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Detrimental effects of self-administered methamphetamine during pregnancy on offspring development in the rat

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Abstract

Background—Methamphetamine (METH) abuse by pregnant women is a commonly observed phenomenon. While the harmful effects of METH are well described for adults, there is only limited knowledge of the effects of METH use during pregnancy on the developing child. In the present study, we investigated how intravenous (*iv*) METH self-administration throughout pregnancy affected rat dams and their offspring through weaning, compared to controls.

Methods—Female rats ($n=16$) were trained to self-administer METH *iv*; every drug infusion by a dam also resulted in a saline injection to a yoked control ($n=16$). When stable levels of self-administration were reached, all females were mated. Daily, 2-hr self-administration sessions continued until litters were born. General health and weight was assessed daily in dams and pups. In addition, pups were evaluated for achievement of age-appropriate developmental milestones (i.e., righting reflex, negative geotaxis, pinna detachment, fur appearance, incisor eruption and eye opening).

Results—Dams self-administered 2–3 mg/kg/day METH throughout gestation without consequence to dam health or weight gain during pregnancy. All females produced viable litters, and litter size and composition did not differ between saline and METH dams. Similarly, maternal pup-directed behavior was not affected by prior METH self-administration. However, despite a lack of weight difference in pups, METH-exposed pups were significantly delayed in reaching all assessed developmental milestones compared to controls.

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Contributors:

D. Rüedi-Bettschen: Design and oversight of the research project, data collection and analysis, manuscript writing
D.M. Platt: contributed to data analysis and manuscript writing. Both authors approved of the final manuscript before submission.

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Conclusion—These results indicate that *in utero* exposure to moderate METH doses can profoundly and adversely affect offspring development, suggesting that even recreational METH use during pregnancy has potential for harm.

Keywords

methamphetamine; pregnancy; self-administration; neurodevelopment; animal model

1. Introduction

Illicit drug use has been increasing steadily in individuals of reproductive age (SAMHSA, 2013). This is especially true for the psychostimulant methamphetamine (METH; Zapata et al., 2008), despite efforts to curb its production and trafficking (Shukla et al., 2012). Notably, approximately 50% of the METH-abusing population are women (Anderson and Choonara, 2007). Because METH is often associated with the experience of increased pleasure during sex, risky sexual behavior, and impaired decision-making, unwanted pregnancies can be considered an expected outcome of METH use (Arria et al., 2009; Kalechstein et al., 2003; Rawson et al., 2002; Zapata et al., 2008). Even when a pregnancy is confirmed, most METH-abusing women delay seeking proper prenatal care until late in their second trimester, often due to fear of involvement from Child Protective Services (Wu et al., 2013). Although METH use tends to decline as pregnancy progresses, especially in heavy users, a significant proportion of women continue to abuse the drug throughout the nine months of gestation (Terplan et al., 2009). Because reported data only represent women that seek treatment, the actual number of pregnant, METH-abusing women is undoubtedly higher (DellaGrotta et al., 2010).

Although the adverse effects of METH are well-described in adults, there is only limited knowledge regarding the impact of METH in pregnant women. Moreover, because METH has been shown to cross the placenta (Won et al., 2001), the developing fetus presumably also is at risk. In clinical studies where METH use during pregnancy was determined via meconium screen or retrospective self-reports, neonates have been reported to be small for their gestational age, presenting with low birth weight and smaller head circumference (Zabaneh et al., 2012). They also exhibited poor movement, low arousal and increased lethargy (LaGasse et al., 2012; Minnes et al., 2012; Smith et al., 2003). Beyond the neonatal stage, METH-exposed children also were more likely to have increased emotional reactivity, anxiety, and diagnoses of attention deficit hyperactivity disorder (Diaz et al., 2014; Kirlic et al., 2013; LaGasse et al., 2012; Minnes et al., 2012; Shah et al., 2012; Smith et al., 2003, 2012; van Dyk et al., 2014; Wouldes et al., 2014). While the METH exposure *in utero* likely plays an important role in affected development, the postnatal environment may be as important to the developing child since a negative postnatal environment characterized by as abuse or neglect has been linked to the development of psychiatric disorders in adulthood (Heim et al., 2004). This is relevant given that METH-taking mothers are more likely to have ongoing legal difficulties, experience domestic violence and experience parenthood as stressful and, thus, are more prone to inferior caregiving (Derauf et al., 2007; Good et al., 2010; Liles et al., 2012).

