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Immediate and lasting effects of chronic daily methamphetamine exposure on activation of cells in hypothalamic-pituitary-adrenal axis-associated brain regions

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Abstract

Rationale—Chronic methamphetamine (MA) abuse leads to dependence and symptoms of withdrawal after use has ceased. Negative mood states associated with withdrawal, as well as drug reinstatement, have been linked to drug-induced disruption of the hypothalamic-pituitary-adrenal (HPA) axis. However, effects of chronic MA exposure or acute MA exposure following withdrawal on neural activation patterns within brain regions that regulate the HPA axis are unknown.

Objectives—In this study, neural activation patterns were assessed by quantification of c-Fos protein in mice exposed to different regimens of MA administration.

Methods—(Experiment 1) Adult male mice were treated with MA (5 mg/kg) or saline once or once daily for 10 days. (Experiment 2) Mice were treated with MA or saline once daily for 10 days and following a 10-day withdrawal period were re-administered a final dose of MA or saline. c-Fos was quantified in brains after the final injection.

Results—(Experiment 1) Compared to exposure to a single dose of MA (5 mg/kg), chronic MA exposure decreased the number of c-Fos expressing cells in the paraventricular hypothalamus, dorsomedial hypothalamus, central amygdala, basolateral amygdala, bed nucleus of the stria terminalis (BNST), and CA3 hippocampal region. (Experiment 2) Compared to mice receiving their first dose of MA, mice chronically treated with MA, withdrawn, and re-administered MA,

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showed decreased c-Fos expressing cells within the central and basolateral amygdala, BNST, and CA3.

Conclusions—HPA axis-associated amygdala, extended amygdala, and hippocampal regions endure lasting effects following chronic MA exposure and therefore may be linked to stress-related withdrawal symptoms.

Keywords

Glucocorticoid; HPA Axis; Methamphetamine

Introduction

The psychostimulant methamphetamine (MA) is abused worldwide at alarming rates. A 2012 report indicated that MA is abused by nearly half a million people in the USA alone (SAMHSA 2012). Chronic MA abuse leads to dependence which is associated with mood and cognitive changes that persist even during periods of abstinence (Li et al. 2013; London et al. 2004; Kalechstein et al. 2003; Rendell et al. 2009). Similar effects have been demonstrated in animal models in which rodents exposed to MA show lasting alterations in stress-related behaviors (Nawata et al. 2012; Jang et al. 2013; Silva et al. 2014) and cognitive impairments (Reichel et al. 2011, 2012).

MA is a potent activator of the HPA axis and has repeatedly been shown to cause large increases in plasma levels of glucocorticoids in rodents (Zuloaga et al. 2014; Zhu et al. 2010; Acevedo et al. 2008). Chronic activation of the HPA axis and subsequent glucocorticoid release into the bloodstream can cause lasting effects on HPA axis function. These effects include changes in basal and stress-induced release of glucocorticoids and alterations within brain regions that regulate the HPA axis (Girotti et al. 2006; Santos et al. 2014). Dysregulation of HPA axis function is strongly linked to changes in emotional (e.g., depression and anxiety), cognitive (e.g., memory), and addiction-related behaviors, including MA seeking (Shoener et al. 2006; Fernández-Guasti et al. 2012; Raber 1998; Nawata et al. 2012). Recent clinical studies have also indicated that MA abuse is associated with alterations in the HPA axis. Individuals that chronically abuse MA show decreased basal stress hormone levels as well as depressive behavior during abstinence (Li et al. 2013; Carson et al. 2012). These effects may be due to MA-induced chronic glucocorticoid exposure which induces permanent effects on HPA axis function.

At present, little is known regarding the brain regions associated with HPA axis function that may be involved in these chronic effects of MA in either humans or rodents. Analysis of immediate early genes, as markers of neural activation, have been utilized to identify brain regions involved in regulating addictive processes (Zahm et al. 2010; McCoy et al. 2011). Acute exposure to MA and other psychostimulants (cocaine and amphetamine) leads to increased immediate early gene expression in several brain regions including the caudate putamen, accumbens, septum, habenula, brainstem, and HPA axis-associated hypothalamic, thalamic, hippocampal, and amygdalar regions (Zuloaga et al. 2014, 2015a; Graybiel et al. 1990; Moratalla et al. 1992; Zahm et al. 2010). Several studies also indicate that chronic exposure to psychostimulants, including MA, blunts the immediate early gene response,

although these studies have largely focused on the caudate putamen and nucleus accumbens (see McCoy et al. 2011). In a previous study, we identified extensive acute MA-induced immediate early gene expression in several brain regions that regulate the HPA axis (Zuloaga et al. 2014), although effects of chronic MA exposure or single MA exposure following a withdrawal period on activation of these brain regions have not been studied. This knowledge is essential to increase understanding of how chronic psychostimulant exposure might affect neural circuitry that controls the HPA axis, which in turn has been implicated in addiction processes (Hildebrandt and Greif 2013). Therefore, in the present study, we investigated effects of repeated MA treatment (10 days, once daily) on protein expression of the immediate early gene c-Fos in order to determine chronic MA effects on the activation of brain regions essential to HPA axis regulation. In order to determine if changes in neural activation persist after a period of withdrawal, in a second study, mice were chronically administered MA or saline and c-Fos responses were compared following re-exposure to MA after a 10-day withdrawal period.

