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, which are 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, stress-induced increases in ventral hippocampal serotonin (5-HT) levels have been linked to coping mechanisms. The goal of this study was to investigate the 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 pretreated with amphetamine (2.5 mg/kg, intraperitoneal) and then subjected to 2 weeks of withdrawal. Amphetamine withdrawal resulted in increased stress-induced behavioral arousal relative to control treatment, suggesting that drug withdrawal induced greater sensitivity to the stressor. When microdialysis was used to determine the effects of restraint on extracellular 5-HT, stress-induced increases in 5-HT levels 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 increase in 5-HT levels in saline-pretreated rats, suggesting that glucocorticoid receptors mediate stress-induced increases in 5-HT levels in the ventral hippocampus. However, mifepristone had no effect on stress-induced increases in 5-HT levels in the central amygdala, indicating that stress increases 5-HT levels in this region independently of glucocorticoid receptors. During amphetamine withdrawal, the absence of stress-induced increases in ventral hippocampal 5-HT levels 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.

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 the 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 hippocampal 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, 2007; Bast, 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 hippocampal 5-HT levels 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 levels 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, whereas 5-HT$_{1A}$ activation is anxiolytic (Cornelio & Nunes-de-Souza, 2007; Christianson *et al.*, 2010; Li *et al.*, 2012).

Rats chronically pretreated with amphetamine show 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 levels are attenuated during acute (24 h) amphetamine withdrawal, possibly because of reduced GR expression along with increased expression of organic cation transporter 3 (OCT3), a polyspecific transporter of organic cations, including monoamines (Barr & Forster, 2011; Barr *et al.*, 2013). In contrast, rats undergoing acute withdrawal from amphetamine show enhanced serotonergic responses in the CeA to the stress-associated neurohormone corticotropin-releasing factor (CRF) (Scholl *et al.*, 2010). Together, these results suggest that there are 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 increases in 5-HT levels in the ventral hippocampus, and augments stress-induced increases in 5-HT levels in the CeA.

Materials and methods

Animals and amphetamine treatment

The male Sprague-Dawley rats (Animal Resource Center, University of South Dakota, Vermillion, SD, USA) used in the experiments were pair-housed in saline ($n = 42$) and amphetamine ($n = 28$) pairs from postnatal week 3 onwards under a reverse light cycle (lights off from 10:00 to 22:00 h) with free access to water and food. Amphetamine or saline treatment was initiated when the rats were at least 8 weeks of age. Rats were injected with amphetamine (2.5 mg/kg, intraperitoneal) or saline once daily for 2 weeks between 11:00 and 14:00 h. This treatment regime increases the anxiety-like behavior of rats at 24 h, 2 weeks and 4 weeks of withdrawal (Barr *et al.*, 2010; Vuong *et al.*, 2010). The rats used in the following experiments had undergone 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 67 rats were used in these experiments.

Surgical procedures

At the end of the first week of withdrawal, surgical prodecures were performed aseptically under isoflurane anesthesia (3% isoflurane; 0.3 mL/min oxygen). Once anesthetised, rats were placed in the stereotaxic frame in the flat-skull position and implanted unilaterally with a guide cannula (20-gauge; Plastics One, Roanoke, VA, USA) cut to project 2 mm dorsal to either the ventral hippocampus or CeA. Probe implantation coordinates relative to bregma were as follows: anteroposterior −2.2 mm, lateral ±4.2 mm and depth −8.2 mm for the

CeA; and anteroposterior −5.3 mm, lateral ±4.4 mm and depth −9.1 mm for the ventral hippocampus (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, USA) and cranioplastic acrylic (Plastics One), with 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, intramuscular; Med-Vet, Libertyville, IL, USA) twice at 12-h intervals, and allowed to recover for 3 days before undergoing further experimental procedures.

Microdialysis and high-performance liquid chromatography procedures

Approximately 12 h prior to experimentation, the rats were lightly anesthetised with xylazine/ketamine anesthesia [80 mg ketamine/10 mg xylazine, intraperitoneal (Ketoset; Fort Dodge Labs, Fort Dodge, IA, USA); xylazine (Vedco, 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, USA) to allow the rats free movement in 10-gallon aquaria. Artificial cerebrospinal fluid (4.295 g of NaCl, 0.1005 g of KCl, 0.062 g of NaH$_2$PO$_4$, 0.0995 g of Na$_2$HPO$_4$, 0.1015 g of MgCl$_2$ and 0.088 g of CaCl$_2$ in 500 mL of distilled H$_2$O; pH 7.1) was then perfused though the probe overnight at a flow rate of 0.12 μL/min. All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

At least 12 h following probe insertion, the flow rate was increased to 0.52 μL/min and dialysates were collected every 20 min. Once three stable consecutive 5-HT baseline samples had been collected, the rats were directly placed in a restraining tube (internal diameter, 6 cm; length, 27 cm) 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 the restraining tubes for 20 min (equivalent of one microdialysis sampling period), before being released back into the testing chamber. Post-restraint samples were collected until three baseline-like samples had been 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 analysed for 5-HT by high-performance liquid chromatography with electrochemical detection. Samples were collected at 20-min intervals and injected into the high-performance liquid chromatography system with a rheodyne injector (Bioanalytical Systems, West Lafayette, IN, USA) with a 10-μL loop. The collection rate of 0.52 μL/min resulted in ~10 μL of dialysate to ensure that the loop was overfilled during each sample period. 5-HT was separated on a Sepstick 3-μm C-18 microbore column (Bioanalytical Systems) with 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 with an LC-4C potentiostat (Bioanalytical Systems) set at 1.0 nA/V. The mobile phase consisted of 0.35 g of EDTA, 3.2 g of NaH_2PO_4, 100 mg of octanalsulfonic acid (soap), 89 mL of methanol, 600 µL of triethylamine and 500 mL of distilled H_2O (pH 5.40). The voltage output was recorded with the CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). 5-HT peaks were identified by comparison with a 5-HT standard (15.8 pg of 5-HT in 10 µL). The 3 : 1 signal-to-noise detection limit of this system for 5-HT was 0.24 ± 0.11 pg. Baseline 5-HT values did not significantly differ between the saline and amphetamine groups for the ventral hippocampus and CeA, respectively. Mean baseline values for ventral hippocampal 5-HT, uncorrected for recovery, ranged from 2.1 ± 0.3 to 3.1 ± 0.9 pg per 10 µL of sample. The baseline values for the CeA, uncorrected for recovery, ranged from 1.5 ± 0.3 to 2.8 ± 0.8 pg per 10 µL of 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 dialysed overnight with artificial cerebrospinal fluid vehicle only (amphetamine, $n = 7$; saline, $n = 9$) 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 after restraint stress. Time spent in locomotion and the amounts of orofacial grooming bouts, rearing and freezing bouts (total immobility for a period of at least 3 s), 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 killed with Fatal Plus (Vortech, Dearborn, MI, USA). Brains were excised and fixed in formalin (Fisher Scientific, Kalamazoo, MI, USA). The brain tissue was frozen and serially sectioned into 60-µm sections at -15 °C in a Jung 1850 cryostat (Lecia Instuments, Heidelberg, Germany), and viewed under a microscope to determine probe placement. Rats were excluded from the study when the probe position was outside the CeA or ventral hippocampus, or when the base of the brain was punctured by the probe.

Statistics

The individual behavioral measures obtained were analysed with separate two-way ANOVAs with one repeated measure (time). Detection of a significant main effect of time in either saline-pretreated or amphetamine-pretreated rats was followed by one-way repeated measures ANOVA, with Student–Newman–Keuls (SNK) *post hoc* tests for multiple comparisons being used to identify which time points differed. Significant effects of either pretreatment or an interaction between pretreatment and time were followed with SNK tests to identify specific time points at which saline-pretreated and amphetamine-pretreated rats 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 concen-

tration for each rat. For each of the microdialysis experiments, the effects of restraint stress on CeA or ventral hippocampal 5-HT levels were determined with a two-way ANOVA with one repeated measure (time). In analyses that revealed a significant interaction between time and treatment, differences among treatments at each time point were identified with Holm–Sidak *post hoc* analysis. Significant effects across time were further analysed within treatment groups with one-way ANOVA with repeated measures followed by Bonferroni *t*-test *post hoc* analysis 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 with SIGMASTAT v.2.03, with the alpha level set at 0.05.