In recent years, a number of preclinical studies have been conducted aiming to understand the relationship between *in utero* METH exposure and detrimental effects on the offspring (McDonnell-Dowling and Kelly, 2015a). Exposure to repeated, high doses of METH (e.g., 10 – 40mg/kg) during pregnancy has been shown to increase dam/pup mortality, significantly reduce birth weight and produce teratogenic effects (Acuff-Smith et al., 1996; White et al., 2014). Notably, repeated administration of more moderate METH doses (e.g., 2.5 – 5mg/kg), before and throughout pregnancy, also has been shown to have profound effects on dams and offspring. For instance, daily, non-contingent administration of METH at moderate doses (5mg/kg) throughout pregnancy has been shown to reduce pup-directed maternal care (Slamberova et al., 2005) and delay pup development (McDonnell-Dowling et al., 2014, 2015b). As adults, METH-exposed offspring demonstrated increased impulsivity (Lloyd et al., 2013), increased nociception (Hrubá et al., 2010a), increased disposition to drug abuse (Slamberova et al., 2012) and disruptions in learning and memory (Hrubá et al., 2010b). These findings illustrate that METH, across a range of doses, can have a multitude of detrimental effects on offspring that can persist across the lifespan.

While studies using non-contingent drug administration are important for pinpointing the effects of prenatal METH exposure and also allow for precise control over dosage, it is well-documented that passive drug administration elicits markedly different effects on neuroadaptation, toxicity, stress hormone levels and behavior when compared to active self-administration (e.g., Dworkin et al., 1995; Fouquet et al., 2001; Galici et al., 2000; Jacobs et al., 2003). In addition, the fixed dose scenario of prior studies does not recapitulate the human reality of variable intakes across time and individuals. Thus, the aim of the present study was to use a model of METH self-administration during pregnancy to study the effects of *in utero* METH exposure on offspring development. While it is true that under a self-administration procedure, intake is likely to vary across animals, we believe that this potential disadvantage is outweighed by the increased translational relevance. In addition, it also was aimed to elucidate possible changes of METH self-administration across gestation and its effects on dam health, behavior and pregnancy outcome.

2. Material and methods

2.1. Subjects and surgery

All experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and the University of Mississippi Medical Center Animal Care and Use Committee. Adult male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), approx. 65 days of age, weighing 300–350g or 200–250g respectively at arrival were housed in single-sex pairs in standard shoebox cages in a temperature and humidity controlled vivarium with a reverse 12-hr light/dark cycle (lights on at 20:00 hrs). Food and water were available *ad libitum*. Behavioral testing occurred during the dark phase.

Female rats were implanted with indwelling jugular vein catheters (3 Fr medical grade polyurethane, Instech Laboratories, Plymouth Meeting, PA) under isoflurane anesthesia. The distal end of the catheter was routed subcutaneously (*sc*) and connected to a vascular access button (Instech Laboratories) implanted in the mid-scapular region, permitting access to the

catheter without exposing it to air. Catheters were flushed daily with heparinized saline (30 IU/ml) to maintain patency. Animals were allowed to recover from surgery for 7 days before starting METH self-administration.

2.2. Apparatus and drugs

Experimental sessions were conducted in operant chambers (Med Associates, Georgia, VT) equipped with an infusion pump, two levers, a white stimulus light above each lever and a house light on the opposite wall. An infusion line was connected to a rotating swivel and routed through a spring tether that was attached to the skin button. Recording of responses and activation of lights and infusion pump were computer-controlled (MED-PC IV, Med Associates, Georgia, VT).

Methamphetamine hydrochloride was purchased from Sigma Aldrich (St. Louis, MO) and prepared in sterile saline for self-administration.

