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Lipopolysaccharide injected to pregnant mice affects behavior of their offspring in adulthood

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We studied consequences of maternal immune response on the course of pregnancy and the behavior of adult offspring. Mice in late gestation (day 16–17) were injected with lipopolysaccharide (LPS). Treatment of pregnant mice with high doses of LPS resulted in fetal resorption or stillbirths. Pregnant mice treated with low doses (100 or 300 μg/kg) of LPS gave birth to normal numbers of pups. However, behavior of the offspring was altered. Adult offspring of dams injected at a dose of 300 μg/kg of LPS traveled longer distances in the open field and spent more time in the central part of the arena, than mice in the control group. Female mice of this group spent more time in open arms of the elevated plus maze, in comparison to female control mice. Results of the Morris water maze test showed impairment of spatial learning and memory in male offspring born to LPS-injected dams. Furthermore, in the nest building test adult mice born from LPS challenged pregnancies constructed worse quality nests, which points to the presence of hippocampal dysfunction. These findings indicate that maternal bacterial infections during pregnancy may alter offspring behavior in adult life.

Key words: prenatal inflammation**,** behavioral tests, anxiety, anxiolytic-like behavior, learning and memory

INTRODUCTION

Numerous clinical studies have indicated that adverse conditions in pregnancy such as maternal bacterial infection with inflammation and products of immune reaction reaching placenta, increase the risk of psychiatric diseases in the offspring adult life (Sham et al. 1992, Wilkerson et al. 2002, Dantzer et al. 2008, Miller et al. 2009, Brown and Derkits 2010). Another effect of activation of the inflammatory pathway in the pregnant female is the increased risk of preterm birth. Several animal models have been developed to investigate mechanisms of the influence of maternal inflammation on the fetus. Administration of lipopolysaccharide (LPS) to pregnant rodent dams is one of the models that are used to study developmental changes in brain mechanisms leading to changes in behavior of the adult progeny (Wang et al. 2006, Beloosesky et al. 2010, Boksa 2010, Burd et al. 2010).

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LPS is a toxic cell membrane component of gram-negative bacteria (Jacob et al. 1977) that activates the immune system *via* macrophages. Binding of LPS to the toll-like receptor-4 (TLR-4) of macrophages activates transcription factors (e.g. nuclear factor kappa B (NFκB), activating synthesis and release of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF-α). Either LPS itself, or products of maternal immune reaction may activate production of cytokines in the placenta and fetal liver (Urakubo et al. 2001). Changes in protein levels of these cytokines in brains of fetuses depend on the gestational day of LPS administration and on its dose. It has been found that the level of cytokines in the placenta and amniotic fluid is increased starting from 2 to 24 hours after treatment of pregnant rats with high doses (2.5 mg/kg) of LPS (Urakubo et al. 2001, Ning et al. 2008). Also in the fetal brain cytokine expression is increased, if high doses of LPS are used (Cai et al. 2000, Liverman et al. 2006), while low doses of LPS do not have that effect (Gayle et al. 2004, Ashdown et al. 2006). It is well established that cytokines in the fetal brain modulate development of neurons and their func-

tioning (for review see Deverman and Patterson 2009). They are involved in both neuroprotective and neurodegeneration processes (Licinio 1997).

LPS and cytokines affect activity of the hypothalamus-pituitary-adrenal (HPA) axis (Grinevich et al. 2001, Ellis et al. 2005, Perez-Nievas et al. 2010) changing levels of stress hormones and their receptors (Liu et al. 1997). These infection-associated changes in the fetus may lead to behavioral modifications or disorders in the adult life of animals. It has been reported that spatial learning and memory were impaired in adult rats that were prenatally exposed to LPS (Lante et al. 2008, Hao et al. 2010). Interestingly, rats that were exposed to *Escherichia coli* on the postnatal day 4 (P4) exhibited memory impairments only at 16 months of age (Bilbo 2010).

Also in adult mice that were born to LPS-treated dams some aspects of learning and memory were impaired (Golan et al. 2005). However, almost all experiments in mice were conducted on various strains of inbred mice and behavioral phenotype is different in different mouse strains (Krackow et al. 2010). For example, the exploratory behavior is high in Swiss and Wild strains compared to that in C57/BL6N and DBA/2, while risk assessment is lower in Swiss and Wild stains compared to C57/BL6n and DBA/2 stains (Parmigiani et al. 1999). Therefore, results of various manipulations (e.g. inducing the immune response) on behavior may depend on the strain.

The aim of this study was to determine whether administration of LPS to outbred mice dams in late gestation affects some aspects of the offspring behavior in their adult life. As the first step, we defined the range of LPS doses that produce toxicity in Swiss mice at gestational days E16–E17.

METHODS

Animals

Swiss mice of both sexes were used in this study. Animals were housed under 12/12 hours light/dark cycle with *ad libitum* access to food and water. All efforts were made to minimize the number of animals and their stress. Our experimental procedures complied with the Polish Law on Experimentation on Animals that implements the European Council Directive 86/609/EEC and also with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved and supervised by a Local Ethics Committee in Warsaw.

Pregnant mice were injected intraperitoneally with a single dose of either 100 μg/kg, 300 μg/kg, 1000 μg/kg or 2000 μg/kg of LPS (*Escherichia coli* serotype 0111:B4, Sigma) on gestational day 16–17.

At the age of 3 weeks pups born from either LPS-treated dams or control untreated dams were weaned and since then they were kept in groups of 4–5 mice of one sex per cage. Each group consisted of individuals from different dams to avoid a litter effect.

Behavioral tests

Starting from the age of about three months 84 mice (48 mice born to LPS treated pregnancies and 36 mice born from untreated, control dams) were subjected to a battery of behavioral tests. Minimum 2 days interval was given between behavioral tests (Paylor et al. 2006).

Open field test

Mice were placed in the apparatus (50 × 50 × 45 cm) and allowed to explore it for 5 minutes. After each test the open field arena was cleaned with 25% ethanol. Movements of animals were recorded with a video camera placed above the apparatus and analyzed off line by the View Point system (Viewpoint SA, France). The distance traveled and time spent by the mice in either the center or periphery of the open field was measured.

Elevated plus maze test

Apparatus used for the elevated plus maze test consisted of two open arms (40 × 6 cm) and two closed arms of the same size connected with a central platform (6 × 6 cm). The maze was placed 50 cm above the ground. Mice were allowed to move freely in this apparatus for 5 minutes. After testing each mouse the apparatus was cleaned with 25% ethanol. Time spent in the open and closed arms as well as time spent on the central platform were analyzed by the View Point system.

The nest building test

Mice were transferred from their cages to individual cages containing wood chip bedding and a pressed

stack of oval cotton pads weighing 3.9 g. They were allowed access *ad libitum* to food and water. After 18 h nests that these mice built from the cotton pads were categorized to grades from 1 (worst) to 5 (best). We followed the procedure described by Deacon (2006).

Morris water maze

Mice were trained in the circular pool (150 cm diameter) in which a visible platform was located in one of its quadrants. Constantly visible cues were present on walls of the room where the experiment was performed. On days 1–4 mice were given 4 trials per day, lasting 1 minute each. Latency to reach the platform was measured in each trial and averaged for each training day. On the day 5 (the probe trial) the platform was removed and mice were subjected to 2 trials lasting 1 minute each. Latency to reach the quadrant where the platform was located during previous training days was measured. Behavior of animals in the pool was recorded and analyzed with the EthoVision system (Noldus Information Technology, the Netherlands).

Statistical analysis

Data were analyzed using either one- or two-way ANOVA followed by *post-hoc* multiple pair-wise comparisons between genders and groups with Holm-Sidak method. Statistical significance level was set at $P<0.05$.

RESULTS

Treatment with high doses of LPS

Seven pregnant mice were injected with high doses of LPS intraperitoneally once, at gestational day 16–17. Three of them were treated with 1000 μg/kg of LPS and 4 were injected with 2000 μg/kg of LPS. All injected females delivered stillbirth pups or did not give birth.

