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Neonatal (+)-methamphetamine increases brain derived neurotrophic factor, but not nerve growth factor, during treatment and results in long-term spatial learning deficits

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Summary

In this study, brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were examined at five time points [postnatal day (P)11, 15, 20, 21, and 68 (the latter with or without behavioral testing)] during and after P11–20 (+)-methamphetamine (MA) (10 mg/kg 4 × day) treatment. BDNF in MA-treated animals was elevated on P15 and P20 in the hippocampus but not in the hypothalamus and was unchanged on P11 and P21. On P68 (1 h after Morris water maze testing) MA-treated offspring showed a trend toward higher levels of BDNF in the hippocampus than saline-treated animals. MA treatment increased NGF levels in the hippocampus but only on P20. No effect of MA treatment was observed in the elevated zero maze. MA-treated offspring had increased latencies, cumulative distances, path lengths, and first bearings in the Morris water maze. The findings indicate that early MA exposure induces hippocampal BDNF increases that precede the later emergence of spatial learning deficits.

Keywords

BDNF; NGF; Spatial learning; Morris water maze; Methamphetamine; Corticosterone; Development; Rat

1. Introduction

With the recent increase in use of methamphetamine (MA) by young adults (Johnston et al., 2005a, b) there is concern regarding fetal exposure to the drug since female users may become pregnant. MA is a powerful psychostimulant that upon administration blocks monoamine transporters for dopamine, serotonin, and norepinephrine and causes release of these neurotransmitters into the synaptic cleft, therefore resulting in a sustained presence of these neurotransmitters in the cleft (Kucaenski and Segal, 1994). While the literature on prenatal MA exposure is limited, there is an association between MA and low birth weight and length, decreased head circumference, and increased fetal distress (Chomchai et al., 2004; Little et al., 1988; Smith et al., 2006). Reductions in the volume of the caudate, putamen, globus pallidus, and hippocampus were observed in children who were prenatally exposed to MA (Chang et al., 2004). Prenatal MA exposure has also been suggested to alter brain ultrasonography (Dixon

and Bejar, 1989) and increase the concentration of glutamine/glutamate and creatine (Smith et al., 2001). Children exposed to MA *in utero* have lower scores on visual motor tests as well as delayed verbal development and deficits in long-term spatial memory compared to age matched controls (Chang et al., 2004).

In rats, exposure to MA from postnatal days (P)11–20 causes long-term spatial learning and memory deficits (Vorhees et al., 1994a, b, 2000; Williams et al., 2002, 2003a, b). This period is approximately analogous to mid-second through third trimester human hippocampal development (Bayer et al., 1993). According to data from Clancy et al. (2007), P11 in a developing rat is equivalent to 182 d of human gestation in cortical development and 134 d in limbic development. Our model may represent a sensitive period to exogenous influences, such as drug exposure, that interfere with neurogenesis and/or synaptogenesis.

In addition, the beginning of the above exposure period is near the end of the stress hyporesponsive period (SHRP), a time when stress-induced increases in corticosterone (CORT) release are attenuated (Sapolsky and Meaney, 1986). The SHRP is presumed to protect the brain from possible neurotoxic effects of high glucocorticoid exposure when neurons that express glucocorticoid receptors are developing, such as in the hippocampus. Increased CORT levels were observed following MA exposure in neonatal rats on P11 or on P15 and P20 following daily dosing beginning on P11 (Williams et al., 2000). In addition, CORT levels were shown to be increased following a single MA dose to separate groups dosed on one of the following days: P1, 3, 5, 7, 9, 11, 13, 15, 17 or 19, or following five consecutive days of MA dosing on P1–5, 3–7, 5–9, 7–11, 9–13, 11–15, 13–17, or 15–19 (Williams et al., 2006). We have also shown that MA administration from P11–15, but not P16–20, causes spatial learning and memory deficits (Williams et al., 2003a). These effects are not limited to MA. For example, MDMA also induces increases in CORT on P11 (Williams et al., 2005) and spatial learning deficits when administered from P11–20 (Broening et al., 2001).