2.3. METH self-administration and mating

Half of the females (one female/cage, randomly chosen) were trained to self-administer METH (0.08 mg/kg/infusion) under a fixed-ratio (FR) schedule, for daily 2-hr sessions. Females were weighed prior to each session and the infusion duration was adjusted via the computer program (maximum of ± 0.5 s) to ensure accuracy of the infusion dose. Completion of the FR on the active lever led to a 4-s drug infusion concomitant with the illumination of the stimulus light above the lever, followed by a 6-s time-out during which the light remained illuminated. Lever presses on the inactive lever were recorded, but had no programmed consequences. Initially, rats were trained under a FR1 schedule until they reached the criteria of a minimum of 3 days with at least 2 days with over 15 infusions/session earned; after which, the FR requirement was increased to 2. The remainder of the females served as yoked saline controls (i.e., they received a saline infusion and light stimuli whenever a METH infusion was delivered).

Once stable levels of self-administration were reached (at least 20 infusions/session with no upward or downward trends in number of infusions over a 3-day period, with the number of infusions within $\pm 20\%$ of the last 3-day mean), a male was introduced to the home cage of a METH-saline yoked female pair and allowed to mate. Males remained with the females until the females were close to delivering their litters, as estimated by time elapsed since first signs of pregnancy (e.g., obvious weight gain, rounded abdomen). METH self-administration was continued daily throughout the mating and gestational periods and ceased the day a litter was born.

2.4. Pup-development and maternal pup-directed behavior

On the day litters were born (designated postnatal day 0, PND0), all pups were sexed, weighed and inspected for anomalies. Litters were culled to four males and four females where possible, keeping the most viable pups. Starting on PND1, pups and dams were weighed daily and their overall health was assessed (i.e., fur appearance of dam, lack of chromodacryorrhea in dam, milk visible in pup's stomach, pups age-appropriately active and warm to the touch). Daily, each pup was assessed for achievement of species-specific

developmental milestones as described in Table 1 (adapted from McDonnell-Dowling et al., 2014). Maternal pup-directed and self-directed behavior (see Table 2) was assessed from PND1–14 for one hour, twice daily - immediately before the transition to the light portion of the cycle (7:00–8:00 hrs) and at the usual start time of the self-administration session (12:00–13:00 hrs) – using the scan sampling method described in Rüedi-Bettschen et al., (2004). Specifically, behavior of each dam was recorded by observers blinded to the treatment assignment every 5 min, resulting in 12 sampling points per dam per hour. Pups were weaned on PND21 and housed in isosexual groups.

2.5. Statistical analysis

Each data set was assessed individually for normality of distribution and homogeneity of variance (D'Agostino-Pearson omnibus test, $p > 0.05$) as well as sphericity (Brown-Forsythe test, $p > 0.05$), and no data sets were rejected for further analysis. Days to birth of a litter after introduction of a male, litter size, composition and weight at birth were analyzed using two-tailed unpaired t-tests, using all dams and pups/litters born of dams with functional catheters (METH $n=14$, saline $n=15$). All further analyses were performed only on dams/litters where 8 pups were present until weaning (METH $n=10$, saline $n=9$). Analysis of pup-related measures was performed using averages of each sex per litter. METH self-administration (measured as mg/kg taken) was analyzed using one-way repeated measures (RM) analysis of variance (ANOVA). Two-way ANOVA was used to analyze pup body weight at birth (sex $\times$ prenatal exposure) and two-way RM ANOVA was used to analyze maternal pup-directed behavior (treatment $\times$ days) and maternal weight gain (treatment $\times$ days). In addition, totals of maternal behavior across the 14-day period were analyzed using separate two-tailed unpaired t-tests to detect differences between prenatal exposure groups, as well as diurnal effects within groups. Three-way RM ANOVA was used to analyze pup weight gain, pup surface righting reflex and negative geotaxis (sex $\times$ prenatal exposure $\times$ days). ANOVAs, were followed by either Dunnett's or Bonferroni multiple comparison tests, as appropriate. In instances where equal sample sizes were required for analysis (i.e., saline-exposed offspring for 3-way ANOVA), an average of the available data was used to create the missing sample. Since sexing was based on visual differences in anogenital distance, measurement of the anogenital distance between saline and METH-exposed pups were analyzed for each sex separately as litter means by t-tests for each day assessed. For pinna detachment, incisor eruption, fur appearance and eye opening, data were treated as present or absent and analyzed using Chi-square tests. GraphPad Prism6 and StatView 5 software (San Diego, CA) was used for statistical analysis. The level of significance was set at $p < 0.05$.

