Published in final edited form as:

Dev Neurosci. 2014 ; 36(2): 108–118. doi:10.1159/000360001.

Long-term effects of early adolescent methamphetamine exposure on depression-like behavior and the hypothalamic vasopressin system in mice

Lauren Joca^a, Damian G. Zuloaga^b, Jacob Raber^{b,c,d}, and Jessica A. Siegel^a

^aDepartment of Psychology, The University of the South, 735 University Avenue, Sewanee, TN 37318, USA

^bDepartment of Behavioral Neuroscience, Oregon Health & Science University, 8131 SW Sam Jackson Park Road, Portland, OR 97239, USA

^cDepartment of Neurology, Oregon Health & Science University, 8131 SW Sam Jackson Park Road, Portland, OR 97239, USA

^dDivision of Neuroscience ONPRC, Oregon Health & Science University, 8131 SW Sam Jackson Park Road, Portland, OR 97239, USA

Abstract

Methamphetamine has neurotoxic effects on the adult human brain that can lead to deficits in behavior and cognition. However, relatively little research has examined the behavioral or neurotoxic effects of methamphetamine in adolescents. The rising rates of adolescent methamphetamine use make it imperative that we understand the long-term effects of methamphetamine exposure on the adolescent brain and how these effects may differ from those seen in adults. In this study, the long-term effects of methamphetamine exposure during early adolescence on behavior and the vasopressin system in the paraventricular nucleus of the hypothalamus in late adolescent and adult male and female C57BL/6J mice were examined. Methamphetamine exposure increased depression-like behavior in the Porsolt forced swim test in both late adolescent and adult male and female mice. Late adolescent male mice exposed to methamphetamine also showed a decrease in the number of vasopressin-immunoreactive neurons in the paraventricular nucleus compared to sex-matched saline-treated controls. Thus, similar to humans exposed to methamphetamine during adolescence, mice exposed to methamphetamine during adolescence show increased depression-like behavior later in life. These changes in behavior may be related to methamphetamine-induced alterations in vasopressin and the hypothalamic-pituitary-adrenal axis, especially in males.

Keywords

methamphetamine; adolescence; vasopressin; depression; mice

Correspondence and should be addressed to Dr. Jessica A. Siegel, Department of Psychology, The University of the South, 735 University Avenue, Sewanee, TN 37383, USA. Telephone: (931) 598-1317. Fax: (931) 598-1145. jasiegel@sewanee.edu.

Conflicts of Interest

The authors have no conflicts of interest to report.

The abuse of methamphetamine (MA) is a growing problem in the United States. MA has neurotoxic effects on the adult human brain that can cause slowed movement [1], altered stress reactivity [2], and a proclivity toward psychotic episodes [3]. Adolescence marks a period of life when individuals are more at risk of developing life-long drug use habits [4, 5], and MA abuse treatment programs report that adolescents account for 20% of all admissions and that the percentage is rising [6]. Moreover, the specific effects of MA on the adolescent brain are not well understood compared to the effects on the adult brain. Brain damage and resulting behavioral changes from MA exposure could manifest differently in adolescents, whose brains are still at a critical stage in development [7].

The existing research on the effects of MA in adolescents shows that adolescents who have been abstinent from MA for an average of 4–11 months show alterations in behavior and increased psychotic symptoms, including increased social anxiety and depression, following an average of 2–3 years of MA exposure [8, 9]. Adolescents in treatment for MA abuse show higher levels of depressive symptoms and suicide ideation than adolescents being treated for other substances of abuse [10]. Adolescent rats self-administer MA at a significantly higher rate than adult rats [11] and show enhanced sensitivity to the rewarding properties of MA compared to adult rats immediately following MA exposure [12]. Furthermore, 14 days of MA exposure in adolescent mice results in long-term deficits in spatial memory and hippocampal plasticity following 21 days of abstinence [13]. Rats show long-term spatial learning impairments 30 days following adolescent MA exposure, but not when exposed to MA during pre-adolescence or adulthood [14], suggesting that adolescence is a time when MA exposure can cause unique and long-lasting cognitive impairments. However, other studies suggest the adolescent brain may be partially protected from the toxic effects of MA. For example, male adolescent mice show less locomotor activation immediately following MA exposure compared to adult male mice [15], and adolescent male rats do not show the same reductions in dopamine transporter (DAT) or tyrosine hydroxylase activity following 7 days of MA exposure as adult male rats [16]. Taken together, these data reflect the potential heightened psychological and behavioral risks adolescents face when using MA and the need for further research on the effects of MA on the adolescent brain.

MA also has effects on the stress response and the hypothalamic-pituitary-adrenal (HPA) axis, and thus MA may exert some of its behavioral and cognitive effects by activating the HPA axis and altering levels of the hormones associated with the HPA axis [2, 8, 17, 18]. HPA axis hyperactivity is associated with cognitive impairments and depression [19–21]. Following an average of 8 months of abstinence, adolescents who used MA for an average of 2.4 years show increased cortisol levels following a social stressor compared to controls [8]. Neonatal MA exposure in rodents from either postnatal day (PND) 11–15 or PND 11–20 is associated with large and prolonged increases in corticosterone and/or adrenocorticotrophic releasing hormone immediately following exposure [17, 22–24]. However, challenges to the HPA axis during brain development can lead to long-term impairments in the system later in life. For example, while neonatal MA exposure in rodents results in immediate increases in corticosterone levels [17, 23, 24], it is often followed by a blunted corticosterone response to stress in adulthood [25]. Maternal separation, which is a

neonatal stressor, decreases the number of vasopressin-immunoreactive cells in the paraventricular nucleus (PVN) of the hypothalamus in adult male rats [26]. Furthermore, we recently showed that exposure to MA from PND 11–20 results in decreased vasopressin-immunoreactive cells in the PVN on both PND 20 and PND 90 in male mice [27]. As release of vasopressin from the PVN modulates adrenocorticotrophic hormone release from pituitary gland and subsequent release of corticosterone from the adrenal gland [28], these data suggest that MA exposure during early brain development could cause behavioral impairments and depression through dysregulation of the HPA axis.

