

Differences in Maternal Behavior and Development of Their Pups Depend on the Time of Methamphetamine Exposure During Gestation Period

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Summary

The present study examined the hypothesis that the extension of noxious effect of methamphetamine (MA) on maternal behavior and postnatal development on the pups may differ in dependence with time of application. Female rats were injected with MA (5 mg/kg) or saline during first (embryonic day (ED) 1-11) or second (ED 12-22) half of gestation. Our results demonstrated that MA exposure on ED 12-22 led to decreased birth weight and weight gained during lactation period relative to rats treated on ED 1-11. Both sexes treated prenatally with MA on ED 1-11 opened eyes earlier compared to animals treated on ED 12-22. As a matter of sensorimotor development application of MA on ED 1-11 impaired the righting reflex, while MA exposure on ED 12-22 impaired the performance of beam balance test in male rats. There were no differences in maternal behavior. Therefore, it seems that MA exposure in the first half of the gestation impaired the early sensorimotor development that is under control of the brain stem, while the MA exposure in the second half of gestation affected the beam balance performance that is dependent on the function of the cerebellum.

Key words

Psychostimulants • Methamphetamine • Maternal behavior • Sensorimotor development • Critical developmental periods

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Introduction

Methamphetamine (MA) as a psychostimulant creates strong feelings of the increased alertness and energy, wakefulness, general wellbeing and confidence, even euphoria and it suppresses appetite (Kish 2008). Due to these effects, low costs and relatively simple production MA belongs to common drugs of abuse, especially in Czech Republic and Slovakia (EMCDDA 2013). A high percentage of MA users represent women (Smeriglio and Wilcox 1999, Smith *et al.* 2008). When abused during pregnancy, MA crosses the placental barrier (Dattel 1990) and may impair the development of the fetus (Rambousek *et al.* 2014, Šlamberová *et al.* 2006).

The negative effect of MA on mothers and their offspring confirm some preclinical studies including those from our laboratory. Rat females, injected with MA during pregnancy, had shorter gestation period, lower weight gain during pregnancy and fewer pups in the litter (Martin 1975, Martin *et al.* 1976). When amphetamine administered during lactation period, maternal behavior was changed: the latencies in the test of retrieving the pups into the nest by mother were prolonged. On the other hand, the time of nursing and nest building were shorter than in control group (Piccirillo *et al.* 1980). Our studies show similar results, in which the pregnant mothers were injected with MA during the only gestation period or gestation and lactation periods. MA-treated mothers care less for their pups, whereas they display more activities of self-care (Šlamberová *et al.* 2005a,b).

In accordance with anorectic effect of MA, mothers and their pups treated with MA during gestation period have been shown to gain less weight than control groups (Acuff-Smith *et al.* 1996).

Development of the pups prenatally exposed to MA has been shown to be impaired as well. The prenatal MA caused increased offspring mortality and delayed development in comparison to control groups (Acuff-Smith *et al.* 1992, 1996, Martin *et al.* 1976). Delayed eye opening have been shown after MA exposure (Martin 1975). In addition, pups displayed impaired development of early locomotion and higher hyperreactivity (Acuff-Smith *et al.* 1992, 1996). Also our studies (Šlamberová *et al.* 2006) demonstrated that pups prenatally exposed to MA had poor performance in several tests of sensorimotor development, such as – righting reflex on surface and in mid-air, beam balance test and rotarod.

Because study of Kellog (1992) showed that drugs administered during prenatal development affects those systems that are evolving at the time of application, and we might expect changes of particular brain structures that develop during the prenatal MA exposure in our experiments. The first half of the gestation represents the period of embryonic development of rats in which the ovum changes to morula (embryonic day – ED 5) and subsequently blastocyst (ED 7). During the periimplantation period, toxic insult generally results either in embryonic death or absence of an effect because of regenerative powers of the pluripotent cells of the embryo in this stage (Christian 2001). The blastocyst implants into endometrium between ED 5-7 (Schlafke *et al.* 1985) and undergoes the process of gastrulation (ED 8.5-9.5). The development of the notochord takes place in the first half of embryonic development, between ED 8.5-9 (Florez-Cossio 1975) and the formation of future brain starts on ED 10.5 (Hoar and Monie 1981). Most defects are probably incompatible with survival at the stage of gastrulation (Van Mierop 1979). It is important to note, that there is considerable variation in embryonic development and by ED 11 may be embryos up to 12 h apart in development (Fujinaga and Baden 1991). Pons and medulla include brainstem motor and sensory nuclei that mature relatively early – ED 10-16 (Rice and Barone 2000). The second half of gestation represents the period of organogenesis. On ED 12, the three-part brain becomes subdivided into five parts and the last part, the cerebellum, starts to form on PD 14 (Campbell *et al.* 1986). Most motor neurons are generated between ED 11-13 (Goulding *et al.* 1993). The most expansive phase

of proliferation in the rat ventricular zone occurs roughly between ED 13-18 (Bayer and Altman 1991).

Teratogenic insult more frequently occur during early organogenesis, which is between ED 7 to ED 12 in rats (Schmidt and Johnson 1997). Acuff-Smith *et al.* (1992) reported that MA in a dose of 50 mg/kg twice daily administered from ED 7-12 produces teratogenic outcomes – anophthalmia and microphthalmia and the olfactory orientation score was lowered. In the further study, the same team of authors reported that there was increased mortality and reduced offspring growth in pups exposed to MA during ED 13-18 (Acuff-Smith *et al.* 1996). In addition, significant decrease in serotonin concentration occurs in nucleus accumbens in rats offspring exposed prenatally to 20 mg/kg of MA twice daily in ED 13-18, but not in ED 7-12 (Acuff-Smith *et al.* 1996).

Based on the above, the aim of the present study was to investigate the difference between the effect of prenatal MA application on maternal behavior and their pups development in the first (ED 1-11) and second (ED 12-22) half of the gestation, which are hypothesized to correspond to first and second trimester of human (Benešová *et al.* 1984, Clancy *et al.* 2007). Our working hypothesis was to show that the extent of noxious effect of MA on offspring development will differ according to the gestational period of MA application.

