I, Curtis E Grace, hereby submit this original work as part of the requirements for the degree of:

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Student Signature: Curtis E Grace

This work and its defense approved by:

Committee Chair: Charles Vorhees, PhD
Charles Vorhees, PhD

Ton Degraw, MD
Ton Degraw, MD

Steve Danzer, PhD
Steve Danzer, PhD

Gary Gudelsky, PhD
Gary Gudelsky, PhD

Michael Williams, PhD
Michael Williams, PhD

James Herman, PhD
James Herman, PhD

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Developmental methamphetamine exposure: long-term effects on stress, learning, and anxiety in rats

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Curtis Edward Grace
B.S., University of Cincinnati, 2002

Committee Chair: Charles V. Vorhees, Ph.D.
Michael T. Williams, Ph.D.
Gary A. Gudelsky, Ph.D.
James P. Herman, Ph.D.
Steve Danzer, Ph.D.
Ton DeGrauw, M.D., Ph.D.
Prenatal methamphetamine (MA) exposure results in cognitive impairments in children. MA has similar effects when exposure occurs neonatally in the rat, a time of development analogous to human second half of pregnancy. Likewise, early stress exposure can cause cognitive deficits and alterations in responses to stress. MA is a potent stimulator of corticosterone (CORT) release in rats but the role of this in the long-term effects of MA is unknown. To better understand the relationship between MA-induced learning deficits and stress, we sought to determine if (a) developmental MA exposure alters later stress responses, (b) whether neonatal MA-induced CORT increases can be inhibited, and (c) if so, whether this would reduce later learning deficits. We found that MA treatment from P11-15 or 11-20 did not alter the adult response to forced swim, confinement, or acute MA treatment. It has been shown previously that pharmacological inhibition of CORT synthesis or adrenalectomy (ADX) to prevent CORT release are either ineffective or cause secondary effects. For example, CORT synthesis inhibitors such as ketoconazole and metyrapone prevent MA-induced CORT release initially, but 24 h later it rebounds resulting in CORT levels higher than in animals treated with MA alone. ADX removes MA-induced CORT responses, but also reduces hippocampal serotonin (5-HT) more than MA alone when treated on P11 and assessed on P12. Adrenal autotransplantation (ADXA) is another method that reduces CORT output. In neonatal rats, we found that ADXA reduced CORT levels in MA-treated rats compared to SHAM-MA by approximately 50%, being more effective on early days and less effective on later days. ADXA was then used to determine if inhibiting CORT release by this method was enough to attenuate adult learning deficits.
We found that rats developmentally exposed to MA displayed long-term deficits in spatial learning in the Morris water maze (MWM) and egocentric learning deficits in the Cincinnati water maze (CWM) as we previously found. ADXA did not attenuate MA-induced cognitive deficits in either task. MA animals also showed reduced locomotor activity. No differences were observed between MA and control animals in novel object recognition learning. In a test of anxiety, MA-treated animals had decreased latency to enter an open zone in the elevated zero maze, but no differences in the primary index of anxiety, time in open, suggesting few if any anxiety effects. Interestingly, we found that the adult CORT responses following forced swim in ADXA animals remained at 50% of control values, demonstrating that full recovery did not occur. As adults, serotonin was reduced in the hippocampus, neostriatum, and entorhinal cortex in MA-treated offspring, indicating that these effects persist and were not altered by ADXA. The data suggest that CORT increases alone may not mediate MA-induced learning deficits. Whether further attenuation of the CORT response would provide a more complete test of the hypothesis remains to be determined. Autotransplanting one adrenal or even one-half of one adrenal instead of two might be more effective. The role of 5-HT reductions also warrants further investigation.
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CHAPTER 1: INTRODUCTION

Methamphetamine Synthesis and Prevalence of Use

Methamphetamine (MA), a phenylethylamine, is a psychostimulant, sympathomimetic drug that belongs to the amphetamine family and is similar in structure to the neurotransmitters dopamine, epinephrine, and norepinephrine (Figure 1). It is this similarity that causes its effects on the central nervous system. MA exists in D- and L-isomers which differ pharmacodynamically (Mendelson et al. 2006). MA was originally synthesized from ephedrine by Nagayoshi Nagai in Japan in 1893 (Nagai N. 1893) and in 1919 was produced in its crystallized form by Akira Ogata. There are a number of methods by which MA can be synthesized but most commence with either phenyl-2-propanone (P2P) or ephedrine and utilize reductive methods for conversion to MA (Allen and Cantrell 1989; Yamamoto 2004). These methods include catalytic reduction via metals such as palladium, dissolving metal reductions using aluminum or amalgams with mercury, metal hydride reductions using LiAlH₄ or NaBH₄ or non-metal reductions such as the Leuckart synthesis which utilize acids (formic acid) and have been previously reviewed (Allen and Cantrell 1989). However, the most common methods used in clandestine laboratories seem to be the Nagai or red phosphorous method, the Birch or “Nazi” method and the P2P method utilizing mercury chloride amalgams, all of which utilize toxic reagents and pose health risks to those exposed (Hammon and Griffin 2007).

Since most of the ingredients in producing MA are available commercially or agriculturally, it can be produced cheaply and easily. It has therefore become a world-wide problem (Anglin et al. 2000; Chomchai et al. 2004; Degenhardt et al. 2008; Hammon
and Griffin 2007; Schifano et al. 2007; Yamamoto 2004). In the midst of World War II (WWII), Japan, Germany, and the United States supplied soldiers with MA to increase alertness and endurance (Anglin et al. 2000). Japan incurred two of the earliest known MA epidemics following WWII and have since experienced a third (Yamamoto 2004). Although stimulants, primarily MA, are the most abused drugs in Japan and comprise almost 90% of drug offenses (16,964 of 19,219), the prevalence of MA abuse in Japan is less than that in the United States (~ 7% in the U.S.) (Yamamoto 2004). MA (desoxyn) has also been used medically to treat a number of conditions, primary ADHD, narcolepsy, or severe obesity (Anglin et al. 2000). The history and spread of MA use in the U.S. is documented and clandestine labs in Mexico and California are still the primary sources for the U.S. (Anglin et al. 2000). Although the problem of abuse in the U.S. originated in the west, it has gradually spread eastward as demonstrated by admissions to treatment centers (Figure 2 and 3) (Substance Abuse and Mental Health Services Administration 2008).

Of importance, MA is primarily abused by adolescents and young adults of childbearing age (Johnston et al. 2008a; Johnston et al. 2008b). Thus, there is potential for MA abuse by pregnant women. Almost 40% of pregnant MA abusers continue to use throughout pregnancy (Smith et al. 2003) and MA has been reported as the primary drug of abuse in almost twenty-five percent of women seeking drug treatment in the U.S. (Terplan et al. 2009). Passive transfer to the fetus is certain since MA readily crosses the placenta (Burchfield et al. 1991; Garcia-Bournissen et al. 2007); therefore there is concern for potential adverse effects caused by in utero MA exposure.

**Effects of human exposure to methamphetamine in utero**
A limited number of studies exist pertaining to the developmental and neurological outcome of MA exposure to the fetus during pregnancy and fewer exist that examine long-term consequences resulting from such exposure. There are several adverse effects observed in children prenatally exposed to MA. Reduced birth weight, length, and head circumference was observed in a study involving the children of intravenous MA users during pregnancy (Little et al. 1988) and these are some of the most commonly observed effects (Dixon and Bejar 1989; Smith et al. 2008). Oro and Dixon demonstrated that children prenatally exposed to MA, cocaine, or both were more likely to be premature and had reduced birth weight, length, and head circumference and had higher occurrence of placental hemorrhage and anemia compared to controls when matched for maternal risk factors (Oro and Dixon 1987). These children also had other complications such as poor sleep patterns, vomiting, tremors, and did not feed well, symptoms likely caused by withdrawal from the drug (Dixon 1989; Oro and Dixon 1987). A Thai study reported similar findings including smaller head circumference, reduced birth weight, and increased agitation, vomiting, temperature instability, and rapid respiration (Chomchai et al. 2004). Using echoencephalography, it was determined that MA/cocaine exposed infants had increased cranial abnormalities and intraventricular hemorrhage compared to normal controls, but these were similar to those observed in ill infants at risk for hypoxic-ischemic injury (Dixon and Bejar 1989). The authors suggested that these infants could be at risk for adverse behavioral and neurological consequences later in life. A follow-up of this exposed group of children demonstrated delays in visual and fine motor coordination during the first year (Dixon 1989). Proton magnetic resonance spectroscopy (1H-MRS) studies in children between 7 and 8 years of
age demonstrated reduced creatine levels in the striatum indicating alterations in energy metabolism, but no reduction in N-acetyl containing compounds (Smith et al. 2001). In children 3-4 years of age, increased total creatine, N-acetyl aspartate, and glutamate/glutamine concentrations in frontal white matter as well as decreased myoinositol in the thalamus were observed by $^1$H-MRS compared to controls, suggesting more rapid, yet abnormal neuronal and glial development (Chang et al. 2009). These children also had reduced performance on the Beery-Visual Motor Integration Test (Chang et al. 2009). Magnetic resonance imaging (MRI) showed that MA exposed children had smaller volumes of brain structures such as the putamen, globus pallidus, and hippocampus compared to non-exposed controls (Chang et al. 2004). The reduced volumes correlated with poorer neurocognitive performance on tests of visual motor integration, attention and psychomotor speed, and long delay spatial and verbal memory tasks, indicating that MA disrupts cognitive processes in these individuals (Chang et al. 2004). With diffusion tensor imaging (DTI), MA-exposed children showed lower diffusivity in frontal and parietal white matter but no change in fractional anisotropy compared to unexposed controls (Cloak et al. 2009), suggesting less structured white matter tracks in these regions. Infants of mothers with positive screens for cocaine and/or amphetamines that were matched with controls for socioeconomic status and race had lower scores on the Fagan Test of Infant Intelligence, a test of novel object recognition memory (Struthers and Hansen 1992). MA exposed infants were tested using the NICU Network Neurobehavioral Scale (NNNS), a test that assesses stress and behavioral and neurological function within the first 5 days of life, and were found to be more lethargic, had decreased arousal, and increased physiological stress (Smith et al. 2008). A case
report of a single prenatally exposed infant discovered cysts in the white matter of the parietal lobe by sonography (Murphy et al. 2007). Illicit drug exposure among Hawaiian births from 1986-2002 showed that infants exposed to MA had higher rates of cardiovascular, CNS, limb, and oral cleft birth defects such as septal defects, microcephaly, polydactyly, syndactyly, and cleft lip or palate (Forrester and Merz 2007).

It should be noted that the existing data for humans exposed to MA in utero are partly confounded since abusers in these studies tend to also abuse other illicit drugs or substances such as alcohol, tobacco, or marijuana (Derauf et al. 2007; Little et al. 1988; Smith et al. 2008). Socioeconomic status is also a problematic issue since many MA-abusing mothers obtain little prenatal care (Chomchai et al. 2004; Derauf et al. 2007; Smith et al. 2008). For instance, follow-up interviews of mothers who used MA were conducted and 72% received no prenatal care and 96% made < 5 prenatal care visits (Chomchai et al. 2004). Also, some reports use surveys to monitor drug use during pregnancy and these can vastly underestimate drug use by pregnant women (Slutsker et al. 1993). Studies following MA-exposed children through adulthood are lacking and therefore the long-term consequences are largely undefined. The available studies, however, provide some insight into the possible developmental and neurological problems associated with in utero MA exposure and suggest that more comprehensive studies are warranted.

Women who abuse MA before pregnancy have been shown likely to continue during pregnancy and studies have documented positive blood and/or urine tests for MA during pregnancy, labor, and delivery (Chang et al. 2004; Chomchai et al. 2004). It has
also been demonstrated that MA readily crosses the placenta in sheep (Burchfield et al. 1991) and can be measured in neonatal human hair (Garcia-Bournissen et al. 2007).

**Adult Exposure to Methamphetamine**

**Humans**

Chronic adult MA abuse results in neurotoxicity and compromised cognition (Chang et al. 2007). Magnetic resonance imaging of adult chronic MA users shows increased globus pallidus and putamen and decreased hippocampal volume (Chang et al. 2005; Thompson et al. 2004). [1H]-Magnetic resonance spectroscopy of MA users reveals reduced N-acetylaspartate/creatinine ratios in the anterior cingulate and reduced creatine in the basal ganglia (Nordahl et al. 2005; Salo et al. 2007; Sekine et al. 2002). Positron emission tomography and autopsy studies show reduced levels of striatal dopamine (DA), tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicular monoamine transporter type 2 (VMAT-2) (Johanson et al. 2006; Kitamura et al. 2007; McCann et al. 2008; Volkow et al. 2001; Wilson et al. 1996). Serotonin transporter (SERT) density is also reduced (Sekine et al. 2006). In chronic MA users, monoamine transporter changes correlate with cognitive/memory impairments (Johanson et al. 2006; Volkow et al. 2001), including impairments of recall, manipulation of information, verbal and non-verbal fluency, attention, and executive function (Gonzalez et al. 2004; Gonzalez et al. 2007; Hoffman et al. 2006; Kalechstein et al. 2003; Moon et al. 2007; Simon et al. 2000).

**Animals**
Acute high-dose MA treatment of rats results in a pattern of neurotoxicity resembling that described in human chronic MA abuse. MA toxicity is thought to occur via several mechanisms. For example the following are observed: (1) hyperthermia that contributes to neurotoxicity; increasing ambient temperature during treatment increases toxicity and lowering ambient temperature has the opposite effect in rats (Broening et al. 1997; Brown et al. 2003; Fukumura et al. 1998; Herring et al. 2008); (2) neostriatal reactive gliosis based on increased expression of glial fibrillary acidic protein (GFAP) (Broening et al. 1997; Fukumura et al. 1998; Herring et al. 2008); and (3) microgliosis (antibody for the rat CD11b receptor) (LaVoie et al. 2004). Hyperthermia, increased GFAP, argyrophilia by silver staining, and microgliosis are also seen in MA-treated mice given a neurotoxic dosing regimen (Fantegrossi et al. 2008; O'Callaghan and Miller 1993; O'Callaghan and Miller 1994; O'Callaghan and Miller 2002; Thomas et al. 2004a; Thomas et al. 2004b). (4) MA causes profound alterations in monoaminergic systems, blocking uptake of monoamines into vesicles via VMAT-2, reversing flow of DAT, and blockade of MAO-induced breakdown of monoamines, resulting in increased monoamine release in the synaptic cleft (Cadet et al. 2007). Specifically, a neurotoxic MA treatment regimen causes DA and 5-HT reductions in the striatum and 5-HT reductions in the hippocampus (Bisagno et al. 2002; Broening et al. 1997; Cappon et al. 2000; Fukumura et al. 1998; Herring et al. 2008; Ott et al. 1971; Wallace et al. 2001) with partial recovery over time (Friedman et al. 1998). Reductions have been observed in striatal DAT (Schroder et al. 2003), TH activity (Kokoshka et al. 2000), and VMAT-2 (Eyerman and Yamamoto 2007), as well as hippocampal reductions in SERT (Schroder et al. 2003) and tryptophan hydroxylase activity (Kokoshka et al. 2000). Similarly in
mice, a neurotoxic dosing regimen of MA causes reductions in TH, DA, 3,4-
dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and DAT binding in
striatum (Itzhak et al. 2002; Miller et al. 2000) and 5-HT, DA, and HVA reductions in
forebrain regions (Fantegrossi et al. 2008). (5) MA-induced DA release is associated with
formation of free oxygen radicals which are neurotoxic (Stokes et al. 1999). In addition,
antioxidants reduce neurotoxicity (Fukami et al. 2004), free radical scavengers attenuate
DA depletion caused by MA (Kondo et al. 1994), and MA exposed mice over expressing
copper-zinc superoxide dismutase are protected from the neurotoxic effects (Cadet et al.
et al. 1994; Mark et al. 2004) and subsequent production of nNOS (Deng and Cadet 1999)
is associated with neurotoxicity which can be prevented by nNOS inhibitors (Itzhak et al.
2000). (7) Lastly, the appearance of cell death (demonstrated by increased TUNEL
staining) in the striatum and hippocampus of mice following high dose MA has also been
reported (Deng et al. 2001).

Neurotoxic MA treatment regimens in rats are reported to also affect behavior.
For example, a MA dosing regimen that caused DA, 5-HT, and/or DAT reductions and/or
GFAP increases resulted in impaired novel object recognition and egocentric learning
(based on self-movement cues), but only minor reductions in locomotor activity (Herring
et al. 2008; Marshall et al. 2007; Wallace et al. 2001). In terms of spatial learning, either
no (Herring et al. 2008; Schroder et al. 2003) or very small transient deficits (Friedman et
al. 1998) are reported. Much less is known about behavioral effects in mice following
MA exposure. In studies using a single, low (1 mg/kg) dose given on multiple days (7
days), groups have observed deficits in novel object learning in mice (Arai et al. 2008; Ito
et al. 2007; Kamei et al. 2006; Mizoguchi et al. 2008). MA-induced dopaminergic reductions are associated with impaired conditioned place preference to cocaine and MA in Swiss Webster mice. These responses were ameliorated by N-acetylcysteine treatment (Achat-Mendes et al. 2005; Achat-Mendes et al. 2007).

**Prenatal exposure to methamphetamine in animals**

*General characteristics*

Models of *in utero* MA exposure in animals exist, primarily in rats. It has been observed that following a single 40 mg/kg dose of MA to mouse dams, MA levels in both the fetal (~99 ng/mg protein) and maternal (~335 ng/mg protein) striatum are highest 60 min post injection (Won et al. 2001). Whole brain levels in the fetus remain elevated 4 h later (~40 ng/mg protein) (Won et al. 2001). Concentrations in adult brainstem and cortex are also higher than fetal levels and therefore, placental transfer of MA to the fetal brain is roughly 20–40 % of adult levels (Won et al. 2001). According to these authors, this represents brain concentrations similar to those of human infants from MA-abusing mothers (Won et al. 2001). However, in the sheep, which is considered a model more relevant to the human placenta, different observations are found. Although the peak concentration of MA (at ~ 15 min) in the ewe was substantially higher than that of the fetus, at 30 min, fetal concentrations were greater than that of the ewe and remained elevated (Burchfield et al. 1991). Further, MA concentrations in organs such as lung, placenta, kidney, and brain were higher than plasma concentrations suggesting that MA accumulated in these tissues (Burchfield et al. 1991). These data suggest that the high concentrations in various areas can lead to damaging effects on postnatal development.
Dams exposed to 5 mg/kg MA b.i.d. from gestational day (GD) 13 to 20 have reduced weight gain compared to controls, but litter size is unaffected (Cabrera et al. 1993). No effects were observed on growth parameters of pups at birth (weight, crown-rump length, or anogenital distance) (Cabrera et al. 1993) although birth weight was reduced in MA animals when exposed throughout gestation (Slamberova et al. 2006). These differences are apparently the result of how soon before birth the drug is discontinued (Cabrera et al. 1993; Hruba et al. 2008; Hruba et al. 2009b; Slamberova et al. 2006; Slamberova et al. 2007). When pregnant females were administered 10, 15, or 20 mg/kg MA b.i.d., from GD13-18, increased mortality in dams and pups, reduced litter size, and reduced gestational length were observed (Acuff-Smith et al. 1996) and at even higher doses (50 mg/kg), mortality is increased in MA progeny (Acuff-Smith et al. 1992). MA exposure throughout gestation and pre-weaning can adversely delay some maturation parameters such as tooth eruption and ear opening (Hruba et al. 2009b). Interestingly, histological evidence demonstrates brain abnormalities such as microgyria, abnormal folding of cortical surfaces, and cerebral and intraventricular hemorrhage as well at low doses (Cui et al. 2006). It is difficult to make direct comparisons between studies in terms of both biochemistry and behavior since drug doses, administration times, and age of postnatal testing widely differ between experiments. In addition, several studies show sex effects that complicate interpretation of the data. In this section, the literature pertaining to prenatal rat and mouse models are reviewed.

**Biochemistry**
Prenatal exposure to MA alters brain neurochemistry, but much less information is available in this model in comparison to exposure in neonatal and adult animals. For DA uptake sites, it appears that in utero exposure to low concentrations of MA cause decreases, while higher concentrations (10 mg/kg) produce increases in pups and adults (Weissman and Caldecott-Hazard 1993). In terms of the extracellular concentrations, prenatal MA (5 mg/kg), administered once daily throughout gestation, results in increased extracellular DA and HVA in the nucleus accumbens following a 1 mg/kg MA challenge in adulthood compared to controls (prenatal saline with adult MA challenge) (Bubenikova-Valesova et al. 2009). DOPAC levels in MA animals are reduced from baseline, but increased compared to controls (Bubenikova-Valesova et al. 2009).

Decreased TH mRNA is also observed in gestationally-exposed females in the ventral tegmental area, which were not observed in males and lasted less than 30 days (Gomes-Da-Silva et al. 2002). Male mice (40 mg/kg b.i.d.) that were administered MA from GD 7-18 had reduced striatal DAT, DA, DOPAC, HVA, and 3-methoxytyramine (3-MT) and DA in the ventral brainstem in adulthood (Heller et al. 2001) but no effects were observed in the hippocampus and neostriatum of rats (Acuff-Smith et al. 1992). Together the data suggest that prenatal MA exposure can alter the developing dopaminergic system.

Alterations in the serotonergic system are also evident. Similar to what is observed with DA uptake sites, 5-HT uptake sites are, in general, decreased by low-dose gestational exposure and increased at higher gestational doses when examined in adulthood (Weissman and Caldecott-Hazard 1993; Weissman and Caldecott-Hazard 1995). However, another study reports no effect on 5-HT uptake sites in prenatal MA
animals as adults (Cabrera et al. 1993). Decreased 5-HT\textsubscript{2} receptor binding is also observed in frontal cortex at lower doses (2 mg/kg) (Sato and Fujiwara 1986). \textit{In vitro} cultures of three-dimensional mesencephalic striatal aggregates from mouse embryos that were taken from dams given MA from GD7-13 showed increased serotonergic, but not dopaminergic markers (Won et al. 2002). 5-HT was increased for at least 2 months and 5-HIAA for 30 days in these aggregates. No alterations in serotonergic markers are observed \textit{in vivo} in rats exposed from GD7-18 at 50 mg/kg b.i.d. and examined on P28 (Acuff-Smith et al. 1992) but 20 mg/kg b.i.d. produced 5-HT depletions on P70 (Acuff-Smith et al. 1996). It is unclear how alterations in the serotonergic system affect long-term brain development, but there is some evidence from \textit{in vitro} experiments that MA and MA-related compounds stimulate cellular growth, differentiation, and morphological remodeling following a neurotoxic insult (Weissman and Caldecott-Hazard 1995).

There are few data available for other neurotransmitters. One group has demonstrated a decrease in norepinephrine (NE) uptake sites in the posterior cortex in adulthood after prenatal exposure (Weissman and Caldecott-Hazard 1993; Weissman and Caldecott-Hazard 1995) and another group showed no effects in the striatum or frontal cortex in pups after prenatal exposure (Sato and Fujiwara 1986). Perhaps effects on NE emerge later in life, but more experiments need to be completed to clarify this.

\textit{Behavior}

Alterations in motor function have been observed following prenatal MA exposure. GD7-12, but not 13-18 exposure of 15 or 20 mg/kg MA b.i.d. causes hypolocomotion in offspring on P14 (Acuff-Smith et al. 1996). No spontaneous
locomotor effects at P15, 30, 60, or 90 (Acuff-Smith et al. 1996) and no early locomotor effects were observed at higher doses (Acuff-Smith et al. 1992). MA (10 mg/kg b.i.d.) throughout pregnancy (first 3 weeks of life) reduced activity and rearing in the open field on P30 (Weissman and Caldecott-Hazard 1993). In contrast, 5 mg/kg MA b.i.d during pregnancy and the neonatal period (GD0-21, 2 day recovery period from birth, then an additional 19 days of exposure) showed that MA male offspring were more active in an activity wheel during the dark cycle over many months (Martin and Martin 1981). As adults, prenatally exposed MA rats (GD0-birth) showed no differences from controls in locomotion in the open field (Schutova et al. 2009), but were hyperactive following a 1 mg/kg MA challenge compared to MA-challenged saline controls (Bubenikova-Valesova et al. 2009; Schutova et al. 2009). These animals also have decreased immobility and increased rearing (Bubenikova-Valesova et al. 2009). Rats administered MA throughout gestation have impaired surface and mid-air righting reflexes and were impaired in rotorod tests as neonates, suggesting early motor problems (Slamberova et al. 2006); similar deficits were observed when exposure continued during lactation (Hruba et al. 2008). Interestingly, these authors show that several of the MA-induced motor deficits are transferred across generations (Slamberova et al. 2007) and differences in maternal care produced by MA administration to the dam may account for some of the motor deficits since cross-fostering to control mothers provides some improvement (Hruba et al. 2008; Hruba et al. 2009b; Pometlova et al. 2009).

There is a relatively small amount of data examining the effects of prenatal MA exposure on anxiety. In one study, there were no differences in anxiety in the open field (time in corners, frequency of corner entry, or time freezing or immobile) or elevated plus
maze (EPM) in adult animals prenatally exposed to MA (5 mg/kg/d) throughout gestation compared to controls, however an acute (1 mg/kg MA) challenge 30 min prior to testing the adult offspring reduced anxiety in both tests regardless of prenatal treatment (Schutova et al. 2009). Although the MA challenge reduced comforting behavior and time spent in corners in the open field and increased time in open and decreased stretch attends in the EPM, these could be the result of increased activity and not a true measure of anxiety. Prenatally exposed MA animals given an acute MA challenge were more exploratory (time spent rearing and sniffing) in the open field compared to controls given the same challenge, indicating pre-exposure increases sensitivity to later exposures of the drug (Schutova et al. 2009).

In terms of spatial learning in the MWM, findings differ depending upon dose and exposure period. We previously examined long-term spatial learning following early (GD7-12) and late (GD13-18) MA exposure (Acuff-Smith et al. 1996). In acquisition and reacquisition phases, no significant deficits in MWM learning were observed following gestational exposure to 15 and 20 mg/kg MA, for either the early or late exposure, but in the shift phase, MA-induced spatial deficits were observed by increased latency to the platform (Acuff-Smith et al. 1996). Deficits were more pronounced in the early group than the late group (Acuff-Smith et al. 1996). No such deficits were observed in animals treated with 2 or 10 mg/kg b.i.d. throughout pregnancy (Weissman and Caldecott-Hazard 1993). However, when low doses (5 mg/kg) were administered throughout gestation, long-term spatial learning deficits were produced, suggesting that the period of exposure may account for differences in effects (Slamberova et al. 2005b); however, these findings were not replicated (Hruba et al. 2009a; Hruba et al.)
Further, pups exposed to MA or SAL during gestation and cross-fostered to dams that continued MA or SAL exposure during lactation were impaired in spatial learning in the MWM (increased latency and cumulative distance) as adults if they received MA from the dam during lactation, i.e., postnatal exposure to MA resulted in MWM deficits regardless of prenatal treatment (Hruba et al. 2009a; Hruba et al. 2009b; Schutova et al. 2008a). Whether this effect is due to the period (pre- vs. pre- and postnatal) of MA exposure or due to differences in maternal care is unclear (Hruba et al. 2009b) since these authors have previously demonstrated that MA impairs maternal behavior (Slamberova et al. 2005a). In addition, these authors also found differences in search strategies between treatment groups; prenatally injected animals (MA or SAL) used different strategies to locate the platform than uninjected controls (Schutova et al. 2008a). Prenatally MA-exposed rats show no deficits in MWM as adults, have been shown to perform better in the MWM retention memory task, but acute MA exposure (1 mg/kg) prior to or following MWM testing in adulthood impairs spatial learning in these animals compared to controls (Schutova et al. 2008b; Schutova et al. 2008a). However, these findings were inconsistent as well (Hruba et al. 2009b). Spatial learning impairment in animals exposed to MA throughout gestation and given MA challenges each day following daily MWM testing suggests that such administration impairs memory consolidation (Schutova et al. 2008a).

Other behavioral data exist, but are few. For instance, MA exposed animals are impaired in a multiple-T water maze; increased errors and latency to the platform were observed (Acuff-Smith et al. 1996), but at higher doses, no effect was observed (Acuff-Smith et al. 1992). Impaired performance on spontaneous alternation and passive
avoidance was also observed (Acuff-Smith et al. 1996). Also, 50 mg/kg MA b.i.d. from GD7-12 causes increased startle amplitude on P27 (Acuff-Smith et al. 1992).

