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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.

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

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

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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.

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.

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.