Published in final edited form as:

Eur J Neurosci. 2014 December ; 40(11): 3684–3692. doi:10.1111/ejn.12735.

Serotonergic responses to stress are enhanced in the central amygdala and inhibited in the ventral hippocampus during amphetamine withdrawal

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Abstract

Withdrawal from amphetamine increases anxiety and reduces the ability to cope with stress, factors that are believed to contribute to drug relapse. Stress-induced serotonergic transmission in the central nucleus of the amygdala is associated with anxiety states and fear. Conversely, increases in stress-induced ventral hippocampal serotonin have been linked to coping mechanisms. The goal of this study is to understand neurobiological changes induced by amphetamine that contribute to stress-sensitivity during withdrawal. We tested the hypothesis that limbic serotonergic responses to restraint stress would be altered in male Sprague-Dawley rats chronically pre-treated with amphetamine (2.5 mg/kg, ip.) followed by two weeks withdrawal. Amphetamine withdrawal resulted in increased stress-induced behavioral arousal relative to control treatment, suggesting that drug withdrawal induced a greater sensitivity to the stressor. When microdialysis was used to determine the effects of restraint on extracellular serotonin, stress-induced increases in serotonin were abolished in the ventral hippocampus and augmented in the central amygdala during amphetamine withdrawal. Reverse dialysis of the glucocorticoid receptor antagonist mifepristone into the ventral hippocampus blocked the stress-induced serotonin increase in saline pre-treated rats, suggesting that glucocorticoid receptors mediate stress-induced serotonin increases in the ventral hippocampus. However, mifepristone had no effect on stress-induced serotonin increases in the central amygdala, indicating that stress increases serotonin in this region independent of glucocorticoid receptors. During amphetamine withdrawal, the absence of stress-induced increases in ventral hippocampus serotonin combined with enhanced stress-induced serotonergic responses in the central amygdala may contribute to drug relapse by decreasing stress-coping ability and heightening stress responsiveness.

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All authors report no conflict of interest.

Keywords

Glucocorticoid receptor; Hippocampus; Amygdala; Microdialysis

Introduction

Amphetamine withdrawal is associated with numerous psychological effects including anxiety and depression (Romanelli & Smith, 2006; Shoptaw *et al.*, 2009). Negative affect during withdrawal increases both sensitivity to stress and drug seeking behaviors in animal models and humans (Weiss *et al.*, 2001; Koob *et al.*, 2004). Although amphetamine appears to strongly affect stress circuitry, the long-term alterations in neuronal functioning induced by chronic amphetamine and withdrawal that contribute to increased stress sensitivity are not fully understood.

The hippocampus is important for generating adaptive behaviors in response to stress (McEwen, 2002; Maras & Baram, 2012). Furthermore, optogenetic activation of the ventral dentate gyrus suppresses anxiety-like behaviors in mice, suggesting that ventral hippocampus activity is associated with reduced anxiety (Kheirbek *et al.*, 2013). This adaptive function is believed to involve increases in serotonin (5-HT) activity and activation of 5-HT_{1A} receptors, which reduce anxiogenic responses to a stressor (Joca *et al.*, 2003; Bast, 2007; Joca *et al.*, 2007; Joels *et al.*, 2008). Local application of the stress hormone corticosterone increases 5-HT levels in the ventral hippocampus, in part, through glucocorticoid receptor (GR) activation (Barr & Forster, 2011). However, it is not known whether stress-induced increases in ventral hippocampus 5-HT are mediated through local GRs.

In contrast, the central nucleus of the amygdala (CeA) is involved in the expression of fear and anxiety (LeDoux, 2000; 2003; Tye *et al.*, 2011), partially via corticosterone activation of mineralocorticoid receptors and GRs in the CeA (Shepard *et al.*, 2000; Tran & Greenwood-Van Meerveld, 2012). Stress increases 5-HT release in the CeA (Mo *et al.*, 2008), and increasing 5-HT can be anxiogenic (Ravinder *et al.*, 2011) or anxiolytic (Inoue *et al.*, 2004) depending on both the subregion of the amygdala targeted and the 5-HT receptor subtype activated. For example, activation of 5-HT_{2C} receptors in the amygdaloid complex is anxiogenic, while 5-HT_{1A} activation is anxiolytic (Cornelio & Nunes-de-Souza, 2007; Christianson *et al.*, 2010; Li *et al.*, 2012).

Rats chronically pre-treated with amphetamine exhibit enhanced anxiety-like behavior for at least 4 weeks following withdrawal (Barr *et al.*, 2010; Vuong *et al.*, 2010), which may result from altered limbic serotonergic function. For example, corticosterone-induced increases in ventral hippocampal 5-HT are attenuated during acute (24 h) amphetamine withdrawal, possibly due to reduced GR expression along with increased expression of organic cation transporter 3 (OCT3), a polyspecific transporter of organic cations, including monoamines (Barr *et al.*, 2011; 2013). In contrast, rats undergoing acute withdrawal from amphetamine show enhanced serotonergic responses in the CeA to the stress-associated neurohormone corticotropin-releasing factor (Scholl *et al.*, 2010). Together, these results suggest alterations in ventral hippocampal and CeA serotonergic transmission following chronic amphetamine

treatment. However, it is not clear whether these changes actually result in altered 5-HT responses to stress during protracted amphetamine withdrawal. Therefore, we tested the hypothesis that amphetamine withdrawal enhances behavioral responses to stressors, attenuates stress-induced 5-HT in the ventral hippocampus and augments stress-induced 5-HT in the CeA.

Materials and Methods

Animals and Amphetamine Treatment

Male Sprague-Dawley rats (Animal Resource Center, University of South Dakota, Vermillion, SD) used in the experiments were pair-housed in saline ($n = 42$) and amphetamine pairs ($n = 28$) from postnatal 3 weeks onwards under a reverse light cycle (lights off from 10:00–22:00 h) with free access to water and food. Amphetamine or saline treatment was initiated when the rats had reached at least 8 weeks of age. Rats were injected with amphetamine (2.5 mg/kg, ip.) or saline once daily for 2 weeks between 11:00 and 14:00. This treatment regime increases anxiety-like behavior of rats when observed at 24 h, 2 weeks and 4 weeks of withdrawal (Barr *et al.*, 2010; Vuong *et al.*, 2010). Rats used in the following experiments were at 2 weeks of amphetamine withdrawal at the time of testing.

The experimental procedures used in this study 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 (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, 2011). A total of sixty seven rats were used in these experiments, with all possible efforts made to minimize the number of animals used and their suffering.

Surgical Procedures

At the end of the first week of withdrawal, surgeries were performed aseptically using isoflurane anesthetic (3% isoflurane, 0.3 mL/min oxygen). Once anesthetized, rats were placed in the stereotaxic frame in the flat-skull position and implanted unilaterally with a guide cannula (20-gauge; Plastics One, Inc. Roanoke, VA, USA) cut to project 2 mm dorsal to either the ventral hippocampus or CeA. Probe implantation coordinates relative to bregma were: AP -2.2 mm, L $\pm$ 4.2 mm, Z -8.2 mm for the CeA and AP - 5.3 mm, L $\pm$ 4.4 mm, Z -9.1 mm for the ventral hippocampus (AP, anterior-posterior; L, lateral; Z, depth; Paxinos & Watson, 1997). The guide cannula pedestal was fixed to the skull with a combination of glass ionomer cement (Fuji Plus dental acrylic, Patterson Dental, Minneapolis, MN) and cranioplastic acrylic (Plastics One, Inc.) using anchoring screws for support. Following the stereotaxic surgery, the rats were returned to their home cage and treated with the analgesic ketoprofen (5 mg/kg, im.; Med-Vet, Libertyville, IL) twice at 12 h intervals and allowed to recover for 3 days before undergoing further experimental procedures.

Microdialysis and HPLC Procedures

Approximately 12h prior to experimentation, the rats were lightly anesthetized with xylazineketamine anesthesia (80 mg ketamine/10 mg xylazine, ip.; Ketaset, Fort Dodge Labs Inc., Fort Dodge, IA, USA; xylazine, Vedco, Inc., St. Joseph, MO, USA) and

implanted with laboratory constructed concentric microdialysis probes (Farmer *et al.*, 1996), which projected -8.2 mm and -9.1 mm below the cortical surface for the CeA and ventral hippocampus, respectively. Membrane lengths of the probes were 2.4 and 3.0 mm for the CeA and ventral hippocampus, respectively. The probes were attached to a liquid swivel (Instech Laboratories, Plymouth Meeting, PA) to allow the animals free movement in 10-gallon aquaria. Artificial cerebrospinal fluid (4.295 g NaCl, 0.1005 g KCl, 0.062 g NaH₂PO₄, 0.0995 g Na₂HPO₄, 0.1015 g MgCl₂, and 0.088 CaCl₂ in 500 mL distilled H₂O; pH 7.1) was then perfused through the probe overnight at a flow rate of 0.12 µL/min. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

At least 12 hrs following probe insertion, the flow rate was increased to 0.52 µL/min and dialysates collected every 20 mins. Once 3 stable consecutive 5-HT baseline samples were collected, the rats were directly placed in a restraining tube (6 cm i.d., 27 cm long), constructed from PVC piping with a narrow channel cut in the top to allow passage of the lines attached to the microdialysis probe. These tubes provided sufficient room for movement associated with respiration, but did not allow the rat to move in any direction. Rats remained in restraining tubes for 20 min (equivalent of 1 microdialysis sampling period), before being released back into the testing chamber. Post-restraint samples were collected until 3 baseline-like samples were obtained.

For experiments in which the GR antagonist was used, mifepristone (2.5 mg/mL for a total delivery of 1.25 ng; Barr & Forster, 2011) or vehicle (5% ethanol and 5% cremophor solution in artificial cerebrospinal fluid) was intracranially delivered by reverse-dialysis at a flow rate of 0.52 µL/min over the 20 min prior to restraint stress.

Dialysates were analyzed for 5-HT using high performance liquid chromatography (HPLC) with electrochemical detection. Samples were collected at 20 min intervals and injected into the HPLC system using a rheodyne injector (Bioanalytical Systems, West Lafayette, IN) with a 10 µL loop. The collection rate of 0.52 µL/min resulted in approximately 10 µL of dialysate to insure that the loop was overfilled during each sample period. Serotonin was separated by a Sepstick 3 µm C-18 microbore column (Bioanalytical Systems) using nitrogen gas to produce a pulseless flow (Bradberry *et al.*, 1993), and detected by a working glassy carbon electrode (Bioanalytical Systems) with an oxidation potential of +0.6 V against an Ag/AgCl reference electrode using a LC-4C potentiostat (Bioanalytical Systems) set at 1.0 nA/V. The mobile phase consisted of 0.35 g EDTA, 3.2 g NaH₂PO₄, 100 mg octanalsulfonic acid (soap), 89 mL methanol, 600 µL triethylamine (TEA) and 500 mL distilled H₂O (pH 5.40). The voltage output was recorded by CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). Serotonin peaks were identified by comparison to a 5-HT standard (15.8 pg 5-HT/10 µL). The 3:1 signal to noise detection limit of this system for 5-HT was 0.24 ± 0.11 pg. Baseline serotonin values did not significantly differ between saline and amphetamine groups for the vHipp and CeA, respectively. Mean baseline values for vHipp 5-HT, uncorrected for recovery, ranged from 2.1 ± 0.3 to 3.1 ± 0.9 pg/10 µL sample. The baseline values for the CeA, uncorrected for recovery, ranged from 1.5 ± 0.3 to 2.8 ± 0.8 pg/10 µL sample.

Behavioral Scoring

To determine whether rats were more behaviorally sensitive to restraint stress during amphetamine withdrawal, separate groups of rats were implanted with probes into the ventral hippocampus and dialyzed overnight with aCSF vehicle only ($n = 7$ amphetamine and $n = 9$ saline) to mimic the experimental conditions used for the neurochemical studies. These groups were digitally recorded for behavioral scoring in a 10 gallon aquarium similar to the microdialysis chamber, 20 min prior to and 40 min post-restraint stress. Time spent in locomotion and the amount of orofacial grooming bouts, rearing and freezing (total immobility for a period of at least 3 seconds) bouts, as measures of stress-related arousal and fear (Feng *et al.*, 2005; Forster *et al.*, 2006), were scored by a trained experimenter blind to treatment using Observer XT 10.5 (Noldus Information Technology, Wageningen, The Netherlands), and collated into 20 min time-bins.

Histology

At the conclusion of experiments, rats were euthanized with Fatal Plus (Vortech, Dearborn, MI, USA). Brains were excised and fixed in formalin (Fisher Scientific, Kalamazoo, MI). The brain tissue was frozen and serially sectioned into 60 μm sections at -15°C in a Jung 1850 cryostat (Lecia Instruments, Heidelberg, Germany) and viewed under a microscope to determine probe placement. Animals were excluded from the study when the probe position was either outside the CeA or ventral hippocampus, or where the base of the brain was punctured by the probe.

Statistics

The individual behavioral measures obtained were analyzed using separate two-way ANOVA with one repeated measure (time). A significant main effect of time within either saline or amphetamine pre-treated rats was followed by one-way repeated measures ANOVA, with Student-Newman-Keuls (SNK) post-hoc tests for multiple comparisons used to identify which time points differed. Significant effects of either pre-treatment or an interaction between pre-treatment and time were followed with SNK tests to identify specific time points in which saline and amphetamine pre-treated animals differed. For microdialysis data, extracellular 5-HT concentrations (uncorrected for recovery) in the three baseline samples were averaged, and post-treatment 5-HT concentrations were calculated as a percentage change from mean baseline concentrations for each rat. For each of the microdialysis experiments, the effects of restraint stress on CeA or ventral hippocampal 5-HT levels were determined by a two-way ANOVA with one repeated measure (time). In analyses that revealed a significant time $\times$ treatment interaction, differences among treatments at each time point were identified using the Holm-Sidak *post hoc* analysis. Significant effects across time were further analyzed within treatment groups using one-way ANOVA with repeated measures followed by the Bonferroni t-test *post hoc* analysis test for multiple comparisons vs a control, where the control was the -40 min time point. The power of the respective tests ($1-\beta$) is indicated following the F values. Analyses were performed using SigmaStat v.2.03, with the alpha level set at 0.05.

Results

Amphetamine withdrawal increases behavioral responses to restraint stress

Amphetamine pre-treated rats undergoing withdrawal exhibited greater behavioral arousal and fear behavior following restraint stress as compared to saline pre-treated rats (Fig. 1). For spontaneous locomotion (Fig. 1A), there was a significant effect of pre-treatment ($F_{1,14} = 9.60$; $P < 0.01$; $1-\beta = 0.79$), a significant effect of time ($F_{2,27} = 35.05$; $P < 0.001$; $1-\beta = 1.00$) and a significant interaction between pre-treatment and time ($F_{2,27} = 9.02$; $P < 0.001$; $1-\beta = 0.94$). One-way ANOVA revealed an effect of time on locomotion for both saline ($F_{2,16} = 4.71$; $P < 0.030$; $1-\beta = 0.59$) and amphetamine ($F_{2,11} = 39.24$; $P < 0.001$; $1-\beta = 1.00$) pre-treated rats. Saline pre-treated rats exhibited greater locomotion in the 20 min immediately after the restraint stress as compared to both pre-stress (SNK $P < 0.05$) and 40 min post-stress (SNK $P < 0.03$) levels (Fig. 1A). Amphetamine pre-treated rats exhibited greater locomotion 20 and 40 min post-stress as compared to pre-stress levels (SNK $P < 0.001$; Fig. 1A). Furthermore, amphetamine pre-treated rats showed greater locomotion at 20 (SNK $P < 0.001$) and 40 (SNK $P < 0.05$) min post-stress compared to saline pre-treated rats (Fig. 1A).

Similar to locomotion, there was a significant effect of pre-treatment ($F_{1,14} = 6.97$; $P < 0.02$; $1-\beta = 0.62$), a significant effect of time ($F_{2,27} = 19.68$; $P < 0.001$; $1-\beta = 1.00$) and a significant interaction between pre-treatment and time ($F_{2,27} = 12.51$; $P < 0.001$; $1-\beta = 0.99$) on the number of rearing bouts exhibited by rats in response to restraint (Fig. 1B). Amphetamine pre-treated rats showed an effect of time on rearing bouts ($F_{2,11} = 14.41$; $P < 0.001$; $1-\beta = 0.29$), with greater rearing being expressed in the 20 min following the stressor as compared to both pre- (SNK $P = 0.001$) and 40 min (SNK $P < 0.005$) post-stress (Fig. 1B). An effect of time on rearing was not apparent for saline pre-treated rats ($F_{2,16} = 1.55$; $P = 0.243$; $1-\beta = 0.21$; Fig. 1B). Consequently, amphetamine pre-treated rats showed greater rearing during the 20 min following restraint stress as compared to saline pre-treated rats (SNK $P < 0.001$; Fig. 1B).

There were also significant effects of pre-treatment ($F_{1,14} = 12.64$; $P < 0.005$; $1-\beta = 0.91$) and time ($F_{2,27} = 3.79$; $P < 0.05$; $1-\beta = 0.50$) on the number of orofacial grooming bouts (Fig. 1C), although there was no significant interaction between pre-treatment and time ($F_{2,27} = 0.88$; $P = 0.472$; $1-\beta = 0.50$). An effect of time was observed for amphetamine pre-treated ($F_{2,11} = 5.52$; $P < 0.05$; $1-\beta = 0.82$) but not saline pre-treated ($F_{1,16} = 1.33$; $P = 0.291$; $1-\beta = 0.92$) rats, with amphetamine pre-treated rats showing greater bouts of orofacial grooming in the 20 min post-stress compared to pre-stress levels (SNK $P < 0.03$; Fig. 1C). Therefore, amphetamine pre-treated rats exhibited more bouts of orofacial grooming in the 20 min following restraint stress than saline pre-treated rats (SNK $P < 0.02$; Fig. 1C).

While the number of bouts of freezing behavior was quite low (Fig. 1D), there was a significant effect of pre-treatment ($F_{1,14} = 8.79$; $P < 0.01$; $1-\beta = 0.75$) and a significant effect of time ($F_{2,26} = 8.47$; $P < 0.001$; $1-\beta = 0.92$), but no interaction between pre-treatment and time ($F_{1,26} = 2.25$; $P = 0.126$; $1-\beta = 0.24$). Amphetamine pre-treated rats showed a significant effect of time for freezing bouts ($F_{2,11} = 7.69$; $P < 0.01$; $1-\beta = 0.65$), and exhibited greater freezing in the 20 (SNK $P < 0.02$) and 40 (SNK $P < 0.001$) min following

the stressor as compared to pre-stress levels (Fig. 1D). An effect of time on freezing was not apparent for saline pre-treated rats ($F_{2,15} = 1.29$; $P = 0.304$; $1-\beta = 0.87$; Fig. 1D). Moreover, amphetamine pre-treated rats exhibited greater freezing behavior 20 (SNK $P < 0.02$) and 40 (SNK $P < 0.02$) min post-stress as compared to saline pre-treated rats (Fig. 1D).

Microdialysis probe placements

Representative placements of probe working membrane surfaces with a 2.4 -3.0 mm length dialysis membrane for the ventral hippocampus and the CeA, respectively, are drawn to scale and illustrated by Fig. 2. Probe locations were similarly distributed in saline and amphetamine pre-treated animals across all experiments. Data from rats in which the probe placements missed the targeted brain region were excluded from subsequent analyses.

Amphetamine withdrawal reduces stress-induced increases in extracellular 5-HT in the ventral hippocampus

This experiment determined if chronic amphetamine treatment induced long-lasting changes in stress-induced serotonergic function in the rat ventral hippocampus, as measured following prolonged withdrawal. Two-way repeated measures ANOVA revealed significant effects of drug treatment ($F_{1,14} = 7.55$, $P < 0.02$; $1-\beta = 0.67$), time ($F_{8,103} = 5.04$, $P < 0.001$; $1-\beta = 0.99$) and an interaction between treatment and time ($F_{8,103} = 5.00$, $P < 0.001$; $1-\beta = 0.99$) (Fig. 3A) on ventral hippocampal 5-HT. Restraint stress increased extracellular 5-HT in the ventral hippocampus of saline pre-treated rats ($F_{8,43} = 3.69$, $P < 0.01$; $1-\beta = 0.89$; Fig. 3A). This effect was rapid, with 5-HT concentrations increased over 600 % relative to pre-stress baseline values in the sample collected at the conclusion of the 20 min restraint period (0 min time point, Bonferroni t-test, $P < 0.002$). In contrast, amphetamine-withdrawn rats showed a modest decline in intra-ventral hippocampal extracellular 5-HT in response to restraint stress ($F_{8,60} = 8.70$, $P < 0.001$; $1-\beta = 1.00$; Fig. 3A). This effect was present in the first sample collected at the end of the restraint period (0 min time point) and, with exception of the 20 min post-restraint sample, persisted for the duration of the experiment with declines in 5-HT ranging from 16% at time 0 min to 44% at time 100 min relative to pre-stress baseline values (Bonferroni t-test, $P < 0.05$). Intra-ventral hippocampal 5-HT was significantly higher in saline pre-treated rats when compared to the amphetamine pre-treated group in the sample collected at the conclusion of the stress (0 min) and the first post-stress sample (20 min; Holm-Sidak $P < 0.05$; Fig. 3A).