Other effects

MA-induced effects upon development of other organ systems have been observed as well. An early preliminary report found that MA (50 mg/kg b.i.d.) exposure from GD7-12 but not GD13-18 resulted in higher incidence of anophthalmia (Vorhees and Acuff-Smith 1990) and further investigation revealed other ocular defects such as microphthalmia and folded retina (Acuff-Smith et al. 1992; Acuff-Smith et al. 1996). Abnormalities in optic nerve myelination (Melo et al. 2006; Melo et al. 2008; Pons et al. 2007) and retinal development have been observed (Pons et al. 2007). It is not entirely clear how eye and retinal defects are produced by prenatal MA exposure, but there is evidence that it may be through oxidative stress mechanisms (Melo et al. 2005). Cardiac hypertrophy, abnormal heart development, and myocardial damage have been observed at birth in Wistar rats following low-dose gestational MA exposure, however this damage recovers, but perhaps at higher doses could permanently alter heart development (Inoue et al. 2004).

With respect to DNA and altered gene expression, prenatal MA exposure has been shown to change striatal expression of ~900 genes (Noailles et al. 2003). Interestingly, substantial changes were altered in genes important for guidance and migration of neurons (i.e., laminin and semaphorin 3) which suggest MA disrupts neuronal organization and thus formation of proper synaptic connections (Noailles et al. 2003). Genes that encode structural proteins and trophic factors were also altered.
(Noailles et al. 2003). Since the function of many of these genes is known, such experiments are insightful in determining mechanisms of MA action as well as how exposure might contribute to later neurological and cognitive problems. Prenatal exposure also results in oxidative DNA damage in the fetal brain and liver without degeneration of striatal dopaminergic nerve terminals, a mechanism distinct from that observed in adult animals (Jeng et al. 2005; Wong et al. 2008). Further experimentation with this lesion model is warranted as well as exploration of gene expression changes from microarray data (Noailles et al. 2003).

There are a number of studies that have examined seizure susceptibility following prenatal MA exposure, primarily by Slamberova et al. For example, 5 mg/kg MA throughout gestation shortens both GABA and NMDA-induced latency to seizure onset in male offspring in adulthood (Slamberova and Rokyta 2005a). Prenatal MA (5 mg/kg) alters seizure susceptibility in adult females and is affected by stage of the estrous cycle (Slamberova and Rokyta 2005a; Slamberova et al. 2008). The authors report increased NMDA-induced seizure duration in MA animals that is more severe during diestrus, but decreased GABA-induced seizure duration (Slamberova and Rokyta 2005b). The findings in these two studies are unclear and should be interpreted with caution since some of the parameters examined do not differ between MA and SAL treated animals, but differ between MA and uninjected controls, suggesting that the effects are perhaps confounded or mediated by injection stress (Slamberova and Rokyta 2005b; Slamberova and Rokyta 2005a). Indeed, the authors suggest that the effects may be due to prenatal stress in a follow-up study (Slamberova et al. 2009). Prenatally exposed adult MA-treated males and diestrus females have reduced thresholds of first fasciculation and
males have reduced clonic flurothyl-induced (GABA<sub>A</sub>) seizure susceptibility
(Slamberova 2005). However, in a subsequent study, flurothyl-induced clonic and tonic-
clonic seizure thresholds were not affected by prenatal MA treatment in either sex
(Slamberova et al. 2008). A 1 mg/kg acute MA challenge prior to seizure induction had a
protective effect regardless of drug exposure (Slamberova et al. 2008) and in another
study, this challenge increased NMDA-induced seizure susceptibility in animals
prenatally exposed to MA (Slamberova et al. 2009).

As can be seen from these studies, findings vary widely. The factors that make
comparisons between studies difficult include, but are not limited to: the dose of MA,
period of exposure, sex of subjects, and testing period. These factors are further
complicated by effects of prenatal stress and alterations in maternal care as well as strain
differences.

**Neonatal exposure to methamphetamine in animals**

**Neonatal rat model**

The neonatal rat has been utilized as a model of in utero human exposure,
particularly of the second half of human pregnancy. This is due to the observation that
comparable regional brain development in a rat continues postnatally compared to human
development. For instance, neurogenesis of granule cells of the dentate gyrus of the
hippocampus continue to develop till approximately P19 and myelination is primarily
postnatal in rodents (Bayer et al. 1993; Rice and Barone S Jr 2000). Mathematical
algorithms that extrapolate regional brain development across species demonstrate that
brain development in the P11 rat is approximately equivalent to 19 weeks post-
conception for limbic and 26 weeks post-conception for cortical structures in humans (Clancy et al. 2007; Clancy et al. 2006). As discussed in this document, MA exposure during the early postnatal period in the rat is defined as exposure up to ~20 days of life and is a model of the second half of intrauterine human development.

In modeling human drug exposure in animals, it is also critical to determine comparable doses between species, i.e., to mimic pharmacokinetic or pharmacological efficacy and/or actual human usage of the drug. Several models have been described, either based on amount of drug in relation to body mass (mg/kg), on administering the drug to approximate human pharmacokinetics (Cho et al. 2001), or by interspecies scaling (Mordenti and Chapell 1989). However, no approach is completely sufficient for a number of reasons. For instance, neonatal (Cappon and Vorhees 2001) and adult (Cho et al. 2001) pharmacokinetic data exist in rats, but not in human fetuses, preventing pharmacokinetic comparison across species at this stage of development. Also, information regarding an average dose taken by pregnant abusers is lacking. MA metabolism in rats is different in duration from humans as well, i.e., $T_{1/2}$ in rats is approximately 1 to 3 h and ~12 h in humans (Cappon and Vorhees 2001; Cho et al. 2001). Lastly, in many studies, MA is administered to rats postnatally whereas humans are exposed in utero and therefore any alteration in drug absorption/clearance by the mother and placenta is bypassed. The model which was used in the following experiments was that described by Mordenti and Chapell where $dose_{human} = \frac{dose_{rat} \times (weight_{human}/weight_{rat})^{0.7}}{weight_{rat}}$ (Mordenti and Chapell 1989). This interspecies dosing equation was used because it takes into account plasma half-life, volume of distribution, and clearance (Mordenti and Chapell 1989). However, concerns with this model have
been raised (Baumann et al. 2007; de la Torre R. and Farre 2004; Lin 1998). Human studies have reported daily MA exposures in abusers ranging from 250-10,000 mg/day, averaging approximately 1600 mg/day (Chang et al. 2005; Simon et al. 2000; Volkow et al. 2001). The reported range (250-10,000 mg) translates to a daily exposure of 4.2 - 166.7 mg/kg given a 60 kg human. Therefore, the dose utilized in the following experiments (10 mg/kg 4x daily) is within the range of human abuse. Furthermore, our lab has demonstrated that MA causes long-term learning and memory impairments using a range of dosages with a neonatal model of exposure in rats that are consistent with the human data (Vorhees et al. 1994a; Vorhees et al. 2000; Vorhees et al. 2007; Vorhees et al. 2008; Vorhees et al. 2009; Williams et al. 2002; Williams et al. 2003c; Williams et al. 2003a; Williams et al. 2003b; Williams et al. 2004b). Although all models are included in the following review of the literature and in Table 1, it should be noted that the experiments performed and described in this manuscript utilize 10 mg/kg MA x 4 daily with 2 h intervals and consisted of P11-15 or P11-20 exposure. A review of the neonatal MA literature follows and is chronologically outlined (Table 1).

General characteristics

MA has anorectic properties, thus it is not surprising that one of the most commonly observed features of neonatal MA exposure in rodents is that of reduced weight gain which recovers after the drug is discontinued (usually in adulthood) (Acevedo et al. 2007; Crawford et al. 2003; Gomes-Da-Silva et al. 1998; Grace et al. 2008; Schaefer et al. 2006; Schaefer et al. 2008; Vorhees et al. 1994a; Vorhees et al. 1996; Vorhees et al. 2000; Vorhees et al. 2007; Vorhees et al. 2009; Williams et al. 2003a; Williams et al. 2003b; Williams et al. 2004b).
Williams et al. 2003c; Williams et al. 2003a; Williams et al. 2004b). It is important to note that although MA induces acute hyperthermia in adult rodents that exacerbates neurotoxicity (Broening et al. 1997; Brown et al. 2003; Fukumura et al. 1998; Herring et al. 2008; Miller and O'Callaghan 2003; O'Callaghan and Miller 1994) it does not in neonates (Armstrong et al. 2001; Cappon et al. 1997). Neonates do not become hyperthermic at P20 whether treated at a typical ambient temperature or in a heated room. Neurotoxicity in terms of increased GFAP at P40 are not seen at typical ambient room temperatures but if placed in a heated room (30°C) show elicited neurotoxicity (Cappon et al. 1997). Neonatal MA exposure in rodents is sometimes associated with increased mortality (Acevedo et al. 2007; Vorhees et al. 1998; Vorhees et al. 2000; Vorhees et al. 2007) but this effect is variable and the cause of death, when it does occur, has never been determined.

**Biochemistry**

Neonatal exposure to MA in the rat appears to primarily affect serotonergic systems. Depletions in hippocampal 5-HT in neonatal rats exposed from P11-15 or P11-20 are observed 24 h following treatment on P16 and P21, but not later on P30; 5-HIAA is decreased at P30, suggesting changes in rate of utilization or turnover (Schaefer et al. 2008). There were no effects on hippocampal 5-HT or 5-HIAA on P12 after P11 treatment (Schaefer et al. 2006). Reductions in 5-HT were also observed in the neostriatum on P21 and 5-HIAA on P16 and P21 (Schaefer et al. 2006; Schaefer et al. 2008). Serotonin depletions were observed in the telencephalon of rats administered a total daily dose of 100 mg/kg from P17-20 and examined 2 weeks later, indicating
depletions may persist (Lucot et al. 1982). Intriguingly, early findings revealed that whole brain (Tonge 1972) and in regions such as the cortex and hippocampus (Tonge 1973), 5-HT and 5-HIAA were elevated 3, 6, and 9 months later following 80 mg/l MA supplied in the drinking water throughout gestation. The discrepancies among these findings could be because of the route of exposure, dose, and/or exposure age. As adults, increases in 5-HT fiber density are found following a single 50 mg/kg exposure in gerbils on P14 in the nucleus accumbens (Lehmann et al. 2003; Lesting et al. 2005), dorsomedial and ventromedial caudate putamen (Lehmann et al. 2003), as well as the prefrontal and entorhinal cortices (Neddens et al. 2003; Neddens et al. 2004). Comparisons across species should be made with caution, since the doses, exposure period, and route of administration are not congruent and regional brain development may be temporally different between rats and gerbils. However, taken together, these data suggest that MA-induced alterations in neonatal 5-HT may disrupt proper development and innervation of serotonergic neurons. Interestingly, several of the affected regions are also important in learning and behavioral tasks in which MA induces deficits. For instance, the hippocampus is important in spatial learning (Morris et al. 1982) and MA-treated neonates are deficient in spatial learning tasks (Vorhees et al. 1994a; Vorhees et al. 2008; Vorhees et al. 2009) and have altered locomotor activity (neostriatum/caudate putamen-dependent) (Acevedo et al. 2007; Vorhees et al. 1994b; Vorhees et al. 2009). It is thus suggestive that long-term learning and behavioral deficits may be attributed to the underlying alterations in 5-HT neuron development or in altered 5-HT levels in these brain regions. Despite such alterations in 5-HT, DA levels are relatively unaffected.
Although similar treatments in adult rats produce striatal DA depletions of 40-60% (Cappon et al. 2000; Wallace et al. 1999) that can be long-lasting, neonatal exposure does not cause initial DA depletions in the neostriatum. For instance, no changes are observed 24 h following treatment on P11, P11-15, or P11-20 when examined on P12, P16, P21, or P30 (Schaefer et al. 2006; Schaefer et al. 2008). Decreases in tyrosine hydroxylase (TH), the rate-limiting enzyme of DA synthesis, were observed on P10 in the nucleus accumbens, prefrontal cortex and striatum in animals treated with an escalating dose from P4-10 (Kaewsuk et al. 2009). TH activity increased in males in caudate and substantia nigra (SN) as well as increased TH mRNA in SN on P30 following treatment from P1-29, suggesting that some effects may be sex-specific (Gomes-Da-Silva et al. 2000). However, no alterations in DA were observed by the same authors (Gomes-Da-Silva et al. 2004). Thus, despite early changes in TH, DA remains unaltered during the neonatal period. Some studies suggest that dopaminergic changes may emerge later in life (Crawford et al. 2003; Lucot et al. 1982; Tsuchida et al. 1996; Wagner et al. 1981). For example, rats treated with MA from P7-10 (25 mg/kg or 50 mg/kg b.i.d.) or P17-20 (50 mg/kg b.i.d.) show DA depletions in caudate when examined 2 weeks following treatment (Lucot et al. 1982). Similarly, rats treated with MA from P10-40 with 25 or 50 mg/kg/day show DA depletions in caudate 2 weeks following treatment (Wagner et al. 1981). It should be noted that this study continued treatment into adolescence and these depletions may be more representative of an adult-like response rather than that of neonates (Wagner et al. 1981). We have seen that neonatal P11-20 exposure reduces DA, DOPAC, protein kinase A (PKA) activity, and D2-like receptor binding in adult striatum (P90), suggesting that there are slow to emerge
effects of early MA exposure (Crawford et al. 2003). Extracellular DOPAC and HVA are increased following neonatal (P14) exposure, while DA levels are similar to animals exposed on P21 and 28 and increased in adults (P56) (Tsuchida et al. 1996). This may suggest that DA uptake processes are immature during the neonatal period, resulting in increased extracellular DA metabolism (Tsuchida et al. 1996). Likewise, the DA transporter (DAT) is not fully developed at this age (Fujita et al. 1993). It is unknown why DA levels are not altered during the neonatal period when large depletions are observed in adults, but the immature state of dopaminergic neurons during this time may be one reason for the protection. Morphologically, neonatal exposure also alters dopaminergic neuron innervation in a number of brain regions in adulthood following a single treatment (50 mg/kg) in gerbils on P14 (Busche et al. 2004; Dawirs et al. 1994; Grund et al. 2007; Neddens et al. 2002). Decreased number, length, and fiber density of DAergic neurons was observed in PFC (Dawirs et al. 1994) and decreased fiber density in nucleus accumbens (Neddens et al. 2002), PFC, and amygdala (Grund et al. 2007). Hence, neonatal treatment can lead to aberrant innervation. It is possible that these later biochemical and morphological perturbations have damaging effects on adult behavior such as altered locomotor activity since altered DA levels were observed in striatal regions, areas known to influence motor function.

There is also evidence for alterations in other neurotransmitter systems such as NE, gamma aminobutyric acid (GABA), glutamate, and acetylcholine following early MA treatment. Early studies that included prenatal and neonatal MA treatment in the drinking water revealed increased NE levels and altered NE metabolism later in life (Tonge 1972; Tonge 1973). Altered NE, primarily increased levels, were also observed
following neonatal treatment in the SN, caudate-putamen, and nucleus accumbens, but in general, these are areas with typically low levels of NE (Gomes-Da-Silva et al. 2004). Other studies showed that neither P10-40 (Wagner et al. 1981), P7-10, nor P17-20 MA treatment had effects on NE in the pons-medulla or telencephalon when examined 2 weeks later (Lucot et al. 1982). These few studies of the effects of neonatal treatment on NE are contradictory and it is unclear what increases in NE signify in these regions. These studies suggest that further study is warranted on the subject.

Neonatal MA effects on GABA are more limited. A single 50 mg/kg MA treatment on P14 in gerbils results in increased GABAergic fiber density in PFC later in life (P90) (Nossoll et al. 1997), an effect which the authors suggest represents compensatory PFC innervation following DAergic denervation (Dawirs et al. 1994). However, in a similar study, no alterations in PFC GABA innervation were observed in animals which were raised in an “enriched” environment following P14 MA exposure but decreased bouton density and increased fiber density was observed in MA animals raised under “impoverished” conditions (Brummelte et al. 2007). The relationship between MA exposure and environmental factors is unclear. These two studies suggest early MA-induced alterations in GABAergic innervation in the PFC which may alter proper signaling. This could influence PFC-related learning such as in working memory tasks. Deficits in working memory are observed in gerbils (Dawirs et al. 1996), but no such findings have been found in rats treated neonatally (Williams et al. 2003c). It is important to reiterate that GABAergic morphological alterations were observed in gerbils treated postnatally and no comparable studies exist in rats. Therefore, interpretation of
these findings is uncertain, especially when considering that the gerbil is not as fully characterized as the rat as a CNS model organism.

Experiments utilizing the same “impoverished” or “enriched” environments in MA-treated gerbils also demonstrate altered acetylcholine innervation in cortical regions (Lehmann et al. 2004) and in the dentate gyrus (Busche et al. 2006). Altered innervation in the dentate could contribute to learning deficits in spatial learning. For glutamate, no differences in somatosensory cortex were observed in rats given 10 mg/kg MA four times a day on P20 (Pu et al. 1996). Little is known about the effects of neonatal MA treatment on glutamate and acetylcholine but both arguably deserve more attention in future experiments.

Neurotrophins, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are important for the survival and maintenance of neurons and can be altered by neonatal MA treatment. In rats treated from P11-20 with MA, hippocampal BDNF was increased on P15 and P20 (Grace et al. 2008; Skelton et al. 2007) and NGF was increased on P20 (Skelton et al. 2007). Furthermore, MA-treated gerbils raised in an enriched environment have decreased BDNF and NGF levels in several brain regions that are increased in animals raised in impoverished conditions (Lehmann et al. 2007). Changes in neurotrophin levels may also affect learning and memory since heterozygous knockout mice of either BDNF or NGF show spatial learning deficits (Chen et al. 1997; Linnarsson et al. 1997), although it is important to note that these animals have decreased neurotrophins throughout life.

Few studies exist that examine morphological changes in brain regions following neonatal exposure (excluding neuron types associated with particular neurotransmitters as
discussed above). Blaesing and colleagues showed that changes in dendritic morphology on P90 occur in PFC following P14 exposure to MA in gerbils (Blaesing et al. 2001). In particular, MA-treated gerbils showed increased spine density and reorganized arrangements of dendritic branching (Blaesing et al. 2001) which seems to corroborate this group’s earlier findings (Nossoll et al. 1997). Furthermore, this exposure results in a disconnection of PFC efferent neurons to cortical and subcortical areas (Bagorda et al. 2006) as well as to the contralateral PFC (Witte et al. 2007); a finding the authors suggest could be a model of schizophrenia. In rats, P11-20 MA treatment also causes long-term (P60) morphological changes in the dentate gyrus and nucleus accumbens (Williams et al. 2004a). Decreased spine density in the dentate and nucleus accumbens as well as decreased dendritic length in the nucleus accumbens was observed (Williams et al. 2004a). This is significant since these regions are important in cognition and morphological changes are observed at an age when we begin behavioral and learning tests. In gerbils, decreased adult neurogenesis that can be reversed by the D2 antagonist haloperidol occurs in the dentate gyrus following neonatal MA exposure (Hildebrandt et al. 1999). Again, these alterations occur in an area important for cognition and the hippocampus in particular is important in spatial learning (Morris et al. 1982). It should be noted that no such studies exist examining the effects of neonatal MA on early neurogenesis, however preliminary studies in our lab suggest that proliferation may be decreased in the hippocampus (unpublished observations). No studies have examined cell death in neonates even though studies in adults show that increased apoptosis occurs (Deng et al. 2001). These studies could be important in determining effects underlying the long-term behavioral and cognitive outcome.
In addition to effects on neurotransmitters and behavior, developmental MA exposure causes stress-like HPA axis changes. For example, in animals treated on P11, P11-15, or P11-20, both CORT and adrenocorticotropic hormone (ACTH) were increased following treatment (Williams et al. 2000). Increased CORT levels also occur on P11, 24 h following the first treatment (of four) and are larger than those seen after exposure to MDMA, methylphenidate, cocaine, or fenfluramine at the same age (Schaefer et al. 2006). MA also increases CORT on P11, 1 h after the last dose (Skelton et al. 2007) and on P16 following P11-15 treatment (Schaefer et al. 2008). Increases in CORT are also observed in neonatal mice after MA treatment (Acevedo et al. 2008). Furthermore, the effects of MA on CORT release are age-dependent. An acute 10 mg/kg MA dose causes increased plasma CORT levels 30 and 105 min later for every age tested between P1-19 (Williams et al. 2006) but the magnitude of the increase followed a U-shaped function (Figure 4) (Williams et al. 2006). This pattern approximates the stress hyporesponsive period (SHRP) (P4-14) when HPA axis responses to stress are lower than at other ages (Sapolsky and Meaney 1986). But when MA is given for 5 days in a row starting on any odd numbered day between P1-15 and examined following a final dose on the 5th day, CORT levels increased progressively with age at 30 min but not at the 105 min time point. This suggests that repetitive dosing triggered feedback mechanisms that terminated the CORT increase more rapidly than after a single dose or altered peak excitability and clearance (Williams et al. 2006). What is especially intriguing about these MA-induced developmental increases in CORT is that they are larger than those observed following forced swim or confinement stress at the same ages (Grace et al. 2008). Neonatal MA exposure may alter the development of the HPA axis since there is
evidence that the adult CORT response to stress is modified long after developmental drug exposure (Skelton et al. 2007; Williams et al. 2003a). MA animals had a reduced response to stress as adults (Skelton et al. 2007; Williams et al. 2003a). It may be that MA stimulates premature development of the HPA axis, but this remains to be tested. Another possible implication of these findings is that MA-induced increases in CORT may contribute to the long-term learning and memory deficits we have found and this concept will be the subject of experiments described below. The biochemical and behavioral effects following increased neonatal stress, in particular increases in CORT, will be discussed in more detail in the following section. In conclusion, neonatal MA exposure results in a variety of biochemical and morphological changes within the brain.

Behavior

Methamphetamine treatment in neonatal rodents produces reproducible changes in behaviors when examined in adulthood in specific domains, including locomotor activity, anxiety, acoustic startle, and learning. Acute adult and neonatal MA exposure induces hyperactivity during drug exposure. P11-20 MA exposure causes a mild hypoactivity in the offspring as adults without subsequent drug exposure (Acevedo et al. 2007; Fujiwara et al. 1987; Vorhees et al. 1994b; Vorhees et al. 2009). For instance, a single 2 mg/kg MA injection in neonatal rats from P2-6 or P17-21 increases locomotor activity 1 min following exposure but no differences in activity in these groups were observed following an acute MA challenge on P35 (Fujiwara et al. 1987). Hypoactivity is observed on P30 following P1-10 or P11-20 exposure (Vorhees et al. 1994b) and MA treatment from P1-10, P6-15, or P11-20 produces dose-dependent, but not exposure age-
dependent, decreases in activity as adults at P60 (Vorhees et al. 2009); but the effects are modest compared to the acute effects of the drug. It may be that the long-term hypoactivity effect is the result of delayed-onset alterations in DA in regions that mediate motor function since we have documented such dopaminergic late-emerging effects (Crawford et al.). Mice neonatally exposed to MA show no differences compared to controls in the rotorod test (Acevedo et al. 2007), indicating no differences in forced-locomotion. Despite the modest reductions in locomotor activity found in rats treated neonatally with MA, we find no transfer of this effect to swimming behavior, as such rats show no effect on time to swim a long straight channel or in swimming speed measured during trials in the MWM (Skelton et al. 2007; Vorhees et al. 1994a; Vorhees et al. 1998; Vorhees et al. 2000; Vorhees et al. 2007; Vorhees et al. 2009; Williams et al. 2003c; Williams et al. 2003b; Williams et al. 2004b).

MA-induced effects on anxiety differ depending upon the animal model and test used. In the open field, gerbils that were administered MA on P14 were more anxious in adulthood as indicated by increased number of voided fecal pellets (Dawirs et al. 1996). In the elevated zero maze, neonatally treated rats have anxiety levels that are similar to controls as adults (Skelton et al. 2007; Williams et al. 2004b), or in some replications show slight reductions in time in the open quadrants (Williams et al. 2003b). C57BL/6 mice treated neonatally with MA show no differences in the open field, elevated plus or elevated zero maze time in open (Acevedo et al. 2007). Exactly why gerbils, mice, and rats differ is not known, but given that the exposure ages, test ages, drug doses, and intervening testing/handling are all different in these experiments may account for the differences. Even in our neonatal MA rat studies, some differences occurred in prior
handling/testing, but our rat data also show the effect to be weak and this may account for its variability. What this suggests is that changes in anxiety from neonatal MA exposure, if they exist, are small and probably not explanatory for other effects, such as the effects on learning and memory.

In rodents and humans, the startle response is used to assess sensorimotor gating by pairing the startle stimulus to a weaker prepulse that modifies the response to the main stimulus (prepulse inhibition or PPI). The neural circuits involved in this phenomenon have been mapped and involve the caudal pontine reticular nucleus as the final output nucleus (Koch 1999) and PPI appears to involve several neurotransmitter systems such as DA and 5-HT since agonists and antagonists of both alter PPI (Geyer et al. 2002). PPI is impaired in a number of human diseases such as schizophrenia and obsessive compulsive disorder (Braff et al. 2001). Rats neonatally treated with MA have increased acoustic startle response in adulthood, but PPI is unaltered (Vorhees et al. 1994a; Vorhees et al. 1996; Vorhees et al. 2009). In mice however the opposite is true, no effects in acoustic startle response are observed, but PPI deficits were seen (Acevedo et al. 2007).

Spatial learning is mediated by the hippocampus. Rats with hippocampal lesions are deficient in spatial memory/navigation tasks, including the MWM (Morris et al. 1982) although other brain regions are also involved. Rodents exposed to MA neonatally show deficits in spatial learning and reference memory in the MWM. This is seen in both MA-treated mice (Acevedo et al. 2007) and rats in the MWM (Skelton et al. 2007; Vorhees et al. 1994a; Vorhees et al. 2000; Vorhees et al. 2007; Vorhees et al. 2008; Vorhees et al. 2009; Williams et al. 2002; Williams et al. 2003c; Williams et al. 2003a; Williams et al. 2003b; Williams et al. 2004b) and Barnes maze (Williams et al.
2003a) but not in working memory in the MWM (Williams et al. 2003c). Originally, it was demonstrated that neonatal MA treatment impaired spatial learning in adult rats when exposed from P11-20 and not P1-10 (Vorhees et al. 1994a) and shown that MA administration four times per day instead of two, given an identical total dose/day, produced more pronounced spatial deficits (Vorhees et al. 2000). Even at lower doses, MA elicits spatial deficits (Williams et al. 2003c; Williams et al. 2004b) and MA-induced deficits last until at least 1 year of age (Vorhees et al. 2007). These deficits do not correlate to altered swimming-based motor function (Williams et al. 2002). Furthermore, no effects were observed in the cued (visible platform) version of MWM or straight channel swimming, indicating that these deficits are not caused by changes in motivation to escape from water or other performance factors (Williams et al. 2003c). However, affected rats have subordinate skill deficits in non-spatial components since, i.e., they show increased thigmotaxis initially, but this non-specific impairment is not present after initial learning, and the spatial deficit persists as shown by the fact that when the platform is moved to a new location the MA-treated offspring are impaired in direct swims to the goal and thigmotaxis is no longer evident (Skelton et al. 2007; Williams et al. 2002).

Additional evidence that the spatial deficits are not caused by non-spatial learning deficits is that MA-treated animals pre-trained in the MWM to find the platform without using spatial cues have increased latencies when tested spatially (Williams et al. 2002). Further assessment of the P11-20 exposure period was performed by division into two exposure periods, one in which rats were treated from P11-15 and another from P16-20; only the P11-15 group showed deficits in MWM, suggesting that the deleterious effects on spatial learning are more closely associated with the first half of the P11-20 treatment interval.
(Williams et al. 2003b). However, this P11-15 effect was revealed using a demanding test method. Instead of testing the rats using a 10 cm platform initially, they were tested using a 5 cm platform. An inspection of their learning curves revealed that both MA and SAL groups had shallow learning curves compared to previous experiments using the 10 cm platform. For this reason, a follow-up experiment was conducted using the 10 cm platform. In this experiment multiple doses (10, 15, 20, or 25 mg/kg, 4 times/day) were used and only the P11-15 exposure period evaluated. In this experiment, MWM deficits were found using the shorter exposure period but only at the highest dose (25 mg/kg) (Vorhees et al. 2008). Differences between these two experiments, such as differences in platform size, may explain why the effect occurred at different doses (Vorhees et al. 2008; Williams et al. 2003b). Nonetheless, the findings suggest that 10 day exposures are more effective than 5 day treatments for inducing spatial learning deficits (Vorhees et al. 2008). Interestingly, MA-induced MWM deficits overlap with the SHRP, suggesting that MA-induced increases in CORT during the SHRP may contribute to such deficits (Vorhees et al. 2009). The experiments in chapter 4 of this dissertation were designed to test this hypothesis.