Methods

Prenatal and postnatal animal care

Adult albino Wistar rats were purchased from Anlab (Prague, Czech Republic) raised in Charles River Laboratories International, Inc. Females (250-300 g) were housed 5 and males (300-350 g) 4 per cage and left undisturbed for a week in a temperature-controlled (22-24 °C) colony room with free access to food and water on a 12 h (light):12 h (dark) cycle. One week after arrival, females were randomly assigned to those, who received MA or saline (SA) on ED 1-11, and those, who were injected on ED 12-22 as MA-treated and SA-treated group. Females were smeared by vaginal lavage to determine the phase of estrous cycle. At the onset of the estrus phase of the estrus cycle females were housed overnight with males (1 female and 1 male per cage). The day of conception was considered as ED 0. The next morning females were smeared again for the presence of sperm and that day was counted as first day of gestation (ED 1). MA-treated groups were injected subcutaneously

(s.c.) with MA in dose of 5 mg/kg/day either in ED 1-11 or ED 12-22 periods. SA females were injected with s.c. saline at the same time and the same volume (1 ml/kg/day) as MA group. All females were weighted daily to see the possible effect of MA on weight gain during the gestation period. The day of birth was counted as postnatal day (PD) 0. The mothers with their litters were not disturbed that day.

Litter characteristics

On PD 1, number of pups and percentage of males and females in each litter was counted. Thereafter, number of pups in each litter was adjusted to 12 and the pups were cross-fostered, so that one mother raised 6 pups from MA and 6 pups from SA-exposed mothers. Whenever possible, the same number of male and female pups was kept in each litter. For identification, prenatally MA-exposed pups were injected intradermally with black

India ink in left foot and prenatally SA-exposed pups in right foot. The birth weight and the weight gain of pups were observed during the whole lactation period. The day of eye opening was recorded. The eyes were considered for open, when both eyes of the pup were fully opened.

The weight gain of mothers during gestation period, the number of pups in each litter and the percentage of males and females in each litter were analyzed using two-way ANOVA (*Drug x Injection period*). Three-way ANOVA (*Drug x Sex x Injection period*) was used to analyze birth weight and weight gain of the pups. Bonferroni post-hoc test was used for comparisons in ANOVA analyses. Chi² test was used for analysis of the eye opening. Differences were considered significant, if $p < 0.05$.

Timetable of experiments is presented in Figure 1. Number of animals of each group used in experiment is shown in Table 1.

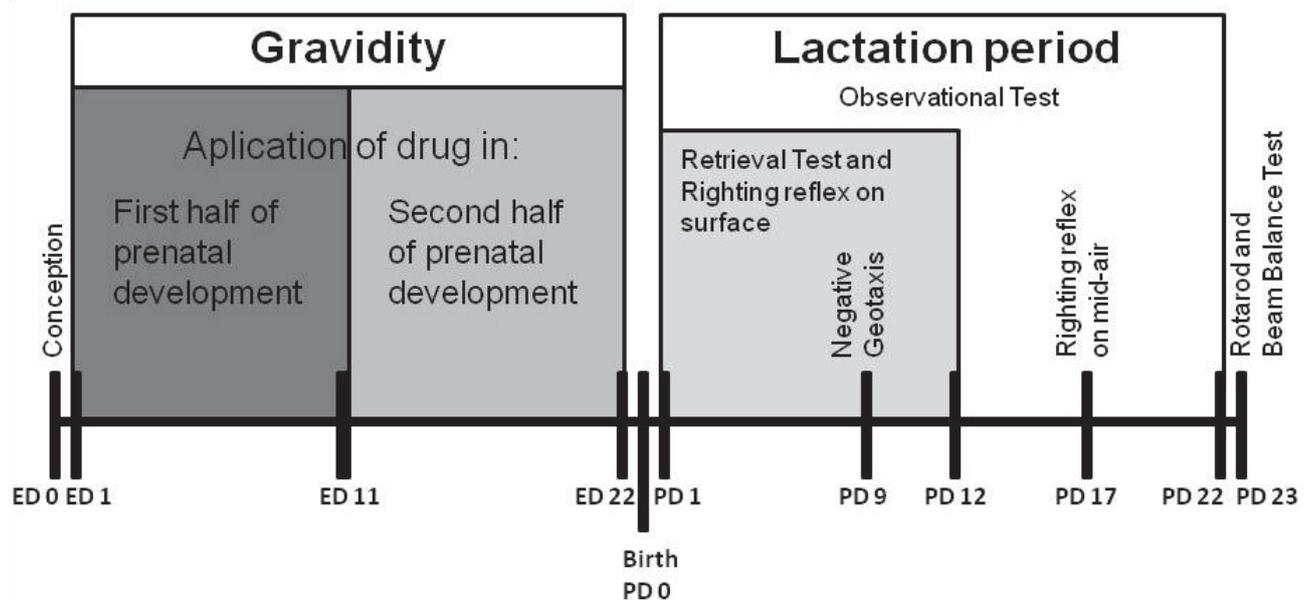


Fig. 1. Timetable of experiments. ED = prenatal day, PD = postnatal day

Table 1. Number of animals per group.

	Mothers		Pups			
	SA	MA	SA		MA	
			Males	Females	Males	Females
ED 1-11	10	11	55	50	61	67
ED 12-22	8	8	49	38	35	34

Maternal behavior

Observational Test

Maternal behavior was observed daily for 50 min in the home cage of each mother and her litter between PD 1 and PD 22. Observations were made during the light phase of light : dark cycle between 08:00-09:00 h (Šlamberová *et al.* 2005a,b, 2007). During each 50 min session, each mother and her litter were observed 10 times for 5 s at 5 min intervals. Eleven types of activities exhibited by the mothers and three types nursing positions (see below) were recorded during each session. Thus, each mother and litter was observed 220 times (22 days x 10 observations per day). During each observation "1" was given, if a behavior occurred, and a "0", if it did not.