The current study examined the long-term effects of early adolescent MA exposure (**PND 30–31**) in late adolescence (**beginning on PND 41**) and adulthood (**beginning on PND 62**) in male and female mice. Previous studies have shown that neonatal MA exposure from PND 11–20 impairs memory in the novel object recognition test [29–31] and the Morris water maze [29, 32–34] later in life. Thus we examined the effects of adolescent MA exposure on memory in these tests. Previous studies have also shown that adult MA exposure impairs social interaction behavior [35], and thus we were also interested in examining the effects of adolescent MA exposure on social behavior in the social interaction test. Finally, we were interested in examining the effects of adolescent MA exposure on depression-like behavior in the Porsolt forced swim test and on the stress response and HPA axis function, as adolescent MA exposure in humans increases depression and suicide ideation compared to adolescents in treatment for other drugs of abuse [10].

Methods

Mice

Seventy-four C57BL/6J mice (37 male, 37 female) were used for this study. The mice were ordered from The Jackson Laboratory (Bar Harbor, ME) and shipped on postnatal day (PND) 24, arrived in our colony on PND 27. Mice were weighed and housed by sex in groups of 4, except for 2 cages with 2 mice per cage (one cage of female mice and one cage of male mice in the late adolescent testing group). Two mice died of natural causes prior to the start of the study after shipping, thus leaving 2 cages with 3 mice per cage (one cage of female mice in the adult testing group and one cage of male mice in the adult testing group). The mice were weighed during the injection period (which began on PND 30) and every 2–3 days throughout the experiment following the injection period (see Figure 1). Mice were provided with food and water *ad libitum*. The mice were kept on a 12 hr light/dark cycle (lights on at 06:00) and all behavioral testing took place during the light cycle in the morning, except for the Morris water maze where testing took place in the morning and afternoon. All procedures and testing protocols were approved by The University of the South's Institutional Animal Care and Use Committee (IACUC).

Injections

(d)-MA hydrochloride (Sigma Aldrich, St. Louis, MO, CAS Number 51-57-0) was dissolved in 0.9% sterile saline to the appropriate concentration. Injections were administered to all mice on PND 30 and 31. This time period is considered early adolescence in mice [7]. The MA treatment consisted of 2 days of 4×7.5 mg/kg MA administered in 2 hour intervals (0.1

mL intraperitoneal injections). A small pilot study in our lab was used to select the dose of MA and previous studies have used a similar injection schedule with lower doses of MA [36, 37] and higher doses of MA on a single day of injections [18]. Intraperitoneal saline injections (0.1 mL) were administered as a control following the same injection schedule. Saline and MA injections were counterbalanced across the sexes and within the housing cages.

Behavioral Testing

The mice were randomly assigned to one of two testing groups: late adolescents and adults. The groups underwent a battery of behavioral testing; the late adolescent age group began on PND 41 and completed testing on PND 52. The adult age group was tested PND 62 through PND 73 (Figure 1). All mice were individually housed 3 days prior to behavioral testing. All mice were tested in the following behavioral tests in the following order: open field, novel object recognition, social interaction, Porsolt forced swim test, and the Morris water maze.

Open Field—Locomotor activity and anxiety-like behavior were assessed in the open field test. The late adolescent group completed the open field test on PND 41. Adult mice were tested on PND 62. The mice were placed in a brightly lit 40 × 40 cm clear, Plexiglas arena. The innermost 20 × 20 cm of the arena were designated as the “center” of the arena. The movement and location of the mice was recorded using Anymaze Video Tracking Software (Stoelting, Wood Dale, IL). The distance traveled within the arena and the percent distance moved in the center of the arena was recorded for a single 10 minute session. The percent distance moved in center was used as a measure of anxiety-like behavior [38]. The arena was cleaned with 70% isopropyl alcohol between mice.

Novel Object Recognition—Object memory was assessed in the novel object recognition test. The mice were habituated for 2 days in the same arenas that were used for open field testing, with no objects present, under dim lighting. The late adolescent group was habituated on PND 42 and 43, the adult group on PND 63 and 64. On each day, the mice underwent a single, 10-minute habituation trial. The arenas were cleaned with 70% isopropyl alcohol between mice.

For the novel object recognition test, the mice were tested in three trials over two days. The late adolescent group was tested on PND 44 and 45. The adult group was tested on PND 65 and 66. Each trial lasted 10 minutes, with a 5 minute inter-trial interval between trials 1 and 2 and a 24-hour inter-trial interval between trials 2 and 3. The same arenas were used for this test as for the open field test and habituation. In trial 1, two identical objects (plastic toy fish) were placed in opposing corners of the arena. In trial 2, one object was replaced by a novel object (plastic toy ball). The position of the novel object was counterbalanced across the treatment groups and the sexes. For trial 3 a second novel object (plastic toy car) replaced the first novel object. All objects were similar in size and texture. The time spent exploring each object was recorded by Anymaze Video Tracking Software (Stoelting, Wood Dale, IL) when the mouse's nose was in the zone of the object but the body was not. The average time exploring all objects and the preference delta score for the novel objects (time spent

exploring new object – time exploring old object) were calculated. The arenas were cleaned with 70% isopropyl alcohol between trials.

Social Interaction—Social behavior was assessed in the social interaction test. The late adolescent group was tested on PND 46 and the adult group was tested on PND 67. Two unfamiliar mice of the same sex and treatment type were tested in a single arena under red light in a single 5 minute trial. The same arenas used in the open field test were used for the social interaction test. The duration of social behaviors of each pair of mice were scored by an experimenter who was blind to the treatment and sex of the mice. Social behaviors were defined as interactions between the mice including sniffing, following, and grooming one another [39]. One social interaction score was obtained for the two mice paired together for the test. There were an odd number of mice in each age group and thus not all of the mice were scored for social interaction.

Porsolt Forced Swim Test—Depression-like behavior was assessed using the Porsolt forced swim test. The late adolescent mice were tested on PND 47. The adult group was tested on PND 68. A 1000 mL glass beaker was filled with 700 mL of tepid water. Each mouse was placed in a beaker for 5 minutes. The amount of time spent immobile versus swimming was quantified using Anymaze Video Tracking Software (Stoelting, Wood Dale, IL). Increased immobility in this test is interpreted as increased depression-like behavior or learned helplessness in rodents [40].