Rats that were administered MA as neonates also show deficits in egocentric route-based learning in the Cincinnati water maze (Figure 5) (Vorhees et al. 2008; Vorhees et al. 2009). This task was originally run under standard lighting conditions and therefore early experiments likely involved a spatial component (extra-maze room cues) as well as learning based on self-movement cues (Vorhees et al. 1994a; Williams et al. 2003c). These earlier experiments in the CWM with standard lighting showed trends toward deficits in MA-treated animals, but were not significant
(Vorhees et al. 1994a; Williams et al. 2003c). To determine whether this task relied primarily on egocentric, rather than allocentric learning, it was run in the dark to eliminate distal cues (Vorhees et al. 2008; Vorhees et al. 2009). MA-treated rats tested under infrared light in the CWM showed significant deficits on multiple parameters (Vorhees et al. 2008; Vorhees et al. 2009) and as with MWM deficits, exposure partially overlapped with the SHRP (Vorhees et al. 2009). The CWM is a test we believe relies upon egocentric learning because visual cues under infrared light are eliminated. This leaves internal self-movement cues involving use of route-based and path integration learning (Etienne et al. 2004) but does not eliminate rote memory (memorizing a path) of specific turn sequences or the possible use of olfactory cues. Two distinct forms of egocentric learning have been described: route-based and path integration (Etienne et al. 2004; Etienne and Jeffery 2004). CWM appears to best fit descriptions of route-based than path integration in that path integration involves the ability to return home by a novel (integrated) path not used on the outbound journey (Etienne et al. 2004; Etienne and Jeffery 2004). No “short-cut” is possible in the CWM precluding a direct test of path integration using this apparatus. Since vector-based learning cannot be demonstrated in the CWM, we surmise that it is best described as a route-based navigation (Etienne et al. 2004; Etienne and Jeffery 2004). Further experiments are needed to support this supposition.

**Stress Effects on the Brain and Cognition**

Hans Selye in 1936 (Selye 1936) described responses to conflict/distressful situations an animal experiences as stress. He defined it as any “nonspecific response of
the body to any demand made upon it” (Selye 1973). A stressor is likewise an agent that produces stress. He described stress as a “general adaptation syndrome” consisting of three phases: alarm reaction, adaptation, and exhaustion (Selye 1985). Three morphological features which are common outcomes to most stressors, were coined the “triad of the alarm reaction” and consist of enlargement of the adrenal cortex, atrophy of the thymus and lymph nodes, and appearance of gastric ulcers (Selye 1973). He also described the action and major components of the HPA axis (Selye 1973; Selye 1985) which have been further described in greater detail (Vazquez 1998).

Upon activation of the HPA axis by a stressor, corticotropin releasing factor (CRF) and arginine vasopressin (AVP) are released into the median eminence of the hypothalamus where they are taken up by the hypophyseal portal system and subsequently stimulate the anterior pituitary to secrete ACTH (Vazquez 1998). ACTH travels through the systemic circulation and stimulates the adrenal cortex, which responds with the release of glucocorticoids, primarily corticosterone in rats or cortisol in humans (Vazquez 1998). There is negative feedback regulation at the level of the pituitary, hypothalamus, and hippocampus (Vazquez 1998) (Figure 6). It has been found that large increases (and decreases) in circulating glucocorticoids can damage the hippocampus and alter learning and memory.

Numerous studies have examined the effects of increased glucocorticoids on the hippocampus. In adult rats, both chronic restraint stress (Watanabe et al. 1992) or CORT injection (Woolley et al. 1990) for three weeks significantly alter hippocampal dendritic morphology. In the CA3 region, reduced apical dendrite length and number of branch points are observed with no alterations in basal dendrites (Watanabe et al. 1992; Woolley
Maternal separation of neonatal rats causes reduction of mossy fiber density in the hippocampus as adults (Huot et al. 2002). Furthermore, prolonged exposure to increased titers of CORT for 12 weeks accelerates hippocampal neuron loss in the CA3 region and increases neuronal damage represented by increased numbers of glial cells (Sapolsky et al. 1985). Prolonged CORT exposure also down-regulates MR and GR (Sapolsky et al. 1984; Sapolsky et al. 1985) and receptor levels remain low after a substantial recovery period, resembling effects observed in aged animals (Sapolsky et al. 1985). Blocking the synthesis of adrenal steroids with cyanoketone prevents the neuronal atrophy caused by stress (McEwen and Magarinos 1997; McEwen 1999). Dendritic atrophy associated with stress may also occur in hippocampal neurons through glutamate, NMDA receptors, GABA, or serotonin mechanisms (McEwen and Magarinos 1997; McEwen 1999). CORT receptors are abundant in the hippocampus and entorhinal cortex (Sapolsky et al. 1983) which are regions important for spatial learning (Morris et al. 1982) and egocentric learning (Etienne et al. 1996; Etienne and Jeffery 2004).

In adult rats, these stress-induced alterations in the hippocampus are associated with impairments in learning and memory (de Kloet 2000; McEwen and Magarinos 1997; McEwen 1999). Effects on hippocampal-dependent spatial learning and memory have shown mixed results depending on the task and form of stress exposure (Conrad 2009), but numerous instances of spatial learning and memory deficits following stress have been reported. For instance, stress causes deficits in Y-maze (Conrad et al. 1996; Wright et al. 2006), radial-arm maze (Nishimura et al. 1999), and MWM learning (Abidin et al. 2004; Moosavi et al. 2007; Radecki et al. 2005) (see (Conrad 2009) for extensive review). Also, glucocorticoid receptor blockade can facilitate spatial learning.
Effects on learning following exogenous administration of CORT vary. For instance, rats that were injected with 26.8 mg/kg CORT (within the range observed following a stressor) in sesame oil once/day for 21 d showed deficits in reference memory in the Barnes maze, but no differences in spatial working memory in the Y- or Barnes mazes (Coburn-Litvak et al. 2003), but spatial working memory deficits were observed when animals were treated for a longer duration (56 d) (Coburn-Litvak et al. 2003).

The neonatal HPA axis responds differently to stressors than it does in the adult. From approximately P4-14, the neonatal rat shows a period of adrenal quiescence in which the adrenal shows reduced responses to stressful perturbations, i.e., the SHRP (Sapolsky and Meaney 1986). The suppressed stress response during this time is thought to be important for protection of developing neurons (Sapolsky and Meaney 1986).

Neonatal stress exposure can also alter adult learning and behavior. Maternally deprived (24 h) rats are impaired in MWM learning as young animals and adults (Oitzl et al. 2000). Exposure to 3 h daily periods of maternal separation for the first 3 weeks of life leads to adult spatial learning and novel object recognition deficits (Aisa et al. 2007). Interestingly, alterations in maternal care are important in these studies since handling of pups improves MWM learning following restraint stress (Garoflos et al. 2005) and reduces anxiety (Knuth and Etgen 2007). Adult alterations in anxiety following neonatal stress exposure, primarily exacerbations, have been demonstrated as well (Huot et al. 2001; Kalinichev et al. 2002; Knuth and Etgen 2007; Romeo et al. 2003; Wigger and Neumann 1999). One commonly observed effect of neonatal stress is alteration in the adult response to stressors (Aisa et al. 2007; Biagini et
including hypersecretion of CORT following stress in adulthood and/or increased anxiety in maternally separated animals, suggesting altered development of the HPA axis.

Similar to some of the effects following a stressor during the neonatal period, administration of exogenous glucocorticoids to the neonate can significantly alter cognition and behavior. The effects on behavior and learning following neonatal exposure are variable and may be dependent upon the concentration of glucocorticoid administration. For instance, the offspring of dams exposed to 200 μg/ml of CORT in the drinking water during lactation show reduced CORT levels in response to restraint stress, reduced anxiety in the elevated plus maze and light-dark tasks, improved passive avoidance learning, and increased hippocampal mineralocorticoid receptors (MR) as adults, suggesting that this amount of early glucocorticoid exposure is beneficial (Catalani et al. 2000). Early (P5-9) and late (P13-17) administration of CORT in the dams’ drinking water improved offspring performance in MWM when tested on P21 and the greatest improvement was observed in the P5-9 group (McCormick et al. 2001). In contrast, rat pups administered 30 or 60 ng/kg/day CORT by oral gavage from P2-16 showed motor deficits in a swimming task as neonates (P7-21) (Pavlovskas-Teglia et al. 1995). CORT administration by polymer implantation (sustained high levels) from P0-12 leads to increased initial acquisition of spatial learning in the radial arm maze in adulthood (Roskoden et al. 2005), whereas subcutaneous injection of CORT (3 x 100 or 3 x 300 μg/0.1 ml) from P12-15 or P22-24 impaired passive avoidance learning in adulthood (Nyakas and Endroczi 1972).
Exposure to dexamethasone (DEX), a synthetic glucocorticoid, leads to adverse effects on learning and behavior. Intraperitoneal injection from P1-3 impaired adult spatial learning and learning in a delayed-matching-to-sample version of the MWM (Kamphuis et al. 2003). The spatial deficit was associated with decreased long-term potentiation (LTP) and facilitation of long-term depression (LTD) in the hippocampus and DEX treatment negatively impacted NMDA receptor complexes in the hippocampus, suggesting the deficits may arise from alteration in hippocampal synaptic plasticity (Kamphuis et al. 2003). Further, a single s.c. injection of DEX on P7 at clinical doses resulted in motor deficits, hyperactivity in the open field, and impaired passive and active avoidance learning (Benesova and Pavlik 1989). Neonatal DEX treatment also reduces neurogenesis in the hippocampus and cortex and causes reduction in brain weight (Kanagawa et al. 2006).

In humans, increases in cortisol are associated with cognitive impairments (McEwen and Magarinos 1997; McEwen 1999) and hippocampal atrophy (Starkman et al. 1992) as well. Atrophy can be reversed following reduction of cortisol and ACTH by pituitary surgery (Starkman et al. 1999). Patients with Cushing’s syndrome are impaired in attention-based working memory tasks and delayed recall using the Luria’s Memory Words test (Revised) (Leon-Carrion et al. 2009). They are also deficient in tests of verbal IQ, verbal learning, and delayed recall (Starkman et al. 2001), as well as, having deficits in other verbal and some visual and spatial learning tasks (Michaud et al. 2009). Cushing’s syndrome patients had similar deficits to advanced-age control subjects (15 years older than Cushing’s patients) (Michaud et al. 2009).
Stress during pregnancy is shown to have adverse effects on developmental and cognitive outcomes. Stressed mothers with high salivary cortisol levels have children that have poorer scores on the Bayley Scales of Infant Development (BSID) at 8 months of age (Buitelaar et al. 2003; Huizink et al. 2003). Increased morning salivary cortisol levels correlated with poorer scores on the Bayley Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) (Buitelaar et al. 2003; Huizink et al. 2003). Niederhofer and Reiter found inferior temperament at 6 months of age and poorer school grades at 6 years, but no changes in intrauterine movement, when the effects of maternal stress were assessed by questionnaire and teacher reports (Niederhofer and Reiter 2004).

The effects of maternal stress during pregnancy caused by an environmental disruption, i.e., severe ice storm resulting in power outages in Quebec, Canada in 1998 on the cognitive abilities of children have been analyzed (Laplante et al. 2008; LaVoie et al. 2004). At 2 years, children exposed had poorer MDI scores and language abilities (Laplante et al. 2004) and at 5.5 years, had higher levels of objective stress (questionnaire to mothers relating exposure categories used in other disaster studies including loss, scope threat, and change), showed reduced cognitive ability (full scale and verbal IQ), and reduced language skills (Peabody Picture Vocabulary Test-Revised) (Laplante et al. 2008). Also, children 14-19 months of age whose mothers were subjected to stressful life events during pregnancy had MDI scores that correlated negatively with the stress events (Bergman et al. 2007). Furthermore, these stress events accounted for 17% of the variance of the Bayley Scales of Infant Development (2$^{nd}$ edition) in these children and were correlated with significant fearfulness scores using the Laboratory Temperament
Assessment Battery (Lab-TAB) (Bergman et al. 2007). In sum, the data suggest that stress, especially early stress, in humans can have lasting CNS effects.

Since early life stress can result in an altered response to stress and cognitive impairment in adulthood, we wanted to determine whether the potent stress effect (CORT release) of MA was the cause of MA-induced cognitive deficits and whether MA similarly altered the adult stress response. Further, stress-induced hippocampal neurotoxicity may be associated with deficits in hippocampal-dependent learning such as the MWM. In the following experiments we determined whether neonatal MA exposure altered the adult response to stress following forced swim, forced confinement, or acute MA injection and determined whether attenuating neonatal MA-induced CORT increases by adrenal autotransplantation (ADXA) reduced the long-term cognitive deficits.
Table 1. Findings from neonatal MA studies in animals

<table>
<thead>
<tr>
<th>Reference</th>
<th>Dose, isomer</th>
<th>Route of admin.</th>
<th>Period of exposure</th>
<th>Frequency of exposure</th>
<th>Species</th>
<th>Control Parameters examined</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tonge 1972) J. Pharm. Pharmacol.</td>
<td>80 mg/L</td>
<td>Drinking water</td>
<td>Prenatal and neonatal till P21</td>
<td>Ad libitum</td>
<td>Rat</td>
<td>Ascorbic acid solution</td>
<td>• ↑ 5-HT and 5-HIAA in 1 chlorpromazine and phenotiazines&lt;br&gt;• ↑ noradrenaline in MA, chlorpromazine&lt;br&gt;• ↑ dopamine in phenyclidine&lt;br&gt;• Monoamines were similar ages</td>
</tr>
<tr>
<td>(Tonge 1973) J. Neurochem.</td>
<td>80 mg/L</td>
<td>Drinking water</td>
<td>Prenatal and neonatal till P21</td>
<td>Ad libitum</td>
<td>Rat</td>
<td>Ascorbic acid solution</td>
<td>• ↑ 5-HT in MA in cortex/hippocampus, ↓ in hypothalamus and amygdala/pons/medulla&lt;br&gt;• ↑ 5-HT in MA+CPZ in cortex/hippocampus and amygdala/pons/medulla&lt;br&gt;• ↑ 5-HT in CPZ in cortex/hippocampus, striatum, thalamus/pons/medulla and amygdala/pons/hypothalamus&lt;br&gt;• ↑ 5-HIAA in MA in cortex/hippocampus; ↑ 5-HIAA in hypothalamus&lt;br&gt;• ↓ 5-HIAA in CPZ and MA in hypothalamus</td>
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<tr>
<td>(Tonge and Leonard 1973) Br. J. Pharmacol.</td>
<td>80 mg/L</td>
<td>Drinking water</td>
<td>Prenatal and neonatal till P21</td>
<td>Ad libitum</td>
<td>Rat</td>
<td>Ascorbic acid solution</td>
<td>• MA, CPZ and phenyclidine&lt;br&gt;no imipramine effects&lt;br&gt;Rates of noradrenaline depletions rates&lt;br&gt;No imipramine effects&lt;br&gt;Rates of noradrenaline depletions rates</td>
</tr>
</tbody>
</table>
-hypothalamus: MA>C>I
-amygdala: PH>C>IM>M
-midbrain: MA>IM>C>P
-pons/medulla: C=PH>M

<table>
<thead>
<tr>
<th>Study</th>
<th>Animal</th>
<th>Treatment</th>
<th>Route</th>
<th>Duration</th>
<th>Tissue</th>
<th>Drug</th>
<th>Analysis</th>
</tr>
</thead>
</table>
| Wagner et al. 1981 | Rat Sprague-Dawley | MA or MA amphetamine or methylphenidate | s.c. | P10-P40 | 2/d, 12 h apart | Saline | Dopamine and norepinephrine levels in caudate, midbrain, hypothalamus, pons-medulla and telencephalon 2 weeks after last injection | ↓ DA in caudate of 25 and 50 mg/kg group
↓ DA in caudate of 50 and 100 mg/kg group
No other differences in DA levels for norepinephrine |
| Lucot et al. 1982 | Rat Sprague-Dawley | 50 or 100 mg/kg/day | s.c. | P7-10 or P17-20 | 2/d, 12 h apart | Saline | Monoamine levels in caudate, telencephalon, midbrain or pons-medulla assessed 2 weeks following treatment | ↓ DA in caudate of 50 at P7-10 and in 100 MA group
↓ 5-HT in telencephalon (P17-20)
↓ DA in caudate and telencephalon of 25, 50 and 100 µg 6-hydroxydopamine; ↓ 5-HT in midbrain only at 100 µg
No MA or 6-HDA affecting norepinephrine
In general 6-HDA depleted norepinephrine more than MA depletion |
| Fujiwara et al. 1987 | Rat Sprague-Dawley | 2 mg/kg, (+) | s.c. | P2-6, P7-11, P12-16, P17-21, P22-26 and P27-31 | 1/d | Saline | Locomotor and rearing activity (1 min after daily injection) and activity following 2 mg/kg i.p. MA | ↑ activity in MA P2-6 group examined at 1st, 3rd, and 5th week
↑ activity and rearing in P22-26 and P27-31 that was higher than P2-6; ↑ stereotypy in MA groups |
| (Pu and Vorhees 1993) Dev. Brain Res. | 10 or 20 mg/kg, (+) | i.p. | P20, 40, 60 or 80 | 4/d, 2 h apart | Rat Sprague-Dawley | Saline | Immunohistochemistry in caudate-putamen for tyrosine hydroxylase or GFAP | • Challenge: No differences between groups; ↑ activity and rearing in 2 groups | • ↓ TH staining in P60 and P80 at both doses | • ↑ GFAP staining in P60 and P80 at both doses | • ↑ GFAP in ventral lateral striatum on P40 MA animal | • No differences in GFAP staining in P20 |  |
| (Dawirs et al. 1994) J. Brain Res. | 50 mg/kg, (+) | i.p. | P14 | 1/d | Gerbil | Saline | Dopaminergic IHC in prefrontal cortex on P90 | • ↓ total number, total length and innervation in both orbital and medial PFC in animals (decreased mature) | • ↓ total number, total length and innervation in both orbital and medial PFC in animals (decreased mature) | • ↓ total number, total length and innervation in both orbital and medial PFC in animals (decreased mature) | • ↓ total number, total length and innervation in both orbital and medial PFC in animals (decreased mature) | • ↓ total number, total length and innervation in both orbital and medial PFC in animals (decreased mature) |  |
| (Tsuchida et al. 1994) Pharmacol. Biochem. Behav. | 2 mg/kg and 4 mg/kg (+) | i.p. | P7-12, P14-19, P21-26, P28-33 or P56-61, all challenged 21 days after last day | 2/d (? interval) | Rat Sprague-Dawley | Saline | Microdialysis in dorsal striatum (21 days after last injection) and HPLC for dopamine and metabolites, activity/stereotypy observation | • ↑ activity scores in older groups after challenge compared to controls | • No basal differences in LSA or HVA between MA and control | • In general, DA was ↓ for 3 days after challenge and DOPAC and HVA were ↓ for 7 days after challenge regardless of pretreatment | • No differences in DA or metabolites between MA and control | • MA pretreatment ↑ dopaminergic activity in P21-26, P28-33 and P56-61 animals compared to controls |  |
| (Vorhees et al. 1994a) Psychopharmacology | 30 mg/kg, (+) | s.c. | P1-10 or P11-20 | 2/d, 8 h interval | Rat Sprague-Dawley CD/VAF | Distilled water | Body weight, spontaneous alternation, passive avoidance, | • ↓ body weight in MA 1-70, ↓ body weight in 11-49 | • ↑ failure to choose only one arm, ↓ spontaneous alternation | • ↑ failure to choose only one arm, ↓ spontaneous alternation | • ↑ failure to choose only one arm, ↓ spontaneous alternation | • ↑ failure to choose only one arm, ↓ spontaneous alternation |  |
macology acoustic startle reactivity, straight channel swimming, MWM, and Cincinnati water maze

<table>
<thead>
<tr>
<th>(Vorhees et al. 1994b) Psycopharmacology</th>
<th>30 mg/kg, (+)</th>
<th>s.c.</th>
<th>P1-10 or P11-20</th>
<th>2/d, 8 h interval</th>
<th>Rat Sprague-Dawley CD/VAF</th>
<th>Distilled water</th>
<th>Locomotor activity, locomotor activity with 10 mg/kg fluoxetine or 2 mg/kg MA</th>
<th>11-20 group (spontaneous alternation)</th>
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<tr>
<td></td>
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<td>• ↑ initial entry latency in but no effects in retention group in passive avoidance</td>
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<td>• ↑ mortality in 1-10 group</td>
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<td>• ↑ Vmax, ↑ Vmean and ↓ startle habituation for M only in both groups</td>
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<td></td>
<td>• ↑ Vmax at all intensities in both groups, ↑ Vmax in P11-20 group at 0, 70, 75, 80, 85 dB</td>
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<td></td>
<td>• No differences in straight swimming times</td>
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<td></td>
<td>• ↑ errors in 11-20 group, no differences in latencies in 1-10 males had ↑ trials lasting</td>
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<td></td>
<td>• ↑ latencies in 11-20 group MWM acquisition and survival</td>
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<td>• ↓ locomotion (# of sectors transitions) in 11-20 males and 11-20 females on P35 or P45 or P60</td>
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<td></td>
<td>• ↓ central distance in both groups, males and females only</td>
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<td></td>
<td>• ↓ peripheral distance in all times and 1-10 males only. Reduction in 11-20 P30 only</td>
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<td></td>
<td>• Fluoxetine challenge: ↓ in 1-10 females (intervals 1 and 3) males (intervals 1 and 3), locomotion in 11-20 males (intervals 1 and 3)</td>
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<tr>
<td>Study</td>
<td>Treatment Details</td>
<td>Age</td>
<td>Interval</td>
<td>Species</td>
<td>Control</td>
<td>Additional Details</td>
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<tr>
<td>Dawirs et al. (1996) J. Neural Transm.</td>
<td>50 mg/kg, i.p.</td>
<td>P14</td>
<td>1/d</td>
<td>Gerbil</td>
<td>Saline</td>
<td>Methamphetamine challenge increased activity in both groups, M and F, at all i</td>
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<tr>
<td>Pu et al. (1996) Synapse</td>
<td>10 mg/kg, i.p.</td>
<td>P20, 40, or adult</td>
<td>4/d, 2 h intervals</td>
<td>Rat Sprague-Dawley</td>
<td>Saline</td>
<td>↑ distance traveled, outer rearing, and # of fecal pellets (anxiety); ↓ Inner crossing field in MA animals; ↑ % number of errors in delayed alternation compared to controls, no differences pretraining</td>
<td></td>
<td></td>
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<tr>
<td>Tsuchida et al. (1996) Pharmacol. Biochem. Behav.</td>
<td>4 mg/kg, i.p.</td>
<td>P14, 21, 28 or 56</td>
<td>1/d</td>
<td>Rat Sprague-Dawley</td>
<td>MA administered to all</td>
<td>Extracellular dopamine, DOPAC and HVA in striatum (microdialysis) collected every 20 min for 180 min.</td>
<td></td>
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<tr>
<td>Vorhees et al. (1996) Neurotoxicol. Teratol.</td>
<td>20 mg/kg, s.c.</td>
<td>P1-10</td>
<td>2/d, at least 8 h interval</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Distilled water</td>
<td>Body weight, acoustic startle reactivity</td>
<td></td>
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</tbody>
</table>

- ↑ distance traveled, outer rearing, and # of fecal pellets (anxiety); ↓ Inner crossing field in MA animals
- ↑ % number of errors in delayed alternation compared to controls, no differences pretraining
- ↓ glutamate positive neurons II-III) in 5/8 adult MA and 3/10 MA showed GFAP
- No effects in P20 and 40
- No differences in baseline P14 group had ↓ baseline compared to P21 and 56; P14 compared to 21 and on P56 compared to 21 and
- ↑ DA in all groups, peak and steadily declines to had greatest increase
- ↓ DOPAC in all groups; P21, 28 and 56 were more than P14
- Slight ↓ in HVA over time and 56; ↑ HVA in P14
- ↓ body weight in MA final
- No effect on Vmax or V for habituation
- ↑ Vmax in MA animals dB, and trends at 75, 80, dB with prepulse
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Dose</th>
<th>Interval</th>
<th>Species</th>
<th>Control</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cappon et al. 1997)</td>
<td>s.c.</td>
<td>10 mg/kg (+)</td>
<td>P20, 40 or 60</td>
<td>4/d, 2 h intervals</td>
<td>Rat Sprague-Dawley CD</td>
<td>Body temperature (total thermal response), neostriatal monoamine levels, GFAP levels</td>
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<tr>
<td>(Nossoll et al. 1997)</td>
<td>i.p.</td>
<td>50 mg/kg (+)</td>
<td>P14</td>
<td>1/d</td>
<td>Gerbil</td>
<td>Saline Electron microscopy of GABA in prelimbic prefrontal cortex (P90)</td>
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<tr>
<td>(Gomes-Da-Silva et al. 1998)</td>
<td>s.c.</td>
<td>10 mg/kg (+)</td>
<td>P1-4, P1-6 and P1-29</td>
<td>2/d, 10 h interval</td>
<td>Rat Wistar</td>
<td>Saline Body weight, eye defects on P5, 7, and 30</td>
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<tr>
<td>(Stadlin et al. 1998)</td>
<td></td>
<td>4 mM (+)</td>
<td>Cultured astrocytes</td>
<td>4, 8, 12, 24 and 48 hr</td>
<td>Mouse C57BL/6</td>
<td>Serum-free media Mesencephalon, striatum and cortex: cell viability by lactate dehydrogenase levels, GFAP and vimentin levels,</td>
</tr>
</tbody>
</table>

- $V_{max}$ MA $> control$ with $\downarrow TTR$ in P20 and 40 MA at 30°C and in P60 MA at 25°C only
- $\downarrow$ neostriatal dopamine, HVA levels in P40 MA at 30°C, P60 MA animals at 25°C only
- $\downarrow$ neostriatal serotonin and HVA levels in P20 and 40 MA animals at 30°C, P60 MA animals only
- $\uparrow$ GFAP levels in P40 MA at 30°C, P60 MA animals at 25°C only, no effects on 7 or 30
- $\downarrow$ weight gain from P24 on 7 and 30
- $\uparrow$ GABAergic fiber density compared to controls
- $\uparrow$ GFAP from 0-8 hr post, then $\downarrow$ GFAP at 48 h in mesencephalic and cortical astrocytes; $\downarrow$ GFAP in striatal astrocytes at 8 h and $\uparrow$ GFAP at 48 h, vacuoles present in all regions
- $\uparrow$ vimentin in mesencephalic and cortical astrocytes
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Route</th>
<th>Age</th>
<th>Mortality</th>
<th>Body Weight</th>
<th>Acoustic Startle</th>
<th>Straight Channel Swimming</th>
<th>MWM</th>
<th>Mortality</th>
<th>Body Weight</th>
<th>TH Activity</th>
<th>TH mRNA</th>
<th>TH mRNA</th>
<th>TH Activity</th>
<th>TH Activity</th>
<th>Mortality</th>
<th>Body Weight</th>
<th>Acoustic Startle</th>
<th>Straight Channel Swimming</th>
<th>MWM</th>
<th>Mortality</th>
<th>Body Weight</th>
<th>TH Activity</th>
<th>TH mRNA</th>
<th>TH mRNA</th>
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<tr>
<td>(Vorhees et al. 1998)</td>
<td>30 mg/kg, (+)</td>
<td>s.c.</td>
<td>P11-20</td>
<td>Distilled water</td>
<td>Mortality, body weight, acoustic startle reactivity, straight channel swimming, MWM</td>
<td>✪↑ mortality in MA rats, ▼ mortality &gt; than SD</td>
<td>▼ body weight in SD males from P12-42 and in ACI P13-49</td>
<td>No acoustic startle effect</td>
<td>No differences in straight channel swimming</td>
<td>Both strains have similar quadrant in MA animals</td>
<td>Both strains have similar quadrant in MA animals in all MWM phases</td>
<td>▼ proliferation in DG in left vs right hemisphere, HAL group similar to controls</td>
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<td>Neurotoxicol. Teratol.</td>
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<tr>
<td>(Hildebrandt et al. 1999)</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>Gerbil</td>
<td>BrdU labeling in dentate gyrus</td>
<td>▼ proliferation in DG in left vs right hemisphere, HAL group similar to controls</td>
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<td>J. Neural Transm.</td>
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<td>(Gomes-Da-Silva et al. 2000)</td>
<td>10 mg/kg, (+)</td>
<td>s.c.</td>
<td>P1-29</td>
<td>Rat Wistar</td>
<td>Tyrosine hydroxylase mRNA in substantia nigra and TH activity in SN and caudate-putamen (P30)</td>
<td>✪↑ TH activity in CPu and males but not females, no overall ↑ TH activity compared to females in both regions</td>
<td>✪↑ TH mRNA in SN of males only</td>
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<td>Treatment</td>
<td>Dose</td>
<td>Time</td>
<td>Species</td>
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<td>Outcomes</td>
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<tr>
<td>(Vorhees et al. 2000)</td>
<td>10 mg/kg or 20 mg/kg, (+)</td>
<td>P11-20</td>
<td>4/d 10mg/kg, 2 h intervals or 2/d 20mg/kg, 6 h interval (given saline at 2 and 4 h)</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
<td>Mortality, body weight, straight channel swimming, MWM cued learning, MWM spatial learning</td>
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<td>(Williams et al. 2000)</td>
<td>15 mg/kg, (+)</td>
<td>P11, P11-15, P11-20</td>
<td>4/d, 2 h intervals</td>
<td>Rat (Sprague-Dawley CD, IGS)</td>
<td>Saline, handled only</td>
<td>Body weight, hippocampus weight, plasma corticosterone and ACTH (at -15, 0, 15, 30 and 60 min)</td>
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</table>
- ↑ mortality in MA animals | |
- ↓ body weight in both MA groups from P13-20 and P28-42 |
- No differences in straight swimming times |
- ↑ latency in MWM cued learning but no difference in 10 x 4/d |
- ↑ latency, path length, cu cumulative distance and cu cumulative distance in MA animals in MWM at 10 x 4/d > 20 x 2/d for acquisition probe |
- ↓ % time in target quadrant in MA groups for acquisition probe |
- ↑ cumulative distance and cu cumulative distance in both MA groups for MWM reversed probe trials |
- ↑ only in first bearing in MA groups for MWM reversed probe trials |
- ↑ latency, path length and cu cumulative distance in 10 x 4/d only in platform phase, no difference in cu cumulative distance in 10 x 4/d on only platform phase, no differences in cu cumulative distance in 10 x 4/d on only platform phase |
- ↓ body weight from P13-20 animals compared to SA handled, in males and females |
- ↑ hippocampal % body weight in MA animals on P11, 15 differences in hippocampal weight only |
- ↑ hippocampal % body weight in females compared to males |
- ↑ CORT in MA at all time points except on SALhandled except on SALhandled except on SALhandled except on SALhandled except on SALhandled except on SALhandled except on SALhandled except on |
- ↑ CORT in MA animals |

