Individual differences in amphetamine sensitization, behavior and central monoamines

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Abstract

Repeated amphetamine treatment results in behavioral sensitization in a high percentage of rats. Alterations to plasma corticosterone, neural monoamines and stress behavior can accompany amphetamine sensitization. Whether these changes occur following repeated amphetamine treatment in the absence of behavioral sensitization is not known. Male Sprague-Dawley rats were treated with amphetamine (2.5 mg/kg, i.p.) or saline once daily for 6 days. Amphetamine-induced locomotion and stereotypy, open-field anxiety behavior, plasma corticosterone and limbic monoamines were measured during withdrawal. Sixty-two percent of amphetamine-treated rats showed behavioral sensitization over the test periods. Only amphetamine-sensitized rats showed increased latency to enter the center of the open-field, as well as increased plasma corticosterone when compared to saline-treated controls. Amphetamine-sensitized rats showed increased dopamine concentrations in the shell of the nucleus accumbens and increased serotonin concentrations in the dorsal hippocampus, which were not observed in amphetamine-treated non-sensitized rats. These findings suggest that anxiety behavior, plasma corticosterone and limbic monoamines concentrations are altered by repeated amphetamine (2.5 mg/kg) treatment, and that these neuroendocrine and behavioral changes are often associated with sensitization to the psychostimulant effects of amphetamine.

Keywords

Anxiety; Corticosterone; Dopamine; Sensitization; Serotonin

1. Introduction

Increased behavioral sensitivity to psychostimulants for extended periods of time suggests that the drugs induce long-term or permanent changes to motor and reward systems within the central nervous system [1]. Behavioral sensitization to amphetamine in rats is characterized by increased motor activity and alterations to neural systems involved in reward [1–5]. Amphetamine-sensitized rats also show increased acquisition to amphetamine self-

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administration when compared to drug naïve controls [6]. This sensitized state can last for months to years following withdrawal of the drug [7,8].

Individual responses to stress are believed to be a major underlying cause of relapse during psychostimulant withdrawal and detoxification [9–12]. Some studies indicate that amphetamine-sensitized rats show increased plasma levels of stress-induced corticosterone release (for example; [13–14] although see [15]). Similarly, amphetamine-treated rats show increased behavioral sensitivity to mild stressors during the withdrawal period when compared to saline-treated controls [12;16,17]. Altered stress responses following sensitization could explain, in part, why stress increases the probability of relapse and drug seeking behavior during a withdrawal period.

Enhanced stress-induced corticosterone levels and behavioral responses in amphetamine-treated rats may also result simply from the pharmacological actions of repeated amphetamine on neural and endocrine stress systems, regardless of whether sensitization occurs. Acute and chronic administration of psychostimulants increases plasma corticosterone concentrations [18;19]. Furthermore, administration of amphetamine causes widespread increases in monoamine release in the central nervous system, including brain regions linked to stress responsiveness such as the hippocampus, medial prefrontal cortex, ventral tegmental area and nucleus accumbens [8]. Following amphetamine administration, exposure to acute and chronic stressors alters limbic serotoninergic activity [20]. This neurotransmitter is strongly linked with fear, anxiety and depression [21–23]. Chronic exposure to psychostimulant treatment is believed to induce intense and repeated activation of stress-related neural and endocrine systems [12,24]. In turn, repeated drug-induced activation of stress pathways may result in long-term neural and endocrine dysfunction, as reflected by increased sensitivity to stress and the induction of anxiety states during drug abstinence.

Currently, it is not known whether alterations in neural, endocrine and anxiety measures are a consequence of behavioral sensitization to amphetamine, or alternatively, result from the pharmacological actions of chronic amphetamine regardless of sensitization. To partially address this question, the goal of this study was to determine whether basal corticosterone levels, anxiety behavior and limbic monoaminergic concentrations of rats that sensitize to the behavioral effects of amphetamine differ from rats that receive amphetamine treatment but do not exhibit behavioral sensitization. The use of male Sprague-Dawley rats as an experimental model offers the opportunity to examine physiological and behavioral differences between amphetamine-sensitized and amphetamine-treated non-sensitized rats, since unselected populations of this strain shows individual variability in the ability to sensitize to amphetamine [25].

2. Materials and Methods

2.1 Animals

Adult male outbred Sprague-Dawley rats (n = 32, mass 300–320 g; Charles River, Raleigh, NC, USA) were housed in pairs in a light controlled room (12-h reverse light/dark cycle: lights off at 8:00 AM). Food and water were available *ad libitum*. All experiments were conducted during the dark phase between the hours of 9:00 AM and 5:00 PM. The following procedures were approved by the Institutional Animal Care and Use Committee of the University of South Dakota, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

2.2 Amphetamine Treatment and Activity Measures

Animals were handled and placed for 40 min in a locomotion chamber ($52 \times 33.5 \times 30$ cm; divided into a 2×3 grid) once daily for five days to habituate to the testing enclosure. Following habituation, rats received either *d*-amphetamine sulfate (2.5 mg/kg salt weight, i.p.; Sigma Chemical Co., St. Louis, MO, USA) or the equivalent volumes of sterile saline (0.9%) (n = 16 per treatment group) administered once daily for six days. With the exception of the first day of administration, rats were placed back into their home cages following injection.

Locomotion testing was conducted in response to the injection on the first day of treatment (first exposure), and 7 and 14 days following the last repeated injection (i.e., 7 and 14 days of withdrawal period). On day 7 of withdrawal, both amphetamine and saline pre-treated rats received 2.5 mg/kg amphetamine. On day 14 of withdrawal, amphetamine and saline pre-treated rats were injected with amphetamine or saline, respectively. Immediately following injection, rats were placed into the locomotion chamber for 70 min under red light illumination, since behavioral sensitization to a variety of amphetamine doses is apparent within 10–60 min of injection (for example; [14,15,26–29]). Two scorers blind to treatment reviewed the behavioral recordings, counting the number of consecutive line-crossings completed by each animal and the total number of rears during the final 60 min of the 70 min testing period [30].

Measures of focused stereotypy intensity were scored during the first exposure test and the day 14 withdrawal testing session. Stereotypy ratings were based on a 0–6 scale, scored over two min for every 10 min interval. Scores were adapted from [30], and intensity ratings and behaviors are summarized in Table 1. The higher ratings (3–6) represent intense focused stereotypy that excludes locomotion [30,31].

2.3 Open Field Testing

On day 11 of withdrawal, rats were placed in an open field chamber (54.5×80 cm) with a center area (21×46 cm) outlined in white, under white room lighting. Behavior was recorded for 30 min using a video camera and VCR. Two scorers blind to treatment reviewed the recordings and measured latency to enter the center of the chamber, and total activity over the 30 min period, which comprised rearing and line crossing. Scoring was performed using a computer-based event recorder (Etholog V. 2.2.5, Sao Paulo, Brazil; [32])

2.4 Collection of Brains and Plasma

Three days following final locomotion test (at day 17 of withdrawal), animals were decapitated and brains collected and frozen at −70°C until sectioned. This method of storage has been shown to preserve monoamine content at levels comparable to that of fresh tissue [33,34]. Trunk blood was collected, and centrifuged at 5,000 rpm. Plasma was drawn off and frozen at −70°C until assayed. Samples were collected three days following amphetamine challenge to ensure complete recovery from the pharmacological effects of amphetamine treatment and the effects of testing procedures.

2.5 Plasma Corticosterone Assay

Measurement of plasma corticosterone was performed using a corticosterone enzyme-linked immunoassay kit, following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Ten µl of plasma and 0.5 µl steroid displacement reagent were diluted with 990 µl of assay buffer for a 100-fold dilution. Duplicates of each sample, controls and standards were assayed. Plasma corticosterone levels were detected by absorbance of samples at 405 nm (wavelength correction set at 595 nm) and compared to known corticosterone standards using automated plate reader reader and KinetiCalc Jr. software (Bio-Tek Instruments, Winooski, VT, USA). Absorbance values were used to calculate maximum binding percent, which ranged

between 19.9 – 20.9 %, and percent of non-specific binding, which was 2.4%; both values were within the manufacturer's range. The detection limit sensitivity of this assay was 27.0 pg/ml.

2.6 Brain Monoamine Analysis

Frozen brains were serially sliced at 300 µm in the coronal plane, at −10°C, and stored at −70° C until microdissection. Limbic and striatal terminal fields (medial prefrontal cortex, central nucleus of the amygdala, dorsal hippocampus, ventral hippocampus, dorsal striatum, shell and core of the nucleus accumbens), and the monoaminergic cell body regions (ventral tegmental area, substantia nigra pars compata, dorsal raphe nucleus, median raphe nucleus) were identified using published guides [35,36], and bilaterally microdissected with a 500 µm id cannula using a dissecting microscope and freezing stage. The microdissected tissue was expelled into 60 µl of sodium acetate buffer (pH 5.0) containing 9.42 pg/µl of internal standard (dihydroxybenzylamine, DHBA), and the cells were lyzed by freeze-thawing.