Pregnant dams treated with either 100 or 300 μg/kg of LPS brought up normal numbers of pups (from 7 to 17). The weight of pups born from dams treated with two doses of LPS did not differ from those of control group mice born to untreated dams (1.70 g in the group

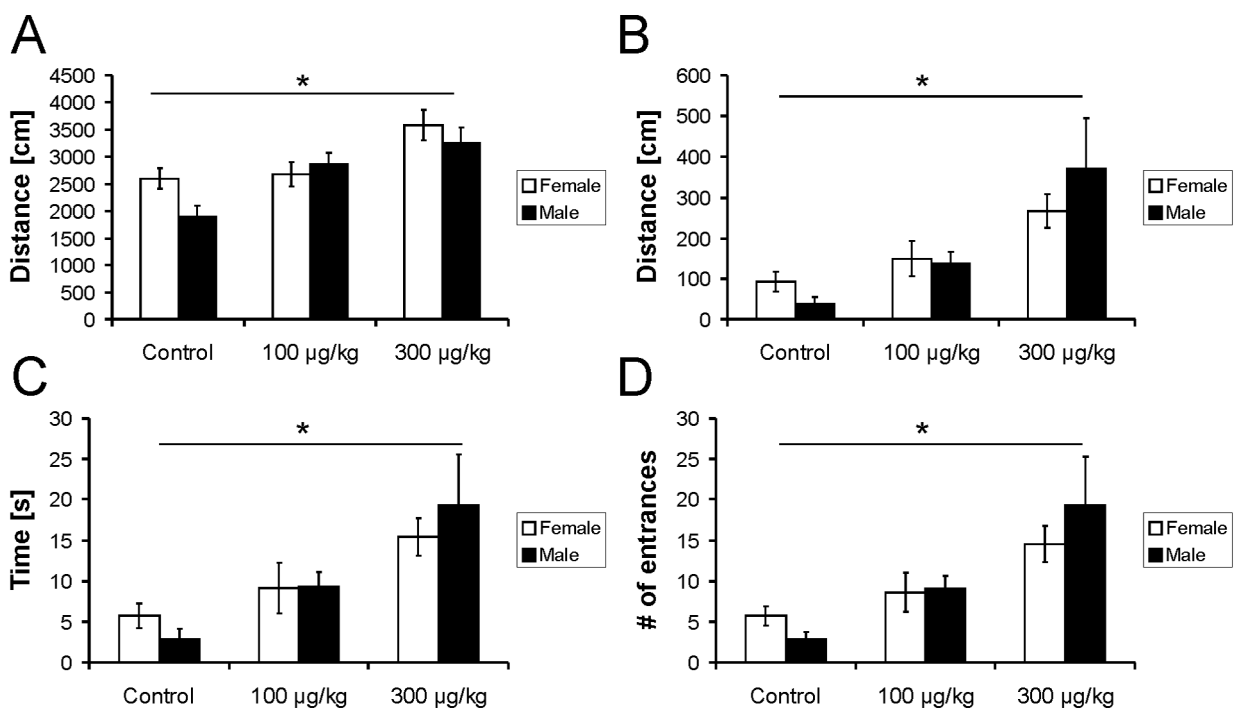

Fig. 1. Activity of mice in the open field test. (A) Total distance traveled by female (light columns) and male (dark columns) mice over the whole area of the apparatus. (B) Distance traveled over the central area of the apparatus. (C) Time spent by females and males in the central area of the apparatus. (D) The number of entrances to the central area of the apparatus by female and male mice. Note that significant differences were observed between control group and offspring born to LPS 300 μg/kg injected dams. * Significant difference (at least $P<0.05$).

treated with 100 µg/kg of LPS; 1.56 g in the group treated with 300 µg/kg of LPS and 1.62 g in the control group). Maternal behavior in LPS-treated dams such as collecting pups in a single place and crouching over pups was the same as in the control dams.