3. Results

3.1. METH self-administration

As depicted in Figure 1, upper panel, female rats maintained substantial levels of METH self-administration prior to mating (group mean: 1.94 ± 0.12 mg/kg/day, $n=10$) and throughout gestation with some fluctuations across time, but without significant differences compared to pre-mating levels ($F(23,208)=1.65$, $p < 0.05$; post hoc test n.s.). Dam weight increased steadily across gestation in both METH self-administering dams and yoked

controls as reflected by a significant main effect of days ($F(24,408)=266.9$, $p<0.01$). There was no significant weight differences between the groups (treatment effect: $F(1,17)=0.02$, n.s.) nor any interaction (treatment $\times$ days: $F(24,408)=1.51$, n.s.; Figure 1 lower panel). All females became pregnant and the number of days between introducing a male and delivery of the litter did not differ between METH (25.79 ± 0.73 days) and yoked saline dams (26.00 ± 0.69 ; $t(27)=0.21$, n.s.).

3.2. Maternal behavior

With the exception of supine nursing at the a.m. observation period, we noted no treatment effects on any maternal behavior (Table 3). In contrast, as described in Table 3, we found significant diurnal and/or day effects for active nursing (i.e., a combined category of kyphosis and licking/grooming of pups) and time spent away from the nest (i.e., a combined category of dam resting and off) – two maternal behaviors that critically impact pup well-being and development, especially at early life stages (Rüedi-Bettschen et al., 2004). As depicted in Figure 2, there were diurnal effects on maternal behavior, with higher levels of active nursing in the am before lights turned on vs. at the usual self-administration time (top panel). Maternal behavior also changed across the first two weeks of pup life. Nursing and licking behaviors decreased across days (am: day effect: $F(13,195)=9.19$, $p<0.01$; self-administration time: day effect: $F(13,208)=1.97$, $p<0.03$), but without an effect of experience (i.e., METH vs saline; am: $F(1,15)=0.57$, n.s.; self-administration time: $F(1,16)=0.01$, n.s.) or a significant interaction of days $\times$ experience (am: $F(13,195)=0.62$, n.s.; self-administration time: $F(13,208)=0.43$, n.s.). Conversely, with increasing pup age, dams were away from their litters more often (bottom panel; am: day effect $F(13,195)=5.49$, $p<0.01$, self-administration time: day effect: $F(13,208)=3.93$, $p<0.01$) without a difference due to prenatal experience (am: $F(1,15)=3.22$, n.s.; self-administration time: $F(1,16)=1.31$, n.s.) or an interaction of days $\times$ experience (am: $F(13,195)=1.48$, n.s.; self-administration time: $F(13,208)=0.41$, n.s.).

3.3. Pup development

On PND0, when all pups in a litter were considered for analysis, no difference was found between offspring of METH dams and their saline yoked control in terms of litter size (11 ± 1 vs. 11 ± 1 , $t(27)=0.03$, n.s.), composition ($44.6\pm4.6\%$ vs. $53.3\pm4.7\%$ males per litter, $t(17)=1.33$, n.s.) and whole litter weight (69.1 ± 3.4 g vs. 70.9 ± 4.9 g, $t(27)=0.31$, n.s.). After culling, remaining pups steadily gained weight across days ($F(20,720)=2487.42$, $p<0.01$), and there was no effect of sex ($F(1,17)=2.10$, n.s), prenatal exposure ($F(1,36)=1.24$, n.s.) or their interactions (data not shown). Body length on PND7 did not differ on the basis of sex ($F(1, 26)=1.45$, n.s.), prenatal exposure ($F(1, 26)=0.71$, n.s.) or their interaction ($F(1, 26)=0.25$, n.s.).

In contrast, several other developmental measures differed between METH- vs. saline-exposed pups. METH-exposed males had a significantly shorter anogenital distance than saline controls on PND1 (3.72 ± 0.08 mm vs. 4.60 ± 0.16 mm, $t(17)=5.10$, $p<0.01$) and 3 (4.92 ± 0.21 mm vs. 5.64 ± 0.08 mm, $t(17)=3.07$, $p<0.01$), but not PND21 (16.82 ± 0.55 mm vs. 17.83 ± 0.55 mm, $t(17)=1.30$, n.s.). Their female siblings, however, had a significantly longer anogenital distance relative to controls only on PND1 (2.03 ± 0.17 mm vs. 1.38 ± 0.14 mm,

$t(17)=2.93$, $p<0.01$; PND3: 2.49 ± 0.15 mm vs. 2.07 ± 0.18 mm, $t(17) = 1.79$, PND21: 10.41 ± 0.50 mm vs. 10.53 ± 0.60 mm, $t(17)=0.16$, both n.s.). When the measure of a particular developmental milestone was presence vs. absence (i.e., pinna detachment, fur appearance, incisor eruption and eye opening), METH-exposed male and female pups were delayed significantly compared to the saline controls. Details including postnatal days measured and statistics are in Table 4.