Brain regions were analyzed for serotonin, its metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine, and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), using high-pressure liquid chromatography. A detailed description of this assay has been published elsewhere [37–38]. Levels of central monoamines were obtained via comparison to known standards, and corrected for recovery using CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). Neurotransmitter and metabolite levels were expressed as pg amine/µg protein.

2.7 Data and Statistical Analysis

The percentage change in amphetamine-induced locomotion from first exposure locomotion test to the day 14 of withdrawal locomotion test was determined for each individual amphetamine pre-treated rat. Rats were allocated into sensitized and non-sensitized groups based on the following criteria that were formed from preliminary data indicating a bimodal distribution of responses over time: 25% or greater increase in locomotion indicated sensitization and a 25 % or greater decrease in locomotion over the testing sessions indicated non-sensitization. In this particular study, 10 of the 16 amphetamine-treated rats were sensitized and 6 of the 16 rats were allocated to the non-sensitized group based on these criteria.

The effects of repeated amphetamine administration on either total locomotion or total rearing was compared across the testing sessions and within each testing session using separate 2-way ANOVAs with one repeated measure (time). Significant effects of time, treatment, or time × treatment interactions were followed by one-way repeated measure ANOVAs. Locomotion and rearing data obtained from day 7 of withdrawal were analyzed independently from the other test days using separate one-way ANOVAs, since all rats received amphetamine injection on this test day. Significant main effects were further analyzed using Student Newman-Keuls (SNK) post-hoc test for multiple comparisons.

To ensure that decreased locomotion exhibited by the non-sensitized group was not due to augmented focused stereotyped behavior that excludes locomotion (thus indicating greater sensitivity to amphetamine), amphetamine-induced stereotypy scores for the first exposure and day 14 withdrawal tests were compared among experimental groups across the testing session. These comparisons were made using separate Kruskal-Wallis ANOVAs on ranks, since the data did not meet assumptions of normality and equal variance.

Open-field behavior (latency and total activity), plasma corticosterone, and monoamine concentrations were all compared between groups using separate one-way ANOVAs. Significant effects were further analyzed using SNK post-hoc tests. All analyses were

performed using SigmaStat (version 2.03; © 1992–1997, SPSS Inc., Point Richmond, CA), with the alpha level set at 0.050.

3. Results

3.1 Amphetamine-Induced Locomotion, Rearing and Stereotypy Intensity

During the first exposure and on day 14 of withdrawal (Fig. 1), sensitized and non-sensitized groups received a single injection of amphetamine, while control rats received a saline injection. For locomotion exhibited during the first exposure test (Fig. 1A), there was a significant effect of treatment $F(2,29) = 76.459$, $p < 0.001$, time $F(5,145) = 10.368$, $p < 0.001$ and a treatment $\times$ time interaction $F(10, 145) = 6.002$, $p < 0.001$. One-way repeated measure ANOVA showed a significant effect for locomotion across time for all three groups (sensitized $p < 0.010$; non-sensitized $p = 0.016$; saline $p = 0.007$). Sensitized rats showed greater locomotion at 30, 40 and 50 min compared to 10 min post-injection ($p < 0.010$). In contrast, post-injection locomotion counts for the non-sensitized rats did not statistically differ at any time point when compared to 10 min post-injection ($p > 0.05$). Saline-treated rats showed a significant decrease in locomotion over the testing session ($p = 0.007$). When locomotion scores were compared between groups, sensitized and non-sensitized groups showed higher locomotion compared to the saline-treated group at each post-injection time point ($p < 0.001$). Furthermore, non-sensitized rats exhibited higher amphetamine-induced locomotion when compared to sensitized rats at 10 min ($p = 0.006$) and 20 min ($p = 0.023$) post-injection.

For locomotion scores at day 14 of withdrawal (Fig. 1B), there was a significant effect of treatment $F(2,29) = 76.459$, $p < 0.001$, a treatment$\times$time interaction $F(10, 145) = 6.002$, $p < 0.001$ and a trend for a significant effect of time $F(5,145) = 10.368$, $p = 0.051$. One-way repeated measure ANOVA showed a significant effect of time on locomotion for saline-treated ($p < 0.001$) and sensitized ($p < 0.001$) rats but not for non-sensitized rats ($p = 0.418$). Sensitized rats had greater locomotion at all post-injection time points compared to 10 min post-injection ($p < 0.001$). Levels of locomotion exhibited by saline-treated rats were lower at all post-injection time points compared to 10 min post-injection ($p < 0.01$). Sensitized and non-sensitized groups showed higher locomotion compared to the saline-treated group at each time point ($p < 0.010$). However, non-sensitized rats exhibited lower post-treatment locomotion when compared to sensitized rats at all time periods after amphetamine injection ($p < 0.010$), with the exception of 10 min post-injection ($p = 0.170$).

Total locomotion within the testing sessions was compared between first exposure and day 14 of withdrawal (Fig. 1C). There was a significant effect of treatment $F(2,29) = 87.568$, $p < 0.001$ and a significant interaction between treatment and time $F(2,29) = 26.348$, $p < 0.001$. Total locomotion scores within the first exposure test were not significantly different between sensitized and non-sensitized groups ($p = 0.156$). However, both amphetamine-treated groups exhibited higher total locomotion compared to saline-treated rats ($p < 0.001$). Sensitized rats showed an increase ($p < 0.001$) in total amphetamine-induced locomotion between first exposure and day 14 of withdrawal. In contrast, non-sensitized rats showed a decrease in total amphetamine-induced locomotion during this period ($p < 0.001$). Thus, total amphetamine-induced locomotion was higher in the sensitized group compared to non-sensitized rats ($p < 0.001$) at day 14 of withdrawal. Saline-treated rats showed a small but significant increase in total locomotion over the testing sessions ($p = 0.044$), but both amphetamine-treated groups exhibited higher total locomotion at day 14 of withdrawal when compared to saline-treated controls ($p < 0.001$).

For rearing during the first exposure test (Fig. 2A), there was a significant effect of treatment $F(2,29) = 17.151$, $p < 0.001$. but neither time $F(5,145) = 2.166$, $p = 0.065$ nor treatment $\times$ time interaction $F(10, 145) = 1.174$, $p = 0.320$ were significant. Analysis of the treatment effect

revealed that non-sensitized rats exhibited greater rearing counts compared to both sensitized and saline-treated rats ($p = 0.001$). At day 14 of withdrawal (Fig. 2B), there was also a significant effect of treatment $F(2,29) = 17.485$, $p < 0.001$. Neither time $F(5,145) = 0.391$, p 0.855 nor interaction between treatment $\times$ time $F(10, 145) = 1.742$, $p = 0.077$ were significant. In contrast to their first exposure, non-sensitized rats exhibited lower amphetamine-induced rearing counts compared to sensitized rats ($p = 0.041$). Both sensitized and non-sensitized rats showed greater rearing counts than saline-treated controls ($p < 0.001$ and $p = 0.014$, respectively).

Total rearing across the testing sessions is summarized in Fig. 2C. There was a significant effect of treatment $F(2,29) = 70.705$, $p < 0.001$, and a significant interaction between treatment and time $F(2,29) = 12.401$, $p < 0.001$. In the first exposure test, non-sensitized rats showed greater total rearing counts than sensitized and saline-treated groups ($p < 0.001$). Rearing counts between sensitized and saline-treated groups were similar ($p = 0.597$). However, sensitized rats showed a significant increase in total amphetamine-induced rearing counts between first exposure and day 14 of withdrawal tests post-treatment sessions ($p < 0.001$), while non-sensitized rats showed a significant decrease in total amphetamine-induced rearing counts across the two testing sessions ($p < 0.001$). This resulted in sensitized rats exhibiting more total amphetamine-induced rearing counts compared to non-sensitized rats ($p = 0.032$). Total rearing counts from saline-treated rats did not significantly differ between the two testing sessions ($p = 0.310$). Both amphetamine-treated groups showed greater total rearing counts at day 14 of withdrawal compared with saline rats ($p < 0.010$).

Stereotypy intensity scores were recorded for the two amphetamine-treated groups and saline controls to ensure that reductions in amphetamine-induced locomotion and rearing observed for non-sensitized rats did not result from heightened focused stereotyped behavior that normally excludes locomotion [5,30,31]. Rats did not show pronounced focused stereotyped behavior regardless of treatment, indicating that locomotion was the predominant behavior exhibited over the testing session (Fig. 3). No significant effects of treatment at any time point were observed for stereotypy ratings during the first exposure or during the day 14 of withdrawal tests ($p < 0.05$ for all comparisons).

All rats were given a single injection of amphetamine at 7 days of withdrawal. In this test, amphetamine-induced locomotion was significantly different among the groups (Fig. 4A; $F_{2,29}, = 4.776$, $p = 0.017$). Sensitized rats exhibited greater amphetamine-induced locomotion when compared to both non-sensitized rats ($p = 0.035$) and saline pre-treated rats ($p = 0.012$). Non-sensitized and saline pre-treated rats showed similar levels of amphetamine-induced locomotion ($p = 0.604$). Amphetamine-induced rearing counts did not differ among the groups in this testing session (Fig. 4B; $F(2,29) = 0.486$, $p = 0.621$)

3.2 Open Field Behavior

Latency to enter the center of the open field on day 11 of withdrawal varied among sensitized, non-sensitized and controls rats (Fig. 5; $F(2,27) = 4.584$, $p = 0.020$). Sensitized rats took a longer time to enter the center region compared to the saline-treated group ($p = 0.018$). Latency to enter the center of the open field for amphetamine-treated non-sensitized rats was not significantly different from either saline-treated ($p = 0.109$) or sensitized rats ($p = 0.389$). Total activity, determined from rearing counts and line-crossing, did not significantly differ between any of the groups during the 30 min observation period $F(2,27) = 0.270$, $p = 0.765$, suggesting that increased latency to enter the center was not a result of decreased activity within the open field.

3.3 Plasma Corticosterone

Plasma corticosterone levels, measured on day 17 of withdrawal, differed among sensitized, non-sensitized and control rats (Fig. 6; $F_{(2,28)} = 4.429$, $p = 0.022$). Sensitized rats had higher plasma corticosterone concentrations than saline-treated rats ($p = 0.021$). In contrast, non-sensitized and saline-treated rats had similar plasma corticosterone concentrations ($p = 0.808$). There was a trend for higher plasma corticosterone levels in sensitized rats compared to non-sensitized rats ($p = 0.061$).

3.4 Neural Concentrations of Dopamine and DOPAC

Figure 7 illustrates significant effects of amphetamine sensitization on concentrations of dopamine in selected brain regions, and the non-significant results are summarized in Table 2. Concentrations of the dopamine metabolite DOPAC were not significantly different in any of the brain regions studied (Table 3).

Dorsal striatal dopamine concentrations were significantly different among groups (Fig. 7A; $F_{(2,27)} = 4.564$, $p = 0.020$). Non-sensitized rats had lower dopamine concentrations in the dorsal striatum when compared to saline-treated controls ($p = 0.022$), but were not significantly different from sensitized rats ($p = 0.079$). Concentrations of dopamine in the dorsal striatum of sensitized rats were similar to values in saline-treated rats ($p = 0.285$).

In the ventral tegmental area, dopamine concentrations also differed among sensitized, non-sensitized and control rats (Fig. 7B; $F_{(2,29)} = 4.856$, $p < 0.016$). Sensitized rats had lower dopamine concentrations in the ventral tegmental area when compared to non-sensitized ($p = 0.020$) and saline-treated rats ($p = 0.027$). However, concentrations of dopamine in the ventral tegmental area of non-sensitized and saline-treated groups were not significantly different ($p = 0.239$).

In the shell of the nucleus accumbens, dopamine concentrations differed among groups (Fig. 7C; $F_{(2,27)} = 3.496$, $p = 0.047$). Dopamine concentrations in the nucleus accumbens shell were higher in sensitized rats when compared to saline-treated controls ($p = 0.030$) or to non-sensitized rats ($p = 0.043$). Dopamine concentrations in the nucleus accumbens of non-sensitized and saline-treated groups were not significantly different ($p = 0.698$).

Sensitized, non-sensitized and saline-treated rats differed in dopamine concentrations within the ventral hippocampus (Fig. 7D; $F_{(2,28)} = 6.585$, $p = 0.005$). Dopamine concentrations in the ventral hippocampus of non-sensitized rats were higher values measured in sensitized ($p = 0.008$) and saline-treated rats ($p = 0.003$). In contrast, ventral hippocampal dopamine concentrations in the sensitized and saline-treated groups were not significantly different ($p = 0.626$).

3.5 Neural Concentrations of Serotonin and 5-HIAA

The significant effects of amphetamine sensitization on serotonin concentrations in selected brain regions are illustrated in Fig. 8, and the non-significant results are summarized in Table 4. Concentrations of the serotonin metabolite 5-HIAA were not significantly different in any of the brain regions studied (Table 5).

Serotonin concentrations in the dorsal raphe nucleus differed among sensitized, non-sensitized and control rats (Fig. 8A; $F_{(2,27)} = 3.681$, $p = 0.040$). Non-sensitized rats had higher serotonin concentrations in the dorsal raphe nucleus than sensitized ($p = 0.032$), and saline-treated rats ($p = 0.040$). However, dorsal raphe serotonin concentrations in sensitized and saline-treated groups were not significantly different ($p = 0.348$).

Serotonin concentrations in the ventral tegmental area also differed among groups (Fig. 8B; F (2,29) = 3.606, p = 0.040). Sensitized rats had lower serotonin concentrations in the ventral tegmental area than saline-treated rats (p = 0.043), and there was a trend for lower serotonin concentrations when sensitized rats were compared to non-sensitized rats (p = 0.057). Ventral tegmental area serotonin concentrations did not differ significantly between non-sensitized and saline-treated groups (p = 0.997).

In the dorsal striatum, there were differences in serotonin concentrations among sensitized, non-sensitized and control rats (Fig. 8C; F(2,27) = 4.363, p = 0.024). Sensitized rats had lower dorsal striatal serotonin concentrations than saline-treated rats (p = 0.028), but were not significantly different from non-sensitized rats (p = 0.747). A trend towards lower serotonin concentrations of dorsal striatal serotonin was evident when non-sensitized rats were compared to saline-treated controls (p = 0.051).

Serotonin concentrations the shell of the nucleus accumbens also differed among sensitized, non-sensitized and control rats (Fig. 8D; F(2,27) = 6.076, p = 0.007). Non-sensitized rats had lower nucleus accumbens shell serotonin concentrations than saline-treated (p = 0.006) and sensitized rats (p = 0.043). Nucleus accumbens shell serotonin concentrations were not significantly different in sensitized and saline-treated groups (p = 0.112).

In the medial prefrontal cortex, serotonin concentrations in sensitized, non-sensitized and saline-treated differed (Fig. 8E; F(2,29) = 5.490, p = 0.010). Decreases in medial prefrontal cortex serotonin concentrations were detected in both sensitized and non-sensitized rats when compared to the saline-treated group (sensitized; p = 0.014, non-sensitized; p = 0.033), but no significant differences were found between sensitized and non-sensitized rats (p = 0.766).

In the dorsal hippocampus, serotonin concentrations differed between groups (Fig. 8F; F(2,27) = 3.916, p = 0.033). Sensitized rats had higher dorsal hippocampal serotonin than non-sensitized rats (p = 0.026), but neither sensitized (p = 0.117) nor non-sensitized (p = 0.119) groups were significantly different from saline-treated rats.

4. Discussion

4.1 Behavioral Sensitization

In rodents, sensitization to repeated amphetamine treatments is often characterized by enhanced locomotor responses to low drug doses and enhanced stereotypy to higher doses of amphetamine [5,7]. In our study, repeated treatment with 2.5 mg/kg amphetamine produced hyperactivity which predominated over focused stereotyped behavior when tested on day 14 of withdrawal. The majority (62%) of amphetamine-treated rats showed sensitization to the locomotor effects of amphetamine as evidenced by increased total amphetamine-induced locomotion and rearing over the testing sessions. These rats also showed greater amphetamine-induced locomotion when compared to saline-treated rats challenged with amphetamine 7 days after the last repeated injection, indicating sensitization to the locomotion effects of amphetamine (Fig. 4A).

In contrast, 38% of amphetamine-treated rats did not show locomotor sensitization. These rats, classified as non-sensitized, exhibited decreased locomotion and amphetamine-induced rearing over the testing sessions. Non-sensitized rats also showed similar amphetamine-induced locomotor responses at 7 days of withdrawal when compared to rats undergoing their first exposure to amphetamine (Fig. 4A), further suggesting that they did not sensitize to the locomotor effects of repeated amphetamine treatment. The absence of locomotor sensitization following repeated amphetamine treatment could not be explained by an increase in amphetamine-induced focused stereotyped behavior that would normally exclude locomotion

[30]. These results suggest that non-sensitized rats are desensitized [39] and may have habituated to the behavioral effects of repeated amphetamine. The proportion of sensitized to non-sensitized rats in our study was similar to that demonstrated for cocaine sensitization studies in rats [40]. Interestingly, the distribution of sensitization measures in the current study was bimodal, such that all the rats treated with amphetamine clearly divided into the two groups (sensitized and non-sensitized). Further studies are required to determine whether this distinct bimodal distribution holds for this strain and other strains of rats.