Behavioral tests

Two way ANOVA showed that there was no gender difference in the open field test within the tested groups of mice ($F_{1,83}$=2.0, P=0.16). Assessment of the mice behavior in the open field test indicated that mice from different groups showed various levels of basal anxiety. When compared to control mice, effects of LPS treatment on locomotion ($F_{2,83}$=11.5, P<0.001) and time spent in the center of the arena ($F_{2,83}$=13.1, P<0.001) were visible in offspring born to LPS 300 µg/kg injected dams. Adult mice born to LPS injected dams traveled longer distances in the whole open field arena and also in its central part than control (Fig. 1A,B). They also spent more time in the central part of the open field than mice from the control group (Fig. 1C) and entered the central part more frequently (Fig. 1D).

In the elevated plus maze test almost all mice of both sexes spent more time in the closed arms of the apparatus than in its open arms, except female mice born to 300 µg/kg and male mice born to 100 µg/kg that spent equal time in closed and open arms (Fig. 2A,B). *Post-hoc* multiple comparisons of the time spent in open arms, following ANOVA ($F_{2,82}$=5.59 P=0.005) by the Holm-Sidak method showed significant sex difference in the group of mice born to LPS 300 µg/kg treated dams. Female mice born to LPS (300 µg/kg) treated dams spent more time in the open arms than female mice from the control group (Fig. 2A). However there was no difference between male mice born to LPS-treated pregnancies and control untreated group of male mice (Fig. 2B).

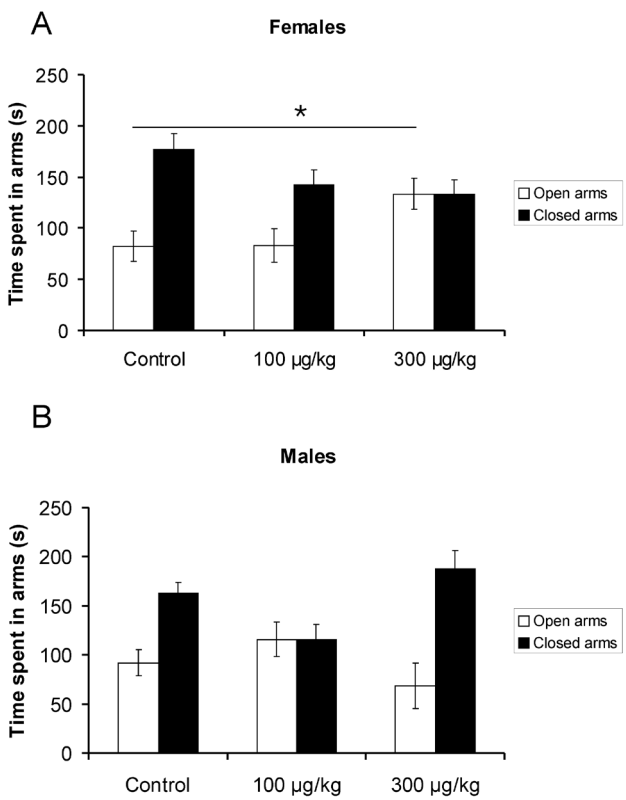

Fig. 2. Activity of mice in the elevated plus maze test. Time spent by females (A) and males (B) in the open (light columns) and closed (dark columns) arms of the apparatus. Note that significant difference was observed only between female mice born to control and LPS 300 µg/kg injected dams. * Significant difference (at least P<0.05).

