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Specific neurodevelopmental damage in mice offspring following maternal inflammation during pregnancy

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Abstract

Intrauterine inflammation is a major risk for offspring neurodevelopmental brain damage and may result in cognitive limitations and poor cognitive and perceptual outcomes.

Pro-inflammatory cytokines, stimulated during inflammatory response, have a pleotrophic effect on neurons and glia cells. They act in a dose-dependent manner, activate cell-death pathways and also act as trophic factors.

In the present study, we have examined in mice the effect of short, systemic maternal inflammation on fetal brain development. Maternal inflammation, induced by lipopolysaccharide (LPS) at gestation day 17, did not affect morphogenic parameters and reflex development during the first month of life. However, maternal inflammation specifically increased the number of pyramidal and granular cells in the hippocampus, as well as the shrinkage of pyramidal cells, but not of the granular cells. No additional major morphological differences were observed in the cerebral cortex or cerebellum. In accordance with the morphological effects, maternal inflammation specifically impaired distinct forms of learning and memory, but not motor function or exploration in the adult offspring. The specific deficiency observed, following maternal inflammation, may suggest particular sensitivity of the hippocampus and other associated brain regions to inflammatory factors during late embryonic development.

Keywords: Cytokine; Hippocampus; Learning; Memory; Morphogenesis; Neurotrophic factors

1. Introduction

Intrauterine inflammation is a major risk for pre-term delivery and offspring neurodevelopmental brain damage, which may result in neurological disorders and mental disability (Dammann et al., 2002; Saliba and Henrot, 2001). Maternal intrauterine infection is thought to affect the immature brain by the induction of pro-inflammatory cytokines. The presence of cytokines in the three relevant maternal/fetal compartments (uterus, fetal circulation, and fetal brain) and the ability of the cytokines to cross boundaries (placenta and blood–brain barrier) between these compartments has been confirmed (Fidel et al., 1994).

The pro-inflammatory cytokines, tumor necrosis factor-α (TNFα), interleukin-1 (IL-1) and IL-6 have been associated with intrauterine infection, pre-term delivery, neonatal infections and neonatal brain damage. IL-1,

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IL-6, and TNFα share several biological functions, even though they are structurally different and bind to different receptors (Dinarello, 2000). In the brain, these cytokines are expressed in both glia cells and neurons. In addition to their function in the immune response, these cytokines modulate neuron development and function. TNFα is involved in the regulation of neurite growth (Neumann et al., 2002), affect neuronal survival (Yang et al., 2002; Barker et al., 2001) and regulate AMPA receptors expression (Beattie et al., 2002). Recently, the involvement of TNFα in the regulation of hippocampal development and function was demonstrated (Golan et al., 2004; