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EFFECTS OF NEONATAL 3,4-METHYLENEDIOXYMETHAMPHETAMINE ON HIPPOCAMPAL GENE EXPRESSION, SPATIAL LEARNING AND LONG-TERM POTENTIATION

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA) is a ring substituted amphetamine similar in structure to mescaline. Exposure to MDMA from postnatal days (P)11-20 has been shown to induce deficits in spatial learning and memory as well as in path integration learning when the animals are tested as adults. These learning and memory deficits emerge at P30 and persist until P360. This dosing regimen has been shown to decrease serotonin and alter serotonin signaling in the adult brain; however these effects were independent of the learning and memory deficits observed. Finally, dosing on P11 has been shown to increase corticosterone levels during the stress hyporesponsive period, a time when there are attenuated CORT levels. While these areas appear to be important in elucidating the mechanisms underlying MDMA, it is important to investigate systems that may not have been previously implicated in MDMA pharmacology. Microarray analysis revealed 71 genes with altered expression (66 up-regulated and 5 down-regulated) in the hippocampus of adult animals treated from P11-20 with MDMA. Real-time PCR analysis verified 8 out of the 24 genes selected for verification. The 8 verified genes were examined in the striatum of adult animals, with the gene encoding angiotensinogen (AOGEN) up-regulated approximately 75%. Following examination in the hippocampus and striatum, the 8 verified genes were examined in the hippocampi of P12 and P21 MDMA-exposed animals. In P12 animals, nuclear orphan receptor 1 was up-regulated by ~600% and AOGEN was down regulated by 50%; while AOGEN was up-regulated by 3 fold on P21. One gene was selected for further investigation. CAPON, the gene that showed the highest up-regulation during microarray analysis, was analyzed with common pathway members nNOS, PSD-95, and the NMDA receptor subunit 1 (NR1). While CAPON protein was unchanged, the remaining proteins were increased in the dentate gyrus of adult animals. Together with the protein changes

associated with NMDA, NMDA signaling was altered since long-term potentiation was decreased in the hippocampus of adult animals. On P11, phosphorylation of CAM-KII and nNOS appear to be unchanged after a single dose of MDMA. The results of these studies implicate the NDMA receptor in MDMA-induced learning and memory deficits.

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CHAPTER 1: INTRODUCTION

3,4-Methylenedioxymenthamphetamine (MDMA), 1-benzo[1,3]dioxol-5-yl-N-methyl-propan-2-amine, is a chemical derivative of amphetamine, and is structurally related to mescaline, methamphetamine, and fenfluramine (Figure 1). MDMA administration primarily activates serotonergic neurons, with secondary activation of noradrenergic and dopaminergic terminals (Rothman et al. 2001). Known as a "club" or designer drug, MDMA is primarily taken at group gatherings known as "raves", which are large, often all night parties consisting of dancing to loud computer generated music combined with computer and laser generated images. Unlike other amphetamine derivatives, MDMA is mildly hallucinogenic with users reporting visual and auditory disturbances and an increase in the intensity of sensory inputs. Psychological effects of the drug, which begin 20-60 min after ingestion, include a general euphoric state, emotional openness, reduction of negativity, and a decrease of inhibitions (Green et al., 2003). Physiological effects of the drug include elevated heart rate, blood pressure, hyperthermia, nystagmus, and insomnia.

History and prevalence of use

MDMA was first synthesized in 1914 as a precursor to potentially therapeutic compounds (Cohen, 1998). The United States Army conducted the first studies on the lethality of MDMA and behavioral changes in animals during the 1950's although the results were not declassified until 1969 (Hardman et al., 1973). The psychoactive effects of MDMA were first described in 1978 by Alexander Shulgin (Shulgin A.T. and Nichols D.E., 1978). Some therapists began to use MDMA to enhance openness and communication with their patients (Grinspoon and Bakalar, 1986). In 1985, MDMA was classified as a Schedule I substance due to a perceived

lack of therapeutic use and high abuse potential (Lawn, 1986). MDMA is also classified as a controlled substance in the United Kingdom and Canada (Green et al., 2003).

Since the inclusion of MDMA in the Monitoring the Future study published by the National Institute on Drug Abuse in 1989, the use of MDMA has increased significantly. In 1989, 1.9% of 19-20 year olds had reported using MDMA; use peaked in 2001 with 11% of respondents reporting using MDMA within the previous year (Johnston et al., 2005a; Johnston et al., 2005b). In 2004, 8% of high school seniors and 14% of college seniors (~21-22 years old) had tried MDMA at least once in their lifetimes. For those 25-26 years old, 20% had tried MDMA in their lifetimes. In 2004, the age group with the highest prevalence of MDMA use within the previous year was 19-20 year olds with 4.2% reporting use, while the lowest were 29-30 year olds with 0.9% reporting use. While use of MDMA is still significant, use has sharply declined in the past few years. With the majority of MDMA users being young adults, including women, the risk of exposure to the fetus becomes a concern, since users could become pregnant and continue to take the drug throughout gestation (Ho et al., 2001).

Adult pharmacology of MDMA

Since the majority of investigation into the mechanism of MDMA action has occurred in adult rats, a brief review of these effects is presented. In the adult rat, MDMA acts primarily on serotonergic neurons, followed by actions on noradrenergic and dopaminergic neurons. Typical doses for MDMA in the adult animal differ in number of injections and amount; however the range of MDMA exposure is 5-60 mg/kg/day and most studies only administer MDMA on one day while a few extend the dosing up to 4 consecutive days. The first study to show the effects of MDMA on serotonin (5-HT) used hippocampal slices which showed that both enantiomers of MDMA release radiolabeled 5-HT equally well (Johnson et al., 1986). Using *in vivo*

microdialysis, it has been shown that peripheral MDMA administration increases central extracellular 5-HT levels (Gudelsky and Nash, 1996; Gough et al., 1991; Yamamoto et al., 1995; Sabol and Seiden, 1998; Shankaran and Gudelsky, 1999; Mechan et al., 2002). *In vitro* assays in which MDMA is applied to brain slices (Johnson et al., 1986; Schmidt, 1987; Schmidt et al., 1987; Berger et al., 1992) or synaptosomal preparations (Berger et al., 1992; O'Loinsigh et al., 2001) have also shown that MDMA administration causes an immediate increase in 5-HT release. A study by Schmidt showed that the primary metabolite of MDMA, methlyenedioxyamphetamine (MDA) is more effective at releasing serotonin and plays a major role in MDMA-related 5-HT release (Schmidt, 1987). MDMA-induced 5-HT release is significantly reduced by prior treatment with the serotonin uptake inhibitor fluoxetine (Gudelsky and Nash, 1996; Mechan et al., 2002). This suggests that MDMA uses a carrier-mediated mechanism, i.e. the serotonin transporter, to increase 5-HT levels through the reversal of the plasma and vesicular membrane transporters (Cole and Sumnall, 2003).

Further evidence for the involvement of 5-HT in MDMA pharmacology is the depletion of 5-HT in animals exposed to MDMA. The first study to show depletions of 5-HT following MDMA administration in rats was by Battaglia et al. (Battaglia et al., 1987) and this effect has been replicated extensively (Green et al., 2003). Reductions in 5-HT appear to persist at least 8 weeks in the hypothalamus, while hippocampal and striatal levels persist at least 16 weeks (Scanzello et al., 1993). In addition to 5-HT deficits, alterations in serotonin transporter (SERT) have been observed. For example, paroxetine binding, a marker of SERT activity, is reduced following MDMA administration (Scanzello et al., 1993; Battaglia et al., 1988). Decreases in paroxetine binding induced by MDMA administration persist until 32 weeks in the cortex and striatum and remained lower than control values in the hippocampus for at least 1 year following

MDMA administration (Battaglia et al., 1988). MDMA not only decreases 5-HT levels and SERT activity, MDMA also decreases the rate limiting enzyme in 5-HT synthesis, tryptophan hydroxylase (TPH). The first study to examine the effects of MDMA on TPH was by Stone et al. showing that TPH activity decreased in the striatum, frontal cortex, hippocampus, and hypothalamus 15 min following MDMA administration (Stone et al., 1987). The reductions in TPH last up to two weeks following MDMA administration (Schmidt and Taylor, 1987). It appears that the reductions in TPH are due to a metabolite of MDMA, as an acute i.c.v. administration produced no reductions in TPH; however a 1 hr infusion of MDMA reduced TPH activity (Schmidt and Taylor, 1988).

MDMA has also been shown to stimulate dopamine (DA) release in the striatum and decrease the *in vivo* levels of its major metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Yamamoto and Spanos, 1988; Yamamoto et al., 1995; Nash and Brodkin, 1991; Nash and Yamamoto, 1992; Gudelsky et al., 1994; Gough et al., 1991; Koch and Galloway, 1997; Sabol and Seiden, 1998). Most data show that MDMA may interact with the dopamine transporter as evidenced by the lack of DA release following the administration of the DA uptake inhibitor GBR12909 both *in vivo* (Nash and Brodkin, 1991) and *in vitro* (Koch and Galloway, 1997), while a contradicting report showed an enhancement of DA release using GBR12909 (Mechan et al., 2002). However, these studies used different routes of MDMA administration: the Nash study used i.c.v. administration for both MDMA and GBR12909; while the Mechan study administered MDMA i.p. and gave GBR12909 s.c. The differences in the results of these studies would again suggest that it may be a metabolite of MDMA that also alters neurochemistry. Pretreatment with fluoxetine or antagonism of the 5-$HT_{2A/2C}$ receptor blocks the MDMA-induced DA release, and agonists of 5-HT receptors enhances DA release, suggesting

that 5-HT release is important for DA release following MDMA administration (Yamamoto et al., 1995; Koch and Galloway, 1997; Gudelsky et al., 1994). MDMA administration has been shown to reduce DA levels in the striatum for up to 10 weeks (Commins et al., 1987; McGregor et al., 2003; Able et al., 2006; Cohen et al., 2005; Clemens et al., 2004). MDMA has also been shown to alter norepinephrine release, however, MDMA administration regimens have not been shown to deplete NE in the rat brain. Recently, MDMA has been shown to increase acetylcholine (ACh) release *in vivo* in the frontal cortex, hippocampus (Nair and Gudelsky, 2006; Acquas et al., 2001) and the striatum (Acquas et al., 2001). Striatal brain slices have also been shown to release ACh following MDMA exposure (Fischer et al., 2000). The release of ACh was prevented in the prefrontal cortex by the administration of the TPH inhibitor PCPA or by inhibiting DA synthesis using α-methyl-p-tyrosine (Nair and Gudelsky, 2006). Hippocampal ACh release was unaffected by either compound, suggesting that MDMA-induced ACh release is 5-HT and DA mediated in the PFC but other mechanisms are responsible for ACh release in the hippocampus. The results of these studies show that MDMA has a large impact on numerous neurotransmitter systems in multiple brain regions.

In addition, MDMA administration has been shown to alter neuroendocrine parameters. Corticosterone (CORT) was shown to be increased beginning at 30 min following MDMA administration, and returned to control levels by 6 h; an effect that was dose dependent (Nash, Jr. et al., 1988). Prolactin levels peak 1 h following MDMA administration, and return to baseline levels by 4 h (Nash, Jr. et al., 1988). Renin and aldosterone are increased in plasma following MDMA exposure (Burns et al., 1996), and it has been shown that MDMA application increases oxytocin and vasopressin levels in hypothalamic cultures (Forsling et al., 2001; Forsling et al., 2002).

MDMA increases free radical production, which was shown by an increase in lipid peroxidation (Sprague and Nichols, 1995; Colado et al., 1997b) and by the alleviation of 5-HT damage by the nitrone radical trapping agent α-phenyl-N-tert-butyl nitrone (PBN) (Colado and Green, 1995). Further evidence of this was shown by the conversion of salicylate to 2,3-dihydroxybenzoic acid (2,3-DHBA) (Colado et al., 1997b) that occurs in the presence of free radicals; this formation can be prevented by ascorbic acid (Shankaran et al., 2001) or manizadol (Shankaran et al., 1999). Administration of sodium ascorbate or L-cysteine prevents the long-term 5-HT depletions caused by MDMA exposure (Gudelsky, 1996) and α-lipoic acid administration prevented damage to 5-HT nerve endings (Aguirre et al., 1999). Contrary to this, another study shows that salicylate does not prevent 5-HT terminal damage (Yeh, 1997). While this one study may cast doubt on the role of free radical production in MDMA-induced 5-HT terminal reduction, most of the evidence supports the theory.

Effects of in utero MDMA exposure in humans

There have been a very limited number of studies performed on the effects of MDMA on fetal development in humans. Two studies were performed by McElhatton et al. that primarily showed that infants exposed to MDMA in utero had a higher incidence of club foot and congenital heart defects (McElhatton et al., 1997; McElhatton et al., 1999). Women who enrolled in this study were generally not polydrug users; a potential confound for most human drug abuse studies. An interesting finding from these studies was that women who abuse MDMA during pregnancy had an increase in "therapeutic" pregnancy terminations. Therefore, a potential confound for human studies is that potentially high exposure groups may not be assessed because of pregnancy terminations. Moreover, the meaning of the club foot defects is unclear since this abnormality is generally regarded as a deformation rather than a malformation

and is therefore unlikely to be chemically-induced. One other Dutch study examined infants prenatally exposed to MDMA and found that one infant out of 43 had cardiac malformations, unfortunately the results were inconclusive due to a small number of subjects enrolled in the study (van Tonningen-van Driel MM et al., 1999). A Canadian study examined the profiles of women who abused MDMA during pregnancy and who called a hotline number for information regarding MDMA use during pregnancy (Ho et al., 2001). The findings of this study show that women who take MDMA during pregnancy are more likely to smoke, consume alcohol and other illicit drugs as well as to procure an abortion as compared to women who reported no MDMA use during pregnancy. Most women who reported MDMA use during pregnancy used the substance once and terminated the use after recognition of the pregnancy. This study may not represent the MDMA using population, as this was a survey of women who called a hotline and not a population-based representative sample.

Pharmacology of prenatal MDMA exposure in animals

General characteristics and behavior

Table 1 summarizes the studies that have examined the effects of prenatal and neonatal exposure to MDMA in animals. The early literature regarding MDMA exposure in the developing animal is problematic because of study design issues. Of the limited data on developmental MDMA exposure, routes of administration, frequency of exposure, strain and species of animal used, and controls included vary widely between studies. This has made synthesis of the effects of developmental MDMA exposure somewhat difficult due to the lack of a consistent model; therefore the data will be summarized but comparison of the results will necessarily be limited. Reduced body weights were observed in the offspring of rat dams exposed twice daily to MDMA (10mg/kg) from embryonic day (E)12-15 (Winslow and Insel,

1990). On postnatal day (P)9, there were no alterations in ultrasonic vocalizations, grid line crossing, or negative geotaxic response in the offspring (Winslow and Insel, 1990). There were no differences in litter size, length of gestation, or body size of rat offspring exposed to MDMA (2.5 or 5 mg/kg) from E6 until E18 compared to the control animals that received distilled water (St Omer et al., 1991). Behaviorally, the offspring showed sex dependent effects of MDMA exposure. In measuring negative geotaxis once a day from P7-10, female offspring exposed to MDMA showed a one day decrease on P7, however no effects were observed in the MDMA-exposed males on any test day or at the other ages tested for females. Male offspring exposed to MDMA showed an increase in olfactory orientation on P9 and P10, and MDMA-exposed females had increased olfactory orientation on P11. Other behaviors examined in that study (surface righting, grip strength, and milk-induced behaviors) were unaffected in MDMA-treated animals (St Omer et al., 1991). MDMA exposure (20 mg/kg) twice a day from E14-17 reduced litter size compared to the saline-exposed control rats (Colado et al., 1997a). Rats exposed from E14-20 to 15 mg/kg MDMA twice daily showed an initial hypoactivity followed by hyperactivity on P21 compared to saline-treated controls (Koprich et al., 2003b). MDMA administration from E14-20 also decreased sucrose preference when the rat offspring were tested at P70 (Galineau et al., 2005). Again, due to the differences in dosing regimens and exposure ages, it is difficult to synthesize what the effects of prenatal MDMA exposure on the behavior of the offspring are and much work is still needed in this area.

Biochemical effects

In rats administered MDMA (2.5 or 5 mg/kg) every other day from E6-18, reductions of 5-HT were observed in the caudate nucleus and the hippocampus at P21 while levels of 5-HT in the frontal cortex and 5-HIAA levels in all regions were unaltered (St Omer et al., 1991). When

MDMA (10^{-3}-10^{-9} M) was applied to synaptosomes from E17 rat embryos a dose dependent decrease in 5-HT was observed (Kramer et al., 1994a). Similarly, E14-17 MDMA (20mg/kg; 2/day) exposure in rats, decreased 5-HT levels in the dorsal telencephalon were observed at P7 (Colado et al., 1997a). Mice exposed to 40 mg/kg MDMA from E5-12 twice a day had a significant increase in 5-HT and 5-HIAA in mesencephalic-striatal tissue cultures (Won et al., 2002), although it should be noted that MDMA is not as effective at inducing 5-HT release in the mouse. While the studies in rats show that MDMA reduces 5-HT, other studies have shown few effects of MDMA exposure on the developing animal. For instance, no changes in 5-HT or DA levels in various brain regions were observed in rat offspring exposed to 20 mg/kg MDMA once/day every other day (Aguirre et al., 1998). Furthermore, when rats were exposed to MDMA (0.5, 1.0 or 10 mg/kg) twice a day from E12-15, no differences were seen in hippocampal levels of 5-HT, 5-HIAA, DA, DOPAC, HVA, or NE when assayed at P21 (Winslow and Insel, 1990). In rats exposed to MDMA (15 mg/kg) twice a day from E14-20, no changes were observed in 5-HT levels in several brain regions on P3 or P21; however, there was an MDMA-induced decrease in striatal 5-HIAA levels and subsequently 5-HT turnover in the striatum on P21 with similar effects seen in DA metabolism and turnover (Koprich et al., 2003b). Although no alterations in DA were observed in animals exposed to MDMA from E14-20, an increase in the immunoreactivity of tyrosine hydroxylase was observed in the striatum, nucleus accumbens, and frontal cortex of MDMA-treated animals on P21 (Koprich et al., 2003b). A dose of 10 mg/kg once a day from E14-20 to rats decreased the 5-HT and 5-HIAA content of the whole brain on P0; however, 5-HT and 5-HIAA, as wells as DA and DOPAC levels, were unaltered at all other days (every other E day from E6 and every 7^{th} day from P0 to P21) examined (Galineau et al., 2005). No changes were observed in DAT or SERT binding from

E18-P70 (Galineau et al., 2005). Regardless of the previous lack of effect, this group did find alterations in DA and 5-HT stimulated release on P70 (Galineau et al., 2005) using microdialysis. No alterations in paroxetine binding were observed in rat offspring exposed to 20 mg/kg MDMA twice a day from E15-18 when examined at P40 (Kelly et al., 2002). This study also observed an increase in glucose utilization in the brain of MDMA-exposed offspring at P90 (Kelly et al., 2002).

Effects of neonatal exposure to MDMA in animals

With regard to brain development, the neonatal rat is equivalent to second to third trimester human development (Bayer et al., 1993; Rice and Barone S Jr, 2000). Neurogenesis in the dentate gyrus proceeds until P19, and myelination in many structures such as the striatum continues well into postnatal life (Figure 2). For the purpose of this discussion, neonatal or postnatal exposure to MDMA is defined as exposure in animals younger than P21 and is a model of the second half of human intrauterine development.

General Characteristics

It has been shown that MDMA administration to neonatal rats decreases the rate of weight gain in animals (Broening et al., 2001; Koprich et al., 2003a; Vorhees et al., 2004; Williams et al., 2003; Broening et al., 1995; Meyer et al., 2004). This anorectic effect of MDMA continues several weeks after administration of the drug is discontinued (Broening et al., 2001; Vorhees et al., 2004; Williams et al., 2003). In adults it has been shown that MDMA causes a hyperthermic effect at ambient room temperatures over 21°C. In developing animals, MDMA does not affect the thermal response of the animals. At higher room temperatures (25° C), MDMA exposure to P10 rats even caused a mild, non-significant decrease in rectal temperatures (Broening et al., 1995). When room temperatures are lowered (10° C), a variable response in

MDMA treatment is observed in animals, that is, hyperthermia initially and then hypothermia starting 6 h after MDMA administration, compared to saline-treated animals (Broening et al., 1995). Rats exposed to MDMA from P1-4 show no alterations in their thermal response regardless of the temperature at which the animals were housed (31 or 37° C) (Meyer et al., 2004).

Behavior

Acutely, MDMA exposure (0.5, 1 or 10 mg/kg) has been shown to cause hyperactivity and diminish ultrasonic vocalizations as well as negative geotaxic response of P10 rats (Winslow and Insel, 1990). Exposure from P0-2 with 10 mg/kg of MDMA also reduced the ultrasonic vocalizations of the pups when examined on P8, 11 and 14, however, there were no effects on activity compared to saline-treated controls (Winslow and Insel, 1990). Object recognition memory was unaffected by P1-4 MDMA (10 mg/kg 2/day) exposure in rats when examined at P68 (Piper and Meyer, 2006). These animals were also given an adult MDMA challenge and the MDMA-treated animals showed a decrease in behaviors associated with serotonin syndrome (head weaving, forepaw treading and low body posture among others) compared to SAL-treated animals (Piper and Meyer, 2006). Recently, several studies have examined the effect of neonatal exposure to MDMA on adult learning and memory. The first of these studies examined learning and memory following either P1-10 or P11-20 MDMA exposure (Broening et al., 2001). Rats were exposed twice a day to three different doses of MDMA (5, 10, or 20 mg/kg/dose) and were tested in the Morris water maze (MWM), a task assessing spatial learning and memory (Morris, 1981; Morris et al., 1982), and the Cincinnati water maze, a task that assesses path integration learning (Vorhees, 1987). Animals treated with MDMA from P1-10 showed no deficits in path integration learning, i.e. animals completed the maze with similar latencies and committed a

similar number of errors in finding the platform, compared to saline-treated littermates (Broening et al., 2001). Rats treated from P11-20 with either 10 or 20 mg/kg of MDMA showed increased latency and errors in the CWM, whereas animals treated with 5 mg/kg performed similarly to saline-treated controls. There were no observed sensorimotor deficits in MDMA-treated animals from either treatment age as evidenced by asymptotic performance in MWM visible platform trials and a straight channel swimming task. During MWM testing, animals treated from P1-10 with MDMA, regardless of dose, showed no deficits compared to saline- treated animals. Following P11-20 MDMA administration the 20 mg/kg MDMA group showed increases in cumulative distance, latency, and path length in the MWM hidden platform acquisition phase, while the 10 mg/kg group had increased path length and cumulative distance, and the 5 mg/kg group showed no effects. During reversal trials, where the platform is moved to the opposite quadrant, MDMA exposure largely had no effect on the performance of the animals, with the only effect being a trend of increased latency in the 20 mg/kg group. For the final phase of the MWM, the platform was moved to the original position and reduced in size from 10 cm x 10 cm to 5 cm x 5 cm, therefore requiring a higher precision of spatial navigation. Animals treated from P11-20 with all doses of MDMA showed increases in latency, path length and cumulative distance compared to saline-treated animals (Broening et al., 2001).

Spatial memory can be assessed by removing the platform from the MWM, starting the animal from a novel start position and determining the amount of time an animal searches in the area of the old platform location as well as the average distance from the platform. The 20 mg/kg MDMA group had a higher average distance from the platform during all phases of the MWM, while the 5 mg/kg group had a higher average distance during the reversal phase and the 10 mg/kg group during the reduced platform phase (Broening et al., 2001).

A subsequent study to Broening et al. examined the effects of injection stress and malnutrition on MDMA-induced learning and memory deficits (Williams et al., 2003). To simulate the weight loss experienced by MDMA-exposed rats, a large litter design was used with 16 animals per litter. To control for injection stress, litters that were only weighed at each treatment time point were included. MDMA exposure from P11-20 induced path integration deficits in the male offspring but not the female offspring. During the acquisition phase of the MWM, MDMA-treated animals (20 mg/kg) had increased latency, path length, and cumulative distance to the platform, while large litter animals and saline-treated animals performed similarly to animals that were weighed only. The reversal trials showed that MDMA-treated animals had an increase in cumulative distance from the platform with trends for increased latency and path length to the platform. Again, saline-treated and large litter control offspring showed no differences compared to weighed-only animals. Finally, during the reduced platform phase of the MWM, MDMA-treated animals showed increased path length and cumulative distance with a trend towards increased latency to the escape platform. In the probe trials of the MWM, MDMA-treated offspring had higher average distances from the platform during the acquisition and reduced phases of the MWM and were similar to saline-treated animals during the reversal phase. During the reversal probe, large litter offspring had a higher average distance from the platform than weighed animals. This was the only measure in which the large litter animals differed from the weighed or saline-treated animals (Williams et al., 2003). The results of this study show that MDMA effects are independent of injection stress and malnutrition/reduced growth.

The Barnes maze is a spatial learning task that does not have the swimming component and can be run as either an appetitive or aversive task (Barnes, 1979). The Barnes maze has also

been used to investigate the spatial learning deficits seen in neonatally MDMA-exposed animals (Vorhees et al., 2004). Half of the animals, counterbalanced for treatment, were either tested first in the MWM (only 1 phase using the 5 cm X 5 cm platform) and then the Barnes maze or vice versa and these tasks were then followed by a working memory test (Vorhees et al., 2004). MDMA-treated animals showed increases in latency, path length, and cumulative distance during MWM testing at all doses administered (5, 10, or 20 mg/kg), however no effects were observed in the Barnes maze or the working memory tasks during this study (Vorhees et al., 2004). Interestingly, if animals were tested in the Barnes maze prior to being tested in the MWM, there was an amelioration of the MWM deficits. This suggests there may be some transference of spatial strategy solving that spatial pretraining might confer and therefore benefit animals in other spatial learning tasks. As in previous studies, no deficits were observed in the visible platform phase of the MWM or in the ability of the animal to swim a straight channel, thereby further solidifying the hypothesis that MDMA-induced learning and memory deficits are not due to sensorimotor deficits (Vorhees et al., 2004).

To examine if P11-20 MDMA exposure interacts with adult MDMA administration, rats that were exposed from P11-20 with 20 mg/kg/dose MDMA (twice daily) or the saline vehicle were subsequently treated with a one day, four-dose regimen of 10 mg/kg MDMA or saline (Cohen et al., 2005) when the animals were adults. Four groups were created in this study (2 neonatal treatments x 2 adult treatments) and these animals were then tested in the following tasks: CWM, MWM, zero maze, a test of anxiety, novel object recognition (NOR), and spontaneous locomotor activity. As shown previously, neonatal MDMA exposure increased latency, path length and cumulative distance in both phases of the MWM that were tested, as well as increased errors committed in escaping the CWM. This study also showed that neonatal

treatment with MDMA induced deficits in time spent with a novel object in the NOR task, which is a hippocampally-mediated non-spatial memory test (Clark et al., 2000). Furthermore, neonatal MDMA induced hypoactivity regardless of adult treatment (Cohen et al., 2005). In the reversal phase of the MWM, adult animals that had been exposed to MDMA as adults and neonates showed deficits, while neonatal MDMA only or adult MDMA only did not produce such deficits.

To summarize the studies utilizing the P11-20 exposure model on later behavior; MDMA treatment from P11-20 disrupts spatial and path integration learning, causes hypoactivity, and disrupts spatial and non-spatial memory when the animals are tested as adults. Anxiety levels and certain types of spatial learning appear to be unaffected by MDMA treatment, although the latter could be more a function of the task instead of a lack of an effect (see Vorhees et al, 2004 for discussion).

Biochemistry

Neonatal MDMA exposure appears to primarily work on serotonergic systems. Exposure twice a day to 10 mg/kg MDMA from P0-2 causes reductions in cortical 5-HT, 5-HIAA and paroxetine binding when the rats were examined on P21 (Winslow and Insel, 1990). This dosing regimen also produced reductions in cortical DA levels. Twice daily exposure to 10 mg/kg MDMA from P1-4 decreased rat hippocampal 5-HT on P25; however all other monoamines in the hippocampus and in the frontal cortex were unaffected (Meyer and Ali, 2002). A one time dose of MDMA (10, 20 or 40 mg/kg) on P10 to rats produces reductions in 5-HT in the frontal cortex, hippocampus, and caudate putamen 24 hours after exposure; however, no monoamine depletions were observed when examined on P17 (Broening et al., 1994; Broening et al., 1995) or on P21 (Winslow and Insel, 1990). Twice a day 10 mg/kg MDMA administration from P1-4 decreases paroxetine binding in the hippocampus and frontal cortex on P60, and decreases

hippocampal SERT binding on P25 compared to saline-treated rats (Meyer and Ali, 2002); however, these effects were not found in a subsequent study when offspring were examined on P70 or P105 (Piper and Meyer, 2006). Twice a day MDMA (20 mg/kg) exposure on P10-13, P15-18, P20-23 or P25-28 did not affect paroxetine binding in the neocortex when rats were examined at P40 (Kelly et al., 2002).

Using the P11-20 model of MDMA exposure, which as previously discussed has been shown to induce learning and memory deficits during adulthood, Koprich et al. have shown depletions in 5-HT and 5-HIAA in the frontal cortex, striatum, hippocampus, and a 5-HIAA reduction in the brain stem on P21 following P11-20 exposure to twice a day MDMA in rats (20 mg/kg) (Koprich et al., 2003a). The dopamine metabolite HVA was increased in the frontal cortex, striatum, hippocampus and brain stem, and DOPAC was also increased in the frontal cortex and striatum (Koprich et al., 2003a). On P11, MDMA (10 mg/kg/dose) to rats induced reductions in hippocampal 5-HT at 1 and 7 h after the first of four doses in the day, as well as at 24, 30, and 78 h after the first dose on P11 (Williams et al., 2005). Reductions in 5-HIAA were seen at 24 and 30 h after the first dose in the hippocampus. In the striatum, reductions in 5-HT do not emerge until 24 h after the first dose, while 5-HIAA reductions were observed 7 h after the first dose. No effects on dopamine were observed in the striatum, however there were reductions in DOPAC seen at 6, 24 and 30 h after the first dose (Williams et al., 2005). The 24 h reductions in 5-HT and 5-HIAA were replicated in a subsequent study (Schaefer et al., 2006). Following behavioral testing (~P105), the study by Broening et al examined monoamine levels in adult animals exposed to 5, 10, or 20 mg/kg of MDMA from P1-10 or P11-20. MDMA exposure induced reductions in 5-HT and NE in the hippocampus and 5-HT reductions in the frontal cortex compared to saline-treated animals (Broening et al., 2001). These reductions were

also observed in a subsequent study where rats did not perform behavioral tasks (Crawford et al., 2006). The hippocampal reductions in 5-HT following MDMA from P11-20 were also observed in animals treated from P1-10 (Broening et al., 2001). Unlike earlier exposure periods, but similar to Kelly et al.'s (2002) findings, paroxetine binding was unchanged 1-78 h following MDMA exposure on P11 (Williams et al., 2005). These results suggest that MDMA works through serotonin systems in adulthood and during development. This hypothesis is further supported by a study by (Crawford et al., 2006) who showed that MDMA-treated animals had enhancement of G protein activity following 5-HT stimulation in the hippocampus and prefrontal cortex, and an increase of G protein activity following R+-8-OH-DPAT stimulation in the hippocampus, suggesting an increase in sensitivity of the 5-HT_{1A} receptor (Crawford et al., 2006).

MDMA, much like methamphetamine, induces the release of the stress hormone CORT during the P11-20 dosing period, this period of administration includes the latter portion of the stress hyporesponsive period (SHRP) (Sapolsky and Meaney, 1986). The SHRP is a period of attenuated stress induced release of CORT, a mechanism believed to protect the developing animal from the possible neurodegenerative effects of high levels of circulating glucocorticoids (Sapolsky and Meaney, 1986). MDMA causes an immediate increase in plasmatic CORT levels in developing rats on P11, and these levels are elevated for 24 h following the first of four doses on P11 (Williams et al., 2005). These CORT alterations do not appear to last until adulthood, as baseline and stressed animals do not appear to have alterations in CORT compared to saline-treated animals (Williams et al., 2003). Finally, Koprich et al. observed increases in brain derived neurotrophic factor (BDNF) on P21 following P11-20 MDMA exposure (20 mg/kg) in rats in the frontal cortex, striatum, brain stem, and hippocampus (Koprich et al., 2003b). BDNF

is a protein that has been shown to be important for neuronal survival and learning and memory (Leibrock et al., 1989; Lindsay et al., 1994; Linnarsson et al., 1997). While these are two very significant areas to continue to explore for the mechanisms underlying MDMA-induced learning and memory deficits, other, unknown mechanisms could be at work. It is the purpose of this dissertation to further explore the learning and memory deficits induced by developmental MDMA exposure as well as mechanisms underlying these deficits.

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Table 1. Studies that have examined the effects of developmental MDMA exposure

Reference	Dose (mg/kg)	Exposure	Species (Strain)	Freq.	Route	Control	Parameter Examined	Findings	Comments
(Winslow and Insel, 1990)	0.5, 1.0, or 10	P10 (all doses) P0-2 (10 mg/kg) E12-14 (10 mg/kg)	Rat (SD)	Once (P10); 1/day or 2/day in prenatal and neonatal study	s.c.	Saline	Ultrasonic vocalizations with TFMPP, 8-OH-DPAT, or DOI challenge; grid cell crossing; temperature; 5-HT, 5-HIAA, and ^{3}H-paroxetine binding in cortex	• ↓ ultrasonic vocalizations during acute MDMA exposure on P10 • ↑ grid cell crossing following acute MDMA exposure of 1.0 and 10 mg/kg on P10 • ↓ latency to geotaxic response following acute MDMA exposure of 1.0 and 10 mg/kg on P10 • ↓ ultrasonic vocalizations at .5 and 3 hours following exposure; no Δ at 6 hours; ↑ at 10 and 24 hours • ↓ ultrasonic vocalizations on P8, 11, and 14 following MDMA exposure on P0-2; no Δ on P5 • no Δ following prenatal exposure • no Δ in grid sell crossing and rate of weight gain on P5, 8, 11, and 14 in P0-2 exposed group • ↓ ^{3}H-paroxetine binding and 5-HT concentration in P0-2 exposed group • ↓ 5-HIAA concentration in group dosed b.i.d. on P0-2; no Δ in group dosed once a day • no Δ in the prenatal exposure group in 5-HT, 5-HIAA, and ^{3}H-paroxetine • ↓ DA in group exposed b.i.d. P0-2 • no Δ in ultrasonic vocalizations following TFMPP, 8-OF-DPAT, or DOI challenge	• Isomer: ±MDMA • Wgt: No • Cross fostering: Yes • Litter effects Yes • N=9-63
(St Omer et al., 1991)	2.5 or 5.0	Every other day E6-18	Rat (CD)	1/day	Orally	Distilled water	Gestation length, litter size, body weight of offspring, surface righting, neg. geotaxis, olfactory orientation, milk induced behavior, forelimb grip strength, passive avoidance, activity, 5-HT & 5-HIAA concentrations	• no Δ in length of gestation, litter size, & body weight of offspring • no Δ in surface righting • ↓ negative geotaxis in females dosed with 2.5 mg/kg on P7 &10. no Δ in animals dosed with 10 mg/kg and males dosed with 2.5 mg/kg • ↑ olfactory orientation in males on P9 &10 & in females on P11 • no Δ in milk induced behaviors on P6 • no Δ in forelimb grip strength • no Δ in passive avoidance resistance • no Δ in figure 8 activity • no Δ in cerebrum 5-HT uptake sites on P29 • ↓ 5-HT in caudate nucleus & hippocampus in	• Isomer: NR • PFC: No • Cross fostering: No • Litter effects Yes • N=4-10

								animals dosed with 10 mg/kg; no Δ in 2.5 mg/mg groups • no Δ in 5-HT in frontal cortex • no Δ in 5-HIAA in frontal cortex, caudate nucleus, & hippocampus	
(Bronson et al., 1994b)	8, 16, 24, & 32	E14 & P1	Chicken	Single	*in ova*	Distilled water	*In ova* motility, distress vocalization, wing extension, tremor, flat body posture, busting forward movements, loss of righting reflex and convulsant like kicking following MDMA challenge	• ↓ in ova motility in 32 mg/kg treated chicks; no Δ in 8 or 16 mg/kg treated chicks • ↑ distress vocalization and wing extension following 24 mg/kg MDMA challenge on P1 • ↑ flat body posture in naïve animals on P1; ↑ flat body posture in animals pretreated with 8 & 16 mg/kg compared to naïve animals; ↓with pretreatment with 32 mg/kg • ↑ bursting activity in naïve animals and animals pretreated with 8 or 16 mg/kg following challenge; no Δ in 32 mg/kg pretreatment group • ↑ loss of righting reflex in animals pretreated with 16 mg/kg following challenge; no Δ in naïve or 8 & 32 mg/kg pretreated group • no Δ in tremors or convulsant like kicking • no Δ in brain, liver, or body weight	• Isomer: NR • PFC: No • Cross fostering: No • Litter effects No • n=19-30
(Kramer et al., 1994b)	10^{-3}-10^{-9} M	E17 synapto-somes	Rat (SD)	Single	Culture media	Baseline levels	5-HT retention	• dose dependent ↓ in 5-HT concentration from MDMA concentrations of 10^{-3}-10^{-7} M with the exception of 10^{-4} (no Δ) • no Δ at concentration of 10^{-8} • ↑ 5-HT retention at MDMA concentration of	• Isomer: NR • PFC: No • Cross fostering: No • Litter effects No • n=3
(Bronson et al., 1994a)	8, 16, or 24	E14	Chicken	Single	*in ova*	Distilled water	Body weight, liver weight, & brain weight	• no Δ in brain, liver, or body weight	• Isomer: NR • PFC: No • Cross fostering: No • Litter effects No • n=20-24
(Broening et al., 1994)	10, 20, & 40	P10	Rat (SD)	Single	Orally	Saline	5-HT, 5-HIAA, DA concentration; 5-HT reuptake sites	• no Δ in 5-HT & 5-HIAA concentration on P17 frontal cortex, hippocampus, & caudate putamen • ↓ 5-HT concentration 3-24 hours after administration in frontal cortex, hippocampus, & caudate putamen • no Δ in DA concentration in caudate putamen	• Isomer: ±MDMA • PFC: No • Cross fostering: No • Litter effects Yes • n=6
(Broening et al., 1995)	20 or 40	P10	Rat (SD)	Single	Orally	Saline	Total thermal response, rectal temperature at different ambient temperatures, 5-HT concentration	• ↑ rectal temperature 2 hours following MDMA administration when animal is placed at 10°C • ↓ temperature at 10°C in animals treated with 40 mg/kg at 6 and 7 hours • ↓ temperature at 25°C in animals treated with 40 mg/kg • no Δ in rectal temperature when animals were placed at 33°C	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects Yes • n=6

								• no Δ total thermal response • no Δ in 5-HT concentration or uptake sites in frontal cortex, hippocampus, and caudate putamen • no Δ in ^{3}H-paroxetine binding	
(Colado et al., 1997a)	20	E14-17	Rat (Wistar)	2/day	s.c.	Saline	Litter size; offspring weight; 5-HT, 5-HIAA, & DA concentration in telencephalon, lipid peroxidation	• ↓ litter size • no Δ in P1 body weight • no Δ in 5-HT, 5-HIAA, or DA concentration • no Δ in lipid peroxidation	• Isomer: ±MDMA • PFC: No • Cross fostering: No • Litter effects No • n=5
(Aguirre et al., 1998)	20	Every other day from E6-20 or P14	Rat (Wistar)	Once	s.c.	Saline	5-HT & DA concentration	• no Δ in 5-HT in hippocampus, frontal cortex, striatum, & hypothalamus • no Δ in DA in striatum & hypothalamus	• Isomer: ±MDMA • PFC: No • Cross fostering: No • Litter effects No • n=10-12
(Whitworth et al., 2001)	10 uM	E17-P13	Rat (SD) thalamo-cortical neuron cultures	Once	Culture media	n/a	5-HT uptake; SERT expression; SERT levels with and without cocaine	• MDMA regulated SERT levels similarly to 5-HT • ↓ 5-HT uptake, SERT expression, & surface expression of SERT when MDMA and cocaine are combined compared to MDMA alone	• Isomer: NR • PFC: No • Cross fostering: No • Litter effects No • n=NR
(Broening et al., 2001)	5, 10, or 15	P1-10 or P11-20	Rat (SD)	2/day	s.c.	Saline	Straight channel swimming; CWM; MWM cued, acquisition, reversal, and reduced platform; 5-HT, DA, & NE concentration at adulthood	• ↓ weight gain during dosing period • no Δ straight channel swimming • no Δ in CWM errors and latency in animals treated P1-10 • ↑ CWM errors and latency in animals treated with 10 & 20 mg/kg on P11-20; no Δ in animals treated with 5 mg/kg • no Δ in MWM in animals treated P1-10 • ↑ path length, cumulative distance from target, and latency to goal in animals treated P11-20 with 20 mg/kg during acquisition phase of MWM • ↑ path length and cumulative distance in 10 mg/kg group treated P11-20 during acquisition phase of MWM; no Δ 5 mg/kg • no Δ reversal phase of MWM • ↑ latency, path length, and cumulative distance in animals treated P11-20 • ↑ average distance and ↓ platform crossings during acquisition, reversal, and reduced phase probe trials in 20 mg/kg group treated P11-20 • ↑ average distance in reversal probe trial in 5 mg/kg group treated on P11-20 • ↓ platform crossing in acquisition and reversal probe trials in 5mg/kg group treated P11-20 • ↑ average distance in reduced probe trials in 10 mg/kg group; ↓ platform crossings on third	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects Yes • N=15

								probe trial during reversal • P1-10 group: ↓ 5-HT (all groups) and NE (10 and 20 mg/kg groups) in hippocampus; no Δ in 5-HT, DA or NE in frontal cortex • P11-20 group: ↓ 5-HT & NE in the hippocampus; ↓ 5-HT in frontal cortex; no Δ in NE or DA in frontal cortex	
(Meyer and Ali, 2002)	10	P1-4	Rat (SD)	2/day	s.c.	Saline	^{3}H-paroxetine binding; 5-HT, 5-HIAA, DA, DOPAC & HVA concentration	• ↓ ^{3}H-paroxetine binding in hippocampus on P25 and 60 • ↓ ^{3}H-paroxetine binding in neocortex on P60; no Δ P25 • ↓ 5-HT concentration in hippocampus; no Δ in 5-HIAA or HVA • no Δ in 5-HT, 5-HIAA, DA,DOPAC, or HVA in neocortex	• Isomer: ±MDMA • PFC: No • Cross fostering: No • Litter effects No • n=6-7
(Won et al., 2002)	40	E5-12	C57Bl/6	2/day	s.c.	Saline	5-HT, 5-HIAA, DA, & DOPAC in reaggregate tissue culture	• ↑ 5-HT in reaggregates • ↑ 5-HIAA in reaggregates and culture media • no Δ in DA in reaggregates • ↑ DOPAC in culture media and reaggregates	• Isomer: ±MDMA • PFC: No • Cross fostering: No • Litter effects No • n=9
(Kelly et al., 2002)	20	E15-18; P10; P15; P20; or P10-13	Rat (SD)	2/day	s.c.	Untreated	^{3}H-paroxetine & SERT binding at P40; glucouse utilization	• no Δ in ^{3}H-paroxetine uptake • no Δ in SERT • ↑ glucose utilization in locus coeruleus, inferior olive, nucleus ambiguous, trigeminal nucleus, subiculum of hippocampus, anterior thalamus, medial hypothalamus, septal nucleus, globus pallidus in animals treated E15-18; no Δ with postnatal treatment	• Isomer: ±MDMA • PFC: No • Cross fostering: No • Litter effects No • n=5-6
(Williams et al., 2003)	20	P11-20	Rat (SD)	2/day	s.c.	Saline, weighed only, and large litters	Straight channel swimming; CWM; MWM acquisition, shifted and reduced hidden platform & probe trials; MWM cued; CORT and ACTH baseline and following forced swim	• ↓ weight gain P12-28 • no Δ straight channel swimming • ↑ latency in males on trials 4 & 5 of CWM • ↑ errors in males during CWM • ↑ latency, path length, and cumulative distance during acquisition phase of MWM • ↑ average distance during MWM acquisition probe trials; no Δ in crosses • ↑ cumulative distance during reversal phase of MWM; no Δ latency and path length • no Δ reversal probe trials • ↑ path length and cumulative distance in reduced platform MWM trials; no Δ latency • ↓ average distance on probe trials during reduced platform trials; no Δ in crossings • no Δ MWM cued trials • no Δ in CORT and ACTH in baseline and forced swim	• Isomer: ±MDMA • Wgt: Yes • Cross fostering: No • Litter effects Yes • N=15
(Koprich et al., 2003b)	15	E14-20	Rat (SD)	2/day	s.c.	Saline	Locomotor activity, tyrosine hydroxylase	• ↓ body weight on P1, no Δ beginning on P3 due to culling method	• Isomer: ±MDMA • Wgt: Yes

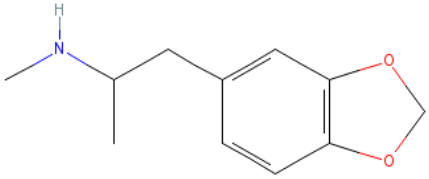
							immunoreactivity; DA, DOPAC, HVA, 5-HT, & 5-HIAA on P3 and P21	• ↑ locomotor activity on P21 • ↑ TH immunoreactivity in str, N.Acc. & frontal cortex on P21 • no Δ in monoamine concentrations in FC on P3 or P21 • ↓ HVA in str on P3 & P21, ↓ DOPAC , 5-HIAA in str on P21; no Δ for rest of monoamines on P3 or P21 in str • ↓ 5-HIAA in N.Acc.; no Δ remaining in remaining monoamines examined	• Cross fostering: No • Litter effects Yes • N=8
(Koprich et al., 2003a)	20	P11-20	Rat (SD)	2/day	s.c.	Saline	DA, DOPAC, HVA, 5-HT & 5-HIAA concentrations on P21; BDNF concentrations on P21	• ↓ weight gain on P21 • ↓ 5-HT in FC; ↓ 5-HT in str of females and in hippocampus of males; no Δ in the brain stem • ↑ 5-HIAA in str, FC, brain stem and hippocampus on P21 • no Δ DA • ↑ DOPAC in str; ↑ DOPAC in FC of females; no Δ DOPAC in brain stem and hippocampus • ↑ HVA in str, frontal cortex, hippocampus and brain stem • ↑ BDNF in hippocampus, str, FC and brain stem	• Isomer: ±MDMA • Wgt: Yes • Cross fostering: No • Litter effects Yes • N=8
(Vorhees et al., 2004)	5, 10, & 20	P11-20	Rat (SD)	2/day	s.c.	Saline	Straight channel swimming; MWM acquisition and probe; Barnes maze counter balanced so ½ of litter were run before MWM and ½ were run after MWM; working memory in MWM	• ↓ weight gain during dosing • no Δ straight channel swimming or cued MWM • no Δ Barnes maze • ↑ latency, path length, cumulative distance in MWM on P60 • ↑ Ave distance in probe trials in 20 mg/kg treated males that went through MWM first • no Δ in working memory • no Δ in MWM for animals that went through Barnes maze first	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects Yes • N=16
(Meyer et al., 2004)	10	P1-4	Rat (SD)	2/day	s.c.	Saline	Body temperature; cleaved caspase-3 on P5; 5-HT, 5-HIAA, DA, DOPAC, HVA concentrations on P25; paroxetine binding on P25 & P60; SERT immunoreactivity on P90	• no Δ temperature at ambient temperatures of 31 or 37 during dosing • ↓ body weight during dosing • ↑ cleavage of Caspase-3 in rostral forebrain and hippocampus on P5 • ↓ 5-HT in the hippocampus on P25; no Δ 5-HIAA • no Δ 5-HT, 5-HIAA, DA, DOPAC or HVA concentrations in the frontal cortex on P25 • ↓ paroxetine binding on P25 & P60 in hippocampus • ↓ paroxetine binding on P60, no Δ on P25 in neocortex	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: No • N=6-7

								• ↓ SERT immunoreactivity in primary visual cortex and somatosensory cortex • ↑ SERT in caudate putamen & N.Acc. shell on P90 • no Δ SERT immunoreactivity on P90 in hippocampus, lateral hypothalamus or frontal cortex	
(Cohen et al., 2005)	20	P11-20	Rat (SD)	2/day	s.c.	Saline	Interaction of neonatal dosing and adult dosing (~P90 1 day 10 mg/kg 4 x day) on MWM, CWM, Zero Maze, NOR, locomotor activity; and 5-HT, 5-HIAA, DA, & DOPAC concentrations	• no Δ zero maze • ↑ latency, path length & cumulative distance in MWM acquisition for neonatal treatment; no interaction with adult treatment • ↑ latency, path length & cumulative distance in reversal phase of MWM in animals treated with MDMA as neonates and as adults • ↑ errors in CWM; no Δ latency & no adult MDMA interaction • ↓ time spent observing the novel object; no adult MDMA interaction • ↓ center and total distance in locomotor activity; no adult MDMA interaction • ↓ 5-HIAA in neonatal animals in hippocampus; no Δ for neonatal treatment for 5-HT • no Δ neonatal treatment on 5-HT, 5-HIAA, DA, or DOPAC in Str and prefrontal cortex	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: Yes • N=15
(Galineau et al., 2005)	10	E13-20	Rat (Wistar)	1/day	s.c.	Saline	DA, DOPAC, HVA, 5-HT & 5-HIAA concentrations at E14, 16, 18, 20, P0, 7, 14, & 21, [^{125}I]PE2I and [^{3}H] MADAM binding at previously mentioned points starting with E18; DA release in striatum after tyramine stimulation at P70; 5-HT release in the hippocampus after fenfluramine stimulation at P70; sucrose preference at P70	• no Δ in whole brain concentrations of DA, DOPAC, or HVA at all times examined • ↓ 5-HT & 5-HIAA in whole brain at P0; no Δ at other points examined • no Δ [^{125}I]PE2I or [^{3}H] MADAM binding in raphe nucleus, hypothalamus, thalamus, hippocampus or cortex • ↓ DA release following tyramine stimulation at P70 • ↓ 5-HT release following FEN stimulation on P70 • ↓ sucrose preference on P70	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: No • N=3-6
(Williams et al., 2005)	10	P11	Rat (SD)	4/day	s.c.	Saline	Thymus weights; CORT; ACTH; DA, DOPAC, 5-HT & 5-HIAA concentrations; paroxetine binding at	• ↓ thymus weight in females at 78 h after 1st dose; no Δ in males & at other time points • ↑ CORT at 1, 7 & 30; no Δ 78 h after 1st dose • ↓ 5-HT in hippocampus at all time points • ↓ 5-HIAA in hippocampus at 6, 24 & 30 h; no Δ at 1 or 78 h	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: Yes • N=8

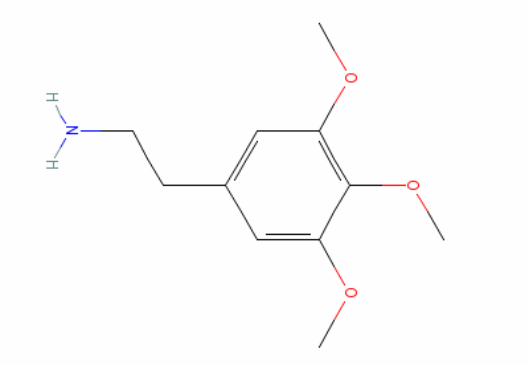
							1, 7, 24, 30, & 78 hours following the 1st dose	• ↓ 5-HT at 24, 30 & 78 h in the str • ↓ 5-HIAA at 24 & 30 h in the str • no Δ in DA in the str; ↓ DOPAC in the str at 1, 6 & 24 h • no Δ in paroxetine binding	
(Crawford et al., 2006)	20	P11-20	Rat (SD)	2/day	s.c.	Saline	5-HT, 5-HIAA, DA, DOPAC concentrations on P90; PKA activity; [^{35}S]GTPγS binding following NPA, 5-HT, or R(+)-8-OH-DPAT stimulation on P90	• ↓ 5-HT in the PFC & hippocampus; no Δ in the str; no Δ 5-HIAA concentrations • ↓ DA in PFC; ↓ str DA in males, no Δ in females; no Δ DOPAC • ↓ PKA activity in PFC & hippocampus; no Δ in the str • no Δ in basal [^{35}S]GTPγS in PFC, str, hippocampus • no Δ in efficacy or potency of NPA-stimulated [^{35}S]GTPγS binding in the PFC or str • ↑ 5-HT stimulated [^{35}S]GTPγS binding in the PFC • ↓ 5-HT stimulated [^{35}S]GTPγS binding in the str • ↑ efficacy of R(+)-8-OH-DPAT and 5-HT stimulated [^{35}S]GTPγS binding in the hippocamus	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: Yes • N=NR
(Piper and Meyer, 2006)	10	P1-4	Rat (SD)	2/day	s.c.	Saline	Object recognition memory; temperature dysregulation & serotonin syndrome following MDMA challenge (10 mg/kg) at P100; SERT binding at P70 & P105	• No Δ in object recognition memory • ↑ temperature dysregulation and serotonin syndrome after MDMA challenge • no Δ in SERT binding in cortex & hippocampus on P70 or P105	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: No • N=11
(Schaefer et al., 2006)	10	P11	Rat (SD)	4/day	s.c.	Saline	Plasmatic CORT concentration; DA, DOPAC, 5-HT & 5-HIAA concentrations 24 hours after 1st dose	• ↑ CORT on P12 • ↓ 5-HT & 5-HIAA in the str & hipp on P12 • no Δ DA in str; ↓ DOPAC	• Isomer: ±MDMA • Wgt: No • Cross fostering: No • Litter effects: Yes • N=8

Figure 1. Comparison of the chemical structure of MDMA, mescaline, fenfluramine and amphetamine

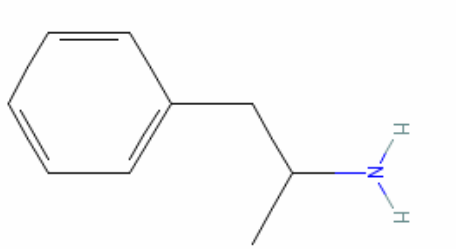
MDMA



Mescaline



Amphetamine



Fenfluramine

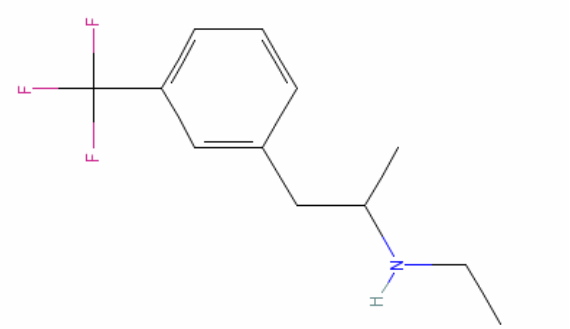


Figure 2. Timetables of rat and human neuorgenesis in the hippocampus. The dentate gyrus of the rat develops during the neonatal period, similar to second and third trimester human brain development. *Adapted from Bayer et al. 1993.*

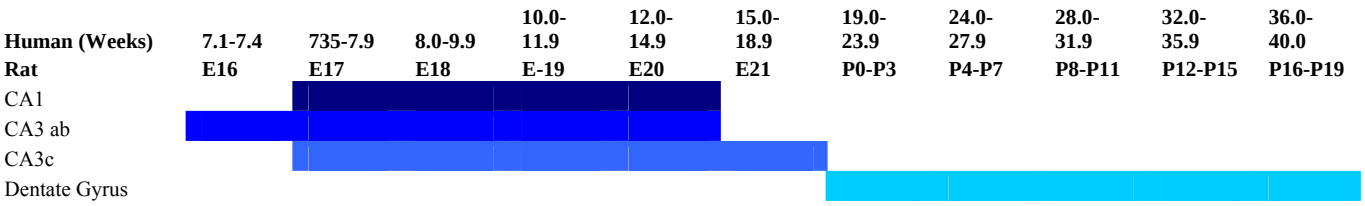
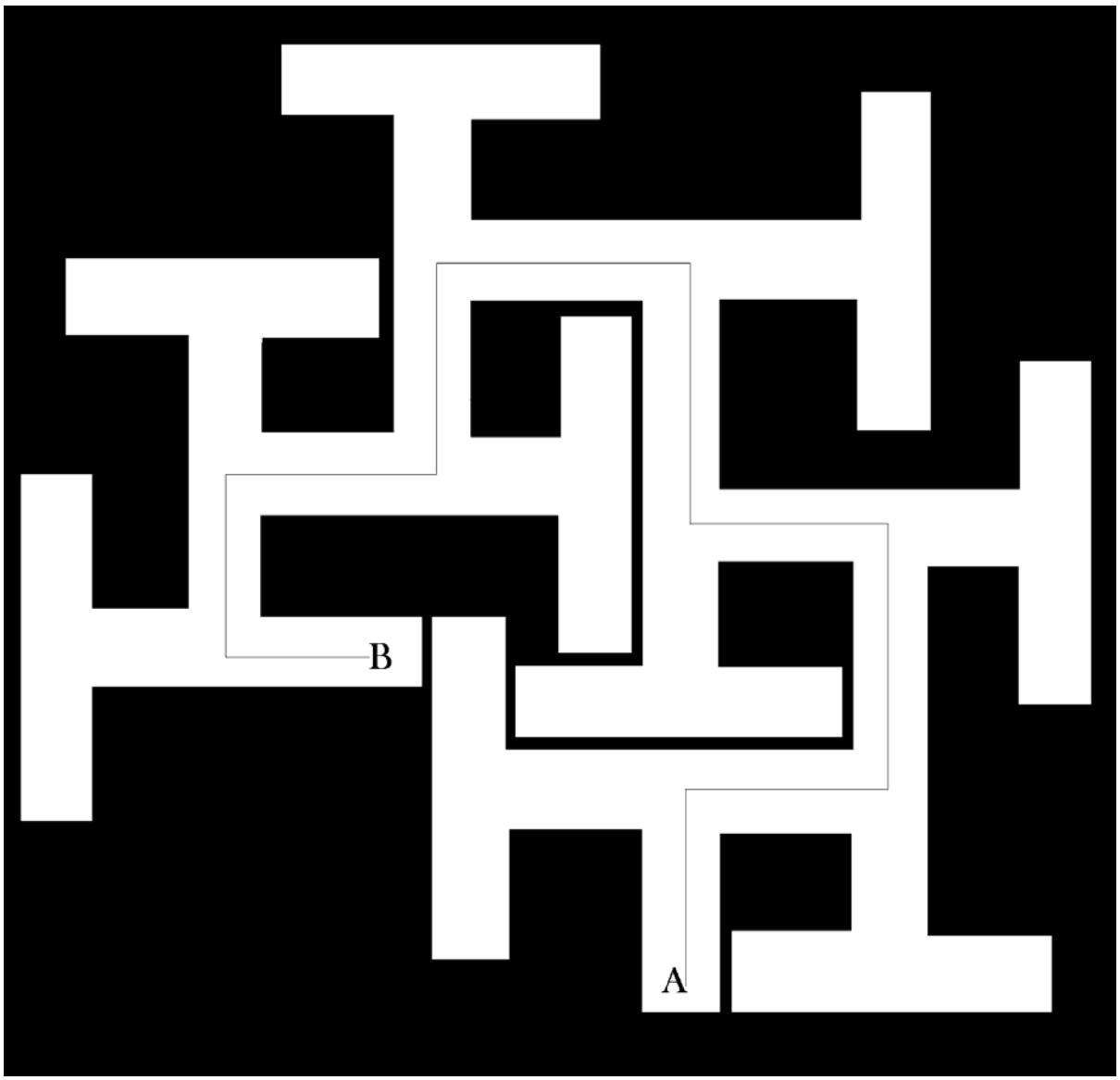

Human (Weeks)
7.1-7.4
735-7.9
8.0-9.9
10.0-11.9
12.0-14.9
15.0-18.9
19.0-23.9
24.0-27.9
28.0-31.9
32.0-35.9
36.0-40.0
Rat
E16
E17
E18
E-19
E20
E21
P0-P3
P4-P7
P8-P11
P12-P15
P16-P19
CA1
CA3 ab
CA3c
Dentate Gyrus

Figure 3. A diagram of the Cincinnati water maze. The CWM is a test of path integration learning. Animals are generally started at point “B” with the escape ladder located at point “A”.


B
A

Hypothesis and Specific Aims

Hypothesis

Exposure to MDMA from P11-20 will produce learning and memory deficits that emerge at an early age (P30) and persist well into adulthood (P360). These learning and memory deficits are likely a result of several independent mechanisms, many of which are not likely to be explored in classical pharmacological studies. It is hypothesized that MDMA exposure should cause a wide array of changes in gene expression, any of which could be further examined in relation to the mechanisms of developmental MDMA exposure in relation to learning and memory.

Specific Aims

1. To determine the emergence and persistence of learning and memory deficits due to P11-20 MDMA exposure.
2. To characterize the gene expression differences between MDMA and saline treated animals.
3. To examine how genes identified by the microarrays interact with other proteins within pathways.
4. To examine how these alterations affect the electrophysiology of the neurons within the hippocampus.

CHAPTER 2: Treatment with MDMA from P11-20 disrupts spatial learning and path integration learning in adolescent rats but only spatial learning in older rats

As submitted to Psychopharmacology (April 4, 2006)

ABSTRACT

Rationale: Previous studies in rats have shown that postnatal day (P)11-20 exposure to ±3,4-methylenedioxymethamphetamine (MDMA; ecstasy) causes learning and memory deficits in adulthood. The emergence and permanence of these learning deficits are currently unknown. *Objective:* This study was designed to investigate learning and memory deficits in adolescent (P30 or P40) and older (P180 or P360) rats exposed to MDMA from P11-20. *Methods:* Within each litter half the animals were exposed to MDMA (20 mg/kg) and half to saline twice a day (8 h apart) from P11-20. In Experiment 1, behavioral testing began on either P30 or P40, whereas in Experiment 2, testing began on either P180 or P360. Offspring were tested in the Cincinnati water maze (CWM), a test of path integration learning (2 trials/day for 5 days), and the Morris water maze (MWM) (three phases, with 5 days of 4 trials/day and a probe trial on the sixth day per phase). *Results:* MDMA-treated rats took longer to find the platform and traveled a greater distance to find the platform at all ages tested in all phases of the MWM. MDMA-treated animals also spent less time in the target quadrant during probe trials. In the CWM, P30 and P40 animals took longer to find the goal and committed more errors in locating the goal, while P180 and P360 MDMA-treated animals performed similarly to saline-treated animals. *Conclusion:* The data suggest that the spatial learning and memory deficits induced by MDMA are long lasting, while the path integration deficits recover over time.

Introduction

The principle abusers of the popular club drug 3,4-methylenedioxymethamphetamine (MDMA) are young adults (Johnston, O'Malley et al. 2005a;Johnston, O'Malley et al. 2005b). Women who use MDMA during these peak reproductive ages may become pregnant and some will continue to use MDMA throughout pregnancy (Ho, Karimi-Tabesh et al. 2001). Maternal use of substituted amphetamines, including MDMA and methamphetamine (MA) result in exposure to the fetus, as these substances cross the placental (Burchfield, Lucas et al. 1991) and the blood brain barriers. To date, there have been few clinical studies of the effects of prenatal exposure to MDMA. The extant clinical data suggest that MDMA exposure may increase the incidence of congenital malformations, namely cardiac malformation and club foot (McElhatton, Bateman et al. 1997;McElhatton, Bateman et al. 1999). No studies have specifically investigated the cognitive and behavioral alterations of children prenatally exposed to MDMA.

The neonatal rat can be used as a model of second and third trimester human development because brain development in some regions is comparable between the two organisms at this stage. For example, the development of the granule cells of the dentate gyrus continues to postnatal day (P)19 in the rat and until birth in the human (Bayer, Altman et al. 1993). Exposure to MDMA from P11-20 in rats induces spatial and path integration learning and memory deficits when the animals are tested during adulthood; however exposure from P1-10 induces no deficits in either learning paradigm (Broening, Morford et al. 2001). In our previous studies using neonatal MDMA exposure (Broening, Morford et al. 2001;Cohen, Skelton et al. 2005;Vorhees, Reed et al. 2004;Williams, Morford et al. 2003), spatial learning was analyzed using the Morris water maze (Morris, Garrud et al. 1982). MDMA-treated animals showed increased latencies, path lengths, and cumulative distances from the platform during

acquisition, reversal (where the platform is moved to the opposite quadrant), and reduced platform (the platform is reduced from 10 cm x 10 cm to 5 cm x 5 cm and moved to the original quadrant) phases of hidden platform training (Broening, Morford et al. 2001;Cohen, Skelton et al. 2005;Vorhees, Reed et al. 2004;Williams, Morford et al. 2003). During the probe (memory) trials of each phase, MDMA-treated animals had a greater average distance from the platform than saline (SAL)-treated animals. The learning and memory deficits in the MWM appear to be independent of sensorimotor changes, as MDMA-treated animals performed similarly to SAL-treated animals in the visible platform phase of the MWM (Broening, Morford et al. 2001;Williams, Morford et al. 2003). Path integration learning has been assessed using a modified Biel maze known as the Cincinnati water maze (CWM; described in Methods and (Vorhees 1987b)). MDMA-treated animals took longer to find the escape and committed more errors compared to their SAL-treated counterparts in the CWM (Broening, Morford et al. 2001;Cohen, Skelton et al. 2005;Williams, Morford et al. 2003). The learning deficits are independent of undernutrition, since MDMA-treated animals showed a decrease in performance in the MWM and the CWM compared to animals raised in a large litter that mimicked undernutrition produced by MDMA exposure (Williams, Morford et al. 2003). The effects of MDMA also are independent of litter composition or maternal behavior (Williams, Morford et al. 2003), since use of either a between or within litter design for MDMA treatment produced the same deficits on MWM performance. Maternal behavior could be an issue because neonates exposed to MDMA from P1-4 exhibit increased activity and ultrasonic vocalizations (Winslow and Insel 1990); behaviors that are known to affect dam/pup interactions. While this exposure period is different from ours, the concern remained, however, the lack of differences between using whole litters treated identically compared to litters split with all treatments within each

litter demonstrates that differences in maternal behavior during the ages of P11-20 do not significantly modify the learning deficits induced by MDMA treatment during this interval. Taken together, the data demonstrate that MDMA exposure during early development alters learning and memory independent of these other factors. Nonetheless, these studies have not examined the emergence or permanence of the learning deficits.

The purpose of this experiment was to examine the emergence and the relative permanence of the MWM and CWM deficits seen in neonatally MDMA-treated animals. Two experiments were performed. The first experiment examined spatial and path integration learning and memory when tested early, during adolescence (P30 and P40), while the second experiment examined learning and memory when tested much later than in previous studies, during later adulthood (P180 and P360).

Methods

Subjects and treatments

Nulliparous female Sprague-Dawley CD, IGS rats were obtained from Charles Rivers Laboratories (Raleigh, N.C.) and allowed to acclimate to the temperature (21°±1) and light cycle (14:10h light-dark cycle with lights on at 0600 h) of the vivarium for a minimum of one week prior to breeding with males of the same strain. Food and water were available *ad libitum* throughout the experiment. Breeding occurred in hanging wire cages and the day a sperm plug was detected was considered embryonic day (E) 0. On E14 females were singly housed in polycarbonate shoebox cages (46 x 24 x 20 cm) and left undisturbed until parturition. Date of birth was considered P0, and on P1 litters were culled to 8 with equal numbers of males and females. Animals were randomly assigned to one of two treatment groups within each litter. Half of the litter, divided equally by sex, received ±MDMA (20 mg/kg, expressed as freebase;

>95% pure and obtained from NIDA) twice a day delivered 8 h apart while the other half received SAL. MDMA or SAL was administered subcutaneously in the dorsum at a dosing volume of 3 ml/kg. Injection sites were varied and no visible signs of skin lesions were observed. The vivarium is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care and all protocols were approved by the Cincinnati Children's Research Foundation's Animal Use and Care Committee. For each experiment, twenty litters were used. For Experiment 1, litters were divided equally so that half of the litter, with one treatment pair per sex, began behavioral testing at P30 while the other half of the litter began testing at P40. Eight groups were created per litter with this design split by age tested, treatment, and sex. Experiment 2 followed the same design with the exception that testing began on either P180 or P360.

Straight Channel Swimming

Beginning on the appropriate day, animals were tested in a 15 x 244 cm straight channel filled to 35 cm with 21 ± 1°C water and with a wire escape ladder mounted on one end. Animals were placed in the channel facing away from the ladder (i.e. facing the back wall of the tank). Latency to escape was measured on 4 consecutive trials with a maximum time of 2 min per trial with no inter-trial interval (ITI).

Cincinnati Water Maze

Three days following straight channel, animals were tested in the CWM for 6 days (Experiment 1) or 5 days (Experiment 2). The CWM, described in detail previously (Vorhees 1987), is a 9 unit multiple-T maze constructed of black acrylic and positioned in a larger square tank. The cul-de-sacs and main channel are 15 cm wide and the walls are 51 cm high. Water depth is 25 cm and maintained at a temperature of 21 ± 1°C. Testing occurred under a single red

25W light bulb to minimize the use of distal cues. Animals were started facing the wall in position B as described in Vorhees (1987) and were allowed 5 min to attempt to escape the maze at position A, where a wire ladder was placed for escape. Animals were administered two trials per day for five days with an ITI of 5 min. Latency to escape the maze and errors of commission were counted for each trial. An error was defined as a whole body entry into a dead-end arm. Repetitive arm entries within a dead-end arm of the maze were each counted as multiple errors; one for entering the first arm of the T and one for each subsequent crossing into an opposite arm.

Morris Water Maze

The MWM is 210 cm in diameter, constructed of stainless steel and painted black. On three walls (arbitrarily representing the N, E, and W points) large geometric figures were mounted. White curtains, which can be opened or closed to reveal or obscure room cues, surround the maze. Each phase consisted of 4 trials per day for 5 days, and on the sixth day a probe trial (30 s) was performed. The time limit for each learning trial was 2 min; animals that failed to find the platform were placed on the platform. The ITI was 15 sec on the platform. The platforms were either 10 x 10 cm or 5 x 5 cm and made of acrylic with a thin nylon mesh attached. The platforms were submerged 1.5-2.0 cm below the water. Water temperature was 21±1° C.

Animals were tested in three phases of the MWM: acquisition, reversal, and reduced. The acquisition phase began 1 (Exp 1) or 2 (Exp 2) days after the cessation of the CWM and subsequent phases began the one day after the probe trail of the previous phase. In each phase, platform position was counter balanced so that during acquisition, half the litters were trained to the platform in the NE quadrant, while the second half was trained to the SW quadrant. For the reversal phase, the platform was moved to the opposite quadrant of the acquisition phase (i.e.,

those trained to the NE were now trained to the SW). For the reduced phase, the platform was moved back to the quadrant used for acquisition. The 10 x 10 cm platform was used in the acquisition and reversal phases, while the 5 x 5 cm platform was used in the reduced phase. Four start positions were used and alternated randomly with the exception that no start position was used twice on a given day and the pattern of start positions was varied on consecutive days. The start positions used for the SW were N, E, SE, and NW. The start positions used for the NE were S, W, NW, and SE. On probe trials, the platform was removed and the animals were given one 30 s trial beginning from a novel start location (NE for SW platform; SW for NE platform).

Performance was recorded using a Polytrack video tracking system (San Diego Instruments, San Diego, CA). Latency, path length, cumulative distance, and first bearing were measured for learning trials. For probe trials, average distance, first bearing, platform crossings, time in target quadrant, and distance in the target quadrant were measured. Cumulative distance is the sum of the distances the animal was from the platform measured in 55 ms intervals. First bearing was calculated by measuring the heading after the first 13 cm of travel relative to a straight line from the start position to the platform.

