


# Prenatal cigarette smoke exposure slightly alters neurobehavioral development in neonatal rats: Implications for developmental origins of health and disease (DoHAD)

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## ABSTRACT

Numerous studies indicate that smoking during pregnancy exerts harmful effects on fetal brain development. The aim of this study was to determine the influence of maternal smoking during pregnancy on the early physical and neurobehavioral development of newborn rats. Wistar rats were subjected to whole-body smoke exposure for  $2 \times 40$  min daily from the day of mating until day of delivery. For this treatment, a manual closed-chamber smoking system and 4 research cigarettes per occasion were used. After delivery the offspring were tested daily for somatic growth, maturation of facial characteristics and neurobehavioral

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development until three weeks of age. Motor coordination tests were performed at 3 and 4 weeks of age. We found that prenatal cigarette smoke exposure did not alter weight gain or motor coordination. Critical physical reflexes indicative of neurobehavioral development (eyelid reflex, ear unfolding) appeared significantly later in pups prenatally exposed to smoke as compared to the control group. Prenatal smoke exposure also resulted in a delayed appearance of reflexes indicating neural maturity, including hind limb grasping and forelimb placing reflexes. In conclusion, clinically relevant prenatal exposure to cigarette smoke results in slightly altered neurobehavioral development in rat pups. These findings suggest that chronic exposure of pregnant mothers to cigarette smoke (including passive smoking) results in persisting alterations in the developing brain, which may have long-lasting consequences supporting the concept of developmental origins of health and disease (DoHAD).

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## KEYWORDS

prenatal, smoking during pregnancy, tobacco, neurobehavioral development, rat

## INTRODUCTION

Numerous studies indicate that smoking during pregnancy may have harmful effects on the newborn. Perinatal exposure to smoking can affect fetal development, resulting in delayed cognitive and motor development, retarded locomotor behavior, and an increase in the incidence of psychiatric abnormalities [12, 13, 30, 31].

Tobacco smoke contains thousands of chemicals [21], and many of these are potentially toxic. The major components in tobacco smoke that have been shown to interfere with brain development are carbon monoxide and nicotine [9]. Nicotine crosses the placenta, enters the fetal circulation and accumulates in the fetal compartments from as early as 7 weeks of gestation in both active and passive smokers [19, 25, 33]. In rats, nicotine has negative effects on brain and lung development [6, 17, 35, 42]. Prenatal nicotine exposure has also been shown to affect the contractility of the uterine arteries, leading to decreased uterine blood flow in animals. Damaging effects have also been shown in first-trimester human fetal brain cell cultures [18]. Nicotine binds to the cholinergic receptors during brain development causing cell death or structural alteration in certain brain areas, which leads to neurobehavioral and functional impairment in offspring [45]. Carbon monoxide also crosses the placenta, where it binds to hemoglobin, producing carboxhaemoglobin, which limits oxygen delivery to the tissues [19]. Therefore, exposure to maternal smoking may lead to fetal hypoxia and ischemia, which affect brain development. Perinatal hypoxia-ischemia increases the risk of future behavioral and neurological deficits. Locomotor ability, learning and memory deficits have been described in juvenile and adult rats subjected to a hypoxic-ischemic insult in the perinatal period [19, 23, 24]. Metals, including lead, cadmium and mercury, which are all neurotoxins, have also been detected in cigarette smoke [4]. Cigarette smoke has well-known damaging effects at all ages, leading to cancerogenesis, vasculopathies and accelerated aging [27, 46]. Recent studies reported that parental smoking during maternal pregnancy may be associated with an increased risk of childhood overweight as well as obesity in adulthood leading to hypertension and other cardiovascular diseases [2, 8, 11, 15, 51]. Maternal smoking during pregnancy has also been



associated with increased risk of wheezing, asthma, airway hyper responsiveness, impaired lung function, and bronchitis [7].