Neurotrophic factors are important for neuronal proliferation, survival, and differentiation during development, and remain expressed during adulthood where they regulate neuronal survival and maintenance. Two neurotrophic factors are brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). BDNF and NGF expression are highest during the perinatal period, with BDNF protein levels reaching peak concentrations in the hippocampus by P14, while NGF levels peak at P7 (Das et al., 2001). In addition to their neurotrophic properties, BDNF and NGF have been implicated in learning and memory. Infusion of NGF for four weeks partially restores age-induced cholinergic atrophy in the nucleus basalis magnocellularis and improves Morris water maze (MWM) performance (Fischer et al., 1987). Mice that carry a heterozygous disruption of NGF have deficits in MWM performance that are alleviated when NGF is infused (Chen et al., 1997). BDNF mRNA has been shown to be increased following three or six days of spatial (Kesslak et al., 1998) or inhibitory avoidance learning (Bekinschtein et al., 2007), as is *trkB* mRNA (Gomez-Pinilla et al., 2001), which is the primary receptor for BDNF. Blocking BDNF using neutralizing antibodies or antisense nucleotides prior to or following inhibitory avoidance learning eliminates memory retention in rats (Bekinschtein et al., 2007; Mu et al., 1999). Furthermore, heterozygous BDNF knockout mice show learning deficits in the MWM (Linnarsson et al., 1997). In adult animals increased BDNF levels in the hippocampus enhance long-term potentiation and facilitate the phosphorylation of the NMDA-NR1 (Suen et al., 1997) and NMDA-NR2 receptor subunits (Lin et al., 1998). While these data come from experiments on BDNF effects in adult brain, they demonstrate its involvement in learning and memory.

There are also interactions between neurotrophins and CORT. BDNF and NGF are altered by increases in CORT. High levels of CORT from P1–7 increase mRNA for NGF in the hippocampus (Roskoden et al., 2004). Adult adrenalectomized (ADX) rats have higher levels

of BDNF mRNA in the dentate gyrus than ADX animals that received CORT replacement (Chao and McEwen, 1994; Schaaf et al., 1997, 1998). Immobilization stress also decreases BDNF mRNA in the dentate in adult animals (Smith et al., 1995). Application of CORT to cultured neurons increases NGF mRNA which is blocked using a glucocorticoid antagonist (Scully and Otten, 1995), while dexamethasone (a synthetic glucocorticoid) treatment increases NGF mRNA in the cerebral cortex and hippocampus (Mocchetti et al., 1996).

The present experiments were designed to explore the effects of neonatal MA treatment on BDNF and NGF concentrations at five different developmental ages: (1) following five days of MA treatment (P11–15; P15 being the age with the highest levels of BDNF expression as demonstrated by Das et al., 2001); (2) after an acute treatment of MA on P11 (the first day of the sensitive period for MA-induced spatial learning deficits); (3) after P11–20 treatment of MA and examined after the last dose on P20; (4) on P21, 24 h after the end of MA treatment; and (5) on P68 either following MWM testing or on P68 with no testing, after P11–20 MA administration. While it is known that adult animals have a sexually dimorphic response to stressors (Beiko et al., 2004), it is not known if sexually dimorphic differences occur throughout development in neurotrophin levels, especially after MA. Therefore, in this study we examined males and females at all ages to be tested.

2. Materials and methods

2.1. Subjects and conditions

Male and nulliparous female Sprague-Dawley CD (IGS) rats were obtained from Charles Rivers Laboratories (Raleigh, NC). Animals were allowed to acclimate to the lighting (14/10 h light/dark cycle; lights on at 0600 h) and ambient temperature (20 ± 1 °C) of the vivarium ($50 \pm 10\%$ humidity) for a minimum of 2 weeks prior to breeding. One male and one female were placed together in hanging wire cages for two weeks, after which females were moved to polycarbonate cages and left undisturbed until parturition. Date of birth was considered postnatal day (P)0. On P1, litters were culled to 8 (Experiments 1, 2 and 3) or 10 (Experiment 4) pups divided equally by sex, with up to two pups fostered from another litter born on the same day. The vivarium is accredited by the Association for the Assessment and Accreditation of Laboratory and Animal Care (AAALAC). All protocols were approved by the Cincinnati Children's Research Foundation's Institutional Animal Care and Use Committee.

2.2. General methods

(+)-Methamphetamine HCl (National Institute on Drug Abuse, Bethesda, MD, expressed as the freebase and greater than 95% pure) was administered subcutaneously at room temperature by injection at a dose of 10 mg/kg in a volume of 3 ml/kg. All injections were given at 2 h intervals and injection sites were varied. At each injection time, animals were briefly removed from the home cage, weighed, dosed, and returned to the litter.

On the day of sacrifice, unanesthetized animals were removed from the home cage, brought to an adjacent room, and rapidly decapitated. Trunk blood was collected in polyethylene tubes containing 0.05 mL of 2% EDTA. Brains were rapidly removed from the skull, the hippocampus, hypothalamus, and striatum (Exp 3) dissected on ice, and frozen on dry ice as detailed previously (Williams et al., 2007). All samples were collected between 1100 and 1300 h in order to control for circadian and ultradian rhythms. Plasma was separated from whole blood by centrifuging the samples at $1300 \times g$ for 25 min followed by collection of the supernatant. Samples were stored at -80 °C until assayed for CORT (plasma), or NGF and BDNF (tissue).

2.2.1. Experiment 1—Within a litter ($N = 10$ litters) equal numbers of animals were dosed with MA or SAL, divided equally by sex, 4 times daily from P11–14 and twice on P15. One h after the final injection, 2 male and female treatment pairs were sacrificed with tissue and plasma collected as described above. Two samples per treatment condition and sex were collected per litter and the mean was used in the analysis. The time point for tissue collection was based on previous reports that neurotrophin changes could be seen as early as 1 h (Smith et al., 1995) with a maximum effect 3–6 h following a stressor (Schaaf et al., 1998). Therefore, tissue collection began 3 h after the initial MA exposure on P15.

2.2.2. Experiment 2—Within a litter ($N = 10$ litters) equal numbers of animals were dosed with MA or SAL, divided equally by sex, 3 times on P11. One h after the final injection, 2 male and female treatment pairs were sacrificed with tissue and plasma collected as described above. Two samples per treatment condition and sex were collected per litter and the mean was used in the analysis. The time point selected for this experiment was based on the results from Experiment 1. Treatment and sex were within litter factors.

2.2.3. Experiment 3—Within a litter ($N = 10$ litters) equal numbers of animals were dosed with MA or SAL, divided equally by sex, 4 times daily from P11–20. One or 24 h after the final injection, a male and female treatment pair was sacrificed with tissue and plasma collected as described above.

2.2.4. Experiment 4—Equal numbers of litters ($N = 24$) were treated with MA or SAL from P11–20. Behavioral testing in the elevated zero-maze began on P60, followed by straight channel swimming on P61, and MWM testing on P64. On P68, following the fifth day of testing in the MWM, animals were either sacrificed immediately (i.e., 0 min) or returned to the home cage and sacrificed at 30, 60, or 120 min following the completion of the last trial of the day. One animal of each sex remained in the home cage during MWM testing and served as a non-tested control, although it should be noted that these animals were transferred each day to the MWM testing suite at the same time as the other animals.

2.3. Corticosterone and neurotrophin assessment

CORT concentrations in plasma were analyzed in duplicate using EIA kits according to the manufacturer's protocol (IDS, Fountain Hills, AZ). BDNF and NGF protein levels were assayed in duplicate via EIA (Promega Corp, Madison WI) according to manufacturer's instructions and compared to total protein levels which were assayed using the Bradford method (Pierce Biotechnology, Rockford, IL). Optical densities for all EIAs were measured on a SpectraMax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA). Results for BDNF and NGF were expressed as ng of neurotrophin per mg of protein in the measured region.

2.4. Behavioral measures

2.4.1. Elevated zero maze—Beginning on the Thursday nearest P60 each animal was administered one 5-min trial in the elevated zero maze apparatus (Shepherd et al., 1994). The maze consisted of a black acrylic circular track 10 cm wide and 105 cm in diameter and 72 cm from the floor (San Diego Instruments). The maze is divided into four quadrants with two quadrants opposite each other having 1 cm clear acrylic curbs on either side to prevent the animal from falling but otherwise open. The other two opposing quadrants are closed by 28 cm tall black acrylic walls on both sides. Overhead fluorescent lights were on during testing. A videocassette recorder attached to an overhead camera recorded each session for later analysis. At the beginning of each trial the subject was placed in a closed quadrant to start the 5-min trial. For each animal, the number of stretch-attends (defined as an event when the animal had its head and shoulders extended into an open quadrant with the remainder of the body in a closed quadrant), head dips, and open entries as well as the total amount of time spent in the

open quadrants of the maze were assessed. The maze was wiped clean of urine and feces with 70% alcohol between animals.