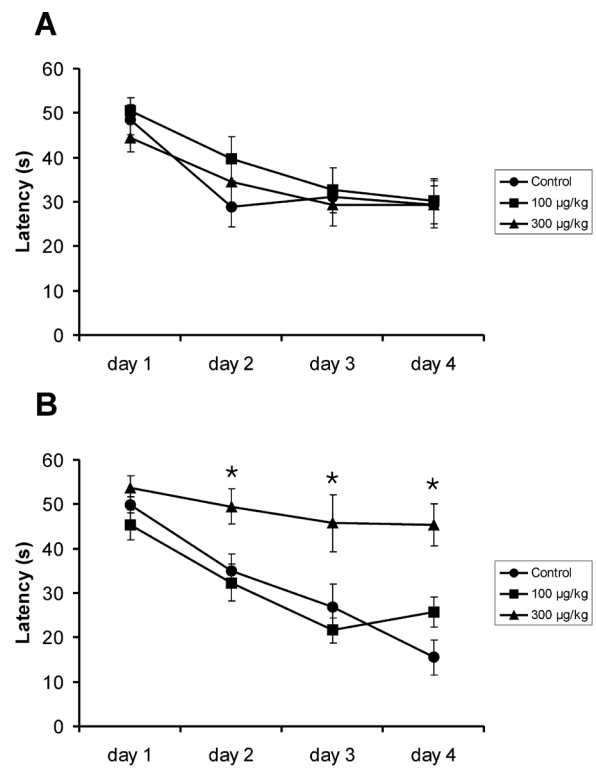

Fig. 3. Performance of mice in the Morris water maze task. (A, B) Latency to reaching the platform during four consecutive days of training. (A) females, (B) males. Note that significant difference was observed in latency to reach the platform on day 2–4 between male offspring born to LPS 300 µg/kg treated dams and those of the control group. *Significant difference (at least P<0.05).

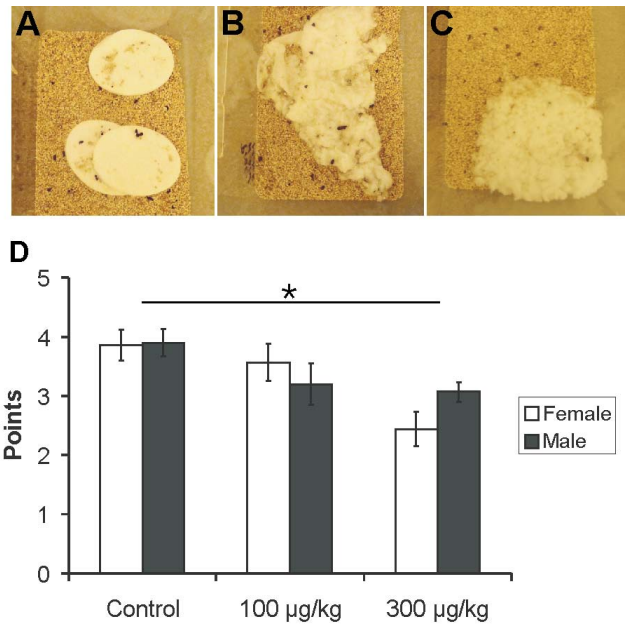

Fig. 4. Performance of mice in the nest building test. Representative pictures of mice nests that were classified as: 1 point (A), 3 points (B), 5 points (C). (D) Average numbers of points scored by nests built by mice from each group. Significant difference was observed in the quality of built nests between offspring born to control and LPS 300 µg/kg treated dams. * Significant difference (at least $P<0.05$).

We used the water maze to test learning and memory functions of the investigated mice. During four training days mice were given four trials each day. Mice from all groups except of adult males born to dams treated with 300 µg/kg of LPS learned to reach the platform (Fig. 3A,B). On the first day of training male mice of control group took more time to reach the platform than on each subsequent day ($P<0.001$). In contrast, males born to LPS 300 µg/kg treated dams did not shorten time to reach the platform in the course of training (Fig. 3B). Holm-Sidak method (all pairwise multiple comparison procedures) showed a significant difference in latency to get the platform on day 2–4 between male offspring born from LPS 300 µg/kg treated dams and those of the control group.

In the probe trials (day 5) almost all mice of both sexes, including those of the control group, increased latency to get to the quadrant where the platform was located before, in comparison to latency of reaching platform on the day 4.

The nest building test was performed to estimate general integrity of hippocampal functions or their disturbance. Nest building is an important natural behavior occurring without intervention or presence of the experimenter. Two way ANOVA showed that there was a statistically significant difference in the quality of constructed nest in the mean values among the different groups ($F_{2,83}=6.96$, $P=0.002$). The mean score of the quality of the nests was significantly higher in the offspring of control dams than in the offspring of dams treated with 300 µg/kg of LPS. None of the mouse born from LPS-treated dams built a nest for the highest score (5 points, Fig. 4D), whereas the majority of control mice (about 70%) built high quality nests, scoring from 4 to 5 points (Fig. 4C).