Amphetamine withdrawal enhances stress-induced extracellular 5-HT in the CeA

This experiment tested whether chronic amphetamine treatment and prolonged withdrawal induced long-lasting changes in stress-induced serotonergic function in the rat CeA. Two-way repeated measures ANOVA showed significant effects of drug treatment ($F_{1,10} = 5.29$, $P < 0.05$; $1-\beta = 0.46$), time ($F_{7,64} = 7.10$, $P < 0.001$; $1-\beta = 1.00$) and an interaction between treatment and time ($F_{7,64} = 3.58$, $P < 0.005$; 0.86) on CeA 5-HT (Fig. 3B). Restraint stress increased extracellular CeA 5-HT in both saline ($F_{6,41} = 7.07$, $P < 0.001$; $1-\beta = 1.00$) and amphetamine pre-treated rats ($F_{4,23} = 3.17$, $P < 0.02$; Fig. 3B; $1-\beta = 0.68$). As for the ventral hippocampus, this effect was rapid, with 5-HT elevated over 70% at the conclusion of restraint (0 min) in the saline pre-treatment group (Bonferroni t-test, $P < 0.05$) and nearly

400% at 20 min post-restraint in the amphetamine pre-treated group (Bonferroni t-test, $P < 0.05$) relative to pre-stress baseline values (Fig. 3B). However, the stress-induced increases in extracellular CeA 5-HT were significantly higher in amphetamine pre-treated rats as compared to saline controls (Fig. 3B). This effect was observed in the first sample collected after the conclusion of the stress (20 min) and the second post-stress sample (40 min; Holm-Sidak $P < 0.05$).

The GR antagonist mifepristone blocks restraint stress-induced 5-HT increases in the ventral hippocampus but not the CeA

The goal of this experiment was to test whether the stress-induced alterations in serotonergic transmission in the rat ventral hippocampus and the CeA were dependent on local GR activation. The GR blocker mifepristone was perfused into either the ventral hippocampus or CeA 20 min prior to the restraint stress, so that the drug would reach the brain as the same time as the stress was initiated. The effects of the GR antagonist were not evaluated in the ventral hippocampus of amphetamine pre-treated rats, since restraint stress in this group failed to induce any increases in hippocampal 5-HT (Fig. 3A). Within saline pre-treated rats, analysis of the effects of the GR antagonist ($n = 5$) or vehicle ($n = 6$) on stress-induced intra-ventral hippocampal 5-HT revealed significant effects of drug treatment ($F_{1,9} = 8.28$, $P < 0.02$; $1-\beta = 0.68$), time ($F_{7,52} = 2.68$, $P < 0.02$; $1-\beta = 0.63$) and an interaction between treatment and time ($F_{7,52} = 2.70$, $P < 0.02$; $1-\beta = 0.64$) (Fig. 3C). When analyzed across time, a 270% increase in ventral hippocampus 5-HT in vehicle-treated rats was evident 20 min after the conclusion of restraint ($F_{6,27} = 2.85$, $P < 0.025$; $1-\beta = 0.62$; Bonferroni t-test, $P < 0.05$) relative to pre-stress baseline values (Fig. 3C). There was also an effect of time in the mifepristone treated group ($F_{4,25} = 2.58$; $P < 0.05$; $1-\beta = 0.53$). However, post hoc comparisons failed to identify a significant difference between pre-stress baseline 5-HT values and any post-stress time points following mifepristone delivery. Vehicle-receiving rats showed higher 5-HT than rats treated with intra-ventral hippocampal mifepristone immediately after stress cessation (0 min time point), which persisted until 40 min post-restraint (Holm-Sidak $P < 0.05$; Fig. 3C), suggesting that local GR antagonism inhibits stress-induced hippocampal 5-HT release in saline pre-treated rats.