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<table>
<thead>
<tr>
<th>Study</th>
<th>Dose</th>
<th>Methodology</th>
<th>Species</th>
<th>Control</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Armstrong et al. 2001)</td>
<td>1, 2, 4, or 8 mg/kg or 0, 1, or 4 mg/kg (2/d of 0, .5, or 2 mg/kg), (+)</td>
<td>i.p.</td>
<td>P10 or P2-8 or P2-9 + 1 or 4 mg/kg MA or P10</td>
<td>1/d</td>
<td>Rat Sprague-Dawley</td>
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<tr>
<td>(Armstrong et al. 2001)</td>
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<td>Saline</td>
<td>Ultrasonic vocalization, temperature</td>
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<tr>
<td>(Armstrong et al. 2001)</td>
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<td>Dose-dependent ↓ in ultrasonic vocalization in acute MA, significant only in 4 and 8 mg/kg doses</td>
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<tr>
<td>(Armstrong et al. 2001)</td>
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<td>No effects on temperature</td>
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<tr>
<td>(Armstrong et al. 2001)</td>
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<td>P2-8+10 challenge and P20 challenge: only 4 mg/kg had ↓ USV in all groups of rats challenged w/saline</td>
</tr>
<tr>
<td>(Blaesing et al. 2001)</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>Saline</td>
<td>Gerbil</td>
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<tr>
<td>(Blaesing et al. 2001)</td>
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<td>Golgi-Collonier silver staining, PFC dendritic morphology, levels III and V</td>
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<tr>
<td>(Blaesing et al. 2001)</td>
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<td>↑ total dendritic length in animals, ↓ apical dendritic length, lateral dendrites originating from soma</td>
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<tr>
<td>(Blaesing et al. 2001)</td>
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<td>Progressively ↑ spine density in animals</td>
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<tr>
<td>(Blaesing et al. 2001)</td>
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<td>↑ spines in all MA dendritic levels, apical &gt; lateral, basal</td>
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<tr>
<td>(Cappon and 15, 20, or 10)</td>
<td>s.c</td>
<td>P1 or P11</td>
<td>1/d: 15 mg/kg</td>
<td>Saline</td>
<td>Rat Sprague-Dawley</td>
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<tr>
<td>(Cappon and 15, 20, or 10)</td>
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<td></td>
<td>Plasma and brain concentrations of</td>
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<tr>
<td>(Cappon and 15, 20, or 10)</td>
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<td>1 x 15: in plasma, MA levels time regardless of age; i.p.</td>
</tr>
<tr>
<td>Study</td>
<td>Dose</td>
<td>Route</td>
<td>Age</td>
<td>Vehicle</td>
<td>Treatment</td>
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<tr>
<td>Vorhees 2001)</td>
<td>20 mg/kg, (+)</td>
<td>2/d, 20 mg/kg (1st and 4th dose); 2nd and 3rd doses were saline</td>
<td>Dawley CD</td>
<td>MA at 5, 10, 15, 20, 30, 60, 240, 480 min post treatment</td>
<td>Animals initially had ( \uparrow ) MA that declined more rapidly; T1/2 plasma ( P1 = 1.9h, P11 = 2.2h ); in brain ( P1 = 4.5h, P11 = 2.5h ).</td>
</tr>
<tr>
<td>Neurotoxicol. Teratol.</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>Gerbil</td>
<td>Saline</td>
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<tr>
<td>(Neddens et al. 2002)</td>
<td>10 mg/kg, (+)</td>
<td>s.c.</td>
<td>P11-20</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
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<td>J. Neural Transm.</td>
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<td>Williams et al. 2002)</td>
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<td>Brain Res.</td>
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<tr>
<td>Crawford et al. 2003 Synapse</td>
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<td>10 mg/kg, (+)</td>
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<td>P11-20</td>
<td>4/d with 2 h intervals</td>
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| Lehmann et al. 2003 Dev. Brain Res.       |          | 50 mg/kg, (+) | i.p. | P14 | 1/d | Gerbil Mongolian Saline Serotonin IHC in caudate-putamen, nucleus accumbens and amygdala |
|                                           |          |               |      |     |     |         |
|                                           |          |               |      |     |     |         |
|                                           |          |               |      |     |     |         |
|                                           |          |               |      |     |     |         |

- Increased latency in naïve and pretrained animals compared to respective controls in MWM novel test
- Increased time in periphery acquisition in naïve and pretrained saline
- Increased % and direct circle swim acquisition in naïve MA compared to pretrained saline
- Increased time in periphery novel test in MA > SAL, naïve > pretrained saline
- Increased % and direct circle swim acquisition in naïve MA compared to pretrained saline
- Decreased body weight in MA rats by 56%
- Decreased PKA activity in all MA males were more affected than females
- Decreased striatal D2-like receptor binding density and affinity, no effect on striatal dopamine and DOPAC in MA animals, no effect on striatal DOPAC in MA compared to ER
- Decreased striatal serotonin IHC in caudate-putamen, nucleus accumbens and amygdala
- No differences between controls in NAcc core or shell
- Increased 5-HT fiber density in IR compared to ER in MA in core only
- Increased 5-HT fiber density in NAcc core and shell; increased 5-HT fiber density in IR compared to ER in MA compared to ER
- Increased 5-HT fiber density in CPu in IR compared to ER compared to ER-MA comparison.
<table>
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<tr>
<th>Study</th>
<th>Dose</th>
<th>Route</th>
<th>Age</th>
<th>Frequency</th>
<th>Species</th>
<th>Treatment</th>
<th>Outcome</th>
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<tr>
<td>(Neddens et al. 2003) Dev. Brain Res.</td>
<td>50 mg/kg, (+)</td>
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<td>P14</td>
<td>1/d</td>
<td>Gerbil</td>
<td>Saline</td>
<td>Serotonin IHC in cerebral cortex (P110)</td>
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<td>• ↑ 5-HT fiber density in b and central amygdala to ER</td>
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<td>• ↑ 5-HT fiber density in caudal cortex compared to somatosensory cortex</td>
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<td>• ↑ 5-HT fiber density in I PFC, orbital PFC and ent cortex</td>
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<td>• ↓ 5-HT fiber density in I compared to IR in insula and ent cortex</td>
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<td>• ↑ 5-HT fiber density in I to ER in insular and ent cortex</td>
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<td>By layer:</td>
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<td>• ↑ in ER-MA in all layer of PFC; ↑ in ER-MA in later layers of PFC</td>
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<td>• ↑ in IR compared to ER and ↓ in IR-MA compared to ER</td>
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<td>• ↑ in III and VI of insular cortex; ↑ in I and II in IR</td>
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<td>No differences in PFC and cortex except layer II</td>
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<td>• ↑ in ER-MA in all layers of entorhinal cortex; ↑ in I and II in ER</td>
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<td>MWM acquisition: ↑ lat path length and trend to larger cumulative distance in groups</td>
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<td>• Dose dependent ↓ in % time spent in quadrant in acquisition phase</td>
</tr>
<tr>
<td>(Williams et al. 2003c) Synapse</td>
<td>5, 10, or 15 mg/kg, (+)</td>
<td>s.c</td>
<td>P11-20</td>
<td>4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
<td>Body weight, straight channel swimming, Cincinnati water maze, MWM cued, MWM hidden platform, MMW working memory</td>
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<td></td>
<td>• ↓ body weight in MA controls from P13-20</td>
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<td></td>
<td></td>
<td>• No differences in straight channel swimming</td>
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<td></td>
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<td>• No effects in MWM cued</td>
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<td>• MWM acquisition: ↑ lat path length and trend to larger cumulative distance in groups</td>
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<td></td>
<td></td>
<td>• Dose dependent ↓ in % time spent in quadrant in acquisition phase</td>
</tr>
</tbody>
</table>
(Williams et al. 2003a)
Dev. Brain Res.

<table>
<thead>
<tr>
<th>(Williams et al. 2003a)</th>
<th>5 mg/kg, (+)</th>
<th>s.c</th>
<th>P11-20</th>
<th>4/d with 2 h intervals</th>
<th>Rat Sprague-Dawley CD, IGS</th>
<th>Saline</th>
</tr>
</thead>
</table>

- Only reached significance in 10 and 15 mg/kg group
- MWM 2nd phase (reversal path length, and cumulative in all MA groups)
- MWM 3rd phase: ↑ lat, P10 and 15 mg/kg groups
- MWM 4th phase: ↑ lat, P10 and 15 mg/kg groups
- MWM 5th phase: ↑ lat, P10 and 15 mg/kg groups
- MWM 6th phase (shifted platform): ↑ lat, pl, and c groups
- No differences in working memory

- ▼ body weight in MA animals P12-20; appetitive version body weight from P49-9 of neonatal treatment
- Barnes aversive: ▼ latency to hole in females compared to MA females took longer; females on days 1 and 2 goal box in MA animals were faster than males
- Barnes appetitive: no significant differences
- ▼ CORT levels in MA animals following forced swim animals had ↑ CORT compared to following forced swim; ▼ CORT levels in all groups following forced swim
- Cliff avoidance: MA animals avoided cliff longer than animals following 20 mg
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Dose</th>
<th>Age</th>
<th>Route</th>
<th>Duration</th>
<th>Animals</th>
<th>Fluid</th>
<th>Test</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Williams et al. 2003b</td>
<td>P11-15 MA and P16-20 SAL (early) or P11-15 SAL and P16-20 MA (late)</td>
<td>10 mg/kg, (+)</td>
<td>s.c.</td>
<td>4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline (P11-20)</td>
<td>Body weight, zero maze, straight channel, MWM</td>
<td>---</td>
<td>No differences in striata or serotonin</td>
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<tr>
<td>Busche et al. 2004</td>
<td>P14</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>1/d</td>
<td>Gerbil</td>
<td>Saline</td>
<td>IHC for dopamine in amygdala, subiculum and entorhinal cortex</td>
<td>---</td>
<td>DA fibre density: lateral nucleus of amygdala &gt; nucleus (amygdale) ≈ ventral &gt; basolateral amygdala &gt; dorsolateral EC ≈ ventral</td>
</tr>
</tbody>
</table>

- No differences in striatal or serotonin
- ↓ body weight in early MA from P11-56, ↓ body weight in MA animals from P16-3
- Zero maze: no treatment effect in MA early, but females had open, head dips and entered treatment effect in MA late; males had ↑ time in open dips compared to females
- No differences in straight path length and cumulative distance in MA compared to controls; males performed better than females further from target.
- MWM acquisition early: no differences in MA compared to controls, but males performed better than females, no treatment effect in probe.
- MWM shifted early: ↑ path length and cumulative distance in MA compared to controls; males performed better than females, no treatment effects in probe. Late: no treatment differences, males performed better than females, no treatment effects in probe.
lateral nucleus (amygdala) 
- EC fibre density in clusters
- Narrow strip of DA innervation along CA1 in ventral subiculum
- ↑ fibre density in both halves of basolateral amygdala in animals
- ↑ fibre density in MA area is exacerbated by IR in animals except the dorsolateral BA1 part of the central nucleus subiculum; basolateral amygdala most affected

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Control</th>
<th>Time</th>
<th>Species</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Gomes-Da-Silva et al. 2004)</td>
<td>10 mg/kg, (±)</td>
<td>s.c.</td>
<td>P1-6, P1-13, or P1-29</td>
<td>2/d, ≈10 h interval</td>
<td>Rat Wistar</td>
</tr>
<tr>
<td>(Lehmann et al. 2004)</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>1/d</td>
<td>Gerbil Mongolian</td>
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<tr>
<td>Exp. Neurol.</td>
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</tbody>
</table>

- ↑ NE levels in SN of MA on P14; ↑ NE levels in SN of males and females on P30
- ↑ SN DOPAC in males on P14, ↓ SN DOPAC in MA males on P30; ↑ DOPAC in MA males vs. females no SN differences
- ↑ NE in CPu of MA females vs. NE in CPu of MA males on P7; NE control females > males and P30 NE control females > males
- ↑ NE in NAcc in P14 MA and P30 MA males and ↑ NE in P7 MA females
- ↑ fiber density in IR control in left medial and orbitofrontal cortex; effects in right hemisphere a less effect
- ↑ fiber density in IR control in LIII-VI in right EC, n

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<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
<th>Age</th>
<th>Route</th>
<th>Duration</th>
<th>Species</th>
<th>Treatment</th>
<th>Technique</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neddens et al. 2004</td>
<td>50 mg/kg, (+)</td>
<td>P14</td>
<td>i.p.</td>
<td>1/d</td>
<td>Gerbil</td>
<td>Saline</td>
<td>Serotonin IHC in cerebral cortex (PFC, insular, frontal, PC and EC) on P110, lateralization</td>
<td>- ↓ fiber density in ER-MA in left forelimb compared to ER; ↑ fiber density in lamina 6 in right</td>
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<td>- ↓ fiber density in IR-MA in left hind limb area compared to IR-MA in right</td>
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<td>- ↓ fiber density in IR-MA in left hind limb area compared to ER in right</td>
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</tbody>
</table>

(Neddens et al. 2004) Brain Res.

| Williams et al. 2004a          | 10 mg/kg, (+) | P11-20 | s.c.  | 4/d with 2 h intervals | Rat Sprague-Dawley | Saline | Golgi-Cox staining in dentate gyrus, nucleus | - ↑ fiber density in ER-MA compared to ER in right PFC |
|                                |               |       |       |                      |                     |        |                                          | - ↑ fiber density in IR-MA (RES/MET) compared to IR-MA in right |
|                                |               |       |       |                      |                     |        |                                          | - ↑ fiber density in IR and compared to ER in right |
|                                |               |       |       |                      |                     |        |                                          | - ER: right > left in PFC, EC left > right |
|                                |               |       |       |                      |                     |        |                                          | - ER-MA, reduced asymmetry right > left in PFC |
|                                |               |       |       |                      |                     |        |                                          | - IR-MA had ↑ left in EC |

(Williams et al. 2004a) 10 mg/kg, (+) s.c. P11-20 4/d with 2 h intervals Rat Sprague-Dawley Saline Golgi-Cox staining in dentate gyrus, nucleus

• ↓ dendritic length in NAc in MA animals
<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Treatment</th>
<th>Age</th>
<th>Methodology</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Williams et al. 2004b)</td>
<td>Rat</td>
<td>5, 2.5, 1.25, .625 mg/kg, (+)</td>
<td>P11-20</td>
<td>4/d with 2 h intervals</td>
<td>CD, IGS accumbens, and cortex (dendritic length, spine density) NAcc in MA animals</td>
</tr>
<tr>
<td>Int. J. Dev. Neurosci.</td>
<td>Rat Sprague-Dawley</td>
<td>Saline</td>
<td>Body weight, zero maze, straight channel swimming, MWM</td>
<td>↓ body weight in MA (15) from P12-20, .625 g body weight on P14-16 ↓ body weight in MA first and continued into adult MA caught up. No effects in zero maze channel MWM acquisition: ↑ first 2.5 an 5 groups only; ↑ cumulative distance in a group; ↑ path length and distance in .625 and 5 gr all measures ↑ in females to males MWM reversal: ↑ first bearing group only; ↑ path length group only; females ↑ in parameters compared to no effect on first bearing</td>
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<tr>
<td>(Lesting et al. 2005)</td>
<td>Gerbil Mongolia</td>
<td>50 mg/kg, (+) i.p.</td>
<td>P14</td>
<td>1/d</td>
<td>Gerbil</td>
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<tr>
<td>(Bagorda et al. 2006)</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>1/d</td>
<td>Gerbil Mongolian</td>
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<td>J. Neural Transm.</td>
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<tr>
<th>(Busche et al. 2006)</th>
<th>50 mg/kg, (+)</th>
<th>i.p.</th>
<th>P14</th>
<th>1/d</th>
<th>Gerbil</th>
<th>Saline</th>
<th>Acetylcholinesterase histochemistry of dentate gyrus</th>
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<tbody>
<tr>
<td>J. Neural Transm.</td>
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<td>• ↑ AChE fiber density in MA and SAL in both hemispheres</td>
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<td>• ↓ in IR-MA</td>
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<td>• ↑ AChE fiber density in molecular layer of left side of septal DG all layers, dense in granular layer of right side</td>
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<td>• ↑ in IR; ↑ in temporal but granular layer of right side</td>
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</tbody>
</table>

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• ↑ AChE fiber density in right septal DG, outer molecular layer, and subgranular layer of septal DG; ↑ in temporal layers except granule layer of temporal DG
• ↓ AChE fiber density in both hemispheres in subgranular layer of temporal DG only

(Butz and Teuchert-Noodt 2006)
J. Neural Transm.

<table>
<thead>
<tr>
<th>25 mg/kg, (+)</th>
<th>i.p.</th>
<th>P14</th>
<th>1/d</th>
<th>Gerbil</th>
<th>Saline</th>
<th>Computerized simulation of fibers, biocytin fiber tracing in PFC</th>
</tr>
</thead>
</table>

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<tr>
<th>10 mg/kg (+)</th>
<th>s.c</th>
<th>P11</th>
<th>4/d with 2 h intervals</th>
<th>Rat Sprague-Dawley</th>
<th>Saline</th>
<th>Body weight, corticosterone, neostriatal and MDMA compared to controls</th>
</tr>
</thead>
</table>

• Computer simulation, juvenile excitatory synaptic connections to inhibitory connections (adult)
• ↑ glutamatergic cortico-density in PFC and PC of IR (juv)
• ↓ glutamatergic cortico-density in PFC of IR-SAL to ER-SAL (juv)
• ↑ glutamatergic cortico-density in PFC in IR-MA compared to IR-SAL (juv)
• ↓ glutamatergic cortico-density in IR-MA compared to ER-SAL (juv)
• No differences in body weight
• ↑ CORT in MA>FEN>MDMA compared to controls
<table>
<thead>
<tr>
<th>J. Neurochem.</th>
<th>MA, (+/-)-MDMA, (+)-fenfluramine, (+/-)-methylphenidate, or cocaine</th>
<th>CD, IGS</th>
<th>hippocampal monoamines (all 24 h after first dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Williams et al. 2006) Stress</td>
<td>10 mg/kg, (+) s.c P1-20, once every other day or in 5 day blocks from P1-20; dosing starting on P1, 3, 5, 7, 9, 11, 13, or 15 and ending on day of sacrifice</td>
<td>1/d or 4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
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<tr>
<td></td>
<td></td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
</tr>
</tbody>
</table>

- ↓ striatal serotonin in FEN>MDMA>MPH controls, COC and MA controls
- ↓ striatal 5-HIAA in MA, all others similar to controls
- ↑ 5-HIAA/5-HT ratio in controls
- No differences in striatal dopamine
- ↓ striatal DOPAC in MA, MA>MDMA, ↑ ratio in controls
- ↓ hippocampal 5-HT and FEN>MDMA, all others similar to controls
- ↑ 5-HIAA/5-HT ratio in controls
- ↓ body weight in MA increments
- ↑ CORT in MA animals from P3-19 with single d-shaped curve
- ↑ CORT in MA animals from P1-19 with single d-shaped curve
- ↑ CORT in MA animals each 5d block, increasing
- ↑ CORT in MA animals only in 1-5, 3-7, and 15-
- ↓ thymus weight in MA 5, 7-11, 9-13, 11-15, 13-19 groups
- ↓ thymus weight only in controls when expressed as % body weight
- Chronic MA CORT > acute 30 min
- Chronic MA CORT < acute 30 min
<table>
<thead>
<tr>
<th>Study (Reference)</th>
<th>Dose</th>
<th>Route</th>
<th>Age</th>
<th>Gender</th>
<th>Species</th>
<th>Treatment</th>
<th>Outcome Measures</th>
<th>Summary</th>
</tr>
</thead>
</table>
| Acevedo et al. 2007 | 5 mg/kg (+)-MA, thioperamide, or immepip | s.c. | P11-20 | 1/d | Mouse C57BL/6J | Saline | Mortality, body weight, open field, plus maze, zero maze, water maze, rotorod, acoustic startle (PPI) | - 15% mortality in MA and THIO females
- No differences in weight
- No differences in anxiety scores in MA and THIO males
- No differences in rotorod
- MA and THIO females spent more time exploring object in location
- MA and THIO animals spent more time exploring novel objects
- Novel object deficits were mimicked by IM
- No differences in water visible platform latency
- % time in quadrant in females that was prevented by IM

| Brummelte et al. 2007 | 50 mg/kg, (+) i.p. | P14 | 1/d | Gerbil Mongolian | Saline | GABAergic immunohistochemistry of PFC | - Homogeneous GABAergic innervation in cortex, somatodendritic GABAergic innervation in layers II, III, and V
- % time in quadrant in females that was prevented by IM
- % time in quadrant in females that was prevented by IM
- GABAergic fibre density in layers I/II and V of impoverished environment mimicked and IM prevented it |

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<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Treatment</th>
<th>Dose</th>
<th>Age</th>
<th>Control</th>
<th>Treatment</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grund et al. 2007</td>
<td>Brain Res.</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>1/d, then 5 mg/kg of methylphenidate from P30-60 (oral)</td>
<td>Gerbil</td>
<td>Saline</td>
</tr>
<tr>
<td>Lehmann et al. 2007</td>
<td>Int. J. Neurosci.</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>1/d</td>
<td>Gerbil Mongolian</td>
<td>Saline</td>
</tr>
<tr>
<td>Skelton et al. 2007</td>
<td>Psychoneuroendocrinology</td>
<td>10 mg/kg, (+)</td>
<td>s.c</td>
<td>P11-14 and twice on P15, 3 times on P11 only, or P11-20</td>
<td>4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
</tr>
</tbody>
</table>

- ↓ density in lamina V of basolateral amygdala in IR-MA compared to MA/water
- No differences in SAL/MA region
- MA/MP were similar to all regions, but ↑ in lamina V of PFC and in basolateral amygdala compared to MA/water
- ↑ hippocampal BDNF in females
- ↑ BDNF in hippocampus of males regardless of treatment
- ↑ BDNF in hippocampus of MA animals
- Trend toward ↑ BDNF in hippocampus in MA animals following MWM
- ↑ NGF in hippocampus of females; no effects on P68
- ↑ NGF in hippocampus of MA animals
- On P68, MWM tested males: hypothalamic NGF compared to females; MA females have hypothalamic NGF compared to saline (tested and naïve males)
- ↑ time in open and stretch posture; females in zero maze, no effects
- No differences in straight path latency, path length, cut off distance, and first bearing behavior between animals, females perform better than males on all measures
- ↑ time in outer annuli and time spent in MA

(Smith et al. 2007)
Brain Res. 0.1-100μM, (+) Membranes and hippocampal slice cultures P60 and P8 1 exposure Rat Sprague-Dawley Buffer + MK-801, Culture medium + PI MK-801 binding (adult hippocampus, cerebellum and cortex), hippocampal
- MA did not affect MK-801 binding to membrane at any concentration, but dextromethorphan inhibited MK-801 binding.
- Hippocampal slices exposed to MK-801 for 24 h did not affect P68.
culture (P8 rat) and cytotoxicity (propidium iodide)

NMDA alone prevents PI uptake; M NMDA exposure PI uptake (all MA concs. except 1:

- With 10 μM NMDA: ↑ PI uptake in CA3 at 100 M effects in dentate gyrus

- With NMDA had ↑ PI uptake attenuate ↑ induced by N (Vorhees et al. 2007) Behav. Pharmacol. 5 mg/kg, (+) s.c P11-20 4/d with 2 h intervals Rat Sprague-Dawley CD, IGS Saline Mortality, body weight, straight channel, MWM (behavior experiments performed on P30, 40, 180, or 360)

- 1 MA died before P175 and SAL animals died before P12-28, MA females significantly reduced up to P35

- No differences in straight channel, MWM (P30 and 40) acquisition and probe combined. Reversal: no differences in path length, cumulative path length, or latency; ↑ first bearing 1 day 1 (P30) and days 2 and 3 combined ↑ first bearing MA animals. Reduced: ↑ path length on day 1 bearing and time in peri combined MA animals; bearing in probe combined MA animals; MWM (P180 and 360) path length on day 2 in combined MA animals; ↑ first bearing combined MA animals in and acquisition probe. ↓ path length, latency, CD bearing in MA animals;
<table>
<thead>
<tr>
<th>Reference</th>
<th>Treatment and Dose</th>
<th>Age</th>
<th>Species</th>
<th>Route</th>
<th>Time</th>
<th>Vehicle</th>
<th>Other Treatments</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Witte et al. 2007)</td>
<td>50 mg/kg, (+) I.P.</td>
<td>P14</td>
<td>Gerbil</td>
<td>I.P.</td>
<td>1/d</td>
<td>Saline</td>
<td>Biocytin labeled contra lateral PFC fiber density (in lamina levels)</td>
<td>• ↑ fiber density in MA and site crossings in probe MA animals. For MA ↑ all parameters; ↑ anterior and site crossings in probe</td>
</tr>
<tr>
<td>J. Neural Transm.</td>
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<tr>
<td>(Acevedo et al. 2008)</td>
<td>5 mg/kg (+)- MA, thioperamide, or Imme p i p</td>
<td>P11-20, P11, 15 and 20 only (pharmacokinetics)</td>
<td>1/d</td>
<td>Mouse</td>
<td>6J</td>
<td>Saline</td>
<td>Corticosterone, histamine levels, MA levels, IHC in hippocampus and cortex (synaptophysin, MAP-2)</td>
<td>• ↑ CORT in MA and THP11, 15 and 20 and in IM+MA on P11</td>
</tr>
<tr>
<td>J. Neurochem.</td>
<td></td>
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66
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<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Route</th>
<th>Age</th>
<th>Dose</th>
<th>Duration</th>
<th>Species</th>
<th>Control</th>
<th>Treatment</th>
<th>Notes</th>
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<tr>
<td>(Brummelte et al. 2008) J. Neg. Results BioMed.</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>1/d, then 5 mg/kg of methylphenidate from P30-60 (oral)</td>
<td>Gerbil</td>
<td>Saline</td>
<td>Dopamine and GABA IHC in PFC and amygdala</td>
<td>• No treatment or rearing region</td>
<td></td>
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<tr>
<td>(Butz et al. 2008) Hippocampus</td>
<td>50 mg/kg, (+)</td>
<td>i.p.</td>
<td>P14</td>
<td>1/d, then 5 mg/kg of methylphenidate from P30-60 (oral)</td>
<td>Gerbil</td>
<td>Saline</td>
<td>Silver staining of lysosomal accumulations in dentate gyrus, computer simulation modeling</td>
<td>• Naturally reared control lysosomal accumulation in molecular layer, sparse outer molecular layer and subgranular layer</td>
<td></td>
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<td></td>
<td>• Modeling suggests occupancy: ↑ synaptic rewiring; ↓ pre-synaptic activity processes (synaptic formation/deletion, addition of postsynaptic elements)</td>
</tr>
<tr>
<td>(Grace et al. 2008) Synapse</td>
<td>10 mg/kg, (+)</td>
<td>s.c.</td>
<td>P11-15</td>
<td>4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline and weighed only</td>
<td>Body weight, corticosterone, neostraiatal and hippocampal BDNF and NGF, adrenal and thymus weight</td>
<td>• ↓ body weight in MA and P12-15; all other treatments compared to controls</td>
<td></td>
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<td></td>
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<td></td>
<td>• P11 (1 treatment), ↑ CORT in MA and ISO (0.5 h and 24 h), ↑ CORT in MA and ISO (6.5 h only)</td>
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<td></td>
<td>• P11-15 (1 treatment on ISO; CORT in MA and ISO (6.5 h only))</td>
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<tr>
<td>(Schaefer et al. 2008)</td>
<td>J. Neurochem.</td>
<td>10 mg/kg (+)-MA or (+/-)-MDMA</td>
<td>s.c.</td>
<td>P11-15 or P11-20</td>
<td>4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
<td>Body weight and P16, 21, and 30 corticosterone, striatal and hippocampal monoamines</td>
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<tr>
<td>compared controls at 0.5 h</td>
<td>change in any group at 1 h</td>
<td>• P11-15 (4 treatments), ↑ MA animals at 6.5 h compared to controls, ↑ CORT in FS compared to WEIGH, ↑ MA animals compared to controls at 24 h</td>
<td>• ↑ hippocampal BDNF in females compared to males</td>
<td>↓ hippocampal NGF in females &gt; males, N &gt; P11</td>
<td>• ↑ neostriatal BDNF in females &gt; males</td>
<td>• ↑ neostriatal NGF in females &gt; males</td>
<td>• ↓ adrenal and thymus weight in MA and P15</td>
<td></td>
<td></td>
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<tr>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>15 and MA from P13-15</td>
<td>MA&gt;MDMA&gt;SAL</td>
<td>• ↓ body weight in MDMA and MA from P13-20, MA&gt;MDMA&gt;MDMA</td>
<td>• ↑ CORT in MA on P16, P21 or 30, no MDMA effects</td>
<td>• ↓ in neostriatal 5-HT and MDMA and MA on P16, MDMA&gt;MA, no effect</td>
<td>• No effects on striatal DA or DA or 5-HT turnover on P16 or 30</td>
<td>• ↓ neostriatal 5-HT and 5-HT in MA and MDMA on P21, no effect on turnover ratio</td>
<td>• ↑ striatal DOPAC and DOPAC</td>
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## Table

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route</th>
<th>Age</th>
<th>Intervals</th>
<th>Animal Type</th>
<th>Condition</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>(Vorhees et al. 2008)</td>
<td>10, 15, 20, 25 mg/kg, (+)</td>
<td>P11-15</td>
<td>4/d with 2 h intervals</td>
<td>Rat Sprague-Dawley CD, IGS</td>
<td>Saline</td>
<td>Body weight, rearing condition (with or without huts), straight channel swimming, Cincinnati water maze, MWM</td>
</tr>
<tr>
<td>(Kaewsuk)</td>
<td>5 or 10</td>
<td>P4-10</td>
<td>1/d</td>
<td>Rat</td>
<td>Tyrosine</td>
<td>Tyrosine hydroxylase inhibition</td>
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</table>

- **↓** hippocampal 5-HT and HIAA in MA and MDMA (MDM >MA) on P16, ↓ hippocampal HIAA and 5-HIAA/5-HT ratio in MA and MDMA on P30.
- **↓** body weight in MA animals reared with enrichment vs those under standard conditions, ↓ body weight in MA animals on P21 and 28, no effect.
- **↓** latency in straight channel, enriched compares to standard.
- CWM: ↑ errors, latency returns in all MA groups, rearing conditions.
- MWM acquisition: ↑ cum. distance, path length, and 25 group only, enriched cd, lower cd, pl, and lat condition; probe showed ↓ % time cd, % distance in 20 females only.
- MWM reversal: ↑ cum. distance, path length, and 25 group only, no condition.
- MWM shifted: ↑ cum. distance, path length, and 25 group only, no condition.
- **↓** tyrosine hydroxylase inhibition.