Materials and methods

Animals

Male C57BL/6J mice (50 days old) were purchased from JAX Laboratories (Bar Harbor, Maine) and maintained on a 12:12 light/dark cycle with lights on at 6:00 a.m. Mice were singly housed for 4 days prior to experiments and were between 60 and 70 days old at the onset of treatment. Rodent chow (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) and water were available ad libitum. All experiments were approved by the OHSU IACUC committee.

Methamphetamine treatment, blood and temperature collection, and perfusion

See Fig. 1 for an experimental timeline. In experiment 1, male mice were injected with (*d*)-MA hydrochloride {[obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute on Drug Abuse drug supply program]} dissolved in saline (5 mg/kg, weight of the salt, ip.) or saline alone between 8:00 and 10:00 a.m. once (acute) or once daily for 10 consecutive days (chronic). The dose and treatment period were chosen based on previous studies performed in neonatal mice that indicate alterations in the HPA axis-associated brain regions (Acevedo et al. 2008; Zuloaga et al. 2013). In experiment 2, male mice were injected (ip.) with MA (5 mg/kg) or saline once daily for 10 consecutive days and following a 10-day period of no treatment were re-administered MA (5 mg/kg, ip.) or saline. For both experiments, at 2 h after the final injection, mice were removed from their cage and body temperatures were measured using a Microtherma 2 rectal thermometer (Thermoworks, Lindon, UT, USA). The 2h time point was chosen based on our study, demonstrating extensive induction in the number of cells expressing c-Fos protein in the mouse brain and peak hyperthermia following administration of MA (Zuloaga et al. 2014). Immediately following temperature measurement, blood was collected from mice by retro-orbital eye bleed as previously described (Johnson et al. 2014) and transferred to EDTA-treated tubes on crushed ice. Following our published protocol (Zuloaga et al. 2014), mice were then injected with an overdose of ketamine (100 mg/kg)/xylazine (10 mg/kg)/acepromazine (3 mg/kg) and, once anesthetized, intra-cardially perfused with 20 ml

phosphate buffered saline (PBS), followed by 40 ml 4 % paraformaldehyde. Brains were removed, stored in 4 % paraformaldehyde overnight, and then transferred to 30 % sucrose. Blood was centrifuged at 5500g for 10 min, and the supernatant was transferred to a new tube and stored at -80 °C until assay. Plasma samples were analyzed using a commercial I^{125} corticosterone radioimmunoassay according to the manufacturer's instructions (MP Biochemicals, LLC, Orangeburg, NY, USA). The intra-assay correlation was 4.2 %.

Brain tissue processing and immunohistochemistry

Fixed brains were coronally sectioned at 40 μ m into three series using a cryostat (Microm HM505E, MICROM international GmbH, Walldorf, Germany) and processed for immunohistochemical detection of c-Fos. Sections were rinsed in phosphate buffered saline (PBS), incubated in 1 % hydrogen peroxide and 0.3 % Triton-X in PBS (PBS-TX) for 10 min, again rinsed in PBS, then incubated in 10 % normal goat serum (NGS) in PBS-TX for 1 h. After rinsing in PBS, sections were incubated in primary antisera (c-Fos rabbit polyclonal: 1:5000, Santa Cruz Biotechnology, sc52) in 4 % NGS and PBS-TX overnight at room temperature. Sections were then rinsed in PBS and incubated for 1 h in biotinylated goat-anti rabbit antibody in PBS-TX (1:500, Vector Laboratories, Burlingame, CA) followed by rinses in PBS and a 1 h incubation in avidin-biotin peroxidase complex (ABC Elite kit, Vector Laboratories, Burlingame, CA). Following rinses in Tris-buffered saline (TBS), sections were developed for visualization of c-Fos-positive cells in a hydrogen peroxide/diaminobenzidine/TBS solution for 10 min, after which sections were rinsed in PBS and immediately mounted on slides. The following day, sections were dehydrated in ethanol, defatted in xylene, and coverslipped with Permount (Sigma Chemical Co., St. Louis MO).