Results

Amphetamine withdrawal increases behavioral responses to restraint stress

Amphetamine-pretreated rats undergoing withdrawal showed greater behavioral arousal and fear behavior following restraint stress than saline-pretreated rats (Fig. 1). For spontaneous locomotion (Fig. 1A), there were significant effects of pretreatment ($F_{1,14} = 9.60$; $P < 0.01$; $1-\beta = 0.79$), time ($F_{2,27} = 35.05$; $P < 0.001$; $1-\beta = 1.00$), and an interaction between pretreatment 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-pretreated ($F_{2,16} = 4.71$; $P < 0.030$; $1-\beta = 0.59$) and amphetamine-pretreated ($F_{2,11} = 39.24$; $P < 0.001$; $1-\beta = 1.00$) rats. Saline-pretreated rats showed greater locomotion in the 20 min immediately after the restraint stress than both pre-stress (SNK, $P < 0.05$) and 40 min post-stress (SNK, $P < 0.03$) levels (Fig. 1A). Amphetamine-pretreated rats showed greater locomotion 20 min and 40 min post-stress than pre-stress (SNK, $P < 0.001$; Fig. 1A). Furthermore, amphetamine-pretreated rats showed greater locomotion at 20 min (SNK, $P < 0.001$) and 40 min (SNK, $P < 0.05$) post-stress than saline-pretreated rats (Fig. 1A).

As was found for locomotion, there were significant effects of pretreatment ($F_{1,14} = 6.97$; $P < 0.02$; $1-\beta = 0.62$), time ($F_{2,27} = 19.68$; $P < 0.001$; $1-\beta = 1.00$) and an interaction between pretreatment and time ($F_{2,27} = 12.51$; $P < 0.001$; $1-\beta = 0.99$) on the number of rearing bouts shown by rats in response to restraint (Fig. 1B). Amphetamine-pretreated 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 than both pre-stress (SNK, $P = 0.001$) and 40 min post-stress (SNK, $P < 0.005$) (Fig. 1B). An effect of time on rearing was not apparent for saline-pretreated rats ($F_{2,16} = 1.55$; $P = 0.243$; $1-\beta = 0.21$; Fig. 1B). Consequently, amphetamine-pretreated rats showed greater rearing during the 20 min following restraint stress than saline-pretreated rats (SNK, $P < 0.001$; Fig. 1B).