Statistical Methods

Because each experiment used a split litter design, offspring were matched on multiple factors by virtue of being littermates(Kirk 1993). To ensure that litter effects were controlled, litter was treated as a random factor (block) in a complete randomized block model analysis of variance (ANOVA). In this model, group, sex, and age at testing were between factors within each block and litter was the blocking factor. Measures taken repetitively on the same animal were treated as repeated measure factors. For maze testing, the ANOVA was a 3 between, 1 within randomized block model. In this model, treatment had 2 levels (MDMA or SAL), sex had

2 levels, and age had 2 levels (P30/P40 for experiment 1 or P180/P360 for experiment 2), while test interval had 5 levels (day). Data were analyzed using SAS Proc Mixed (SAS Institute, Carey, N.C.). Each model was checked for best fit against covariance matrix models provided by Proc Mixed. Autoregressive (AR(1)) covariance structure was optimal in most cases, however in a few cases compound symmetry (CS) was the better fit. Proc Mixed provides adjusted degrees of freedom and do not match those used in standard ANOVAs and therefore can be fractional or different even among multiple dependent variables within a given behavioral test (i.e., latency, path length and cumulative distance in the MWM). Significant interactions were analyzed using one-way simple-effect slice ANOVAs at each level of the repeated measure factor. For the sake of clarity, F values will only be shown for effects involving treatment. Significance was considered at $p \leq 0.05$ and trends at $p \leq 0.1$. Data are presented as the least square means ± the standard error of the mean derived from the least square ANOVA from Proc Mixed.

Results

Body Weights

For experiment 1, a main effect of treatment [$F(1,149)=208.54$, $P<0.0001$, Figure 1A] was observed during the dosing period with MDMA-treated animals weighing less than SAL-treated animals. A treatment x day interaction was also observed [$F(9,1382)=54,25$, $P<0.0001$] and showed that beginning on P12, MDMA-treated animals weighed significantly less than SAL-treated counterparts. Following weaning, a main effect of treatment was observed for body weight [$F(1,85.6)=7.05$, $P=0.0095$, Figure 1B], with MDMA-treated animals weighing significantly less than SAL-treated animals. There were significant sex (males weighed more

than females) and day (all animals gained weight over time) effects observed both in dosing and postweaning weights.

For experiment 2, a main effect of treatment [$F(1,137)=320.06$, $P<0.0001$, Figure 1C] was observed during dosing with MDMA-treated animals weighing less than SAL-treated animals. A treatment x day interaction [$F(9,1398)=13.84$, $P<0.0001$] was observed with MDMA-treated animals having significantly lower body weights beginning on P11 than SAL-treated animals. For adult weights, no significant main effect or interaction with treatment was observed (Figure 1D). Main effects of sex and day were observed during dosing and adulthood with effects similar to those in Experiment 1.

Experiment 1: P30 and P40 testing

Straight Channel Swimming

No main effect for treatment was observed in latency to escape the 244 cm straight channel.

Cincinnati Water Maze

MDMA-treated animals took longer to reach the escape ladder [$F(1,131)=11.55$, $P=0.0009$, Figure 2A] and committed more errors [$F(1,133)=8.45$, $P=0.0043$, Figure 2B] than SAL-treated animals. No treatment x age effect was observed. A trend for a treatment x day interaction was observed for latency [$F(5,551)=2.07$, $P=0.0674$] but not for errors. *Post hoc* analysis showed that MDMA-treated animals took longer to reach the escape ladder on days 1-4 compared to SAL-treated animals, and asymptotic levels were achieved by day 5 for both treatments (not shown).

Morris Water Maze-Acquisition Phase

Path length is shown as the representative measure (Figure 3A). MDMA-treated animals took longer to find the hidden platform [$F(1,125)=17.21$, $P<0.0001$], traveled a greater path [$F(1,124)=23.77$, $P<0.0001$], had greater cumulative distances from the platform [$F(1,126)=27.58$, $P<0.0001$], and started at a greater first bearing to the platform [$F(1,125)=24.12$, $P<0.0001$] compared to SAL-treated animals. A trend for a treatment x day interaction for cumulative distance [$F(4,415)=2.35$, $P=0.0533$] was the only interaction seen during the acquisition phase. This interaction was not followed up because it was not supported by comparable effects on in any other measure. For all measures during all three phases of the MWM in this experiment, there was a main effect of day, with animals performing better over consecutive days of training.

During probe trials, MDMA-treated animals had a higher degree of first bearing [$F(1,104)$, $P=0.0032$], crossed the platform site less [$F(1,104)=8.24$, $P=0.005$], and spent less time [$F(1,104)=4.27$, $P=0.0413$] and traveled less distance in the target quadrant [$F(1,104)=5.24$, $P=0.0241$] than SAL-treated animals. Percent time in target quadrant is shown in Figure 4A. For time in the target quadrant, there was a treatment x sex interaction [$F(1,104)=4.33$, $P=0.0399$] and a treatment x age interaction [$F(1,104)=3.13$, $P=0.0800$]. For the treatment x age interaction, effect-slice ANOVA revealed treatment effects at P30 ($P=0.007$), however no effect on P40. Treatment x sex analysis revealed that females showed a treatment effect ($P=0.0041$), while males did not. For distance in the target quadrant, a trend for a treatment x age interaction was observed [$F(1,104)=3.33$, $P=0.0711$] and showed that P30 animals were affected by MDMA treatment ($P=0.0039$) while P40 animals showed no treatment effect.

Morris water maze-Reversal Phase

As seen during the acquisition phase, MDMA-treated animals had longer latencies [$F(1,137)=21.55$, $P<0.0001$], greater path lengths [$F(1,135)=22.06$, $P<0.0001$, Figure 3B] and cumulative distance from the platform [$F(1,135)=18.83$, $P<0.0001$], and had larger first bearings [$F(1,117)=16.94$, $P<0.0001$] than SAL-treated animals. Treatment x age effects were seen for latency [$F(1,134)=4.53$, $P=0.0351$], path length [$F(1,135)=7.18$, $P=0.0083$], and cumulative distance [$F(1,135)=6.73$, $P=0.0105$]. For latency, slice-effect ANOVA revealed significant treatment effects at P30 ($P<0.001$) and P40 ($P=0.0401$). For path length, MDMA treatment increased path length on P30 ($P<0.0001$) and showed a trend toward increased path length at P40 ($P<0.0798$). MDMA increased the cumulative distance from the platform in P30 animals ($P<0.0001$) but not on P40.

During reversal phase probe trials, MDMA treatment increased first bearing [$F(1,112)=7.78$, $P=0.0062$], decreased platform crossings [$F(1,112)=5.26$, $P=0.0237$], and decreased time [$F(1,112)=4.57$, $P=0.0347$, Figure 4B] as well as distance [$F(1,112)=4.91$, $P=0.0281$] in the target quadrant. There was also a trend towards a treatment effect in average distance from the platform [$F(1,112)=3.38$, $P=0.0686$]. Treatment x age interactions were observed for first bearing [$F(1,112)=10.59$, $P=0.0015$], platform crossings [$F(1,112)=7.25$, $P=0.0082$], and time [$F(1,112)=6.48$, $P=0.0123$] and distance [$F(1,112)=7.65$, $P=0.0066$] in the target quadrant. MDMA treatment increased first bearing ($P<0.001$), platform crossings ($P=0.0004$), and time ($P=0.0009$) as well as distance ($P=0.0004$) in the target quadrant on P30; however no effects of treatment were seen on P40.

Morris water maze-Reduced platform trials

Treatment effects were seen for latency [$F(1,136)=26.77$, $P<0.0001$], path length [$F(1,135)=24.47$, $P<0.0001$, Figure 3C], cumulative distance [$F(1,136)=22.74$, $P<0.0001$], and

first bearing [F(1,117)=14.37, P=0.0002]. Treatment x age interactions were observed for latency [F(1,136)=6.45, P=0.0122], path length [F(1,135)=7.27, P=0.0079] and cumulative distance [F(1,136)=7.59, P=0.0067]. For latency, MDMA treatment increased time to reach the platform at P30 (P<0.0001) and P40 (P=0.0333). For path length, MDMA treatment increased the distance to reach the platform on P30 (P<0.0001) and showed a trend toward increased path length on P40 (P=0.055). Similar effects were seen for cumulative distance (P30, P<0.0001; P40, P=0.08).

For probe trials during the reduced phase, MDMA treatment increased first bearing [F(1,109)=9.83, P=0.0022], reduced platform crossings [F(1,109)=6.33, P=0.0133], and time [F(1,109)=4.42, P=0.0378, Figure 4C] as well as distance [F(1,109)=5.03, P=0.0269] in the target quadrant. No treatment x age interactions were observed for any parameter.

Experiment 2:-P180 and P360 testing

Straight Channel swimming

No effects of treatment were seen for straight channel swimming.

Cincinnati Water Maze

No effects of treatment were observed for latency and errors in the CWM (Figure 5). An effect of day was observed for both measures, showing that the animals, regardless of treatment, learned the task. A main effect of age was observed for latency. Further investigation showed that P360 animals took longer to reach the platform than P180 animals.

Morris water maze--Acquisition

Similar to the effects observed in the young animals, MDMA-treated animals took longer to reach the platform [F(1,130)=9.08, P=0.0031], traveled a greater path to the platform [F(1,130)=9.22, P=0.0029, Figure 6A], remained farther away from the platform

[F(1,131)=11.86, P=0.0008], and started with a higher degree of first bearing [F(1,129)=38.19, P<0.0001] compared to SAL-treated animals. An age effect was observed for latency, path length, and cumulative distance that demonstrated that P180 animals found the platform faster and with a shorter distance traveled than P360 animals. A main effect of day was observed for this and all phases of the MWM in experiment 2 showing that all animals learned the task over days. No treatment x age interaction was observed for the acquisition phase of the MWM.

For the probe trial, neonatal MDMA treatment increased average distance from the platform [F(1,121)=7.11, P=0.0087], first bearing [F(1,121)=5.85, P=0.0171], and decreased platform crossings [F(1,121)=6.96, P=0.0094]. For platform crossings, an interaction of treatment x age [F(1,121)=4.46, P=0.0367] was observed and showed that MDMA-treated animals crossed the platform fewer times when tested at P180 compared to SAL-treated animals; however there was no treatment effect at P360. A main effect of sex was observed for average distance, first bearing, and platform crossings with males being closer to the platform, having a smaller first bearing, and crossing the platform more often than females.

Morris Water Maze-Reversal phase

Consistent with the acquisition phase of this experiment and the previous experiment, MDMA treatment increased latency [F(1,129)=15.53, P=0.0001] to reach the platform and also increased the path taken to the platform [F(1,130)=6.47, P=0.0121, Figure 6B] and the distance from the platform [F(1,131)=10.12, P=0.0018]. MDMA-treated animals also started with a greater first bearing to the platform [F(1,129)=50.91, P<0.0001]. For all measures, a sex effect was observed with males performing better in the maze compared to females. An age effect was observed for latency, path length and cumulative distance with younger animals finding the platform more efficiently than older animals. For path length, a treatment x sex x age interaction

was observed [F(1,130)=6.47, P=0.0453]. This interaction was minor and did not materially change the interpretation of the findings based on the main effects noted above.

In the probe trial, a main effect of treatment was observed for first bearing [F(1,128)=19.85, P<0.0001], platform crossings [F(1,128)=3.95, P=0.0489], as well as time [F(1,28)=4.29, P=0.0403, Figure 7B] and distance [F(1,128)=5.41, P=0.0216] in the target quadrant. MDMA-treated animals were farther off course, spent less time and traveled less in the target quadrant, and crossed the platform fewer times than SAL-treated animals. A treatment x sex x day interaction was observed for distance [F(1,128)=4.13, P=0.0441] and time in the target quadrant [F(1,128)=4.47, P=0.0364]. Subsequent analysis showed that MDMA treatment decreased time and distance in the target quadrant for males at P180 (P=0.011 and P=0.0050, respectively) and females at P360 (P=0.0178; P=0.0471, respectively).

Morris Water Maze-Reduced Phase

Main effects of treatment were observed with MDMA-treated animals having a longer latency [F(1,130)=25.30, P<0.0001], path length [F(1,128)=17.23, P<0.0001, Figure 6C], cumulative distance [F(1,129)=23.31, P<0.0001], and first bearing [F(1,129), P<0.0001]. Cumulative distance [F(1,129)=4.93, P=0.0281] and path length [F(1,128)=8.21, P=0.0049] showed a treatment x sex x age interaction that indicated that MDMA-treated females at P180 and MDMA-treated males at P360 had greater path lengths and were further from the platform than SAL-treated counterparts. Main effects of sex and day were observed on all measures, with males performing better than females and all animals had improved performance over days.

For probe trials, main effects of treatment were observed with MDMA-treated animals having greater average distances from the platform [F(1,123)=23.85, P<0.0001], decreased platform crossings [F(1,123)=4.57, P=0.0345], larger first bearings [F(1,123)=13.57, P=0.0003],

and decreases in time [F(1,123)=15.15, P=0.0002, Figure 7C] as well as distance [F(1,123)=23.85, P<0.0001] in the target quadrant. A treatment x age interaction was observed only for distance traveled in the target quadrant [F(1,123)=11.77, P=0.0120]. A main effect of sex was observed with males performing better than females during the probe trials.

Discussion

We have previously shown that MDMA treatment from P11-20 causes spatial and path integration learning and memory deficits when the animals are tested at P60 (Broening, Morford et al. 2001;Vorhees, Reed et al. 2004;Williams, Morford et al. 2003) and at approximately P90 (Cohen, Skelton et al. 2005). The purpose of this study was to further elucidate the behavioral effects of MDMA by testing younger (P30 and P40) and older (P180 and P360) rats in the MWM and the CWM. We found that MDMA treatment caused deficits in the hidden platform and probe trial phases of the MWM at all ages tested. Interestingly, path integration deficits, examined by the CWM, were seen in the animals tested at P30 or P40, but not in animals tested at P180 or P360. The path integration deficits do not appear to be the result of the SAL-treated animals performing more poorly in this task as they age, as can be seen by comparing Figure 2 to Figure 5. These data suggest that MDMA's effects on path integration learning are transient and persist only into early adulthood. Currently, the brain region or regions responsible for path integration learning are not entirely defined, although several regions have been implicated (see (Etienne and Jeffery 2004)). Since the MWM effects are persistent in this study and the path integration effects show recovery, it would appear that path integration and spatial learning occur in separate brain regions. While treatment effects were still significant in the MWM, age was also a factor, with P180 animals performing better than P360 animals in hidden platform

training. Interestingly, older animals appear to perform better in the learning trials of the task; however, they do not perform as well during the probe/memory trials.

This is the first study to examine path integration learning in younger rats (i.e., P30) using the CWM. As previously stated, the younger animals treated with saline performed similarly to their older counterparts. Path integration is thought to be one of the more basic types of learning found in organisms from ants to humans (Etienne and Jeffery 2004). Since path integration learning is more basic, it is likely that animals would acquire this skill at a younger age. The differences seen in the MDMA-treated animals at P30 compared to SAL-treated animals at this age show that the CWM is effective in detecting learning deficits in younger animals. The CWM may also be more effective in detecting learning deficits caused by amphetamine analogs. For example, adult animals treated with the potent 5-HT depleting agent fenfluramine fail to show deficits in the MWM, however they show deficits in CWM learning (Skelton, Blankenmeyer et al. 2004;Williams, Morford et al. 2002). It should be noted that developmental exposure to fenfluramine induces both CWM and MWM deficits (Morford, Inman-Wood et al. 2002). Administration of MDMA to adult animals produces CWM deficits while producing only memory deficits in the MWM (Able, Gudelsky et al. 2006;Cohen, Skelton et al. 2005;Sprague, Preston et al. 2003).

While MDMA-treated animals do not perform as well in the MWM as SAL-treated animals, the MDMA-treated animals do learn the task. Learning curves for treatments by age are shown in Figure 8. Similar learning curves were seen for the CWM. Interestingly, there are no effects of sex in the younger animals in the MWM while the older animals show sex-related learning differences; males performed better than the females in the MWM. This sex difference is likely due to the activational effects of gonadal steroids since the P30 and P40 animals were

tested prior to puberty or during the transition from puberty to adulthood. Therefore, the incomplete maturation of the gonadal hormones may prevent the sexually dimorphic effects seen in young-adult and older animals (tested beginning after P60) (Broening, Morford et al. 2001;Vorhees, Reed et al. 2004;Williams, Morford et al. 2003). Slight weight differences were seen in the younger animals; however these did not appear to affect the motor performance of MDMA-treated animals. For example, all animals performed similarly in straight channel swimming, a measure that has been previously shown to uncover motor deficits in offspring exposed to valproic acid (Vorhees 1987a). Offspring exposed to MDMA have also been shown not to be affected in the visible platform phase of the MWM (Broening, Morford et al. 2001;Vorhees, Reed et al. 2004;Williams, Schaefer et al. 2005), which further supports that MDMA does not induce deficits to sensorimotor systems.

The mechanisms related to MDMA-induced learning and memory deficits are still unclear. P11-20 MDMA exposure slightly decreases hippocampal serotonin levels when examined in adult animals, however these differences appear to be unrelated to the learning and memory deficits, since animals that were treated from P1-10 showed similar 5-HT reductions but no learning and memory deficits (Broening, Morford et al. 2001). Because of this finding, we did not measure neurotransmitter content in the current animals following behavioral testing. A possible mechanism involving serotonin (5-HT) signaling, however, does exist. Firstly, recent data has shown that following MDMA exposure on P11, hippocampal 5-HT levels drop sharply (Williams, Schaefer et al. 2005). Secondly, MDMA treatment from P11-20 increases the activity of 5-HT receptors, in particular 5-HT_{1A}, in the hippocampus (Crawford, Williams et al. 2006). Thirdly, depletion of 5-HT with the tryptophan hydroxylase inhibitor p-chlorophenylalanine (PCPA) from P10-20 has been shown to cause deficits in the radial arm maze in adult animals

(Mazer, Muneyyirci et al. 1997). Similar depletions have been shown to alter synaptic architecture and decrease long-term potentiation responses in brain slice preparations (for review, see (Sodhi and Sanders-Bush 2004)). We suggest that early 5-HT perturbation permanently increases 5-HT signaling, whereas the 5-HT reductions seen in adulthood may not as important as the changes in neurotransmitter receptor signaling that occur following the early reductions in 5-HT (Skelton, Blankenmeyer et al. 2004;Williams, Morford et al. 2002). Alterations in brain derived neurotrophic factor (BDNF) levels may be another potential influence for the learning deficits following MDMA administration, since it has been reported that P11-20 MDMA exposure increases BDNF levels in the hippocampus and striatum on P21 (Koprich, Campbell et al. 2003). This finding is important since BDNF is known to play a role in learning and memory function, neuronal plasticity, and survival (Figurov, Pozzo-Miller et al. 1996;Linnarsson, Bjorklund et al. 1997) and therefore disruptions to this system may influence learning ability. Another possible mechanism for the long-term learning and memory deficits may involve the hypothalamic-pituitary-adrenal axis. In rodents, the stress hyporesponsive period (SHRP) begins at approximately P2 and extends to P14 (Sapolsky and Meaney 1986). This is during a time of significant hippocampal neurogenesis, and the SHRP is thought to protect the developing neurons from the aversive effects of high levels of glucocorticoids. MDMA administration on P11 produces marked increases in corticosterone (CORT) levels (Williams, Schaefer et al. 2005), which could lead to alterations in neural development. Finally, preliminary data suggest that the NMDA receptor may play a role in MDMA induced learning and memory deficits (Skelton, Williams et al. 2005).

In conclusion, this study extends the characterization of MDMA induced learning and memory deficits to adolescent and older rats. Interestingly MDMA-treated animals continued to

have spatial learning deficits that lasted to one year of age, however path integration learning was only impaired in the younger animals.

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Figure 1. Mean (± SEM) body weights during dosing (A & C) and testing (B & D) for Experiment 1 (A & B) and Experiment 2 (C & D). Rats were treated with 20mg/kg MDMA or SAL twice daily from P11-20. MDMA administration produced significant decreases in weight gain from P11-20, however rats quickly return to control levels. *P<0.05

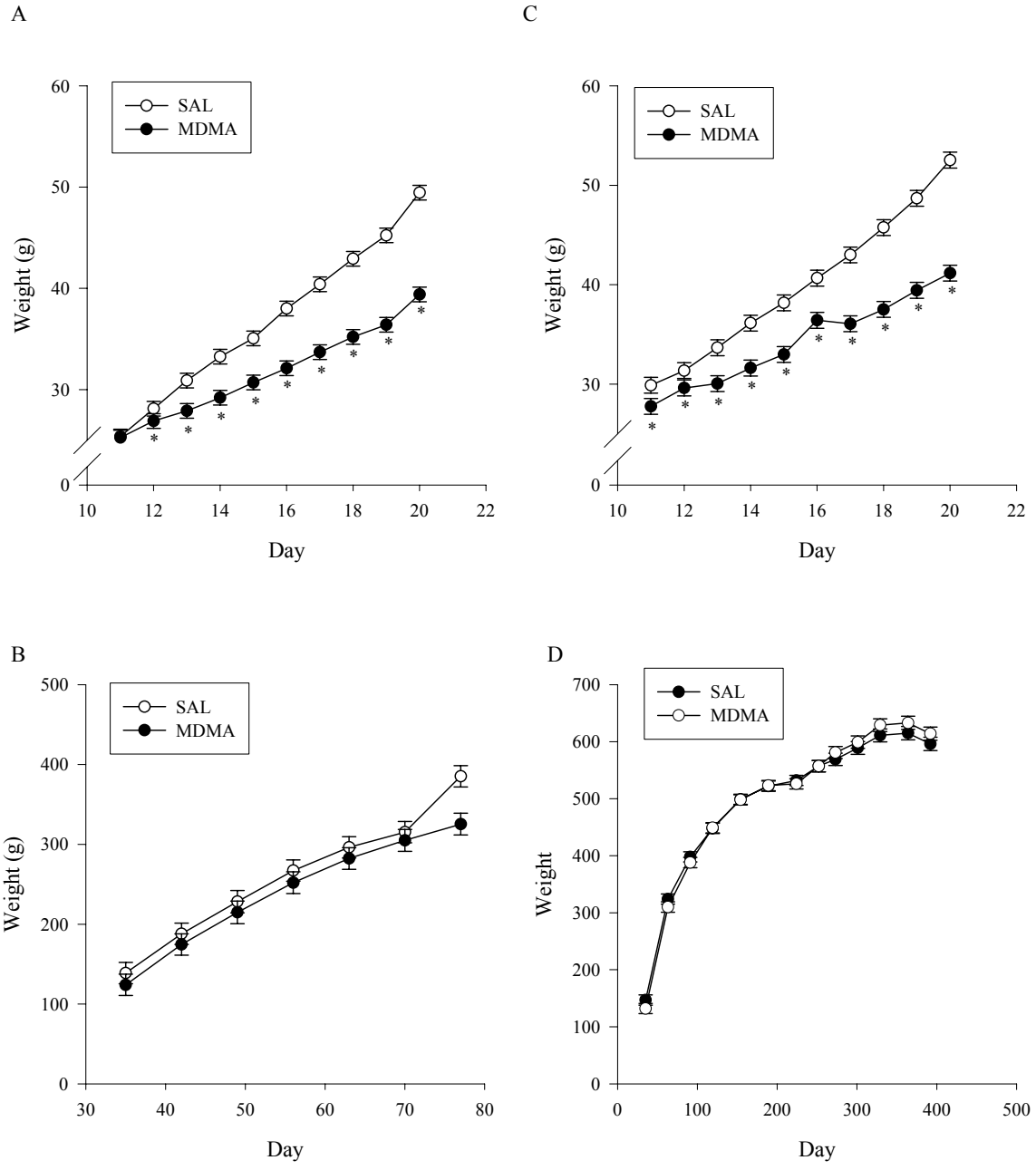

A
SAL
MDMA
Weight (g)
Day
C
SAL
MDMA
Weight (g)
Day
B
SAL
MDMA
Weight (g)
Day
D
SAL
MDMA
Weight
Day

Figure 2. Effects of P11-20 MDMA treatment in the CWM on P30 & P40. A main effect of treatment indicates that MDMA treatment increases A) latency and B) errors to complete the CWM. *** ($P<0.001$)

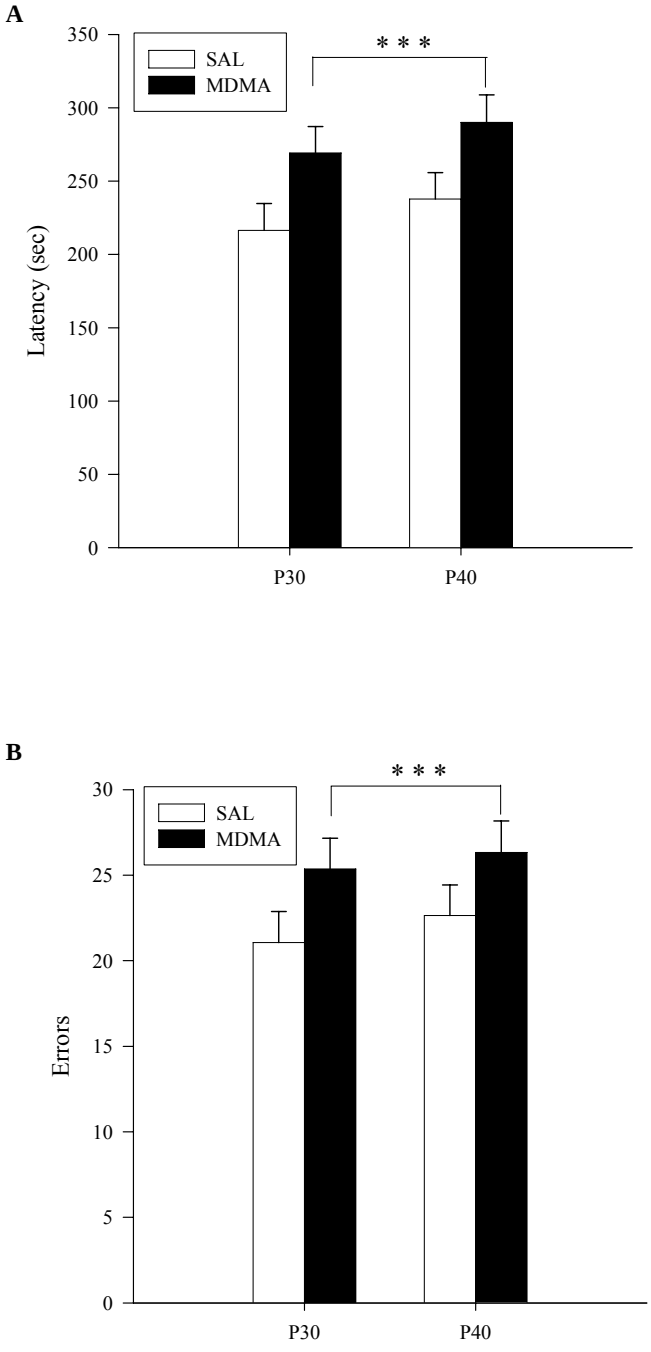

A
SAL
MDMA
* * *
Latency (sec)
350
300
250
200
150
100
50
0
P30
P40
B
SAL
MDMA
* * *
Errors
30
25
20
15
10
5
0
P30
P40

Figure 3. Mean path lengths to the hidden platform of the MWM. Rats treated with MDMA from P11-20 show a main effect of treatment with increased path length during acquisition (A), reversal (B), and reduced platform (C) phases of the MWM. *** $P<0.001$; †$P<0.10$

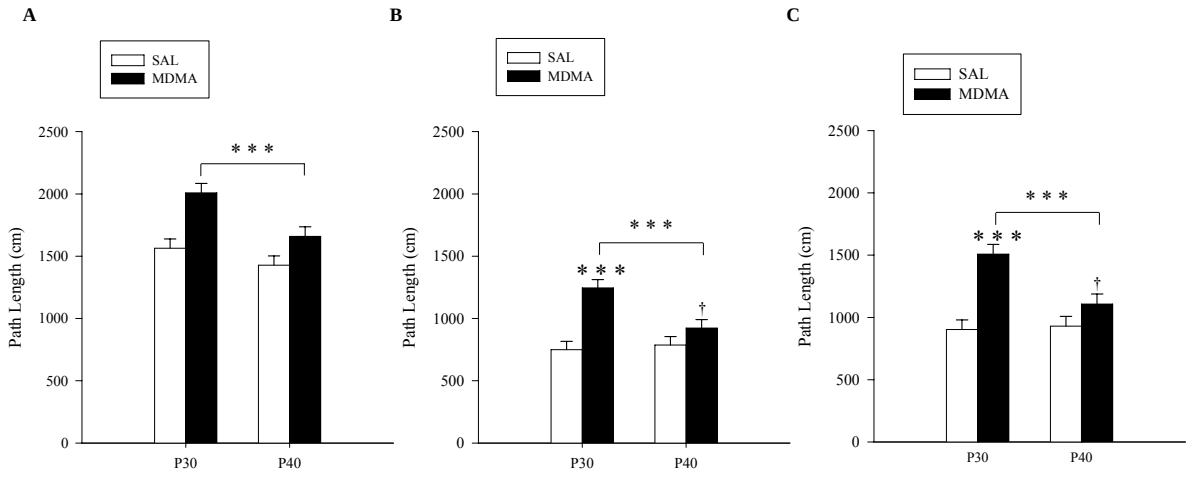

A
B
C
SAL
MDMA
Path Length (cm)
2500
2000
1500
1000
500
0
P30
P40
* * *
†

Figure 4. Percent time in target quadrant during probe trials. A main effect of P11-20 treatment with MDMA shows a decreased time in the target quadrant during acquisition (A), reversal (B), and reduced platform (C) phases of the MWM. *P<0.05, **P<0.01, ***P<0.001

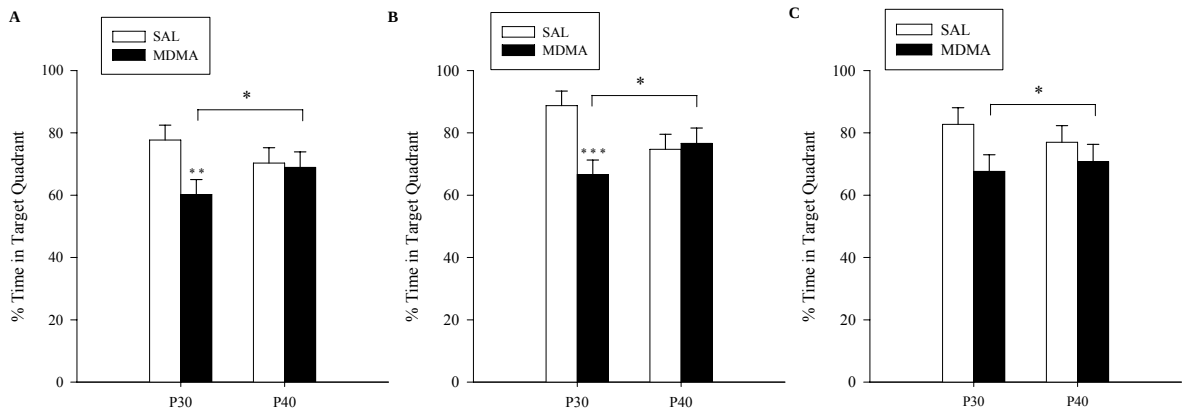
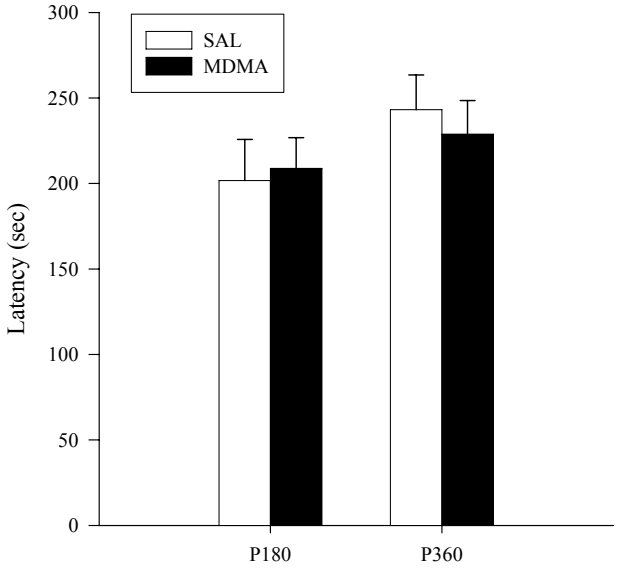

A
SAL
MDMA
% Time in Target Quadrant
100
80
60
40
20
0
*
**
P30
P40
B
SAL
MDMA
% Time in Target Quadrant
100
80
60
40
20
0
*
***
P30
P40
C
SAL
MDMA
% Time in Target Quadrant
100
80
60
40
20
0
*
P30
P40

Figure 5. P180 and P360 performance in the CWM. No main effect of treatment was observed for latency (A) or errors (B).

A


SAL
MDMA
Latency (sec)
300
250
200
150
100
50
0
P180
P360

B

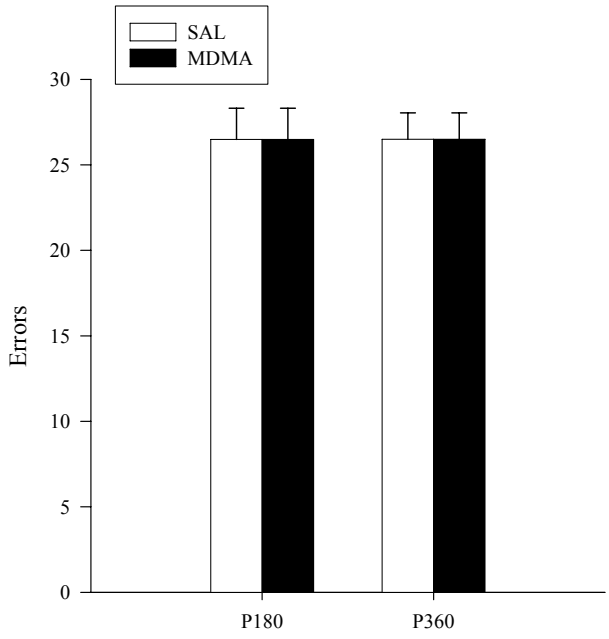

SAL
MDMA
Errors
30
25
20
15
10
5
0
P180
P360

Figure 6. Mean path lengths to the hidden platform of the MWM. Main effects of treatment were observed with MDMA treated animals having greater path lengths in the acquisition (A), reversal (B), and reduced platform (C) phases of the MWM.

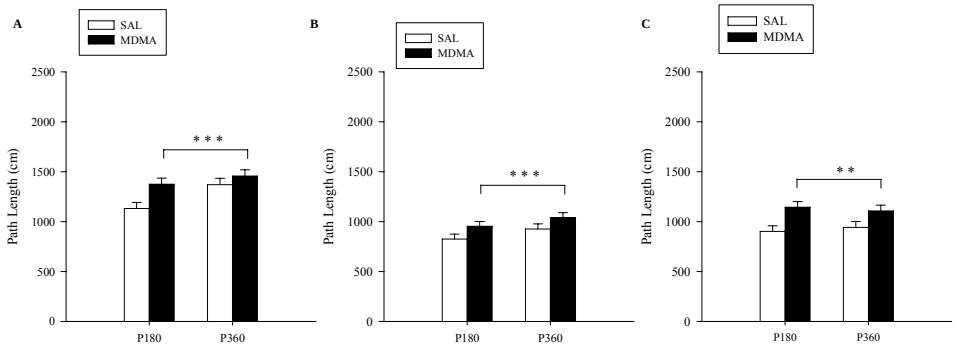

A
SAL
MDMA
Path Length (cm)
2500
2000
1500
1000
500
0
* * *
P180
P360
B
SAL
MDMA
Path Length (cm)
2500
2000
1500
1000
500
0
* * *
P180
P360
C
SAL
MDMA
Path Length (cm)
2500
2000
1500
1000
500
0
* *
P180
P360

Figure 7. Percent time in the target quadrant during probe trials. A main effect of treatment was observed during reversal (B) and reduced platform (C) phases of the MWM. No main effect of treatment was observed during the acquisition. ***P<0.001, *P<0.05

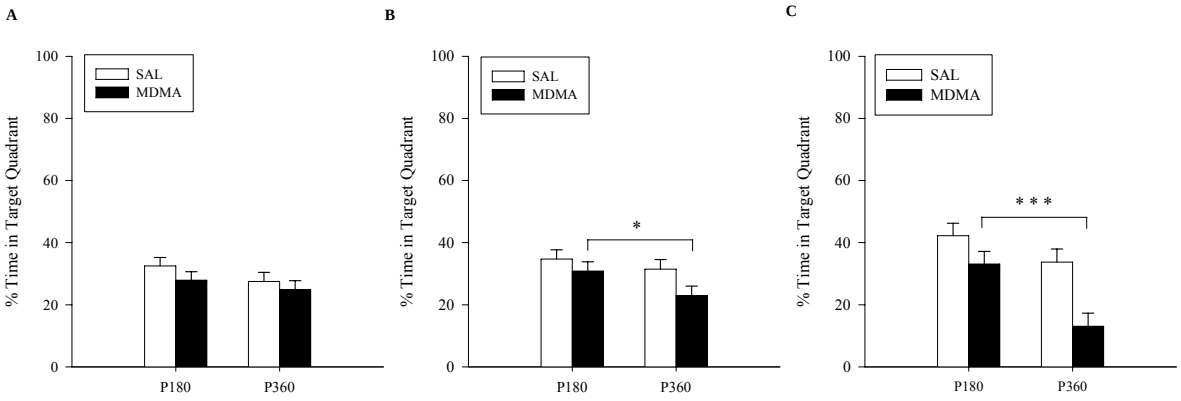
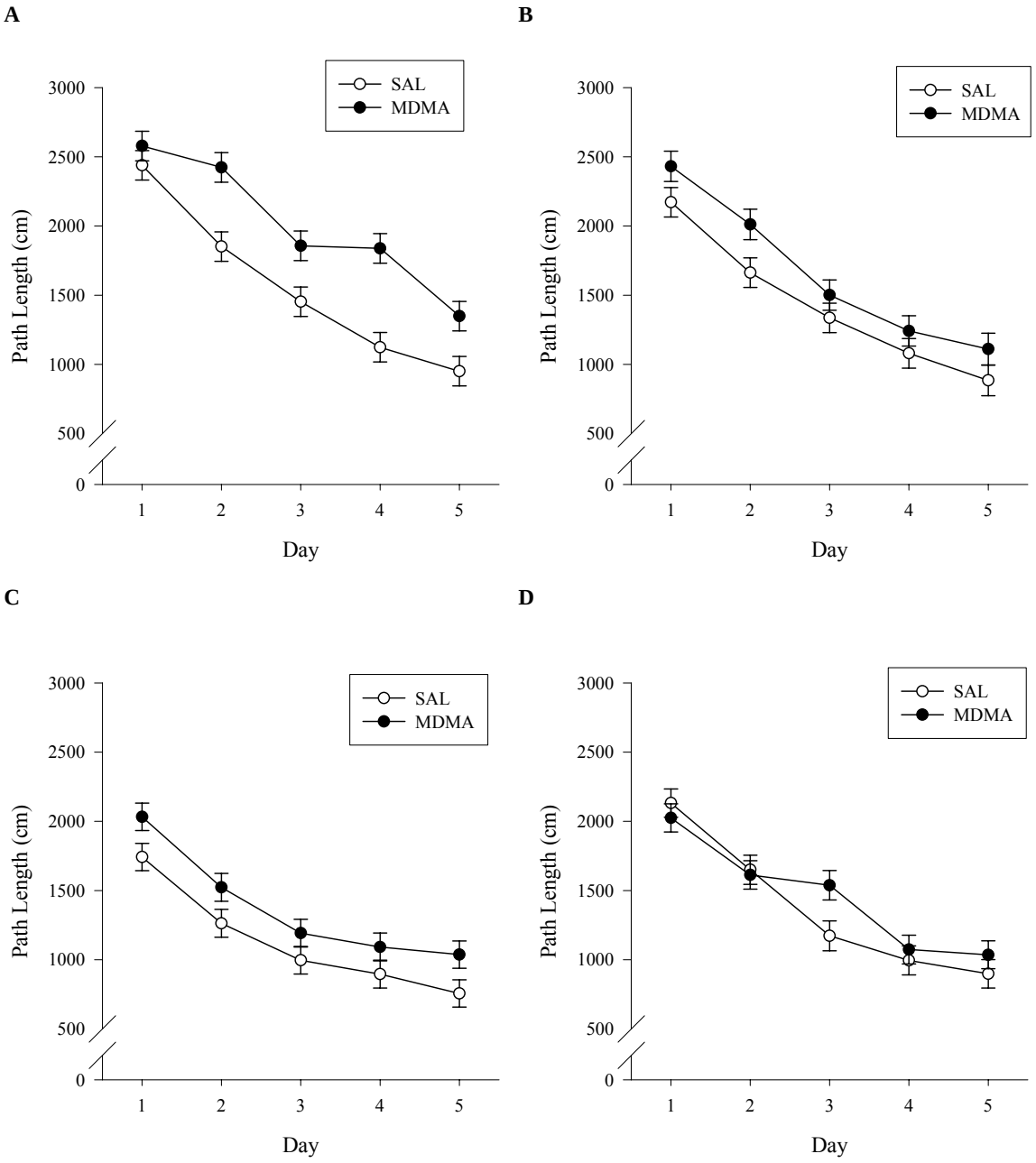

A
B
C
SAL
MDMA
% Time in Target Quadrant
100
80
60
40
20
0
P180
P360
*
* * *

Figure 8. Learning curves for P30 (A), P40 (B), P180 (C) and P360 (D) animals. Path length analysis by day show that SAL and MDMA treated animals improve their performance each day, although MDMA treated animals did not learn the task as well.


A
B
C
D
SAL
MDMA
Path Length (cm)
Day
3000
2500
2000
1500
1000
500
0
1
2
3
4
5

CHAPTER 3: Neonatal exposure to ±3,4-methelynedioxymethamphetamine alters NMDA receptor subunit 1, nNOS and PSD95 expression and LTP in adult rats

As submitted to Science (May 9th, 2006)

Abstract

Differences in adult hippocampal gene expression after postnatal day (P)11-20 (±)3,4-methylenedioxymethamphetamine (MDMA; 20 mg/kg x 2/day) exposure were examined using Affymetrix microarrays. MDMA-treated animals had 66 up- and 5 down-regulated genes by >1.2 fold. Of genes analyzed further, 8/24 were confirmed by RT/RT-PCR in the hippocampus but only 1/24 in the striatum. Of the 8 altered genes, two were up-regulated on P12 and one on P21. CAPON showed the greatest change in expression by microarray and RT/RT-PCR. Immunohistochemistry revealed a trend in CAPON protein expression change in the hippocampus but larger effects were found among related proteins that are part of the NMDA receptor complex, i.e., nNOS, PSD-95 and NMDA-NR1α . Brain slice electrophysiology showed that MDMA treatment reduced the amplitude of EPSPs in the dentate gyrus following an LTP-inducing stimulus. The results are the first to demonstrate long-term gene and protein expression changes as well as electrophysiological changes after developmental stimulant exposure that neuroanatomically and temporally match spatial and path integration learning and memory deficits previously shown to be induced by P11-20 MDMA treatment.

Use of 3,4-methylenedioxymethamphetamine (MDMA) is an ongoing problem throughout the industrialized world but the long-term effects of use remain largely unknown. A related concern is the effect of MDMA on brain development and later function if exposure occurs during gestation. We identified a period in brain development when MDMA exposure results in impairments in learning and memory of the offspring as adults, long after drug exposure has ended(Broening et al., 2001; Vorhees et al., 2004; Williams et al., 2003; Cohen et al., 2005). MDMA acts primarily on 5-HT terminals and causes 5-HT reductions in adult exposed animals as well as adult animals treated developmentally(Green et al., 2003). The loss of 5-HT seen in animals treated with MDMA developmentally cannot be correlated with learning and memory deficits, as animals that do not have learning and memory deficits still show 5-HT reductions(Broening et al., 2001; Skelton et al., 2004). While drugs such as alcohol(Goodlett and Horn, 2001) and cocaine(Harvey, 2004) have been studied more extensively than MDMA, mechanisms underlying the pathophysiology of these drugs have thus far been elusive.

In this study, microarray analysis using the Affymetrix U34A RG gene chips (~8,800 transcripts) was performed on the hippocampi of adult rats treated from P11-20 with 20 mg/kg MDMA or the saline vehicle twice daily (doses spaced 8 h apart). At the onset of the experiment, 6 animals from each treatment condition were analyzed. Two MDMA and one saline array were later removed due to inconsistent hybridization. Following normalization using Robust Microarray Average software, Wilcoxon-Mann-Whitney nonparametric analyses of expression level changes revealed significant alteration in the expression of 171 genes in the hippocampus of MDMA-treated vs. saline-treated animals. Of the 171 genes shown to be changed, 133 were up-regulated and 38 were down-regulated with a range of expression change of 1.01-1.51 fold. Applying a 1.2 fold increase threshold for up-regulated genes to be analyzed

further led to the inclusion of 66 genes. Similarly, applying a 1.2 fold decrease threshold led to 5 genes identified as down-regulated. The results are summarized in Table 1.

To verify the results of the microarray experiment, 24 genes were selected for confirmation using quantitative real-time RT-PCR (RT/RT-PCR). Genes chosen for confirmation were selected based on a search of the literature for evidence of involvement in learning and memory processes. Genes not selected at this time were excluded due to being housekeeping genes, an EST or whose function was insufficiently understood. For the verification experiment, an additional 8 animals (4/group) were treated as above and the hippocampus and striatum removed at P60. Of the 24 genes selected, 5 showed significantly altered expression by RT/RT-PCR ($p<0.05$, Fig. 1a), while 3 showed trends ($p<0.10$). Of these 8 genes, 5 were up-regulated both by microarray and RT/RT-PCR, while 3 showed divergent expression (up in microarray/down in RT/RT-PCR). The expression profile of the remaining 16 genes is shown in Figure 1b. When the 8 altered genes were examined in the striatum, only one gene (angiotensinogen, AOGEN), showed a change in gene expression (Figure 1C), suggesting that the remaining expression changes in the hippocampus are region-specific.

Following validation of candidate genes, we examined if these gene expression changes seen in the adults were apparent during or immediately following the drug administration period. RT/RT-PCR was performed on the hippocampi of treated animals on P12 (after 1 day of dosing) or P21 (after 10 days of dosing). On P12, two of the eight candidate genes (Nuclear orphan receptor 1 ((*NOR1*) and *AOGEN*) were altered in hippocampus with NOR1 showing a six-fold increase and AOGEN showing an ~50% decrease. On P21, AOGEN was the only gene with an altered expression (>3-fold increase in hippocampus).

CAPON (carboxyl-terminal PDZ ligand of nNOS), the gene with the highest expression change by both microarray and RT/RT-PCR, was examined by immunohistochemistry in an additional set of adult animals exposed to MDMA or saline from P11-20. CAPON is an adaptor protein associated with PSD95 (Jaffrey et al., 1998), a protein linked to NMDA receptors. Along with CAPON, proteins associated with it, including NMDA receptor subunit 1 alpha (NR1α), were examined. A 14% decrease was seen in the amount of CAPON in the dentate gyrus and CA1-CA3 regions (Figure 3), a non-significant change ($p \approx 0.15$). nNOS immunoreactivity was increased by 46.2% ($p<0.01$) in the dentate gyrus and by 25.2% ($p<0.08$) in CA1-CA3 regions (Figure 3). Whereas, PSD95 and NR1α showed 31.3% ($p<0.01$; Figure 3) and 18 % ($p\leq0.05$) increases, respectively, in the dentate gyrus, they were unchanged in the CA1-CA3 regions.

As adults, animals treated with MDMA from P11-20 showed long-term potentiation (LTP) assessed in the dentate gyrus following perforant path stimulation of brain slices using a multielectrode array system (MED64) (Figure 4). Following baseline stimulation, in which there were no differences between MDMA- and SAL-treated animals, a theta burst stimulus (TBS) was applied. Following the TBS, EPSPs were recorded for 30 min. While both MDMA- and SAL-treated animals showed increases in EPSP amplitude after the TBS, the MDMA-treated animals had a reduction in amplitude when compared to SAL-treated animals ($p < 0.01$; Figure 4).

We previously showed that MDMA treatment of rats from P11-20 causes spatial and path integration learning deficits in the offspring as adults long after the end of drug treatment (Broening et al., 2001; Vorhees et al., 2004; Williams et al., 2003). In adult animals, MDMA is known to alter monoamines, especially reducing 5-HT (Green et al., 2003). However, unlike the large 5-HT changes seen after adult MDMA exposure, in P11-20 MDMA-treated animals

changes in hippocampal and frontal cortical monoamines of adult offspring are small and uncorrelated to changes in learning(Broening et al., 2001), although during and immediately following drug administration monoamines are perturbed (Williams et al., 2005). In order to develop new leads on long-term effects of MDMA on the developing brain, we used Affymetrix gene chips to screen for expression changes that could be detected at the age when the learning and memory deficits had been shown to occur. Expression changes greater than or equal to 1.2 fold in the hippocampus of P60 rats after P11-20 MDMA exposure were examined. This threshold was used for several reasons: (1) the effects of interest were presumed to be residual, reflecting a state long after the acute effects of the drug are past, (2) many genes have multiple regulatory sites only one or a few of which may be altered by early MDMA treatment, and (3) the hippocampus contains a heterogeneous cell population of which MDMA may only affect a subset. Because of the relatively low threshold, a number of significant changes were found. To sort these, additional criteria were needed.

Of the total number of expression changes, 24 were known to be involved in learning and memory. Of these, 8 showed a significant change by RT/RT-PCR. While most of the verified genes matched the microarray profile, a few showed divergent changes. These may be false positives or the product of interaction effects. We also examined the expression of the 8 genes 24 h following the first (P11) and last treatment day (P20). Two genes were altered on P12, while only one was altered on P21. AOGEN was the only gene altered at all three time points in the hippocampus. AOGEN is the precursor for Angiotensin II (AngII) and Angiotensin IV, both of which have been shown to facilitate learning and memory in normal and hippocampally-lesioned rats (see(Gard, 2002; Wright et al., 2002)). However, the expression changes in

AOGEN were in opposite directions at different ages, thereby casting doubt on whether it was a reliable marker of later cognitive changes.

Genes not selected for verification were primarily EST's, housekeeping genes, and genes not known to be involved in neuronal function. These genes were not pursued because they were low probability candidates for association with cognitive outcome, however, we cannot rule out the possibility that some of these could someday be unexpectedly found to be associated with learning and memory.

Insulin-like growth factor 1 (IGF1) plays a role in protecting the brain from injury and has been found to be expressed in the brain following hypoxic-ischemic injury (Guan et al., 1996), and IGF1 knockout mice display a loss of cells in the dentate gyrus (Cheng et al., 2001; Guan et al., 1996), a region undergoing rapid neurogenesis during our exposure period (P11-20). IGF1 administration has also been shown to improve the performance of diabetic rats in the Morris water maze (Lupien et al., 2003), therefore, IGF1 remains a possible candidate in the long-term effects of early MDMA exposure. NOR1 is a ligand-activated transcription factor, although its ligand is not identified, and a member of the Nurr77/NGFI-B subfamily of nuclear receptors. NOR1 was first found in neuronal cultures undergoing apoptosis (Ohkura et al., 1994). Further characterization of this gene, using antisense oligonucleotides in primary cell culture, showed that inhibiting NOR1 induced cell migration and increased the number of cells with processes (Ohkura et al., 1996). After 1 week of NOR1 inhibition, many of the cultured cells resembled neurons, suggesting that NOR1 inhibits neuron outgrowth (Ohkura et al., 1996). NOR1 is also activated by drugs such as cocaine and morphine (Werme et al., 2000a), as well as by neuroleptics such as haloperidol and clozapine (Werme et al., 2000b). Moreover, AngII administration up-regulates the transcription of NOR1 in the adrenal gland (Fernandez et al.,

2000) and therefore could be linked to increased adrenal cortical output observed after P11 MDMA exposure (Williams et al., 2005).

The Kv1.1 potassium channel is encoded by the KCNA1 locus and has been shown to be activated by IGF1 in HEK293 cells (Gamper et al., 2002). Mice lacking the Kv1.1 gene show increases in neuronal excitability in the CA3 region of the hippocampus (Herson et al., 2003) and eventually die of seizure disorders (Smart et al., 1998). This suggests that the Kv1.1 gene is more closely linked to epilepsy than to learning and memory mechanisms and appears to be an unlikely candidate for mediating MDMA's developmental effects. Phospholemman, also known as Fxyd1, is an accessory protein of Na,K-ATPase that reduces the affinity of the Na,K-ATPase for Na^+ and K^+ (Crambert et al., 2002). Whether this gene could be linked to MDMA-induced learning and memory deficits is unknown, but we have recently shown that Na,K-ATPase KO mice have spatial learning and memory deficits (Moseley et al., unpublished). Glial-cell line-derived neurotrophic factor (GDNF) and its primary receptor GDNF receptor alpha have been implicated in the survival of dopaminergic neurons (Lin et al., 1993). GDNF administration reduces apoptosis in striatal dopaminergic neurons on P14 (Oo et al., 2003) and thus has been proposed as a possible treatment for Parkinson's disease (Kirik et al., 2004). GDNF infusion blocks the ability of cocaine and morphine to increase tyrosine hydroxylase immunoreactivity in the striatum and blocking GDNF via a neutralizing antibody or genetic knockout increases the rewarding effects of cocaine (Messer et al., 2000). However, GDNF appears to play a more significant role in striatal function than in hippocampal function. Hence, all 8 of the confirmed genes altered in hippocampus of MDMA-treated offspring are implicated in learning and memory processes to one degree or another. One of them, however, CAPON, provided a lead to

further investigation because of its known involvement with NMDA receptors and they have a well-established role in spatial learning and memory.

Using immunohistochemistry, we examined the protein changes in the hippocampus of CAPON and the related proteins of this receptor complex, PSD95, nNOS and NMDA, focusing on the NMDA-NR1 subunit because of its known role in learning and memory (Cain et al., 1996; Morris et al., 1986; Morris, 1989). Protein levels of CAPON were reduced in the dentate gyrus, while PSD95, nNOS, and NR1 were elevated in the dentate gyrus. The genes for PSD95, nNOS, and NR1 did not show significant changes in the microarray analysis of the whole hippocampus, presumably because of subregional specificity as demonstrated with the immunohistochemical analysis. NMDA receptors have been shown to be intimately involved in spatial learning and memory and LTP. Inhibiting NMDA receptors has been shown to induce deficits in spatial learning and LTP (Lisman, 2003; Ahlander et al., 1999), and paradoxically so have treatments that increase NMDA receptor expression. Female rats that have been ovariectomized show both spatial learning deficiencies and increased NMDA-NR1 receptor immunoreactivity (El Bakri et al., 2004). With increases and decreases in NMDA receptor levels causing learning and memory deficits, it is reasonable to hypothesize that LTP would perform similarly, since LTP is believed to be the cellular mechanism of learning. This inverted U shaped curve of learning and NMDA receptor levels is similar to that of corticosterone (CORT). It is possible; however, that other neurotransmitter systems, such as GABA, could be altered by MDMA treatment and that the changes in NMDA receptors could be a result of compensatory increases in GABAergic inhibitory pathways.

nNOS is activated by stimulation of NMDA receptors and has been suggested to play a role in adult MDMA-induced 5-HT depletion. Administration of the nNOS inhibitors N^{ω}-nitro-

L-arginine methyl ester (L-NAME) or S-methyl-L-thiocitulline (S-MTC) prevent the MDMA-induced 5-HT depletion in the striatum of rats and L-NORAG partially alleviated the 5-HT deficits in the frontal and parietal cortices of adult rats (Zheng and Laverty, 1998). Increases in nNOS were seen in animals treated on P3 and P4 with a 5-HT depleting dose of *p*-chloroamphetamine(Tagliaferro et al., 2003) and in adult rats treated with a 5-HT depleting regimen of p-chlorophenylalanine(Ramos et al., 2002).

Long-term potentiation is dependent on functional NMDA receptors(Brown et al., 1988; Lisman, 2003). To assess LTP in adult rats exposed to MDMA from P11-20, we used a multi-electrode array system. This system measures extracellular currents in the region where the electrode is placed on the slice. MDMA-treated animals showed a decrease in LTP amplitude compared to saline-treated animals, further supporting the hypothesis that developmental MDMA exposure induces long-lasting changes in NMDA receptor signaling. It has also been suggested that LTP is the cellular mechanism of some types of learning (Lisman, 2003; Brown et al., 1988). The results of this study combined with the learning and memory deficits seen in comparably MDMA-treated animals provides converging evidence that these cellular changes are important in the cognitive deficits observed.

While the developmental effects of several drugs of abuse, such as alcohol and cocaine, and other chemical agents have been studied extensively in animals and humans, few have been linked to plausible underlying mechanisms of action in part because the task of identifying changes far removed from the time of initial insult, especially when they occur during the dynamic changes of development, have been difficult to parse from the mature brain. To surmount this difficulty, we used gene chip microarrays to search for the lasting effects stemming from early MDMA exposure. The results identified for the first time a set of cellular

changes that may underlie the learning and memory deficits seen after developmental MDMA exposure. The plausibility of the findings turns on the observations that (1) effects of MDMA were in the brain region known to be involved in spatial and path integration learning, (2) key proteins in this region are undergoing rapid change during the period when the drug was administered, (3) the protein expression changes were long lasting, (4) the protein changes were present when the learning deficits were observed, and (5) the protein changes were accompanied by electrophysiological changes in LTP that are known to accompany types of learning deficits induced by developmental MDMA.

Materials and Methods

Subjects

Male and nulliparous female Sprague-Dawley CD IGS rats (Charles Rivers Laboratories, Raleigh, N.C.) were bred following a minimum two week acclimation to the vivarium. The date a sperm plug was detected was considered E0 and females were removed from breeding cages after two weeks with the male. Date of birth was considered postnatal day (P)0 and on P1 litters were culled to eight pups with an even number of males and females within the litter. Beginning on P11, male pups were subcutaneously injected twice daily with either 20 mg/kg d,l-MDMA HCl (expressed as the free base) dissolved in saline or saline alone in a dosing volume of 3 ml/kg. MDMA was obtained from the National Institute on Drug Abuse and was greater than 95% pure. This was a within litter design, so that each litter had an equal number of SAL- and MDMA-treated animals. Animals were weaned by the dam and removed from the dam's cage and housed in same sex pairs on P28. Three time points were examined for this study: P12, 21, and 60. For animals used in microarray or RT/RT-PCR studies, animals were removed from the

home cage room on the day of sacrifice, brought to an adjacent suite and decapitated. The hippocampus and striatum were dissected on ice and placed in *RNAlater* (Ambion, Austin, TX) for processing. Animals for immunohistochemistry were sacrificed with an overdose (65 mg/kg) of sodium pentobarbital injected i.p. Following respiratory arrest, animals were perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA). The brain was removed and placed in 4% PFA overnight followed by 30% sucrose until sectioning. All procedures were approved by the Cincinnati Children's Research Foundation's (CCRF) Laboratory Animal Use and Care Committee and the vivarium is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

RNA isolation and Microarray analysis

RNA was isolated from the collected tissue using RNABee (Tel-Test, Friendswood, TX) reagent according to the manufacturer's instructions. Briefly, tissue was homogenized in RNABee to which chloroform was added. Following brief mixing, the mixture was centrifuged at 12,000 x g at 4°C for 15 min. The supernatant was collected and an equal volume of isopropanol was added. Following a brief incubation, the sample was centrifuged for 10 min at the same force. The resulting pellet was washed with 75% ethanol followed by a 5 min centrifugation at 7,500 x g. The pellet was then dissolved in DEPC-treated TE and RNA quantified by UV spectrometry. Ten μg samples of RNA from P60 animals were sent to the microarray core of CCRF for analysis on Affymetrix RG34A gene chips. Data were normalized with Robust Microarray Average (RMA) software and then analyzed with Gene Spring software version 6.0 (Silicon Genetics, Redwood City, CA). Each chip represented an individual animal and 6 samples from each treatment group were used. One sample from each treatment group was labeled and hybridized to two chips to minimize technical variance. Of the six samples analyzed, one SAL

chip and two MDMA chips were removed due to problems with the labeling reaction, leaving the final numbers for analysis 4 MDMA-treated and 5 SAL-treated. Wilcoxon-Mann-Whitney non-parametric analyses were used to determine significant differences. A $p<0.05$ was considered significant with no correction for multiple tests; individual genes were ranked according to largest fold difference from control.

Real-Time Reverse Transcription PCR

cDNA libraries were created from samples that were not used in the microarray analysis. Reverse transcription was performed using the Superscript II reverse transcription kit from Invitrogen (Carlsbad, CA) following the manufacturer's instructions. 2 μg of RNA were added to a reaction containing 0.5 mM DNTP's and 1.25 mM oligo d(T) (Invitrogen, Carlsbad, CA). The RNA was denatured by heating at 65°C for 5 min and then placing samples in an ice bath for 1 min. Reaction buffer and 5 mM DTT were added to the reaction and incubated for 1 min at 42°C. Superscript reverse transcriptase was added and the sample was incubated for 1 h at 42° and then 70° for 15 min to stop the reaction. The RT reaction was then diluted to 1:5 for subsequent analysis. No RT controls were used to examine the samples for genomic DNA contamination.

Real-time PCR was carried out on the DNA Engine Opticon System by MJ Research (Reno, NV). A standard curve was generated using standard dilutions of a PCR amplified region of the β-actin gene. Primers that amplified a smaller product within this section of the gene were then used to generate a standard curve for each set of PCR reactions. Primers used for real time PCR analyses are shown in Table 2. For each reaction, 10 μl of QuantiTech SYBR green mix (Qiagen) were added to 500 nM of each primer and 2 μl of the RT reaction. The PCR reaction consisted of an initial incubation at 96° for 15 min followed by 45 cycles of 96°C for 30 s, 57°C

for 30 s, 72°C for 45 s, and 74° for 10 s, with a fluorescence reading at the end of each cycle. A threshold level of fluorescence was set and the cycle number that each sample crossed the threshold was determined and compared to the standard curve to calculate the relative amount of each gene in the sample. The loading control value, which was determined by running the sample with the same β-actin primers used to generate the standard curve, was divided from the sample value and then the SAL samples were averaged and all samples were divided by that value. Two-way analysis of variance was used to analyze the values with a $p < 0.05$ to determine significance.

Immunohistochemistry

Free floating sagittal sections (50 μm) were used for immunohistochemistry. Sections were quenched in PBS+0.3% H_2O_2 + 0.1% Triton X-100 for 30 min then blocked for 30 min using the serum from the animal that was used to generate the secondary antibody. Primary antibodies were diluted in PBS + Triton X-100 and incubated with the sections overnight at 4° C. CAPON (Abcam, Cambridge, MA) was diluted 1:500, nNOS (Chemicon, Temecula, CA) 1:2000, NR1α (Chemicon) 1:750, and PSD-95 (Chemicon) 1:500. Following washes with PBS + 0.1% Triton X-100, sections were incubated with the secondary antibody (Vector Labs, Burlingame, CA) in PBS + Triton X-100. Sections were then washed with PBS + Triton and then incubated with the avadin-biotin solution from Vector. Sections were developed using DAB with nickel chloride enhancement, dehydrated, and cover slipped. Photos were taken with a Nikon Eclipse E600 microscope. Optical density measurements were taken using Scion Image (NIH) and a one-tailed t-test was used to determine significance.

Long-Term Potentiation

Animals were transferred to the Environmental Health Effects Laboratory at Wright Patterson Air Force Base the morning of assessment. Animals were rapidly decapitated and the brains were immediately removed and placed in ice-cold aCSF (124 mM NaCl, 3 mM KCl, 1.25mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM dextrose, 26 mM $NaHCO_3$, and 2 mM $CaCl_2$). The cerebellum and the cortex rostral to the optic chiasm were removed free hand. The ventral side of the brain was cut on each side at a 20-30° angle and then sectioned on a vibratome filled with ice-cold aCSF that was constantly oxygenated. Each brain was sectioned at 300 μm beginning at the level of the hippocampus. Sections were then transferred to an aCSF bath at 30° C for 1 h. For recording, sections were placed on a MED64 (AlphaMed Sciences, Tokyo, Japan) array chamber with constant infusion of warm (32° C) aCSF and under constant humidity. Slices were allowed to acclimate to the conditions of the chamber for 10-15 min, at which point a baseline recording was obtained. Sections were stimulated alternately from two channels with a pair pulse stimulus (60-90 mA) originating in the entorhinal cortex every 10 s and recordings were collected at the dentate gyrus (Sarvey et al., 1989). Stimulating current was decided by applying a current until the resulting EPSP was between 300-500 mA maximum amplitude. Maximum amplitude of the EPSP was recorded using the MED64 Performer 2.0 Software (AlphaMed Sciences, Tokyo, Japan). After a 5 min baseline recording, a theta burst stimulation was applied every 200 ms for 2 s at 1.5 x the stimulating current (mA) used to generate the recording EPSP. Following the tetanizing stimulus, EPSPs were recorded for 30 min. Data were analyzed using a general linear model (Proc GLM, SAS, Cary, NC) with treatment as a between factor and time as a repeated measure with the pre- and post-stimulation periods analyzed separately.

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Table 1. Genes altered in adult hippocampus by P11-20 MDMA exposure

Fold Change		Description	P-value	Accesion Number
1.52	↑	CAPON	0.0352	NM_138922
1.46	↑	EST	0.0076	BG665046
1.44	↑	Secreted acidic cystein-rich glycoprotein (osteonectin) (Sparc)	0.0078	NM_012656
1.44	↑	nuclear receptor subfamily 4, group A, member 3 (Nr4a3)	0.0177	NM_031628
1.43	↑	NF1-B2; nuclear factor I/B	0.0352	AB012231
1.43	↑	prolactin- and FGF-2-responsive; NonO/p54nrb homolog	0.0352	AF036335
1.42	↑	EST	0.0076	BF393884
1.4	↑	KCNA1	0.0177	M26161
1.4	↑	3-hydroxy-3-methylglutaryl-CoA synthase	0.0177	M33648
1.4	↑	EST	0.0078	BG666091
1.4	↑	EST	0.0025	AW918825
1.39	↑	glutathione transferase subunit 8.	0.0078	X62660
1.38	↑	EST	0.0026	AA800184
1.36	↑	Activated leukocyte cell adhesion molecule (Alcam)	0.0352	NM_031753
1.36	↑	EST	0.0026	BI300357
1.36	↑	EST	0.0025	AI639009
1.35	↑	p21 (CDKN1A)-activated kinase 3 (Pak3)	0.0076	NM_019210
1.35	↑	retroviral-like ovarian specific transcript 30-1	0.0025	U48828
1.34	↑	Protein-L-isoaspartate (D-aspartate) O-methyltransferase (Pcmt1)	0.0352	NM_013073
1.34	↑	frizzled homolog 1 (Drosophila) (Fzd1)	0.0025	NM_021266
1.33	↑	RalA binding protein 1 (Ralbp1)	0.0358	NM_032067
1.33	↑	EST	0.0177	BQ202936
1.31	↑	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) (Dab2)	0.0076	NM_024159
1.31	↑	Angiotensinogen (Agt)	0.0181	NM_134432
1.31	↑	EST	0.0177	AA799450
1.3	↑	f-spondin	0.0078	M88469
1.3	↑	Microtubule-associated protein tau (Mapt)	0.0181	NM_017212
1.3	↑	EST	0.0076	AA848355
1.29	↑	190 kDa ankyrin isoform; ankyrin 3 (G)	0.0358	AF069525
1.29	↑	Mx1 protein	0.0076	X52711
1.29	↑	EST	0.0076	AW915638
1.28	↑	Cytochrom P450 Lanosterol 14 alpha-demethylase (Cyp51)	0.0005	NM_012941
1.28	↑	system N1 Na+ and H+-coupled glutamine transporter (Hnrpu)	0.0076	NM_057139
1.28	↑	TFIII Zn finger transcription factor 4; alternatively spliced form*	0.0076	NM_053583
1.28	↑	ID3	0.0177	NM_013058
1.28	↑	Lipoprotein lipase (Lpl)*	0.0352	NM_012598
1.28	↑	EST	0.0352	U67992
1.27	↑	CBP/P300-interacting transactivator 2	0.0181	NM_053698
1.27	↑	solute carrier family 15 (H+/peptide transporter), member 2	0.0352	NM_031672
1.27	↑	CBP/P300-interacting transactivator 2	0.0181	NM_053698
1.26	↑	mitogen activated protein kinase kinase kinase 1 (Map3k1)	0.0177	NM_053887
1.26	↑	Milk fat globule-EGF factor 8 protein (Mfge8)	0.0352	NM_012811
1.26	↑	EST	0.0177	AI638939
1.26	↑	EST	0.0177	AA891311
1.26	↑	EST	0.0025	AA957811
1.25	↑	Metabotropic glutamate receptor 3	0.0352	M92076
1.25	↑	FGF receptor activating protein 1 (Frag1)	0.0076	NM_053895
1.25	↑	v-jun sarcoma virus 17 oncogene homolog (avian) (Jun)	0.0352	NM_021835

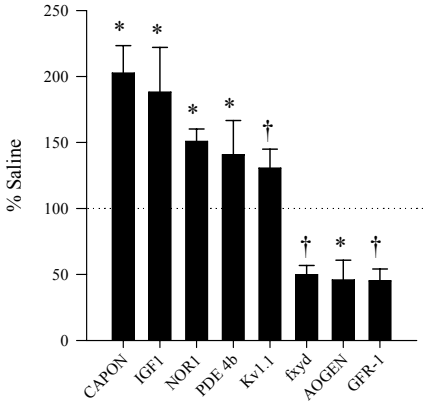
1.24	↑	Glypican 3 (Gpc3)	0.0006	NM_012774
1.24	↑	glutathione S-transferase, theta 2 (Gstt2)	0.0026	NM_012796
1.24	↑	EST	0.0352	BG666091
1.23	↑	EST	0.0005	BQ201485
1.22	↑	protein tyrosine phosphatase type IVA, member 2 (Ptp4a2)	0.0181	NM_053475
1.22	↑	EST	0.0078	BF400606
1.22	↑	EST	0.0358	AI007614
1.22	↑	EST	0.0026	AW916321
1.21	↑	cAMP phosphodiesterase	0.0076	J04563
1.21	↑	FXYD domain-containing ion transport regulator 1 (Fxyd1)	0.0025	NM_031648
1.21	↑	B-cell translocation gene 1 (Btg1)	0.0025	NM_017258
1.21	↑	CD24 antigen (Cd24)	0.0078	NM_012752
1.21	↑	VL30 element	0.0358	M91235
1.21	↑	EST	0.0025	BQ202940
1.21	↑	EST	0.0352	BQ783114
1.2	↑	regulated endocrine-specific protein 18 (Resp18)	0.0025	NM_019278
1.2	↑	lamina-associated polypeptide 1C (Lap1c)	0.0025	NM_145092
1.2	↑	EST	0.0177	AA860047
1.23	↓	Suppressor of K+ transport defect 3 (Skd3)	0.0025	NM_022947
1.22	↓	myosin heavy chain, polypeptide 7 (Myh7)	0.0025	NM_017240
1.21	↓	EST	0.0181	BF287675
1.2	↓	casein kinase 1 delta	0.0358	NM_139060
1.2	↓	EST	0.0177	M83745

Table 2. Primers used for RT/RT-PCR

Gene	Forward	Reverse
Actin	gacgttgacatccgtaaagacc	gcagtaatctccttctgcatcc
α5 integrin	gctcatcgtttctatcccacc	gctcatcgtttctatcccacc
Angiotensinogen	tgctgcccagaaaatcaac	gagaagcctctcatcttccc
CAPON	tggaaggtaaagcagggaag	ataacggtgggagcaacag
c-met	ccagcccattactacaaaacac	acacagccaaaatgccctc
f-spondin	cacaaggagaagcaaccag	aaaaaggaggcggacaaac
Fxyd	cattcacctacgattaccacac	aagttccctcctcttcgtc
GFRα	ccccaacaacaacaaccac	gctacattcagcttctcactc
Glypican	aatcaacaccaccgaccacc	atctctaccacacctgccatac
IGF-1	ggagtgtgaaaaagcaagagg	gcagatgaggaaaactgagg
Kv1.1	atcttcaaactctcccgcc	cactagaaaacagtatgacgcc
Lumican	tcccctcactcaaagcagtc	cacatccagcaaatacccatcc
MAP Tau	atcttcaaactctcccgcc	cactagaaaacagtatgacgcc
IRS2	acctttcctctaccaccacc	gctgccttcactgcttttcc
NF1-B2	ctaaacccatcactacatccac	acacttgaaaaggaaccaagc
NOR1	tgaacacctgcccctctatc	aacacaaccccaacacaatac
p47	gcaaccccaagttcagtgtc	agcccccagcataaaacctc
PCMT1	ctctttcttcctccacacattc	gtaacaaagtccaaaacaagcg
Pde4b	ctctttcttcctccacacattc	gtaacaaagtccaaaacaagcg
RESP 18	attccaccacattgtacccc	aaaccaccttcccctccttc
rPTP	accacaaccaagttgcttcttc	tcatcaccatcttcaccacctc
5-HT receptor 2a	atgacacagtgacagagggag	acaaaagagccgcaggaaag
SPARC	ggagcaggacatcaacaag	ttatccccaggcagaacag
Trh degrading enzyme	gtgcctaagcatccttatgctg	cttcaaccacacatcttcccac
TRHR	aatgactcaacccatcagaac	ggcaaacagaattacaaccac

Figure 1. (A) P11-20 MDMA exposure causes the up-regulation of 5 genes and the down regulation of 3 genes in the hippocampus as verified by RT/RT-PCR. (B) Genes that were not significantly altered by P11-20 MDMA dosing. (C) The angiotensinogen gene is up-regulated in the striatum due to P11-20 MDMA exposure * $p<0.05$ † $p<0.10$.

A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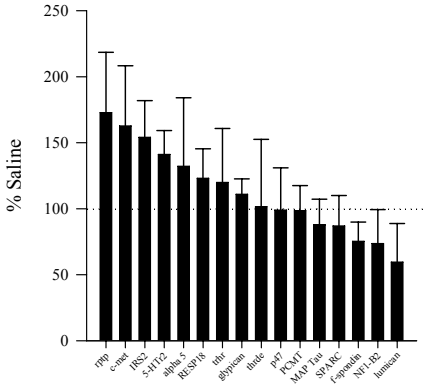
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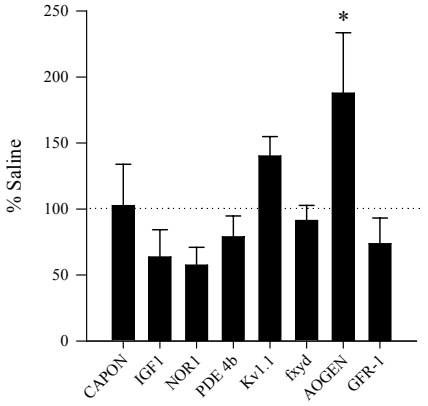
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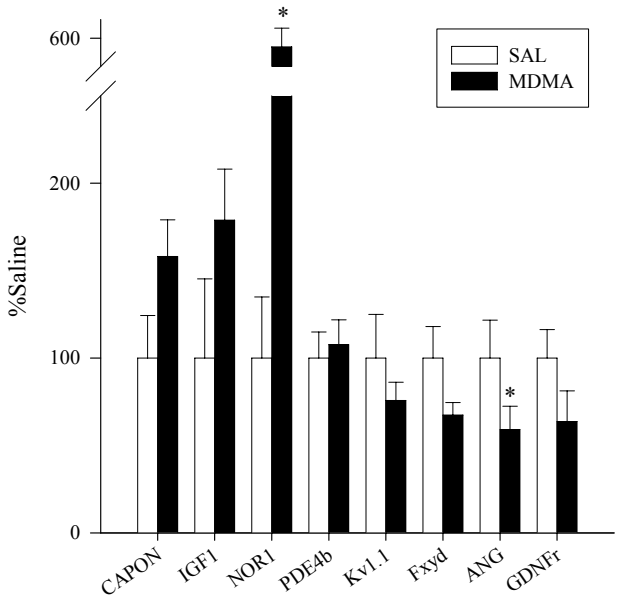
AOGEN
GFR-1

Figure 2. Gene expression changes 24 hours after (A) P11 or (B) P11-20 MDMA exposure.
*$p<0.05$

A


600
200
100
0
%Saline
SAL
MDMA
*
*
CAPON
IGF1
NOR1
PDE4b
Kv1.1
Fxyd
ANG
GDNFr

B

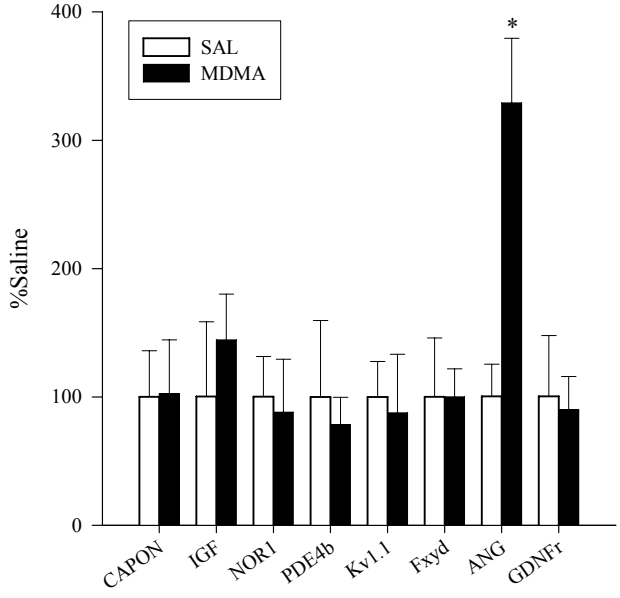

400
300
200
100
0
%Saline
SAL
MDMA
*
CAPON
IGF
NOR1
PDE4b
Kv1.1
Fxyd
ANG
GDNFr

Figure 3. Immunoreactive changes in adult rats in the dentate gyrus exposed to MDMA or saline from P11-20. *$p<0.05$

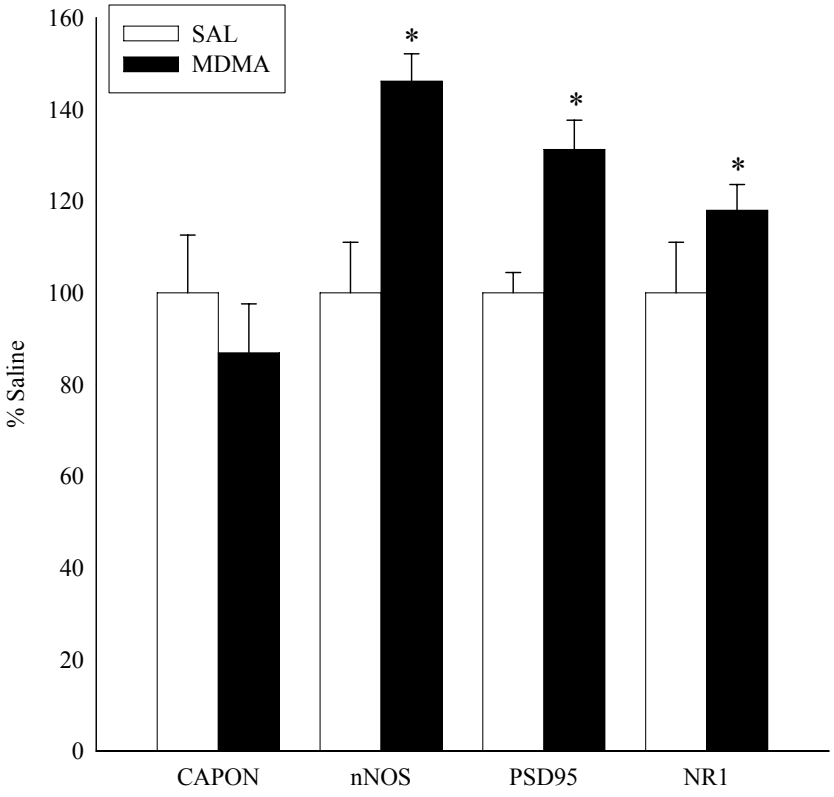
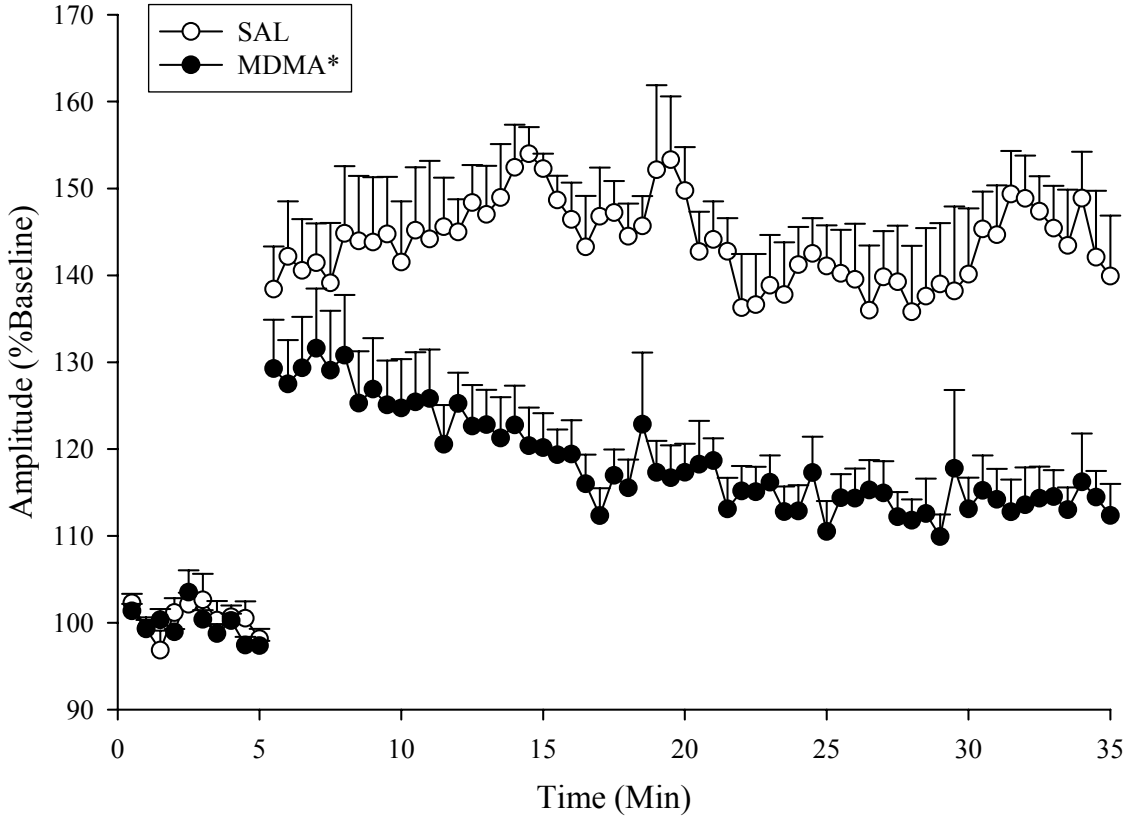

160
140
120
100
80
60
40
20
0
% Saline
SAL
MDMA
*
*
*
CAPON
nNOS
PSD95
NR1

Figure 4. Exposure to MDMA from P11-20 induces reductions in LTP measured in brain slice preparations using the MED64 system. *A main effect of treatment was observed ($p<0.001$).


SAL
MDMA*
Amplitude (%Baseline)
Time (Min)
170
160
150
140
130
120
110
100
90
0
5
10
15
20
25
30
35

CHAPTER 4: P11 EXPOSURE TO 3,4-METHYLENEDIOXYMETHAMPHETAMINE DOES NOT INCREASE CAM-KII OR NEURONAL NITRIC OXIDE SYNTHASE PHOSPHORYLATION IN THE RAT HIPPOCAMPUS

Abstract

The popular club drug, 3,4-methylenedioxymethamphetamine has been shown to disrupt learning and memory function in developmentally exposed rats. Further, MDMA treatment from P11-20 increases the expression of neuronal nitric oxide synthase (nNOS), post-synaptic density 95 (PSD95), and the NMDA receptor subunit 1 (NR1) in the dentate gyrus of adult animals. These changes are believed to be related to the decrease in the ability of MDMA-exposed animals to maintain LTP in brain slice electrophysiology. The purpose of this study was to examine the immediate effects of MDMA on the downstream signaling targets of the NMDA receptor, namely calcium-calmodulin kinase II (CAM-KII) and nNOS. A single dose of MDMA on P11 failed to alter the phosphorylation of CAM-KII or nNOS in the hippocampus of these animals compared to animals that received saline only. PSD95 and CAPON levels were also unaltered in the hippocampus. The results of this study suggest that NMDA does not have a role in early MDMA mechanisms.

Introduction

Previous studies from this lab have shown that MDMA administration from postnatal day (P)11-20 causes learning and memory deficits when the animals are examined as adults (Broening et al., 2001; Cohen et al., 2005; Vorhees et al., 2004; Williams et al., 2003). This is a period that is analogous to second and third trimester human brain development (Bayer et al., 1993). Exposure to MDMA during this period has been shown to deplete serotonin (5-HT) in adult animals, a finding that cannot be correlated with the learning and memory deficits, because animals that were treated from P1-10 have no learning and memory deficits but show similar 5-HT depletions (Broening et al., 2001; Crawford et al., 2006). While the serotonergic depletions in adulthood cannot be correlated to learning and memory deficits, recent data suggest that 5-HT signaling could be permanently altered by P11-20 MDMA signaling. MDMA administration on P11 has been shown to deplete 5-HT in the hippocampus up to 72 h following dosing (Williams et al., 2005), and P11-20 administration decreased 5-HT on P21 (Koprich et al., 2003). These early 5-HT changes could alter the signaling of 5-HT in the brain, as animals treated with MDMA from P11-20 have increased binding of [^{35}S]GTPγS under 5-HT or R(+)-8-OH-DPAT stimulation, suggesting alteration of the 5-HT$_{1A}$ receptor (Crawford et al., 2006).

While these findings have provided an insight into the possible mechanisms of the learning and memory deficits discovered in MDMA-treated animals, we have recently identified another system that could be perturbed by this drug. Using microarray analysis, we demonstrated 71 genes with altered expression in the hippocampus of adult animals that had been exposed to MDMA from P11-20 (Skelton et al., 2005). One of the genes shown to be increased in the hippocampus was CAPON, an accessory gene of the NDMA receptor that is

hypothesized to facilitate the interaction of neuronal nitric oxide synthase (nNOS). Analysis of protein levels failed to show a difference in CAPON expression, however, nNOS, the NMDA receptor subunit 1 (NR1), and PSD-95 protein levels were all increased in the hippocampus of these animals (Skelton et al., 2005). Additionally, long-term potentiation was disrupted in animals treated with MDMA from P11-20, further supporting the hypothesis that NMDA transmission is disrupted in these animals. In adult animals, the NMDA receptor antagonist MK-801 prevents MDMA induced 5-HT deficits; however, MK-801 causes hypothermia which has been shown to prevent 5-HT deficits related to MDMA exposure (Farfel and Seiden, 1995). Using another NMDA receptor antagonist, ACEA1021, which prevents hyperthermia but does not induce hypothermia, also prevented 5-HT depletions in MDMA-treated animals (Russell and Laverty, 2001). Further, it has been shown that animals given MDMA then tested in a passive avoidance paradigm have decreased NR1, NR2B and phospho-CAM-KII levels than control animals; however no changes were observed if the animals were not tested in passive avoidance (Moyano et al., 2004; Moyano et al., 2005). Decreased levels of PSD-95 were observed in these animals regardless of previous avoidance testing (Moyano et al., 2004; Moyano et al., 2005).

Various studies have been preformed using nNOS inhibitors in an attempt to prevent MDMA-induced 5-HT deficits, although these studies have produced mixed results. Small attenuations of the 5-HT depleting effect of MDMA were observed using N-nitro-L-arginine methyl ester (L-NAME), N-Ω-nitro-L-arginine (L-NORAG) and N-nitro-L-arginine (NA) prior to MDMA treatment (Zheng and Laverty, 1998; Taraska and Finnegan, 1997). When MDMA was co-infused with malonate, the nNOS inhibitor S-MTC prevented 5-HT and DA depletions (Darvesh et al., 2005). This study also observed increases in nitric oxide metabolites following MDMA exposure. The purpose of the present study was to examine the effects of P11 exposure

to MDMA on downstream targets of the NDMA receptor, specifically the phosphorylation of nNOS and CAM-KII.

Materials and Methods

Subjects

Male and nulliparous female Sprague-Dawley CD IGS rats (Charles Rivers Laboratories, Raleigh, N.C.) were bred following a minimum two week acclimation to the vivarium. The date a sperm plug was detected was considered E0 and females were removed from breeding cages the following day (E1). Date of birth was considered postnatal day (P)0 and on P1 litters were culled to eight pups with an even number of males and females within the litter. Beginning on P11, male pups were subcutaneously injected once with either 20 mg/kg d,l-MDMA HCl (expressed as the free base) dissolved in saline or saline alone in a dosing volume of 3 ml/kg. All injections occurred between 0900 and 1100 h to control for circadian and ultradian rhythms. MDMA was obtained from the National Institute on Drug Abuse and was greater than 95% pure. This was a within litter design, so that each litter had an equal number of SAL- and MDMA-treated animals. Animals were decapitated 0.5, 1, 2, and 4 h after administration of MDMA and the hippocampus was dissected by hand and frozen on dry ice until preparation. All procedures were approved by the Cincinnati Children's Research Foundation's Animal Use and Care Committee and the vivarium is fully accredited by the Association for Association for the Assessment and Accreditation of Laboratory Animal Care.

Western blotting

Hippocampi were homogenized in ice cold NP-40 buffer (50mM Tris, 150mM NaCl, 1% NP-40) using a polytron homogenizer. The samples were centrifuged for 5 min at 12,000 x g and 4 ºC and the supernatant was collected. Protein concentrations were determined using the

Bradford method (Pierce Biotechnology, Rockford, IL) and for each sample 300 μg of total protein was incubated with 2 X sample buffer and incubated at 95 ºC for 5 min. Polyacrylimide gels were loaded with the samples and electrophoresed for 30 min at 130 V. Products were then transferred at 40 V to a nitrocellulose membrane for 1.5 h in transfer buffer (25mM Tris, 192mM glycine, 10% methanol). The membrane was blocked in phosphate buffered saline containing 3% bovine serum albumin and 0.05% Tween-20. Primary antibodies were diluted in the blocking buffer and were incubated with the membrane for 1 h at room temperature under gentle agitation. Primary antibody dilutions were as follows: phospho-CAM-KII (Upstate Biotechnology; 1/2000); PSD-95 (Oncogene; 1/2000); CAPON (Abcam; 1/1000); and phospho-serine (Abcam 1/4000). The membrane was washed three times for 5 min with PBS+Tween20 (0.05%) followed by a 30 min incubation with the appropriate secondary antibody conjugated to alkaline phosphotase. The membrane was then washed three times with PBS and the chemilumenscent signal was generated using DuoLux substrate. The membrane was exposed to film and band intensity was measured using ImageJ Software (NIH, Bethesda, MD). Actin levels were used as loading controls and protein levels were adjusted as necessary.

Immunoprecipitation

To assess the phosphorylation of nNOS, samples (400 μg) were incubated overnight with nNOS antibody (1/2000 dilution). Sepharose beads conjugated to recProtein G were incubated with the samples for 1 h. The beads were collected by centrifugation at 12,000 x g for 10 min followed by washing with immunoprecipitation buffer and subsequent centrifugation. Protein loading dye was added to the beads and incubated at 95 ºC for 5 min. Electrophoreses and western blotting was performed on the supernatant following centrifugation as described above using the antibody against phosphoserine.

Statistics

Data were analyzed using a general linear model ANOVA (PROC GLM, SAS, Cary, N.C.). Treatment and time were considered within litter factors. Interactions of time and treatment were analyzed using Student t-Test with significance considered to be $p < 0.05$ and a trend considered $p < 0.10$.

Results

Phosphorylated nNOS and CAM-KII

There were no main effects of treatment on the levels of nNOS in the hippocampus of P11 animals treated with MDMA (Figure 1). No time by treatment effects were observed. There was no main effect of treatment or any interaction of time and treatment for the phophorylated levels of CAM-KII in P11 animals (Figure 2).

PSD-95 and CAPON levels

There was no MDMA related effect on PSD-95 levels in the hippocampi of P11 animals (Figure 3). Similarly, CAPON levels were unchanged in the hippocampi of MDMA-treated animals (Figure 4).

Discussion

It has been previously shown that MDMA exposure from P11-20 alters spatial and path integration learning and memory, even when the animals are tested as early as P30 (Broening et al., 2001; Vorhees et al., 2004; Williams et al., 2003; Chapter 2). This dosing period has also been shown to diminish LTP in the adult rat and increase the expression of pathway members of the NMDA receptor signaling pathway such as NR1, nNOS and PSD-95 (Skelton et al., 2005). With the changes in the NMDA receptor complex in these animals, the purpose of this study was to further elucidate the function of the NMDA receptors in MDMA mediated alterations in brain

function. The results of this study show that there are no apparent immediate effects of MDMA on the NDMA receptor as evidenced by the lack of changes in the phosphorylation of CAM-KII in the hippocampi of MDMA-treated animals. There is also no apparent effect of MDMA on nNOS activity, as there were no changes in the phosphorylation of this protein as well. To further support the hypothesis that MDMA does not effect the NMDA receptor following one dose of MDMA on P11 are the lack of changes in two other structural proteins involved in NDMA signaling, namely PSD-95 and CAPON , (Jaffrey et al., 1998; Jaffrey et al., 2002).

The results of the current study suggest that there could be other mechanisms responsible for the changes in the NDMA receptor complex seen in adult animals. This hypothesis is supported by several recent findings. In animals treated from P11-20, brain derived neurotrophic factor levels are increased in the hippocampus on P21, suggesting that MDMA alters the fundamental development of the brain during this period of rapid development (Koprich et al., 2003). During the development of the brain, many neurotransmitters themselves have a neurotrophic function. Accordingly, MDMA treatment on P11 reduces 5-HT up to 72 h after a 4-dose regimen (Williams et al., 2005). Animals treated on P11-20 with fenfluramine show learning and memory deficits when they are tested as adults, these animals also show the most profound serotonin deficits during neonatal administration (Schaefer et al., 2006; Morford et al., 2002). Further evidence for MDMA altering the development of the hippocampus is found by the six-fold increase of the Nuclear orphan receptor 1 (NOR1) 24 h following 2 doses of MDMA on P11 (Skelton et al., 2005). NOR1 is involved in neuron outgrowth, as NOR1 expression reduced neuron outgrowth and NOR1 is highly expressed in neurons undergoing apoptosis (Ohkura et al., 1994; Ohkura et al., 1996). These data suggest that MDMA alters the

fundamental development of the brain rather than altering specific neurotransmitter systems similar to what occurs after MDMA exposure in adult rats.

One possible reason for the lack of activation of NMDA related proteins could be the immaturity of the NDMA receptor on P11. This is shown by the inability of MK-801 to prevent morphine dependency in P1 or P7 animals, however MK-801 prevents morphine dependency in P14 animals (Zhu and Barr, 2001). It appears that during early expression of the NDMA receptor, the predominate subunit is the NR2B, while in adults the NR1 is the predominant subunit (Takai et al., 2003). This alteration of subunit composition could be the reason CAM-KII is activated in adult animals exposed to MDMA but not in neonatal animals exposed to MDMA (Moyano et al., 2004; Moyano et al., 2005). Similarly, nNOS is tightly linked to the NMDA receptor and the immaturity of the receptor may prevent the alteration in nNOS during the development of the animal.

It has been suggested that the effects of prenatal ethanol exposure are mediated through the alteration of the NDMA receptor (Ikonomidou et al., 2000). This suggests that the NMDA receptor system is sensitive to agents that may not directly act on the glutamatergic system. It could be possible that alterations of other neurotransmitter systems, such as GABA, are affected by MDMA at this age and this then has a secondary effect on NMDA receptors. Further studies are required to elucidate the relationship between NMDA and MDMA. It is unlikely that MDMA is neurotoxic at this age, as another substituted amphetamine, methamphetamine, given to animals on P20 did not produce increases in GFAP 3 days later, while administration on P60 did increase GFAP levels (Pu et al., 1996).

In conclusion, the results of this study suggest that MDMA does not alter early NMDA receptor signaling and that later alterations of this complex may be related to secondary effects.

It would be useful to examine the effects of MDMA treatment on later time points during and following the cessation of dosing.