First, it was noted whether a mother was nursing. Three different positions were recognized as nursing: a) arched nursing (when the mother is arched over her pups with legs splayed), b) blanket nursing (when the mother is over her litter, but did not have her back arched and there was no obvious extension of her legs), c) passive nursing (when the mother is lying on her side or back with one or more suckling pups). The first two nursing positions were designed as active and the third one as passive nursing. In addition to nursing, 11 maternal activities were recorded: 1) mother in or out of the nest, 2) mother in contact with any of her pups, 3) mother is licking or grooming any of her pups, 4) mother is carrying pups, 5) mother is manipulating nest shavings, 6) mother is resting with eyes closed, 7) mother is eating, 8) mother is drinking, 9) mother self-cares (eating, drinking and self-grooming), 10) mother is rearing, 11) mother is sniffing with head raised.

The occurrence of each activity (maximum 10 in each session) was counted in each of 22 sessions. Two-way ANOVA (*Drug x Injection period*) with Repeated Measure (*Days*) was used to analyze each maternal activity separately. Bonferroni post-hoc test was used for comparisons in ANOVA analyses. Differences were considered significant, if $p < 0.05$.

Retrieval Test

After the Observational test ended, the mother and pups were tested for Retrieval test (Šlamberová *et al.* 2005a,b, 2007). The Retrieval test was conducted daily within PD 1-12 between 09:00-10:00 h. Each mother and litter was tested 12 times. All pups were removed from

their mothers and placed in a separate cage for 5 min. The cage with pups was placed on a heating pad to prevent chilling. After this separation, the entire litter was returned to their mothers and the pups were scattered all around the cage. The mother was then observed for 10 min and the following latencies were recorded: 1) to carry the first pup, 2) to return the first pup into the nest, 3) to return all pups into the nest. Unusual types of behavior were recorder as well: 1) removing a previously returned pup from the nest, 2) caring of the pups randomly in the cage before placing them into the nest, 3) extensive disruption of the nest shavings. During each observation "1" was given, if a behavior occurred, and a "0", if it did not.

Latencies were analyzed by Two-way ANOVA (*Drug x Injection period*) with Repeated Measure (*Days*). Bonferroni post-hoc test was used for comparisons in ANOVA analyses. Differences were considered significant, if $p < 0.05$.

Battery of tests of the pups development

Righting reflex on surface

Righting reflex on surface was tested daily within PD 1-12 (Altman and Sudarshan 1975, Hrubá *et al.* 2009). Each pup was turned to supine position and the time that it took for the pup to right with all four paws containing the surface of the testing table was recorded.

Three-way ANOVA (*Drug x Sex x Injection period*) with Repeated Measure (*Days*) was used to analyze differences in righting reflex on surface. Tuckey post-hoc test was used for comparisons in ANOVA analyses. Differences were considered significant, if $p < 0.05$.

Negative Geotaxis

Negative geotaxis was tested on PD 9 (Altman and Sudarshan 1975, Hrubá *et al.* 2009, Meek *et al.* 2000). Each pup was placed facing downward on a screen inclined at 30° angle. Each animal was given three trials and the best latency of turning the face upward (180° rotation) was recorded. If the pup was slid off the board, it was replaced in the downward position.

Three-way ANOVA (*Drug x Sex x Injection period*) was used to analyze differences in negative geotaxis. Bonferroni post-hoc test was used for comparisons in ANOVA analyses. Differences were considered significant, if $p < 0.05$.

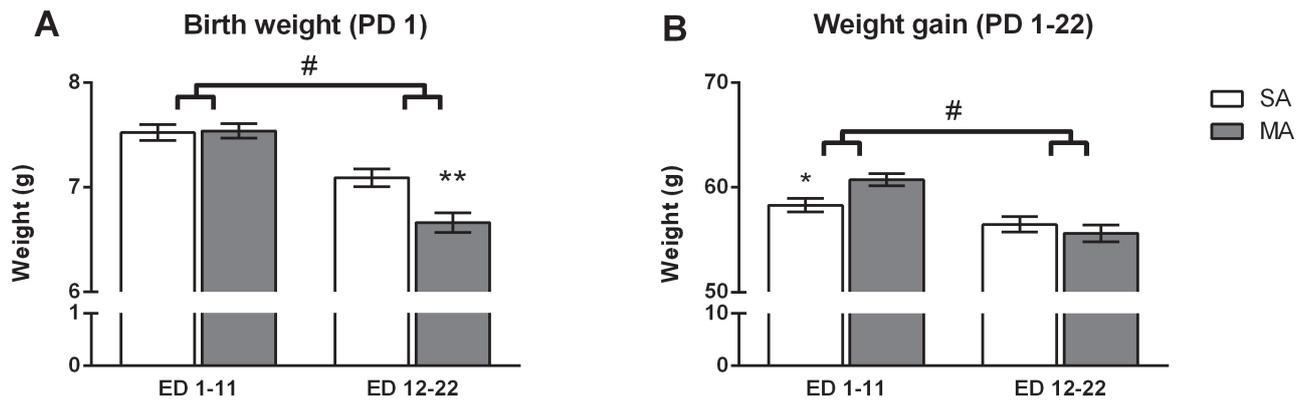


Fig. 2. Effect of prenatal MA exposure on: **A)** birth weight – PD 1, **B)** weight gain of the pups during the lactation period – PD 1-22. Values are means \pm SEM ($n=69-128$). MA = methamphetamine, SA = saline, ED = embryonic day, PD = postnatal day. * $p<0.05$ MA vs. saline of the same injection period, ** $p<0.01$ MA vs. saline of the same injection period, # $p<0.001$ all pups of ED 1-11 vs. all pups of ED 12-22 regardless the sex and prenatal treatment

Righting reflex in Mid-Air

The righting reflex in mid-air was tested on PD 17 (Altman and Sudarshan 1975, Hrubá *et al.* 2009). Each pup was held on its back 40 cm above soft pad, then released and position when reaching the soft pad was observed. A score of “1” was given, when a pup reached the ground at once with all four paws and a “0”, when it did not.