DISCUSSION

In this study we investigated the effect of LPS, a major component of the cell wall of Gram-negative bacteria, inducing inflammatory responses that influence the course of pregnancy in mice and brain functions of the offspring, when they grew up. We found that intraperitoneal injection of LPS in high doses (1000 µg/kg or more) to mouse dams on gestational day 16–17 induced fetal resorptions or stillbirths. Maternal exposure to LPS at the dose of 300 µg/kg or less was not toxic and pups were normally born and grew to adulthood. However, maternal infection can affect the behavior of adult offspring.

Behavioral changes evoked by prenatal exposure to LPS

LPS treatment of pregnant mothers disturbs various developmental processes of the brain and changes behavioral of adult offspring born from those pregnancies (Fortier et al. 2004, Romero et al. 2007, Coyle et al. 2009). To assess the balance of the anxiety-motivated behavior and exploratory activity motivated by curiosity of the adult mice born from LPS influenced pregnancies, we analyzed their behavior in the open field and elevated plus maze tests. We found that mice born to dams treated with 300 µg/kg of LPS showed higher locomotor activity in the open field test than mice from the control group. What more, they entered the center of the open field more frequently and for a longer time. These data indicate that LPS-treated mice are hyperactive and less anxious than control animals. In a recent study Girard and coauthors (2009) have reported that they did not observe differences in the

open field test between control rats and adult offspring born from LPS-treated pregnancies. However, it should be noted that in that study rats were investigated when they were 15 to 25 days old, therefore they were immature. Thus, discrepancy between our results and those of Girard and others (2009) may depend on the age the animals tested and/or on the species differences. Our preliminary results (not shown in this study) demonstrate clear age-dependent differences in behavior of P30 and P90 mice tested in the open field test. Development of mental impairment with age in mice prenatally exposed to LPS was shown by Wang and colleagues (2010).

In the elevated plus maze test behavior of the male and female mice prenatally exposed to LPS was strikingly different. Namely, females spent more time in open arms than control females, whereas males spent more time in closed arms than control males. Therefore, while behavior of females prenatally exposed to LPS showed their reduced level of anxiety, behavior of males showed stronger anxiety-driven reactions. We have no explanation yet for this finding, but sex differences in the anxiety-motivated behavior have been described earlier (Meng and Drugan 1993, Alonso et al. 2000, Faraday 2002). Many behavioral measures are also highly dependent on the investigated strain (Mineur and Crusio 2002, Wahlsten et al. 2003, Milner and Crabbe 2008, Bailey and Crawley 2009, Miller et al. 2010). For example, some mouse strains exhibited a higher anxiety level in the open field test, but not in the elevated plus maze (Montkowski et al. 1997). These authors suggested that the open field is a stronger fear-inducing stimulus than the elevated plus maze. Our results provide new evidence supporting this conclusion.

A number of studies demonstrated that prenatal exposure to LPS might impair memory and learning (Golan et al. 2006, Lante et al. 2008, Coyle et al. 2009). We used the Morris water maze task to investigate learning and memory abilities in the 3–4 months old offspring of mothers exposed to LPS in pregnancy. We found that male progeny of pregnant mice injected with LPS exhibited memory and learning deficits when tested in the Morris water maze, while females were not impaired. Gender differences in the prenatal influence of LPS have been found by Wang and coworkers (2010). One possible explanation is that in the Swiss strain that we used, impairment of mental abilities due to exposure to LPS in the fetal period starts earlier in males than in females. Another possibility is that males are more vulnerable during development. These hypotheses require further investigation. Interestingly, adult progeny of mice injected with LPS during early gestation (day 8) did not show deficits in spatial learning and memory (Coyle et al. 2009), probably because at that stage neural plate is composed of proliferating stem and precursor cells and there are no neurons (Peeters et al. 1996).