Since stress-induced increases in extracellular 5-HT were detected in the CeA of both amphetamine and saline pre-treated rats, we evaluated the effects of the GR blocker mifepristone in both groups (Fig. 3D). There were significant effects of drug treatment ($F_{1,13} = 7.86$, $P < 0.020$; $1-\beta = 0.687$), time ($F_{7,84} = 12.84$, $P < 0.001$; $1-\beta = 1.00$), and a significant interaction between treatment and time ($F_{7,84} = 4.05$, $P < 0.001$; $1-\beta = 0.93$). There were significant increases in extracellular 5-HT across time in both saline ($F_{7,45} = 6.28$, $P < 0.001$; $1-\beta = 0.99$) and amphetamine pre-treated rats ($F_{6,37} = 7.382$, $P < 0.001$; $1-\beta = 0.99$). In both groups, this effect was evident at 20 min post restraint (Bonferroni t-test, $P < 0.05$) with 5-HT increases of approximately 60% and 330% in saline and amphetamine pre-treated rats, respectively, relative to pre-stress baseline values. Furthermore, CeA 5-HT was significantly higher in amphetamine pre-treated rats when compared to the saline pre-treated group in the first two post-stress samples (20 and 40 min; Holm-Sidak $P < 0.05$). Intra-CeA administration of the GR antagonist mifepristone did not block the restraint stress-induced increases in 5-HT in either amphetamine or saline pre-treated rats (Fig. 3D).

Discussion

The results of these experiments show that stress-induced behaviors associated with arousal and fear are enhanced following long-term withdrawal from chronic amphetamine. This is accompanied by alterations to stress-induced changes in serotonergic transmission within limbic regions known to modulate behavioral responses to stress. Specifically, amphetamine withdrawn rats subjected to acute restraint stress had higher levels of locomotion, rearing, orofacial grooming and freezing than saline pre-treated rats following removal of the stressor. Stress and stress-related hormones increase both non-ambulatory and ambulatory activity in rodents (as reviewed by Lowry & Moore, 2006) which is thought to represent an increase in behavioral arousal. Thus, increases in locomotion, grooming and rearing in response to stress are interpreted as increased stress-induced behavioral arousal during amphetamine withdrawal. Neurochemical measurements showed that exposure to restraint stress resulted in an increase in ventral hippocampal extracellular 5-HT in the saline pre-treated group. However, this effect of restraint on ventral hippocampus 5-HT was completely absent in rats pre-treated with amphetamine. Furthermore, restraint-induced increases in ventral hippocampal 5-HT in saline pre-treated rats was blocked by local delivery of the GR antagonist mifepristone, suggesting that the response was due to local activation of GRs. In contrast, both saline and amphetamine pre-treated rats exhibited increases in extracellular 5-HT within the CeA following restraint, but this response was markedly higher in the amphetamine pre-treated group. Delivery of intra-CeA mifepristone failed to block the serotonergic response to the stressor in either group, suggesting that the effect was independent of local corticosterone activation of GRs. It is not clear at this stage whether 5-HT in the ventral hippocampus or CeA relates to behavioral arousal following stress. However, 5-HT in the CeA is directly related to the expression of freezing behavior (Forster *et al.*, 2006), thus heightened stress-induced 5-HT in the CeA may underlie increased freezing following stress during amphetamine withdrawal. Together, the current findings indicate that during long-term amphetamine withdrawal, behavioral reactivity to stressors is increased, and that this effect may be mediated, in part, through the disruption of stress-related limbic serotonergic responses.

Expression of anxiety-like behaviors persists for at least 4 weeks following drug cessation (Barr *et al.*, 2010), and there is evidence that during acute amphetamine withdrawal there is a disruption in corticosterone-mediated serotonergic transmission in the ventral hippocampus (Barr *et al.*, 2013), which is required for stress adaptation (Graeff *et al.*, 1996; Joca *et al.*, 2003; Keck *et al.*, 2005; Joca *et al.*, 2007). Therefore, we first tested if long-term amphetamine withdrawal is associated with increased behavioral responsiveness to an acute stressor. Our results show that in response to restraint stress, rats chronically pre-treated with amphetamine exhibit higher levels of arousal and fear-associated behaviors than saline pre-treated controls at two weeks following cessation of drug treatment. This finding, combined with established work showing that rodents exhibit increases in depressive-like and anxiety-like behaviors during both acute and long-term withdrawal from psychostimulants (Harris & Aston-Jones, 1993; Cryan *et al.*, 2003; Perrine *et al.*, 2008; Barr *et al.*, 2010; Vuong *et al.*, 2010; El Hage *et al.*, 2012) and that stress exposure can reinstate drug-seeking behavior (Capriles & Cancela, 1999), is consistent with a link between long-lasting effects of

psychostimulant withdrawal and the ability to respond appropriately to stress. Furthermore, these behavioral changes mimic negative withdrawal-induced effects exhibited by humans that are believed to contribute to drug relapse (Koob & Le Moal, 2008).

Acute (24 h) withdrawal from chronic amphetamine treatment dramatically decreases corticosterone-induced increases in extracellular 5-HT within the ventral hippocampus of anesthetized rats (Barr & Forster, 2011). Here we show that after two weeks of withdrawal from chronic amphetamine, increases in ventral hippocampal 5-HT in response to restraint stress are virtually abolished. Previous research demonstrates that corticosterone rapidly increases 5-HT in the ventral hippocampus via local GRs (Barr & Forster, 2011). The current results add to this by showing that restraint-stress induced 5-HT responses within the ventral hippocampus of saline pre-treated animals were similarly dependent on local GR activation. Fairly rapid genomic responses to corticosterone have been reported, but slower than the time frame indicated by the effects in the current study. For example, glucocorticoid receptor translocation to the nucleus in rat CA1 cells occurs by 30 min after plasma corticosterone reaches peak levels (Sarabdjitsingh *et al.*, 2010) and corticosterone-induced transcriptional changes in rat hippocampal slices are evident 1 hour after systemic corticosterone injection (Morsink *et al.*, 2007). Thus, the current effects may be due to non-genomic mechanisms.

