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# SEROTONIN IN THE VENTRAL HIPPOCAMPUS MODULATES ANXIETY-LIKE BEHAVIOR DURING AMPHETAMINE WITHDRAWAL

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**Abstract—Withdrawal from amphetamine is associated with increased anxiety and sensitivity to stressors which are thought to contribute to relapse. Rats undergoing amphetamine withdrawal fail to exhibit stress-induced increases in serotonin (5-HT) release in the ventral hippocampus and show heightened anxiety-like behaviors. Therefore, we tested the hypothesis that reducing 5-HT levels in the ventral hippocampus is a causal mechanism in increasing anxiety-like behaviors during amphetamine withdrawal. First, we tested whether reducing 5-HT levels in the ventral hippocampus directly increases anxiety behavior. Male rats were bilaterally infused with 5,7-dihydroxytryptamine (5,7-DHT) into the ventral hippocampus, which produced a 83% decrease in ventral hippocampus 5-HT content, and were tested on the elevated plus maze (EPM) for anxiety-like behavior. Reducing ventral hippocampus 5-HT levels decreased the time spent in the open arms of the maze, suggesting that diminished ventral hippocampus 5-HT levels increases anxiety-like behavior. Next, we tested whether increasing 5-HT levels in the ventral hippocampus reverses anxiety behavior exhibited by rats undergoing amphetamine withdrawal. Rats were treated daily with either amphetamine (2.5-mg/kg, i.p.) or saline for 2 weeks, and at 2 weeks withdrawal, were infused with the selective serotonin reuptake inhibitor paroxetine (0.5 μM) bilaterally into the ventral hippocampus and tested for anxiety-like behavior on the EPM. Rats pre-treated with amphetamine exhibited increased anxiety-like behavior on the EPM. This effect was reversed by ventral hippocampus infusion of paroxetine. Our results suggest that 5-HT levels in the ventral hippocampus are critical for regulating anxiety behavior. Increasing 5-HT levels during withdrawal may be an effective strategy for reducing anxiety-induced drug relapse.** 

**Key words: psychostimulant, withdrawal, anxiety, hippocampus, serotonin, rat.**

## INTRODUCTION

Amphetamines are widely abused, and discontinuance of use is associated with a clinical withdrawal syndrome that includes anxiety disorder and other dysphoric symptoms in at least 87% of individuals within 24 h of abstinence (Cantwell and McBride, 1998; Schuckit et al., 1999; Srisurapanont et al., 1999a,b; Romanelli and Smith, 2006; Shoptaw et al., 2009; Srisurapanont et al., 2011). These dysphoric symptoms can last for weeks, are difficult to manage, can lead to self-harm, and may lead to cessation of treatment and relapse (Koob, 2000, 2003; Romanelli and Smith, 2006; Gossop, 2009). In rats, 24 h withdrawal from amphetamine also increases anxiety-like behaviors (Vuong et al., 2010) and this effect persists for at least 4 weeks following cessation of drug administration (Barr et al., 2010). Thus, rat models used to study the neurobiology of anxiety states during drug withdrawal may provide an important tool for developing pharmacotherapies designed to treat dysphoric states during amphetamine abstinence in order to disrupt the chronic cycle of addiction.

Serotonin (5-HT) is associated with the regulation of anxiety, stress, and mood (Graeff et al., 1996; Holsboer, 2000; Millan, 2003). Stressors, stress hormones such as corticosterone, and exposure to the mildly anxiogenic elevated plus maze (EPM) all increase extracellular 5-HT in the hippocampus (Wright et al., 1992; Keck et al., 2005; Barr and Forster, 2011; Li et al., 2014). The entirety of the hippocampus expresses high concentrations of glucocorticoid receptors (De Kloet et al., 1975; Reul and de Kloet, 1985; Chao et al., 1989), and the effects of stress on 5-HT release in the hippocampus appear to involve corticosterone activating glucocorticoid receptors within the ventral hippocampus to stimulate 5-HT release (Barr and Forster, 2011; Li et al., 2014).

Increases in hippocampal 5-HT levels are believed to reduce anxiety-like behaviors of rats (Guimaraes et al., 1993; Graeff et al., 1996), indirectly suggesting that 5-HT levels in the hippocampus are related to adaptive coping. In support of this, rats bred for high anxiety-like

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*Abbreviations:* 5,7-DHT, 5,7-Dihydroxytryptamine; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; EPM, elevated plus maze; HPLC, high-performance liquid chromatography; OCT 3, organic cation transporters type 3; PMCo, posteromedial cortical amygdaloid nucleus; SNK, Student–Newman–Keuls.

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behavior have reduced stress-induced hippocampal 5-HT release (Keck et al., 2005). Similarly, rats going through amphetamine withdrawal fail to demonstrate corticosterone-induced or stress-induced extracellular 5-HT release within the ventral hippocampus (Barr and Forster, 2011; Li et al., 2014) at the same withdrawal periods when increased anxiety-like behavior is observed (Barr et al., 2010; Vuong et al., 2010). Furthermore, amphetamine withdrawal is associated with reduced glucocorticoid receptor expression and increased expression of organic cation transporters type 3 (OCT 3) in the ventral hippocampus, the latter of which clear extracellular 5-HT (Barr and Forster, 2011; Barr et al., 2013). Combined, these molecular changes are thought to lead to dampened extracellular 5-HT levels in response to stress during amphetamine withdrawal (Li et al., 2014).