2.4.2. Straight channel—On the day following elevated zero maze testing, each animal was administered four timed trials in a straight channel apparatus to test for swimming ability. The straight channel is 15 cm wide $\times$ 244 cm long $\times$ 50 cm in height and composed of grey PVC plastic. The apparatus was filled with water (22 ± 1 °C) to a depth of 35 cm. A wire escape ladder was placed at one end. At the beginning of each trial the animal was placed facing the back wall of the channel at the opposite end from the ladder. A trial began when the animal was released and ended when the escape ladder was grasped.

2.4.3. Morris water maze—Testing in the MWM began three days following straight channel trials. The MWM is used to test the spatial learning and memory ability of rodents (D'Hooge and De Deyn, 2001; McNamara and Skelton, 1993; Morris, 1981). The maze used herein consisted of a tank 210 cm in diameter and painted flat black with 50 cm high sides and filled to a height of 32 cm as described previously (Vorhees and Williams, 2006). The escape platform (10 cm $\times$ 10 cm) was made from clear acrylic with wire mesh attached to the top for traction and was submerged 2 cm below the water surface; water temperature was 22 ± 1 °C. Animals were administered four trials per day for five days. The tank was divided into four equal quadrants, with cardinal points designated with compass headings and north arbitrarily the point furthest from the experimenter. The escape platform was placed in the center of the SW quadrant and animals were started from one of four start positions (NW, N, E, and SE) in pseudo-random order with the restriction that one trial began from each start position per day. Each trial had a time limit of 120 s with a 15 s inter-trial interval (ITI). If the animal did not find the platform in the allotted time it was manually placed on the platform for the ITI. Swim paths and latencies were recorded by an overhead camera and video-tracking system (Polytrack System, San Diego Instruments, San Diego, CA).

Principal measures recorded were latency, path length, cumulative distance from the platform (recorded every 55 ms) and angle of deviation (first bearing) from a direct line to the platform (measured during the first 13 cm of recorded path at the beginning of each trial). Supplemental analyses of time spent in specific annuli and number of direct swim events to the target were also analyzed. For annuli determination, a 20 cm wide virtual ring was drawn inside the maze, using Polytrack software, that spanned the inside and outside corners of the platform; this area hereafter is referred to as the target annulus. The ring between the outer wall of the maze and the target annulus was designated the outer annulus and the remainder, the inner annulus. For direct swims (Saucier et al., 1996) a 36 cm corridor was demarcated using Polytrack software from the start position directly to the target with the start position and target being in the center of the corridor (see Fig. 1).

Probe trials were not performed because the goal of this study was to assess BDNF during the learning phase of the MWM. Previous studies have shown that MA causes deficits in reference memory on probe trials in the MWM (Vorhees et al., 2000; Williams et al., 2002, 2003b), and that MA does not induce sensorimotor deficits in animals exposed neonatally (Vorhees et al., 2000; Williams et al., 2002, 2003b).

2.5. Statistics

Analysis of variance (ANOVA) was used to determine treatment effects. Neurotrophin levels in the hippocampus, striatum, and hypothalamus were expressed as a percent of total protein in these regions. Treatment was a within litter factor for Experiments 1, 2, and 3 and a between litter factor for Experiment 4. All analyses included sex as a within factor and the MWM had additional within factors of day and trial. Direct swim events were calculated as proportions

expressed graphically as a percent of total swim events and significance was determined using Fisher's test for uncorrelated proportions (Walpole and Meyers, 1985). A $p \leq 0.05$ level of confidence was used to determine significance and trends were noted at $p < 0.10$.

3. Results

3.1. Corticosterone

Experiment 1—Rats were treated four times/day with MA or SAL from P11–14, treated twice on P15 and examined 1 h after the second dose. MA-treated animals showed a trend toward decreased CORT in plasma 1 h following the last injection of MA compared to SAL-treated animals ($F(1,9) = 4.13, p < 0.07$; Fig. 2A).

Experiment 2—Rats were administered MA three times on P11 and sacrificed 1 h after the last dose. There was a main effect of treatment ($F(1,8) = 48.74, p < 0.0001$; Fig. 2B) showing significantly higher levels of CORT in MA-treated animals on P11 relative to SAL-treated animals.

Experiment 3—No main effect of MA treatment on CORT was observed on P20 or on P21. A treatment $\times$ sex interaction ($F(1,37) = 6.71, p < 0.02$) was observed on P20 with males treated with MA having higher levels of CORT than SAL-treated males (Fig. 2C).

Experiment 4—Rats were treated with MA or SAL from P11–20 and sacrificed on P68 at various times either after five days of MWM training or no testing. For the animals that were untested, CORT values were not normally distributed and therefore were log transformed prior to ANOVA. There was a significant neonatal treatment effect for CORT in untested animals ($F(1,22) = 7.41, p < 0.02$; Fig. 3A) with MA-treated animals having lower CORT levels than SAL-treated animals. Females, regardless of neonatal treatment, had higher resting CORT levels than males ($F(1,22) = 11.65, p < 0.003$). For tested animals, there was no significant effect of treatment or the time $\times$ treatment interaction. There was a main effect of time ($F(3,66) = 32.81, p < 0.0001$; Fig. 3C) such that animals had higher CORT levels 0 and 30 min after removal from the MWM compared to 60 and 120 min after testing; the latter levels were comparable to those of untested animals. There was also a main effect of sex ($F(1,22) = 5.57, p < 0.03$; Fig. 3B) with females again having higher CORT levels than males and a sex $\times$ time interaction ($F(3,66) = 4.15, p < 0.01$; Fig. 3D) with female CORT levels higher than males at 0, 60 and 120 min after MWM testing.

3.2. Brain derived neurotrophic factor

Experiment 1—For the hippocampus, a main effect of treatment ($F(1,9) = 13.44, p < 0.005$; Fig. 4) was observed on P15 after P11–15 MA dosing. MA-treated animals had higher levels of BDNF in the hippocampus compared to SAL-treated animals. No significant effect of MA treatment was observed in the hypothalamus.

Experiment 2—In rats treated on P11, no main effect of treatment was observed for BDNF in the hippocampus or hypothalamus. There was a main effect of sex with males having higher levels of BDNF in the hippocampus ($F(1,9) = 169.18, p < 0.0001$; Fig. 5A) and hypothalamus ($F(1,9) = 7.91, p < 0.02$; Fig. 5B) than females.

Experiment 3—In the hippocampus on P20, MA treatment increased the level of BDNF ($F(1,38) = 7.04, p < 0.02$, Fig. 5C) compared to controls. No effects were seen in the hypothalamus or striatum. No effects of MA treatment were seen on P21.

Experiment 4—Among P68 tested animals, there was a treatment $\times$ time interaction observed ($F(3,51) = 3.48, p < 0.02$; Fig. 6). Animals treated with MA showed a trend ($p < 0.10$) towards higher BDNF levels in the hippocampus 60 min following MWM training compared to SAL-treated animals. There was no difference in BDNF levels between animals tested in the MWM and untested animals. There was no MA effect among P68 untested animals.

Correlations for CORT and BDNF were examined for each experiment and on P11 (Experiment 2), there was a significant correlation ($r = 0.53, p < 0.001$), however no other significant correlations were observed for CORT and BDNF.

3.3. Nerve growth factor

Experiment 1—After P11–15 exposure, no effect of treatment or treatment $\times$ sex interaction was seen for NGF. P15 animals showed a sex difference in the hippocampus with higher NGF levels in females compared to males ($F(1,9) = 18.39, p < 0.002$; Fig. 7A).

Experiment 2—On P11, no significant NGF differences were noted.

Experiment 3—MA treatment increased the level of NGF in the hippocampus on P20 ($F(1,38) = 7.06, p < 0.02$; Fig. 7B). No effects were seen in the hypothalamus or striatum. No effects of MA treatment were seen on P21.

Experiment 4—On P68, males that were tested in the MWM had lower levels of NGF in the hypothalamus compared to females tested in the MWM ($F(1,18) = 11.11, p < 0.004$). A sex $\times$ treatment interaction was also observed for the tested animals ($F(1,18) = 5.18, p < 0.04$; Fig. 7C) with MA-treated females having lower levels of NGF than SAL-treated females. No main effect of time was observed following testing for NGF. In animals not tested in the MWM, a trend towards lower levels of NGF ($F(1,18) = 3.04, p < 0.01$) was seen in males in the hypothalamus, and there was a sex $\times$ treatment interaction for this region ($F(1,18) = 6.09, p < 0.02$; Fig. 7D). MA-treated females had lower levels of NGF in the hypothalamus compared to SAL-treated females.

Correlations for CORT and NGF were examined for each experiment and on P11 (Experiment 2), there was a significant correlation ($r = 0.36, p < 0.05$), however no other significant correlations were observed for CORT and NGF.

3.4. Behavior

3.4.1. Elevated zero maze—Beginning on P60, animals were tested in the elevated zero maze. The main effect of treatment and the treatment $\times$ sex interaction were not significant for any of the measures. There was a sex effect observed with females spending significantly more time in the open ($F(1,20) = 12.68, p < 0.002$), exhibiting more stretch attends ($F(1,20) = 15.72, p < 0.001$), and showing a trend toward more open arm entries ($F(1,20) = 3.83, p < 0.06$) compared to males (not shown). No sex difference was seen for the number of head dips.

3.4.2. Straight channel—A day following the elevated zero maze, animals were tested for motoric function using straight channel swimming. No differences in swimming performance were observed between treatment groups or between sexes in this task.

3.4.3. Morris water maze—Beginning on P63, animals were tested in the MWM for five consecutive days with four trials per day. There was a significant main effect of treatment for latency to reach the goal ($F(1,22) = 18.52, p < 0.0003$; Fig. 8A) with MA-treated animals taking longer. MA-treated offspring also swam farther ($F(1,22) = 14.13, p < 0.001$; Fig. 8B), had greater cumulative distances from the goal ($F(1,22) = 18.82, p < 0.0003$; Fig. 8C), and had larger

angles of deviation from a direct line to the goal ($F(1,22) = 31.85, p < 0.0001$; Fig. 8D) compared to SAL-treated animals. A main effect of sex was observed with females taking longer ($F(1,22) = 15.16, p < 0.0008$), traveling farther ($F(1,22) = 14.89, p < 0.0009$), having greater cumulative distance ($F(1,22) = 16.39, p < 0.0005$), and beginning with a larger angle of first bearing ($F(1,22) = 6.20, p < 0.03$) compared to males.

Further analyses showed that MA-treated offspring spent a larger percentage of time in the outer annulus ($F(1,22) = 10.30, p < 0.004$; Fig. 9A), with a corresponding decreased percentage of time in the target ($F(1,22) = 7.55, p < 0.012$; Fig. 9A) and inner annuli ($F(1,22) = 9.11, p < 0.006$; Fig. 9A) compared to SAL-treated animals. MA-treated animals also had significantly fewer direct swims to the target compared to SAL-treated animals ($Z = -4.606, p < 0.01$; Fig. 9B and C).

4. Discussion

We have previously shown that MA exposure from P11–20 causes deficits in MWM learning and memory (Vorhees et al., 1994a, b, 1996, 1999, 2000; Williams et al., 2002, 2003a, b). Consistent with these findings, we showed that MA exposure caused learning deficits when the animals were tested here. The results of this study also confirmed our previous work that showed CORT was increased on P11, regardless of the time point assessed (Williams et al., 2000). Neurotrophins are important factors in learning and memory and have been shown to be altered by changes in CORT levels, making them potential candidates as contributors to MA-induced spatial learning deficits. High levels of CORT from P1–7 increase NGF mRNA levels in the hippocampus (Roskoden et al., 2004), and the synthetic glucocorticoid, dexamethasone, increases NGF mRNA expression in adult animals (Mocchetti et al., 1996; Scully and Otten, 1995). However, high levels of CORT in adult animals have also been shown to decrease BDNF mRNA levels (Chao and McEwen, 1994; Schaaf et al., 1998; Smith et al., 1995). The results of the present study show increased BDNF following five and 10 days of MA exposure and increases of hippocampal NGF after 10 days of exposure. There were no changes in BDNF or NGF after 1 day of MA exposure on P11, suggesting that a prolonged exposure to MA is required to alter neurotrophic levels at this age. Whether this effect is a direct effect of MA or indirect through the increase in CORT is not yet clear. Consistent with CORT-induced changes, a previous study showed altered NGF levels after seven days of elevated CORT (Roskoden et al., 2004). Other explanations for the lack of changes on P11 are that neurotrophins may be resistant to change during some periods of development (Danzon et al., 2004) or that levels were altered during a period of time we did not assess (i.e., we cannot rule out the possibility that different time points during the dosing period would demonstrate MA-induced differences in neurotrophic factors).

BDNF and NGF have been suggested to be important in learning and memory. Mice that have heterozygous mutations in either NGF (Chen et al., 1997) or BDNF (Linnarsson et al., 1997) show learning and memory deficits. Spatial learning has also been shown to increase the mRNA levels of BDNF in the hippocampus after three and six days of training (Kesslak et al., 1998). Inhibitory avoidance (IA) training increases BDNF levels 12 h after the training phase (Bekinschtein et al., 2007). Blocking BDNF using neutralizing antibodies or antisense oligonucleotides 12 h after IA training eliminates retention performance seven days later (Bekinschtein et al., 2007). Our results show no significant change in BDNF levels between animals that were tested in the MWM for five days and those that were untested. There were also no differences in BDNF or NGF levels at early time points following MWM testing. It is possible that we examined BDNF levels too soon after MWM training. The Bekinschtein et al. (2007) study found BDNF increased in the dorsal hippocampus 12 h following IA training, while the latest point we examined was 2 h following the last trial. In the Kesslak et al. (1998) study, animals were administered eight trials per day, so animals had 24 and 48 trials

by the time BDNF mRNA was assayed, compared to the 20 trials that our animals experienced. The Kesslak et al. study also examined mRNA, whereas in this study we examined protein levels. This suggests that either extensive learning (i.e., more MWM trials) is necessary to induce an effect on BDNF levels or there is regulation of BDNF mRNA translation that leads to dissociation between mRNA and protein. Another potential difference between the two studies is the age of animals at testing. The Kesslak et al. study used rats that weighed between 175–225 g (approximately 46–52 days old) and this is during the transition from puberty to adulthood. The changes in hormone levels during puberty may be a contributing factor since testosterone and estrogen can alter BDNF levels (Bimonte-Nelson et al., 2003; Franklin and Perrot-Sinal, 2006).

We observed an increase in CORT levels in males following MA administration on P20; however no effects were seen in females of this age. We previously showed that MA raises CORT levels in P20 animals regardless of sex (Williams et al., 2000). It is unclear why a sex difference was obtained this time and not previously. However, sexually dimorphic effects were also seen in BDNF, NGF and adult CORT levels as well as in MWM learning. On P11, males had higher BDNF levels in the hippocampus and hypothalamus relative to females. In contrast to the BDNF effects, females had greater hippocampal NGF levels at P15 and greater hypothalamic NGF levels at P60. For both neurotrophins, no sex effects were seen on P20 or P21. Whether the differences in developmental neurotrophic levels influence the sexually dimorphic ability of animals to solve the MWM is unknown, however it is of interest that the differences in BDNF and NGF in the hippocampus occur during a period of hippocampal development that is sensitive to drug-induced changes in MWM learning (Williams et al., 2003a). It is also known that testosterone decreases NGF levels in aged males, while dihydrotestosterone, which cannot be aromatized to estrogen, has no effect (Bimonte-Nelson et al., 2003). This suggests that estrogen may be involved in neurotrophin expression.

Another sexually dimorphic effect was observed in the CORT response of animals following MWM testing. In agreement with a previous report (Beiko et al., 2004), we found that female rats had significantly higher CORT values than males following five days of MWM learning. It is important to note that despite differences in procedure between the two studies (i.e., 12 trials performed on a single day with pretraining (Beiko et al., 2004) compared to our procedure of administering four trials per day for five days), both studies found increased levels of CORT in female rats vs. male rats. Since female rats had poorer behavioral performance than their male counterparts it suggests a relationship between CORT levels and MWM performance. However, it is likely that such a relationship will be dependent upon an optimal level of CORT since CORT was increased in all animals at 0 and 30 min following MWM testing with a return to baseline levels (compared to non-tested animals) by 60 min. Others have also suggested that an optimal level of CORT is necessary for maximal learning and memory and that higher levels of CORT disrupt these processes (de Quervain et al., 1998). One of the initial experiments addressing the role of CORT relative to MWM training concluded that CORT facilitates memory storage only on the early trials (Sandi et al., 1997). Comparison of male and female learning under conditions of equal levels of CORT would be useful for determining the role that CORT plays in the sexually dimorphic learning and memory effects in the MWM.