In terms of righting reflex, the time it took pups to right themselves when placed on their back on a surface decreased across PND1–3 in all pups ($F(2,72)=63.09$, $p<0.01$) and overall METH-exposed pups were significantly slower than controls ($F(1,36)=14.89$, $p<0.01$), more so on day 1 and 2, as indicated by a significant prenatal exposure $\times$ days interaction ($F(2,72)=6.16$, $p<0.01$, Figure 3). There was no significant main effect of sex ($F(1,36)=0.11$, n.s.), or interaction including sex ($F(2,72)=0.07$, n.s.).

Similarly, all pups showed a decrease in the time to turn to a heads-up position in the negative geotaxis assessment across days ($F(3,108)=35.74$, $p<0.01$) and METH-exposed offspring took overall longer than controls ($F(1,36)=44.19$, $p<0.01$), but more pronounced on days 7–9, as indicated by a prenatal exposure $\times$ days interaction ($F(3,108)=5.75$, $p<0.01$, Figure 4). There was no significant main effect of sex ($F(1,36)=0.08$, n.s.) or interaction including sex ($F(3,108)=1.23$, n.s.).

4. Discussion

The present study focused on the potential use of intravenous METH self-administration by pregnant rat dams, rather than investigator-administered drug, as a means to expose offspring to the drug *in utero*. Multiple outcomes were tracked and included pregnancy results, maternal health and behavior, and offspring health and development. By and large, dams and the pregnancy itself were unaffected by METH. In contrast, METH-exposed offspring showed delays in achieving key developmental milestones compared to their saline-exposed counterparts.

To our knowledge, this is the first study to demonstrate that intravenous self-administration of METH is a feasible means to expose developing offspring to the drug. Moreover, this study shows that METH self-administering females can be successfully mated and produce viable litters (McDonnell-Dowling and Kelly, 2015a). Under the parameters of the present study, dams self-administered stable levels of METH before and throughout pregnancy. This is in contrast to human findings showing that METH abusing women have a tendency to reduce their drug use as their pregnancy progresses (SAMHSA, 2013). It is important to note, however, that the TEDS database only reports on women that seek treatment for their METH use implying that there is a population of women that does not seek treatment and likely maintains steady levels of intake. It also appears that it is mainly the heavy METH users that reduce their intake, while the percentage of women abusing METH at a more moderate level remains the same or even increases during the last trimester (SAMHSA, 2013).

Like control dams, METH-administering dams achieved adequate weight gain throughout pregnancy. This observation is in contrast to the human (Good et al., 2010; Smith et al., 2006) and rodent literature (Acuff-Smith et al., 1996; Cho et al., 1991; McDonnell-Dowling et al., 2014, 2015b, 2016) showing that METH use/administration during pregnancy can lead to inadequate weight gain seen in the present study. Multiple reasons could account for the similarities in weight gain. First, dams in our study self-administered approximately 2–3 mg/kg of METH per session. When placed in the context of human levels of intake and scaled from rodents to humans, this dose of daily self-administered METH is most comparable to an individual just initiating use or a more recreational user (Golub et al., 2005). It may simply be the case that our level of intake is not high enough to exert effects on weight gain (cf. Acuff-Smith et al., 1996; Cho et al., 1991). Second, under self-administration conditions, dams can actively determine how much drug they self-administer on any given day; in essence, giving them “control” over the effects of the drug. This is in contrast to the vast majority of other preclinical studies of the developmental effects of METH which used passive/investigator-administered METH injections. Non-contingent drug exposure has been shown to increase the adverse effects of drugs compared to contingent administration of the same dose (Dworkin et al., 1995; Galici et al., 2000). In the few studies that did utilize comparable METH doses but passive administration, maternal weight gain was either lower (Cho et al., 1991; McDonnell-Dowling and Kelly, 2015b, 2016) or unchanged (Slamberova et al., 2005). Finally, compared to METH abusing women, rats in our model have the advantage of having access to METH without having to compromise on food provision or having to endure a stressful home environment – both factors that could contribute to weight loss. It has been reported that METH abusing women also often experience difficult home environments characterized by partners that also abuse drugs, domestic violence and economical struggles (Good et al., 2010; Woudes et al., 2012).