Corticosterone may increase ventral hippocampal 5-HT via a non-genomic membrane GR receptor (Orchinik *et al.*, 1991). Certainly, the ability of corticosterone to increase excitatory neurotransmitters in the hippocampus has been attributed to membrane GRs, which are not sensitive to mifepristone (Venero & Borrell, 1999). However, stress-induced (current study) and corticosterone-induced (Barr & Forster, 2011) increases in ventral hippocampus 5-HT are blocked by mifepristone, suggesting alternative non-genomic mechanisms. Zeise *et al.* (1992) show that corticosterone decreases GABA-mediated inhibitory postsynaptic potentials in the hippocampus, an effect sensitive to mifepristone and dependent on cytosolic events. It has been suggested that corticosterone may alter GABAergic inhibition in the hippocampus by GR-mediated effects on intracellular signaling that are independent of genomic mechanisms (Teschmacher *et al.*, 1996). Therefore, the possibility that increased stress-induced 5-HT may be a result of GR-mediated reduction of GABAergic inhibition of serotonergic terminals in the ventral hippocampus warrants further investigation. Combined, these findings suggest that increases in ventral hippocampus 5-HT in response to stress are mediated through corticosterone activation of local GRs, with the blunted response following prolonged amphetamine withdrawal resulting in part from a reduction in GR expression in the ventral hippocampus caused by chronic amphetamine treatment (Shilling *et al.*, 1996; Barr & Forster, 2011).

However, other complementary mechanisms besides a reduction in GRs likely contribute to the lack of serotonergic response during prolonged amphetamine withdrawal. This is because amphetamine pre-treated rats still show some ventral hippocampal GR expression (Barr & Forster, 2011), which suggests that some corticosterone-induced or stress-induced 5-HT should be retained in the ventral hippocampus of amphetamine pre-treated rats. Repeated amphetamine administration does not impact the firing rate of dorsal raphe neurons that innervate the ventral hippocampus (Heidenreich and Rebec, 1989), implying

that this is unlikely to contribute to the attenuated 5-HT response in the ventral hippocampus during withdrawal. In addition, synthesis of 5-HT in either the raphe nuclei or ventral hippocampus does not differ between amphetamine and saline-pre-treated animals (Barr *et al.*, 2013). However, 5-HT clearance in the ventral hippocampus is increased in amphetamine pre-treated rats (Barr *et al.*, 2013). This effect is not mediated by the serotonin transporter (SERT), but instead by the organic cation transporter 3 (OCT3), a corticosterone-sensitive monoamine transporter (Grundemann *et al.*, 1998) that is widely distributed in the brain Gasser *et al.*, 2009) and shows increased expression and function in the ventral hippocampus following chronic amphetamine treatment and acute withdrawal (Barr *et al.*, 2013). Therefore, the current finding of an absence in stress-induced increases in ventral hippocampal 5-HT concentrations during prolonged amphetamine withdrawal is most likely due to a combination of decreased GR activation and increased OCT3-mediated 5-HT clearance.

The finding that stress-induced 5-HT responses in the ventral hippocampus are blunted during amphetamine withdrawal is significant, because decreased hippocampal responsiveness is associated with attenuated HPA axis negative feedback that contributes to maladaptive stress responses (McEwen, 2002; Herman & Mueller, 2006). Furthermore, increases in ventral hippocampus 5-HT have been implicated in the ability to cope with stressors (reviewed in Joca *et al.*, 2007). Thus, our results showing that amphetamine pre-treated rats exhibit virtually no changes in ventral hippocampal 5-HT when subjected to an acute stressor, combined with the earlier report that intra-ventral hippocampus corticosterone at physiological levels fails to increase extracellular 5-HT (Barr and Forster, 2011) suggests that chronic amphetamine induces a long-lasting changes in of ventral hippocampal serotonergic function that may lead to altered ability to cope with stress during withdrawal. In contrast to the ventral hippocampus, stress-induced CeA 5-HT was augmented in rats undergoing amphetamine withdrawal when compared with saline pre-treated controls. This greater response in amphetamine pre-treated rats may not be mediated by local GR activation, since intra-CeA delivery of the GR antagonist mifepristone at the same concentration that blocked stress-induced 5-HT in the ventral hippocampus failed to block stress-induced increases in CeA 5-HT in either control or amphetamine pre-treated rats. It is possible that the enhanced stress-induced CeA 5-HT response during prolonged amphetamine withdrawal might be mediated by corticotropin-released factor (CRF). This is because during acute restraint stress, the CeA releases CRF (Merali *et al.*, 1998), and stress-induced increases in CeA 5-HT depend on the activation of CRF receptors (Mo *et al.*, 2008), presumably via those located in the dorsal raphe (Lowry *et al.*, 2000; Forster *et al.*, 2006). A consequence of chronic amphetamine treatment is an increase in dorsal raphe expression of CRF₂ receptors that persist during long-term drug withdrawal (Pringle *et al.*, 2008), and intra-dorsal raphe injections of a CRF₂ antagonist suppress anxiety-like behaviors during withdrawal (Vuong *et al.*, 2010). Furthermore, CRF₂ receptor activation of the dorsal raphe increases 5-HT in the CeA to a greater degree in amphetamine pre-treated rats as compared to saline controls (Scholl *et al.*, 2010). Therefore, future research should test whether the enhanced CeA 5-HT in response to stress during prolonged amphetamine withdrawal is similarly mediated by increased CRF₂ receptor activation in the dorsal raphe nucleus, and if

blockade of these receptors reduces both the heightened CeA 5-HT and behavioral responses to stress.