In human epidemiological studies it is difficult to prove that smoking during pregnancy has the potential to cause fetal brain damage, because of the many co-variables that operate together with smoking. Such factors are low socio-economic status, poor prenatal care or co-abuse of different other drugs. Using an animal model, these problems can be resolved. A number of methods for the administration of tobacco smoke during pregnancy have been employed to assess the potential of nicotine and other cigarette smoke components in causing altered neuronal development in rodents. A recent study demonstrates significant increase in locomotor activity that was observed as early as 4 weeks of age persisting through 4 months of age in a mouse model [50]. These findings are similar to reports in humans where investigators found an increased risk for attention-deficit hyperactivity disorder, aggression and other childhood behavior problems [5, 8]. However, we know little about the effects of prenatal smoke exposure on the early development of the offspring.

Development is reflected among others by the maturation of neurological reflexes and motor coordination. The appearance of certain neurological reflexes is known to be influenced by various factors [20, 43, 44]. Although the maturation of neurological reflexes represents hallmarks of nervous system development [1, 2, 10, 15, 20, 43, 44], relatively little is known about the maturation of these reflexes following prenatal exposure to smoking. The aim of the present study was to investigate the influence of maternal smoking during pregnancy on the early physical and neurobehavioral development of newborn rats. Our hypothesis was that maternal smoking during pregnancy interferes with development and therefore results in a delayed physical and motor development and changed locomotor behavior, which could be traceable with a battery of tests at early stages of the life of rat pups.

## MATERIALS AND METHODS

### Animals

Male and female Wistar rats aged 2–4 month were chosen to be mated, and randomly distributed in smoking and non-smoking group. All animals were maintained under 12 h light/dark cycle with free access to food and water. Animal housing, care and application of experimental procedures were in accordance with the institutional guidelines under approved protocols (No.: BA02/2000-17/2012, University of Pécs). Two female rats and a male rat were mated in one cage. Animals were exposed to whole-body smoke exposure for  $2 \times 40$  min daily from mating until delivery. A TE2 closed-chamber manual smoking system (Teague Enterprises, Woodland) and 4 3R4F research cigarettes (College of Agriculture, University of Kentucky, Lexington, KI) were used to deliver cigarette smoke to animals. This model represents an animal model for passive smoking. Offspring of these female rats were the prenatal smoke-exposed group (PSE).

The control group (2 females and 1 male rat) was housed in another chamber of the same type, but were not exposed to cigarette smoke. Altogether four litters were used, 2 PSE and 2 control. Animals were cross-fostered immediately after birth to minimize litter differences and effects of possible changes in maternal behavior due to smoke exposure.



## Neurobehavioral testing

The assessment of neurobehavioral development was started on the first postnatal day and was carried out daily between 6 and 9 am by the same person (BM) until postnatal day 21 (PND), in a blinded fashion. Neurobehavioral maturation and development of motor coordination were tested using standardized procedures. Weight was measured daily until 3 weeks of age, as well as maturation of physical characteristics such as eye opening, incisor eruption and ear unfolding. Pups were tested for the following signs and reflexes: (1) *Surface righting reflex*: rats were placed in supine position and the time in seconds to turn over to prone position and place all four paws in contact with the surface was recorded. (2) *Negative geotaxis*: animals were placed head down on an inclined grid (45°) of 30 cm. The forelimbs of the pups were placed in the middle of the grid. The day they began to turn around and climb up the board with their forelimbs reaching the upper rim was observed. In cases the animal did not turn around and climb up the board within the observed 30 s, the test was considered negative. From the day of the appearance of the negative geotaxis, the time in seconds to reach the upper end of the board was recorded daily. (3) *Crossed extensor reflex*: the left rear paw was pinched and the animal was observed for the extension of the right leg. The day of disappearance of the crossed extensor reflex in its pure form, when it was replaced by a more complex behavioral response, was noted. (4) *Sensory reflexes*: the ear and the eyelid were gently touched with a cotton swab and the first day of the ear twitch reflex and the contraction of the eyelid were recorded. (5) *Limb placing*: the back of the forepaw and the hind paw was touched with the edge of the bench while the animal suspended, and the first day of lifting and placing the paws on the table was noted. (6) *Limb grasp*: the fore- and hindlimbs were touched with a thin rod, and the first day of grasping onto the rod was recorded. (7) *Gait*: the animals were placed in the center of a white paper circle of 13 cm in diameter, and the day they began to move off the circle with both forelimbs was recorded. In cases when the animal did not leave the circle for 30 s, the test was considered to be negative. From the day of the appearance, the time in seconds to move off the circle was recorded daily. (8) *Auditory startle*: the first day of the startle response to a clapping sound was observed. (9) *Air righting*: the pups were dropped head down onto a bed of shavings from a height of 50 cm, and the day of first landing on four feet was recorded.