Morris Water Maze—Spatial learning and memory was evaluated using the Morris water maze on PND 48 through 52 for the late adolescent group and PND 69 through 73 for the adult group. The maze (circular tub, 122 cm diameter) was divided into four virtual quadrants by the Anymaze Video Tracking Software (Stoelting, Wood Dale, IL). The maze was filled with opaque water and all testing occurred under bright lighting. The mice were trained to locate a submerged platform hidden in one of the four quadrants in order to escape. On days 1 and 2 of testing, white curtains surrounded the maze and the platform was marked by a beacon for the visible component of training (non-spatial training; visible platform sessions). The curtains and beacon were removed on days 3 through 5 so that the mice had to use spatial cues from around the room to locate the platform (spatial training; hidden platform sessions). Each test day consisted of 2 sessions, one in the morning and one in the afternoon. Each session included three 60-second trials with a 10–15 minute inter-trial interval. If the mouse did not find the platform in 60 seconds then it was led to the platform by the experimenter and allowed to remain there for 3 seconds before being removed from the maze. During the visible sessions, the platform was moved to a different quadrant for each session. During the hidden sessions, the platform remained in the same quadrant. Mice were placed in the water maze facing the wall of the pool at nine different drop locations around the pool. The drop location was alternated for each trial. Swimming patterns were recorded using the Anymaze Video Tracking Software (Stoelting, Wood Dale, IL). The speed and escape latency were measured.

Spatial memory was assessed in probe trials following the last training session on days 3, 4, and 5. Probe trials occurred approximately 1 hour following the last hidden-session training trial. The platform was removed from the pool and mice were given a single 60-second trial.

The percent time in the target quadrant (the quadrant where the platform was previously located) and cumulative distance from the previous platform location was recorded. A smaller cumulative distance indicates swimming closer to the previous platform location and thus better spatial memory.

Vasopressin Immunohistochemistry and Quantification

On PND 53 or PND 54 for the late adolescent group and on PND 74 or PND 75 for the adult group, mice were deeply anesthetized with a cocktail containing 100 mg/kg ketamine, 10 mg/kg xylazine, and 2 mg/kg acepromazine and were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (pH = 7.4). Brains were removed and post-fixed overnight in 4% paraformaldehyde at 4° C, transferred to 30% sucrose solution, and embedded in cryoprotectant. Serial coronal sections (50 µm) were collected onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA) through the paraventricular nucleus (PVN) of the hypothalamus (Bregma -0.19 mm to -2.44 mm) with a 150 µm inter-section distance [41]. Sections were incubated for 2 hours at room temperature in a blocking solution (5% normal donkey serum in PBS containing 0.2% Triton X-100), followed by incubation overnight at 4° C in rabbit anti-vasopressin (1:100; ImmunoStar, Hudson, WI) primary antibody. Following 4 washes with PBS containing 0.2% Triton X-100 (10 min each) and 1 wash with PBS (15 min), sections were incubated for 2 hours at room temperature in donkey anti-rabbit (1:100) for fluorescent visualization (Life Technologies, Grand Island, NY). Following 4 washes in PBS containing 0.2% Triton X-100 (10 min each) and 1 wash with PBS (15 min), the sections were covered with anti-fade solution containing a DAPI counterstain (Vectashield, Vector Laboratories) and were cover slipped (Fisher Scientific).

Quantification of vasopressin expressing cells was performed using an Olympus IX81 confocal microscope (Olympus, Center Valley, PA, USA) by a researcher blinded to the experimental group of the animals. Three to five bilateral sections containing the PVN were captured using a 10x objective (UPlan FL, Olympus) from 4 mice per treatment, sex, and age group. Previous studies from our lab show that this group size is large enough to detect significant effects of developmental MA exposure on vasopressin expression [27]. However, due to tissue damage in one brain, only sections from 3 mice from the adult saline male group were quantified. Vasopressin-immunoreactive cells were counted within PVN containing sections of each animal using Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

Statistical Analysis

For each behavioral and cognitive outcome measure and the number of vasopressin-immunoreactive cells in the PVN, a 3-way analysis of variance (ANOVA) was conducted to test for main effects of, and interactions between, age, sex, and treatment. Any significant interactions were further explored in 2-way or 1-way ANOVAs. Performance in the Morris water maze and differences in weight were assessed over time with a repeated measure ANOVA, with session and day as the repeated factor, respectively. All statistical analyses were conducted using SPSS software (IBM, Armonk, NY). Data are presented as means ±

standard error of the mean (SEM). A significance level of $p = 0.05$ was used for all statistical tests.

Results

Weights

The weights of the mice were recorded from the day they arrived in the colony (PND 27) until the first day of behavioral testing (PND 41; Table 2). A repeated measure ANOVA was used to assess potential weight differences between the groups on each day that weights were obtained. All mice gained weight over the time period measured, as indicated by a main effect of day ($F(1, 70) = 1113.74, p < 0.01$). There were no differences between the treatment groups in weight on any day except for the second day of injections (PND 31) and on PND 37, as indicated by a 2-way interaction between day and treatment ($F(1, 70) = 11.78, p < 0.01$). Post-hoc analyses revealed that on the second day of injections (PND 31), the saline treated mice weighed significantly more than the MA-treated mice ($F(1, 72) = 17.41, p < 0.01$; Table 2). On PND 37, however, the MA-treated mice weighed significantly more than the saline-treated mice ($F(1, 72) = 5.76, p = 0.02$; Table 2). Other than these two days there were no significant differences in weight between the saline- and MA-exposed mice. Overall male mice weighed more on average (17.1 ± 0.2 grams) than female mice (15.1 ± 0.1 grams), as indicated by a main effect of sex ($F(1, 70) = 132.63, p < 0.01$).

Open Field

There was no main effect of age or treatment, nor any interactions between the factors, on total distance moved (Table 1). There was a main effect of sex on total distance moved in the open field ($F(1, 66) = 13.43, p < 0.01$). Female mice moved a significantly greater distance (3401.43 ± 112.33 cm) overall than male mice (2882.77 ± 84.4 cm). There was no main effect of treatment or age for percent distance moved in the center of the open field. There was, however, a trend for a 2-way interaction between treatment and age group ($F(1, 66) = 1.49, p = 0.05$). The adult MA mice trended toward moving a greater percent distance in the center of the arena compared to adult saline mice (Table 1). There was a significant main effect of sex on percent distance moved in center in the open field test ($F(1, 66) = 4.49, p = 0.04$). The female mice (13.92 ± 0.56 %) moved a greater percent distance in the center of the open field arena than male mice (12.21 ± 0.58 %).

