cm × 90 cm, with 40 cm high walls. The open-field arena was divided into a 6 × 6 grid of equally-sized squares using black tape (36 total squares) with 4 squares forming the center; 12 squares forming the middle perimeter; and 20 squares forming the outer perimeter. The test started by placing a rat in the center. The behavior of each rat in the open-field arena was recorded on video and scored afterwards using Anymaze Software (Stoelting, Woods Dale, IL, USA).

2.4. Social interaction test

Anxiety-like behavior was measured utilizing the SI test (File, 1980) that was further modified and validated measure of anxiety-associated behaviors (Sanders and Shekhar, 1995) and is sensitive to current pharmacological treatments for anxiety disorders [acute benzodiazepine (Johnson et al., 2010) and chronic selective serotonin reuptake inhibitor (SSRI) treatments (Lightowler et al., 1994)]. The apparatus consists of a solid wooden box with an open roof approximately 0.9 m long × 0.9 m wide with walls 0.3 m high. A video camera was fixed above the box, and all behavioral tests were videotaped under low red light conditions (approximately 100 lx) and in a familiar environment. The “experimental” rat and an unfamiliar “partner” rat are both placed individually in the center of the box and allowed to habituate to the environment for a 5-minute period 24 h prior to each SI test. During the SI test, the two rats are placed together in the center of the box, and the total duration (sec) of non-aggressive physical contact (grooming, sniffing, crawling over and under, etc.) initiated by the “experimental” rat is quantified over a 5-minute duration. Videotaped sessions were scored at a later time by an investigator (Stephanie Fitz), who was blind to any drug treatment.

2.5. Fear conditioning protocol

The fear-conditioning chamber has a grid floor composed of 6 stainless steel rods connect to a shock generator (Kinder Scientific, Poway, CA, USA). The fear conditioning protocol was 4 days long and was implemented 7 days after 5,7-DHT or vehicle injections and was finished on day 11. On day 7, rats were placed in the conditioning chamber and allowed to habituate for 10 min. On day 8, test day 1, the rats were placed back in the conditioning chamber and underwent 10 trials, using a 120 s inter-trial interval, of a tone conditioned stimuli (CS: 80 dB, 20 s) co-terminating with a single shock unconditioned stimuli (UC: 0.80 mA, 500 ms). On day 9, test day 2, the rats where given 10 trials, using a 120 s inter-trial interval, of tone (CS only). On day 10, test day 3, rats underwent an extinction paradigm of 40 trials, using a 120 s inter-trial interval, of tone CS only. All sessions were video-recorded and the total time spent freezing during the tones on all 3 test days was scored blind by the investigator Stephanie Fitz.

2.6. Ex vivo processing of brain tissue for later HPLC analyses and immunohistochemistry

2.6.1. Experiment 1—After the final behavior test on day 11, all rats were anesthetized with isoflurane and decapitated; their brains were then removed and frozen and were coronally sectioned at 300 μm on a Leica cryostat for verification of the cannulae placement then later stored in a −80 °C freezer.

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2.6.2. Experiment 2—After the final behavior test on day 11, the rats were anesthetized with isoflurane, then perfused transcardially with 0.05 M phosphate buffered saline (PBS; 250 ml), followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; 250 ml). Brains were removed and post-fixed for 24 h in the same fixative, rinsed for 24 h in 0.1 M PB, then placed in cryoprotectant (30% sucrose in 0.1 M PB) for an additional 4–5 days. To maintain a consistent plane for coronal sections brains were placed in a rat brain matrix (ASI Instruments, Model No. RBM-4000C, Warren, MI, USA) and cut with a razor blade at the caudal border of the mammillary bodies. Brains were frozen in a beaker of liquid isopentane pre-cooled by surrounding the beaker with dry ice. Serial coronal sections (30 μm) were cut using a cryostat and were immediately placed in cryoprotectant consisting of 27% ethylene glycol and 16% glycerol in 0.05 M PB to yield six alternative sets of sections. Sections were stored at −20 °C until immunohistochemical processing for the serotonin transporter. All solutions had a pH of 7.4.

2.7. High performance liquid chromatography with electrochemical detection (HPLC-ED) sample analysis of 5-HT, norepinephrine and dopamine in BLC and 5-HT in DRN

In Experiment 1, the 300 μm coronal brain sections were placed on an inverted glass petri precooled with dry ice placed underneath dish. The BLC and central amygdala (CeA) region −3.00 mm from bregma (rostral side of section) and dorsal raphe nucleus (DRN) −8.00 mm bregma (rostral side of section) were respectively micropunched with Harris Aluminum Micro-Punches with a tip diameter of 2.0 mm and 1.0 mm (cat. no. 15089-4, Ted Pella, see red dashed circle in Fig. 1a) and placed into 1 ml Epindorph tubes with 5 ml of 0.1 N perchloric acid then stored at −80 °C, were analyzed for 5-HT, norepinephrine, and dopamine content using HPLC/EC, as previously described (Li et al., 1998) with modifications. Samples were loaded into a 5 ml sample loop and injected onto an analytical column (BDS Hypersil C18, 3 mm, 2 × 150 mm; Thermo Fisher Scientific, Waltham, MA) with a mobile phase consisting of: 50 mM sodium phosphate, 0.1 mM EDTA, 400 mg/L sodium octyl-sulfate, and 10% methanol at pH 6.0. Monoamines were oxidized at 350 mV using an amperometric detection system (Decade II detector with VT-03 ISAAC cell; Antec Leyden, Boston, MA) at a sensitivity setting of 0.1 nA/V. Output from the detector was analyzed with a computer program (ChromPerfect, Justice Innovations, Inc., Palo Alto, CA), and levels were determined by comparison with a standard curve.

2.8. Immunostaining the serotonin transporter in the BLC

In Experiment 2, immunostaining for the serotonin transporter (SERT) was done on the 30 μm coronal brain sections of perfused rats to determine if 5,7-DHT had effectively lesioned local serotonergic fibers and terminals. Immunostaining for SERT using a primary antibody directed against SERT (rabbit anti-SERT polyclonal serum antibody, cat. no. 24,330, Immunostar, Hudson WI, USA; diluted 1:1500). Free-floating sections were washed in 0.05 M PBS for 30 min, then incubated in 1% H_2O_2 in PBS for 20 min. Sections were then washed 10 min in PBS and 20 min in PBS with 0.3% Triton X-100 (PBST). Sections were then incubated 12-16 h in PBST with primary antibody solution at room temperature. After a 30-minute wash in PBST, sections were incubated 2 h in the biotinylated swine anti-rabbit IgG secondary antibody (cat no. BA-1000, Vector Laboratories, Burlinghame, CA, USA; diluted 1:500). Sections were washed again for 30 min in PBST then incubated 1.5 h in an

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avidin-biotin complex provided in a standard Vector Elite kit (cat no. PK-6100, Vector Laboratories; diluted 1:500). Substrates for chromogen reactions were SG (SK-4700, Vector Laboratories, Burlingame, CA, USA) in PBS containing 0.003% H_2O_2, pH 7.4. Substrate reactions were run for 15 min. All sections were mounted on clean glass slides, dried overnight, dehydrated and mounted with coverslips using DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA). All washes and incubations were done in 12-well polystyrene plates with low frequency shaking on an orbital shaker.

2.8.1. Photography—Photomicrographs were obtained using a Leica brightfield microscope using N plan 5×, 10×, 20× and 40× objective lenses (model DMLB, Leica Microsystems, Buffalo Grove, IL, USA), a SPOT digital camera (RT color, Diagnostics Instruments Inc., Sterling Heights, MI, USA) and SPOT 4.0.6 for Windows digital imaging software (Silicon Graphics, Mountain View, CA, USA) or a Nikon 90i microscope and a Nikon DS-Fi1 digital camera with NIS Elements 3.00 imaging software (A.G. Heinze Inc., Lake Forest, CA, USA). Photographic plates were prepared in CorelDraw 11.633 for Windows (Eden Prairie, MN, USA).

2.9. Statistical analyses

The following dependent variables were analyzed using a two-tailed independent Student's *t*-test (open-field, social interaction, and 5-HT, norepinephrine, and dopamine concentrations). Fear conditioned freezing behavior was analyzed using a one way ANOVA with repeated measures with *drug treatment* as main factor and time as the repeated measures. In the presence of significant main effects, between-subjects post-hoc tests were conducted using two-tailed independent Student's *t*-tests. Statistical significance was accepted with p 0.05. All statistical analyses were carried out using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and all graphs were generated using SigmaPlot 12.0 for Windows (SPSS Inc.) and figure-plate illustrations were done using CorelDraw version 12 for Windows.

3. Results

3.1. Open-field behavior and social interaction test

Rats receiving intra-BLC injections of vehicle (n = 8) or 5,7-DHT (n = 11) did not show differences in general locomotor associated behaviors (i.e., distance traveled, $t_{(16)} = -0.7$, $p = 0.477$) in the open-field (one less n for vehicle group due to malfunctioning video). Although the 5,7-DHT-treated rats did not show a preference for spending more time in the center regions of the open-field (center time, $t_{(7)} = 0.5$, $p = 0.630$; data not shown), they did show an increase in social interaction, compared to vehicle-treated controls $t_{(17)} = -2.8$, $p = 0.012$ (Fig. 1a).

3.2. Fear conditioning behaviors

On acquisition day all rats displayed increased freezing over time with repeated pairings of the conditioned stimulus (tone) with the unconditioned stimulus (shock). However, 5,7-DHT-treated rats had reduced acquisition of fear on test day 1, which was evidenced by their freezing ~50% of duration of the tones 3–5, compared to vehicle-treated rats, which displayed ~80–95% freezing during tones 3–5 (*treatment × time effect,* $F_{(4,68)} = 6.5$, $p <$

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0.001, Fig. 1b). On test day 2 (evidence of consolidation), there was a significant *treatment × time effect,* $F_{(4,68)} = 3.0$, $p = 0.024$ detected, with 5,7-DHT treated rats showing ~20–30% less freezing during tones 1–5 (Fig. 1c). On test day 3, the fear recall was ~80% freezing in the control rats and markedly reduced to ~40% freezing in the 5,7-DHT treated rats (*treatment × time effect,* $F_{(19,323)} = 6.6$, $p < 0.001$, Fig. 1d).

3.3. Histological verification of cannulae placements

Histological verification of injection site location was done on 300 μm coronal brain sections as they were being sectioned. The distribution of injection sites was done using a Leica Stereozoom microscope at 10× magnification. The injection sites from *Experiment 1* were located within the BLC complex of all vehicle-treated rats, and all 5,7-DHT-treated rats except one, which had one injection site in the BLC and one on the BLC/CeA border. The injection sites from *Experiment 2* were located within the BLC complex of all vehicle-treated rats except one unilateral injection in the CeA, and all 5,7-DHT treated-rats except two, which had unilateral injections into the CeA. The behaviors in those rats did not differ significantly from other vehicle or 5,7-DHT rats so were included in the final analyses. All cannula placements are illustrated on a coronal brain section from a standard rat stereotaxic atlas (Paxinos and Watson, 1997) in Fig. 1e (for neurochemical data in Fig. 1g) and 1f (for immunohistochemical data in Fig. 1h).

3.4. Effects of 5,7-DHT injections into the BLC on local 5-HT concentrations and terminal fields

3.4.1. Experiment 1—Using HPLC detection of 5-HT, norepinephrine, and dopamine concentrations in 2.0 mm diameter micropunches of the BLC/CeA area, in 300 μm-thick coronal brain sections, we determined that 5,7-DHT injections into the BLC/CeA area (see Fig. 1e) reduced local concentrations of 5-HT by ~40% ($t_{(6)} = 1.9$, $p = 0.050$), but did not alter local norepinephrine ($t_{(6)} = 3.8$, $p = 0.714$) or dopamine ($t_{(5)} = 0.6$, $p = 0.641$) concentrations, or 5-HT concentrations in the dorsal raphe nucleus ($t_{(5)} = 0.7$, $p = 0.525$) (Fig. 1g).

3.4.2. Experiment 2—Contrary to some other reports, following 5,7-DHT injections into the BLC/CeA area (see Fig. 1f) we did not observe obvious loss of serotonergic fibers or terminals in the BLC region when assessing SERT immunostaining in the BLC region (Fig. 1h).

4. Discussion

Here we show that depletion of serotonin within the BLC using 5,7-DHT decreased anxiety-associated behaviors in a social interaction test, but also reduced acquisition of cue-induced fear conditioned freezing (as well as an expected proportionate reduction in recall during extinction sessions). In *Experiment 1, ex vivo* analyses of microdissected tissue revealed that 5,7-DHT reduced local 5-HT concentrations in the BLC/CeA by ~40% without altering local norepinephrine or dopamine concentrations, or 5-HT concentrations in the DRN. The level of 5,7-DHT-induced depletion of 5-HT in the amygdala ranged from 40 to 80% depletion, which is very consistent with other published studies using this technique in the

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amygdala (File et al., 1979; Izumi et al., 2012; Sommer et al., 2001; Tran et al., 2013). Our more modest reduction of 5-HT in the BLC/CeA are most likely due to our decision to include a larger diameter to capture the BLC and the CeA since one 5,7-DHT injection was located on the BLC/CeA border. Yet in *Experiment 2*, the 5,7-DHT injections into the BLC did not produce any clear evidence of loss of SERT immunoreactive fibers. Some authors have reported that 5,7-DHT does produce site-specific destruction of serotonergic terminals, yet this may depend on the doses used (we used 5 μg per side, but others have used from to 4-16 μg 5,7-DHT), or the timing of tissue assessment post-5,7-DHT injections (we assessed this at 11 days post-injection, but others whom have assessed this 2 weeks post-injection have observed significant decreases in SERT binding or SERT-immunoreactive fibers (Sommer et al., 2001; Lieben et al., 2006; Tran et al., 2013). Collectively, our data suggest that our low dose and shorter timeline for assessing the lesions was long enough to show local reductions in 5-HT concentrations and disrupted fear conditioned behavioral responses, but not long enough to observe a significant loss of SERT-immunoreactive fibers.

In 2012, Izumi and colleagues conducted a contextual fear conditioning study using injections of a higher dose of 5,7-DHT (8 μg per side) into the amygdala. In these studies, injections of 5,7-DHT were done 3 days after a contextual fear conditioning paradigm where the rats received 3 days of repeated footshock (no tone pairings) and freezing was assessed 2 weeks after 5,7-DHT injection for 5 consecutive days when placed in the same footshock box. In this study, they show a depletion of 5-HT (but not catecholamines) in the amygdala 14 days after injection. The depletion of serotonin in the amygdala following contextual fear conditioning reduced later recall of fear-associated freezing (Izumi et al., 2012). Collectively, our results alongside Izumi's study provide evidence that 5-HT in the amygdala plays a role in both threat learning and threat recall. The decrease in anxiety-associated behaviors after 5,7-DHT injections into the amygdala in our experiments is consistent with File and colleagues, who also observed anxiolytic-like behaviors in a SI test following intra-amygdala 5,7-DHT injections (File et al., 1981). Overall, these data demonstrate that 5-HT plays a critical role in the regulation of anxiety states and threat memory acquisition/recall through actions within the BLC, and that disruption of serotonergic activity in the amygdala contributes to aberrant anxiety states and fear memory.

The vast majority of forebrain projecting serotonergic cell bodies are localized in the dorsal (DRN) and median (MnR) raphe nuclei. Neurons within these regions send projections to the various parts of the amygdala, including BLC (Vertes, 1991). The specific origin of serotonergic fibers in the BLC primarily originate from the midline DRN, with far fewer projections originating in the MnR [evidenced with the retrograde tracer cholera toxin B (CTB) injections into the BLC region and CTB + *tryptophan hydroxylase* double immunohistochemistry co-localization in the brainstem raphe (Hale et al., 2008)]. Consistent with the DRN and MnR being the origin of serotonergic fibers in the BLC, File and colleagues showed that 5,7-DHT injections into the DRN/MNR led to marked 44% depletion of 5-HT in limbic regions (e.g., hippocampus) and significantly increased social interaction scores (File et al., 1979). Yet, although the effects of depleting 5-HT in the BLC produces consistent anxiolytic effects and diminished threat learning in the experiments conducted here, exactly how 5-HT release in the BLC increases anxiety and enhances threat