## Motor coordination test

Rat pups were tested for the development of more complex motor behavior once a week on the third and fourth week of age.

*Grid walking*: rats were placed on a stainless steel grid floor (20 cm × 40 cm with mesh size of 4 cm<sup>2</sup>) elevated 1 m above the floor. For a 1-min observation period, the total number of steps was counted. *Foot-fault test*: The number of foot-fault errors, when the animals misplaced a forelimb or hindlimb that it fell through the grid, was also recorded during a 1-min period.

## Open-field activity

Animals were observed for locomotor and spontaneous exploratory behavior in an open field at 5 weeks of age as previously described [24, 28, 34]. Pups were placed in an open field consisting of a 42 cm × 42 cm box with 21 cm high walls around. Rats were placed individually in the center, always facing the same direction, and were video-recorded for 5 min. Recordings were



evaluated in blinded fashion. The following parameters of locomotor activity were measured: head lifting, grooming, walking by the wall, or time spent the middle of the box. The time spent in the first zone next to the wall was also measured.

## Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM). The results in appearance of physical and neurological signs as well as daily weights were compared with Student's *t*-test. Improvements in daily performance in righting reflex, negative geotaxis and gait were evaluated by two-way ANOVA repeated measures, whereas daily values were compared by *t*-test. Statistical significance is reported at  $P \leq 0.05$ .

## RESULTS

Only one pup from the prenatal smoke-exposed (PSE) group died on the first day. No animal died in the control group. There was no significant difference in weight gain between the prenatally smoke-exposed and the control group (Fig. 1).

### Neurological signs and reflexes

Most neurological signs appeared slightly later in prenatally smoke-exposed pups. The delay of ear unfolding was significant. In addition, several neurological reflexes, such as hind limb placing, eyelid reflex and forelimb grasp developed noticeably later (Fig. 2). PSE animals had significantly better performances in negative geotaxis throughout the test period (Fig. 3).

### Motor coordination

Among the many motor coordination tests available, the most reliable indicator in our previous studies had been the grid walking/foot fault test [14, 28, 32]. In the grid-walking test, PSE animals had significantly lower total number of steps on the third week of age (Fig. 4). The number of foot faults in ratio to the total number of steps was lower in the PSE group in case of

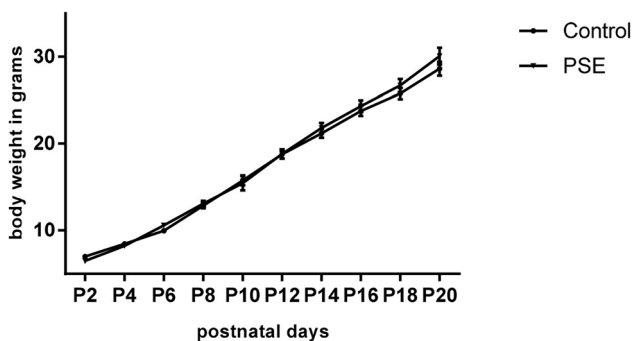


Fig. 1. Weight gain of control and prenatal smoke pups; Control vs. PSE (prenatally smoke-exposed). Values are expressed as average grams  $\pm$  SEM



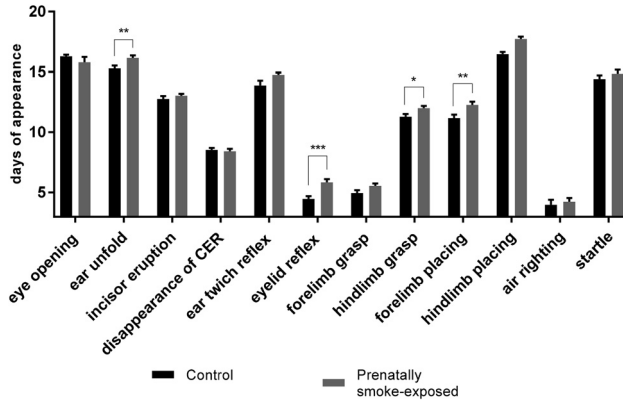


Fig. 2. Appearance of somatic signs and neurological reflexes. Values are expressed as average days  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