While indirect correlative evidence is suggestive, it is not clear whether disruptions in 5-HT transmission specific to the ventral hippocampus are a mechanism underlying elevated anxiety. The goals of our study were to test directly if reducing 5-HT content in the ventral hippocampus increases anxiety-like behavior, and to establish whether restoring 5-HT levels in the ventral hippocampus of rats undergoing amphetamine withdrawal reverses heightened anxiety-like behavior.

## EXPERIMENTAL PROCEDURES

### Animals

Male Sprague-Dawley rats (Animal Resources Center, The University of South Dakota) were housed in pairs from weaning (3 weeks old) with access to food and water *ad libitum*, and maintained on a reverse 12-h light/12-h dark cycle (lights off at 10:00 a.m.) at a constant temperature of 22 °C and 60% relative humidity. Rats were used in the following studies once they reached early adulthood (8 weeks old). All procedures were approved by the Institutional Animal Care and Use Committee of the University of South Dakota, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edn., 2011).

### Experiment 1 – effects of reduced serotonin in the ventral hippocampus on anxiety-like behavior

To test a direct link between reduced hippocampal 5-HT content (Barr et al., 2010; Barr and Forster, 2011) and heightened anxiety during amphetamine withdrawal (Barr et al., 2010; Vuong et al., 2010), this experiment determined whether 5-HT lesions of the ventral hippocampus would cause increased anxiety-like behavior in drug-naïve rats to mimic amphetamine withdrawal.

*Drug preparation.* Ascorbic acid vehicle (0.1%) was prepared by dissolving ascorbic acid (Sigma–Aldrich, St. Louis, MO, USA) in artificial cerebrospinal fluid (aCSF); neurotoxin was prepared by dissolving 5 mg of 5,7-dihydroxytryptamine (5,7-DHT) (Sigma–Aldrich, St. Louis, MO, USA) in 1 ml 0.1% ascorbic acid vehicle. Desipramine (Enzo Life Science, Farmingdale, NY, USA) was dissolved in distilled water; GBR-12909 (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in 1:1 distilled water and dimethyl sulfoxide (DSMO). Aliquots of the ascorbic acid vehicle and 5,7-DHT were stored at −80 °C while desipramine and GBR-12909 were freshly prepared prior to use.

*Serotonin lesion procedures.* Rats underwent aseptic stereotaxic recovery surgery at the end of the light phase of the light cycle, by being anesthetized with isoflurane (induced at 4–5%, maintained at 2.5% in 0.3 L $O_2$), and mounted in a small mammal stereotaxic frame (Kopf, Tujunga, CA, USA) with the incisor bar set at −3.7 mm. Body temperature was maintained at a constant 36 ± 0.5 °C by an electronic heating pad (Harvard Apparatus, Holliston, MA, USA). Desipramine (20-mg/kg, i.p.) and GBR-12909 (10 mg/kg, i.p.) were administered 20 min before ventral hippocampus infusions to block the transport of 5,7-DHT into norepinephrine and dopamine terminals (Jonsson, 1980; Kusljic and van den Buuse, 2004). Small trephine holes were drilled in the skull above the ventral hippocampus (−5.2 mm posterior, ±4.5 mm lateral, and −7.8 mm ventral from bregma; (Paxinos and Watson, 1997) Bilateral ventral hippocampal infusions of 5,7-DHT (5 μg/μl; 0.5 μl per side; (Kusljic and van den Buuse, 2004); $n = 8$) or vehicle (0.1% ascorbic acid; 0.5 μl per side; (Kusljic and van den Buuse, 2004); $n = 7$) were delivered through a 30-gauge stainless steel cannula at a flow rate of 0.5 μl/min using a microsyringe pump (Stoelting, Wood Dale, IL, USA). Following infusions, the cannulae were left in position for an additional 2 min to minimize backflow along the cannula track. Rats received the analgesic ketoprofen (5-mg/kg, i.m.; Fort Dodge Animal Health, Overland Park, KS, USA) at the end of surgery. Rats were allowed to recover for 2 weeks before behavioral testing to ensure the full extent of the 5-HT lesion (Kusljic and van den Buuse, 2004).

*EPM testing.* EPM was used to determine the influence of 5-HT lesions in modulating anxiety-like behavior. The maze (Noldus Information Technology, Wageningen, The Netherlands), elevated 1 m from the ground, consisted of perpendicular, intersecting runways (12 cm wide × 100 cm long) connected by a central zone, and contained two opposing closed arms with high walls on three sides (40 cm high) and two open arms with no walls. Rats were tested in the dark (active) phase using red light illumination throughout the entire process, between 11:00 a.m. and 1:00 p.m. After being placing in the central region (facing a closed arm), a rat was allowed to explore freely for 5 min. Time spent in open arms and total distance moved in EPM during the 5-min test period were recorded and scored by automated software (Ethovision XT v5.1; Noldus Technologies).

*Monoamine analysis.* Rats were decapitated the day following EPM testing during the dark phase of the light cycle (between 2 and 3 pm). Brains were rapidly removed, frozen on dry ice and stored at −80 °C until