There were also significant effects of pretreatment ($F_{1,14} = 12.64$; $P < 0.005$; $1-\beta = 0.91$) 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 effect of an interaction between pretreatment and time ($F_{2,27} = 0.88$; $P = 0.472$; $1-\beta = 0.50$). An effect of time was observed for amphetamine-pretreated ($F_{2,11} = 5.52$; $P < 0.05$; $1-\beta = 0.82$) but not saline-pretreated ($F_{1,16} = 1.33$; $P = 0.291$; $1-\beta = 0.92$) rats, with amphetamine-pretreated rats showing more bouts of orofacial grooming in the 20 min post-stress than prestress (SNK, $P < 0.03$; Fig. 1C). Therefore, amphetamine-pretreated rats showed more bouts of orofacial
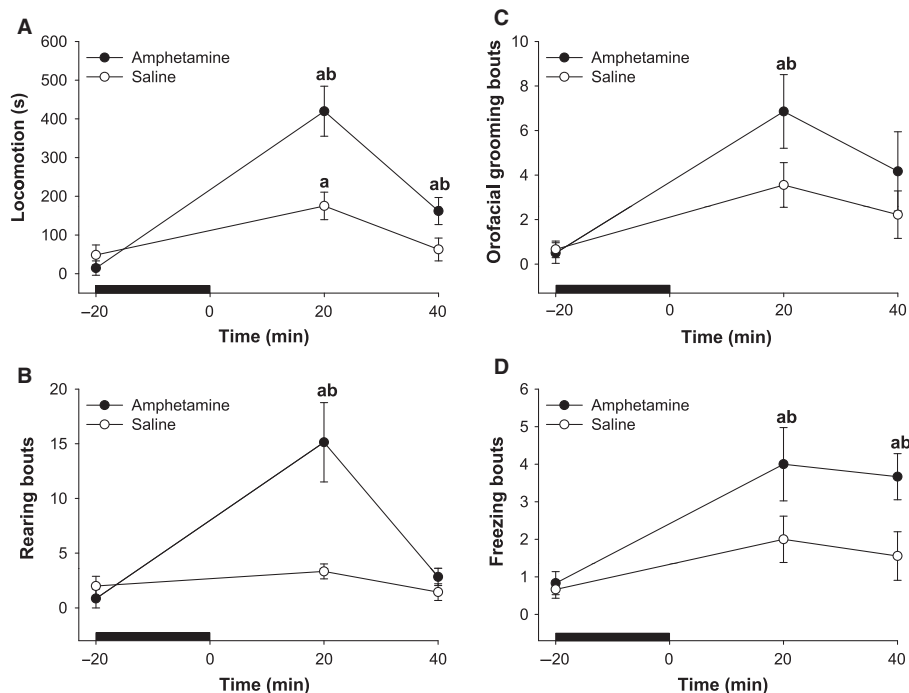
FIG. 1. Amphetamine withdrawal increases spontaneous (A) locomotion, (B) rearing, (C) orofacial grooming and (D) freezing responses immediately following restraint stress. Restraint was applied for 20 min, as marked by the horizontal bar. Values for behavior are expressed as the mean ± standard error of the mean. [a]Significantly different from pre-stress levels. [b]Significant differences between the amphetamine (closed circles; $n = 7$) and saline (open circles; $n = 9$) treatment groups. $P < 0.05$.

grooming in the 20 min following restraint stress than saline-pre-treated rats (SNK, $P < 0.02$; Fig. 1C).

Although the number of bouts of freezing behavior was quite low (Fig. 1D), there was a significant effect of pretreatment ($F_{1,14} = 8.79$; $P < 0.01$; $1-\beta = 0.75$) and a significant effect of time ($F_{2,26} = 8.47$; $P < 0.001$; $1-\beta = 0.92$), but no effect of an interaction between pretreatment and time ($F_{1,26} = 2.25$; $P = 0.126$; $1-\beta = 0.24$). Amphetamine-pretreated rats showed a significant effect of time on freezing bouts ($F_{2,11} = 7.69$; $P < 0.01$; $1-\beta = 0.65$), and showed greater freezing behavior in the 20 min (SNK, $P < 0.02$) and 40 min (SNK, $P < 0.001$) following the stressor than prestress (Fig. 1D). An effect of time on freezing was not apparent for saline-pretreated rats ($F_{2,15} = 1.29$; $P = 0.304$; $1-\beta = 0.87$; Fig. 1D). Moreover, amphetamine-pretreated rats showed greater freezing behavior 20 min (SNK, $P < 0.02$) and 40 min (SNK, $P < 0.02$) post-stress than saline-pretreated rats (Fig. 1D).

Microdialysis probe placements

Representative placements of probe working membrane surfaces with a 2.4–3.0-mm-long dialysis membrane for the ventral hippocampus and the CeA, respectively, are drawn to scale and illustrated in Fig. 2. Probe locations were similarly distributed in saline-pretreated and amphetamine-pretreated rats 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 levels in the ventral hippocampus

