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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 reduced 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-DHT into the ventral hippocampus, which produced a 83% decrease 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 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, ip.) 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 is critical for regulating anxiety behavior. Increasing 5-HT levels during withdrawal may be an effective strategy for reducing anxiety-induced drug relapse.

Keywords

psychostimulant; withdrawal; anxiety; hippocampus; serotonin; rat

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1. 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 hours 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 hours 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 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, that 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.

2. Experimental Procedures

2.1. 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 (8^{th} edn., 2011).

2.2. 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.

2.2.1. 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-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 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.

2.2.2. 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, ip.) and GBR-12909 (10 mg/kg, ip.) were administered 20 minutes 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 (5μg/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). 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, im.; Fort Dodge Animal Health, Overland Park, KS, USA) at the end of surgery. Rats were allowed to recover for two weeks before behavioral testing to ensure the full extent of the 5-HT lesion (Kusljic and van den Buuse, 2004).

2.2.3. Elevated Plus Maze Testing—Elevated plus maze (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 3 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 center 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).

2.2.4. 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 sectioning. Frozen brains were sectioned at 300 μm thickness in a cryostat (North Central Instrument, Plymouth, MN, USA) maintained at −10 °C. The ventral hippocampus was microdissected on a freezing plate (Physitemp Instruments, Inc., Clifton, NJ, USA) using a 20 gauge cannula, as were brain regions located either adjacent to the targeted delivery site or along the cannula track that may have been affected by drug diffusion. These additional regions included the substantia nigra (SN), the posteromedial cortical amygdaloid nucleus (PMCo), dorsal hippocampus, cortex immediately above the hippocampus, and dorsal thalamus. The serotonin cell body regions of the dorsal and medial raphe nuclei (dRN and mRN) were also microdissected using a 23 gauge cannula, to examine possible feedback effects on monoaminergic activity at the level of the cell bodies following lesions of 5-HT terminals in the ventral hippocampus. All samples were expelled into 60 μL sodium acetate buffer (pH: 4.95) containing the internal standard alpha-methyl-dopamine, and stored at −80°C until further analysis.

Samples were thawed to lyse cells and 2 μl ascorbate oxidase (1 mg/mL) was added to each sample followed by centrifugation at 15,000 × g for 3 min. The supernatant (45 μl) was injected using an autoinjector for HPLC analysis (Waters 717 Plus Autosampler, Waters Corp., Milford, MA, USA). Serotonin, norepinephrine, and dopamine were separated using a Nova-Pak C_{18} 4 μm column (Waters Corp.) and electrochemically detected using an LC-4C detector (BioAnalytical Systems, Inc., West Lafayette, IN, USA) and a glassy carbon electrode set at a potential of + 0.6 V vs. an Ag/AgCl reference electrode. The mobile phase contained 14 g citric acid, 8.6 g sodium acetate, 110 mg 1-octane-sulfonic acid, 150 mg EDTA disodium salt, and 100 mL methanol in 1 L deionized water (Renner and Luine, 1986, Ling et al., 2009). The remaining protein pellet was dissolved into 0.4 N NaOH for three days and protein content was measured using the Bradford assay (Bradford, 1976, Watt et al., 2007).

Monoamine concentrations (pg) were obtained by calculating peak height compared to known standards, and were corrected for recovery using CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). The final monoamine value (pg/μg) was obtained by normalizing pg amine to tissue protein content (μg).

2.3. Experiment 2 – Effects of Paroxetine Treatment in the Ventral Hippocampus on Anxiety-like Behavior during Amphetamine Withdrawal

The purpose of this experiment was to determine whether increasing 5-HT levels in the ventral hippocampus of amphetamine withdrawn rats would reduce anxiety-like behaviors. Local infusion of the selective serotonin reuptake inhibitor paroxetine was employed, because we previously showed that expression of the serotonin transporter (SERT - the substrate for paroxetine) in the ventral hippocampus is not affected by amphetamine pretreatment and withdrawal (Barr et al., 2013). In addition, paroxetine infused directly into the ventral hippocampus of amphetamine pretreated rats undergoing withdrawal raises extracellular 5-HT levels to the same degree as in saline pretreated rats (Barr et al., 2013).