Chi² test was used to analyze differences in righting reflex in mid-air. Differences were considered significant, if $p<0.05$.

Beam Balance Test

The beam balance test on PD 23 was used to examine vestibular function and sensorimotor coordination engaged in maintenance of the balance on the narrow bar (Hrubá *et al.* 2009, Murphy *et al.* 1995). A wooden bar 40 cm long with a diameter of 1 cm was suspended 80 cm above padded soft surface. The pup was placed on the bar being held by the nape of its neck and its forepaws were allowed to touch the bar. Time of fore- and hindlimb grasping reflex was recorded with a limit of 120 s. Rats were subjected to three consecutive trials.

Three-way ANOVA (*Drug x Sex x Injection period*) with Repeated Measure (*Trials*) was used to analyze differences in performance in beam balance test. Bonferroni post-hoc test was used for comparisons in ANOVA analyses. Differences were considered significant, if $p<0.05$.

Rotarod

Rotarod performance was examined on PD 23 to test the sensorimotor coordination and dynamic postural reactions necessary for active moving to maintain the

balance on the rotating cylinder (Hrubá *et al.* 2009, Šlamberová *et al.* 2006). Pups were positioned on a rugged cylinder (11.5 cm in diameter, rotating at constant speed of 6 rpm) in the opposite direction of cylinder rotation, so they were able to walk forward. The duration of balance on the rotarod was determined for 120 s. Rats were subjected to maximum 6 trials until successfully accomplished the task. Number of falls was recorded.

Three-way ANOVA (*Drug x Sex x Injection period*) with Repeated Measure (*Trials*) was used to analyze differences in test of rotarod. Bonferroni post-hoc test was used for comparisons in ANOVA analyses. Differences were considered significant, if $p<0.05$.

Results

Litter characteristics

Drug treatment did not influence the length of the gestation in any of the groups. There was no significant difference between groups of dam in weight gain during both gestation periods. The number of pups and the percentage of males and females in all litters were not significantly altered by the drug treatment. Drug exposure during the first half of the gestation (ED 1-11) did not influence the birth weight of the pups. In the second half of the gestation (ED 12-22), the birth weight of the prenatally MA-treated pups was lower compared to SA-treated pups [$F(1,385)=7.63$, $p<0.01$]. The difference in birth weight between the two drug administration periods was observed (ED 1-11 vs. ED 12-22). The administration of the MA or SA during the second half of the embryonic development caused lower birth weight in both MA and SA-treated pups in comparison to groups of the first half of embryonic

development [$F(1,385)=65.86$, $p<0.001$] (Fig. 2A). SA-treated pups in ED 1-11 gained less weight during the lactation period [$F(1,385)=5.50$, $p<0.05$]. The pups exposed to MA or SA during ED 12-22 gained less weight during the lactation in comparison to the pups exposed during ED 1-11 [$F(1,385)=24.62$, $p<0.001$] (Fig. 2B). In the day of eyes opening, there were no significant differences between MA- and SA-treated groups in both of

injection periods. Sex differences were observed between MA-treated males and females of ED 12-22. On the PD 13 [$\chi^2=22.60$; $p<0.01$] and 14 [$\chi^2=59.97$; $p<0.0001$] more MA-treated females had their eyes opened compared to MA-treated males. Further, more of MA-treated males and females of both treatments of ED 1-11 had their eyes opened on PD 13 and PD 14 in comparison to the same sex and drug treated pups of the ED 12-22 (Table 2).

Table 2. Differences in eyes opening.

	Males ED 1-11		Females ED 1-11		Males ED 12-22		Females ED 12-22	
	SA	MA	SA	MA	SA	MA	SA	MA
PD 13	7.27	13.11 #	21.57 #	20.89	2.04	0	5.26	14.71 +
PD 14	61.81	70.49 ##	82.35 ##	79.10 #	32.65	28.57	39.47	55.88 +

Values are percent of all pups of the same sex and prenatal drug exposure which had their eyes fully opened on the corresponding day ($n=34-67$). MA = methamphetamine, SA = saline, ED = embryonic day, PD = postnatal day. + $p<0.05$ males vs. females ED 12-22 of the same drug exposure, # $p<0.05$ pups ED 1-11 vs. ED 12-22 of the same sex and drug exposure, ## $p<0.001$ pups ED 1-11 vs. ED 12-22 of the same sex and drug exposure

Maternal behavior

Observational Test

There were no differences in any of the maternal and non-maternal activities between MA- and SA-treated dams in the period of injection ED 1-11 (Table 3A). The incidence of mothers manipulating shavings and grooming pups during the observation was too low for statistical analysis. When administered MA during ED 12-22, there were no significant differences in maternal activities in comparison to SA-treated mothers (Table 3B). In non-maternal activities, eating was decreased in MA-treated mothers relatively to SA-treated group [$F(1,29)=12.85$, $p<0.01$]. There were no differences in other non-maternal activities between MA- and SA-treated groups. The incidence of mother sniffing during the session was too low for statistical analysis. When the effect of gestational periods of injections was compared, we observed increased incidence in following activities – active nursing [$F(1,29)=31.69$, $p<0.001$], time spent in nest [$F(1,29)=9.90$, $p<0.01$], time spent in contact with pups [$F(1,29)=26.44$, $p<0.001$] and sleeping [$F(1,29)=20.70$, $p<0.001$]. In all of these activities the incidence of activities were more frequent in the group of mothers injected during second half of gestation in comparison to the mothers injected in the first half of gestation regardless of the treatment.

Retrieval Test

We displayed no significant differences in any of the observed categories – latency to carry the first pup, returning the first pup into the nest and returning all pups into the nest. There was no interaction between drug treatment and postpartum days in the latency to carry the first pup or returning the pups into the nest. No unusual behaviors, as defined in Methods, were exhibited by any of the mothers during the 12 days of testing. No differences were found between gestational periods of injections.