Microscopy

Quantification of c-Fos-positive cells was performed using an Olympus IX81 microscope (Olympus, Center Valley, PA, USA) equipped with Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA), as described (Zuloaga et al. 2014). Images of discrete brain regions were captured bilaterally within two sections using a $\times 10$ objective. Regions were identified using the mouse brain atlas of Franklin and Paxinos (1997). c-Fos immunoreactive cells (identified by dark brown nuclear label) were quantified bilaterally within fixed area frames: suprachiasmatic nucleus (SCN; Bregma -0.46 to -0.58; 740 $\times$ 420 μ m), paraventricular hypothalamic nucleus (PVH; Bregma -0.82 to -0.94; box, 275 $\times$ 450 μ m), paraventricular thalamic nucleus (PVT; Bregma -1.46 to -1.58; box, 790 $\times$ 410 μ m), BNST (Bregma 0.02 to -0.10; box, 335 $\times$ 620 μ m), central amygdala (CEA; Bregma -1.34 to -1.46; circle, 575 μ m diameter), basolateral amygdala (BLA; Bregma -1.34 to -1.46; circle, 450 μ m diameter), medial amygdala (MEA; Bregma -1.34 to -1.46; circle 450 μ m diameter), dorsomedial hypothalamus (DMH; Bregma -1.46 to -1.58; 440 $\times$ 550 μ m), dentate gyrus (Bregma -1.70 to -1.82; box, 850 $\times$ 420 μ m), CA1 (Bregma -1.70 to -1.82; box, 850 $\times$ 420 μ m), CA3 (Bregma -1.70 to -1.82; box, 850 $\times$ 420 μ m), and the medial caudate-putamen (Bregma 1.1 to 0.98; box, 850 $\times$ 550).

Statistical analysis

All data are reported as means $\pm$ standard error of the mean (SEM). Data were analyzed using GraphPad Prism v.4 and SPSS v.16.0 software (Chicago, IL). For plasma corticosterone,

body temperature, and c-Fos analyses, 2-way ANOVAs were utilized with drug treatment (MA, saline) and treatment period as between-subject factors. Bonferroni corrected post hoc comparisons were performed when statistically appropriate.

Results

Body weight

Experiment 1 (acute vs. chronic)—Mice treated with MA for 10 days showed a greater decrease in body weight (-0.775 ± 0.27 g) compared to mice treated with saline for 10 days (-0.13 ± 0.13 g; ($t(14)=2.24$; $p<0.05$).

Experiment 2 (chronic/withdrawal)—A 2-way ANOVA revealed a significant effect of both treatment ($F(1,89)=25.52$, $p<0.001$) and time ($F(2,89)=35.99$, $p<0.001$) as well as a treatment $\times$ time interaction ($F(2,89) = 9.6$, $p<0.001$). Specifically, mice treated with MA showed a greater decrease in body weight compared to mice treated with saline for 10 days ($p<.01$). Ten days after the treatment period, mice treated with MA regained body weight, although they remained slightly below the body weight of saline-treated mice ($p<.05$; see Fig. 2).

Plasma corticosterone

Experiment 1 (acute vs. chronic)—A 2-way ANOVA revealed a significant main effect of drug treatment ($F(1, 28)=21.84$, $p<0.001$). Mice treated with MA showed higher plasma corticosterone levels than mice treated with saline (Fig. 3a). No significant main effect of treatment period or interaction between drug treatment and treatment period was found in experiment 1 (Fig. 3a), indicating that there were no differences between chronic and acute MA treatment on plasma corticosterone levels.

Experiment 2 (chronic/withdrawal)—A 2-way ANOVA revealed a significant main effect of final drug treatment ($F(1, 26)=22.40$, $p<0.001$), with mice treated with MA as their final injection showing greater plasma corticosterone levels than mice treated with SA as their final injection (Fig. 3b). There was also a trend toward a main effect of prior chronic drug treatment, as mice treated with MA for 10 days, and subsequently withdrawn, showed higher plasma corticosterone levels following a final injection of either saline or MA ($F(1,26)=3.25$, $p=0.08$).

Body temperature

Experiment 1 (acute vs. chronic)—Two-way ANOVA revealed a significant effect of drug treatment ($F(1,28)=14.77$, $p<0.001$) and treatment period ($F(1,28)=6.04$, $p<0.05$). MA treatment increased body temperature and chronic treatment (both saline and MA) decreased the temperature response (Fig. 3c).

Experiment 2 (chronic/withdrawal)—There was a significant main effect of final drug treatment ($F(1,26)=37.49$, $p<0.001$) and chronic drug treatment ($F(1,26)=6.76$, $p<0.05$). Specifically, body temperature was elevated in mice treated with MA as their final injection. Furthermore, of mice receiving MA as the final treatment, those that had received prior

chronic MA treatment showed a decreased body temperature compared to mice that had received prior chronic saline treatment (Fig. 3d).

c-Fos immunohistochemistry

Experiment 1—A significant main effect of drug treatment on the number of c-Fos immunopositive cells was found in the PVH ((F(1,28)=190.2, $p<0.001$; Fig. 4a), SCN ((F(1,28)= 4.403, $p<0.05$; Fig. 4b), DMH ((F(1,28)=202.0, $p<0.001$; Fig. 4c), PVT ((F(1,28)=30.91, $p<0.001$; Fig. 4d), CEA ((F(1,28)=274.5, $p<0.001$; Fig. 5a), BLA ((F(1,28)=23.85, $p<0.001$; Fig. 5c), BNST ((F(1,28)=113.5, $p<0.001$; Fig. 5d), CA3 ((F(1,28)=21.45, $p<0.01$; Fig. 6b), and caudate-putamen ((F(1,28)=147.5, $p<0.001$; Fig. 6d). There was an overall increase in the number of c-Fos-positive cells in MA-treated compared to saline-treated in each of these regions except the SCN, which showed a slight decrease in c-Fos-expressing cells following MA treatment. There was also a trend toward an increase in c-Fos-expressing cells in MA-treated mice in the CA1 region ((F(1,28)=4.13, $p=0.052$; Fig. 6a). The MEA (Fig. 5b) and DG (Fig. 6c) showed no significant effect of drug treatment.