Novel Object Recognition

For the average total time exploring all objects during the novel object recognition test, there were no main effects of sex or treatment, but there was a main effect of age group ($F(1, 66) = 9.58, p < 0.01$). There was also a sex $\times$ age group interaction for average time exploring all objects ($F(1, 66) = 6.36, p = 0.01$). Post-hoc analyses indicated that among the adult mice, the males (96.8 ± 12.78 seconds) spent a greater average time exploring the objects compared to the females (60.55 ± 11.83 seconds; $F(1, 36) = 4.33, p = 0.045$). For trial 2, there were no main effects or interactions for the delta preference score (time exploring new object – time exploring old object; Table 1). For trial 3, there was no main effect of sex or treatment for the delta preference score, but there was a main effect of age group ($F(1, 66) = 4.41, p = 0.04$). The late adolescent mice showed significantly more preference for the novel

object compared to the adult mice (Table 1). There were no significant interactions for the delta preference score in Trial 3.

Social Interaction

There were no significant effects of treatment, sex, or age on the duration of the social behaviors in the social interaction test (Table 1). There were also no interactions between any of these factors on social behaviors in the social interaction test.

Porsolt Forced Swim Test

There was a main effect of treatment on the percent time spent immobile in the Porsolt forced swim test ($F(1, 66) = 6.86, p = 0.01$). MA-treated mice spent a significantly greater amount of time immobile compared to the saline-treated mice (Figure 2). There were no main effects of age or sex or significant interactions between them for percent time spent immobile in the Porsolt forced swim test.

Morris Water Maze

For the visible platform non-spatial sessions of the Morris water maze, there was a session $\times$ age group interaction for swim speed ($F(3, 198) = 4.32, p < 0.01$). Post-hoc analysis showed that in the fourth session, late adolescent mice swam faster (9.69 ± 0.49 cm/sec) than the adult mice (8.07 ± 0.50 cm/sec; $F(1, 72) = 5.33, p = 0.02$). There were no main effects or interactions between any other factors for speed in the visible sessions. For the latency to find the platform in the visible sessions, there was a 3-way interaction between session, treatment, and age group ($F(3, 198) = 4.96, p < 0.01$). However, post-hoc analyses only revealed that for visible session 3 ($F(1, 70) = 1.75, p < 0.01$) and session 4 ($F(1, 70) = 10.18, p < 0.01$), the late adolescent mice had a shorter latency to escape than the adult mice (data not shown). For swim speed in the hidden platform spatial sessions, there were no main effects or interactions. For latency to find the platform in the hidden platform sessions, there was a main effect of session, indicating that all groups reduced the latency to escape from the maze over the sessions ($F(5, 330) = 24.19, p < 0.001$). However, there were no main effects of sex, treatment, or age group, nor any interactions between them, for latency to find the platform during the hidden sessions (Table 1).

There were no significant effects of treatment, sex, or age on cumulative distance from the platform location or percent time spent in the target quadrant during the probe trials (Table 1). There were also no interactions between these factors on cumulative distance from the platform location or percent time spent in the target quadrant during the probe trials.

Vasopressin Immunohistochemistry

For the average number of vasopressin-immunoreactive cells in the PVN, there were no main effects of treatment, sex, or age group, but there was a 3-way interaction between treatment, sex, and age group ($F(1, 23) = 4.62, p = 0.04$). Because previous data suggest sex differences in the effects of MA [29], we explored the interaction in the male and female mice separately. Among the female mice, there was no main effect of treatment or age group, or an interaction between them, on the average number of vasopressin-immunoreactive cells in the PVN. Among the male mice, there was a marginally significant

interaction between treatment and age group ($F(1, 11) = 4.85, p = 0.05$). Post-hoc analyses revealed that while there was no effect of treatment among the adult male mice, there was an effect of treatment among the late adolescent male mice ($F(1, 6) = 14.07, p = 0.01$), with MA-exposed late adolescent male mice showing fewer vasopressin-immunoreactive cells in the PVN compared to saline-exposed late adolescent male mice (Figure 3).

Discussion

The purpose of this study was to assess the effect of early adolescent MA exposure on cognition, behavior, and the PVN vasopressin system in late adolescence and adulthood. Consistent with our predictions, we found higher depression-like behavior in the forced swim test among the MA treatment group compared to the saline treatment group. We also found that early adolescent MA exposure decreased the number of vasopressin-immunoreactive cells in the PVN of late adolescent male mice. To the best of our knowledge this is the first study to examine the effects of adolescent MA exposure on depression-like behavior, and our findings suggest this is a good model of the increased depression seen in human adolescents exposed to MA.

All mice exposed to MA showed significantly greater depression-like behavior in the Porsolt forced swim test than mice exposed to saline regardless of sex or age. However, upon examining the data, it became clear that the main effect was driven by greater differences among the treatment groups in the male mice. Furthermore, previous data suggest that there are sex differences in the effects of MA on cognitive function [29]. Thus we were interested in exploring the differences between the sexes in this data and examined the effects of treatment among the male and female mice separately in an exploratory analysis. Two-way ANOVAs with treatment and age group as factors revealed that there was no main effect of treatment or age group on the percent time immobile in the Porsolt forced swim test in female mice. However, there was a treatment $\times$ age group interaction among the male mice ($p = 0.04$). Post-hoc analyses showed that while there was no effect of treatment among the adult male mice, the late adolescent MA-exposed male mice spent significantly more time immobile compared to the late adolescent saline-exposed male mice ($p = 0.02$), suggesting increased vulnerability to the effects of MA among males at an early age. This result must be interpreted with caution, however, as the effect seems to be caused by a decrease in percent time immobile among the late adolescent saline-exposed male mice rather than an increase among the late adolescent MA-exposed male mice. Nonetheless, this result suggests that future studies should be aware of, and examine, potential sex differences in the effects of early adolescent MA exposure.

In general the findings from the Porsolt forced swim test in this study are interesting as they parallel findings in human MA users. Adolescents in treatment for MA use show higher levels of depressive symptoms and suicide ideation compared to adolescents being treated for other substances of abuse [10]. At neurotoxic levels, MA use has been shown to reduce the density of DAT in the reward pathway and frontal cortex, as well as reduce the density of serotonin transporters in the adult brain [42]. Reduced serotonin and DAT density could disrupt neurotransmission and lead to dysfunction in the serotonin and dopamine neurotransmitter systems, both of which are implicated in depression [43, 44]. However,

previous research suggests that adolescent rodents are resistant to the neurotoxic effects of MA on DAT and tyrosine hydroxylase activity compared to adult rodents [16]. This resistance to the neurotoxic effects of MA on the dopamine system may be due to increased vesicular monoamine transport of dopamine into vesicles and increased levels of functionally active DAT in the adolescent compared to the adult rodent brain [45]. These findings suggest that the increased levels of depressive-like behaviors among the MA-exposed mice in the current study are a result of something other than MA's effects on the DAT system.