2.3.1. Amphetamine Treatment—Rats in early adulthood (8–10 weeks) were injected with amphetamine (2.5 mg/kg, ip.) or saline daily during the dark phase of the light cycle (between 11:00 a.m. to 1:00 p.m.) for 14 days. This procedure increases expression of anxiety-like behaviors at 24 hours, 2 weeks, and 4 weeks withdrawal (Barr et al., 2010, Vuong et al., 2010). Rats used in this experiment were at 2 weeks of withdrawal at the time of testing for anxiety-like behavior.

2.3.2. Stereotaxic Surgery—After one week of withdrawal, rats underwent aseptic stereotaxic recovery surgery (as described for Experiment 1) for guide cannula implantation. Burr holes were made in the skull above the ventral hippocampus (− 5.6 mm posterior and ± 4.6 lateral from bregma, (Paxinos and Watson, 1997), and single guide cannulae (22 gauge, 8 mm total length; Plastics One, Roanoke, VA, USA) were implanted on each side of the brain. Guide cannulae were fixed to the skull with a coating of cranioplastic cement (Plastics One), with dental screws inserted into the skull serving as anchor points. Rats received analgesic ketoprofen (5 mg/kg, im.; Fort Dodge Animal Health) at the end of surgery and were allowed 3 days recovery before undergoing acclimation to infusions.

2.3.3. Acclimation to Intracranial Infusion Procedure—All rats were acclimated to the infusion procedure during the dark phase of the light cycle from 11:00 a.m. to 2:00 p.m. over a three day period prior to the testing day, in order to minimize anxiety behavior as a result of handling or the infusion process. The rat was held and gently restrained, with a 30 gauge stainless-steel infusion cannula (2 mm longer than the guide) then inserted through the guide cannula into the ventral hippocampus. Artificial cerebrospinal fluid (0.5 μl) was infused over 1 min using a microinfusion pump (Stoelting, Wood Dale, IL, USA). The infusion cannula was kept in place for an additional 1 min to ensure complete diffusion and to minimize black flow. After bilateral aCSF infusion, rats were returned to their home cages and kept in the testing room for 40 min to acclimate to the waiting time required between infusion and EPM testing.

2.3.4. Drug Infusion and Elevated Plus Maze Testing—At two weeks withdrawal, rats received bilateral infusion of either vehicle (0.5 μl aCSF) or paroxetine (0.5 μM in 0.5 μl aCSF; AK Scientific, Union City, CA, USA; n = 6–8 rats per treatment group). This concentration of paroxetine was chosen because it elicits maximal but similar increases in ventral hippocampal extracellular 5-HT in saline and amphetamine pretreated rats during withdrawal (Barr et al., 2013). Behavioral testing was conducted 40 min after infusion to allow for maximum increases of extracellular 5-HT levels in the ventral hippocampus (Barr et al., 2013), with EPM trials performed as described for Experiment 1.

2.3.5. Histology—At the completion of EPM testing, rats were euthanized with sodium pentobarbital (1.0 ml Fatal-plus, ip.; Vortech Dearborn, MI, USA). Brains were removed and fixed in 10% buffered formalin (Fisher Scientific) for three days. Brains were then sectioned at 60 μm using a microtome (Cambridge Instrument, Buffalo, NY, USA) and mounted on slides. Infusion cannula placement was determined by two experimenters blind to treatment who evaluated the sections using a light microscope.

2.4. Data analysis

All data were analyzed in SigmaStat 3.5 with significance set *a priori* at $p \leq 0.05$. Grubb's test was used to identify any statistical outliers (Lowry et al., 2001), which resulted in the removal of 4 monoamine data points from HPLC analysis but no outliers in the behavioral data were identified. Each of the 4 monoamine outliers was a different monoamine in a different brain region from a different rat (each rat contributed 24 monoamine data points in total), and did not involve any data from the ventral hippocampus. Thus, the removal of 1 of 24 monoamine data points of the 4 rats did not warrant removing their behavioral data from the analysis. Behavioral and monoamine data for Experiment 1 were analyzed using separate one-way ANOVA. Correlations between ventral hippocampal serotonin levels and time spent in open arms of the EPM in Experiment 1 were performed using linear regression ANOVA. Behavioral data from Experiment 2 were analyzed with 2-way ANOVA (pre-treatment x intracranial infusion), with significant main effects or interactions further assessed by Student-Newman-Keuls (SNK) *post hoc* tests for multiple comparisons.

3. Results

3.1. Experiment 1 – Effects of Reduced Serotonin Content in the Ventral Hippocampus on Anxiety-like Behavior

3.1.1. Monoamine Levels Following 5-HT Lesion of the Ventral Hippocampus —One rat with only a partial (< 40%) 5-HT depletion following 5,7-DHT infusion in the ventral hippocampus was excluded from the following analyses (with the exception of the linear regression analysis, where correlations were made between all 5-HT levels and EPM behavior for all individuals). The remaining animals had reductions in 5-HT content ranging from 74% – 94% in the ventral hippocampus, with an average of 83% 5-HT depletion (Fig. 1A). Serotonin levels in the ventral hippocampus of 5,7-DHT treated rats were significantly lower compared to controls ($F_{(1, 13)} = 90.23$, $P < 0.001$; Fig. 1A). There were no significant effects of 5,7-DHT infusion on norepinephrine levels (Fig. 1B) and dopamine levels (Fig. 1C) in the ventral hippocampus.

Monoamine concentrations in most brain regions surrounding or related to the ventral hippocampus were not affected by 5,7-DHT infusion with the exception of the PMCo (Table 1). Specifically, the rats treated with 5,7-DHT had a 44 % reduction in 5-HT content in the PMCo, which was significantly less 5 as compared to vehicle controls ($F_{(1, 12)} = 5.56$, P = 0.036; Table 1). This indicates some ventral diffusion of the toxin from the ventral hippocampus to the PMCo.

3.1.2. Reduced Serotonin Content Increases Anxiety-like Behavior—Rats with 5,7-DHT lesions expressed increased anxiety-like behavior during EPM testing (Figs. 2–3). Specifically, 5-HT lesioned animals exhibited decreased time in open arms ($F_{(1,13)} = 5.34$, P = 0.021; Fig. 2A) as compared to vehicle-treated controls. This difference was not as a result of decreased locomotion following 5-HT lesion, since the total distance moved in the entire maze was not significantly different between the two treatment groups (Fig. 2B).

Reduced 5-HT content in the ventral hippocampus was associated with greater anxiety-like behaviors, as evidenced by a positive linear relationship ($r^2 = 0.777$) between 5-HT concentration and the duration of time spent in the open arms ($F_{(1, 7)} = 28.9$, P < 0.001; Fig. 3A) in the rats treated with 5,7-DHT. In contrast, there was a negative but non-significant trend for a relationship between the levels of 5-HT in the PMCo and time spent in the open arms in 5,7-DHT lesioned rats ($F_{(1, 7)} = 5.45$, P = 0.052; $r^2 = 0.358$; Fig. 3B).

3.2. Experiment 2 – Effect of Paroxetine Treatment the Ventral Hippocampus on Anxiety-like Behavior during Amphetamine Withdrawal

Drug Infusion Cannula Placement: Drug infusion cannulas were similarly distributed in the ventral hippocampus among both amphetamine and saline pre-treated animals (Fig. 4). Anterior-posterior measurement for cannula placement in the ventral hippocampus ranged from −4.16 mm to −6.30 mm from bregma for saline pretreated rats (Fig. 4A) and from −4.16 mm to −6.04 mm from bregma for amphetamine pretreated rats (Fig. 4B).

3.2.2. Effect of Paroxetine Treatment in the Ventral Hippocampus of Amphetamine Pretreated Rats Reduces Anxiety-like Behavior—A significant effect of ventral hippocampal infusion was present when comparing time spent in open arms, ($F_{(1, 23)} = 4.4$, P = 0.046), and there was a significant interaction between pre-treatment and ventral hippocampus infusion ($F_{(1, 23)} = 10.8$, P = 0.003; Fig. 5A). *Post hoc* analysis demonstrated that amphetamine-pretreated rats receiving vehicle infusion spent significantly less time in open arms as compared to saline pre-treated rats (SNK, P = 0.015; Figure 5A). In contrast, amphetamine pre-treated rats that received paroxetine infusions spent more time in open arms than amphetamine pre-treated rats infused with vehicle (SNK, P = 0.001; Fig. 5A). There was no significant difference in time spent in the open arms either between saline pre-treated animals infused with paroxetine compared to vehicle or between amphetamine and saline pre-treated rats that received paroxetine infusions (Fig. 5A).

Total distance moved by animals in the EPM was not significantly affected by pre-treatment or ventral hippocampus infusion, and there was also no significant interaction between the two factors. Thus, altered general locomotion is not an explanation for differences in time spent in open arms associated with amphetamine or paroxetine treatments (Fig. 5B).

4. Discussion

Rats undergoing serotonergic lesions in the ventral hippocampus showed a marked reduction in 5-HT content within this region and also exhibited decreased time spent in the open arms of the EPM, suggesting that reduction in ventral hippocampal 5-HT concentrations increased the expression of anxiety-like behavior. This hypothesis is further supported by the strong linear relationship between individual 5-HT levels in lesioned rats and open arm duration, with the greatest anxiety-like behavior exhibited by rats with the lowest concentration of ventral hippocampal 5-HT. Further support for a role for ventral hippocampus 5-HT in mediating anxiety is derived from our finding that increasing 5-HT via local paroxetine infusions attenuated the expression of anxiety-like behavior by amphetamine-withdrawn rats. Importantly, neither the paroxetine infusion nor 5-HT lesion affected locomotion within the EPM. The latter finding is consistent with an earlier report that 5-HT lesion in the ventral hippocampus did not affect spontaneous locomotion (Kusljic and van den Buuse, 2004), and suggests the effect of 5-HT manipulations on open arm behavior observed here was not due to deficits in locomotion. Combined, our results provide direct evidence for a role of 5-HT in the ventral hippocampus in dampening anxiety states, and indicate that the blunted 5-HT responses to either corticosterone or stress in the ventral hippocampus of amphetamine pretreated rats (Barr and Forster, 2011, Li et al., 2014) contribute heavily to the previously observed increases in anxiety during amphetamine withdrawal (Barr et al., 2010, Vuong et al., 2010).

Within the hippocampus, the lesioning procedure was selective to 5-HT in the ventral subregion, since 5,7-DHT infusion affected neither ventral hippocampal norepinephrine and dopamine levels nor any monoamine in the dorsal hippocampus. The regions surrounding the ventral hippocampus and the serotonergic cell body regions projecting to the ventral hippocampus also remained unaffected by 5-HT lesion with the exception of the PMCo, which showed a partial 5-HT lesion, probably as a result of diffusion from the ventral hippocampus. Unlike the ventral hippocampus, there was no significant relationship between PMCo 5-HT concentrations and anxiety-like behavior, and we are unaware of any evidence in the literature linking the PMCo to anxiety. Instead, this subregion of the amygdala appears to function primarily in olfactory signal integration (Gutierrez-Castellanos et al., 2014), with a role in directing appropriate chemosensory investigation behavior during rodent reproductive interactions (Maras and Petrulis, 2008). Thus, it appears most likely that increased anxiety-like behavior in 5,7-DHT treated rats was mediated by the ventral hippocampus and not the PMCo.

In the ventral hippocampus, reduced stress-induced 5-HT release has been observed in rats selected for high anxiety (Keck et al., 2005), and amphetamine pretreated rats with increased anxiety-like behavior and have deficits in ventral hippocampal 5-HT release in response to corticosterone and stress that are long lasting (Barr et al., 2010, Vuong et al., 2010, Barr and

Forster, 2011, Barr et al., 2013, Li et al., 2014). Until now, it was not clear whether 5-HT deficits in this region were actually responsible for increased anxiety states. The current study shows that reducing 5-HT content in ventral hippocampus directly contributes to the enhanced anxiety and that pharmacologically increasing 5-HT levels in the ventral hippocampus of amphetamine pretreated rats rescues anxiety to control levels during amphetamine withdrawal. Therefore, the current study provides direct evidence for a role of 5-HT levels in the ventral hippocampus in dampening anxiety states.

Serotonin levels in the ventral hippocampus may mediate the expression of anxiety through the activation of 5-HT_{1A} receptors, which are densely distributed throughout the hippocampus (Kia et al., 1996, Hensler, 2003). These receptors are linked to G-proteins, which inhibit the production of adenylyl cyclase and open of potassium ion channels to dampen firing of target cells (Markstein et al., 1986, Hensler, 2003). Increased anxiety-like behavior is associated with decreased 5-HT_{1A} receptor expression in the ventral hippocampus and other brain regions (Fuss et al., 2013), while reduced anxiety-like behavior has been reported in transgenic mice with global overexpression of 5-HT_{1A} receptors (Kusserow et al., 2004). Furthermore, infusions of 5-HT_{1A} receptor agonists into the hippocampus have anxiolytic effects (Kataoka et al., 1991, Carli et al., 1993, Stefanski et al., 1993). Thus, it appears that 5-HT_{1A} receptors may be anxiolytic in the hippocampus (Joca et al., 2007). Therefore, attenuated 5-HT levels in the ventral hippocampus, as seen either following 5,7-DHT infusion or during amphetamine withdrawal, may result in increased anxiety-like behavior through a reduction in 5-HT actions on 5-HT_{1A} receptors in this region. This possibility should be tested in future studies, as should the potential for targeting ventral hippocampus 5-HT_{1A} receptors directly to ameliorate anxiety associated with amphetamine withdrawal.

Interestingly, infusion of paroxetine in the ventral hippocampus of saline pre-treated rats had no effect on anxiety-like behavior. We previously established that the concentration of paroxetine used in the current study elevates extracellular 5-HT levels in the ventral hippocampus of saline pre-treated rats for at least 240 mins (Barr et al., 2013). Combined, these and current findings imply that increasing 5-HT levels in the ventral hippocampus is only effective in reducing anxiety-like behaviors of rats with existing reductions in 5-HT function (Barr and Forster, 2011, Barr et al., 2013, Li et al., 2014) and correspondingly heightened anxiety behavior. Furthermore, the effectiveness of increasing extracellular 5-HT levels in the ventral hippocampus of amphetamine pretreated rats only may be a function of increased expression or sensitivity of postsynaptic 5-HT receptors (such as the 5-HT_{1A} receptor as discussed above) as a compensation for reduced 5-HT levels observed during withdrawal. Thus, determining the expression and sensitivity of 5-HT_{1A} receptors in the ventral hippocampus during amphetamine withdrawal would be an important future direction.

Reduced 5-HT levels during amphetamine withdrawal may derive from overcompensation of negative feedback mechanisms initiated in response to the pharmacological actions of chronic amphetamine to increase extracellular 5-HT concentrations. For instance, the organic cation transporter 3 (OCT3) is a polyspecific low-affinity but high-capacity transporter that functions in the clearance of cations including 5-HT (Grundemann et al.,

1998, Koepsell et al., 2007, Daws, 2009), and is believed to contribute to the regulation of extracellular 5-HT especially when concentrations are elevated (Koepsell et al., 2007, Baganz et al., 2008, Baganz et al., 2010). Expression and function of OCT3 in the ventral hippocampus are both increased following chronic amphetamine treatment, possibly to assist in synaptic clearance of excessive drug-evoked 5-HT release. The persistence of OCT3-mediated clearance beyond drug cessation (Barr et al., 2013) may partially explain reductions in extracellular 5-HT release during amphetamine withdrawal in response to either corticosterone or an acute stressor (Barr and Forster, 2011, Barr et al., 2013, Li et al., 2014). Expression of glucocorticoid receptors in ventral hippocampus is also decreased after chronic amphetamine administration, which dampens glucocorticoid receptor-mediated 5-HT release either in response to exogenous corticosterone or during stress conditions (Barr and Forster, 2011, Li et al., 2014). Combined, lowered corticosterone-mediated 5-HT release and greater synaptic clearance within the ventral hippocampus may contribute to reductions in 5-HT levels during amphetamine withdrawal, and represent potential targets for the reversal of anxiety states

In summary, our results demonstrate a direct association between 5-HT levels in the ventral hippocampus and the expression of anxiety-like behavior. Previous work suggests that 5-HT in ventral hippocampus plays an important role in the regulation anxiety-like behavior via 5-HT_{1A} receptors, and our study adds to this by suggesting that 5-HT in the ventral hippocampus may be necessary to dampen excessive anxiety states. Overall, these results can offer new insight into future treatment options for the reversal of anxiety during amphetamine withdrawal.

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Highlights

- Serotonin lesion of ventral hippocampus increases anxiety
- Restoring serotonin in ventral hippocampus reduces anxiety in amphetamine withdrawal
- Ventral hippocampal serotonin thus dampens anxiety states

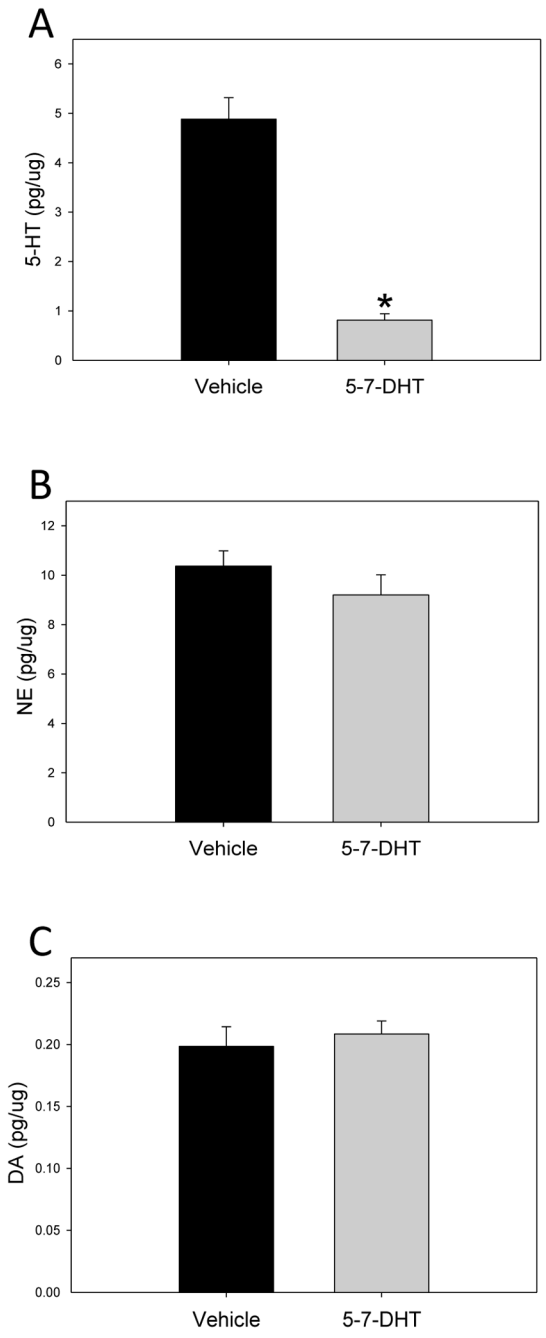

Figure 1.

Concentration of (**A**) serotonin (5-HT), (**B**) norepinephrine (NE), and (**C**) dopamine (DA) in the ventral hippocampus for 5,7-DHT and vehicle-infused rats two weeks following treatment (n=7–8 per group, mean ± SEM). * significant difference compared to vehicle infusion group, $P < 0.05$.

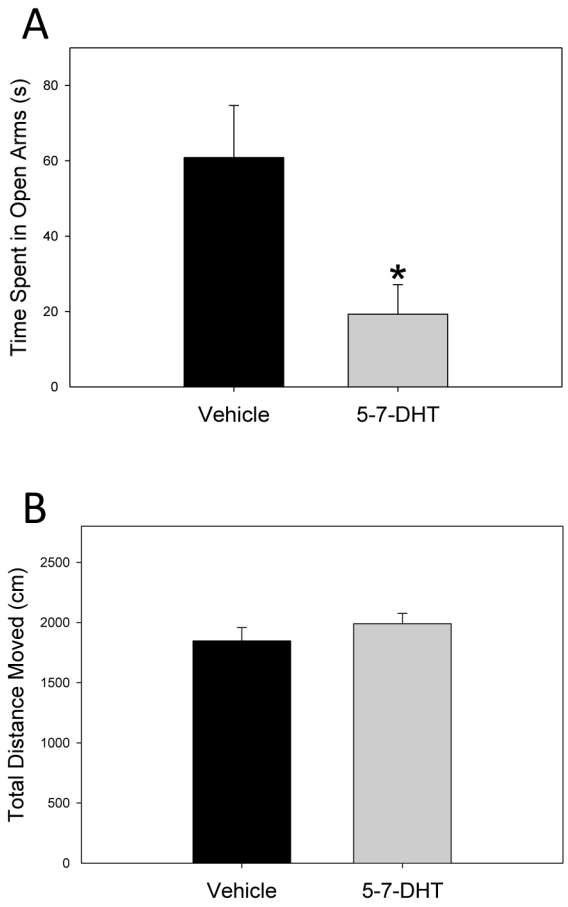

Figure 2.

Effects of 5,7-DHT ventral hippocampus infusions on (**A**) time spent in open arms and (**B**) total distance moved during the 5 min elevated plus maze test (n=7–8 per group, mean ± SEM). * significant difference compared to vehicle infusion group, $P < 0.05$.

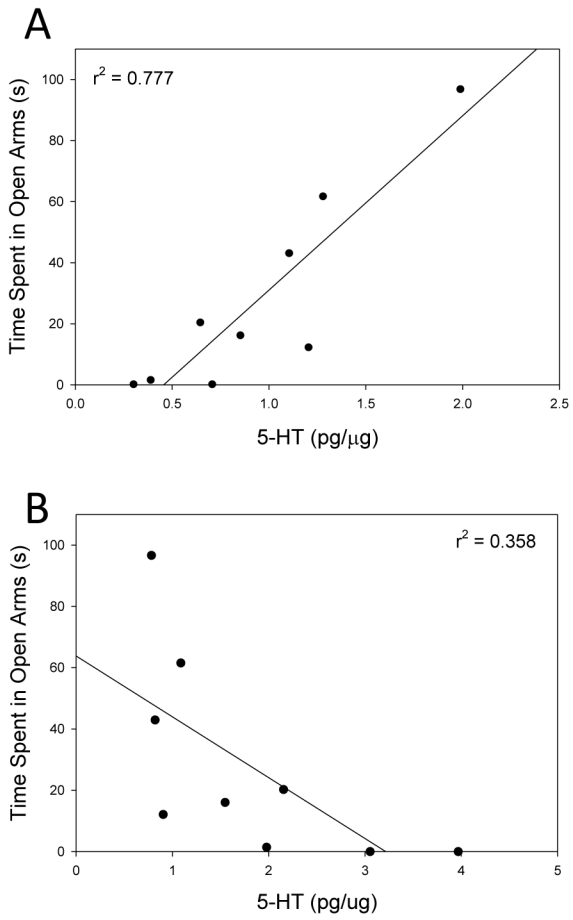

Figure 3.

Linear relationships between time spent in open arms of the elevated plus maze and concentrations of serotonin (5-HT) in the (**A**) ventral hippocampus ($P < 0.05$) and (**B**) Posteromedial Cortical Amygdaloid Nucleus (PMCo; $P > 0.05$) of rats with serotonergic lesions.

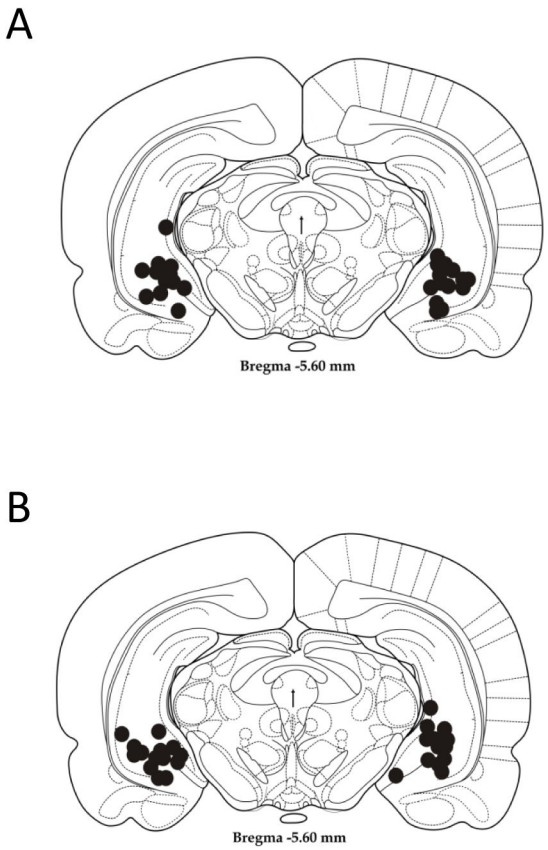

Figure 4.

Representative schematic diagrams of drug infusion cannula placements in the ventral hippocampus (dark dots) for (**A**) saline and (**B**) amphetamine pre-treated groups. Figures were adapted from Paxinos and Watson (1997).

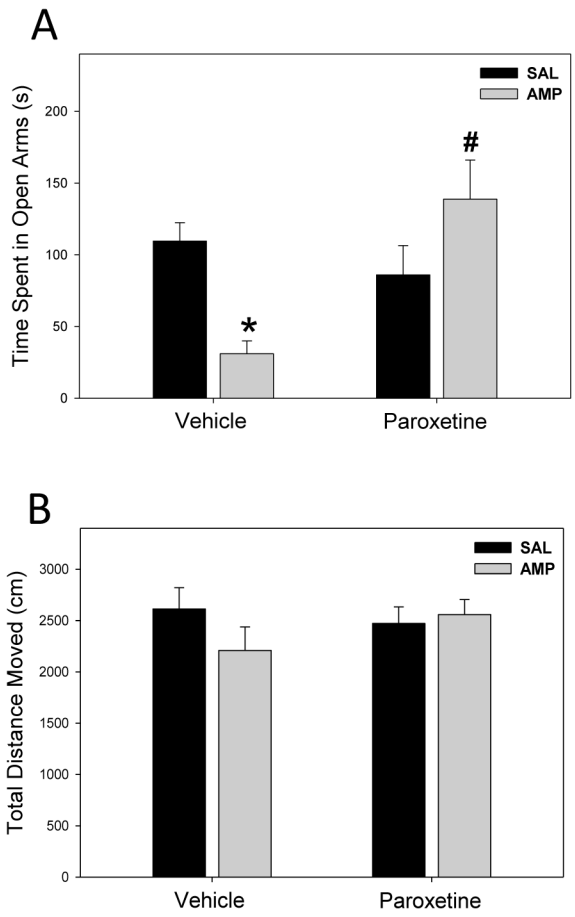

Figure 5.

Effects of chronic amphetamine treatment and ventral hippocampus paroxetine infusion on (**A**) time spent in open arms and (**B**) total distance moved during the 5 min elevated plus maze test (n=6–8 per group, mean ± SEM). * significant difference compared to saline group; #significant difference compared to vehicle infusions within amphetamine pre-treatment, $P < 0.05$.b

Table 1

Concentration of 5-HT, NE, and DA in the Brian Region Surrounding Ventral Hippocampus Two Weeks after 5,7-DHT or Vehicle Infusion

	Monoamine Levels (pg/μg)	
	Vehicle	**5,7-DHT**
Dorsal Hippocampus		
5-HT	2.52 ± 0.34	2.05 ± 0.19
NE	7.20 ± 0.48	6.83 ± 0.41
DA	0.22 ± 0.02	0.18 ± 0.02
Dorsal Raphe Nuclei		
5-HT	15.30 ± 0.71	13.96 ± 0.77
NE	14.05 ± 0.96	12.83 ± 1.02
DA	2.16 ± 0.30	2.18 ± 0.15
Median Raphe Nuclei		
5-HT	40.08 ± 7.40	35.37 ± 7.24
NE	31.41 ± 7.52	30.45 ± 6.81
DA	8.34 ± 2.56	11.61 ± 4.82
Cortex		
5-HT	1.31 ± 0.11	1.23 ± 0.07
NE	2.96 ± 0.33	3.80 ± 0.31
DA	0.21 ± 0.06	0.17 ± 0.01
Dorsal Thalamus		
5-HT	7.35 ± 0.79	6.87 ± 0.48
NE	5.39 ± 0.36	5.37 ± 0.32
DA	0.29 ± 0.02	0.30 ± 0.02
Posteromedial Cortical Amygdaloid Nucleus		
5-HT	3.45 ± 0.48	1.94 ± 0.39 *
NE	5.15 ± 0.56	4.84 ± 0.47
DA	1.02 ± 0.17	0.95 ± 0.08
Substantia Nigra		
5-HT	12.65 ± 1.50	11.44 ± 1.15
NE	4.99 ± 1.12	4.51 ± 0.43
DA	6.72 ±30.97	8.05 ± 0.69

Shown are mean ± SEM (n=7–8). 5-HT = serotonin. DA = dopamine. NE = norepinephrine.

* significantly different from vehicle control, $P < 0.05$