The aim of this experiment was to determine whether 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 levels. Restraint stress increased extracellular 5-HT levels in the ventral hippocampus of saline-pretreated rats ($F_{8,43} = 3.69$; $P < 0.01$; $1-\beta = 0.89$; Fig. 3A). This effect was rapid, with 5-HT levels being increased by > 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 levels 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 the exception of the 20-min post-restraint sample, persisted for the duration of the experiment, with declines in 5-HT levels ranging from 16% at 0 min to 44% at 100 min relative to pre-stress baseline values (Bonferroni t-test, $P < 0.05$). Intra-ventral hippocampal 5-HT levels were significantly higher in saline-pretreated rats than in amphetamine-pretreated rats 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 increases stress-induced extracellular 5-HT levels in the CeA

The aim of this experiment was to determine 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 revealed significant effects of
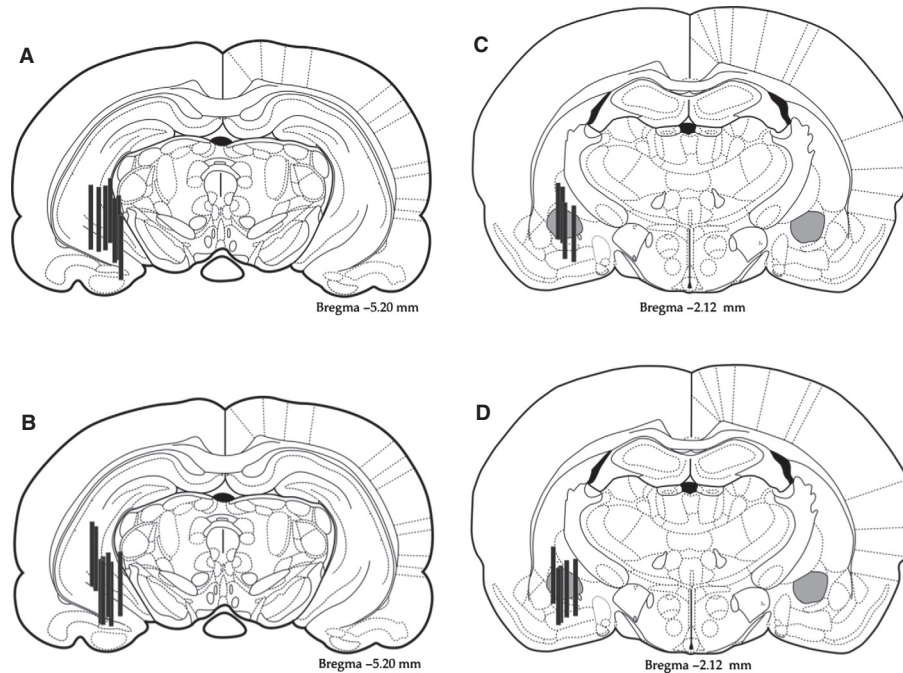
FIG. 2. Illustrations from Paxinos & Watson (1997), showing representative locations of the working surface of probes implanted in the ventral hippocampus of (A) saline-pretreated and (B) amphetamine-pretreated rats, and the CeA (gray area) of (C) saline-pretreated and (D) amphetamine-pretreated rats.
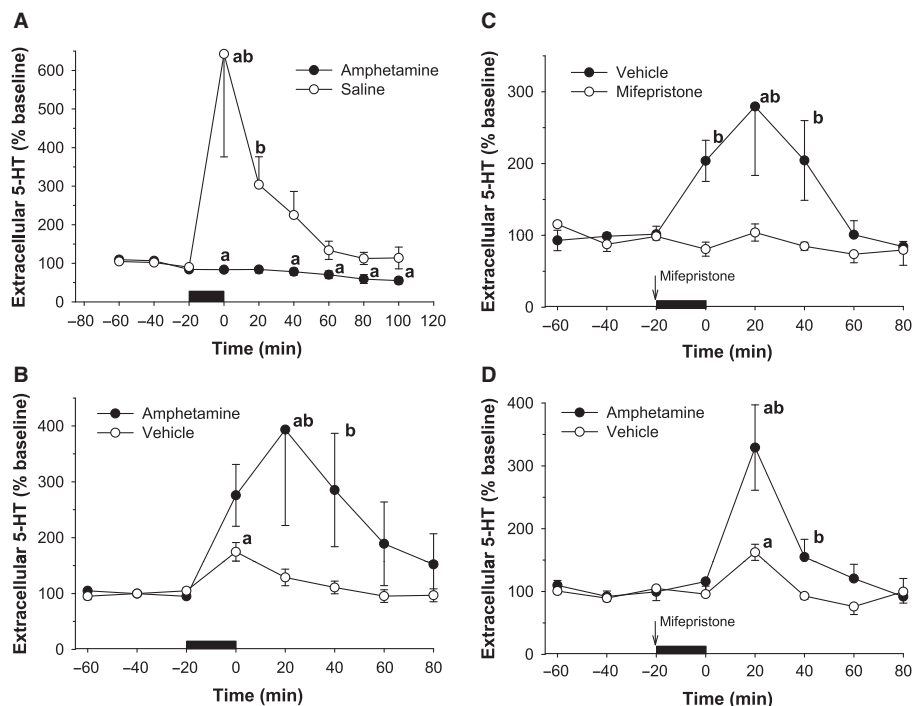