MA's effects on the vasopressin cells in the PVN may cause increased depressive-like behavior among the MA-exposed male mice. In agreement with previous data examining the effects of neonatal MA exposure on the vasopressin system in male mice [27], our data show that MA exposure during early adolescence decreased the number of vasopressin-immunoreactive cells in the PVN in the late adolescent male mice. Previous studies in rodents have shown that MA exposure immediately increases corticosterone levels [17, 22, 23] and heavy use of MA in humans alters the levels of hormones associated with the HPA axis [2, 8]. Taken together these data suggest that MA exposure during early adolescence may lead to dysregulation of the HPA axis, resulting in a decrease in the vasopressin-immunoreactive cells later in adolescence among male mice. It may be the case that the immediate increases in corticosterone levels following MA exposure lead to subsequent impairments in the HPA axis. Furthermore, the impairments in the HPA axis and vasopressin system seem to recover with abstinence, as there was no significant decrease in vasopressin-immunoreactive cells in the PVN in the adult male mice.

Previous research shows that female mice are more vulnerable to the long-term cognitive and neurotoxic effects of postnatal MA exposure [29, 30, 46]. Females also have a higher propensity of using MA during adolescence [10] and show a greater plasma corticosterone release following neonatal and adult MA exposure [22, 47] compared to males. This might be related to reduced negative feedback in females, resulting from reduced activation of glucocorticoid receptor-positive neurons in the CA3 region of the hippocampus and cingulate cortex [47]. To the best of our knowledge this is the first study examining the effects of MA exposure during early adolescence on depression-like behavior and the vasopressin system in rodents, and our findings suggest that in adolescence the female mice were not more vulnerable to the effects of MA, and if anything, the male mice may have shown an increased vulnerability to the effects of MA on depression-like behavior. Differential brain growth patterns between males and females could cause disparities in sensitivity to the effects of MA during early adolescence. Studies also suggest sex differences in the vasopressin system. For example, adult male mice show denser vasopressin innervations originating from the PVN compared to adult female mice [48] and early adolescent male rats show higher levels of vasopressin mRNA in the amygdala compared to early adolescent female rats [49]. The potential differences in the vasopressin system between male and female mice during early adolescence may result in the system being more vulnerable to MA among males. Further research is warranted to examine the relationship between MA-induced changes in the vasopressin system, and the mechanism underlying the sex differences in the effects of early adolescent MA exposure.

Contrary to our hypotheses, MA did not affect memory in either the novel object recognition test or the Morris water maze, did not have profound effects on anxiety-like behavior, and did not alter social behavior in the social interaction test. The results of the open field showed that adult MA exposed mice moved a greater percent distance in the center of the arena compared to the adult saline exposed mice, which would be interpreted as less anxious behavior. However, these data were not statistically significant and represented only a trend. Further study, perhaps with a larger sample size, is needed to determine whether this result is related to early MA exposure. Studies in adult rats treated with a single day of $4 \times 5\text{mg/kg}$ injections of MA showed significant deficiencies in object recognition memory for up to 3 weeks after MA exposure [50]. These data suggest that adolescent animals may be resistant to MA's effects on memory, although further research is needed to see if there are effects immediately after MA exposure in early adolescence that dissipates over time with abstinence. Previous research has also shown that MA exposure in adult rats decreases social interaction [35]. This suggests that adolescents are potentially partially protected from the effects of MA with the specific dosing paradigm used in the current study, as our mice exposed to MA during early adolescence showed no deficits in social interaction later in life. This relative resistance to the behavioral effects of MA may be due to the differences in the dopamine system in adolescence compared to adulthood described above [16, 45], although future research examining the DAT and dopamine function following early adolescent MA exposure are warranted. Different doses of MA during adolescence may have effects on memory and social behavior, and future research is warranted to examine this possibility. Indeed, other research has shown that adolescent rodents have increased susceptibility to the long-term memory impairments caused by MA compared to younger and older rodents [14], suggesting that different doses and different cognitive and behavioral tests may reveal more effects of adolescent MA exposure in mice.

Future studies might increase the dose of MA in adolescence to see if more severe behavioral and cognitive effects can be induced. The dose of MA used in the current study was based on previous studies using similar dosing paradigms [36, 37]. It is difficult, however, to know if this dose parallels the doses of MA used by adolescents. According to King et al. (2010), adolescents in treatment for MA abuse use on average about 0.57–1.17 grams of MA per day [9]. Extrapolating human self-administered doses of MA to rodents is a challenge considering the pharmacokinetic differences between species. Doses of MA that are self-administered in adult humans (average 1.6 grams per day) are sufficient to cause long-term reductions in striatal DAT levels [1]. In adult mice, 20 mg/kg reduces striatal DAT levels to 50% while 40 mg/kg reduces levels to approximately 25% of control values [51]. We based our dose of 7.5 mg/kg MA (4 injections per day for 2 day) on previous studies in rodents showing 10 mg/kg dose (4 injections for only one day) induced decreases in dopamine levels in adult mice [18] while 5 mg/kg dose (4 injections for 2 days) in adolescence induced decreases in the dopamine system in adult mice [37]. Based on the finding that these doses impair dopamine function, we predicted that our dose was also neurotoxic and thus had similar effects to those doses used in human MA abusing populations. However, the lack of significant effects on some of the cognitive tests in our study suggests higher doses may be needed in future studies.

From the results of our study, we cannot distinguish between the effects of adolescent MA exposure that are truly related to age and those that are related to abstinence. In other words, the adolescent mice began behavioral testing 10 days after MA exposure while the adult mice began testing 31 days after MA exposure. Thus differences between the age groups could be due either to the age of testing or due to differences in abstinence. Future studies should examine the same adult ages as the current study, but include the same drug treatment in adulthood that ends 10 days before testing in order to begin to address this question.