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learning is complex. Factors that contribute to this are: 1) the amygdala contains all of the 5-HT receptor subtypes (5-HT_{1-7}); and 2) these receptors mediate both excitatory and inhibitory actions of 5-HT and some receptor subtypes are expressed on both GABAergic interneurons and glutamatergic projection neurons (McDonald and Mascagni, 2007). Application of 5-HT in the BLC region initially produces inhibitory responses by depolarizing GABAergic interneurons, and leads to increased inhibition of excitatory pyramidal neurons (Rainnie, 1999). However, there is evidence that stress-related conditions leading to repeated or prolonged release of 5-HT can lead to loss of local inhibition. For example, extracellular levels of 5-HT increase rapidly in the BLC during conditioned fear (Zanoveli et al., 2009) and during exposure to inescapable stress (Amat et al., 1998) and if this stress is chronic (i.e., inescapable stress, but arguably also occurring during fear conditioning paradigms), 5-HT concentrations remain high in the BLC, which appears to lead to a net loss of local GABA inhibition and subsequent increase in excitation of glutamatergic projection neurons. As mentioned in the introduction, this is support by studies showing that serotonin increases GABAergic tone in BLC by exciting local GABAergic interneurons via the postsynaptic 5-HT_{2A} receptor (Jiang et al., 2009; McDonald and Mascagni, 2007; Rainnie, 1999), but stress downregulates the 5-HT2A receptor and reduces serotonin's effects on local GABAergic tone (Jiang et al., 2009). In general, this could lead to net increases in the excitability of amygdala glutamatergic projection neurons, leading to enhanced fear conditioned behavior, so an overall increase in local 5-HT levels should also enhance fear conditioning. This hypothesis is supported by work done by Bosker and Ravinder, where a single systemic treatment with serotonin reuptake inhibitor treatment in rats increased extracellular 5-HT in the amygdala by ~150% (Bosker et al., 2001) and also enhanced acquisition of fear associated freezing responses, and increased fear conditioned freezing responses (Ravinder et al., 2013). Moreover, acute systemic injection of the SSRIs citalopram or fluoxetine, which increases 5-HT concentrations in the brain, including amygdala (Bosker et al., 2001), administered prior the training enhances the acquisition of auditory fear conditioning (Burghardt et al., 2004; Ravinder et al., 2013). Acute treatment with SSRIs also enhances fear-potentiated startle in humans (Grillon et al., 2007). Finally, complete loss of the SERT gene throughout development (i.e., $SERT^{-/-}$ knockout) produces rats that are anxious at baseline (Olivier et al., 2008). The loss of the SERT disrupts clearance of 5-HT in the CNS, which is evidenced by high baseline concentrations of extracellular 5-HT in limbic regions such as the hippocampus (Homberg et al., 2007; Olivier 2008). Within the BLC, the net effect is that $SERT^{-/-}$ rats have reduced local inhibition, which leads to enhanced evoked action potentials on local glutamatergic projection neurons (Johnson et al., 2012). This loss of inhibition in the BLC most likely contributes to high baseline anxiety (Olivier et al., 2008), enhanced threat learning, and resistant extinction of fear conditioned freezing behaviors (Johnson et al., 2012).

5. Conclusions

The present data, in combination with data showing that pharmacologically increasing 5-HT with SSRIs enhances fear conditioning in rodent and in humans, further support an important role for 5-HT in the modulation of anxiety-like behavior and fear-associated

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memories through its actions within the BLC. Furthermore, our data are consistent with previous experiments where increasing or depleting 5-HT levels in the BLC region respectively enhances or diminishes fear conditioned behaviors. These data provide the first evidence showing the impairment of fear acquisition due to reduced 5-HT levels within the BLC. These data are also supportive of the hypothesis that increased 5-HT activity within the amygdala may be an important mechanism in the pathophysiology of PTSD (Wellman et al., 2007; Zanoveli et al., 2009).

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Abbreviations

5-HT	serotonin
5,7-DHT	5,7-dihydroxytryptamine
BLC	basolateral and lateral amygdala nuclei complex
CeA	central amygdala
HPLC-ED	High performance liquid chromatography with electrochemical detection
SERT	serotonin transporter