FIG. 3. (A) Restraint stress increased ventral hippocampal 5-HT levels in saline-pretreated (n = 7) but not amphetamine-pretreated (n = 9) rats. (B) Restraint stress augmented the increase in CeA 5-HT levels in amphetamine-pretreated (n = 5) as compared with saline-pretreated (n = 7) rats. (C) The increase in ventral hippocampal 5-HT levels in saline-pretreated rats was blocked by local delivery of the GR antagonist mifepristone (n = 5) as compared with vehicle (n = 6). (D) Increases in CeA 5-HT levels were still present in both amphetamine-pretreated (n = 7) and saline-pretreated (n = 8) rats following local administration of the GR antagonist mifepristone. All measures were obtained following a 2-week period of drug withdrawal. The stress was applied for 20 min during the sample period marked by the horizontal bar. Amphetamine-pretreated rats are represented by closed circles, and saline-pretreated rats by open circles. Values for 5-HT are expressed as the mean ± standard error of the mean percentage change from baseline, which was determined from the average of three pre-stress samples. [a]Significantly different from baseline levels. [b]Significant differences between treatment groups. $P < 0.05$.

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; $P < 0.005$; $1-\beta$ = 0.86) on CeA

5-HT levels (Fig. 3B). Restraint stress increased extracellular CeA 5-HT levels in both saline-pretreated ($F_{6,41}$ = 7.07; $P < 0.001$; $1-\beta$ = 1.00) and amphetamine-pretreated ($F_{4,23}$ = 3.17; $P < 0.02$;

$1-\beta = 0.68$; Fig. 3B) rats. As for the ventral hippocampus, this effect was rapid, with 5-HT levels being increased by > 70% at the conclusion of restraint (0 min) in saline-pretreated rats (Bonferroni *t*-test, $P < 0.05$) and by nearly 400% at 20 min post-restraint in amphetamine-pretreated rats (Bonferroni *t*-test, $P < 0.05$) relative to pre-stress baseline values (Fig. 3B). However, the stress-induced increases in extracellular CeA 5-HT levels were significantly higher in amphetamine-pretreated rats than in saline controls (Fig. 3B). This effect was observed in the first sample collected after the conclusion of the stress (20 min) and in 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 in the CeA

The aim of this experiment was to determine 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 the CeA 20 min prior to restraint stress, so that the drug would reach the brain at the same time as the stress was initiated. The effects of the GR antagonist were not evaluated in the ventral hippocampus of amphetamine-pretreated rats, as restraint stress in this group failed to induce any increases in hippocampal 5-HT levels (Fig. 3A). In saline-pretreated rats, analysis of the effects of the GR antagonist ($n = 5$) or vehicle ($n = 6$) on stress-induced increases in intra-ventral hippocampal 5-HT levels 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). Analysis across time revealed a 270% increase in ventral hippocampal 5-HT levels in vehicle-treated rats 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 mifepristone-treated rats ($F_{4,25} = 2.58$; $P < 0.05$; $1-\beta = 0.53$). 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-treated rats showed higher 5-HT levels 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-pretreated rats.