In summary, the findings from this study suggest that MA exposure during early adolescence increases depression-like behavior in male and female mice and decreases vasopressin-immunoreactive cells in the PVN in late adolescent male mice. MA-induced alterations in the vasopressin system and HPA axis may contribute to increases in depression-like behavior following adolescent MA exposure. Future studies with this rodent model of MA exposure during early adolescence are warranted to better understand the association between MA-induced decreases in vasopressin cells and increases in depression-like behavior.

Acknowledgments

The authors would like to thank Jackman Corley for his help with the immunohistochemistry for this study. This work was supported by a Faculty Research Grant from The University of the South, a National Institute of Drug Abuse Grant T32DA007262, and the development account of Dr. Raber.

References

1. Volkow ND, Chang L, Wang GJ, Fowler JS, Leonido-Yee M, Franceschi D, Sedler MJ, Gatley SJ, Hitzemann R, Ding YS, Logan J, Wong C, Miller EN. Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. *Am J Psychiatry*. 2001; 158:377–82. [PubMed: 11229977]
2. Li SX, Yan SY, Bao YP, Lian Z, Qu Z, Wu YP, Liu ZM. Depression and alterations in hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid axis function in male abstinent methamphetamine abusers. *Hum Psychopharmacol*. 2013; 28:477–83. [PubMed: 23913817]
3. Nakama H, Chang L, Cloak C, Jiang C, Alicata D, Haning W. Association between psychiatric symptoms and craving in methamphetamine users. *Am J Addict*. 2008; 17:441–6. [PubMed: 18770088]
4. Doremus-Fitzwater TL, Varlinskaya EI, Spear LP. Motivational systems in adolescence: possible implications for age differences in substance abuse and other risk-taking behaviors. *Brain Cogn*. 2010; 72:114–23. [PubMed: 19762139]
5. Spear LP, Varlinskaya EI. Sensitivity to ethanol and other hedonic stimuli in an animal model of adolescence: implications for prevention science? *Dev Psychobiol*. 2010; 52:236–43. [PubMed: 20222058]
6. Rawson R, Gonzales R, McCann M, Ling W. Use of methamphetamine by young people: is there reason for concern? *Addiction*. 2007; 102:1021–2. [PubMed: 17567381]
7. Spear LP. The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev*. 2000; 24:417–63. [PubMed: 10817843]
8. King G, Alicata D, Cloak C, Chang L. Psychiatric symptoms and HPA axis function in adolescent methamphetamine users. *J Neuroimmune Pharmacol*. 2010; 5:582–91. [PubMed: 20358305]
9. King G, Alicata D, Cloak C, Chang L. Neuropsychological deficits in adolescent methamphetamine abusers. *Psychopharmacology (Berl)*. 2010; 212:243–9. [PubMed: 20640405]
10. Rawson RA, Gonzales R, Obert JL, McCann MJ, Brethen P. Methamphetamine use among treatment-seeking adolescents in Southern California: participant characteristics and treatment response. *J Subst Abuse Treat*. 2005; 29:67–74. [PubMed: 16135335]

11. Anker JJ, Baron TR, Zlebnik NE, Carroll ME. Escalation of methamphetamine self-administration in adolescent and adult rats. *Drug Alcohol Depend.* 2012
12. Zakharova E, Leoni G, Kichko I, Izenwasser S. Differential effects of methamphetamine and cocaine on conditioned place preference and locomotor activity in adult and adolescent male rats. *Behav Brain Res.* 2009; 198:45–50. [PubMed: 18996417]
13. North A, Swant J, Salvatore MF, Gamble-George J, Prins P, Butler B, Mittal MK, Heltsley R, Clark JT, Khoshbouei H. Chronic methamphetamine exposure produces a delayed, long-lasting memory deficit. *Synapse.* 2013; 67:245–57. [PubMed: 23280858]
14. Vorhees CV, Reed TM, Morford LL, Fukumura M, Wood SL, Brown CA, Skelton MR, McCrea AE, Rock SL, Williams MT. Periadolescent rats (P41–50) exhibit increased susceptibility to D-methamphetamine-induced long-term spatial and sequential learning deficits compared to juvenile (P21–30 or P31–40) or adult rats (P51–60). *Neurotoxicol Teratol.* 2005; 27:117–34. [PubMed: 15681126]
15. Zombeck JA, Lewicki AD, Patel K, Gupta T, Rhodes JS. Patterns of neural activity associated with differential acute locomotor stimulation to cocaine and methamphetamine in adolescent versus adult male C57BL/6J mice. *Neuroscience.* 2010; 165:1087–99. [PubMed: 19932887]
16. Kokoshka JM, Fleckenstein AE, Wilkins DG, Hanson GR. Age-dependent differential responses of monoaminergic systems to high doses of methamphetamine. *J Neurochem.* 2000; 75:2095–102. [PubMed: 11032899]
17. Williams MT, Inman-Wood SL, Morford LL, McCrea AE, Ruttle AM, Moran MS, Rock SL, Vorhees CV. Prewaning treatment with methamphetamine induces increases in both corticosterone and ACTH in rats. *Neurotoxicol Teratol.* 2000; 22:751–9. [PubMed: 11106868]
18. Grace CE, Schaefer TL, Herring NR, Graham DL, Skelton MR, Gudelsky GA, Williams MT, Vorhees CV. Effect of a neurotoxic dose regimen of (+)-methamphetamine on behavior, plasma corticosterone, and brain monoamines in adult C57BL/6 mice. *Neurotoxicol Teratol.* 2010; 32:346–55. [PubMed: 20096350]
19. Aisa B, Tordera R, Lasheras B, Del Rio J, Ramirez MJ. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology.* 2007; 32:256–66. [PubMed: 17307298]
20. Piwowarska J, Chimiak A, Matsumoto H, Dziklinska A, Radziwon-Zaleska M, Szelenberger W, Pachecka J. Serum cortisol concentration in patients with major depression after treatment with fluoxetine. *Psychiatry Res.* 2012; 198:407–11. [PubMed: 22429479]
21. Lok A, Mocking RJ, Ruhe HG, Visser I, Koeter MW, Assies J, Bockting CL, Olf M, Schene AH. Longitudinal hypothalamic-pituitary-adrenal axis trait and state effects in recurrent depression. *Psychoneuroendocrinology.* 2012; 37:892–902. [PubMed: 22094110]
22. Acevedo SF, Pfankuch T, van Meer P, Raber J. Role of histamine in short- and long-term effects of methamphetamine on the developing mouse brain. *J Neurochem.* 2008; 107:976–86. [PubMed: 18786166]
23. Grace CE, Schaefer TL, Herring NR, Skelton MR, McCrea AE, Vorhees CV, Williams MT. (+)-Methamphetamine increases corticosterone in plasma and BDNF in brain more than forced swim or isolation in neonatal rats. *Synapse.* 2008; 62:110–21. [PubMed: 17992688]
24. Schaefer TL, Skelton MR, Herring NR, Gudelsky GA, Vorhees CV, Williams MT. Short- and long-term effects of (+)-methamphetamine and (+/-)-3,4-methylenedioxymethamphetamine on monoamine and corticosterone levels in the neonatal rat following multiple days of treatment. *J Neurochem.* 2008; 104:1674–85. [PubMed: 17996031]
25. Williams MT, Blankenmeyer TL, Schaefer TL, Brown CA, Gudelsky GA, Vorhees CV. Long-term effects of neonatal methamphetamine exposure in rats on spatial learning in the Barnes maze and on cliff avoidance, corticosterone release, and neurotoxicity in adulthood. *Brain Res Dev Brain Res.* 2003; 147:163–75. [PubMed: 14741761]
26. Desbonnet L, Garrett L, Daly E, McDermott KW, Dinan TG. Sexually dimorphic effects of maternal separation stress on corticotrophin-releasing factor and vasopressin systems in the adult rat brain. *Int J Dev Neurosci.* 2008; 26:259–68. [PubMed: 18367364]