Several brain regions also showed a significant effect of treatment period; PVH ((F(1,28)=11.32, $p<0.01$; Fig. 4a), DMH ((F(1,28)=9.502, $p<0.01$; Fig. 4c), CEA ((F(1,28)= 45.01, $p<0.001$; Fig. 5a), MEA ((F(1,28)=7.619, $p<0.05$; Fig. 5b), BLA ((F(1,28)=35.92, $p<0.001$; Fig. 5c), BNST ((F(1,28)=22.26, $p<0.001$; Fig. 5d), CA1 ((F(1,28)=14.89, $p<0.001$; Fig. 6a), CA3 ((F(1,28)=7.619, $p<0.05$; Fig. 6b), and caudate-putamen((F(1,28)=11.8, $p<0.01$; Fig. 6d). Each of these regions showed a decrease in c-Fos-positive cells following chronic treatment. For several of these regions, this effect was largely driven by an attenuated increase in the number of c-Fos-positive cells in mice chronically treated with MA (e.g., PVH, CEA, and BNST). In other regions, this effect appeared driven by decreases in c-Fos-positive cells following chronic treatment of both MA and saline compared to acute treatment groups (e.g., BLA, CA1, DMH, MEA, and CA3). The SCN (Fig. 4b), PVT (Fig. 4d), and DG (Fig. 6c) showed no significant effect of treatment period.

Drug treatment was affected by chronic exposure, as reflected by a significant interaction, in the PVH ((F(1,28)=7.622, $p<0.05$; Fig. 4a), CEA ((F(1,28)=33.65, $p<0.001$; Fig. 5a), BNST ((F(1,28) =9.25, $p<0.01$; Fig. 5d), and caudate-putamen ((F(1,28) = 7.9, $p<0.01$; Fig. 6d). This interaction was largely the result of a dramatic attenuation in c-Fos-positive cells following chronic MA exposure compared to acute MA exposure.

Experiment 2—Final treatment with MA significantly increased the number of c-Fos immunopositive cells in the PVH ((F(1,27)=433.4, $p<0.001$; Fig. 4e), CEA ((F(1,27)= 389.8, $p<0.001$; Fig. 5e), BNST ((F(1,27)=266.0, $p<0.001$; Fig. 5h), BLA ((F(1,27)=39.45, $p<0.001$; Fig. 5g), DMH ((F(1,27)=331.4, $p<0.001$; Fig. 4g), PVT ((F(1,27)=75.81, $p<0.001$; Fig. 4h), CA1 ((F(1,27)=14.74, $p<0.05$; Fig. 6e), CA3 ((F(1,27)=35.43, $p<0.001$; Fig. 6f), DG ((F(1,27)= 23.71, $p<0.01$; Fig. 6g), and caudate-putamen ((F(1,27)= 111.5, $p<0.001$; Fig. 6h) compared to final treatment with saline. No significant effect of final treatment was found in the SCN (Fig. 4f), but there was a trend toward a decrease in c-Fos

immunopositive cells in the MEA after final treatment of MA compared to saline (($F(1,27)=3.32$, $p=0.079$; Fig. 5f).

Chronic MA treatment also affected c-Fos immunoreactivity (as reflected by a significant main effect of treatment period) in four brain regions: the CEA (($F(1,27)=21.88$, $p<0.001$; Fig. 5e), BNST (($F(1,27)=20.80$, $p<0.001$; Fig. 5h), and DMH (($F(1,27)=6.679$, $p<0.05$; Fig. 4g). In the CEA and BNST, this effect was primarily driven by a decrease in c-Fos-positive cells in mice chronically treated with MA and re-exposed to MA compared to mice receiving their first dose of MA. In the DMH, chronic treatment with MA increased c-Fos immunoreactivity following a final injection of either MA or saline compared to mice repeatedly treated with saline. The BLA (($F(1,27)=4.051$, $p=0.054$; Fig. 5g) and MEA (($F(1,27)=4.03$, $p=0.055$; Fig. 5f) also showed marginal effects of treatment period. In the BLA, this trend is largely due to an attenuated c-Fos response in mice chronically treated with MA and re-exposed to MA compared to mice receiving their first dose of MA. In the MEA, mice chronically treated with MA showed a trend toward an increase in c-Fos-positive cells compared to mice chronically treated with saline, regardless of whether they received MA or saline as their final treatment. The PVT (Fig. 4h), SCN (Fig. 4f), CA1 (Fig. 6e), and DG (Fig. 6g) showed no significant effect of treatment period.