Functionally, the CeA has been implicated the expression of fear and anxiety-like behaviors, and its activity has been linked to the reinstatement of drug-seeking behavior (Lu *et al.*, 2005; Nawata *et al.*, 2012). A heightened and persistent expression of stress-induced anxiety is usually observed during the period of drug withdrawal, indicating the susceptibility to relapse when exposed to stressors (Erb, 2010). Evidence for the amygdala contributing to this response is supported by the report that footshock-induced methamphetamine reinstatement is due to increased CRF in the amygdala (Nawata *et al.*, 2012). Little is known about the exact role 5-HT in the CeA might play in the increased behavioral response to stress as observed in amphetamine pre-treated rats of the current study, or in anxiety states as previously seen during amphetamine withdrawal (Barr *et al.*, 2010; Vuong *et al.*, 2010). However, previous studies show that both unconditioned and conditioned fear responses are associated with increased amygdalar 5-HT levels in rats (Yokoyama *et al.*, 2005; Forster *et al.*, 2006), and humans suffering from posttraumatic stress disorder have reduced 5-HT transporter expression in the amygdala (Murrough *et al.*, 2011) which would allow for greater extracellular 5-HT during stress. Furthermore, stimulation of 5-HT_{2C} receptors in the amygdala has been shown to be anxiogenic (Cornelio and Nunes-de-Souza, 2007; Christianson *et al.*, 2010). Combined, these results suggest that the presence of an augmented serotonergic response in the CeA to stress during amphetamine withdrawal may contribute to increased stress susceptibility and anxiety.

In conclusion, the current results suggest that withdrawal from chronic amphetamine results in long-lasting and opposing effects on ventral hippocampus and CeA 5-HT responses to stress. These alterations are proposed to be mediated by GRs and OCT3 in the ventral hippocampus and by CRF₂ receptors on serotonergic neurons projecting to the CeA, which requires further testing. The alterations to serotonergic function in these two limbic regions are thought to result in heightened stress responsiveness and anxiety. Therefore, future efforts to normalize stress-induced 5-HT function in the ventral hippocampus and CeA may reduce stress sensitivity and anxiety states during withdrawal, and thus prevent drug relapse.

Acknowledgements

We would like to thank Nate Vinzant and Ashley Cook for their assistance with amphetamine treatments. This work was supported by NIH grant R01 DA019921 (GLF), NSF grant IOS 1257679 (MJW) and NSF grant IOS 0921874 (KJR).

Abbreviations

CeA	Central nucleus of the amygdala
CRF	Corticotropin releasing factor
GR	Glucocorticoid receptor
HPLC	High pressure liquid chromatography
OCT3	Organic cation transporter 3

5-HT	Serotonin
SERT	Serotonin transporter
SNK	Student Newman-Keuls

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Figure 1.

Amphetamine withdrawal increases spontaneous (A) locomotion, (B) rearing, (C) orofacial grooming and (D) freezing responses immediately following restraint stress. Restraint was applied for 20 min as marked by the horizontal bar. Values for behavior are expressed as the mean $\pm$ SEM. ^aSignificantly different from pre-stress levels; ^bSignificant differences between amphetamine (closed circles; $n = 7$) and saline (open circles; $n = 9$) treatment groups; ($P < 0.05$).

Figure 2.

Illustrations from Paxinos & Watson (1997) showing representative locations of the working surface of probes implanted in the ventral hippocampus of (A) saline, (B) amphetamine pre-treated rats and the central nucleus of the amygdala (grey area) of (C) saline and (D) amphetamine pre-treated rats .

Figure 3.

(A) Restraint stress increased ventral hippocampus 5-HT levels in saline ($n = 7$) but not amphetamine ($n = 9$) pre-treated rats. (B) Restraint stress augmented the increase in CeA 5-HT levels in amphetamine ($n = 5$) pre-treated rats as compared to saline ($n = 7$) pre-treated rats. (C) The increase in ventral hippocampal 5-HT levels within saline pre-treated rats was blocked by local delivery of the GR antagonist mifepristone ($n = 5$) compared to vehicle ($n = 6$) (D) Increases in CeA 5-HT were still present in both amphetamine ($n = 7$) and saline ($n = 8$) pre-treated rats following local administration of the GR antagonist mifepristone. All measure were obtained following a two week period of drug withdrawal. The stress was applied for 20 min during the sample period marked by the horizontal bar. Amphetamine pre-treated rats are represented by closed circles and saline by open circles. Values for 5-HT are expressed as the mean $\pm$ SEM percent change from baseline, which was determined from the average of three pre-stress samples. ^aSignificantly different from baseline levels; ^bSignificant differences between treatment groups; ($P < 0.05$).