Many studies have focused on pre-existing differences that may predict amphetamine sensitization in rats and addiction susceptibility in humans. For example, individuals that have high sensation-seeking scores also express higher self-report measures associated with the reinforcing effects of amphetamine [41]. Likewise, rats that have high locomotor counts in response to a novel environment (high responders) or have high novelty preference show greater locomotor responses to an initial administration of amphetamine, and greater amphetamine self-administration when compared low novelty responders [26,42,43]. These high responders also exhibit greater sensitization following repeated amphetamine [26,27, 44]. However, this is a dose-dependent effect, since high and low responders show equal levels of sensitization at amphetamine doses higher than 1.0 mg/kg i.p. [26,43]. While this variable should be examined in the future, the 2.5 mg/kg dose used in our study probably precludes novelty responses as a predictor of amphetamine sensitization. Furthermore, a significant difference between the current study and those examining the relationship between high/low responders and amphetamine is that rats in the current study were habituated to the testing enclosures prior to testing.

4.2 Open Field Behavior and Plasma Corticosterone

Sensitized rats took a significantly longer time to enter the center of the brightly-lit open field compared to saline-treated controls, indicating increased anxiety. While further testing in other anxiety paradigms is required, these results suggest that in addition to showing elevated stress responses to mild stressors [12,16,17], sensitized rats show increased anxiety-like behavior during the withdrawal period. However, the latency for non-sensitized animals to enter the center was not different from saline-treated controls or sensitized rats. Similarly, plasma corticosterone levels following drug treatment and withdrawal were increased in sensitized rats compared to saline-treated controls, but corticosterone levels in non-sensitized rats were not different from sensitized or saline-treated rats. These results highlight the importance of comparing amphetamine sensitized and non-sensitized rats to determine the effects of amphetamine sensitization on anxiety and endocrine measures. The findings suggest that for both anxiety-like behavior and plasma corticosterone, non-sensitized rats could represent a state intermediate to that of saline-treated and amphetamine-sensitized rats.

Previous studies have shown that amphetamine-treated rats exhibit increased plasma corticosterone, behavioral and neurochemical responses to stress when compared to saline controls [12,13,45], although some of these measures appear to be dependent on amphetamine dose [15]. Corticosterone has been proposed to facilitate the rewarding effects of amphetamine, and high circulating levels of corticosterone may potentiate amphetamine sensitization ([46]; although see [47]). Future research should examine whether pre-existing individual differences in anxiety behavior and corticosterone levels prior to drug exposure can predict the degree of amphetamine sensitization.

4.3 Neural Dopamine and DOPAC Concentrations

Following amphetamine treatment and withdrawal, no changes were seen in concentrations of the dopamine metabolite, DOPAC, in any brain region studied. This suggests that the changes in dopamine concentrations observed were a result of altered synthesis and storage of dopamine

rather than alterations to dopaminergic activity. While both sensitized and non-sensitized rats had different dopamine concentrations in various striatal or limbic sites when compared to saline-treated controls, there were no instances where dopamine concentrations in a given brain region were significantly changed in both sensitized and non-sensitized rats. This suggests that the changes in dopamine concentrations were related to either the processes of behavioral habituation or sensitization to repeated amphetamine.

Higher dopamine concentrations in the nucleus accumbens shell of sensitized rats suggest that synthesis or storage of dopamine in this region increased during the sensitization process. This could explain why psychostimulant-sensitized rats show heightened amphetamine-induced nucleus accumbens shell dopamine release during withdrawal when measured by microdialysis [48–50]. The current results also show that repeated amphetamine treatment in the absence of sensitization does not appear to affect dopamine concentrations in the shell of the nucleus accumbens, which is in line with previous findings in an amphetamine sensitization-resistant line of rats [51,52]. Our findings add support to the hypothesis that dopamine in the nucleus accumbens shell could underlie sensitization to repeated amphetamine treatment [53–55] and may be involved in increased reward and motivation leading to addiction [53,54,56]. In contrast to this hypothesis, Roman High-Avoidance rats sensitized to lower (1 mg/kg) doses of amphetamine show attenuated amphetamine-induced (0.2 mg/kg) dopamine release in the shell of the nucleus accumbens during withdrawal [52]. The contrasting results regarding sub-region changes to accumbal dopamine following sensitization highlight the impact of differences in amphetamine dose, sensitization methodology and rat strain on the study of the underlying mechanisms of amphetamine sensitization.

Dopaminergic activity in the ventral tegmental area is thought to be related to increased amphetamine-induced dopamine release in the nucleus accumbens in sensitized rats [1,55, 57]. Following a period of withdrawal, dopamine responses to acute cocaine in the ventral tegmental area are diminished [1,58]. The current study demonstrates that only sensitized rats had decreased dopamine concentrations in the ventral tegmental area, which could explain decreased drug-induced release of dopamine in this region as measured by microdialysis in the withdrawal period [1,58]. In contrast, only non-sensitized rats had decreased dopamine concentrations in the dorsal striatum. Interestingly, rats that show reduced locomotion to repeated amphetamine treatment in a similar fashion to the non-sensitized rats of the current study have lower dopamine content in the striatum in the absence of amphetamine exposure, when compared to rats that sensitize to amphetamine [39]. Since dopamine in the dorsal striatum is required for some of the motor behaviors induced by amphetamine [31,59,60], future research should test the possibility that decreased dopamine concentrations in the dorsal striatum may play a role in habituation to the motor effects of amphetamine.

4.4 Neural Serotonin and 5-HIAA Concentrations

Similar to the dopaminergic systems, concentrations of the serotonin metabolite, 5-HIAA, did not significantly change in any of the brain regions studied as a response to amphetamine treatment and withdrawal. Therefore, the changes in serotonin concentrations appear to be a result of altered synthesis or storage of serotonin rather than alterations in serotonergic activity. With the exception of the medial prefrontal cortex, changes in serotonin concentrations detected in the sensitized and non-sensitized groups did not occur in the same direction (i.e., increase or decrease) within the same brain region. This again suggests that the majority of alterations to serotonin concentrations were related either to amphetamine habituation or to amphetamine sensitization.

Non-sensitized rats had decreased serotonin concentrations in the shell of the nucleus accumbens when compared to saline-treated and sensitized rats. Direct administration of serotonin to the nucleus accumbens inhibits acute amphetamine-induced locomotion [61,62]

via complex interactions with a number of serotonin receptor subtypes [63]. However, it is not known how serotonin function in the nucleus accumbens relates to chronic amphetamine treatment and withdrawal. Our current findings suggest that decreased serotonin concentrations in the nucleus accumbens may be an adaptation to repeated amphetamine treatment. Therefore, decreased serotonergic function in this region may inhibit sensitization to the locomotor activation effects of amphetamine, but this possibility requires testing.

Amphetamine-induced increases in serotonin are thought be responsible for amphetamine depression of glutamatergic excitatory transmission in the ventral tegmental area of drug-naive rats [64]. In our study, ventral tegmental area serotonin concentrations were lower in sensitized rats when compared to saline-treated controls. Conceivably, such reductions in serotonin could attenuate amphetamine-depression of excitatory transmission in the ventral tegmental area of these rats, leading to heightened responses to amphetamine.

Sensitized rats also had lower serotonin concentrations in the dorsal striatum compared to the saline-treated group. Central serotonin depletion results in increased locomotor responses to amphetamine [65]. While this effect cannot be attributed to serotonin depletion in a specific brain region, Rebec and colleagues [66] showed that enhanced amphetamine-locomotion in serotonin depleted rats was paralleled by altered neuronal responses in the dorsal striatum, and proposed that the effect of serotonin depletion on amphetamine-induced locomotion is mediated by the dorsal striatum. Our results add to this by suggesting that sensitization to the locomotor effects of amphetamine is associated with decreased serotonin levels in the dorsal striatum.

While no differences in serotonin concentrations in the ventral hippocampus were detected, serotonin in the dorsal hippocampus was higher in sensitized rats compared to non-sensitized rats. Our findings provide support for the hypothesis that serotonin in the dorsal hippocampus, but not ventral hippocampus, mediates amphetamine-induced locomotion [67]. Furthermore, it appears that sensitization to the locomotor effects of amphetamine is reflected by differences in dorsal hippocampal serotonin concentrations.

Decreased serotonin concentrations in the medial prefrontal cortex in both sensitized and non-sensitized rats suggest that these changes were due to repeated amphetamine treatment, and not associated with sensitization. Decreased serotonin activity in the frontal cortex during psychostimulant withdrawal has been shown previously [68]. Combined with our current results, this suggests that decreased serotonin concentrations and function in the frontal cortex results from the pharmacological actions of repeated amphetamine. Since elevations in medial prefrontal cortex serotonin activity are thought to be involved in reducing expression of acute fear behavior [69], reduced serotonin function in this region could result in prolonged fear behavior of amphetamine-treated rats. This speculation is partly supported by the finding that cocaine-treated rats undergoing withdrawal show poor extinction of fear behavior in a conditioned foot-shock paradigm [70].