Battery of tests of the pups development

Righting reflex on surface

Righting reflex on surface did not shown any sex differences in any of injection schedules. As shown in Figure 3, prenatally MA-treated pups were slower in righting reflex during the first postnatal day compared to SA-treated pups, when drug was administered during the first half of prenatal development [$F(1,236)=6.56$, $p<0.01$] (Fig. 3B). No differences were found between groups of pups, when MA or SA was administered during second half of prenatal development in any of the test days. The pups treated with MA on ED 12-22 were slower in righting in first two postnatal days than pups exposed to MA on ED 1-11 [$F(3,2310)=5.22$, $p<0.05$] (Fig. 3B, C). SA-treated pups on ED 1-11 were slower in the first postnatal day than pups SA-treated on ED 12-22.

Table 3. Effect of MA administration during the first and the second half of gestation on maternal and non-maternal activities of rats.

A) Observational test ED 1-11	vs. ED 12-22	SA	MA
Maternal activities			
<i>Nursing</i>		4.45 ± 0.30	4.65 ± 0.25
<i>Active nursing</i>	##	3.03 ± 0.32	2.81 ± 0.26
<i>Passive nursing</i>		1.42 ± 0.23	1.84 ± 0.19
<i>In nest</i>	#	4.47 ± 0.38	4.50 ± 0.31
<i>In contact with pups</i>	##	5.42 ± 0.32	5.62 ± 0.26
<i>Manipulating shavings</i>		NA	NA
<i>Grooming pups</i>		NA	NA
Non-maternal activities			
<i>Self-grooming</i>		0.76 ± 0.14	0.72 ± 0.11
<i>Drinking</i>		0.64 ± 0.08	0.53 ± 0.07
<i>Eating</i>		1.30 ± 0.14	0.95 ± 0.11
<i>Sleeping</i>		0.96 ± 0.24	1.55 ± 0.19
<i>Sniffing</i>		0.48 ± 0.09	0.33 ± 0.07
<i>Rearing</i>	##	0.44 ± 0.13	0.41 ± 0.10
B) Observational test ED 12-22			
		SA	MA
Maternal activities			
<i>Nursing</i>		6.52 ± 0.34	7.30 ± 0.44
<i>Active nursing</i>		4.85 ± 0.30	4.83 ± 0.38
<i>Passive nursing</i>		1.67 ± 0.37	2.47 ± 0.47
<i>In nest</i>		5.90 ± 0.36	6.69 ± 0.46
<i>In contact with pups</i>		7.26 ± 0.30	8.15 ± 0.38
<i>Manipulating shavings</i>		0.41 ± 0.07	0.17 ± 0.09
<i>Grooming pups</i>		1.08 ± 0.08	0.83 ± 0.10
Non-maternal activities			
<i>Self-grooming</i>		0.80 ± 0.11	0.58 ± 0.14
<i>Drinking</i>		0.55 ± 0.05	0.40 ± 0.07
<i>Eating</i>		1.54 ± 0.08	1.10 ± 0.10 *
<i>Sleeping</i>		2.47 ± 0.31	3.69 ± 0.39
<i>Sniffing</i>		NA	NA
<i>Rearing</i>		0.53 ± 0.08	0.27 ± 0.10

Values are shown as means ± SEM (n=8-11) and represent the frequency of behavior during 1 h observation every day over the lactation period. MA = methamphetamine, SA = saline, ED = embryonic day, NA = not analyzed measures. * p<0.01 vs. saline, # p<0.01 ED 1-11 vs. ED 12-22 regardless the drug exposure, ## p<0.001 ED 1-11 vs. ED 12-22 regardless the drug exposure

Negative Geotaxis

There were no significant differences between MA- and SA-treated groups in both of the injections schedules as well as between sexes. When compared the injection schedule, MA-treated pups in ED 1-11 [$F(1,386)=2.44$, $p<0.05$] were able to manage the test of negative geotaxis in lower time in comparison to pups

injected with MA during ED 12-22. There was no difference between SA-treated groups. The effect of the injection period was shown regardless the drug treatment or gender. All pups treated during first half of embryonic development passed the test faster than pups treated during second half of embryonic development [$F(1,382)=8.58$, $p<0.01$] (Fig. 4).