In order to test if non-spatial learning strategies in the MWM were affected by MA exposure we examined not only traditional measures (i.e., latency, path length, and cumulative distance), but also swim patterns (i.e., time in annuli and direct swims). Analyses of swimming patterns by annuli show that MA-treated animals spend more time in the outer annulus of the maze. Cain et al. (1996) have shown this pattern is characteristic of animals who have failed to learn fundamental aspects of the task, i.e., locating the escape platform requires learning to swim away from the wall. The increase in thigmotaxis in MA-treated animals suggests that this fundamental strategy is altered along with spatial mapping difficulties (Williams et al.,

2002). Another measure used for this study was analysis of direct swims to the platform. This assesses the animals' ability to learn the general direction of the goal even if it does not know the exact position of the platform. Control animals increased their direct swims across days whereas MA-treated animals deviated from a direct path more frequently. These effects support the hypothesis that MA-treated animals have a compound deficit in which they acquire both the spatial and non-spatial strategies of the MWM more slowly than controls. On the other hand, MA-treated animals are not so impaired that they cannot perform the task since they eventually reach asymptotic performance levels that are comparable to those of controls. Rather, it is the rate of learning in MA-treated offspring that is slower than that of controls.

In conclusion, the results indicate that neonatal MA treatment alters levels of CORT, BDNF, and NGF and these changes precede later spatial learning impairments. Future investigations will be required to determine if these early changes are in fact causative in the resultant learning deficits.

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Figure 1.

Diagrams for annuli swimming and direct swim analysis. (A) The target annulus for analysis of target annuli swims was drawn by drawing a 20 cm ring that encompassed the distal and proximal corners of the platform. (B) The corridor for direct swims was a 36 cm channel directly from the start location to the platform.

Figure 2.

Neonatal CORT following MA exposure. (A) Levels of CORT in plasma (mean $\pm$ SEM) show a slight decrease on P15 following MA exposure from P11–15 ($N = 10$ per treatment). (B) CORT levels are increased following MA exposure on P11 ($N = 10$ per treatment). (C) On P20, CORT levels are increased following MA exposure in males only ($N = 10$ per treatment).

*** $p < 0.001$, † $p < 0.07$.

Figure 3.

Adult CORT interactions. (A) Adult CORT levels are decreased as a result of P11–20 MA treatment. (B) Female animals had higher levels of CORT compared to males. (C) CORT levels are increased immediately following MWM training and decline over time. (D) Females generally have higher CORT following MWM training. ** $p < 0.01$, * $p < 0.05$; $N = 12$ per treatment.

Figure 4. Neonatal BDNF following MA exposure. MA treatment increases hippocampal BDNF protein levels in P15 animals. Animals were exposed to MA from P11–15 and sacrificed after final exposure on P15, $* < 0.05$; $N = 10$ per age group.

Figure 5.

Neonatal sex effects in BDNF following MA exposure on P11, female rats had lower levels of hippocampal (A) and hypothalamic (B) BDNF compared to males. MA treatment increased BDNF in P20 animals regardless of sex following P11–20 exposure (C). *** $p < 0.001$, * $p < 0.05$. $N = 10$ per age group.

Figure 6.

BDNF levels following MWM training. BDNF levels remain relatively stable following MWM testing with MA treated animals having a slight increase in BDNF that approached significance ($N = 12$ per treatment).

Figure 7.

NGF changes due to sex and training. (A) Females have higher levels of hippocampal NGF compared to males ($N = 10$ per treatment). (B) MA treatment increases NGF levels in the hippocampus on P20 ($N = 10$ per treatment). (C) Trained female MA-treated animals ($N = 12$ per treatment) have lower hypothalamic NGF levels than SAL-treated females. (D) NGF ($N = 12$ per treatment) levels are lower in untrained females treated with MA compared to untrained females. ** $p < 0.01$.

Figure 8. Neonatal MA treatment induces MWM deficits. P11–20 MA treatment caused increases in latency (A), path length (B), cumulative distance (C) and first bearing (D) to the platform. *** $p < 0.001$, ** $p < 0.01$ ($N = 12$ per treatment).

Figure 9.

MA treatment alters strategies in the MWM. (A) P11–20 MA treatment increases time spent in the periphery of the MWM. (B) MA treatment decreases the percent of direct swims to the platform from the start. (C) MA treatment decreases total direct swims by day. *** $p < 0.001$, ** $p < 0.01$ ($N = 12$ per treatment).