Pup-directed maternal care did not differ between METH dams and controls. Again, this is in contrast to other rodent studies (McDonnell-Dowling and Kelly, 2016; Slamberova et al., 2005), but is likely attributable to the daily dose and/or the mode of delivery (active v. passive) in the present study. There also is the possibility that subtle changes in maternal care were undetected by our method/schedule of scoring. This could be especially true during the birthing process and the first few postnatal hours because METH is accumulated in milk (Bartu et al., 2009) and could be present during the first nursing bout(s). This METH, in turn, would be excreted by the pups and consumed by the dam during pup-grooming, possibly inducing subtle effects on maternal care. Importantly, though, the similarities between METH-taking and control dams in terms of health and behavior suggest that any differences observed in offspring development and behavior cannot be due to inadequate weight gain and/or altered maternal care (Stern and Lonstein, 2001) and, more likely, are due to METH itself. A possible means to control for the impact of even subtle changes in the postnatal environment (i.e., maternal care) lies in cross-fostering the offspring between exposure groups. We have not been able to do so in the current study, as logistical limitations made it impossible to start a sufficient number of females at the same time to allow for cross-fostering.

METH-exposed litters were indistinguishable from control litters in terms of size, composition and individual pup weight/length until weaning. The latter results are in

contrast to the human literature, where *in utero* METH-exposed newborns were found to be small for their gestational age (Minnes et al., 2012; Smith et al., 2003) and similar findings of decreased birthweight have been reported in other rodent studies (McDonnell-Dowling et al., 2014, 2015b; Slamberova et al., 2005). While this lack of difference can likely be explained by factors already described (e.g., daily dose, mode of administration, maternal health), it also suggests that moderate doses of METH throughout pregnancy, provided that it is self-administered, do not hinder appropriate growth and weight development of offspring after birth. It should be noted, though, that as we only estimated gestational day 0 based on dam weight gain and appearance, it remains possible that there were differences in gestational age at birth.

Although weight and length were similar between METH- and saline-exposed pups, METH-exposed offspring were significantly delayed in reaching some species-specific developmental milestones including the somatic milestones of pinna detachment, fur appearance, incisor eruption and eye opening, as well as milestones of a more behavioral nature (e.g., surface righting reflex, negative geotaxis). Interestingly, there were no marked differences in the effects of METH as a function of sex. That is, if a milestone was delayed in male pups, it was also delayed similarly in female pups. The finding of METH-induced delays in development is in agreement with human studies (Himes et al., 2014; Woules et al., 2014) and other rodent studies where non-contingent injections and higher doses of METH have been used (Cho et al., 1991; McDonnell-Dowling et al., 2014). However, if one excludes studies that used METH doses leading to teratogenic effects (Acuff-Smith et al., 1996), the level of impairment is similar across studies irrespective of the METH dose administered. This suggests a threshold phenomenon where METH across a broad range of doses is enough to delay development, with very high doses producing teratogenic effects.

In summary, the current study demonstrates that, under the conditions used herein, METH self-administration during gestation is a feasible way to expose the developing offspring to the drug without affecting multiple indices of maternal behavioral and physical health. This approach leads to offspring that, while of similar size and weight as controls, are delayed in achieving species-specific developmental milestones. Placing our findings in the context of a human METH user, these data show that even moderate doses of METH corresponding to early METH use or a more recreational user, still have marked effects on offspring development. The extent to which this model produces more long-lasting effects in offspring remains to be determined.

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Highlights

- Prenatal methamphetamine exposure is achieved via maternal *iv* self-administration.
- Methamphetamine self-administration has no effect on pregnancy outcome.