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mg/kg (+)-MA, 2mg/kg melatonin, melatonin (30 min pretreatment) + MA

P4-5 with 5 mg/kg and P6-10 with 10 mg/kg

Wistar

hydroxylase, GAP-43 and synaptophysin protein levels and immunohistochemistry in prefrontal cortex, dorsal striatum, nucleus accumbens, and substantia nigra

PFC, and striatum of MA which is reversed by melatonin treatment

• ↓ # of TH immunoreactive fibers in NAcc, PFC, and MA animals; small ↑ in immunoreactive nerve fibers in striatum in melatonin group; Mel+MA ↑ fibers in all

• ↓ TH in substantia nigra group, Mel and Mel + MA controls; similar result of immunohistochemistry

• ↓ synaptophysin in PFC of MA group, which was reversed in melatonin (similar IHC)

• TH and synaptophysin in striatal nerve terminals, which was reversed in melatonin

• ↓ GAP-43 in NAcc of MA group, reversed in Mel + MA group


10 or 25 mg/kg s.c.
P1-20, P6-15, or P11-20 4/d with 2 h intervals

Rat Sprague-Dawley CD, IGS

Saline

Body weight, locomotor activity, acoustic startle (PPI), straight channel, Cincinnati water maze, MWM

• ↑ mortality in 25 MA group

• ↓ body weight in 10 and 2nd day of treatment till end of treatment period, which persisted into adulthood

• ↓ activity (horizontal activity) and 25 groups, females > males than males

• No sensorimotor gating (PPI)

• No differences in straight channel

• CWM errors and latency 20>P6-15>P1-10, 25 MA>Mel>MA>SAL, P1-10 no dif...
Start returns: 25 MA>10 MA>SAL, no effect of dose period. 
- MA>10MA>SAL, 25 MA group>controls, both MA groups>controls, no difference. 
- Acquisition probe: ↑ avg distance and initial error; ↓ % distance in target quadrant, % time in target, MSD, ↓ crossovers in 25 MA group.


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*Neuropsychopharmacology* **32**, 531-541.


McCann U. D., Kuwabara H., Kumar A., Palermo M., Abbey R., Brasic J., Ye W.,
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*Synapse* **62**, 91-100.

Effects of neonatal corticosterone treatment on maze performance and HPA axis in

122.

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Myelination changes in the rat optic nerve after prenatal exposure to methamphetamine.
*Brain Res.* **1106**, 21-29.

of axon size and myelin occupancy in rats prenatally exposed to methamphetamine.
*Brain Res.* **1222**, 61-68.


Nagai N. (1893) "Kanyaku maou seibun kenkyuu seiseki (zoku)".


vulnerable to both environmental and pharmacological epigenetic challenges. *Brain Res.* **1021**, 200-208.


fenfluramine, or methylphenidate administration in the neonatal rat. *Journal of Neurochemistry.*


Figure 1. Structural comparison of MA with neurotransmitters in which MA primarily affects.
amphetafine  
methamphetamine  
dopamine  
epinephrine  
norepinephrine  
serotonin
Figure 2. Primary MA/amphetamine admission rates per 100,000 people age 12 and older to treatment by state from 1996-2001. Admissions were highest in western states such as California in 1996 and admissions increased eastward by 2001. Adapted from Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2008.
Figure 3. Primary MA/amphetamine admission rates per 100,000 people age 12 and older to treatment by state from 2002-2006. Admissions continued to increase in western states and admissions increased eastward, demonstrating the spread of MA abuse in the U.S. Adapted from Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2008.
Figure 4. Acute exposure to MA in the neonatal rat produces elevations in plasma corticosterone that can be described as a U-shaped function. These increases, although significant, are blunted during the stress hyporesponsive period (P4-14). From Williams et al., 2006.
Figure 5. Diagram of the Cincinnati water maze. This task is used as a test of egocentric learning in which animals begin in the start position (S) and navigate to the goal (G) under infrared lighting. Errors and latency to find the goal are measured.
**Figure 6.** Diagram of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis by a stressor leads to release of CRF and AVP from the median eminence of the hypothalamus, subsequent stimulation of the anterior pituitary to secrete ACTH, and stimulation of the adrenal cortex to release glucocorticoids which have negative feedback regulation at the level of the pituitary, hypothalamus, and hippocampus. *From Vazquez, 1998.*
Hypothesis

Developmental exposure to stress leads to altered behavior, learning deficits, and altered responses to stress, effects hypothesized to be mediated by corticosterone release. Methamphetamine (MA) also causes a large release of corticosterone when given during development. It was hypothesized that developmental MA exposure leads to long-term changes in adult learning and memory and in stress reactivity and that inhibiting the drug-induced corticosterone release will attenuate the drug’s long-term effects.

Specific Aims

1. Test the hypothesis that developmental MA exposure will cause altered adult responses to stressors (forced swim, forced confinement and MA challenge).

2. Develop a model of adrenal autotransplantation that will reduce MA-induced corticosterone release while causing no secondary effects on brain serotonin beyond those known to be caused by MA alone.

3. Use the adrenal autotransplantation method to test whether attenuating MA-induced corticosterone release during development attenuates the later cognitive deficits.
CHAPTER 2

Effects of neonatal methamphetamine treatment on adult stress-induced corticosterone release in rats

Curtis E. Grace, Tori L. Schaefer, Nicole R. Herring, Michael T. Williams, and Charles V. Vorhees

Division of Neurology, Dept. of Pediatrics, Cincinnati Children’s Research Foundation and University of Cincinnati College of Medicine, Cincinnati, Ohio

As submitted to Hormones and Behavior (December, 21 2009)
Abstract

Neonatal (+)-methamphetamine (MA) exposure results in long-term learning and memory impairments in rats and similar observations have been made in animals neonatally exposed to stress. Animals exposed to stress early in life have an altered response to stress in adulthood, suggesting that there are alterations in hypothalamic-pituitary-adrenal axis development. MA produces increases in corticosterone in neonates and these effects last for at least 24 h. We therefore determined whether neonatal MA exposure altered the adult stress response. Rats were administered 10 mg/kg MA, saline vehicle, or were weighed-only four times daily from postnatal day 11-15 or 11-20. In adulthood, corticosterone levels were measured just prior to and following acute stressor exposure. The stressors were: (1) 15 min forced swim, (2) 15 min confinement, and (3) acute MA treatment (10 mg/kg), with forced swim and confinement counterbalanced. As adults, there were main effects of stressor, with forced swim having the largest effect, a stress order effect caused by forced swim having a greater effect when it was given first in the P11-15 group. Nonetheless, there was no main effect of neonatal MA treatment, i.e., MA did not alter corticosterone levels compared to controls regardless of stressor type, nor did neonatal MA alter later stress reactivity to an acute MA challenge. The data do not support the concept that early MA exposure alters later adult stress reactivity.

Key words: Methamphetamine, neonatal methamphetamine, stress, forced swim test, corticosterone, confinement stress
Introduction

Methamphetamine (MA) is a commonly abused psychostimulant among males and females. If female abusers become pregnant, passive transplacental exposure of the fetus to MA can occur if use of the drug continues. However, some continue to use during pregnancy. For example, as of the latest available data (2006), 24% of pregnant women seeking drug treatment from federally-supported drug treatment centers (which are 83% of all drug treatment centers) reported MA as their primary drug of use (Terplan et al, 2009). The report also showed that this 24% has steadily increased from 1991-2006; extrapolation of this trend suggests that the percentage may be even higher today. Despite such prevalence, relatively little is known about the effects of prenatal MA exposure on their children’s neurodevelopment or behavior.

What human data are available suggest that there is reason for concern over the outcome of infants exposed to MA in utero. For example, case-control retrospective studies indicate that prenatally MA-exposed infants exhibit reduced birth weight, length, and head circumference, higher occurrence of placental or intraventricular hemorrhage, and anemia (Chomchai et al, 2004; Dixon, 1989; Dixon and Bejar, 1989; Little et al, 1988; Oro and Dixon, 1987; Smith et al, 2008), and poor sleep, vomiting, tremors, poor feeding, and drug withdrawal symptoms (Chomchai et al., 2004; Dixon, 1989; Oro and Dixon, 1987).

Newer studies indicate that in utero MA-exposed infants and children show deficits in visual motor integration, attention, psychomotor speed, spatial and verbal memory (Chang et al, 2004; Chang et al, 2009), reduced novel object recognition memory on the Fagan Test of Infant Intelligence (Struthers and Hansen, 1992), and reduced
arousal and quality of movement in newborns (Smith et al., 2008). Magnetic resonance imaging (MRI) studies of in utero MA exposed children reveal decreased volume of the hippocampus, putamen, and globus pallidus (Chang et al., 2004), changes in white matter diffusivity using diffusion tensor imaging (DTI-MRI) with no changes in fractional anisotropy (Cloak et al, 2009). Moreover, magnetic resonance spectroscopy (MRS) reveals higher total creatine, N-acetyl aspartate, and glutamate/glutamine in frontal white matter (Chang et al., 2009).

We developed an animal model for second-half of pregnancy-equivalent effects of MA using the neonatal rat. The model is based on observations that, for example, cells in the dentate gyrus continue to proliferate until approximately postnatal day (P)19 in the rat which is approximately equivalent to post-conception day 240 in humans (Bayer et al, 1993). Another approach uses interspecies scaling algorithms. One such model shows that P11 rat brain corresponds with human gestation at 26 weeks for cortical structures and at 19 weeks for limbic structures (Clancy et al, 2006;Clancy et al, 2007).

On the basis of such comparisons, we demonstrated that MA treatment from P11-15 results in Cincinnati water maze (CWM) egocentric learning deficits (Vorhees et al, 2008) and P11-20 MA treatment in Morris water maze (MWM) spatial learning deficits (Williams et al, 2002;Williams et al, 2003b). These exposures also produce morphological changes in the dentate gyrus and nucleus accumbens (Williams et al, 2004).

The MA exposure period used in these experiments overlaps with the stress hyporesponsive period (SHRP), which in rats is from approximately P4-14, and is a period in development when the adrenal gland responds minimally to environmental
perturbations, resulting in small or no elevations in corticosterone (CORT) (Sapolsky and Meaney, 1986). However, depending upon the stressor, circulating CORT levels may be increased from basal levels during this time, but do not approach levels observed in the adult. For instance, 30 min after novelty, saline injection, or adrenocorticotropic hormone (ACTH) injection in 24 h maternally deprived pups, CORT was elevated compared to control animals (Levine et al, 1991). Previous studies using several different stressors such as maternal separation or endotoxin exposure have documented that exposure to stress during the SHRP can result in alterations in hypothalamic-pituitary-adrenal (HPA) axis reactivity in adulthood (Aisa et al, 2007;Biagini et al, 1998;Felszeghy et al, 2000;Hodgson et al, 2001;Kalinichev et al, 2002;Kamphuis et al, 2002;Shanks et al, 1995;Wigger and Neumann, 1999). For example, maternal separation during the SHRP can lead to hypersecretion of CORT following stress in adulthood and/or increased anxiety (Aisa et al., 2007;Biagini et al., 1998;Kalinichev et al., 2002;Wigger and Neumann, 1999). Furthermore, alterations in HPA axis reactivity have been associated with cognitive deficits in the MWM and novel object recognition (Aisa et al., 2007). We have previously demonstrated alterations in stress reactivity following MWM testing of rats neonatally exposed to MA (Skelton et al, 2007). We have also demonstrated that a single dose of MA increased CORT 30 min after administration on any single day between P1-19, and these levels remained elevated 105 min later (Williams et al, 2006). In the same study, MA given 4 times/day for 4 days with a final dose on the fifth day increased CORT 30 min later, and the effect increased as developmental age increased. However, by 105 min post-treatment the increase in CORT had disappeared, indicating that repeated dosing triggers feedback mechanisms that
terminate the response earlier (Williams et al., 2006). ACTH is also increased by MA during and just following the SHRP (Williams et al., 2000). Given that MA increases CORT during the SHRP more than does a physical or psychological stressor (Grace et al., 2008), we sought to determine if MA given during or started during the SHRP altered HPA axis reactivity when the animals were adults. Prior to this experiment, we showed that animals treated with 5 mg/kg MA from P11-20 showed reduced CORT release following 15 min forced swim in adulthood compared to controls, suggesting an alteration in HPA axis development (Williams et al., 2003a). We therefore determined whether treatment with MA from P11-15 or P11-20 would alter HPA axis reactivity after acute stressor application using forced swim, confinement, or a challenge dose of MA in adulthood.

Methods

Animals and conditions

Male (251-275g) and nulliparous female (151-175g) Sprague-Dawley CD IGS rats were obtained from Charles River Laboratories (Raleigh, NC) and the male offspring were the subjects of the experiment. Rats were acclimated to the vivarium (14 h dark: 10 h light, lights on at 600 h with temperature and humidity controlled) for at least one week prior to breeding and were housed in polycarbonate cages. Stainless steel enrichment enclosures were placed in cages starting on embryonic day 1 and remained in the cages throughout the experiment (Vorhees et al., 2008). P0 was designated as the day on which the litter was born. On P1, pups were removed from their mothers, weighed, sexed, and culled to 10 of which at least 4 were males. On P7, pups were marked for identification by ear punch and on P28 were separated from the dam and housed in pairs. Animals had
ad libitum access to food and water and all protocols were approved by the Institutional Animal Care and Use Committee. The vivarium is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Treatments

Males from each litter were placed into one of three treatment groups: (1) weighed-only (WEIGH), (2) saline (SAL), or (3) 10 mg/kg (+)-methamphetamine HCl (expressed as the freebase, NIDA, > 95% pure) in a volume of 3 ml/kg. Any additional males from each litter received 10 mg/kg MA to allow for potential losses. Each treatment was administered 4 times per day at 2 h intervals from either P11-15 or P11-20 since we previously showed that different learning impairments are observed following these regimens (Vorhees et al., 2008; Williams et al., 2003b). SAL or MA was administered by s.c. injection in the dorsum. 20 litters were assigned to each treatment interval but catheter clogging reduced the number of usable animals per group. Offspring were weighed on P1, P7, prior to each treatment and weekly thereafter.

Surgical procedures

Jugular catheters were surgically implanted between P92-200. Rats were moved to a surgical suite and anesthetized using isoflurane gas. The neck region was shaved and swabbed with betadine and alcohol. Using sterile procedures and continuous isoflurane administration, an incision was made lateral to the midline and the jugular vein exposed and occluded with a silk suture superior to the catheter insertion point. An incision was made in the jugular vein and a catheter introducer (Becton-Dickinson and Co., Franklin Lakes, NJ) was inserted to guide the catheter (Braintree Scientific Inc., Braintree, MA) to a point just superior to the aorta. The inner diameter of the catheter port was 0.0584 cm,
the catheter body 0.0279 cm, and the intravascular tippet 0.0305 cm (Braintree Scientific Inc.). The catheter was pre-filled with 10 IU/ml heparinized saline and connected to a 3 ml syringe using a 23 gauge blunt needle (Braintree Scientific Inc.) which remained connected to the catheter until it was firmly secured. The position of the catheter was kept consistent and secured using another silk suture fastened around a node on the catheter and at the opening of the jugular incision. The suture posterior to the incision site was used to anchor and further secure the catheter in place. After securing the catheter, blood was drawn through the catheter to check for patency. Once patency was achieved, the 3 ml syringe was disconnected from the catheter and a port plug inserted (Braintree Scientific Inc.). The catheter was tunneled from the opening of the body wall in the neck and exited through an incision in the dorsum between the shoulders. It was secured using sutures and the incision in the neck was closed. Sensorcaine® was applied to incision sites. Both exterior suture sites were sealed (GLUEture®) and the animal was placed in an incubator at 28°C until anesthetic recovery. Post-surgery animals were housed singly to prevent damage to the catheter and returned to the home room. Approximately 0.2 ml of a heparinized glycerol (50 IU/ml) catheter lock solution was injected in the catheter to maintain patency until blood was drawn.

**Blood collection and plasma isolation**

On the day following surgery, a blood sample was collected. This continued for 3 days to ensure proper catheter function and to familiarize animals to the procedure. Blood drawn on the third day following surgery was used to determine baseline CORT levels. On days 4, 5, and 6 post-surgery, one of 3 stressors was administered. On these days, blood was taken at 6 time points as follows: 0 (immediately upon removal from the
home cage and prior to stressor exposure), 15 (15 min following the stressor), 30, 60, 90, and 120 min. Following each blood collection, catheters were flushed with 10 IU/ml heparinized saline. Blood was collected on ice and plasma separated from whole blood by centrifugation at 1300 RCF at 4°C for 10 min and stored at –80°C until CORT levels were measured. The red blood cells were resuspended in physiologic saline and reintroduced back in the animals through the catheter after the subsequent blood sample. Approximately 0.2 ml of blood was obtained in each draw and 0.1 ml of plasma was collected with an identical volume (0.1 ml) of saline used to resuspend the red blood cells. After the final red blood cell replacement on each day, the catheter was again kept patent by a 0.2 ml injection of 50 IU/ml heparinized glycerol.

Stressors

All stressors were applied between 1000 and 1145 h. On days 4 and 5 post-surgery, animals were subjected to one of two stressors, forced swim (FS) or confinement. FS was conducted in a 15 cm diameter by 46 cm tall PVC cylinder filled with water to a depth of 35 cm (22 ± 1°C). Forced confinement (FC) was performed in the same apparatus without water and both stressors were administered for 15 min. A counter-balanced design was employed in the order of FS or FC on day-1 and the other half in the order FC or FS on day-2. On day-3 all animals were given an injection of 10 mg/kg MA, s.c.

Corticosterone assessment

Plasma corticosterone was assayed using Octeia ELISA kits (IDS, Fountain Hills, AZ) and each sample was diluted 1:3 and assayed according to the manufacturer’s
protocol. The ELISAs were measured on a SpectraMax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Statistics

Corticosterone data were analyzed using general linear model analysis of variance (ANOVA) because there were two repeated measure factors in the design which can be problematic for mixed linear models. The model for the corticosterone x stress type was a 3 treatment x 2 stress type x 2 order x 6 time point ANOVA (2-between, 2-within). The model for the day-3 MA challenge corticosterone data was a 3 treatment x 2 order x 6 time point ANOVA (2-between, 1-within). For these analyses, variance-covariance matrices found to be significantly non-spherical were corrected using Greenhouse-Geisser adjusted F-ratios. For body weight data, mixed models were used as there was only one repeated measure factor. In this case, the variance-covariance matrix was tested for best fit to structural models and the autoregressive-1 (AR1) model chosen in conjunction with Kenward-Roger adjusted degrees of freedom. Significant interactions were analyzed using simple-effect slice ANOVAs. Significance was set at $p \leq 0.05$. Data were analyzed using SAS version 9.1 (SAS Institute, Cary, NC).

Results

Body weights

Body weights were recorded during the treatment periods and weekly thereafter. In the P11-15 group, there were overall effects of treatment $F(2, 38) = 7.67$, $p < 0.002$, day, $F(4, 144) = 126.48$, $p < 0.0001$, and there was a treatment x day interaction $F(8, 144) = 13.21$, $p < 0.0001$. MA-treated animals had reduced weight gain compared to SAL or WEIGH animals. Further examination of the treatment x day interaction revealed that
MA-treated animals had significantly reduced weight gain compared to SAL- or WEIGH-treated animals on P13-15 (Figure 1A). This reduction was transient. Adult weights were similar between treatment groups (Figure 1B) and there were no differences observed between the WEIGH animals and the SAL animals.

For P11-20 dosing weights, there were main effects of treatment, $F(2, 52.3) = 35.95, p< 0.0001$, and day, $F(9, 438) = 138.69, p< 0.0001$, and a treatment x day interaction, $F(18, 438) = 10.57, p< 0.0001$. MA-treated animals had reduced weight gain compared to SAL or WEIGH animals. Further analysis of the treatment x day interaction showed that MA-treated animals had reduced weight gain from P12-20 (Figure 1C) compared to SAL or WEIGH animals. This MA-induced weight reduction was transient. There was no treatment or treatment x day interactions on adult body weights (Figure 1D). No differences were noted between SAL and WEIGH animals.

**Corticosterone**

Plasma CORT levels were initially increased, but then showed the expected decrease from negative feedback over time following stressors or MA challenge in adulthood after P11-15 (Figure 2 A-F) or P11-20 (Figure 3 A-F) exposure to MA, SAL, or WEIGH. For animals treated from P11-15, there were main effects of stressor, $F(1, 31) = 37.98, p< 0.0001$, and time, $F(5, 155) = 18.06, p< 0.0001$, but no effect of treatment. FS produced greater increases in CORT levels compared to confinement. There were also stressor x order, $F(1, 31) = 9.63, p< 0.004$, and stressor x time interactions, $F(5, 155) = 12.42, p<0.0001$. The stressor x order interaction showed that the stressors applied on day-2 increased CORT levels more than when the stressors were applied on day-1, i.e., FS on day-2 had overall CORT levels that were ~173% greater
than levels after FS on day-1 (Figure 2A and E) and overall CORT levels for confinement on day 2 were ~137% greater compared to day-1 levels (Figure 2B and D). For the stressor x time interaction, beginning at 0 min, the confinement group had increased CORT levels compared to FS (p< 0.02). From 15-90 min (p< 0.05), the FS group had increased levels of CORT compared to confinement, but no differences were observed at 120 min. The data for MA (Fig 2C and F) were analyzed separately and showed only a main effect of time, F(5, 130) = 72.85, p< 0.0001.

For CORT levels in the P11-20 group, there were main effects of stressor, F(1, 46) = 150.18, p< 0.0001, and time, F(5, 230) = 99.74, p< 0.0001. As previously shown, greater increases in CORT levels were observed following FS compared to confinement. There were also interactions of stressor x order, F(1, 46) = 4.00, p<0.05, stressor x time, F(5, 230) = 42.62, p< 0.0001, and stressor x time x treatment, F (10, 230) = 2.86, p< 0.003. The stressor x order interaction demonstrated that CORT levels in the FS animals on day-1 were ~122% greater than the CORT levels when FS was on day-2 (Figure 3A and E), and no differences were observed between confinement groups. The stressor x time interaction revealed that regardless of treatment, CORT levels in the FS group were significantly elevated compared to the confinement group beginning at 15 min and lasting the duration (120 min) of the experiment, p< 0.0001 for all time points. The stressor x time x treatment interaction was attributable to reduced CORT levels in the WEIGH animals in the FS group at 60 min, regardless of order. The analysis of MA challenge revealed main effects of order, F(1, 46) = 8.24, p< 0.006, and time, F(5, 230) = 54.17, p< 0.0001. The order effect was attributable to increased CORT following MA in the
animals that received FS followed by confinement; CORT levels were ~137% greater than animals that received confinement followed by FS.

In terms of the magnitude of the CORT response for each stressor, peak levels of CORT in the WEIGH group were examined for each neonatal dosing interval, regardless of order. For P11-15, WEIGH animals in FS had the highest CORT levels between 30 and 90 min with peak levels at 60 min (~3813% of 0 min values). WEIGH animals after confinement had CORT levels that were highest between 15 and 60 min with peak levels at 15 min (~762% of 0 min values), and following MA challenge, CORT levels were increased between 30-120 min with peak levels at 60 min (~1754% of 0 min values). For P11-20 dosing, the WEIGH group had the highest levels of CORT following FS between 30 and 90 min with peak levels at 60 min (~3652% of 0 min values), whereas following confinement CORT levels were increased between 15 and 60 min with peak levels at 15 min (~775% of 0 min values). Lastly, the highest CORT levels after MA challenge in WEIGH animals were between 30 and 120 min with peak levels at 60 min (~715% of 0 min values).

Discussion

It has been well-documented that early life experiences, especially exposure to stress can have lasting effects on HPA axis and reactivity that lasts into adulthood (Aisa et al., 2007; Biagini et al., 1998; Felszeghy et al., 2000; Hodgson et al., 2001; Kalinichev et al., 2002; Kamphuis et al., 2002; Shanks et al., 1995; Wigger and Neumann, 1999). However, the present data demonstrate that following each stressor that was applied in adulthood, no differences were observed between neonatal treatment groups, i.e., all groups showed the normal increases in CORT levels following acute stressors exposure.
Therefore even a potent pharmacological agent such as MA with stressor-like effects on adrenal CORT release that surpass those caused by acute stressors during early development (Grace et al., 2008), does not produce a long-term alteration in HPA axis reactivity to acute exposure to FS or FC. We previously demonstrated neonatal MA exposure caused reduced adult CORT response following forced swim stress (Williams et al., 2003a), however that effect was not seen in the present experiment perhaps because of experimental designs differences between these studies. In the previous experiment rats were extensively tested behaviorally prior to testing the effects of FS (Williams et al., 2003a), e.g., in the Barnes maze. Similarly, neonatal MA exposure reduced the CORT response in adulthood following MWM testing (Skelton et al., 2007). These data suggest that exposure to extended behavioral testing interacts with the effects of adult stress to modify the effects the CORT response to an acute stressor. Perhaps this is a two-hit phenomenon, where prior testing (which involves some level of stress) is required to blunt HPA axis reactivity since animals in the present experiment lacked this additional experience. The effect may be due to exposure to a novel environment since animals in previous experiments were exposed to several testing environments prior to assessment of CORT levels. MA-induced neonatal increases in CORT are likely to be affected by drug dose, timing, and later experience leading to long-term reactivity patterns (Skelton et al., 2007; Williams et al., 2003a).