27. Zuloaga DG, Siegel JA, Acevedo SF, Agam M, Raber J. Developmental methamphetamine exposure results in short- and long-term alterations in hypothalamic-pituitary-adrenal-axis-associated proteins. *Dev Neurosci.* 2013; 35:338–46. [PubMed: 23860125]
28. Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci.* 1997; 20:78–84. [PubMed: 9023876]
29. Acevedo SF, de Esch IJ, Raber J. Sex- and histamine-dependent long-term cognitive effects of methamphetamine exposure. *Neuropsychopharmacology.* 2007; 32:665–72. [PubMed: 16710318]
30. Siegel JA, Craytor MJ, Raber J. Long-term effects of methamphetamine exposure on cognitive function and muscarinic acetylcholine receptor levels in mice. *Behav Pharmacol.* 2010; 21:602–14. [PubMed: 20729719]
31. Siegel JA, Park BS, Raber J. Long-term effects of neonatal methamphetamine exposure on cognitive function in adolescent mice. *Behav Brain Res.* 2011; 219:159–64. [PubMed: 21238498]
32. Vorhees CV, Inman-Wood SL, Morford LL, Broening HW, Fukumura M, Moran MS. Adult learning deficits after neonatal exposure to D-methamphetamine: selective effects on spatial navigation and memory. *J Neurosci.* 2000; 20:4732–9. [PubMed: 10844042]
33. Vorhees CV, Skelton MR, Williams MT. Age-dependent effects of neonatal methamphetamine exposure on spatial learning. *Behav Pharmacol.* 2007; 18:549–62. [PubMed: 17762523]
34. Williams MT, Morford LL, Wood SL, Wallace TL, Fukumura M, Broening HW, Vorhees CV. Developmental D-methamphetamine treatment selectively induces spatial navigation impairments in reference memory in the Morris water maze while sparing working memory. *Synapse.* 2003; 48:138–48. [PubMed: 12645039]
35. Slamberova R, Mikulecka A, Pometlova M, Schutova B, Hrubá L, Deykun K. The effect of methamphetamine on social interaction of adult male rats. *Behav Brain Res.* 2010; 214:423–7. [PubMed: 20600345]
36. Good RL, Radcliffe RA. Methamphetamine-induced locomotor changes are dependent on age, dose and genotype. *Pharmacol Biochem Behav.* 2011; 98:101–11. [PubMed: 21163294]
37. Good RL, Liang LP, Patel M, Radcliffe RA. Mouse strain- and age-dependent effects of binge methamphetamine on dopaminergic signaling. *Neurotoxicology.* 2011; 32:751–9. [PubMed: 21798282]
38. Clement Y, Calatayud F, Belzung C. Genetic basis of anxiety-like behaviour: a critical review. *Brain Res Bull.* 2002; 57:57–71. [PubMed: 11827738]
39. File SE, Seth P. A review of 25 years of the social interaction test. *Eur J Pharmacol.* 2003; 463:35–53. [PubMed: 12600701]
40. Matsuo N, Takao K, Nakanishi K, Yamasaki N, Tanda K, Miyakawa T. Behavioral profiles of three C57BL/6 substrains. *Front Behav Neurosci.* 2010; 4:29. [PubMed: 20676234]
41. Franklin, K., Paxinos, G. The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 1997.
42. Cruickshank CC, Dyer KR. A review of the clinical pharmacology of methamphetamine. *Addiction.* 2009; 104:1085–99. [PubMed: 19426289]
43. Dunlop BW, Nemeroff CB. The role of dopamine in the pathophysiology of depression. *Arch Gen Psychiatry.* 2007; 64:327–37. [PubMed: 17339521]
44. Owens MJ, Nemeroff CB. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin Chem.* 1994; 40:288–95. [PubMed: 7508830]
45. Volz TJ, Farnsworth SJ, Rowley SD, Hanson GR, Fleckenstein AE. Age-dependent differences in dopamine transporter and vesicular monoamine transporter-2 function and their implications for methamphetamine neurotoxicity. *Synapse.* 2009; 63:147–51. [PubMed: 19021208]
46. Siegel JA, Park BS, Raber J. Methamphetamine exposure during brain development alters the brain acetylcholine system in adolescent mice. *J Neurochem.* 2011; 119:89–99. [PubMed: 21824143]
47. Zuloaga DG, Johnson LA, Agam M, Raber J. Sex Differences in Activation of the Hypothalamic-Pituitary-Adrenal Axis by Methamphetamine. *J Neurochem.* 2014
48. Rood BD, Stott RT, You S, Smith CJ, Woodbury ME, De Vries GJ. Site of origin of and sex differences in the vasopressin innervation of the mouse (*Mus musculus*) brain. *J Comp Neurol.* 2013; 521:2321–58. [PubMed: 23239101]

49. Forbes-Lorman RM, Rautio JJ, Kurian JR, Auger AP, Auger CJ. Neonatal MeCP2 is important for the organization of sex differences in vasopressin expression. *Epigenetics*. 2012; 7:230–8. [PubMed: 22430799]
50. Marshall JF, Belcher AM, Feinstein EM, O'Dell SJ. Methamphetamine-induced neural and cognitive changes in rodents. *Addiction*. 2007; 102(Suppl 1):61–9. [PubMed: 17493054]
51. Yu J, Cadet JL, Angulo JA. Neurokinin-1 (NK-1) receptor antagonists abrogate methamphetamine-induced striatal dopaminergic neurotoxicity in the murine brain. *J Neurochem*. 2002; 83:613–22. [PubMed: 12390523]

Figure 1.