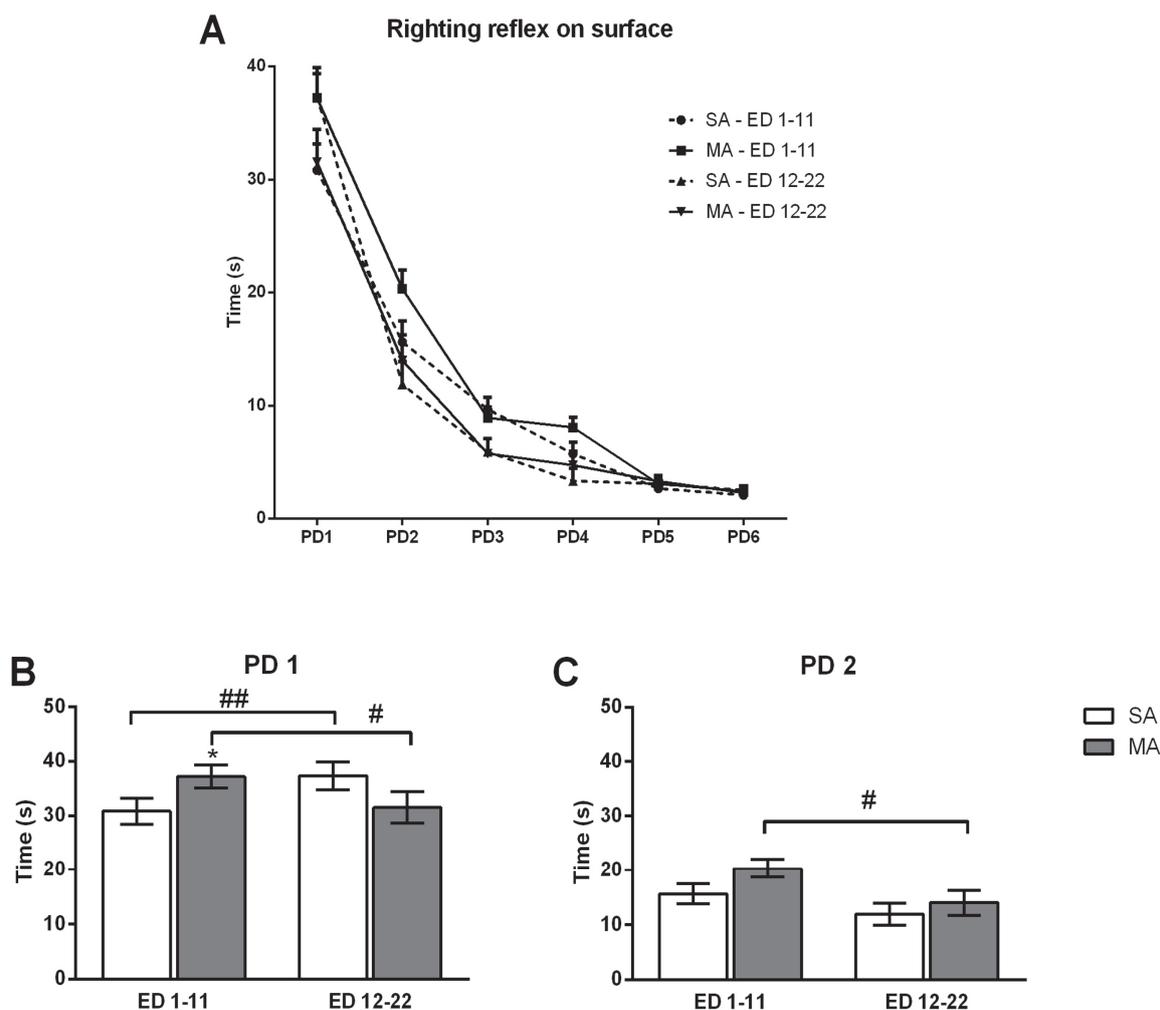


Fig. 3. A) Effect of prenatal MA exposure on righting reflex on surface in first 6 postnatal days. **B)** Effect of prenatal MA exposure on righting reflex on surface in first postnatal day. **C)** Effect of prenatal MA exposure on righting reflex on surface in second postnatal day. Values represent the time required for rotating from the resupine position to the position on four paws and are shown as means \pm SEM ($n=69-128$). MA = methamphetamine, SA = saline, ED = embryonic day, PD = postnatal day. * $p<0.01$ MA vs. saline of the same injection period, # $p<0.05$ pups ED 1-11 vs. ED 12-22 of the same drug exposure, ## $p<0.01$ pups ED 1-11 vs. ED 12-22 of the same drug exposure

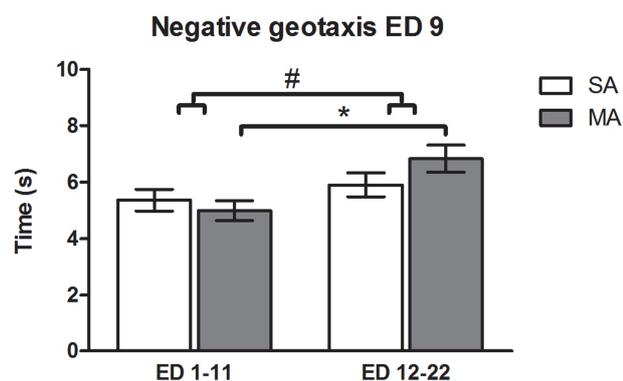


Fig. 4. Effect of prenatal MA exposure in the test of negative geotaxis on the PD 9. Values are means \pm SEM ($n=69-128$) and represent the time required for turning from the position of negative geotaxis into position of positive geotaxis. MA = methamphetamine, SA = saline, ED = embryonic day. * $p<0.05$ pups ED 1-11 vs. ED 12-22 of MA exposure, # $p<0.01$ all pups of ED 1-11 vs. all pups of ED 12-22 regardless the sex and prenatal treatment

Righting reflex in Mid-Air

Drug administration, sex and injection period did not induce any differences in righting reflex in mid-air.

Beam Balance Test

No differences in time spend on the bar were shown between MA- and SA-treated groups on ED 1-11 (Fig. 5A). When MA was administered during prenatal period of ED 12-22, only males had poor performance in the test compared to SA-exposed males [$F(1,151)=4.02$, $p<0.05$] (Fig. 5B). The groups did not vary significantly across the gestational periods of injections.

Rotarod

In the test on the rotating rod, there were no differences in the time spend on the cylinder between

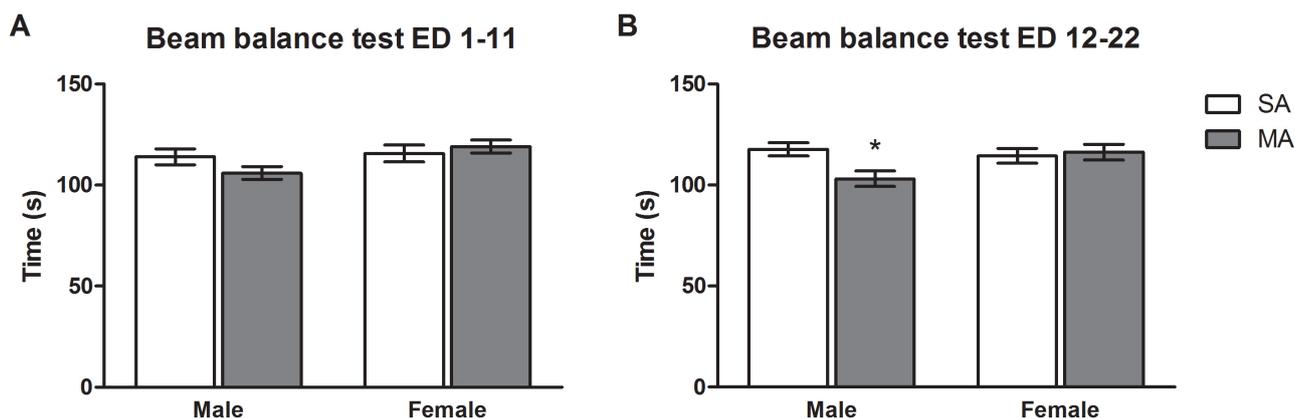


Fig. 5. Effect of prenatal MA exposure in beam balance test, when MA applied during: **A)** first half of embryonic development, **B)** second half of embryonic development. The graph shows the average time that animals endure to balance on the beam. Values are means \pm SEM ($n=34-67$). MA = methamphetamine, SA = saline, ED = embryonic day. * $p<0.05$ MA vs. saline of the same sex and injection period

MA- and SA-treated groups in both of the injection schedules. The test did not show any sex differences between groups. No differences were found between gestational periods of injections.