Aside from the early MA treatment, the data also show that a single day of stress exposure affects CORT levels following stress on subsequent days and that this differs between those given MA or SAL on P11-15 versus those treated on P11-20. Animals treated from P11-15 show exacerbated CORT levels as adults on the second day of stress
regardless of the stressor, suggesting sensitization. However, animals treated from P11-20 showed the opposite following FS but no differences following confinement. CORT levels after FS in the P11-20 groups were lower on the second day compared to the first, suggesting habituation. In addition, CORT levels following MA were altered by stressor order in the P11-20 groups but not in the P11-15 groups. The mechanism of these differences is unknown, but suggest that the window of neonatal exposure is critical as has been shown with other developmental insults (Stanwood and Levitt, 2004).

An examination of the WEIGH controls also revealed that the time range and peak of increases in CORT were different for each stressor, but similar across P11-15 and P11-20 exposure conditions. FS and MA challenge produced peak CORT levels 60 min after stress exposure, but confinement produced a peak 15 min afterward. The CORT peak after confinement was also smaller than the peaks after FS or MA challenge and the latter effects lasted longer. Unexpectedly the effect on CORT following the first stressor was greater than after MA challenge. Presumably this is because the MA challenge was given third (Grace et al., 2008) but why a cross-sensitization was not seen is unclear.

We have shown that MA exposure produces protracted increases in CORT during the SHRP (Schaefer et al, 2006;Schaefer et al, 2008;Williams et al., 2000;Williams et al., 2006). We examined the effects of early MA on adult stress reactivity in order to understand whether such changes contribute indirectly to the long-term learning and memory deficits observed in early MA-treated rats (Vorhees et al., 2008;Williams et al., 2002;Williams et al., 2003a). It is known that early HPA axis changes can have long-term effects on learning and memory (Croiset et al, 2000;Lupien and McEwen, 1997) and corticosteroids regulate cell proliferation in brain regions that are important for learning.
and memory (Gould et al, 1991; Lupien and McEwen, 1997). Since no alterations in adult stress reactivity were observed in the present experiment following developmental MA exposure, it may be that altered HPA-axis development is not the principal factor in how early MA induces its long-term learning and memory impairments. It may be that there is a complex interplay between early MA exposure and some combination of later experience (e.g., from behavioral testing) that is required to induce changes in stress reactivity or perhaps there is an effect but it is not revealed by responses to an acute stressor. Whatever the case, the present data demonstrate that developmental MA exposure does not alter corticosterone release in response to FS, FC or MA challenge suggesting that the HPA axis threshold or magnitude of response was not permanently altered by early drug exposure.
REFERENCES


Figure 1. Body weights during dosing and after weaning. Neonatal rats were weighed-only or treated with 10 mg/kg MA or saline 4 times daily from P11-15 (A-B) or P11-20 (C-D). (A) MA-treated rats had reduced weight gain from P13-15 compared to either control. Following the dosing period, MA animals rebounded and gained weight at rates comparable to controls (B). Similar observations were made for the P11-20 group except the decreased weight was observed beginning on P12 (C), but no differences were detected in adulthood (D). *** = p< 0.001, ** = p< 0.01, * = p< 0.05 compared to WEIGH or SAL.
Fig. 1

A. B. C. D.
Figure 2. Plasma CORT levels in adult rats following one of three stressors after P11-15 treatment with MA, SAL, or were only weighed (WEIGH). A-C: groups given forced swim (FS), followed by confinement and then MA on three successive days. D-F: groups given confinement, FS, and MA on three successive days. CORT levels increased following exposure to the stressor and began to show a return to baseline after 120 min. Arrows indicate when the stressor was administered after the 0 min blood sample was taken. The first day of stress produced elevated CORT levels compared to the second (A vs E and B vs D).
Fig. 2

**A.** FS

**B.** Confinement

**C.** MA

**D.** Confinement

**E.** FS

**F.** MA
Figure 3. Plasma CORT levels in adult rats following one of three stressors after P11-20 treatment with MA, SAL, or were weighed-only (WEIGH). A-C: group given FS, confinement, and MA on three successive days; D-F: groups given confinement, FS, and MA on three successive days. Each stressor increased CORT levels acutely with levels showing a return to baseline after 120 min. No effects of early MA treatment were observed. Arrows indicate when exposure to the stressor occurred.
CHAPTER 3

Neonatal methamphetamine-induced corticosterone release in rats is inhibited by adrenal autotransplantation without altering the effect of the drug on hippocampal serotonin

Curtis E. Grace¹, Tori L. Schaefer¹, Gary A. Gudelsky², Michael T. Williams¹, and Charles V. Vorhees¹

¹Division of Neurology, Dept. of Pediatrics, Cincinnati Children’s Research Foundation and University of Cincinnati College of Medicine, Cincinnati, Ohio
and
²College of Pharmacy, University of Cincinnati, Cincinnati, Ohio

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Abstract

Rat neonatal methamphetamine exposure results in corticosterone release and learning and memory impairments in later life; effects also observed after neonatal stress. Previous attempts to test the role of corticosterone release after methamphetamine using corticosterone inhibitors were unsuccessful and adrenalectomy caused reductions in hippocampal serotonin greater than those caused by methamphetamine alone. Here we tested whether adrenal autotransplantation could be used to attenuate methamphetamine-induced corticosterone release without also altering the effects of the drug on serotonin. Adrenal autotransplantation surgery occurred on postnatal day 9 followed by methamphetamine or saline treatment from postnatal day 11-20 (10 mg/kg/dose x 4/day). Plasma corticosterone and hippocampal serotonin and 5-hydroxyindoleacetic acid were determined 30 min following the first treatment on each day between postnatal days 11-20. Adrenal autotransplantation attenuated neonatal methamphetamine-induced corticosterone release by ~70% initially, ~55% midway through treatment, and ~25% by the end of treatment. Methamphetamine reduced serotonin and 5-hydroxyindoleacetic acid in the hippocampus to the same degree as in sham-surgery rats. The data show that neonatal adrenal autotransplantation is an effective method for partially reducing treatment-induce corticosterone release while providing sufficient corticosterone to sustain normal growth and development. The method should be applicable to other models of developmental stress/corticosterone release.

Key words: methamphetamine, corticosterone, serotonin, adrenal autotransplantation
1. **Introduction**

Methamphetamine (MA) is one of the most widespread drugs of abuse [20,21]. Recent data show that 24% of pregnant women entering drug treatment programs report MA as their primary drug of abuse [42]. Prospectively ascertained data in humans suggest that ~40% of pregnant MA users continue to use throughout pregnancy [7,37], and since MA readily crosses the placenta [4,15] there is passive exposure of the fetus. Infants born to women who used MA during pregnancy are reported to have reduced birth weight, length, and head circumference and increased rates of anemia and hemorrhage [7,12,13,26,32,38]. Children exposed to MA *in utero* also show deficits in visual motor integration, attention, psychomotor speed, spatial and verbal memory [5,6], novel object recognition memory on the Fagan Test of Infant Intelligence [41], as well as reduced arousal and quality of movement in newborns [38]. Magnetic resonance imaging (MRI) studies of *in utero* MA-exposed children reveal decreased volume of the hippocampus, putamen, and globus pallidus [6], and changes in white matter diffusivity using diffusion tensor imaging (DTI-MRI) with no changes in fractional anisotropy [10]. Magnetic resonance spectroscopy (MRS) data show higher total creatine, N-acetyl aspartate, and glutamate/glutamine in frontal white matter [5].

Algorithms that compare brain development across species reveal that P11 brain development in rats is comparable to humans at 26 weeks of gestation for cortex and 19 weeks of gestation for limbic structures [8,9]. Rats treated with MA neonatally exhibit later deficits in spatial learning and memory, egocentric learning, have augmented acoustic startle reactivity, and other effects [45,46,47,48,49,52,55,56,58] as well as decreased spine density in the dentate gyrus and nucleus accumbens and increases in
apical dendritic branching in the parietal cortex [53]. These animals also show reductions in 5-HT levels in the hippocampus and neostriatum during and immediately following drug exposure and at P90; however, dopamine (DA) levels are unaffected during dosing, but depletions emerge by P90 [11,35]. Neonatal MA treatment also causes increased release of ACTH and corticosterone [1,36,54,57] lasting for at least 24 h [34,35]. This effect of MA is more potent than corticosterone released in response to stressors such as forced swim or isolation at the same age [16]. The increase in neonatal MA-induced corticosterone release occurs during a period of normal hypothalamic-pituitary-adrenal quiescence referred to as the stress hyporesponsive period (SHRP) (approximately P4-14) [33] when despite dampened responsiveness, exposure to stressors can have long-lasting affects, an observation that may be important in understanding how neonatal MA leads to long-term effects. For example, prolonged stress that triggers increases in corticosterone during the SHRP sometimes lead to long-term alterations in hypothalamic-pituitary-adrenal (HPA) axis reactivity, increased startle reactivity, and spatial learning deficits in the Morris water maze [2,14,18,22,23,52]; effects similar to those caused by neonatal MA treatment as described above.

Previous experiments using bilateral adrenalectomy (ADX) effectively prevented P11 MA-induced corticosterone release but caused secondary effects on 5-HT in which hippocampal 5-HT levels in ADX-MA treated animals were reduced more than those in SHAM-MA treated animals (unpublished observations). This is a potential confound since hippocampal 5-HT changes may be involved in the MA-induced learning deficits [27]. In order to avoid this we sought an alternative to ADX.
Here we describe a method of attenuating MA-induced neonatal corticosterone release that may be useful for testing hypotheses concerning the role of adrenal responses to neonatal MA treatment or other drugs/stressors. We chose adrenal autotransplantation (ADXA) because experiments using corticosterone synthesis inhibitors (ketoconazole or metyrapone), while initially blocking MA-induced corticosterone release, exhibited later corticosterone rebound 24 h later (unpublished observations). Partial restoration of the adrenal cortex function following ADXA has the advantage of attenuating MA-induced corticosterone release while still allowing sufficient corticosterone for normal growth and develop and reducing the compensatory mechanisms (increased release of CRF and ACTH) known to accompany ADX [50,51].

2. Materials and Methods

2.1 Subjects and Conditions

Male (251-275 g) and nulliparous female (151-175 g) Sprague-Dawley CD IGS rats (Charles River Laboratories, Raleigh, NC) were acclimated to the vivarium for at least one week prior to breeding. The offspring were the subjects of this experiment and a total of 34 litters were used. Environmentally-enriching stimuli (stainless steel enclosures) [46] were placed in cages animals throughout the experiment. Food and water were provided ad libitum and the housing room was maintained on a 14:10 h light-dark cycle (lights on at 600 h). Litters were culled to 12 with 4 animals removed from each litter for tissue collection at each assessment age (i.e., each day between P11 to P21) with each litter contributing to 3 time points. The offspring removed at each sampling were randomly assigned as follows: sham surgery (SHAM)-saline (SAL), SHAM-MA, ADXA-SAL, or ADXA-MA. Therefore, half of the litter received SHAM surgery or
ADXA and half MA or SAL so that the 4 surgery/treatment groups were represented in each sampling per litter. Allocation of pups to time points for sacrifice was as follows: The 3 sampling periods were pseudo-randomized in clusters so that times of sacrifice within a litter would occur on successive days. For example, litters were sacrificed on P11, 12, and 13 or P12, 13 and 14, or P18, 19, and 20, etc. until all time points were filled. Cluster order was also pseudo-randomized for each litter. We have previously shown that MA causes equal corticosterone release in male and female pups at these ages [54], therefore offspring were sampled randomly. Litters with <12 pups at birth had up to two pups of equivalent age fostered from litters of the same age. Protocols were approved by the Institutional Animal Care and Use Committee, and the vivarium is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.2 Treatments

(+)-Methamphetamine HCl (expressed as the freebase and > 95% pure, National Institute on Drug Abuse, Bethesda, MD) was administered subcutaneously at a dose of 10 mg/kg in a volume of 3 ml/kg or an equal volume of SAL to controls 4 times each day at 2 h intervals except on the last day when they received one dose 30 min prior to tissue collection. The 10 mg/kg dose was based on previous experiments using allometric scaling [29]. A recent study estimated that among a group of MA users in treatment rearrested for drug use relapse had plasma or urine blood concentrations at the time of testing that pharmacokinetic modeling showed intake values of the median users to be 52 mg/dose and the heavy user 350-600 mg/dose or for a 60 kg human, ~1 mg/kg for the median users and 5.8-10.0 mg/kg for the heavy MA users [16,28]. Since T_{1/2} in humans
is 10-12 h but in rats is ~1-1.5 h, rats must be dosed more frequently to compensate for their more rapid rate of clearance. Thus, the dose of MA used in the present experiment (10 mg/kg x 4 doses/day) represents a model for a heavy MA user based on interspecies scaling. Body weights were recorded prior to each dose.

2.3 Surgical Procedures

Adrenal autotransplantation or sham surgery was performed on P9. Half of each litter had ADXA surgery and the other half sham surgery. The incision site was swabbed with 70% ethanol and betadine and animals anesthetized with isoflurane. For the ADXA animals, a bilateral approach was used to excise the adrenals, after which the adrenals were placed back into the peritoneum. The sham operation involved the primary incision, locating the adrenal glands, but leaving them intact. After surgery, the body cavity was sutured, the dermis stapled, and the site swabbed with warm saline and betadine to prevent infection.

2.4 Plasma and Tissue Collection

On the day of sacrifice, animals were taken to an adjacent room and decapitated; blood was collected in polyethylene tubes containing 2% EDTA (0.05 ml), and stored on ice until centrifuged. Plasma was isolated from whole blood by centrifugation at 1300 RCF for 25 min and the supernatant collected and stored at -80 °C until assayed. Brains were removed and the hippocampus dissected over ice with the aid of a brain block (Zivic-Miller, Pittsburgh, PA). The brain was sliced coronally at the optic chiasm and immediately caudal to the mammillary body and the hippocampi were dissected bilaterally from this section. Hippocampal tissues were frozen on dry ice and stored at -80 °C until assayed.
2.5 *Corticosterone Assay*

Plasma samples were thawed and assayed with Octeia Corticosterone ELISA kits (IDS, Fountain Hills, AZ). Each sample was diluted 1:5 and assayed according to the manufacturer’s protocol. The ELISAs were measured and quantified on a SpectraMax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA).

2.6 *High Performance Liquid Chromatography (HPLC)*

Hippocampi were weighed and homogenized using a hand-held glass homogenizer in a volume of 0.2 N perchloric acid 50 times that of the tissue. The homogenate was centrifuged for 5 min at 12,000 × g, the supernatant collected and stored on ice, and 20 μl aliquots were injected into a C18-column (MD-150, 3x150mm; ESA, Chelmsford, MA). The column was connected to a Coulochem electrochemical detector (25 A, Chelmsford, MA) and an integrator recorded the heights of each peak. The mobile phase consisted of 35 mM citric acid, 54 mM sodium acetate, 50 mg/L of disodium ethylenediamine tetraacetate, 70 mg/l of octanesulfonic acid sodium salt, 6% v/v methanol, and 6% v/v acetonitrile, with a final pH of 4.0. The flow rate was 0.4 ml/min and quantities of each sample were calculated from standard curves for 5-HT and 5-HIAA concentrations.

2.7 *Statistics*

Data were analyzed using mixed linear analyses of variance (ANOVA) (SAS Proc Mixed, SAS Institute 9.1, Cary, NC) unless otherwise specified. The covariance matrix for each data set was examined using best fit statistics and in most cases the best fit was to an autoregressive-1 (AR(1)) covariance structure. Mixed model ANOVAs used Kenward-Roger adjusted degrees of freedom; these do not match those obtained from
general linear model ANOVA and can be fractional. Measures taken repetitively on the same animal, such as day, were repeated measure factors. If significant interactions were observed, analyses at each level of the repeated measure factor were performed using slice effect ANOVAs. Since different animals were sacrificed each day, body weight data were analyzed using separate ANOVAs for each day. Mortality data were analyzed using Fisher tests of uncorrelated proportions.

3. Results

3.1 Body Weight

In the P11 group, there was an effect of surgery, F(1,363) = 10.5, p<0.001; the ADXA groups had reduced weight compared to the SHAM groups. This effect was also significant for the P12 through P19 groups (p-values from p< 0.001 to p < 0.03). No effect of surgery was observed on P20. There were also effects of drug and these began on P12, F(1,335) = 24.8, p<0.0001, and were significant on all days through P20, e.g., on P20 the treatment main effect was F(1,41) = 89.8, p<0.0001. Regardless of surgery, MA-treated groups had reduced body weight compared to the SAL-treated groups (Fig. 1). There were no surgery x treatment interactions.

3.2 Mortality

Offspring mortality is shown in Table 1. Only the ADXA-MA groups treated from P11-20 showed a significant increase in mortality.

3.3 Corticosterone

There were significant main effects of drug, F(1,294) = 184.8, p< 0.0001, surgery, F(1,294) = 62.1, p<0.0001, day, F(9,294) = 6.1, p< 0.0001, and the interactions of surgery x drug, F(1,294) = 31.5, p< 0.0001, and drug x day, F(9,294) = 2.3, p< 0.02.
There were no drug x surgery x day effects (Fig. 2A). The drug x day interaction revealed that beginning on P12, the MA-treated groups had increased levels of corticosterone compared to the SAL-treated animals regardless of surgical condition (Fig. 2B). Analysis of the surgery x drug interaction revealed that SHAM-MA animals had increased corticosterone compared to SHAM-SAL; ADXA-MA had increased corticosterone compared to ADXA-SAL; and ADXA-MA corticosterone levels were reduced compared to SHAM-MA (Fig. 3A). No differences were observed between the SHAM-SAL and ADXA-SAL groups. On P11, ADXA-MA animals had corticosterone levels that were ~30% of SHAM-MA levels; the difference between these groups gradually diminished over the course of treatment reaching ~75% of SHAM-MA levels by P20 (Fig 3B). The combined ADXA groups had reduced corticosterone levels compared to the combined SHAM groups (Fig. 3 inset); in addition, corticosterone levels increased in all groups over time.

3.4 **Hippocampal 5-HT and 5-HIAA**

5-HT levels by drug and surgical condition are shown in Fig. 4A. For 5-HT there were significant main effects of drug, \(F(1,276) = 641.5, p < 0.0001\), and day, \(F(9,276) = 7.4, p < 0.0001\), but not surgery. The combined MA-treated groups had reduced 5-HT levels compared to the combined SAL-treated groups (Fig. 4B). There was also a significant interaction of drug x day, \(F(9,276) = 5.7, p < 0.0001\), in which MA-treated groups had reduced 5-HT levels that varied in intensity as treatment progressed. There were no other significant effects or interactions for 5-HT.

For 5-HIAA (Fig. 5A), there were main effects of surgery, \(F(1,277) = 10.5, p < 0.001\), and drug, \(F(1,277) = 291.0, p < 0.0001\). The combined ADXA groups had
increased levels of 5-HIAA compared to the combined SHAM groups. The drug main effect was attributable to the fact that the combined MA-treated groups had reduced 5-HIAA levels compared to combined SAL-treated groups. There were interactions of surgery x drug, $F(1,277) = 4.6, p< 0.03$, and drug x day, $F(9,277) = 14.4, p<0.0001$. The drug x day interaction revealed that 5-HIAA was increased on P11 and decreased from P13-20 in the combined MA-treated groups compared to combined SAL-treated groups (Fig. 5B). The surgery x drug interaction is shown in Fig. 5C. The two MA-treated groups have nearly identical averages however the ADXA-SAL had increased 5-HIAA levels compared to SHAM-SAL. There was no surgery x drug x day interaction.

4. **Discussion**

MA significantly increases corticosterone from P12-20 (Fig. 2B). ADXA effectively attenuated this effect, reducing the increases in corticosterone to ~51% of SHAM-MA levels averaged across the 10 days of treatment (Fig. 3A). However, the degree of corticosterone release inhibition varied. Corticosterone levels in ADXA-MA animals were ~30% of SHAM-MA levels on P11 and rose to ~75% by P20. These data suggest that neonatal adrenal engraftment occurs more rapidly than in adults. In adult rats, adrenal autotransplantation has been reported to reduce plasma corticosterone levels beginning 1-5 weeks post-surgery, with progressively rising levels for 6-9 weeks [30,31,39,40,44]. In neonatal rats, the ADXA method was partially effective at blocking MA-induced corticosterone increases in the present experiment. Accordingly, this method appears useful for testing hypotheses concerning the role of corticosterone release in mediating or contributing to a number of the long-term effects of early MA exposure. Mortality from the ADXA procedure itself as reflected in the ADXA-SAL
groups was not significantly above SHAM-SAL. Moreover, the combination of ADXA
and MA-treatment did not increase mortality at any age from P11-19 but an increase was
seen on P20. This increase was unexpected and given that the P11-19 groups had only
one less day of treatment, it seems unlikely that the P11-20 increase is reliable.

The method uses adrenal autotransplantation to temporarily interrupt and
gradually reinstate adrenal function. The regenerative properties of the adrenal gland
have been investigated previously. For example in adult rats, adrenal cortex grafts,
regardless of size, exhibit regeneration [3,30,31,39,44]. Histological evidence shows
initial necrosis of the adrenal cortex and medulla, followed by proliferation and
differentiation of the cortex and formation of an adrenal capsule [3,30,31,39,44]
resembling normal morphology by 180 days [44]. There is also evidence that adrenal
cortex autografts become reinnervated [43], but the adrenal medulla degenerates entirely
[39].

MA exposure from P11-20 depletes hippocampal 5-HT levels regardless of
surgery, replicating and extending previous findings [34]. 5-HT levels were also
significantly reduced on P11, which was not observed previously [34]. It is possible that
such reductions, especially in a brain region important in spatial learning, may contribute
to the long-term cognitive changes in MA-treated offspring. Future experiments will be
needed to address this. Hippocampal 5-HIAA levels were also reduced in MA-treated
animals regardless of surgery on most days of treatment (P13-20). This is likely due to
the reduction in 5-HT presumably resulting from MA inhibition of 5-HT synthesis since
MA is an established tryptophan hydroxylase inhibitor [17,19,24,25]. It is less clear why
5-HIAA levels were initially increased in MA animals on P11, but may be related to the
fact that MA initially causes a large release of monoamine followed by depletion, a
pattern consistent with this finding (Fig. 5C). More importantly, both 5-HT and 5-HIAA
levels were similar between ADXA-MA and SHAM-MA groups demonstrating that
ADX eliminated the discrepancy previously observed between ADX-MA and SHAM-
MA groups in 5-HT levels (unpublished observations).

5. Conclusions

Adrenal autotransplantation provides an effective method of attenuating
corticosterone release in neonatal rats. This model could be utilized for examining the
effects of early exposure to stress or other drugs on brain development and function. Of
particular interest in the present context is determining whether the MA-induced
corticosterone release in neonates contributes to later learning and other behavioral
effects.

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Conflict of Interest Statement

The authors’ declare no conflict of interest concerning the research reported
herein.
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Table 1. Mortality data (number deceased/total) for surgery/treatment pairs for each exposure period

<table>
<thead>
<tr>
<th>Exposure Period (postnatal age in days)</th>
<th>SHAM-SAL</th>
<th>ADXA-SAL</th>
<th>SHAM-MA</th>
<th>ADXA-MA</th>
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**p< 0.01 compared to SHAM-SAL
Figure 1. Body weight of animals treated with MA or SAL following ADXA or SHAM operation on P9. ADXA significantly reduces weight gain from P11-19 and MA significantly reduces weight gain from P12-20. Data are represented by surgery condition and treatment group. N = 8-13 per surgery treatment pair, per day.
Fig. 1
Figure 2. Corticosterone levels in rats exposed to MA or SAL from P11-20 following ADXA or SHAM surgery. (A) Representation of each surgery/treatment group. Groups sizes SHAM-SAL = 7-13; SHAM-MA = 7-11; ADXA-SAL = 7-12; ADXA-MA = 7-10 per day. (B) Same data as in (A) except with the two MA-treated and two SAL-treated groups combined across surgical condition. SAL = 14-25; MA = 14-21 per day. ***p < 0.001, **p < 0.01, *p < 0.05 compared to SAL.
Figure 3. (A) Corticosterone levels averaged across treatment age. CORT levels are increased in SHAM-MA and ADXA-MA animals compared to their respective controls. ADXA significantly attenuated MA-induced CORT levels compared to SHAM-MA animals. Group sizes SHAM-SAL = 96; SHAM-MA = 92; ADXA-SAL = 94; ADXA-MA = 85. Inset: effect of treatment without regard to surgical condition. (B) CORT levels as a percent SHAM-MA values. ***p < 0.001.
Fig. 3

A. Corticosterone (ng/ml)

Day 11 12 13 14 15 16 17 18 19 20

B. CORT (% SHAM-MA)

Day 11 12 13 14 15 16 17 18 19 20

SHAM-MA

ADXA-MA

SHAM-SAL

SHAM-MA

ADXA-SAL

ADXA-MA
**Figure 4.** Hippocampal 5-HT levels: 5-HT was measured 30 min following the first dose of the last day of treatment. (A) Effects on 5-HT for each surgery/treatment group. MA reduced 5-HT on all treatment days. Group sizes SHAM-SAL = 8-12; SHAM-MA = 7-11; ADXA-SAL = 7-12; ADXA-MA = 7-9 per day. (B) Same data as in (A) except with the two MA-treated and two SAL-treated groups averaged together to show the main effect of drug treatment. SAL = 15-24; MA = 14-20 per day. ***p < 0.0001 compared to SAL.
Fig. 4

A. SHAM-SAL, SHAM-MA, ADXA-SAL, ADXA-MA

B. SAL, MA

Day 11 12 13 14 15 16 17 18 19 20

5-HT (ng/mg tissue)

0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40
Figure 5. Hippocampal 5-HIAA levels: (A) Effects on 5-HIAA for each
treatment/surgery group. Group sizes SHAM-SAL = 8-12; SHAM-MA = 7-11; ADXA-
SAL = 7-12; ADXA-MA = 7-9 per day. (B) Same data as in (A) except with the two
MA-treated and two SAL-treated groups averaged together to show the main effect of
drug treatment. SAL = 15-24; MA = 14-20 per day. (C) Surgery x drug interaction
revealed that 5-HIAA was reduced in SHAM-MA compared to SHAM-SAL and ADXA-
MA compared to ADXA-SAL animals. 5-HIAA levels were also increased in ADXA-
SAL compared to SHAM-SAL animals. SHAM-SAL = 91; SHAM-MA = 88; ADXA-
SAL = 89; ADXA-MA = 80. ***p < 0.001, *p < 0.05 vs. SAL.
CHAPTER 4

Effects of inhibiting neonatal methamphetamine-induced corticosterone release in rats by adrenal autotransplantation on later learning, memory, and plasma corticosterone levels

Curtis E. Grace, Tori L. Schaefer, Devon L. Graham, Matthew R. Skelton, Michael T. Williams, and Charles V. Vorhees

Division of Neurology, Dept. of Pediatrics, Cincinnati Children’s Research Foundation and University of Cincinnati College of Medicine, Cincinnati, Ohio

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Abstract

Rationale: Neonatal rat methamphetamine (MA) exposure has been shown to cause long-term behavioral impairments similar to some of those observed following neonatal stress. The mechanism by which MA induces impairments is unknown but may be related to early increases in corticosterone release. We previously developed a method to attenuate MA-induced corticosterone release using adrenal autotransplantation (ADXA) in neonatal rats. This exposure period corresponds to the second-half of human pregnancy. Objective: To determine whether inhibition of neonatal MA-induced increases in corticosterone attenuates the long-term behavioral deficits associated with early MA treatment. Results: ADXA successfully attenuated MA-induced plasma corticosterone increases by ~50% during treatment (P11-20) but did not attenuate the long-term behavioral effects of MA-treatment. MA-treated rats, regardless of surgery, showed increased errors and latencies in the Cincinnati water maze test of egocentric learning and increased latency, path length, and cumulative distance in three phases of Morris water maze spatial learning and reference memory. MA-treated offspring were hypoactive, had subtle reductions in anxiety in the elevated zero maze but not in the light-dark test. ADXA had no effect on MA-induced long-term 5-HT reductions in the neostriatum or entorhinal cortex or on 5-HIAA reductions in the hippocampus. Conclusions: Fifty percent attenuation of neonatal MA-induced elevations in corticosterone does not alter the long-term egocentric or allocentric learning deficits or other behavioral effects of neonatal MA exposure. Because the ADXA effect was partial, the data cannot rule out the possibility that a more complete block of MA-induced corticosterone release might not prevent later cognitive deficits.
Introduction

Methamphetamine (MA) abuse has become a worldwide problem (Johnston et al., 2008a; Johnston et al., 2008b; Srisurapanont et al., 2001). MA is abused primarily by adolescents and young adults (Johnston et al., 2008a; Johnston et al., 2008b). Some percentage of female users will become pregnant. Approximately 40% of pregnant MA users continue to use throughout pregnancy (Smith et al., 2003). One out of every 4 pregnant women seeking drug treatment in the U.S. reports MA as their primary drug of abuse (Terplan et al., 2009). Since MA readily crosses the placenta, (Burchfield et al., 1991; Garcia-Bournissen et al., 2007) fetal exposure in such cases is inevitable.