Final MA treatment was affected by prior chronic drug treatment and withdrawal, as reflected by a significant interaction in the CEA (($F(1,27)=18.30$, $p<0.001$; Fig. 5e), BNST (($F(1,27)=23.41$, $p<0.001$; Fig. 5h), BLA (($F(1,27)=21.28$, $p<0.001$; Fig. 5g), CA3 (($F(1,27)=11.96$, $p<0.01$; Fig. 6f), and caudate-putamen (($F(1,27)=14.8$, $p<0.001$; Fig. 6h). In each of these regions, there was a greater MA-induced elevation in the number of c-Fos-positive cells in mice not previously exposed to MA compared to mice chronically treated with MA. In CA3, prior treatment with MA eliminated the elevation in c-Fos normally induced by final treatment with MA. In the caudate-putamen, chronic treatment with MA, compared to chronic saline treatment, increased the number of c-Fos immunoreactive cells in mice that were given a final injection of saline (Fig. 6h). See Fig. 7 for a summary of the effects of acute MA, chronic MA, and re-exposure to MA after a 10-day period of withdrawal from chronic MA exposure on the number of c-Fos expressing cells in distinct brain regions.

Discussion

The main findings of the study indicate that neural activation is decreased following chronic MA exposure, compared to acute exposure, in several HPA axis-associated hypothalamic, hippocampal, amygdala, and extended amygdala brain regions. Furthermore, within specific hippocampal and amygdala/extended amygdala regions, MA-induced neural activation remains reduced following re-exposure after a 10-day period of withdrawal. These lasting changes in HPA axis-associated brain regions induced by chronic MA exposure may be associated with stress-related withdrawal symptoms reported in rodents and humans (Nawata et al. 2012; Li et al. 2013).

Chronic activation of the HPA axis resulting from repeated stress has been shown to generate mixed effects on immediate early gene expression in HPA axis-associated brain

regions. In general, this variability is the result of the type of stressor utilized. Restraint and immobilization stress generally reduce c-Fos expression following repeated exposure (Umemoto et al. 1994; Girotti et al. 2006; Melia et al. 1994). In contrast, stressors such as forced swim and cold exposure have been shown to induce opposite effects, augmenting activation of HPA axis-associated brain regions (Melia et al. 1994; Bhatnagar and Dallman 1998). Similar results are found in terms of repeated stress-induced plasma ACTH and glucocorticoid release. Prior exposure to stress results in a blunted release of ACTH and corticosterone when a rodent is re-exposed to the same stressor (Girotti et al. 2006; Armario et al. 2004; Belda et al. 2004). This blunted HPA axis responsivity to a homotypic stressor has been postulated to occur via changes in neural activity in brain regions that regulate pituitary-adrenal functions (Girotti et al. 2006). Evidence for this comes from studies which indicate that when a rodent is exposed to a novel stressor following prior/heterotypic stress exposure, this habituation is not seen (Armario et al. 1988; Hashimoto et al. 1988; Hauger et al. 1988). Therefore, prior/repeated HPA axis activation does not appear to cause an overall suppression of HPA axis activation, but rather, suppressed responses to the same stressor.

Our current findings showing decreased immediate early gene protein expression are in accord with these previous studies involving repeated homotypic stress, particularly those involving restraint and immobilization. Reduction in neural activation has previously been reported following repeated exposure to psychostimulants (e.g., cocaine, amphetamine, MA). In general, these studies have primarily focused on examining alterations in immediate early gene responses within the nucleus accumbens, caudate putamen, cerebellum, and cerebral cortex with few studies examining HPA axis-associated regions (McCoy et al. 2011; Hamamura et al. 1999). In one study, Zahm et al. (2010) reported that self-administration of cocaine *increased* c-Fos immunoreactivity in the extended amygdala on the 6th day of exposure compared to the 1st day. In contrast, no differences were found in these areas following repeated exposure when cocaine was administered by the investigator. Repeated cocaine self-administration also *decreased* c-Fos immunolabeling in the PVH, while repeated injections had no effect on c-Fos in this region (Zahm et al. 2010). In a study involving MA self-administration, repeated administration appeared to decrease c-Fos immunoreactivity in the CEA and BNST (Cornish et al. 2012). This finding contrasts with the earlier described findings of Zahm et al. (2010) following cocaine self-administration in these same regions and highlights the potentially unique effects of these two psychostimulants. Data reported in this study support the previous report by Cornish et al. (2012) that indicates chronic exposure to MA can suppress activation of the CEA and BNST. Furthermore, we also identified several other regions that show suppression in neural activation following repeated daily MA exposure. These include the PVH, DMH, BLA, and CA3. The attenuation in c-Fos immunoreactivity in the caudate-putamen is in accord with previous findings, which indicate similar effects following repeated injections of MA and cocaine (McCoy et al. 2011; Hope et al. 1992).