Discussion

The aim of the present study was to determine the difference in the effect of MA exposure during the first and second half of gestation on maternal behavior and the postnatal consequences of prenatal exposure of MA on development of the pups. It is assumed that the first and second halves of gestation period in rats correspond to first two trimesters in the development of neural system in human (Benešová *et al.* 1984, Clancy *et al.* 2007). We expected that application of MA during first and second half of embryonic development will cause functional changes that correspond to the brain structures that are developing during the time of the drug exposure.

Abnormal maternal behavior may affect prenatal and/or postnatal development of the pups. Although, we observed no difference between MA- and SA-treated groups in maternal behavior in either of the injection schedules, it is contradictory to our previous studies (Šlamberová *et al.* 2005a,b). Previously we demonstrated attenuated active nursing, when MA was administered only during the gestation period and increased passive nursing, when was administered during 9 weeks of pre-mating, gestation and lactation periods. In addition, the time spent in contact with pups and pups' grooming by MA-treated mothers have been shown to be decreased and latencies in retrieval test prolonged (Šlamberová *et*

al. 2005a,b). The difference between our previous and the present studies might be due to the schedule and shorter period of MA application in the current study. Similar conclusions are mentioned in study of effect of cocaine on maternal behavior, where Vernotica *et al.* (1996) found that maternal behavior was affected acutely during the intoxication period, while 16 h after cocaine injection, when plasma level of cocaine falls to non-detectable levels, the drug-injected mother displayed maternal behavior comparable to saline-injected mothers.

We observed increased incidence in maternal and non-maternal activities (active of nursing, time spent in nest, time spent in contact with pups and sleeping) in the group of mothers exposed to MA as well as saline during second half of gestation in comparison to the mothers injected in the first half of gestation. Since we did not come across any study, which would compare maternal behavior depending on administration of any drug during gestation, we can only speculate if we would like to interpret these differences. The maternal behavior of rats during the progression of the postpartum period is not static, but rather is dynamic. It changes in response to developing behavioral and physiological needs of the pups (Grota and Ader 1969). Pups are more active during the second half of gestation and are able to find their mother by themselves (Šlamberová *et al.* 2001). Among the brain structures critically involved in postpartum maternal responsiveness, it is widely believed that the medial preoptic area (mPOA) acts as a primary locus of integration, orchestrating the effective expression of maternal behavior to the developmental stage of the pups across postpartum (Pereira and Morrell 2009). We can only guess whether the stress caused by injection during

gestation or the needs of pups contributed to the changes in neural circuits and patterns in maternal behavior.

The pharmacokinetics of MA significantly changes throughout the gestation (Rambousek *et al.* 2014, White *et al.* 2011). White *et al.* (2011) confirmed decreased systemic clearance of MA at the end of gestation period. The reduced clearance lengthens the time of MA exposure. However, there is still lack of information about the pharmacokinetic changes of MA in first and second halves of embryonic development of pups. Further investigation is necessary for understanding the prenatal effect of MA.

Some preclinical studies including ours (Acuff-Smith *et al.* 1996, Hrubá *et al.* 2009, Šlamberová *et al.* 2006) and clinical studies (Smith *et al.* 2006) reported interference of prenatal MA exposure with somatic growth. The possible explanation for decreased weight might be an anorectic effect of MA (Bittner *et al.* 1981). While drug exposure throughout gestation is well known to alter somatic growth, there is an assumption that exposure to certain drugs during only a part of development period is equally capable of altering somatic growth (Dobbing and Sands 1971, Smith and Chen 2010). In the present study, we have shown that the exposure to MA during second half of prenatal development resulted in decreased birth weight and weight gain during lactation period compared to MA exposure during the first half of prenatal development. Our results are in agreement with studies of Acuff-Smith *et al.*, in which MA administered during ED 7-12 did not influence the weight of the pups (Acuff-Smith *et al.* 1992, 1996), whereas the reduced offspring growth occurred when MA was administered during ED 13-18 (Acuff-Smith *et al.* 1996). The periods of injection are comparable to ours. However, the doses of MA used in those studies are significantly higher (50 mg/kg) (Acuff-Smith *et al.* 1992) or gradually increasing (0, 5, 10, 15 or 20 mg/kg) (Acuff-Smith *et al.* 1996); both scheduled twice a day. We injected MA in a dose of 5 mg/kg once a day during ED 1-11 or ED 12-22.

The developing visual system is extremely vulnerable to the effects of prenatal exposure to neurotoxic drugs (Dominguez *et al.* 1991). Nevertheless, the present study did not confirm the findings of our previous studies that pups prenatally exposed to MA opened their eyes later than saline-exposed pups (Hrubá *et al.* 2009, Šlamberová *et al.* 2006). It might be also attributable to shorter period of injection in the recent study. On the other hand, the delay of eye opening was apparent when the injection periods were compared.