A limited number of human prenatal MA studies exist. Findings include reduced birth weight, length, and head circumference and higher rates of placental and intraventricular hemorrhage and anemia (Chomchai et al., 2004; Dixon, 1989; Dixon and Bejar, 1989; Little et al., 1988; Oro and Dixon, 1987; Smith et al., 2008). Additionally, increased agitation, vomiting, temperature instability, and rapid respiration have been noted (Chomchai et al., 2004). Magnetic resonance imaging studies report that exposed children have smaller volumes of the hippocampus, putamen, and globus pallidus, and reduced attention, visual motor integration, psychomotor speed, and spatial and verbal memory (Chang et al., 2004). Changes in white matter diffusivity have also been reported (Cloak et al., 2009) as have reduced object recognition scores on the Fagan Test of Infant Intelligence (Struthers and Hansen, 1992).

We have developed an animal model of MA exposure comparable to second-half of human pregnancy using the neonatal rat. This model is based on observations that cells in the dentate gyrus continue to proliferate at approximately postnatal day (P)19 in
the rat which is approximately equivalent to post-conception day 240 in humans (Bayer et al., 1993). We also incorporate interspecies scaling algorithms and one such model shows that P11 rat brain is approximately equivalent to human gestation at 26 weeks for cortical structures and at 19 weeks for limbic structures (Clancy et al., 2006; Clancy et al., 2007). MA treatment from P11-20 produces reductions in neostriatal and hippocampal serotonin (5-HT) that are apparent within 24 h following the first MA treatment without affecting dopamine (DA) (Schaefer et al., 2008). P11-15 MA treatment also reduces hippocampal and neostriatal 5-HT 24 h after the P15 dose (Schaefer et al., 2008) whereas P11-only exposure does not reduce hippocampal or neostriatal 5-HT (Schaefer et al., 2006). Yet neither P11-15 nor P11-20 causes any 5-HT reductions when examined on P30 (Schaefer et al., 2008). This is in contrast to the profound effects of adult MA exposure. For example, adult rats treated with MA on a single day show large reductions in neostriatal DA and 5-HT that last for months (O'Callaghan and Miller, 2002). In a separate experiment DA reductions were apparent at P90 following P11-20 MA treatment (Crawford et al., 2003). P11, 11-15, or 11-20 MA treatment also induces large increases in plasma corticosterone (CORT) that last at least 24 h after the last dose (Schaefer et al., 2006; Schaefer et al., 2008; Williams et al., 2000). Since early stress induces changes in brain development and behavior, (Chapillon et al., 2002; Sanchez et al., 2001) this effect of the drug may be important.

Behaviorally, long-term deficits in spatial learning and memory in the Morris water maze (MWM) are seen after P11-20 MA treatment (Vorhees et al., 1994; Vorhees et al., 2000; Williams et al., 2002; Williams et al., 2003c). An experiment comparing P11-15 to P16-20 MA treatment on MWM performance showed disproportionately more
effects after the P11-15 than after P16-20 exposure (Williams et al., 2003b). Follow-up experiments have revealed that 10-day exposures result in more severe MWM deficits than shorter intervals (Vorhees et al., 2008) whereas the effects on a different maze, the Cincinnati water maze (CWM), result in reliable effects after P11-15 or P11-20 MA treatment (Vorhees et al., 2008). By contrast, P1-10 MA treatment causes no deficits in MWM performance (Vorhees et al., 1994). Taken together, the data show that exposure intervals in which MA results in MWM and CWM deficits overlap. Intriguingly, the susceptible periods of MA-induced MWM and CWM deficits overlap with the stress hyporesponsive period (SHRP).

The SHRP is a period from approximately P4-14 when the adrenal gland is resistant to environmental stressor-induced CORT release (Sapolsky and Meaney, 1986). The SHRP is not a refractory period, but rather a phase of diminished responsiveness. For example, maternally separated neonatal rats show elevated CORT levels 30 min after exposure to novelty, saline injection, or adrenocorticotropic hormone (ACTH) exposure compared to controls but the levels are below those seen in adult stressed rats (Levine et al., 1991). Similarly, MA increases plasma ACTH and CORT in neonatal rats but to an even greater extent than do stressors such as forced swim or confinement (Grace et al., 2008; Williams et al., 2000; Williams et al., 2006).

In the following experiments, we tested whether the heightened CORT response caused by neonatal MA exposure during the SHRP contributes to the learning and behavioral deficits observed later in life. Adrenal autotransplantation (ADXA) has been previously shown in adults to inhibit the CORT response for a period of days with
eventual recovery. Therefore, ADXA was performed on P9 followed by MA exposure from P11-20. We chose ADXA because we have previously demonstrated that it reduces CORT in neonates by ~50% from P11-20 (unpublished observation). Using alternative (unpublished observation) methods such as complete adrenalectomy (ADX) or pharmacological inhibition were not as successful and/or caused secondary effects (unpublished observation). Pharmacological inhibition of CORT synthesis using ketoconazole or metyrapone initially blocks the CORT response to MA, but CORT rebounds 24 h later even with continuing inhibitor treatment (unpublished observation). Complete bilateral ADX overcomes the problems with synthesis inhibitors and completely blocks MA-induced CORT release, but also causes depletions in hippocampal 5-HT greater than those seen in SHAM-MA treated animals (unpublished observation). This potential confounder is avoided using the ADXA method (unpublished observation) and was therefore used herein.

Materials and Methods

Subjects and Conditions

Male (251-275 g) and nulliparous female (151-175 g) Sprague-Dawley CD IGS rats were obtained from Charles River Laboratories (Raleigh, NC) and acclimatized to the vivarium for at least one week prior to breeding. The offspring were the subjects of this experiment. Environmental stimuli (semicircular stainless steel enclosures) were placed in the cage upon receipt of the animals and remained in the home cage for the duration of the experiment (see (Vorhees et al., 2008) for details). Food and water were provided ad libitum and the housing room was maintained on a 14 h light: 10 h dark cycle (lights on at 600 h EST). Litters were culled with preferential retention of males up
to 10. Litters with < 8 males at birth had no more than 2 pups fostered from other litters born on the same day. For simplicity and because there are rarely MA-induced sex differences in later behavior, males were used (unpublished observation). Within each litter, 4 animals were assigned to the SHAM operation groups and 4-6 to the ADXA group. The extra 2 ADXA animals were assigned to the MA treatment condition in the event of mortality (unpublished observation). Hence, each litter contained 2 pups for each of 4 groups: 2 SHAM-SAL, 2 SHAM-MA, 2 ADXA-SAL, and 2 ADXA-MA. Extra ADXA-MA animals were removed from the experiment at weaning (P28) if not needed as replacements such that the final design contained 4 animals/treatment/litter. Animals were housed in pairs after being separated from the dam. Behavioral testing began on P60. Protocols were approved by the Institutional Animal Care and Use Committee. The vivarium is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Surgical Procedures

The ADXA procedure was derived from (Okamoto et al., 1992) in adult rats and adapted here for P9 rats. Our procedures differed in that specific placement of the adrenals under the cutaneous maximus was not performed and antibiotics were not given following surgery. Rats were transported in their home cage to a surgical suite and half of the litter was subjected to severing of the adrenal glands via a dorsal route and then the adrenals were placed into the peritoneum for spontaneous engraftment. The remaining animals in each litter were given the same operation except that the adrenals were untouched (SHAM). Animals in both surgery groups (ADXA or SHAM) were anesthetized with isoflurane and the dorsum was swabbed with 70% ethanol and betadine.
prior to surgery. After surgery the wound was sutured, the dermis stapled, and the
dorsum swabbed with warm saline. Following surgery, subjects were returned to the
home cage. Staples were removed on P21.

_Treatments_

(+)-Methamphetamine HCL (expressed as the freebase and > 95% pure, National
Institute on Drug Abuse, Bethesda, MD) was injected at 10 mg/kg in a volume of 3 ml/kg
s.c. or an equal volume of saline to controls. Animals were dosed four times per day at 2
h intervals from P11-20. Sites of injection were varied in order to prevent skin irritation.

_Body Weights_

Animals were weighed prior to each dose from P11-20, on P21, and weekly
thereafter.

_Behavioral Experiments_

Animals were assigned to one of 2 different pathways for behavioral testing. One
pup of each group/litter was assigned to receive the pathway A set of tests and one pup of
each group/litter was assigned to receive the pathway B set of tests.

_Pathway A tests_

_Light/Dark Exploration (P60)_

Animals were placed in 41 x 41 cm locomotor test chambers (Accuscan
Instruments, Columbus, OH) with a black acrylic enclosure placed inside. The enclosure
filled half of the chamber with an opening of 10 cm x 6.5 cm (w x h) facing the open side
of the chamber. Animals were placed in the middle of the open side. Rats were tested
for 10 min and data were collected every minute. The number of transitions from light to
dark (open to closed) and total time spent on each side were recorded. Chambers were cleaned with 70% ethanol between subjects.

**Novel Object Recognition (P61-64)**

On P61, subjects were placed in circular, polyethylene arenas (91 cm in diameter with 51 cm high walls) and allowed to habituate for 10 min/day to the test chambers for 3 days prior to testing; chambers were cleaned with 70% ethanol between trials. On the fourth day, testing was conducted in two phases. During familiarization, two identical objects were placed along a line bisecting the arena 41 cm apart and 25 cm from the sides. Rats were placed in the center and allowed to explore until 30 s of combined object exploration time was accumulated, after which the animals were removed. Exploration was scored using a video camera above the arena. If an animal did not accumulate 30 s of exploration time within 10 min, it was removed and not tested further. Object exploration was scored when the animal was oriented toward and within 1 cm of the object and when climbing on the object only if the rat was actively exploring it. Retention was tested 1 h after familiarization by being placed back in the test arena with a new object and an exact copy of the original object and allowed to accumulate 30 s of object investigation time.

**Straight Channel (P65)**

Each subject was placed in a 244 cm straight channel containing room temperature water. The animal was placed facing the wall at the opposite end of that containing an escape platform. Latency to reach the escape was recorded for 4 trials with a maximum of 2 min/trial.

**Cincinnati Water Maze (P66-83)**
On the day following straight channel, rats were tested in the CWM, a multiple-T water maze as previously described (Vorhees, 1987; Vorhees et al., 1991; Vorhees et al., 2009). The task was performed under infrared light and a CCD camera was mounted above the maze and attached to a monitor in an adjacent room. Rats were acclimated to the dark for 5 min before testing. Two trials were given per day for 18 consecutive days with a maximum of 5 min/trial and an intertrial interval (ITI) of 5 min. Latency to reach the escape and the number of errors were recorded. An error was committed when an animal left the direct path to the goal and entered a cul-de-sac. An error was scored when the head and shoulders crossed an imaginary boundary at the “stem” of the cul-de-sac and whenever it crossed into either arm of a “T”. Animals that did not complete the task within 5 min were given an error score equal to the maximum number of errors committed by the worst performing animal + 1 to correct for cases where an animal stopped searching before reaching the time limit.

Morris Water Maze Working Memory (P84-104)

A short-term working memory version of the MWM task was employed for 21 days (P84-104) adapted from (Morris et al., 1986). Animals were to locate a submerged platform (10 cm diameter) in a tank (210 cm diameter) of water (21 ± 1°C) based on the positions of distal cues in the room. The platform was submerged 1.5-2.0 cm below the water level. Each rat was given 2 trials/day with a time limit of 2 min/trial. The ITI was 15 s on the platform. If the platform was not located within 2 min, the animal was placed on the platform. Start and platform positions were pseudo-randomized each day but start positions were always a cardinal direction and platform positions always an ordinal direction. For example, on the first day of testing, animals were placed in the north
position and the platform was situated at the southeast position. Both positions were fixed for both trials on a given day. Animals were tracked and data collected using AnyMaze software (Stoelting, Wood Dale, IL).

**Pathway B tests**

*Elevated Zero Maze (P60)*

The elevated zero maze (EZM) test began on P60 and was run in the morning to control for ultradian and circadian rhythm effects. The maze is 105 cm in diameter with a 10 cm wide circular runway divided into four equal quadrants with 2 quadrants being open and two being closed with black side walls. Both open and both closed quadrants were positioned across from each other. The runway was mounted 72 cm above the floor. The open quadrants had 1 cm clear acrylic curbs to prevent slipping and the closed quadrants had 28 cm high black acrylic walls. A dimmed halogen floor lamp was used as the lighting source. Each animal began the task in the middle of a closed quadrant and was tested for 5 min. Latency to enter the open, time spent in the open, and number of head dips were recorded from video recordings made using an overhead camera and DVD recorder. DVDR malfunction resulted in several trials being lost. The apparatus was cleaned with 70% ethanol between subjects.

*Locomotor Activity (P61)*

On the day following EZM, animals were placed in 41 x 41 cm locomotor chambers (Accuscan Instruments, Columbus, OH) and movements were recorded in 5 min intervals for 1 h. The number of vertical and horizontal movements as well as the amount of time spent in the center vs. periphery were extracted and analyzed. Chambers were cleaned with 70% ethanol between animals.
Straight Channel (P62)

Morris Water Maze Hidden Platform (P63-80)

Subjects were placed in a 210 cm diameter tank of water (21 ± 1°C) and required to find a submerged platform. The platform was submerged 1.5-2.0 cm below the water. There were three phases of the MWM hidden platform test each consisting of 6 days of platform trials with 4 trials/day, and on the sixth day a single probe trial with no platform present was performed and the animal started from a novel position. The three phases were: Acquisition (P63-68) with a 10 cm diameter platform in the southwest quadrant; reversal (P69-74) with a 7 cm platform in the northeast quadrant; and shift (P75-80) with a 5 cm platform in the northwest quadrant. Probe trials lasted for 30 s; learning trials were up to a maximum of 2 min with an ITI of 15 s on the platform. Curtains were opened during hidden platform trials so that distal room cues were revealed. Data were collected using video tracking software (AnyMaze, Stoelting, Wood Dale, IL).

Morris Water Maze Cued (P81-82)

MWM cued testing began the day following hidden platform trials and was conducted for 2 days. Subjects were placed in the tank and were tested for latency to reach a 10 cm diameter platform submerged 1.5-2.0 cm below the water with a yellow plastic ball mounted on a brass rod to mark its location. Curtains were drawn closed around the tank to minimize distal cues. On each day, subjects were given 4 trials with the locations of the platform and starting positions randomized ((2 min trial limit with ~30 s ITI) (15 s on the platform + 15-20 s to reposition the platform)).

Tissue Collection
On P109 after the end of behavioral testing, brains were removed and assayed for DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and norepinephrine (NE) in neostriatum, 5-HT, 5-HIAA, and NE in the hippocampus, and DA, DOPAC, 5-HT, 5-HIAA, and NE in entorhinal cortex for animals in pathway A only. Animals were taken into an adjacent suite, decapitated, and brains removed and dissected over ice with the aid of a brain block (Zivic-Miller, Pittsburgh, PA). The brain was sliced coronally at the optic chiasm and caudal to the mammillary body and the hippocampus dissected bilaterally. The neostriatum was dissected from the 2 mm section rostral to the optic chiasm and entorhinal cortex 2 mm caudal from the mammillary body. Tissues were stored at -80°C until assayed.

Corticosterone levels following an acute stressor were obtained in order to estimate the adrenal response. Litters assigned to pathway B were placed in 46 cm tall x 15 cm in diameter clear acrylic cylinders filled to a depth of 35 cm (22 ± 1 °C) for 10 min. Rats were removed, decapitated and blood collected in polyethylene tubes containing 2% EDTA (0.05 ml). Plasma was isolated from whole blood by centrifugation at 1300 RCF for 25 min and the supernatant collected and stored at -80°C.

Monoamine Assays

HPLC reagents were obtained from Sigma Chemical (Sigma-Aldrich Co., St. Louis, MO) unless otherwise specified. Frozen tissues were weighed, thawed, and sonicated in 0.1 N perchloric acid. Samples were centrifuged for 14 min at 13,000 x g at 4°C. The supernatant for each sample was transferred to a new vial for injection onto a Supelco Supelcosil™ LC-18 column (15 cm x 4.6 mm, 3 μm) (Sigma-Aldrich Co.). The
HPLC system consisted of a Waters 717plus autosampler (Waters Corp., Milford, MA) and ESA 5840 binary pump and Coulochem III electrochemical detector. The potential settings were E1 at -150 mV and E2 at +250 mV, with a guard cell potential set at +350 mV. MD-TM mobile phase (ESA Inc., Chelmsford, MA) was used and consisted of 75 mM sodium dihydrogen phosphate (monohydrate), 1.7 mM 1-octanesulfonic acid sodium salt, 100 μl/l triethylamine, 25 μM EDTA, and 10% acetonitrile (pH = 3.0). The pump flow rate was 0.7 ml/min, and the samples were run at 28°C. Standards for DA, DOPAC, HVA, NE, 5-HT, and 5-HIAA were prepared in 0.1 N perchloric acid. All neurotransmitters were run on a single chromatogram.

Corticosterone Assay

Plasma samples were thawed and assayed with Octeia Corticosterone ELISA kits (IDS, Fountain Hills, AZ). Each sample was diluted 1:5 and assayed according to the manufacturer’s protocol. The ELISAs were measured and quantified on a SpectraMax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Statistics

Data were analyzed using mixed linear analyses of variance (ANOVAs) (SAS Proc Mixed, SAS Institute, SAS v.9.1, Cary, NC). The covariance matrix for each data set was checked using best fit statistics and in each case the best fit was to the autoregressive-1 covariance structure. Proc Mixed uses Kenward-Roger adjusted degrees of freedom that do not match those obtained from general linear model ANOVAs and can be fractional. Measures taken repetitively on the same animal, such as day, were repeated measure factors. Significant interactions were analyzed using slice effect
ANOVA at each level of the repeated measure factor. Data are presented as least square (LS) mean ± LS SEM.

Results

Body Weight

During treatment, there were effects of surgery, $F(1,138) = 28.4$, $p< 0.0001$, drug, $F(1,138) = 274.6$, $p< 0.0001$, day, $F(9,1333) = 502.9$, $p< 0.0001$, surgery x day, $F(9,1333) = 3.9$, $p<0.0001$, and drug x day, $F(9,1333) = 96.9$, $p< 0.0001$. MA-treated and ADXA animals had decreased body weight gain but no drug x surgery interaction was observed. The surgery x day interaction revealed that ADXA animals had reduced weight gain compared to SHAM animals (P11-20). The drug x day interaction showed that MA animals had reduced weight gain beginning on P12 that lasted throughout the dosing period. For post weaning weights, there were main effects of surgery, $F(1,138) = 4.2$, $p< 0.04$, drug, $F(1,138) = 21.2$, $p< 0.0001$, and week, $F(9,1112) = 2853.3$, $p< 0.0001$, but no interactions. The ADXA animals weighed less than the SHAM animals and MA-treated animals weighed less than SAL-treated animals. Representative body weights are shown in Table 1.

Pathway A tests

Light/Dark

There were no significant effects of drug, surgery, or interactions for time in light, time in dark, latency to dark entry or number of dark entries. For example, time (s) in light ls means ± SEM were SHAM-SAL = 204.1 ± 18.7, SHAM-MA = 204.0 ± 19.1, ADXA-SAL = 177.0 ± 19.1 and ADXA-MA = 197.1 ± 18.7.

Novel Object Recognition
During familiarization there was no preference for one side over the other. During retention, there were no significant effects of surgery, drug, or interactions among these.

**Straight Channel Swimming**

There was a main effect of trial, $F(3,359) = 96.8$, $p < 0.0001$, but no effect of drug, surgery, or interaction of these on latency to swim the straight channel.

**Cincinnati Water Maze**

For errors (Figure 1A-C), there were main effects of drug, $F(1,78.4) = 10.8$, $p < 0.002$, and day, $F(17,1125) = 50.2$, $p < 0.0001$. Both MA-treated groups committed more errors than SAL-treated groups regardless of surgery condition (Figure 1C). For latency (Figure 1D-F) to the platform, there were main effects of drug, $F(1,79.1) = 10.7$, $p < 0.002$, and day, $F(17, 1122) = 48.05$, $p < 0.0001$; a similar pattern as errors was seen. MA-treated groups had longer latencies to the platform (Figure 1F) than SAL-treated groups regardless of surgical condition. No main effect of surgery or interactions between surgery and drug were obtained for errors or latency.

**Morris Water Maze Working Memory**

For the trial-dependent, matching-to-sample (working memory) version of the MWM, there was a surgery x drug x day interaction, $F(20,1345) = 1.9$, $p < 0.01$ for latency. The ADXA-MA group showed an increased percent change in latency between trial-1 and trial-2 only on day 6 and the SHAM-MA group showed decreased percent change compared to SHAM-SAL on days 15 and 17. Analysis of swim speed showed a main effect of day, $F(20,1352) = 2.7$, $p < 0.0001$, and interactions of surgery x day, $F(20,1352) = 1.6$, $p < 0.05$, and drug x day, $F(20,1352) = 2.0$, $p < 0.01$. The ADXA
groups had decreased percent change in speed on days 10, 16, and 18 and increased percent change on day 20 compared to the SHAM groups. In addition, the MA groups had decreased percent change on day 5 and 10 and increased percent change on day 20. All these effects were sporadic and small and showed no consistent pattern of effects (not shown).

**Pathway B tests**

*Elevated Zero Maze*

Latency to first open quadrant entry, time in open, and number of head dips were analyzed. For latency, there was a main effect of drug, $F(1,49) = 4.5, p< 0.04$; the MA-treated groups had decreased latency to first open entry compared to the SAL-treated groups (Figure 2). There were no main effects or interactions on time in open or head dips.

*Locomotor Activity*

Total, central, and peripheral distances, horizontal activity, and vertical activity were analyzed. For total distance, there were no surgery x treatment x day interactions, (Figure 3 A and B), but there were main effects of drug, $F(1,72.8) = 5.1, p< 0.03$, time, $F(11,674) = 92.5, p< 0.0001$, and interactions of surgery x time, $F(11,674) = 1.9, p< 0.04$, and drug x time, $F(11,674) = 2.3, p< 0.01$. MA-treated groups were hypoactive compared to SAL-treated groups (Figure 3C inset). Further analysis of the drug x time interaction revealed that the MA-treated groups had reduced locomotion from 10-20 min and at 55 min compared to SAL-treated groups (Figure 3C). Similar results were obtained for horizontal activity. For peripheral distance, there were main effects of drug, $F(1,72.8) = 10.4, p< 0.002$, time, $F(11,679) = 65.2, p< 0.0001$, and interactions of surgery
x time, $F(11, 679) = 2.1, p < 0.02$, and drug x time, $F(11, 679) = 4.2, p < 0.0001$. MA-treated groups moved less in the periphery than did SAL-treated groups (not shown). The drug x time interaction showed decreased peripheral distance in MA-treated groups from 10-20 and 50-55 min compared to SAL-treated groups (not shown). For center distance, an overall effect of time was observed, $F(11, 658) = 83.5, p < 0.001$, but no treatment-related effects were seen. For vertical activity, there were main effects of drug, $F(1, 55.9) = 11.24, p < 0.001$, and time, $F(11, 679) = 3.5, p < 0.0001$. MA-treated groups had increased vertical movements compared to SAL-treated groups (not shown).

*Morris Water Maze Hidden Platform*

Spatial learning in the MWM was analyzed for path length, latency to the target platform, cumulative distance, and mean speed. For acquisition path length (Figure 4A-C), there were main effects of drug, $F(1, 54.7) = 8.6, p < 0.005$, and day, $F(4, 201) = 97.2, p < 0.0001$, and the interaction of drug x day, $F(4, 201) = 3.5, p < 0.01$. Further analysis of the interaction showed that on days 1 and 2, MA-treated groups had increased path length compared to SAL-treated groups, regardless of surgery (Figure 4C). Similar findings were observed for latency and cumulative distance (not shown). For swim speed, there were main effects of drug, $F(1, 55.7) = 4.5, p < 0.04$, and day, $F(4, 212) = 4.4, p < 0.002$. MA-treated groups swam slower compared to SAL-treated groups (not shown). There were no other significant effects.

For reversal path length (Figure 4D-F), there were main effects of drug, $F(1, 74) = 8.6, p < 0.005$, and day, $F(4, 220) = 66.9, p < 0.0001$, and a drug x day interaction, $F(4, 220) = 2.8, p < 0.03$. The interaction was attributable to increased path length in MA-treated
groups on days 1 and 2 (Figure 4F). Analyses of latency and cumulative distance were similar. For swim speed, the only main effect was day, \( F(4,210) = 13.0, p< 0.0001 \).

For shift path length (Figure 4G-I), there were main effects of drug, \( F(1,53.3) = 9.5, p< 0.003 \) (Figure 4I), and day, \( F(4,204) = 66.7, p<0.0001 \), but no interactions. The MA-treated animals had longer path lengths than the SAL-treated animals, regardless of surgery. Similar findings were observed for latency and cumulative distance. For speed the only main effect was for day, \( F(4,214) = 12.3, p< 0.0001 \).

For probe trials, platform site crossovers, initial heading error, and average distance from the target were analyzed. For acquisition probe initial heading error, there was a main effect of drug, \( F(1,53) = 10.7, p< 0.002 \), which was attributable to increased heading error in MA-treated groups compared to SAL-treated groups (Figure 5A). There were no effects for crossovers or average distance.

For reversal probe crossovers, there was a main effect of surgery, \( F(1,53) = 6.0, p< 0.02 \). For initial heading error, there was a main effect of drug, \( F(1,53) = 4.6, p< 0.04 \). SHAM groups had fewer crossovers than the ADXA groups (not shown) and MA-treated groups had increased initial heading errors than SAL-treated groups (Figure 5B).

For shift probe initial heading error, there was a main effect of drug, \( F(1,53) = 18.6, p< 0.0001 \), in which MA-treated groups had greater heading errors than SAL-treated groups (Figure 5C). There were no effects for average distance from the target on acquisition, reversal, or shift probe trials.

*Morris Water Maze Cued*

There was a latency main effect of day, \( F(1,71) = 29.6, p< 0.0001 \), but no effects of drug, surgery, or interactions.
Corticosterone

Corticosterone levels were measured following 10 min forced swim in adulthood to determine the extent of which ADXA attenuated CORT release. There was an overall effect of surgery, $F(1,45) = 17.7, p< 0.001$, in which the ADXA groups had plasma CORT levels that were significantly lower than SHAM groups (Figure 6B). CORT levels were 50.2% of their respective controls (Figure 6A). There were no effects of drug or interactions.

Monoamines

Monoamines were analyzed in the hippocampus, neostriatum, and entorhinal cortex (Figure 7). In the hippocampus (Figure 7A-B), there was a main effect of drug for 5-HIAA, $F(1,40) = 5.1, p< 0.03$, and a surgery x drug interaction for NE, $F(1,40) = 4.0, p< 0.05$. 5-HIAA was decreased in the MA-treated groups compared to SAL-treated groups (Figure 7B) and NE was increased in the ADXA-MA group (Figure 7A) compared to the ADXA-SAL group. For neostriatal 5-HT (Figure 7C-D), there was a main effect of drug, $F(1,39) = 2.6, p< 0.05$, in which the MA-treated groups had reduced 5-HT compared to the SAL-treated groups (Figure 7D). No neonatal surgery x drug interactions were observed. In the entorhinal cortex (Figure 7E-F), there were main effects of drug for both 5-HT, $F(1,39) = 5.7, p< 0.02$, and 5-HIAA levels, $F(1,38) = 4.6, p< 0.04$. 5-HT and 5-HIAA levels were reduced in the MA-treated groups compared to the SAL-treated groups (Figure 7F).