Achievement of developmental milestones in exposed offspring is delayed

Figure 1. METH self-administration by dams prior to and throughout gestation

Upper panel: Daily METH self-administration of dams, expressed as mg/kg/session (n=10). Open circle denotes last stable day of self-administration before mating, followed by the last 2 days of self-administration before becoming pregnant.

Lower panel: Body weight in g of METH self-administering (closed circle, n=10) and saline-yoked control dams (open circle, n=9) at start of self-administration (SA), throughout mating and gestation until a litter was delivered (PND0).

Data points are expressed as Mean ± SEM. GD0 denotes beginning of gestation.

Figure 2. Maternal behavior

Visualization of maternal behavior scores obtained during 1-hr observations either immediately before the light/dark transition (triangles) or at the METH self-administration session starting time (circles) in METH dams (closed symbol, $n=9$) compared to yoked saline controls (open circle, $n=9$). Upper panel depicts active nursing behavior, combining scores of kyphosis and licking (Rüedi-Bettschen et al., 2004). Lower panel shows dams away from nest/pups, engaged in non pup-related behaviors. Data is shown as Mean $\pm$ SEM.

Figure 3. Pup surface righting reflex

Surface righting reflex in offspring (males and females combined) of METH self-administering dams (n=10, shaded bars) and saline controls (n=9, white bars). Cutoff time was 60s. Data is expressed as Mean $\pm$ SEM. Asterisks indicate main effect of prenatal exposure.

Figure 4. Pup negative geotaxis reflex

Time spent turning head-up on a 30 degree incline after head-down placement in offspring (males and females combined) of METH self-administering dams (n=10, shaded bars) and saline controls (n=9, white bars). Cutoff time was 60s. Data is expressed as Mean $\pm$ SEM. Asterisks indicate main effect of prenatal exposure.

Table 1

Developmental milestones assessed in pups.

Developmental assessment	PND	Criteria
Somatic development		
Body weight	1 – 21	
Body length	7	From snout to base of tail.
Anogenital distance	1, 3, 21	From base of genitals to top of the anus.
Pinna detachment	1 – 3	Both pinnas detached completely.
Fur appearance	3 – 6	Fur on head and back at least.
Incisor eruption	7– 12	All four incisors erupted.
Eye opening	12 – 16	Both eyes open completely.
Behavioral development		
Surface righting reflex	1 – 3	Time to right with all four paws on surface.
Negative geotaxis	7 – 10	Time to turn heads-up on a 30° incline.

Table 2

Maternal behavior assessed PND1–14.

Maternal Behavior	Description
Off	Dam is in no physical contact to pups.
Mouthing	Dam repositions pup in the nest.
Retrieval	Dam retrieves pup from outside the nest.
Hovering	Dam is over pups, but not immobile; engaged in other activities.
Licking	Dam is licking/grooming pups.
Kyphosis	Dam nursing in an arched-back, immobile posture.
Prone nursing	Dam nursing lying very flat on the pups, no or little limb support.
Supine nursing	Dam passively nursing, lying on the side or back.
Dam resting	Dam is outside the nest.
Dam drinking/eating	Dam is eating/drinking; inside or outside the nest.
Dam self-grooming	Dam is self-grooming; inside or outside the nest.
Dam nest-building	Dam is rearranging nest or carrying nesting material.

All maternal behaviors were scored using the scan-sampling method outlined in Rüedi-Bettschen et al, (2004), resulting in 12 samples/hour/dam.

Table 3

Maternal behavior – total scores across PND1–14.