Prenatally MA-treated pups of both sexes opened their eyes earlier when the drug was administered during ED 1-11 compared to the same sex pups treated during ED 12-22. The critical period for eye development begins around ED 10 when the optic vesicle arises from walls of the embryonic forebrain. By the ED 13, a well-developed optic cup is formed and obliteration of the intraretinal space occurs (Palmowski and Tulsi 1987). Embryonic development of retina begins on about ED 13 and maximum thickness of retina is reaches on PD 5, whereas retinal volume does not reach its peak up until PD 12 (Braekevelt and Hollenberg 1970). In this respect, the injection period ED 12-22 seems to prolong the embryonic development of eyes and thereafter delay the day of eyes opening. Acuff-Smith *et al.* (1996) found, that folded retina occurrence is attributed to gradually increasing doses of MA exposure during ED 13-18. However, the same study showed occurrence of anophthalmia and microphthalmia after gradually increasing doses (0, 5, 10, 15 or 20 mg/kg) of MA during ED 7-12, but not ED 13-28. In present study, both, MA as well as saline injections, caused differences in eye opening, therefore, the role of stress should not be ignored. The prenatal, as well as neonatal stress might be a strong modulator of eyes opening (Ellenbroek *et al.* 2005).

From our previous studies it is known that prenatal exposure to MA affects sensorimotor coordination (Šlamberová *et al.* 2006). The present study is answering question whether timing on prenatal MA administration is important factor for changes in sensorimotor development. The battery of tests in different postnatal days was used. The righting reflex on surface examines especially tactile maturation that develops prior to motor skills of the pups and is under control of the brain stem (Pellis and Pellis 1994). When the pups were exposed to MA during the only gestation period, they were slower in righting in first five postnatal days (Šlamberová *et al.* 2006), whereas the pups were treated with the drug during prenatal and/or postnatal period, their performance was impaired even on PD 12 compared to control pups (Hrubá *et al.* 2008). In the recent study the duration of prenatal MA exposure was reduced to eleven days either on ED 1-11 or on ED 12-22. Animals exposed to MA during the first half of gestation were slower in righting in first postnatal day than saline-exposed animals, while this effect was not apparent, when MA was administered during the second half of gestation. Administration of MA in ED 1-11 increased the time of righting reflex during first two

postnatal days compared to pups treated with MA in ED 12-22. These findings are in agreement with study of Acuff-Smith *et al.* (1996), in which the gradually increasing doses of MA caused delayed development of early locomotion when the drug was injected during ED 7-12, but not during ED 13-18.

The test of negative geotaxis is an automatic, stimulus-bound orientation movement considered diagnostic of vestibular and/or proprioceptive function (de Castro *et al.* 2007). In consistence with work of Hrubá *et al.* (2009), we expected the impaired performance by prenatally MA-treated pups in turning. Unexpectedly, prenatally MA-treated pups did not differ from control groups in both of the injection period. Therefore, the assumption that MA attenuates the physiological effect of acute stress (Söderpalm *et al.* 2003) represented by the manipulation during the test, may play an important role in this case. In addition, the effect of the prenatal injection periods regardless prenatal drug exposure and gender was proved to be significant. Pups treated with MA or saline during first half of prenatal development turned faster into the position of negative geotaxis than the animals treated during the second half of prenatal development.

Further, test of righting reflex in mid-air did not display any significant differences between groups in drug exposure, injection period or gender in the present study, what is contradictory to our previous studies. The limitation might be explained by the duration of prenatal exposure of MA. When MA was injected during the only gestation period (Šlamberová *et al.* 2006) or gestation and lactation periods (Hrubá *et al.* 2008), less MA-treated pups were able to successfully reach the ground. Another explanation of insignificant result of our study might be the late postnatal day of testing. Although two of our studies (Hrubá *et al.* 2008, Šlamberová *et al.* 2006) showed differences when pups were tested on PD 17, as in present study, other study displayed differences on testing day PD 15 but not earlier or later (Šlamberová *et al.* 2007). This explanation is supported by the study of Mesquita *et al.* (2007), in which pups stressed during neonatal period displayed differences until PD 12 but not later.

Rotarod and beam balance test refer about sensorimotor development at the end of the lactation period on the PD 23 that requires fully developed cerebellar coordination. MA exposure did not influence the latency of remaining on the rotating cylinder in any of the groups and injection periods. On the other hand, MA exposure during the second half of gestation impaired the performance in beam balance in male rats, while this

effect of MA was not significant after MA exposure during the first half of gestation. Interestingly, our previous studies (Hrubá *et al.* 2009, Pometlová *et al.* 2009) display reverse results, particularly, the prenatally MA-treated pups had poor performance in rotarod test, whereas their performance on bar did not differ from control groups. These discrepancies are somewhat difficult to interpret. The basic differences between rotarod and beam balance test is that in the rotarod test the pup has to keep moving against the direction of the cylinder rotation to prevent falling – this engages dynamic postural reactions; while in the beam balance test fine motor movements are necessary for holding balance on the narrow bar (Pometlová *et al.* 2009). The expected and observed changes on rotarod and beam balance test might be explained by increased muscle weakness, which is caused by inhibition of transmission at the neuromuscular junction (Gerald and Gupta 1977).

In the recent paper, our results do not fully correspond to our previous research. First of the most essential differences might be the shorter period of MA application in the current study. Our former research focused on the entire gestation and/or lactation period (Šlamberová *et al.* 2005a,b, 2006) whereas in the recent study MA was administered for 11 days either during first or second half of gestation. The schedule of experiments may cause the difference especially in the test of righting reflex in mid-air. The role of prenatal stress should not be ignored. However, the evidence suggesting specific periods of heightened vulnerability to stress during pregnancy is inconsistent (Class *et al.* 2011), so it is difficult to estimate the share of stress on the results. In comparison to the findings of other authors, we see the differences not only in manipulating with animals, but also in doses of MA (Acuff-Smith *et al.* 1992, 1996).

In conclusion, it is suggested that MA exposure between ED 1-11 forwarded the process of eyes opening and impaired the early sensorimotor development, while the MA exposure between ED 12-22 decreased the birth weight and affected the beam balance performance tested at the end of lactation. Thus, MA exposure in the first half of the gestation impaired the early sensorimotor development that is under control of the brain stem, while the MA exposure in the second half of gestation affected somatic development necessarily for locomotion and also disordered function of the cerebellum.

Conflict of Interest

There is no conflict of interest.

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