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Ref Type: Abstract

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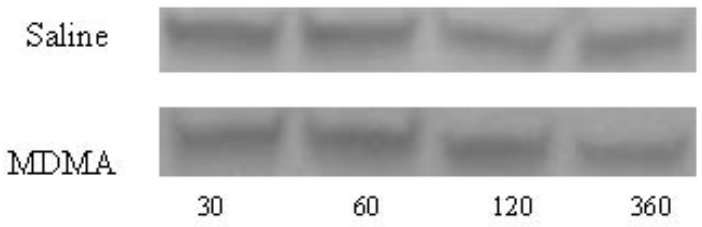
Figure 1. Levels of phosphorylated neuronal nitric oxide synthase. No differences were observed between MDMA and SAL treated animals


Saline
MDMA
30
60
120
360

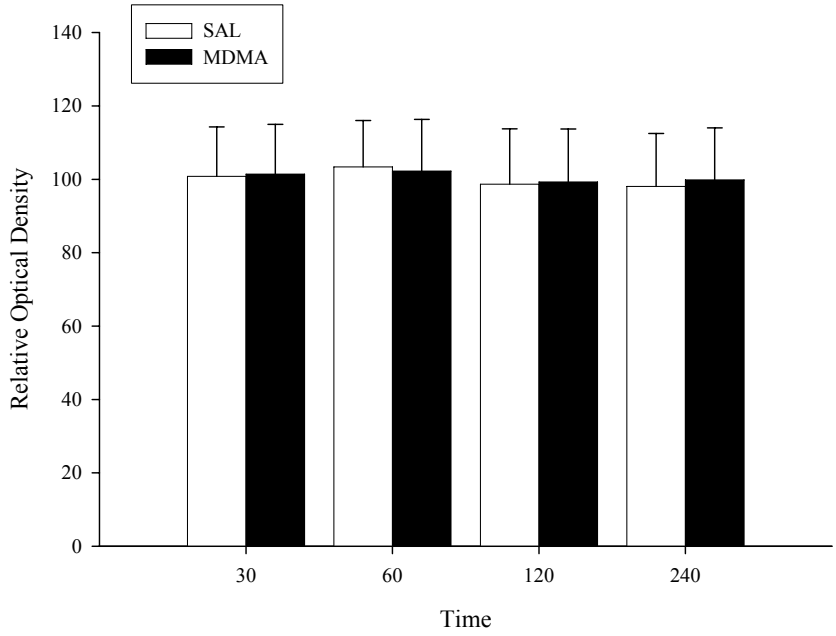
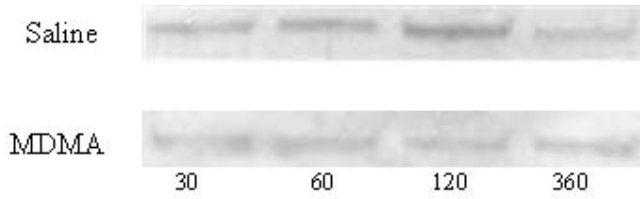

SAL
MDMA
Relative Optical Density
140
120
100
80
60
40
20
0
30
60
120
240
Time

Figure 2. Phosphorylation of CAM-KII is unaltered by MDMA treatment on P11. Animals were administered 1 dose of MDMA and hippocampal levels of phospho-CAM-KII were examined.


Saline
MDMA
30
60
120
360

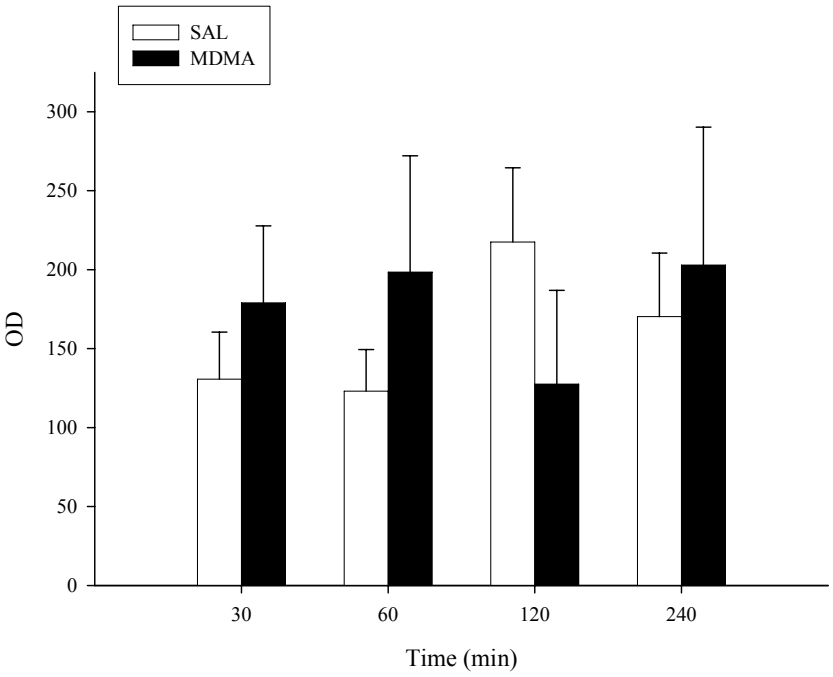
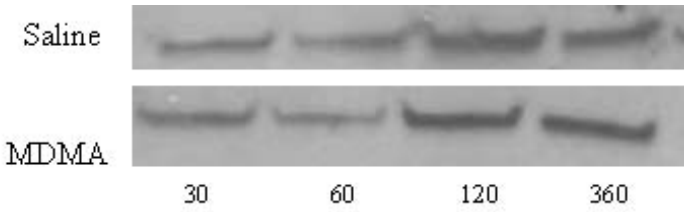

SAL
MDMA
OD
300
250
200
150
100
50
0
30
60
120
240
Time (min)

Figure 3. MDMA treatment does not alter PSD-95 levels in the hippocampus up to 4 hours following a single dose on P11.


Saline
MDMA
30
60
120
360

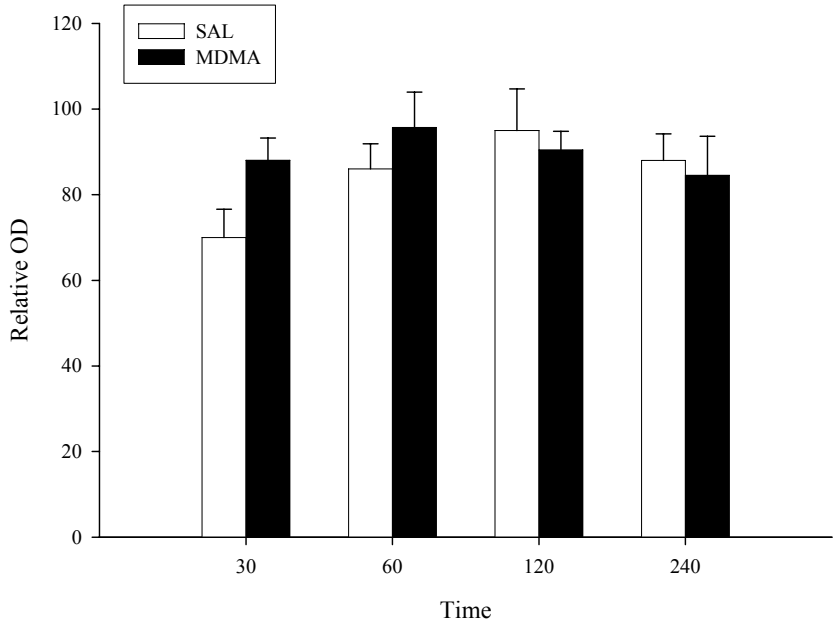
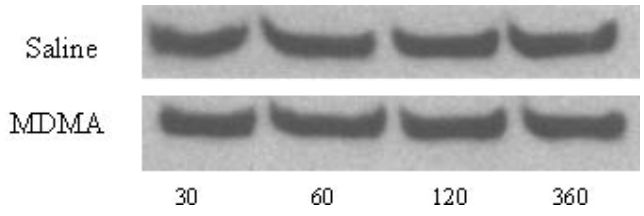
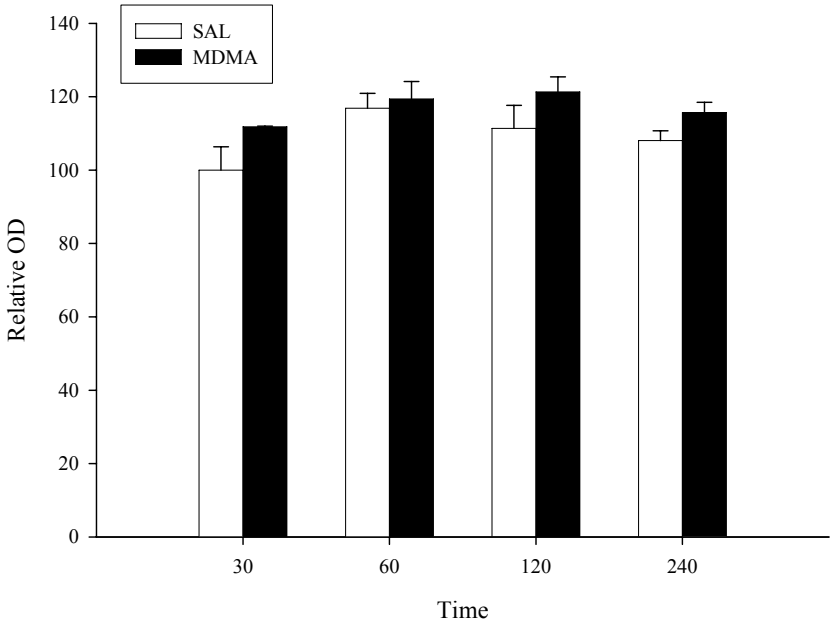

SAL
MDMA
Relative OD
120
100
80
60
40
20
0
30
60
120
240
Time

Figure 4. A single dose of MDMA on P11 dose not alter hippocampal CAPON levels up to 4 hours after administration.


Saline
MDMA
30
60
120
360


SAL
MDMA
Relative OD
140
120
100
80
60
40
20
0
30
60
120
240
Time

CHAPTER 5: DISCUSSION

Conclusions

The results of the present studies suggest that P11-20 MDMA exposure causes spatial learning and memory deficits that emerge very early and persist well into adulthood; while path integration learning deficits emerge early but dissipate later in life. Further, microarray analyses of P11-20 MDMA-treated animals reveal a very small subset of genes that have altered expression levels in the adult hippocampus. It is also apparent that the majority of gene expression changes in the hippocampus occur sometime after the cessation of dosing, and compared to the striatum the gene expression changes of interest were unique to the hippocampus. Upon further examination of protein levels, it was shown that the pathway members of CAPON, namely NMDA receptor subunit 1 (NR1), neuronal nitric oxide synthase (nNOS), and post-synaptic density 95 (PSD-95) are increased during adulthood in the dentate gyrus of P11-20 MDMA-treated animals. Further evidence supporting the alteration of NMDA signaling was found in the decrease of long-term potentiation (LTP) observed in the brain slices of MDMA-treated animals. Finally, it has been shown that MDMA administration does not appear to alter the signaling of the NDMA receptor, as phosphorylation of nNOS and calcium calmodulin kinase II (CAM-KII) appear to be unaltered in the hippocampus of P11 animals given a single dose of MDMA. Collectively, the results of these studies provide evidence for a long-term alteration of the NMDA receptor system as a result of developmental MDMA exposure. While these studies suggest the alteration of NMDA receptors by developmetal MDMA exposure, the lack of NMDA receptor activation on P11 along with the gene expression changes, such as the up-regulation of NOR1, suggest that there may be a fundamental change in

brain organization rather than an alteration in any single neurotransmitter pathway. Further studies will be required to examine this possibility.

MDMA and the NMDA receptor

In the hippocampus, the NDMA receptor complex is highly expressed during the second week of life, followed by a decrease in expression that lasts until adulthood, when the receptor is again highly expressed (Takai et al., 2003). During the early period of high expression, it appears that the complex is immature with the NMDA receptor subunit 2B predominately controlling the receptor (Takai et al., 2003). It has been suggested using models of fetal alcohol syndrome that prolonged NDMA receptor activation during drug exposure may have deleterious effects on future CNS function (Ikonomidou et al., 2000; Ikonomidou et al., 2001). The results in Chapter 3 suggest that developmental MDMA exposure may permanently alter the expression of the NDMA receptor, and in turn, alter cellular mechanisms that are hypothesized to be the foundation of learning and memory formation. The NMDA receptor is not fully functional during the period of MDMA exposure since pretreatment with MK-801 did not attenuate morphine dependence in P7 animals, however MK-801 did attenuate the dependence in P14 and P21 animals (Zhu and Barr, 2001). It is unlikely that MDMA exposure in neonates causes glutamate excitotoxicity, as similar drugs such as methamphetamine, which is neurotoxic in adult animals, does not induce neurotoxicity in developmentally exposed animals (Pu and Vorhees, 1993). Neurotoxicity is generally defined by various markers such as increased expression of glial fibrillary acidic protein (GFAP), increased silver staining and agyrophilia, and animals exposed to MA on P20 do not show GFAP alterations 3 days after administration (Pu and Vorhees, 1993). In fact, MA induced neurotoxicity is not observed until adulthood, as P40 rats do not show any increases in GFAP, but P60 rats do (Pu and Vorhees, 1993). One of the main

reasons that the neonate is not sensitive to the neurotoxic effects of substituted amphetamines is likely that the neonate does not have a hyperthermic response to these compounds (Broening et al., 1994; Broening et al., 1995). Therefore, the more reasonable hypothesis regarding MDMA and NMDA in the neonatal brain would be that MDMA induces a permanent change in glutamate signaling via the NMDA receptor. This is further supported in the deficits in LTP seen in the adult animals exposed to MDMA from P11-20. While LTP is not exclusively NMDA receptor mediated (Brown et al., 1988), the combination of LTP deficits and increases in NMDA receptors seen in the brain of these animals would suggest that glutamate signaling is at least partly responsible for these deficits. To further support this hypothesis, it has been shown that the early 5-HT perturbations induced by MDMA exposure appear to permanently alter 5-HT signaling (Crawford et al., 2006; Williams et al., 2005). Many neurotransmitters have a neurotrophic effect in the immature brain, helping to guide, differentiate and prune the developing neurons. Brain derived neurotrophic factor is increased in the hippocampus on P21 after P11-20 MDMA administration (Koprich et al., 2003), which further suggests that MDMA alters the overall development of the brain rather than specific neurotransmitter systems.

In adult animals application of MDMA to the nucleus accumbens has been shown to decrease glutamate efflux (White et al., 1994). It has been proposed that this depression of glutamate release allows DA release that is modulated through the 5-HT_{2A} receptor (Obradovic et al., 1996). It appears that the effects of MDMA on glutamate levels are transient, as multiple doses of MDMA have no apparent effect on glutamate levels. Antagonism of the NMDA receptor using MK-801 has been shown to prevent the decreases of 5-HT induced by MDMA exposure; however MK-801 prevents the hyperthermia associated with MDMA exposure as well (Farfel and Seiden, 1995). When hyperthermia is maintained during the dosing period following

MK-801, 5-HT depletions are present. The glycine specific antagonist to the NMDA receptor, ACEA 1021, reduces 5-HT depletions in rats; however this also abolishes hyperthermia induced by MDMA (Finnegan et al., 1989; Russell and Laverty, 2001). However, while MK-801 causes hypothermia, ACEA 1021 maintains a normal body temperature, which can still deplete 5-HT levels (Broening et al., 1995).

In adult animals, a single dose of MDMA decreases NR1 and NR2B levels as well as membrane phosphorylated CAM-KII, total CAM-KII levels, and CAM-KII activity 90 min after administration with or without passive avoidance learning (Moyano et al., 2004). Decreases in NR1, NR2B, PSD-95, and phosphorylated CAM-KII levels are found in animals given MDMA (10 mg/kg x 8 doses) for one day following a secondary MDMA challenge and passive avoidance learning (Moyano et al., 2005). No differences were seen if the animals did not undergo learning with MDMA challenge. This suggests that there is a temporary activation of NMDA receptors during MDMA administration that returns to normal function after time.

Nitric oxide and MDMA

In the adult rat, there have been several studies using various nNOS ihibitors that have related nitric oxide generation with MDMA administration. N^G-nitro-L-arginine- methyl ester (L-NAME) treatment prior to MDMA administration slightly alleviated the 5-HT depletions in the parietal cortex and hippocampus, while N^G-nitro-L-arginine (NA) did not prevent MDMA induced 5-HT deficits (Taraska and Finnegan, 1997). Deficits in 5-HT induced by MDMA were partially recovered by pretreatment with another nNOS inhibitor N^{ω}-nitro-L-arginine (L-NORAG) in the frontal and parietal cortex, while complete deficits were still observed in the hippocampus, striatum, occipital cortex, and cerebellum (Zheng and Laverty, 1998). MDMA increased nNOS activity in the frontal and parietal cortex and the striatum 6 h after MDMA

administration, but levels returned 7 h after administration (Zheng and Laverty, 1998). L-NAME or S-methyl-L-thiocitrulline (S-MTC) administration prevented 5-HT and DA depletions in the striatum by infusion or i.p. injection of MDMA and malonate, that enhances the DA-depleting effects of MDMA (Darvesh et al., 2005). Nitrotyrosine levels as well as nitric oxide metabolite levels were increased following MDMA dosing in these animals (Darvesh et al., 2005).

In mice deficient in nNOS, there were no effects in locomotor activity following MDMA administration in the first five days of administration; however, if animals were challenged 45 days following MDMA exposure, nNOS KO mice showed significantly decreased activity compared to WT (Itzhak et al., 2004). This effect is similar to the decrease in locomotor activity following 7-nitroindazole (7-NI) pretreatment with a single day MDMA challenge and then waiting 10, 31 or 61 days to assess locomotor compared to MDMA alone (Anderson and Itzhak, 2003). 7-NI pretreatment has also been shown to be neuroprotective against the effects of MDMA; however, 7-NI also produced hypothermia in the mice, while pretreatment with two other nNOS inhibitors, S-MTC and AR-R17477AR, prevented some of the DA reductions in the mice co-administered MDMA without reducing hyperthermia (Colado et al., 2001). While the nNOS KO mice showed no alterations in 5-HT or DA depletions induced by MDMA compared to WT mice, there was an amelioration of DAT depletion induced by MDMA in nNOS KO mice (Itzhak et al., 2004).

The results in Chapter 3 would suggest that nNOS is altered in the hippocampus of MDMA-treated animals. This could be further elucidated by challenging animals with an NMDA receptor agonist and measuring NO levels and nNOS activity in the brain. Following one dose of MDMA in P11 animals, there is no alteration of nNOS activation in the hippocampus as evidenced by the unchanged levels of phosphorylation in these animals. This is

likely related to the immaturity of the NMDA receptor, as nNOS activation is almost completely dependent on NMDA receptor activation and the resultant calcium influx.

Future Directions

Many of the possible areas in which these studies could be continued derive from the microarray analysis of the MDMA-treated animals. The foremost area of future exploration would be the brain renin-angiotensin system (RAS). Brain angiotensinogen is primarily transcribed in the glial cells, as circulatory angiotensin cannot cross the blood-brain barrier. Angiotensinogen has at least 2, perhaps 3 active peptide fragments. Angiotensinogen is cleaved to an inactive angiotensin I (AngI) which is subsequently cleaved to the primary active peptide, angiotensin II (AngII) by the Angiotensin I converting enzyme (ACE). Subsequent cleavage produces AngIII and AngIV, of which AngIV has been shown to be neuroactive and the role of AngII has yet to be completely deduced since it may be cleaved to AngIV prior to acting on neuronal receptors (Wright et al., 2002). AngII primarily binds to AT1 and AT2 while AT4 is the primary receptor for AngIV. AT4 receptors are highly expressed in the hippocampus, and AngIV has been well linked with learning and memory formation as well as LTP (Gard, 2002; Wright et al., 2002). Perfusion of brain slices with the NDMA receptor antagonist, D,L-AP5, abolished LTP in the hippocampus, while co-perfusion of D,L-AP5 with the AT4 receptor agonist Nle1-AngIV prevented the actions of D,L-AP5 (Davis et al., 2006). This could be potentially significant to MDMA-treated animals, as the LTP deficits seen in these animals could be related to alterations of the brain RAS in addition to alterations in NMDA transmission. LTP mediated through the RAS does not appear to be independent of glutamate signaling, as AMPA receptor antagonism diminishes LTP in Nle1-AngIV treated slices (Davis et al., 2006). AOGEN has also been linked to anxiety, as ACE inhibitors mimic anxiolytic drugs such as diazepam

while AngII administration increased anxiety immediately after administration followed by a decrease in anxiety like behaviors (Barnes et al., 1990; Costall et al., 1990; Georgiev et al., 1987; Tsuda et al., 1992). It has been shown repeatedly that administration of AngII, that could be cleaved to AngIV, facilitates learning and memory, and can rescue function in animals with deficits in learning and memory (for review see (Gard, 2002; Wright et al., 2002)). Another potentially beneficial tool for the study of AOGEN interaction with MDMA is the development of the brain specific AOGEN deficient transgenic rat (Schinke et al., 1996). The use of this animal in addition to pharmacological manipulation of the AT4 receptor could shed further light for the mechanisms of the learning and memory deficits induced by MDMA.

Another possible area of investigation is the nuclear orphan receptor 1 (NOR1). Interestingly, it has been shown that NOR1 mRNA is up regulated by AngII in adrenal cortical cells (Fernandez et al., 2000; Romero et al., 2004), suggesting that AngII or AngIV may work through NOR1 activation in the MDMA-treated animals. Neuronal outgrowth is inhibited by NOR1 expression, and NOR1 is expressed in neuronal cultures undergoing apoptosis (Ohkura et al., 1994; Ohkura et al., 1996). As shown in Chapter 3, NOR1 is up-regulated six-fold in the hippocampus 24 h following MDMA administration on P11. This is similar to the activation of NOR1 by morphine and cocaine and neuroleptics such as haloperidol and clozapine (Werme et al., 2000b; Werme et al., 2000a). It would be interesting to measure NOR1 mRNA in the hippocampus of the AOGEN deficient rats with and without MDMA treatment. With the up-regulation of NOR1 in the P11 animals, it may also prove beneficial to examine the neuronal growth of MDMA-treated animals during the dosing period. The up-regulation of NOR1 also supports the hypothesis that developmental MDMA exposure does not affect a particular neurotransmitter system, but alters brain development as a whole. Testing animals that were

treated with MDMA with an inhibition of NOR1, either pharmacologically or through antisense technology would be a logical next step in this research.

It should be stressed that the pharmacology of MDMA differs between rats and mice, with mice expressing more of a dopaminergic effect from MDMA expression while rats show primarily 5-HT deficits (Green et al., 2003). This makes the utilization of knock-out technologies difficult, since this is primarily done in mice, with few knock-out or transgenic rats available at this time.

The lack of permanence in the Cincinnati water maze (CWM) is an interesting finding from this dissertation and warrants further investigation. The first study that would likely need to be undertaken would be to elucidate the brain regions responsible for path integration learning using lesion studies. Once the regions thought to be under control of path integration learning were discovered, the next possible step would be to examine the differences in younger animals treated with MDMA from P11-20 versus the older animals that did not have spatial learning and memory deficits. Possible methods of examining these deficits would include microarray analysis, monoamine signaling alterations and perhaps Golgi staining to observe neuron growth. While it has been shown that deficits in the CWM are independent of 5-HT depletions (Skelton et al., 2004; Williams et al., 2002), again, it could be that long-term alterations in neurotransmitter signaling are more important than monoamine depletions (Crawford et al., 2006; Schaefer et al., 2006; Williams et al., 2005).

Finally, although not discussed in great detail in this dissertation, the effects of corticosterone (CORT) may play a great role in the MDMA related learning and memory deficits in these animals. It has been shown that CORT modulates learning in an inverse U-shaped manner, with learning deficits observed in animals with low or high levels of CORT (reviewed in

(Lupien and McEwen, 1997)). MDMA administration on P11 increases plasmatic CORT levels during a time when rats are hyposensitive to environmental stressors (Sapolsky and Meaney, 1986). Since some of the genes found on the microarray screen are linked to CORT expression, it is logical that these are products of CORT increases. For example, in cell culture, it has been shown that glucocorticoid receptor activation antagonizes NOR1 activity (Martens et al., 2005). It has also been shown that inhibiting CORT increases during fenfluramine (FEN) administration blocks deficits observed in the CWM (Skelton et al., 2004; Williams et al., 2002). A possible test for this hypothesis would be to adrenalectomize the neonates before dosing and provide constant levels of CORT replacement with a low, physiological and high CORT replacement group. Examining gene expression changes of the selected genes in these rats would demonstrate if the gene expression changes were due to CORT or were directly altered by MDMA.

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