4.5 Conclusions

Our results suggest that following repeated amphetamine (2.5 mg/kg) treatment, the changes in dopamine and serotonin concentrations in many limbic regions are associated with sensitization rather than with repeated amphetamine administration in the absence of sensitization. The findings also suggest that the specific alterations in limbic dopamine and serotonin concentrations detected in non-sensitized rats could reflect habituation to the effects of repeated amphetamine administration. Although it remains to be seen whether similar effects are observed with different doses of amphetamine, the current study should help direct future examination of the neural circuitry involved in the individual propensity for sensitization and stress-induced relapse. Future work should also examine the possibility that pre-existing

individual differences in levels of anxiety-like behavior, plasma corticosterone or limbic monoamine markers could predispose animals to sensitize to the effects of amphetamine.

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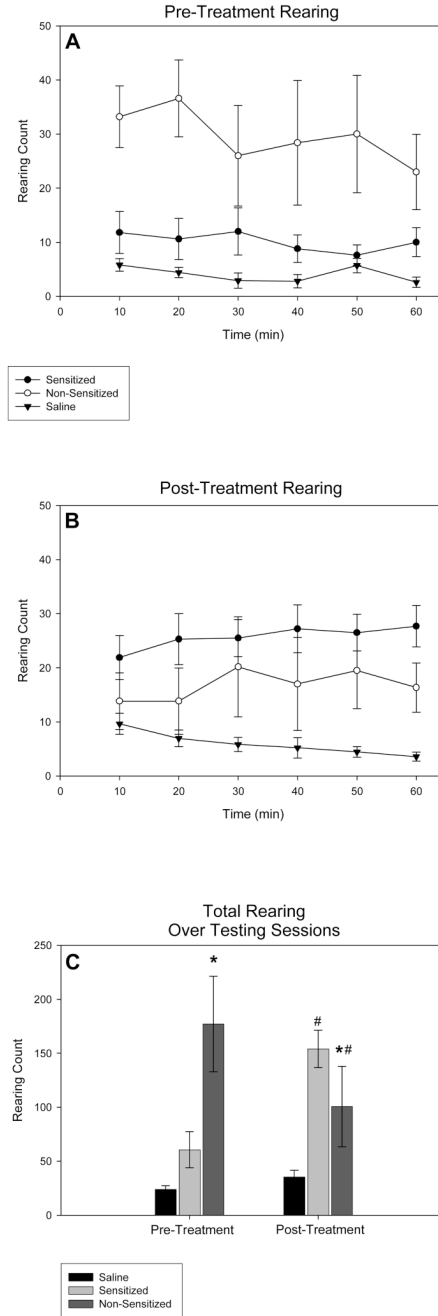
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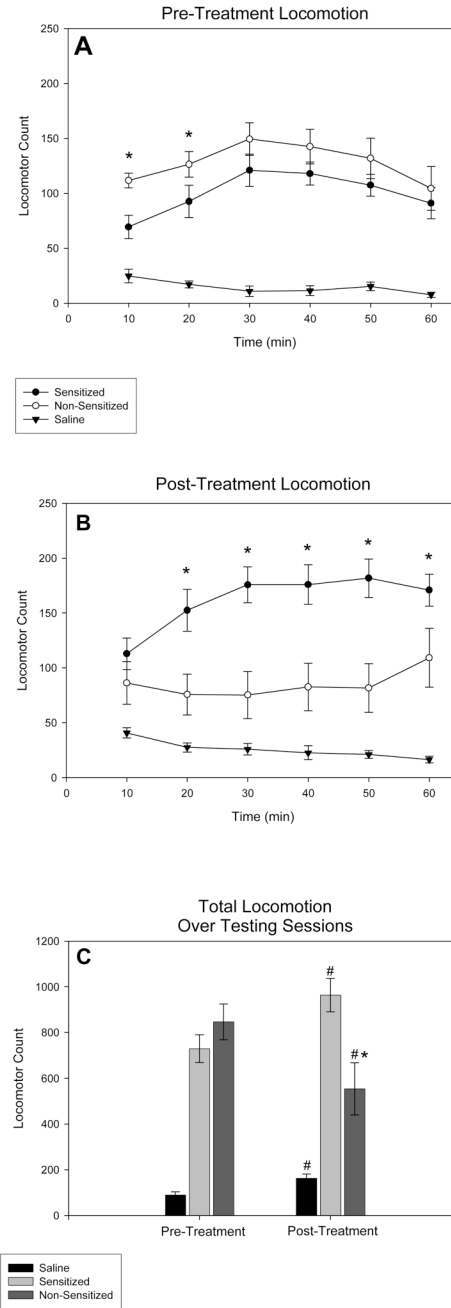
Fig. 1.
Locomotion counts over time (**A**) following first injection and (**B**) at 14 days of withdrawal, in response to a challenge of 2.5 mg/kg, i.p. amphetamine (sensitized and non-sensitized groups), or saline. (**C**) Total locomotion during first exposure and at 14 days of withdrawal, during the 60 min observation period. * represents significant differences between sensitized and non-sensitized rats, # represents significant differences from pre-test. Both amphetamine-treated groups were significantly higher than saline-treated rats at all time points and testing sessions (see Results for statistics). (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).

Fig. 2.
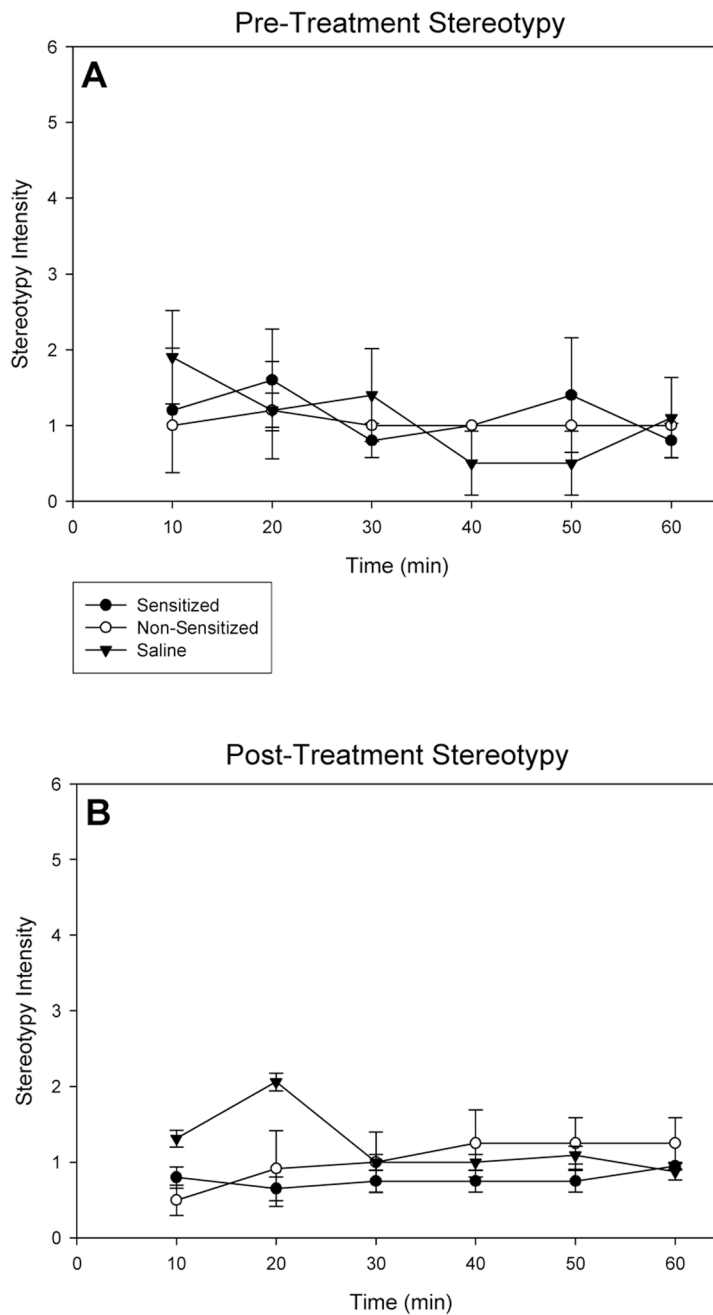Rearing counts over time (**A**) following first injection and (**B**) at 14 days of withdrawal, in response to a challenge of 2.5 mg/kg, i.p. amphetamine (sensitized and non-sensitized groups), or saline. (**C**) Total rearing during first exposure and at 14 days of withdrawal, during the 60 min observation period. * represents significant differences between sensitized and non-sensitized rats, # represents significant differences from pre-test. Both amphetamine-treated groups were significantly higher in total rearing than saline-treated rats at 14 days of withdrawal, but only non-sensitized rats were significantly higher compared to saline-treated rats in the first exposure test (see Results for statistics). (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).