Within the CEA, BLA, BNST, and CA3, there was continued suppression in number of cells expressing c-Fos after a re-exposure to MA following a 10-day withdrawal period. This indicates that these HPA axis-associated amygdala, extended amygdala, and hippocampal regions endure lasting effects of a prior chronic MA exposure and therefore may be linked to stress-related withdrawal symptoms. Amygdala and hippocampal regions are critical for the

regulation of the brain corticotropin-releasing factor (CRF) system. The brain CRF system has been implicated in addiction processes specifically by regulating changes in stress-related behaviors (Zorrilla et al. 2013). Alterations in the CRF system contribute to excessive drug self-administration and drug seeking (Nawata et al. 2012; Buffalari et al. 2012). The CEA and BNST are central to the brain CRF addiction circuitry and receive CRF secreted from neurosecretory cells in the PVH. CA3 also contains abundant neurons that express CRF (Chen et al. 2001). Although the BLA does not receive direct inputs from CRF-producing cells in the PVH, it has been shown to express the corticotropin-releasing hormone receptor 1 (CRHR1; Justice et al. 2008) which is implicated in addiction processes (Zorrilla et al. 2013; Moffett and Goeders 2007). The BLA has also been implicated as a key area in regulating drug associated memories (Jian et al. 2014); therefore, alterations in immediate early genes may reflect changes in these memory processes.

Blunted immediate early gene response after a prolonged period (10 days) of withdrawal from MA is a novel finding. Few studies have examined effects of re-exposure to methamphetamine after a withdrawal period of greater than 24 h (McCoy et al. 2011). In a recent study, mice receiving repeated (5 days) daily MA (2 mg/kg) exposure exhibited *increased* c-Fos in the caudate-putamen upon re-exposure to MA after a 7-day withdrawal period when compared to saline pre-treated mice challenged with MA (Jedynak et al. 2012). In contrast, our treatment regimen (5 mg/kg daily for 10 days preceded by 10-day withdrawal) induced an attenuated c-Fos response in the caudate-putamen following re-exposure to MA. This indicates that lasting effects of chronic MA on immediate early gene expression in the caudate-putamen is dose- and timing-specific, with an extended exposure to a higher dose inducing an opposite effect of the lower and shorter duration dose. The treatment regimen utilized by Jedynak et al. 2012 can induce locomotor sensitization in mice and the elevated immediate early gene expression in the caudate-putamen likely reflects this response. The attenuation in the number of c-Fos-positive cells reported in the current study is in accord with other studies that indicate that higher doses of MA administered over an extended period of time can produce this effect in the striatum (McCoy et al. 2011). Furthermore, we report that these effects persist after a significant withdrawal period.

Alterations in the number of c-Fos expressing cells in the present study may reflect changes within the CRF system or potentially alterations in HPA axis negative feedback, specifically through modification of glucocorticoid receptor (GR) within affected brain regions. Brain CRF and GR circuitry have been shown to be altered by MA exposure (Martin et al. 2012; Lowy 1990; Zuloaga et al. 2013; Kabbaj et al. 2003). Furthermore, a recent study in our laboratory indicated extensive MA-induced activation of GR-containing cells in the PVH and CEA (Zuloaga et al. 2014). At present, the link between MA-induced c-Fos in HPA axis-associated brain regions reported here and alterations in brain CRF and GR circuitry is unclear. Further studies are therefore warranted to determine the phenotype of cells expressing c-Fos in order to elucidate the potential function of these cells in stress and addiction processes. Beyond being a marker for neural activation, c-Fos per se has also been specifically implicated in mediating changes in neural plasticity and contributes to development of locomotor sensitization following repeated cocaine exposure (Zhang et al. 2006). Therefore, evaluating changes in c-Fos following repeated exposure to

psychostimulants such as MA may be a useful tool for identifying discrete brain regions and circuits involved in the regulation of addiction associated behaviors.

Although chronic effects of MA on the activation of HPA axis-associated brain regions were not accompanied by significant alterations in corticosterone levels at 2 h after the final MA injection, mice treated with MA prior to 10 days of withdrawal did show a trend toward greater corticosterone levels following the final injection. The lack of significant effects may be due to the particular time point at which plasma corticosterone levels were assessed. For this study, blood was taken at 2 h after the final injection because this time point was ideal for obtaining peak expression of c-Fos following MA (Zuloaga et al. 2014; Giardino et al. 2011). However, the peak levels of corticosterone following MA exposure occur at around 60 min and generally decline thereafter (Zuloaga et al. 2014). Therefore, corticosterone levels observed here likely represent declining/recovery levels and not peak levels. In a previous study, we reported that corticosterone levels were back to baseline at 2 h post-injection in males administered 1 mg/kg MA. The higher dose of MA used in the present study likely contributed to the fact that levels were still above baseline, but were likely well past peak concentrations. Ongoing studies in our laboratory will determine effects of chronic MA on the time course of plasma corticosterone induction as well as its relationship to MA levels in plasma and brain. What is apparent is that unlike the attenuated c-Fos induction in several HPA axis-associated brain regions following chronic MA exposure, there is not an attenuation in corticosterone release. As a result, this repeated exposure to high levels of glucocorticoids may produce harmful effects on the brain (e.g., cell death, decreased neurogenesis) similar to those induced by chronic stress (Uno et al. 1989; Pham et al. 2003). These chronic elevations in glucocorticoids may also be linked to behavioral modifications reported following chronic MA exposure, including altered anxiety- and depression-related behaviors (Nawata et al. 2012; Jang et al. 2013; London et al. 2004; Zuloaga et al. 2015b).