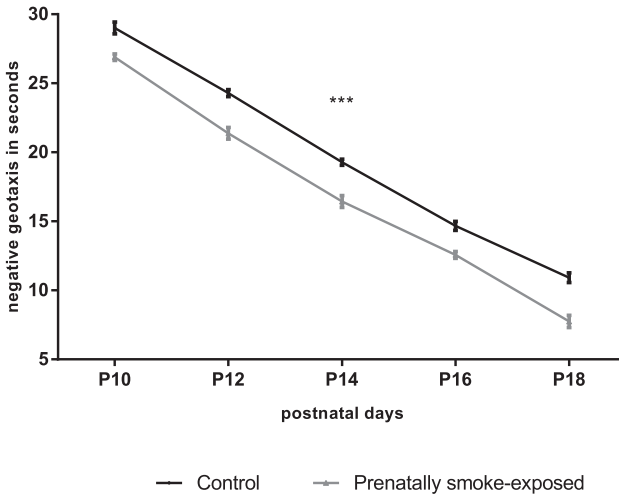


Fig. 3. Daily performance of negative geotaxis reflex of control and PSE pups. Values are expressed as average seconds  $\pm$  SEM. \*\*\* $P < 0.0005$

the left and right forelimb and the right hind limb on the third week of age. PSE pups made significantly less foot faults in ratio to the total number of steps with both of their hind limbs at 4 weeks of age.

### Open field activity

In the open field test, general activity and movement pattern did not show gross differences between the groups. The number of head lifting decreased in both groups during the trials



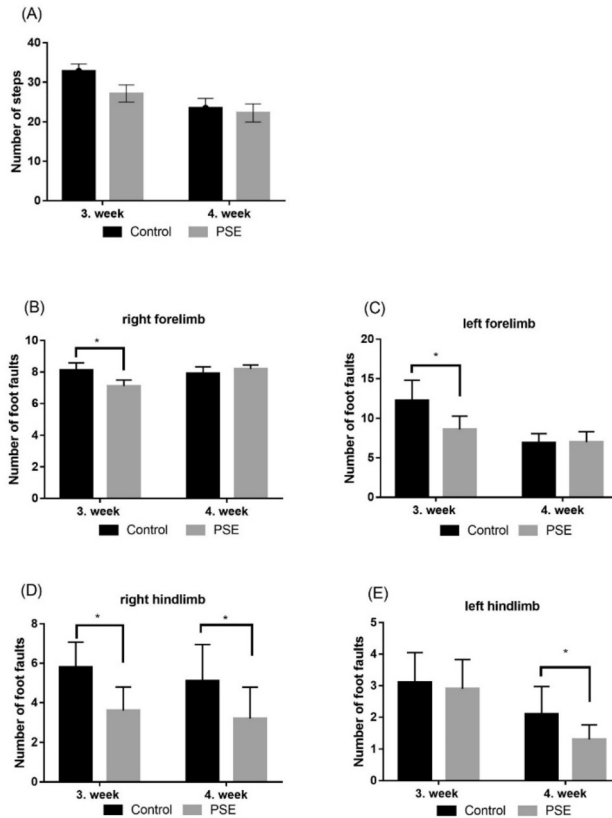


Fig. 4. Performances in motor coordination tests of control and prenatally smoke-exposed (PSE) animals. (A) Number of total steps in the grid-walking test. (B) Number of foot faults in ratio to number of total steps with the right forelimb. (C) Number of foot faults in ratio to number of total steps with the left forelimb. (D) Number of foot faults in ratio to number of total steps with the right hind limb. (E) Number of foot faults in ratio to number of total steps with the left hind limb. Values are expressed as average  $\pm$  SEM. \* $P < 0.05$

monitoring but no significant differences could be observed between the control and PSE animals. The time spent with grooming increased with every trial, but there was no difference between the two groups. Similar pattern was observed in the speed and movement pattern of the animals: no significant differences were found in total rest time, walking near the wall or a subject in the center of the box (data not shown).