In addition, the nest building test showed disorganization of one of natural behaviors that is highly dependent on the integrity of hippocampus (Deacon 2006) in LPS-exposed group. Impairment of performance in the water maze test in male offspring of LPS-treated mothers was similar, though less severe than in animals with large hippocampal lesions (Deacon et al. 2002). Therefore, disturbance of hippocampal functions in LPS-exposed male mice must be one of the important mechanisms of impairment of their learning. These results are similar to those of blocking glucocorticoid (GR) receptors in the hippocampus by administration of the GR antagonist (de Kloet et al. 1999, McGaugh and Roozendaal 2002), which impaired consolidation of new information.

Possible mechanisms of the influence of maternal exposure to LPS on brain development and functioning in the offspring

We found that higher doses of LPS (1000 μg/kg and 2000 μg/kg) were toxic and almost always resulting in the death of all newborn pups. Either the pups' immune system was not fully developed yet and it could not cope effectively with the injected LPS or products of immune reaction of their mother or the inflammatory processes involving placenta hampered survival and development of the fetuses.

There are several possible mechanisms by which LPS-injected in pregnant mice dams influences fetuses. Ning and coauthors (2008) have investigated levels of one of pro-inflammatory cytokines, TNF-α in the maternal serum, amniotic fluid and brain of fetuses. Pregnant mice were injected with LPS (500 μg/kg) on gestational day 17 and were sacrificed 90 minutes later. At that short time the level of TNF-α in the maternal serum as well as in the amniotic fluid and fetal brain increased, even though expression of TNF-α mRNA in the fetal liver and brain did not change. This indicates that the TNF-α protein may be transferred from maternal circulation to the amniotic fluid and

then the fetal brain. Interestingly, when pregnant mice were first treated with a very low dose of LPS (10 μg/kg) and then injected with a high dose (500 μg/kg) 4–24 h later, the level of TNF-α in fetuses was lower. Most probably the maternal immune system was mobilized by the first dose and inactivated the second dose of antigen. Administration of LPS increased also expression of other pro-inflammatory cytokines such as IL-1β and IL-6 in the fetal mouse brain (Gayle et al. 2004, Golan et al. 2005, Liverman et al. 2006).

These LPS induced factors could influence brain development, as already two days after LPS administration to pregnant rat dams, the rate of apoptosis was highly increased in the subventricular zone of E20 fetal brains (Paintlia et al. 2004). Some physiological and structural changes in the brain of rat offspring may continue after birth. Decreased rate of neurogenesis was observed in the hippocampal dentate gyrus of two-weeks old rats born to dams injected with 100 μm/kg of LPS at gestational days 15/16 or 18/19 (Cui et al. 2009). However, the rate of dentate gyrus neurogenesis in two month old (P60) offspring has not been changed. On the other hand, some consequences of exposure to LPS and/or inflammatory processes may develop with age. For example, dendritic length of pyramidal neurons of the hippocampal area CA1 was normal at P10 and P35, but reduced at P60 in rats born to mothers treated with LPS during pregnancy (Baharnooni et al. 2009). Maternal exposure to LPS is also associated with development of hypomyelination or dopaminergic and serotoninergic dysfunctions in later life of the offspring (Paintlia et al. 2004, Rousset et al. 2006, Wang et al. 2006, Wang et al. 2009).

Thus, administration of LPS to pregnant mice at mid-gestation may affect various developmental processes *via* activation of the mother immune system and possibly activation of the fetal immune system itself. The adverse effects of exposure to LPS on the fetal brain development may last long, influencing also processes that start and continue later in adult life.

CONCLUSIONS

In conclusion, we found that adult mice of both sexes born from dams treated with LPS (300 μg/kg) during pregnancy show hyperactivity and lower level of anxiety-driven behavior in the open field. Male mice born to LPS-treated dams tested in the water maze were not able to retrieve information about placement of the platform during 4 training days. This syndrome of hyperactivity, reduced anxiety and impaired learning ability resembles the attention deficit hyperactivity disorder (ADHD) in humans. Therefore, LPS model may be useful in further investigation of brain mechanisms of this disorder.

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