Importantly, future studies aimed at investigating effects of chronic MA on basal and stress-induced corticosterone release will be essential to elucidate potential lasting effects of MA on normal HPA axis function. In the current study, corticosterone levels in mice given a final injection of saline did not significantly differ between chronic MA- and chronic saline-treated mice, although there was a trend toward elevated corticosterone in chronic MA-treated mice. Corticosterone levels at 2 h post-injection are a reflection of basal levels since injections of saline (particularly in mice that have been repeatedly handled and injected) induce only small increases in corticosterone that return to baseline prior 2 h (Zuloaga et al. 2014; Goel and Bale 2010). Regardless, true basal corticosterone levels collected from unhandled mice would provide an ideal measure of effects of chronic MA on normal HPA axis function.

Body temperature responses were decreased in mice chronically exposed to MA both immediately after the chronic treatment period and following re-exposure after 10 days of withdrawal. Based on our previous report, the 2-h time point at which temperature was measured likely represents a peak MA-induced hyperthermic response (Zuloaga et al. 2014). Decreased body temperature in chronic MA-treated mice is consistent with previous reports which indicate persistent suppression of MA-induced hyperthermia following repeated MA exposure (Danaceau et al. 2007; Myles et al. 2008; Myles and Sabol 2008). The neural

regulation of this phenomenon is unclear. Hypothalamic regions have been implicated in the regulation of body temperature including several that were investigated in the present study; PVH, DMH, and SCN (Inenaga et al. 1987; Stephan and Nunez 1977; Zaretskaia et al. 2002). However, none of these regions showed a lasting change in neural activation, indicating that adaptation may occur in other brain regions or organs that regulate temperature. Further studies which focus on adaptation to MA-induced hyperthermia are needed to investigate this mechanism.

In summary, the primary findings of this study indicate that several HPA axis-associated brain regions show a decrease in neural activation following chronic exposure to MA compared to the first exposure. Select regions, specifically extrahypothalamic areas including amygdala and hippocampal sub-regions, show a suppression in neural activation even after an extended period of withdrawal. This suggests that these brain regions endure lasting effects of a prior chronic MA exposure and therefore may be linked to short- and long-term alterations in the HPA axis and stress-related behaviors associated with MA withdrawal (Li et al. 2013; Carson et al. 2012; Nawata et al. 2012).

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Experiment 1. (Acute/Chronic) **Experiment 2. (Chronic/Withdrawal)**

Fig. 1. Timelines of MA administration for experiments 1 and 2

Fig. 2.

Chronic MA administration decreases body weight. Body weight is shown as change from pre-treatment weight. MA decreases body weight following 10 days of treatment. Weight in MA-treated mice increases to near saline levels following 10 subsequent days of no treatment. (*T1*) body weight prior to treatment, (*T2*) body weight after the 10th injection of MA or saline, and (*T3*) body weight after 10 days of no injections

Fig. 3.

MA exposure affects plasma corticosterone levels and body temperature. **a** MA treatment increased plasma corticosterone levels in mice treated with MA once (acute) or once daily for 10 days (chronic). **b** Plasma corticosterone levels were significantly increased in mice receiving a final injection of MA compared to a final injection of saline. There was also a trend toward mice that were chronically treated with MA to show elevated plasma corticosterone levels ($p=.08$). **c** The elevation in body temperature following MA administration was blunted by repeated daily administration of MA. **d** This blunted temperature response to MA persisted following re-exposure to MA after 10 days of withdrawal. *** $p<.001$; * $p<.05$ compared to groups receiving their first dose of MA

Fig. 4.

Effects of MA treatment on the number of c-Fos-positive cells in hypothalamic/thalamic regions. **a–d** Experiment 1: The number of c-Fos-positive cells in the PVH, SCN, DMH, and PVT in mice treated with MA or saline once (acute) or once daily for 10 days (chronic). **e–h** Experiment 2: The number of c-Fos-positive cells in the PVH, SCN, DMH, and PVT in mice treated with MA or saline for 10 days and following 10 days of no treatment were administered a final injection of MA or saline. Asterisks (***) $p < .001$, ** $p < .01$, * $p < .05$ indicate a significant effect of drug (MA, saline) treatment (**a–d**) or final drug treatment (MA, saline; **e–h**). [^] indicates brain regions that showed a significant effect of treatment period ($p < .05$). #, brain region that showed a significant interaction indicating an attenuation in the number of c-Fos-positive cells (**a–d**) in chronic MA compared to acute MA mice or (**e–h**) in chronic MA mice receiving a final injection of MA compared to chronic saline mice receiving a final injection with MA. Representative images of the PVH from MA and saline-treated mice in experiment 1 (**i**) and experiment 2 (**j**)

Fig. 5.