Maternal Behavior	Morning			Self-administration time		
	METH	Saline	p	METH	Saline	p
Off	26.11 ± 3.75 *	33.00 ± 6.23 *	t(16)=0.95, n.s.	72.89 ± 6.18	53.89 ± 7.41	t(16)=1.97, n.s.
Mouthing	0 ± 0	0 ± 0		0 ± 0	0 ± 0	
Retrieval	0 ± 0	0 ± 0		0 ± 0	0 ± 0	
Hovering	37.22 ± 1.93	29.67 ± 4.34	t(16)=1.59, n.s.	40.33 ± 2.01	32.00 ± 5.15	t(16)=1.51, n.s.
Licking	12.11 ± 1.81	9.33 ± 1.87	t(16)=1.07, n.s.	11.33 ± 1.05	14.11 ± 2.69	t(16)=0.96, n.s.
Kyphosis	74.89 ± 7.15 *	64.22 ± 9.50 *	t(16)=0.89, n.s.	27.22 ± 4.09	25.56 ± 4.46	t(16)=0.28, n.s.
Prone nursing	7.22 ± 2.85	5.78 ± 1.99	t(16)=0.44, n.s.	1.67 ± 0.99	3.78 ± 1.26	t(16)=1.32, n.s.
Supine nursing	10.22 ± 2.38	4.33 ± 1.35	t(16)=2.15, p<0.05	7.67 ± 2.63	5.11 ± 2.69	t(16)=0.68, n.s.
Dam resting	5.67 ± 2.56	7.56 ± 2.91 *	t(16)=0.46, n.s.	9.33 ± 2.03	17.36 ± 3.66	t(16)=1.98, n.s.
Dam eating	13.00 ± 1.68 *	13.22 ± 2.62 *	t(16)=0.07, n.s.	26.11 ± 1.57	24.22 ± 3.29	t(16)=0.52, n.s.
Dam self-grooming	7.00 ± 1.38	8.33 ± 1.87	t(16)=0.57, n.s.	7.22 ± 1.16	5.11 ± 1.09	t(16)=1.34, n.s.
Dam nest-building	2.11 ± 0.90 *	4.33 ± 1.39	t(16)=0.20, n.s.	13.00 ± 4.20	7.33 ± 2.13	t(16)=1.20, n.s.
Dam away	31.78 ± 5.51 *	40.56 ± 7.59 *	t(16)=0.95, n.s.	82.22 ± 5.85	71.56 ± 10.07	t(16)=0.92, n.s.
Active nursing	87.00 ± 6.04 *	73.56 ± 10.04 *	t(16)=1.15, n.s.	38.56 ± 4.37	39.67 ± 5.93	t(16)=0.15, n.s.
Self-directed	30.00 ± 4.30 *	35.11 ± 7.97	t(16)=0.64, n.s.	23.88 ± 6.23	21.47 ± 5.36	t(16)=0.29, n.s.

Maternal Behavior score totals for across PND1–14 during the a.m. observation ("morning") and observations during the former self-administration time ("self-administration") in METH and saline control dams.

Dam away consists of the combined scores of *off* and *dam resting*. *Active nursing* combined scores of *kyphosis* and *licking*. *Self-directed* scores combine *dam eating*, *self-grooming* and *resting*. Data are expressed as Mean ± SEM, followed by statistical information. n.s. denotes non-significance.

* indicate significant differences between the a.m. observation and observations during the former self-administration time within the prenatal exposure group.

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Table 4
Summary of „present-absent” assessment for achievement of developmental milestones

Assessment	Males				Females			
	METH	Saline	X ²	p	METH	Saline	X ²	p
	%	%			%	%		
Pinna								
PND1	10	9	1.99	0.05	5	25	5.95	0.02
PND2	20	90	5.96	0.01	20	88	5.70	0.01
PND3	98	100		n.s.	98	100		n.s.
Fur								
PND3	0	2.7		n.s.	0	14		n.s.
PND4	3	61	5.55	0.01	0	45	4.82	0.01
PND5	25	88	5.56	0.01	28	100	4.44	0.01
PND6	80	100	2.87	0.01	88	100	2.99	0.01
Incisor								
PND7	0	9		n.s.	0	6		n.s.
PND8	3	9	0.71	n.s.	3	6		n.s.
PND9	8	36	3.06	0.01	10	49	3.71	0.01
PND10	65	82	3.78	0.01	43	89	4.15	0.01
PND11	98	100	3.18	0.01	68	97	3.29	0.01
PND12	98	100		n.s.	95	100		n.s.
Eyes Open								
PND 12	0	0		n.s.	0	0		n.s.
PND13	3	8		n.s.	3	11		n.s.
PND14	23	57	3.08	0.01	26	57	2.67	0.01
PND15	68	87	1.97	0.05	78	86	2.12	0.03
PND 16	100	100		n.s.	100	100		n.s.

Percentage of pups that have achieved the milestone on any postnatal day for METH- and saline-exposed offspring, followed by statistical values (calculated from raw data, p < listed number). Milestones assessed were pinna unfolding, fur appearance, incisor eruption and eye opening. n.s. denotes non-significance.