Fig. 3.
Stereotypy scores as a measure of intensity over time (**A**) following first injection and (**B**) at 14 days of withdrawal, in response to a challenge of 2.5 mg/kg, i.p. amphetamine (sensitized and non-sensitized groups), or saline. (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).
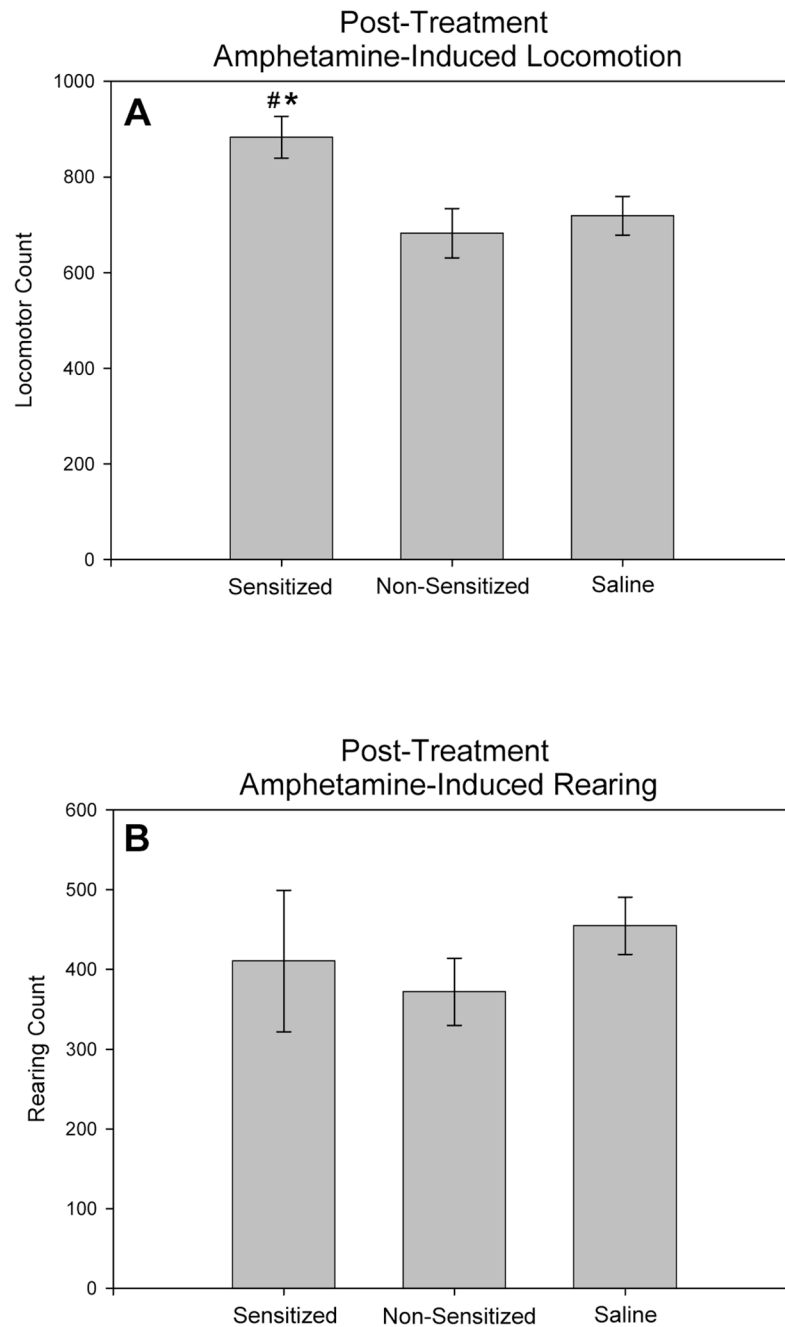
Fig. 4.
Total amphetamine-induced locomotion (**A**) and total amphetamine-induced rearing (**B**) during 60 mins of observation in response to amphetamine-challenge (2.5 mg/kg, i.p.) at 7 days of withdrawal, for amphetamine pre-treated (sensitized and non-sensitized) and saline pre-treated rats. * represents significant differences from saline