As stress-induced increases in extracellular 5-HT levels were detected in the CeA of both amphetamine-pretreated and saline-pretreated 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 an 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 levels across time in both saline-pretreated ($F_{7,45} = 6.28$; $P < 0.001$; $1-\beta = 0.99$) and amphetamine-pretreated ($F_{6,37} = 7.382$; $P < 0.001$; $1-\beta = 0.99$) rats. In both groups, this effect was evident at 20 min post-restraint (Bonferroni *t*-test, $P < 0.05$), with increases in 5-HT levels of approximately 60% and 330% in saline-pretreated and amphetamine-pretreated rats, respectively, relative to pre-stress baseline values. Furthermore, CeA 5-HT levels were significantly higher in amphetamine-pretreated rats than in saline-pretreated rats 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 levels in either amphetamine-pretreated or saline-pretreated 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 in stress-induced changes in serotonergic transmission within limbic regions that are 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-pretreated 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 levels in saline-pretreated rats. However, this effect of restraint on ventral hippocampal 5-HT levels was completely absent in amphetamine-pretreated rats. Furthermore, restraint-induced increases in ventral hippocampal 5-HT levels in saline-pretreated rats were blocked by local delivery of the GR antagonist mifepristone, suggesting that the response was attributable to local activation of GRs. In contrast, both saline-pretreated and amphetamine-pretreated rats showed increases in extracellular 5-HT levels within the CeA following restraint, but this response was markedly higher in amphetamine-pretreated rats. 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), so stress-induced increases in 5-HT levels 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 of 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, 2007; Keck *et al.*, 2005). Therefore, we first tested whether 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 pretreated with amphetamine show higher levels of arousal and fear-associated behaviors than saline-pretreated controls at 2 weeks following cessation of drug treatment. This finding, combined with established work demonstrating that rodents show 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 shown 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 levels within the ventral hippocampus of anesthetised rats (Barr & Forster, 2011). Here, we show that after 2 weeks of withdrawal from chronic amphetamine, increases in ventral hippocampal 5-HT levels in response to restraint stress are almost completely abolished. Previous research has demonstrated that corticosterone rapidly increases 5-HT levels 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-pretreated rats were similarly dependent on local GR activation. Fairly rapid genomic responses to corticosterone have been reported, but they occurred in a slower time frame than that indicated by the effects in the current study. For example, GR 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 h after systemic corticosterone injection (Morsink et al., 2007). Thus, the current effects may be attributable to non-genomic mechanisms.

Corticostone may increase ventral hippocampal 5-HT levels via a non-genomic membrane GR receptor (Orchinik et al., 1991). Certainly, the ability of corticosterone to increase the levels of 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 hippocampal 5-HT levels are blocked by mifepristone, suggesting alternative, non-genomic mechanisms. Zeise et al. (1992) have shown that corticosterone decreases GABA-mediated inhibitory postsynaptic potentials in the hippocampus, an effect that is sensitive to mifepristone and is dependent on cytosolic events. It has been suggested that corticosterone may alter GABAergic inhibition in the hippocampus through GR-mediated effects on intracellular signaling that are independent of genomic mechanisms (Teschemacher et al., 1996). Therefore, the possibility that increased stress-induced 5-HT levels may result from GR-mediated reduction of GABAergic inhibition of serotoninergic terminals in the ventral hippocampus warrants further investigation. Combined, these findings suggest that increases in ventral hippocampal 5-HT levels 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 GR activation probably contribute to the lack of serotonergic response during prolonged amphetamine withdrawal. This is because amphetamine-pretreated 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-pretreated rats. Repeated amphetamine administration does not affect the firing rate of dorsal raphe neurons that innervate the ventral hippocampus (Heidenreich & 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 the ventral hippocam-