## DISCUSSION

Perinatal injuries and exposure to toxic agents can lead to many long-term damages, even at older age, but little is known about immediate consequences [26, 37, 38, 40, 47].



The prenatal period is one of the critical age windows – in addition to early postnatal life and adolescence – that is open to plastic changes and the influence of external agents [34]. Adverse effects such as chemicals in tobacco smoke can cause severe damage with permanent disabilities. Most studies regarding the negative effects of perinatal treatments focus mainly on long-term effects, and most examinations are carried out on adult animals following perinatal injuries. Less data is available on short-term effects and early neurobehavioral changes.

In the present study we showed detectable changes in the maturation of physical parameters and neurologic development of rat pups after prenatal smoking. Although we did not find any difference in birth weight, growth was slightly more dynamic in the prenatally smoke-exposed group. Certain physical reflexes and signs of PSE pups, like eyelid reflex and ear unfolding appeared later compared to the control pups. We also observed a delay in reflexes, indicating neural maturity, like in case of limb placing and grasping reflexes. Maternal exposure to tobacco smoke may lead to some neuromuscular and behavioral deficits in nursing pups.

On the other hand, we observed a remarkably better motility in PSE pups in negative geotaxis testing. In addition, the number of foot faults was lower in case of the pups prenatally exposed to smoke. These findings are in agreement with several studies demonstrating detrimental effects of prenatal smoke exposure [12, 31]. Other studies indicate that rats exposed to variable prenatal stress evolved, in addition to several behavioral anomalies, increased locomotor behavior and stereotypic-like behaviors [49]. In contrast to these, several studies where only one important component of tobacco smoke, nicotine was exposed to pregnant rats, rat pups showed deficits in righting reflexes and the negative geotaxis test [36, 41]. During prenatal smoke exposure only a small amount of nicotine crosses the placenta, which means a lower risk to neurobehavioral deficits. These findings suggest that PSE has more similarities with prenatal stress exposure.

Several toxic agents have been shown to cause altered neurobehavioral development [39]. One of the mechanisms of neurotoxicity is excitotoxicity caused by excessive levels of monosodium glutamate (MSG). MSG is a food additive widely used as a flavoring substance. In contrast to adults, the immature blood-brain barrier in new born rat pups allows significant transport of MSG into the nervous system. In our previous experiments, where MSG was given on postnatal days 1,3,5,7, and 9, mortality was markedly higher in MSG-treated pups than in the control group [28]. We found minor delays in the appearance of certain neurological reflexes (forelimb placing and grasping, air righting) and body weight and length were also significantly lower in the MSG-treated pups. MSG treatment also caused worse performance in motor coordination tests.

Both pre- and postnatal stress models are widely used in neuro-endocrinological and psychiatric research [3, 16, 29]. These stress models may provide important correlation with human psychiatric disorders. Maternal separation of rat pups is a well-known model for early life events as it induces long-lasting changes in several stress-related systems [16, 34]. Interestingly, weight gain and reflex development was faster in male pups exposed to perinatal stress. We found a subtle enhancement in male rats, but only slight delay in females.

Previously we have also shown major retardation in the development of neurological signs and reflexes in animal models of perinatal pathologies like asphyxia and hypoxia. Almost all reflexes were delayed and showed a severe, from one to even 4-day delay, and the appearance of some physical signs was also delayed [22, 28]. Compared to these other insults, prenatal exposure to smoking did not result in such marked differences. We suppose that maternal smoking during pregnancy may cause an increased vulnerability to harmful impacts later in adulthood.





According to the “Developmental Origins of Health and Disease (DOHaD)” hypothesis, which posits that environmental exposures during multiple sensitive periods of development (especially the in utero period) have a lasting impact on health and disease risk, we suppose that maternal smoking during pregnancy may cause an increased vulnerability to harmful impacts later in adulthood [48].

*Conflict of interest:* No conflict of interest.

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