controls, # represents significant differences between sensitized and non-sensitized rats. (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).
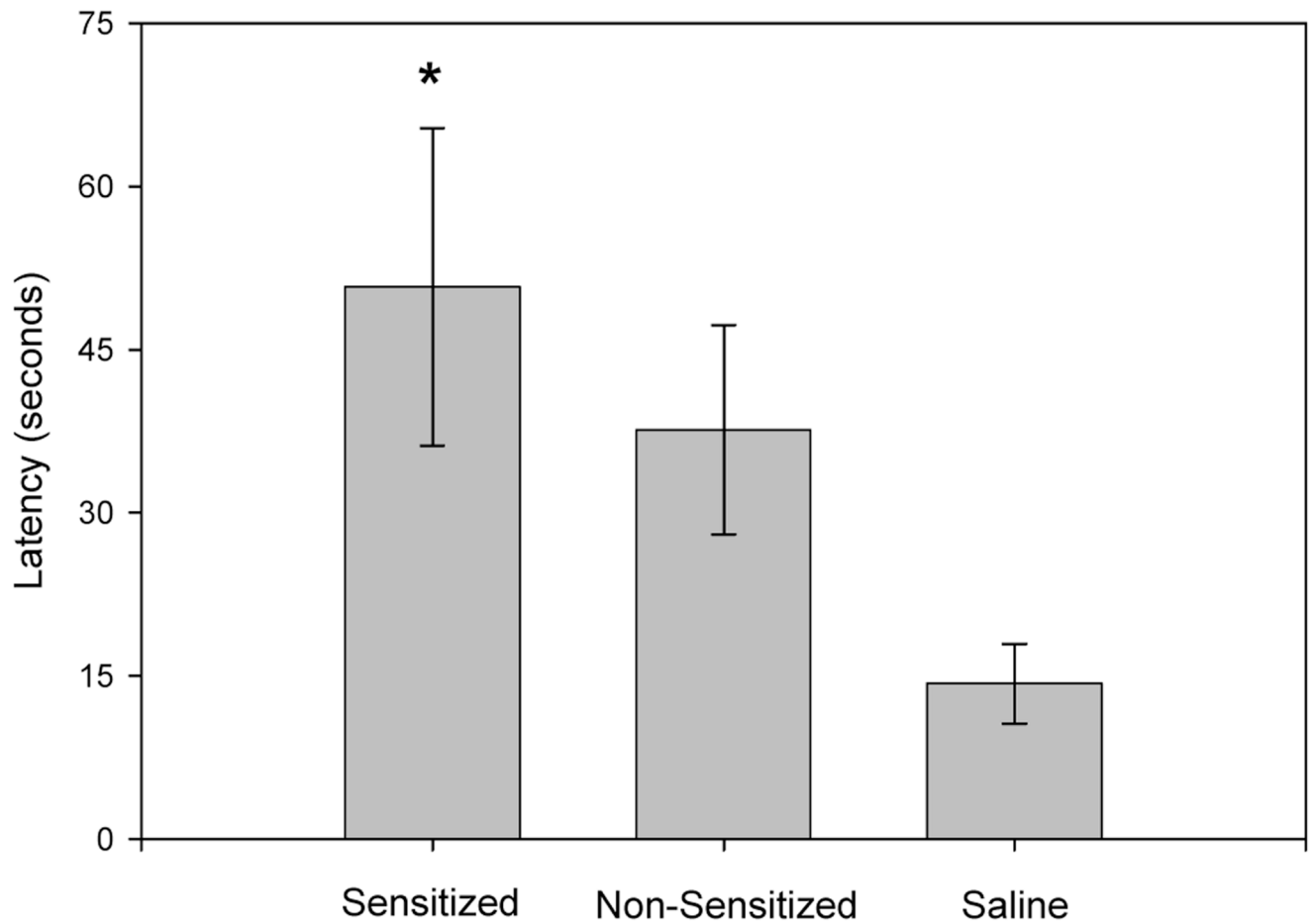
Fig. 5.
Latency to enter center of open field, at 11 days of withdrawal. * represents significant difference from saline-treated controls. (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).
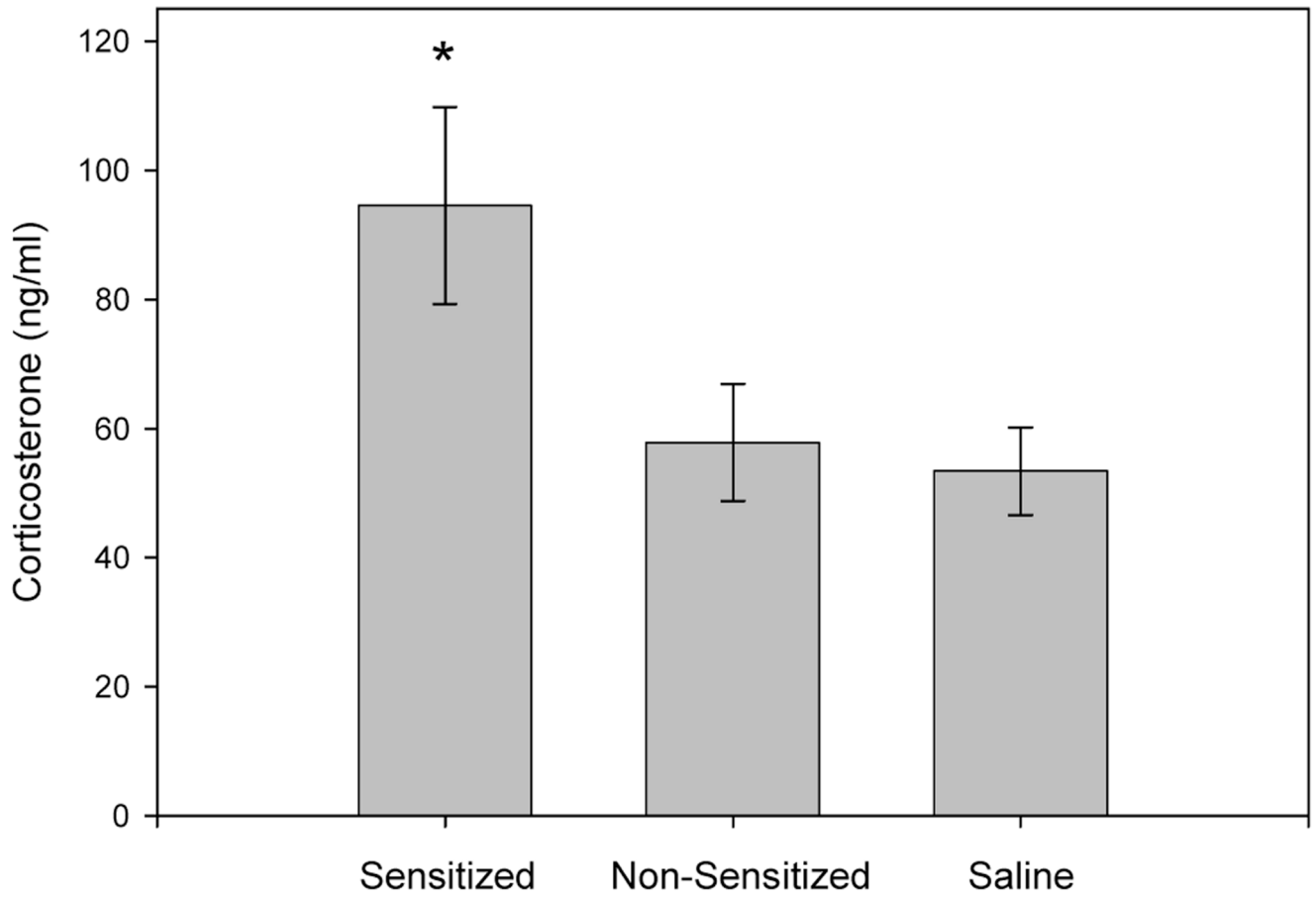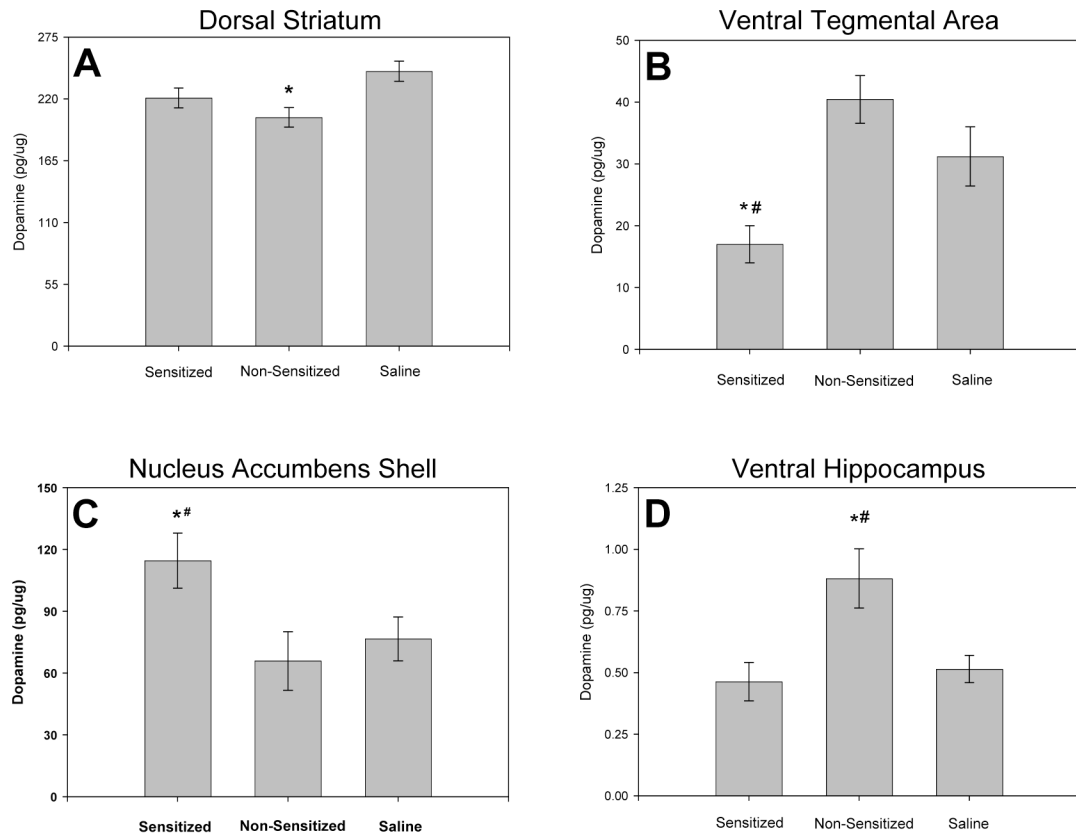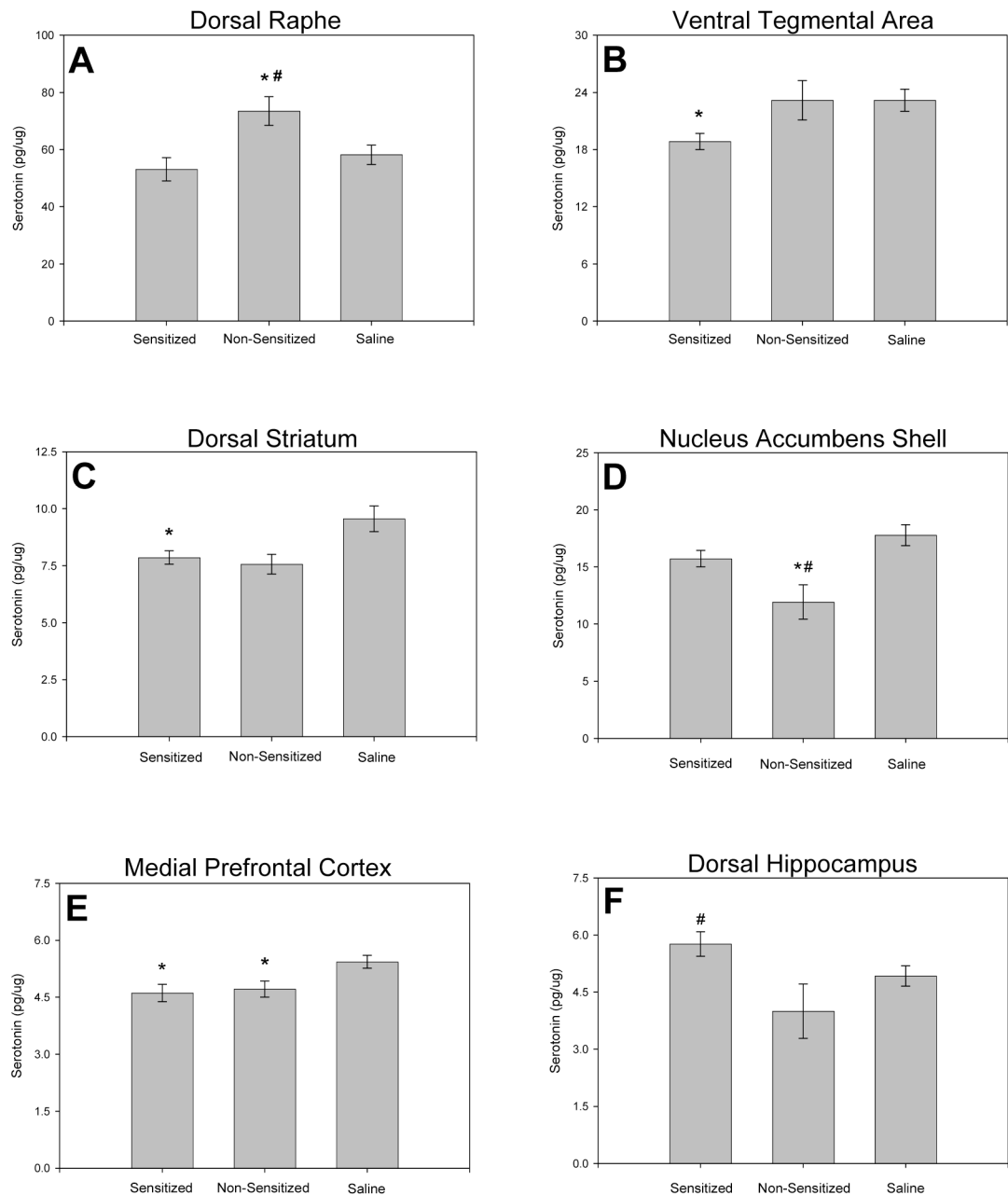
Fig. 6.
Plasma corticosterone concentrations at 17 days of withdrawal. * represents significant differences from the saline-treated group. (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).