pus does not differ between amphetamine-pretreated and saline-pretreated animals (Barr et al., 2013). However, 5-HT clearance in the ventral hippocampus is increased in amphetamine-pretreated rats (Barr et al., 2013). This effect is not mediated by the serotonin transporter, but instead by OCT3, a corticosterone-sensitive monoamine transporter (Grudemann 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 of stress-induced increases in ventral hippocampal 5-HT levels during prolonged amphetamine withdrawal is most likely attributable 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 hypothalamic–pituitary–adrenal axis negative feedback that contributes to maladaptive stress responses (McEwen, 2002; Herman & Mueller, 2006). Furthermore, increases in ventral hippocampal 5-HT levels have been implicated in the ability to cope with stressors (reviewed in Joca et al., 2007). Thus, our results showing that amphetamine-pretreated rats show almost no changes in ventral hippocampal 5-HT levels when subjected to an acute stressor, combined with the earlier report that intra-ventral hippocampal corticosterone at physiological levels fails to increase extracellular 5-HT levels (Barr & Forster, 2011), suggests that chronic amphetamine induces long-lasting changes in ventral hippocampal serotonergic function that may lead to an altered ability to cope with stress during withdrawal. In contrast to the ventral hippocampus, stress-induced CeA 5-HT levels were increased in rats undergoing amphetamine withdrawal as compared with saline-pretreated controls. This greater response in amphetamine-pretreated rats may not be mediated by local GR activation, as intra-CeA delivery of the GR antagonist mifepristone at the same concentration that blocked stress-induced increases in 5-HT levels in the ventral hippocampus failed to block stress-induced increases in CeA 5-HT levels in either control or amphetamine-pretreated rats. It is possible that the enhanced stress-induced CeA 5-HT response during prolonged amphetamine withdrawal might be mediated by CRF. This is because, during acute restraint stress, the CeA releases CRF (Merali et al., 1998), and stress-induced increases in CeA 5-HT levels 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 CRF_2 receptors that persists during long-term drug withdrawal (Pringle et al., 2008), and intra-dorsal raphe injections of a CRF_2 antagonist suppress anxiety-like behaviors during withdrawal (Vuong et al., 2010). Furthermore, CRF_2 receptor activation of the dorsal raphe increases 5-HT levels in the CeA to a greater degree in amphetamine-pretreated rats than in saline-pretreated controls (Scholl et al., 2010). Therefore, future research should investigate whether the increase in CeA 5-HT levels in response to stress during prolonged amphetamine withdrawal is similarly mediated by increased CRF_2 receptor activation in the dorsal raphe nucleus, and whether blockade of these receptors reduces both the heightened CeA 5-HT and behavioral responses to stress.

Functionally, the CeA has been implicated in 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). Heightened and persistent expression of stress-induced anxiety is usually observed during the period of drug withdrawal,

indicating the susceptibility to relapse in the presence of stressors (Erb, 2010). Evidence for the amygdala contributing to this response is provided by a report that footshock-induced methamphetamine reinstatement is attributable to increased CRF levels in the amygdala (Nawata *et al.*, 2012). Little is known about the exact role that 5-HT in the CeA might play in the increased behavioral response to stress as observed in amphetamine-pretreated rats in the current study, or in anxiety states as previously seen during amphetamine withdrawal (Barr *et al.*, 2010; Vuong *et al.*, 2010). However, previous studies have shown 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 post-traumatic stress disorder have reduced 5-HT transporter expression in the amygdala (Murrough *et al.*, 2011), which would allow for greater extracellular 5-HT levels during stress. Furthermore, stimulation of 5-HT$_{2C}$ receptors in the amygdala has been shown to be anxiogenic (Cornelio & Nunes-de-Souza, 2007; Christianson *et al.*, 2010). Combined, these results suggest that the presence of an augmented serotonergic response to stress in the CeA 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 hippocampal and CeA 5-HT responses to stress. These alterations are proposed to be mediated by GRs and OCT3 in the ventral hippocampus and by CRF$_2$ receptors on serotonergic neurons projecting to the CeA, which requires further investigation. The alterations in serotonergic function in these two limbic regions are thought to result in heightened stress responsiveness and anxiety. Therefore, future efforts to normalise 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

Abbreviations

5-HT, serotonin; CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; GR, glucocorticoid receptor; OCT3, organic cation transporter 3; SNK, Student Newman–Keuls.

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