Timeline of injections and behavioral testing. Mice arrived in the colony on postnatal day (PND) 27 and injections occurred during early adolescence from PND 30–31. The late adolescent group went through the behavioral testing battery from PND 41–52, and the adult group went through the behavioral testing battery from PND 62–73.

Figure 2.

Late adolescent male mice exposed to methamphetamine showed increased depression-like behavior in the Porsolt forced swim test compared to age and sex-matched controls. Mice exposed to methamphetamine during early adolescence showed more immobility (greater depression-like behavior) in the forced swim test compared to mice exposed to saline, regardless of sex and age (main effect of treatment; $p < 0.05$). $N = 9-10$ mice per treatment, sex, and age group.

Figure 3.

Late adolescent male mice exposed to methamphetamine showed decreased vasopressin-immunoreactive cells in the paraventricular nucleus compared to age and sex-matched controls. The figure shows representative images of vasopressin-immunoreactive cells in the paraventricular nucleus of the hypothalamus in male mice. Among the male mice, there was a significant interaction between treatment and age group ($p < 0.05$). Late adolescent saline-exposed male mice (A) had significantly more vasopressin-immunoreactive cells than late adolescent methamphetamine-exposed male mice (B). There were no significant differences in the number of vasopressin-immunoreactive cells between adult saline-exposed male mice (C) and adult methamphetamine-exposed male mice (D).

Behavioral and cognitive performance of mice treated with methamphetamine or saline

Table 1

Behavioral Test	Measure	Late Adolescents				Adults			
		Saline Males	MA Males	Saline Females	MA Females	Saline Males	MA Males	Saline Females	MA Females
Open Field	Total Distance Moved (cm)	2717.5 ± 216.9	2955.2 ± 168.9	3104.6 ± 203.5 *	3247.7 ± 153.4 *	2910.1 ± 171.7	2941.7 ± 132.8	3643.9 ± 224.9 *	3588.6 ± 270.8 *
Open Field	% Distance Moved in Center	12.1 ± 1.3	10.60 ± 1.0	14.5 ± 1.1 *	13.5 ± 0.8 *	12.5 ± 1.4	13.5 ± 0.8 **	12.3 ± 1.4	15.2 ± 1.1 #*
Novel Object Recognition	Delta Trial 2 (sec) ^a	7.9 ± 5.4	-1.9 ± 7.9	15.5 ± 3.5	16.21 ± 17.0	9.19 ± 6.5	14.3 ± 6.1	6.8 ± 3.7	15.8 ± 7.1
Novel Object Recognition	Delta Trial 3 (sec) ^a	38.7 ± 17.9 **	36.8 ± 8.7 **	44.4 ± 7.5 **	43.1 ± 9.1 **	26.8 ± 12.0	33.4 ± 9.1	9.4 ± 4.3	30.3 ± 11.1
Social Interaction	Duration of Social Interaction (sec)	36.7 ± 4.7	28.7 ± 4.7	33.2 ± 4.6	27.4 ± 2.2	44.2 ± 7.9	41.4 ± 5.5	32.9 ± 5.7	32.9 ± 4.2
Morris Water Maze	Average Latency to Escape Hidden Sessions (sec)	29.5 ± 3.3	32.3 ± 2.8	34.0 ± 4.8	36.2 ± 3.6	33.4 ± 3.1	30.2 ± 2.6	30.9 ± 2.4	37.2 ± 3.3
Morris Water Maze	Average Cum. Distance Probe Trials (cm)	2688.0 ± 135.4	2754.1 ± 12.9	2682.2 ± 176.3	2782.8 ± 199.5	2873.3 ± 171.5	2713.0 ± 102.8	2610.2 ± 107.4	2742.5 ± 174.8
Morris Water Maze	Average % Time Target Quadrant Probe Trials	25.9 ± 3.0	26.4 ± 2.1	29.0 ± 3.6	28.1 ± 3.4	26.1 ± 3.5	28.7 ± 2.4	29.1 ± 2.0	28.6 ± 3.3

Note: Cum. = cumulative; MA = methamphetamine.

All Measures shown as group means ± SEM.

^aDelta preference score calculated as time exploring the novel object - time exploring the old object.

Adult MA mice **higher** than adult saline mice, $p < 0.10$.

* Females higher than males, $p < 0.05$.

** Late adolescent mice higher than adult mice, $p < 0.05$.

Table 2

Weights of mice treated with methamphetamine or saline

	Saline	Methamphetamine
Postnatal day 27	12.92 ± 0.17	12.95 ± 0.18
Postnatal day 28	13.56 ± 0.20	13.61 ± 0.20
Postnatal day 29	14.25 ± 0.19	14.34 ± 0.20
Postnatal day 30	15.14 ± 0.24	15.34 ± 0.23
Postnatal day 31	15.81 ± 0.22 *	14.53 ± 0.21
Postnatal day 32	16.61 ± 0.27	16.79 ± 0.30
Postnatal day 33	17.39 ± 0.27	17.84 ± 0.27
Postnatal day 34	17.58 ± 0.29	18.50 ± 0.25 *
Postnatal day 35	18.17 ± 0.28	18.82 ± 0.26
Postnatal day 36	18.42 ± 0.29	18.82 ± 0.24
Postnatal day 37	18.72 ± 0.32	19.24 ± 0.28
Postnatal day 38	19.11 ± 0.34	19.68 ± 0.31
Postnatal day 39	19.50 ± 0.38	20.03 ± 0.34
Postnatal day 40	19.58 ± 0.37	20.08 ± 0.33
Postnatal day 41	19.78 ± 0.39	20.24 ± 0.36

All Measures shown grams for group means ± SEM. Injections occurred on postnatal day 30 and 31.

* Higher than comparison group, $p < 0.05$.