Fig. 7.
Dopamine concentrations in the limbic and nigrostriatal systems at 17 days of withdrawal. Dopamine concentrations in (**A**) the dorsal striatum, (**B**) the ventral tegmental area, (**C**) the shell of the nucleus accumbens, and (**D**) in the ventral hippocampus, for sensitized rats, non-sensitized rats and saline-treated controls. * represents significant differences from the saline-treated group, # represents significant differences between sensitized and non-sensitized groups. (Amphetamine-sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).

Fig. 8.
Serotonin concentrations in the limbic and nigrostriatal systems at 17 days of withdrawal. Serotonin concentrations in (**A**) the dorsal raphe, (**B**) the ventral tegmental area, (**C**) the dorsal striatum, (**D**) the shell of the nucleus accumbens, (**E**) the medial prefrontal cortex, and (**F**) in the dorsal hippocampus, for sensitized rats, non-sensitized rats and saline-treated controls. * represents significant differences from the saline-treated group, # represents significant differences between sensitized and non-sensitized groups. (Amphetamine sensitized: N=10, non-sensitized: N=6, saline: N=16; means ± S.E.M. are shown for all groups).

Table 1

Stereotypy intensity ratings and associated behaviors.

Rating	Behaviors expressed over 2-min observation periods
0	Inactive/Locomotion only
1	Intermittent, non-oral-based stereotypy such as repetitive head bobbing, grooming with limbs and rearing
2	Continuous, non-oral based stereotypy expressed over wide area of enclosure
3	Continuous, non-oral-based stereotypy expressed in restricted area of enclosure
4	Pronounced, oral-based stereotypy directed at self in a restricted area such as repetitive licking, biting and grooming with mouth
5	Intermittent, oral-based stereotypy directed externally such as repetitive licking or biting of walls and floor of enclosure
6	Continuous, oral-based stereotypy directed externally

Table 2

Basal concentrations of dopamine within the limbic and nigrostriatal systems 17 days following repeated amphetamine (2.5 mg/kg, ip.) or saline pre-treatment.

Region	Treatment	DA Concentrations (pg/μg tissue) Mean±SEM	F	p
Central Nucleus of the Amygdala	Sensitized	10.7±4.7		
	Non-Sensitized	29.8±2.8	3.667	0.055
	Control	24.3±5.9		
Medial Prefrontal Cortex	Sensitized	1.3±0.1		
	Non-Sensitized	1.2±0.1	2.198	0.133
	Control	1.6±0.1		
Dorsal Raphe Nucleus	Sensitized	7.0±0.7		
	Non-Sensitized	10.6±0.8	2.156	0.138
	Control	9.4±1.0		
Median Raphe Nucleus	Sensitized	3.3±0.5		
	Non-Sensitized	5.0±0.3	2.982	0.071
	Control	4.6±0.4		
Nucleus Accumbens Core	Sensitized	142.3±7.5		
	Non-Sensitized	150.9±4.5	0.363	0.700
	Control	146.9±5.8		
Substantia Nigra	Sensitized	8.4±2.1		
	Non-Sensitized	5.0±1.1	0.524	0.599
	Control	8.2±2.0		

Table 3

Basal concentrations of DOPAC within the limbic and nigrostriatal systems 17 days following repeated amphetamine (2.5 mg/kg, ip.) or saline pre-treatment.

	DOPAC Concentrations (pg/µg tissue)			
Region	**Treatment**	**Mean±SEM**	**F**	**P**
Central Nucleus of the Amygdala	Sensitized	3.1±1.4		
	Non-Sensitized	5.5±0.4	2.003	0.181
	Control	5.3±0.4		
Medial Prefrontal Cortex	Sensitized	0.6±0.0		
	Non-Sensitized	0.6±0.1	1.596	0.227
	Control	0.7±0.1		
Dorsal Raphe Nucleus	Sensitized	2.2±0.3		
	Non-Sensitized	3.3±0.4	2.161	0.146
	Control	2.6±0.3		
Median Raphe Nucleus	Sensitized	2.8±0.7		
	Non-Sensitized	3.2±0.3	0.219	0.805
	Control	2.7±0.4		
Ventral Tegmental Area	Sensitized	5.2±0.6		
	Non-Sensitized	7.8±1.1	2.035	0.149
	Control	7.0±0.8		
Nucleus Accumbens Shell	Sensitized	27.9±3.2		
	Non-Sensitized	19.1±2.3	2.006	0.163
	Control	23.3±1.7		
Nucleus Accumbens Core	Sensitized	41.8±3.9		
	Non-Sensitized	35.8±1.2	0.712	0.500
	Control	38.6±2.4		
Substantia Nigra	Sensitized	3.1±0.6		
	Non-Sensitized	2.1±0.3	1.238	0.310
	Control	3.2±0.4		
Dorsal Striatum	Sensitized	23.1±2.4		
	Non-Sensitized	19.5±0.5	1.891	0.173
	Control	23.7±1.1		

Table 4

Basal concentrations of serotonin within the limbic and nigrostriatal systems 17 days following repeated amphetamine (2.5 mg/kg, ip.) or saline pre-treatment

		5-HT Concentrations (pg/μg tissue)		
Region	**Treatment**	**Mean±SEM**	**F**	**p**
Central Nucleus of the Amygdala	Sensitized	21.4±2.0		
	Non-Sensitized	23.3±2.3	0.965	0.397
	Control	26.3±2.6		
Median Raphe Nucleus	Sensitized	52.1±4.3		
	Non-Sensitized	68.2±6.3	1.590	0.226
	Control	58.1±3.4		
Nucleus Accumbens Core	Sensitized	9.6±0.2		
	Non-Sensitized	9.3±0.4	0.777	0.472
	Control	10.1±0.4		
Substantia Nigra	Sensitized	15.6±1.2		
	Non-Sensitized	14.0±1.3	1.017	0.378
	Control	16.5±1.0		
Ventral Hippocampus	Sensitized	9.9±0.6		
	Non-Sensitized	8.0±0.7	2.130	0.140
	Control	8.7±0.5		

Table 5

Basal concentrations of 5-HIAA within the limbic and nigrostriatal systems 17 days following repeated amphetamine (2.5 mg/kg, ip.) or saline pre-treatment.

	5-HIAA Concentrations (pg/μg tissue)			
Region	**Treatment**	**Mean±SEM**	**F**	**p**
Central Nucleus of the Amygdala	Sensitized	16.1±01.3		
	Non-Sensitized	20.3±02.0	1.712	0.201
	Control	18.7±01.2		
Medial Prefrontal Cortex	Sensitized	4.4±00.2		
	Non-Sensitized	4.8±00.1	1.079	0.353
	Control	4.9±00.3		
Dorsal Raphe Nucleus	Sensitized	61.7±04.2		
	Non-Sensitized	80.9±06.5	1.423	0.261
	Control	70.7±05.4		
Median Raphe Nucleus	Sensitized	82.0±11.2		
	Non-Sensitized	106.9±04.9	0.972	0.393
	Control	89.1±07.7		
Ventral Tegmental Area	Sensitized	17.0±00.6		
	Non-Sensitized	18.4±00.8	1.559	0.227
	Control	18.7±00.7		
Nucleus Accumbens Shell	Sensitized	8.4±00.5		
	Non-Sensitized	7.5±00.7	2.679	0.086
	Control	9.2±00.4		
Nucleus Accumbens Core	Sensitized	8.1±00.4		
	Non-Sensitized	8.4±00.6	0.359	0.701
	Control	8.6±00.4		
Substantia Nigra	Sensitized	12.3±00.6		
	Non-Sensitized	13.5±01.3	0.288	0.752
	Control	14.1±01.5		
Dorsal Striatum	Sensitized	6.4±00.5		
	Non-Sensitized	6.2±00.2	2.720	0.085
	Control	7.5±00.4		
Dorsal Hippocampus	Sensitized	7.8±00.5		
	Non-Sensitized	7.6±00.6	0.0815	0.922
	Control	7.6±00.4		
Ventral Hippocampus	Sensitized	8.3±00.9		
	Non-Sensitized	7.0±00.5	0.951	0.400
	Control	7.7±00.3		