Effects of MA treatment on the number of c-Fos-positive cells in the extended amygdala. **a–d** Experiment 1: The number of c-Fos-positive cells in the CEA, MEA, BLA, and BNST in mice treated with MA or saline once (acute) or once daily for 10 days (chronic). **e–h** Experiment 2: The number of c-Fos-positive cells in the CEA, MEA, BLA, and BNST in mice treated with MA or saline for 10 days and following 10 days of no treatment were administered a final injection of MA or saline. Asterisks ($***p < .001$, $**p < .01$, $*p < .05$) indicate a significant effect of drug (MA, saline) treatment (**a–d**) or final drug treatment (MA, saline; **e–h**). $\wedge$ indicates brain regions that showed a significant effect of treatment period ($p < .05$). #, brain regions that showed a significant interaction indicating an attenuated c-Fos induction (**a–d**) in chronic MA compared to acute MA mice or (**e–h**) in chronic MA mice receiving a final injection of MA compared to chronic saline mice receiving a final injection with MA. Representative images of the CEA from MA and saline-treated mice in experiment 1 (**i**) and experiment 2 (**j**)

Fig. 6.

Effects of MA treatment on the number of c-Fos-positive cells in the hippocampus and caudate-putamen. **a–d** Experiment 1: The number of c-Fos-positive cells in CA1, CA3, DG, and caudate-putamen in mice treated with MA or saline once (acute) or once daily for 10 days (chronic). **e–h** Experiment 2: The number of c-Fos-positive cells in CA1, CA3, DG, and caudate-putamen in mice treated with MA or saline for 10 days and following 10 days of no treatment were administered a final injection of MA or saline. Asterisks (***) $p < .001$, ** $p < .01$, * $p < .05$) indicate a significant effect of drug treatment (MA, saline; **a–d**) or final drug treatment (MA, saline; **e–h**). [^] indicates brain regions that showed a significant effect of treatment period ($p < .05$). #, brain region that showed a significant interaction indicating an attenuation in c-Fos (**a–d**) in chronic MA compared to acute MA mice or (**e–h**) in chronic MA mice receiving a final injection of MA compared to chronic saline mice receiving a final injection with MA. @ indicates a significant attenuation in c-Fos in chronic saline-treated mice receiving a final injection of saline compared to chronic MA-treated mice receiving a final injection of saline. Representative images of CA3 from MA and saline-treated mice in experiment 1 (**i**) and experiment 2 (**j**)

	CEA	BLA	BNST	CA3	CPU	PVH	DMH	CA1	PVT	DG	MEA	SCN
Acute MA	++++	++	++++	++	++++	++++	++++	+	++	+	≈	-
Chronic MA	++	+	++	+	++	+++	+++	≈	++	+	≈	-
Re-exposure	++	+	++	+	++	++++	++++	+	++	+	≈	≈

Lasting attenuation following chronic MA (under CEA, BLA, BNST, CA3, CPU)
 Short-term attenuation following chronic MA (under PVH, DMH)
 No attenuation following chronic MA (under CA1, PVT, DG)
 No effect of MA or attenuation by MA (under MEA, SCN)

Fig. 7.

Summary of the effects of MA exposure on the number of c-Fos-positive cells in HPA axis-associated brain regions. Comparison of acute MA exposure, chronic MA exposure, and re-exposure to MA after a 10-day period of withdrawal following chronic MA exposure. +, ++, +++, ++++ indicate a small to large induction of c-Fos after MA treatment compared to saline treatment. - indicates a small decrease in c-Fos after MA treatment compared to saline treatment. ≈ indicates similar c-Fos levels in MA and saline treated mice. The data represent patterns of c-Fos based on data from experiments 1 and 2. Acute MA refers to patterns of c-Fos following the 1st exposure to MA in mice from experiments 1 and 2. Chronic MA refers to mice from experiment 1 that were treated with MA once daily for 10 days and c-Fos was assessed following the 10th injection. The re-exposure group refers to mice treated for 10 days with MA and re-exposed to MA after a 10-day withdrawal period (experiment 2). *CEA* central amygdala, *BLA* basolateral amygdala, *BNST* bed nucleus of the stria terminalis, *CA3* Cornu Ammonis Region 3, *CPU* caudate-putamen, *PVH* paraventricular hypothalamus, *DMH* dorsomedial hypothalamus, *CA1* Cornu Ammonis Region 1, *PVT* paraventricular thalamus, *DG* dentate gyrus, *MEA* medial amygdala, *SCN* suprachiasmatic nucleus