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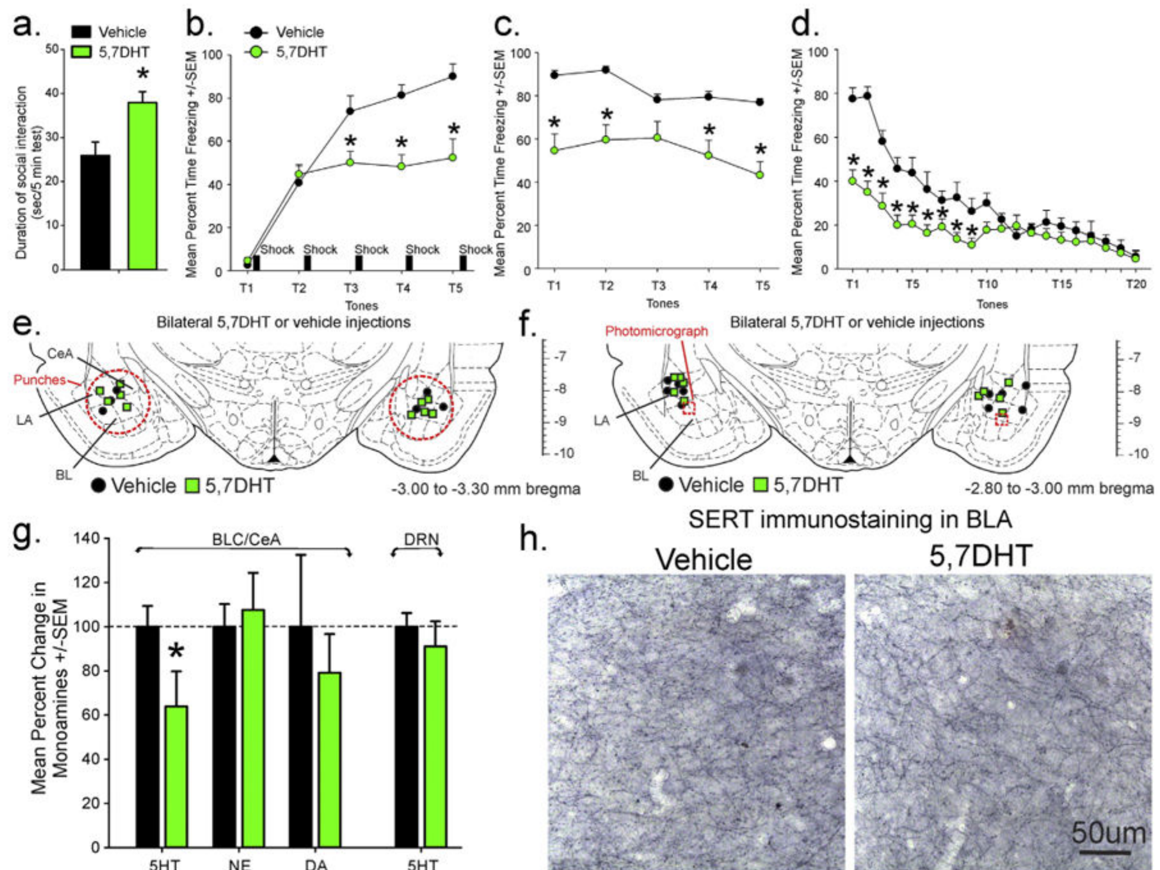

Fig. 1.

a) Bar graph illustrates social interaction (SI) time for each treatment group (n = 8,11). Line graphs in b–d) represent freezing behaviors during standard fear conditioning protocol using tone as the conditioned stimulus and shock as the unconditioned stimulus on b) acquisition day, and c) tone only on day 2 for evidence of consolidation, and d) tone only on day 3 for recall and extinction. Data are presented as means ± SEM. *, represent significant difference with an independent 2-tailed Student's *t*-test, *p* 0.05 for bar graphs and an independent 2-tailed Student's *t*-test, *p* 0.05 protected by a one way ANOVA with repeated measures for line graphs (n = 8,11). e–f) Schematic representations of the bilateral injection sites as determined by histology for HPLC measures of monoamines in micropunched BLC/CeA and DRN (n = 3,6) and immunohistochemistry of SERT-ir fibers in BLC (n = 5,5). Injection site placements are illustrated as symbols (with black circle indicating vehicle injections, and green squares indicating 5,7-DHT injections). Illustrations of coronal brain sections are based on the rat brain atlas of Paxinos and Watson (1997). Numbers to bottom right of the section indicate the distance posterior from bregma; the vertical scale on the right of the section represents the distance ventral from bregma (in mm). The basolateral amygdala complex (BLC) consists of the lateral amygdaloid nucleus (LA) and basolateral amygdaloid nucleus (BL)). Solid lines represent white matter tracts and dashed lines illustrate subdivisions of the BLC. Abbreviations: BL, basolateral amygdaloid nucleus; CeA, central amygdaloid nucleus; ec, external capsule; LA, lateral amygdaloid nucleus; opt, optic tract. g) Bar graph illustrates concentrations of 5-HT, norepinephrine (NE), and dopamine (DA) in

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the BLC/CeA, and 5-HT in the dorsal raphe nucleus (DRN). h) Two representative photomicrographs from the BLC region from a vehicle-injected rat (left) and a 5,7-DHT-injected rat (right).