Discussion

In the present experiment, it was determined that attenuation of the CORT response to MA was produced using ADXA, but did not reduce the long-term cognitive
deficits seen in MWM or CWM learning/memory. These data suggest that large increases in plasma CORT during neonatal MA exposure may not be responsible for the later cognitive deficits. Despite a substantial literature demonstrating long-term alterations in HPA-axis reactivity (Aisa et al., 2007; Biagini et al., 1998; Felszeghy et al., 2000; Hodgson et al., 2001; Kalinichev et al., 2002; Shanks et al., 1995; Wigger and Neumann, 1999), deficits in spatial learning, deficits in novel object recognition (Aisa et al., 2007), and alterations in anxiety (Huot et al., 2001; Kalinichev et al., 2002; Knuth and Etgen, 2007; Romeo et al., 2003; Wigger and Neumann, 1999) following neonatal exposure to physical or psychological stressors, the present data did not demonstrate a beneficial effect on later behavior by reducing the magnitude of the MA-induced release of CORT during an early and sensitive period of brain development. Another possible explanation for the lack of attenuation of the cognitive deficits by ADXA is that there may be a threshold at which CORT elicits its adverse effects. It is conceivable that the CORT reductions by ADXA are not sufficient to be below such a threshold and therefore do not provide neuroprotection. In this regard, we have shown that MA exposure during the neonatal period produces a larger increase in CORT than physical or psychological stressors (Grace et al., 2008). Therefore the ~50% reduction in CORT increase caused by MA may still be within the range of that produced by stressors that have been shown to elicit long-term changes in behavior. Experiments to determine if a further reduction in CORT release following MA may alleviate the learning and memory changes will require different approaches or modification of the ADXA method to make it more complete. Alternatively, the type of stressor involved may be important at least for causing alterations in long-term HPA-axis reactivity. Here we examined CORT levels following
an acute forced swim stressor in adulthood and observed no effects of MA treatment, suggesting normal HPA axis reactivity to stress. We have observed a similar absence of long-term MA effects on CORT release in adult offspring following forced swim, confinement to a small space, or acute MA treatment (unpublished observation). These data, combined with those in the present experiment, suggest that a pharmacological neonatal stressor such as MA may be different from physical or psychological stressors at least in terms of altering the developing HPA axis. Alternatively, ADXA may have induced secondary/compensatory changes that offset any beneficial effect of reducing MA-related CORT increases. For example, it is known that ADX causes compensatory increases in the release of ACTH and CRF (Dallman et al., 1987) which may have deleterious consequences. With the removal of CORT, changes in both the mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) are likely to occur. It is conceivable that alterations in MR and GR by ADXA could affect later cognition and future experiments will be designed to test this hypothesis. Another important issue in future studies testing the possible involvement of early drug-induced increases in plasma CORT levels will be to assess these potential compensatory systems to determine if they change in ways that effectively cancel the beneficial effect of preventing large increases in CORT release.

The positive control group (SHAM-MA) replicated our previous findings and since the ADXA treatment (ADXA-MA) failed to prevent this effect, this group too effectively replicated our previous findings. We have previously demonstrated that deficits in hippocampal-dependent spatial learning and reference memory in the MWM emerge in adulthood following neonatal exposure to MA (Vorhees et al., 2008; Vorhees
et al., 2009; Williams et al., 2002; Williams et al., 2003a; Williams et al., 2003b), an effect seen here in both the SHAM-MA and ADXA-MA treated groups. In the acquisition phase, MA-treated animals had reductions in mean speed compared to controls, which we do not typically see. However, these changes cannot account for the navigational impairment for several reasons. First, MA animals had increased path lengths to the target platform regardless of the time it took them to get there. Second, the straight channel showed no differences in maximal swimming speed across groups. Third, there were no differences observed between groups in the cued (visible platform) version of the MWM showing that speed differences were not instrumental in determining the outcome on hidden platform trials. Taken together, these data represent consistent evidence of spatial learning deficits in MA-treated groups not confounded by unrelated performance differences.

We have previously demonstrated that neonates exposed to MA have deficits in egocentric learning in the CWM (Vorhees et al., 2009) and this effect was also seen here. Egocentric learning is the ability to navigate to a destination using self-motion cues and routes rather than distant landmarks and is thought to involve the hippocampus (Etienne et al., 1996; Etienne and Jeffery, 2004), grid and border cells in the entorhinal cortex, and head direction cells in the presubiculum. MA-treated animals showed long-term alterations in monoamines in both the hippocampus (5-HIAA) and entorhinal cortex (5-HT and 5-HIAA), which suggest that the serotonergic system may correlate to changes in egocentric learning; however, further analysis showed that such correlations were not significant (not shown). However, the changes in the serotonergic system seen after
testing are smaller than those induced during the period of drug administration, limiting the likelihood of finding high correlations between these variables.

We found that rats developmentally exposed to MA have reduced locomotor activity prior to maze testing. Several experiments using early stress exposure find no differences in spontaneous locomotion in adulthood (Aisa et al., 2007; Knuth and Etgen, 2007), demonstrating that effects may depend upon the stressor involved. However, as discussed, these effects do not appear to affect MWM or CWM learning or memory since no changes in swimming speed or straight channel swimming times were found and therefore represent a separate effect of the drug independent of its effects on learning and memory.

MA-treated animals also showed a subtle reduction in the elevated zero maze test of anxiety; but no effect in light/dark exploration. Taken with our previous observations (Skelton et al., 2007), long-term effects on anxiety by neonatal MA exposure appear to be minor. A number of studies have demonstrated that neonatal maternal separation increases anxiety in the elevated plus maze in adulthood (Huot et al., 2001; Kalinichev et al., 2002; Knuth and Etgen, 2007; Romeo et al., 2003; Wigger and Neumann, 1999) again suggesting that neonatal MA exposure works through different mechanisms than maternal separation. A possible explanation for this is that the increases in CORT may not be associated with a particular event as with maternal separation. For example, rats that exhibit CORT increases following maternal separation also incur a psychological event of prolonged maternal deprivation of care in the form of nursing, grooming, and physical contact. During drug treatment, the animals were only briefly separated from
the dam and remained with their littermates and hence did not experience the same type of deprivation as in maternal separation experiments.

Several studies have found that following adrenal autotransplantation in adult rats, plasma CORT levels are detectable 1 to 5 weeks post-transplantation (Nabishah et al., 1998; Okamoto et al., 1992; Srougi et al., 1978; Srougi and Gittes, 1978; Vendeira et al., 1992) and are similar to controls by 6 to 9 weeks (Nabishah et al., 1998; Srougi and Gittes, 1978). The regenerative properties of the adrenal gland have been examined and grafts containing adrenal cortical tissue, regardless of size, have the ability to regenerate (Belloni et al., 1982; Nabishah et al., 1998; Okamoto et al., 1992; Srougi et al., 1978; Vendeira et al., 1992). Histological evidence shows that following ADXA, early necrosis of cells in the adrenal occurs, followed by cortical cell proliferation, differentiation into zones, formation of an adrenal capsule, and return of function (Belloni et al., 1982; Nabishah et al., 1998; Okamoto et al., 1992; Srougi et al., 1978; Vendeira et al., 1992) which resembles normal morphology by 180 days (Vendeira et al., 1992). The timeline by which these processes occur differ slightly between experiments. Necrosis occurs and regeneration begins within one week (Srougi et al., 1978; Srougi and Gittes, 1978; Vendeira et al., 1992). Organization into zones occurs by 15 days and encapsulation by 30 days (Vendeira et al., 1992). There is also evidence that adrenal autografts are reinnervated (Ulrich-Lai and Engeland, 2000). The adrenal medulla does not regenerate (Srougi and Gittes, 1978), possibly due to its increased sensitivity to anoxia; CORT levels remain low in animals in which only adrenal medulla was engrafted (Nabishah et al., 1998). Despite the evidence concerning ADXA in adult rats, nothing is known about this approach in neonatal rats. We recently demonstrated that in P9 rats, ADXA causes
rapid re-engraftment and restoration of partial function in as little as 2-3 days
(unpublished observation).

The mechanisms by which MA causes long-term learning and memory deficits are unknown. A candidate other than increased CORT release is reduction of brain 5-HT. Hippocampal serotonin has been shown to be reduced throughout the period of neonatal MA exposure (Schaefer et al., 2006; Schaefer et al., 2008). The current experiment demonstrates that depletions in 5-HT in the neostriatum and entorhinal cortex and 5-HIAA in the hippocampus and entorhinal cortex persist into adulthood. Even with alterations in the serotonergic system in brain regions that are known to be involved in allocentric and egocentric learning, how serotonin mediates such effects remains to be determined. However, a test of the role of 5-HT in contributing to the long-term effects of MA is a logical step in future experiments in order to understand how MA induces cognitive deficits.

Acknowledgment

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(+)-fenfluramine or (+/-)methylphenidate administration in the neonatal rat.
J.Neurochem. 98, 1369-1378.


Table 1. Offspring body weight (g) as a function of age

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<th></th>
<th>P11</th>
<th>P15</th>
<th>P20</th>
<th>P56</th>
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<td>26.7 ± 0.8</td>
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#p< 0.01 combined ADXA groups vs. combined SHAM groups; ##p< 0.001 vs. SHAM; *p< 0.01 combined MA groups vs. combined SAL groups.
Figure 1. Cincinnati water maze: The MA-treated groups show significant deficits in CWM performance regardless of surgery. A-C: Errors; D-F: latency to reach the escape platform. Learning curves for the SHAM and ADXA groups are shown separately in the left panels for each dependent measure and the main effect of drug treatment with surgical conditions combined are shown in the right-hand panel; **p < 0.01 or ***p < 0.001 combined MA-treated groups vs. combined SAL-treated groups.
Fig 1.
Figure 2. Elevated zero-maze: The combined MA-treated groups had reduced time to first entry to an open quadrant compared to the combined SAL-treated groups *p < 0.05.
Fig 2.

Latency to open (s)

SAL

MA

*
Figure 3. Locomotor activity: Ls mean ± SEM total distance moved (cm) during a 60 min test session. A: Activity for SHAM and B: ADXA per treatment group. MA groups had reduced total distance (C inset) compared to SAL groups, and specifically MA groups had reduced total distance from 10-20 min and at 55 min (C). *p< 0.05, **p< 0.01, ***p< 0.001, all compared to SAL controls.
Fig 3.
Figure 4. Morris water maze learning trials: Ls mean ± SEM path length (cm) averaged by day (4 trials/day) in neonatally MA vs. SAL-treated rats having ADXA or SHAM surgery. Left and middle panels show each treatment group as a function of surgical condition. Right panels show the main effect of drug treatment with the two MA-treated and two SAL-treated groups merged. Top row: acquisition; middle row: reversal; bottom row: shift. Panels C and F are shown by day to illustrate a significant drug x day interaction. Panel I is shown averaged across days to illustrate a drug main effect with no drug x day interaction. *p < 0.05, **p < 0.01, ***p < 0.001 combined MA-treated groups vs. combined SAL-treated groups.
Fig 4.

**Acquisition**

- **SHAM**
  - Path length (m) vs Day
  - Graph showing a downhill trend with data points and error bars.

- **ADX A**
  - Path length (m) vs Day
  - Graph showing a downhill trend with data points and error bars.

**Reversal**

- Path length (m) vs Day
  - Graph showing a downhill trend with data points and error bars.

**Shift**

- Path length (m) vs Day
  - Graph showing a downhill trend with data points and error bars.

- Additional bar graph showing a comparison with error bars and symbols indicating statistical significance.

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**Legend:**
- SAL
- MA

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**Figure 5.** Morris water maze probe trials: Ls mean ± SEM average heading error to the former location of the platform 24 h after the last platform trial for each phase of MWM testing. There were no significant main effects of surgical condition and no interactions between drug and surgical condition. There were significant main effects of drug. For clarity the main effect of drug (with surgical conditions averaged together) is shown. *p < 0.05, **p < 0.01, ***p < 0.001 combined MA-treated groups vs. combined SAL-treated groups.
Fig 5.
Figure 6. Corticosterone levels following forced swim: Ls mean ± SEM (ng/ml) plasma CORT levels in rats following completion of behavioral testing (P106). Rats were euthanized immediately following 10 min of forced swim. ADXA reduced CORT levels by 50.2% in both ADXA-SAL and ADXA-MA animals compared to their respective SHAM controls (A). The main effect of decreased CORT in ADXA animals is shown, regardless of surgery (B). ***p< 0.001 compared to SHAM controls.
Fig 6.

A. SHAM-SAL
SHAM-MA
ADXA-SAL
ADXA-MA

50.2 %

B. SHAM
ADXA

50.2 %
**Figure 7.** Monoamine concentrations: DA and 5-HT in neonatally MA vs. SAL treated rats as a function of ADXA vs. SHAM surgery following the completion of behavioral testing (P106). A, C, E: monoamines in each region analyzed by group. B, D, F: main effect of drug with surgical conditions combined. *p < 0.05 combined MA-treated groups vs. combined SAL-treated groups.
CHAPTER 5

Discussion

Long-term HPA axis Reactivity

From the data in chapter 2, it was determined that neonatal MA exposure does not alter the adult response to stress, suggesting that altered development of the HPA axis does not account for the long-term cognitive deficits (Vorhees et al. 2008; Williams et al. 2002; Williams et al. 2003) observed. Early MA exposure did not evoke an altered stress response in the treated offspring that in turn interfered with learning, despite evidence from the early stress field that early exposure to stressors can have lasting effects on HPA axis reactivity that last to adulthood (Aisa et al. 2007; Biagini et al. 1998; Felszeghy et al. 2000; Hodgson et al. 2001; Kalinichev et al. 2002; Kamphuis et al. 2002; Shanks et al. 1995; Wigger and Neumann 1999). This finding stands in contrast to two studies in which we found a blunted CORT response in early MA-treated animals in later life (Skelton et al. 2007; Williams et al. 2003). However, both of these studies involved tests of learning given before testing the effects on CORT release after an acute stress exposure. It may be that the cumulative effect of MA exposure and learning, or learning in an aversive environment may be essential before a blunted CORT response in MA-treated offspring is seen (Skelton et al. 2007; Williams et al. 2003). Animals were also assessed for stress reactivity at different times; rats were tested at a later age in the current study, and we cannot rule out that alterations in stress reactivity could have occurred earlier. Nonetheless, the experiments in chapter 2 suggest that adult reactivity to stress is intact following neonatal MA exposure when examined in animals without
prior water maze experience or that changes in CORT are not required for cognitive deficits.

Neonatal CORT Attenuation and Behavior

Because of the CORT elevation following neonatal MA exposure (Grace et al. 2008) we determined whether attenuating the CORT increases would prevent later learning deficits. In chapter 3, we developed a model of attenuated CORT release by using ADXA. In this model, we utilized the ability of the adrenal cortex to regenerate. Thus the adrenal was excised and placed in the peritoneum to interrupt adrenal function with a gradual reinstatement of function as has previously been reported in adult rats, but which we did for the first time in P9 rats (Belloni et al. 1982; Nabishah et al. 1998; Okamoto et al. 1992; Srougi et al. 1978; Vendeira et al. 1992). To our knowledge, this was the first study to demonstrate recovery of adrenal function (as evidenced by CORT release) following ADXA in neonates. Although in adults CORT release is eventually restored to near normal levels, we showed in chapter 4 that neonatal ADXA continues to suppress CORT following forced swim stress well into adulthood by ~ 50%. We chose this method because previous studies in which CORT synthesis inhibitors were given prior to MA treatment caused a large rebound in CORT release after 24 h even though CORT was initially attenuated (Schaefer et al. 2010) and even if administration of the synthesis inhibitor was continued. We chose not to use adrenalectomy to prevent neonatal CORT release for several reasons. First, although ADX effectively removes the CORT response in neonates, we observed a confounding effect on hippocampal 5-HT at 24 h; levels were reduced in ADX-MA animals compared to SHAM-MA on P12, i.e.,
both groups showed reduced hippocampal 5-HT but the ADX-MA 5-HT reductions were larger than those in the SHAM-MA group 24 h after treatment. This suggests an effect due to absence of glucocorticoids or mineralocorticoids in the system (unpublished observation), or other adrenal hormones such as norepinephrine (NE) and epinephrine. This potential confound with 5-HT raised the possibility of an interaction between 5-HT and the HPA axis (Hanley and van de Kar 2003). By contrast, we showed that ADXA removed this confounding effect (chapter 3) and MA-treated animals, regardless of surgery, had reduced 5-HT levels throughout the treatment period, an effect that extends our previous findings (Schaefer et al. 2006). Second, ADX can slow growth. Third, ADX requires additional manipulation once weaned from the dam; due to lack of aldosterone animals must be supplied with constant access to salt (generally in the drinking water) to survive. Fourth, neonatal ADX causes reduced branching of dendrites in the hippocampus which could potentially alter hippocampal-dependent learning such as in the MWM (Hashimoto et al. 1989). Using the ADXA model and expanding the days on which we assessed its effects on hippocampal 5-HT, we demonstrated that MA exposure reduced 5-HT levels throughout the entire treatment period. This finding has since led us to focus efforts on determining whether MA-induced 5-HT reductions are associated with later cognitive deficits since reductions in 5-HT can cause altered hippocampal morphology and spatial learning deficits (Lauder and Krebs 1978; Lauder 1990; Mazer et al. 1997; Whitaker-Azmitia et al. 1996; Yan et al. 1997).

Behavioral and cognitive deficits in MA-treated animals were still apparent following neonatal attenuation of CORT release using the ADXA model (chapter 4). Several studies have demonstrated that increased stress in neonates or exogenous
glucocorticoid administration can adversely affect later behavior and learning (Aisa et al. 2007; Benesova and Pavlik 1989; Huot et al. 2001; Kalinichev et al. 2002; Kamphuis et al. 2003; Knuth and Etgen 2007; Nyakas and Endroczi 1972; Oitzl et al. 2000; Pavlovskaya-Teglia et al. 1995; Romeo et al. 2003; Wigger and Neumann 1999). We therefore determined whether attenuation of MA-induced CORT increases would attenuate long-term cognitive and behavioral impairments of the drug. As shown in chapter 4, no beneficial effects of ADXA were observed for CWM or MWM. Further, locomotor activity was reduced in MA animals, consistent with our previous findings and ADXA did not alter this effect. A latency reduction to first open quadrant entry was also observed in the EZM, but no effect on time spent in the open quadrants (the main index of anxiety in this test), nor any change in the light/dark task, suggesting long-term anxiety effects of MA are minimal if any. No effects of the drug were observed in novel object recognition. This is the first time we have tested P11-20 MA treated rats for this type of learning and we had no specific predictions as to whether or not it would be affected. Although novel object recognition is a largely hippocampally-dependent behavior as is the MWM and the MWM was affected, there are data showing some non-overlap between these two forms of learning, hence the absence of a novel object learning effect in the MA-treated offspring does not suggest a contradiction. The hippocampus has subregion specificity and it may be that neonatal MA affects the hippocampus in some subregions more than others, an idea we have not yet explored.

We found long-term (P109-110) reductions in hippocampal 5-HT and 5-HIAA and 5-HIAA reductions in the entorhinal cortex. This may be important because these regions are associated with allocentric and egocentric learning, but unfortunately
correlations between these reductions and learning deficits were not significant nor did any of them approach statistical significance. We showed that increased CORT levels during the neonatal period occurred following MA exposure, but ADXA did not reduce CORT to levels of controls. Larger reductions in MA-induced CORT increases may be necessary to further test the role of CORT in these effects.

**Alternative Explanations**

Findings from these experiments suggest that MA-induced long-term cognitive deficits are not the direct result of CORT increases, although CORT may indirectly affect other neurotransmitter systems. Other mechanisms are suggested in the adult literature. For instance, MA causes profound alterations in monoaminergic systems, blocking uptake of monoamines into vesicles via VMAT-2, reversing flow of DA via DAT, and blockade of MAO-induced breakdown of monoamines, resulting in increased monoamine release in the synaptic cleft (Cadet *et al.* 2007). MA adult treatment causes DA and 5-HT reductions in the striatum and 5-HT reductions in the hippocampus (Bisagno *et al.* 2002; Broening *et al.* 1997; Cappon *et al.* 2000; Fukumura *et al.* 1998; Herring *et al.* 2008; Ott *et al.* 1971; Wallace *et al.* 2001). Reductions in dopaminergic markers such as DAT (Schroder *et al.* 2003), TH activity (Kokoshka *et al.* 2000), and VMAT-2 (Eyerman and Yamamoto 2007), as well as hippocampal reductions in SERT (Schroder *et al.* 2003) are observed in adults. Neonatal reductions in DA are not observed following MA exposure (Schaefer *et al.* 2008), suggesting that dopaminergic neurons are not fully developed at this time. However, DA depletions in the striatum do emerge in adulthood (Crawford 2003), which may affect adult behavior, especially motor
function. MA-induced DA release in adult brain is associated with formation free oxygen radicals as well (Stokes et al. 1999). Antioxidant treatments and free radical scavengers reduce such neurotoxicity in adult brain (Fukami et al. 2004; Kondo et al. 1994). Excitotoxicity by MA-induced glutamate release (Abekawa et al. 1994; Mark et al. 2004) and increased nNOS (Deng and Cadet 1999) are also associated with adult MA-induced neurotoxicity (Itzhak et al. 2000). No studies have examined the effects on glutamate in the neonate. MA-induced cell death (increased TUNEL staining) in the striatum and hippocampus of mice (Deng et al. 2001) as well as up-regulation of pro-apoptotic (BAD, BAX, and BID) and down regulations of anti-apoptotic (Bcl-2 and Bcl-XL) genes are observed in adults (Itzhak and Achat-Mendes 2004) but no such data exist for neonates.

MA also alters inflammatory cytokines that are factors contributing to neuronal injury. For instance, interleukin-6 (IL-6) is involved in dopaminergic neurotoxicity since IL-6 KO mice show protection (Cadet et al. 2003). Tumor necrosis factor-α (TNF-α) plays a role as well since neurotoxicity is exacerbated in TNF-α KO mice administered MA; and exogenous TNF-α administration reduces MA toxicity (Nakajima et al. 2004). IL-6 mRNA is up-regulated in the hippocampus, striatum, and frontal cortex and TNF-α mRNA is up-regulated in hippocampus and frontal cortex following MA administration in mice (Goncalves et al. 2008). Further, systemic injection of interferon-gamma (IFN-γ) protects against MA-induced reductions in DAT (Hozumi et al. 2008). MA-induced inflammatory responses are important in adult neurotoxicity, but have not been examined in neonates. If cytokines were involved in neonatal MA neurotoxicity, perhaps CORT levels are increased to suppress neuronal inflammation. Further investigation of the effects of cytokines following MA exposure in neonates is warranted.
MA-induced increases in CORT may indirectly affect neurotransmitter systems as well. Stress can influence dopaminergic markers. For example, stress affects the synthesis and activity of TH in the ventral tegmental area (VTA). CORT administration increases D₁ receptor mRNA in the nucleus accumbens and striatum, and increases D₁ receptor binding in the VTA and substantia nigra (SN) (Czyrak et al. 2003). Further, co-localization of D₁ and GR are found in PFC, striatum, VTA, and SN (Czyrak and Chocyk 2001). There is also evidence that DA regulates CORT release as well since active D₁ receptors are located in the paraventricular nucleus of the hypothalamus and stimulation of these receptors may lead to increased corticotrophin releasing hormone (CRH) release (Czyrak et al. 2003). Neonatal MA administration also increases NE in SN, caudate-putamen and nucleus accumbens (Gomes-Da-Silva et al. 2004). The HPA axis is involved in regulation and stimulation of norepinephrine and epinephrine. Noradrenergic neurons from the locus ceruleus project to the hypothalamus and hippocampus and are involved in regulation of the HPA axis (Cooper et al. 1996). Hypophysectomized rats show reduced epinephrine levels. In addition, ACTH or DEX administration restores both phenylethanolamine-N-methyltransferase (PNMT), the enzyme which converts NE to epinephrine from the medulla, and epinephrine (Wurtman 2002). 5-HT regulates and is regulated by CORT. ADX reduces tryptophan hydroxylase activity and increases 5-HT₁ receptors in the hippocampus and in turn, 5HT₁a receptor stimulation can increase HPA axis activity (Chaouloff 1995).

Future Studies
The experiments examining the use of ADXA for attenuating MA-induced CORT release during the neonatal period determined that long-term MWM and CWM learning deficits were not attenuated. This could be due to insufficient suppression of the CORT response. We found that ADXA-MA animals had a significant reduction in plasma CORT levels compared to SHAM-MA animals (Chapter 3) and CORT levels were reduced by approximately 50% for the entire treatment period (Chapter 3). However, this 50% reduction was not uniform across the exposure interval. In the first few days of treatment, CORT levels in the ADXA-MA groups were 70% lower than in the SHAM-MA group and the degree of reduction declined as treatment continued. By P15 it was about a 50% reduction and by the end of treatment on P20 it was only a 30% reduction. Essentially, the adrenals were recovering over time. Since we do not know the most critical days within the P11-20 window for the effects of MA on cognitive development it is not possible to determine what effect the increasing CORT response may have had, but one could hypothesize that there may be a threshold that, if exceeded, causes long-term learning and memory deficits. Therefore, further suppression of the CORT response may be more effective, perhaps even dramatically so. Returning smaller amounts of adrenal tissue is known to result in lower levels of CORT release following ADXA in adult rats (Okamoto et al. 1992). It therefore might be worthwhile determining whether similar findings would be observed in neonates by analyzing MA-induced CORT levels following removal of the adrenals and replacing both, one, or half of one and conduct an adrenal tissue dose-response experiment. If smaller adrenal grafts produced greater inhibition of the MA-induced CORT release, it might be worth trying to repeat the learning and memory experiment.
One common substrate where the neurotoxic effects of both increased glucocorticoids and MA converge is glutamate. In adult brain, increased glucocorticoid exposure increases expression of NMDA receptors in the hippocampus (Bartanusz et al. 1995) and results in increased glutamate release that is reduced by ADX (Lowy et al. 1993). Further, phenytoin, which blocks release of glutamate, also prevents glucocorticoid-induced neuronal atrophy in adult brain (McEwen and Magarinos 1997). MA-induced glutamate release (Abekawa et al. 1994; Mark et al. 2004) has been shown to increase nNOS in adult brain (Deng and Cadet 1999), which is associated with neurotoxicity and can be prevented by nNOS inhibitors (Itzhak et al. 2000). It is possible that the MA effects on glutamate are due to the ability of the drug to increase CORT. Therefore, it could be hypothesized that MA-induced cognitive deficits are due to glutamatergic excitotoxicity. However, little information is available about MA effects on glutamate levels in neonatal rats. Since the adult and neonatal MA response are different, it would be interesting to determine if glutamate release was altered by MA by microdialysis, however this procedure may be difficult in neonates. It would also be important to determine if excitotoxicity and increased nNOS occurs in neonates. If alterations were observed, it would be interesting to determine if NMDA receptor antagonists could protect MA-treated animals from the long-term cognitive deficits. NMDA receptor antagonists are promiscuous and can affect other neurotransmitter systems. Therefore selectivity of the NMDA antagonist used is critical. One possible antagonist is memantine, which also affects nicotinic receptors, but has shown promise in adult mice at preventing MA neurotoxicity (Chipana et al. 2008) and is sufficiently safe that it is a marketed drug. If alterations in glutamate occur, future studies could include
memantine treatment prior to MA exposure (Chipana et al. 2008). If pretreatment were effective, one could then test to determine if memantine attenuates long-term deficits in MWM and CWM learning. On the other hand, irreversible NMDA antagonists, such as MK-801, are not suitable as they are developmental neurotoxins (Ikonomidou et al. 1999).

In chapters 3 and 4, as well as in previous studies (Schaefer et al. 2006; Schaefer et al. 2008), neonatal MA exposure causes depletions in 5-HT during development, especially in the hippocampus. 5-HT depletions during development can be detrimental since 5-HT is a neurotrophic factor that promotes serotonergic neuron survival and supports neuron development during early stages of life. 5-HT depletions can cause reduced spine density, retarded migration, delays in proliferation of neurons, and deficits in spatial learning in the radial arm maze (Lauder and Krebs 1978; Lauder 1990; Mazer et al. 1997; Whitaker-Azmitia et al. 1996; Yan et al. 1997). In this regard, we have observed altered hippocampal morphology following neonatal MA exposure (Williams et al. 2004). The hippocampus, striatum, and entorhinal cortex are important in learning and memory and are innervated by 5-HT fibers (Sodhi and Sanders-Bush 2004). It is possible that MA-induced reductions in 5-HT could affect later cognition. One might hypothesize that prevention of neonatal 5-HT depletions by administration of a selective 5-HT reuptake inhibitor (SSRI) might reduce long-term allocentric and egocentric learning deficits. Subcutaneous injection of citalopram prior to MA exposure might represent one approach. Citalopram is selective, has a high affinity for SERT, and has the least effect on cytochrome P450 activity, which can affect drug metabolism (Hemeryck and Belpaire 2002). We have previously demonstrated that citalopram reduces 5-HT depletions in
neonatal MDMA-treated rats (unpublished observation). Therefore, if MA-induced 5-HT reductions were blocked by citalopram, we could determine if doing so prevents or attenuates long-term cognitive deficits.

In conclusion, the known developmental effects of MA may occur through a number of mechanisms and involve multiple neurotransmitter systems. It may be that MA-induced increases in CORT release play a role in MA toxicity and later cognitive decline, but may not be the primary mechanism based on the current findings. Whether based on morphology, alterations in neurotransmitter systems, or CORT, it may be that the combination of multiple MA-induced insults is required to induce cognitive deficits from this drug. Perhaps studies preventing multiple effects of MA exposure, such as attenuating CORT elevations and 5-HT reductions, will be necessary to establish the principal causes of the long-term effects.


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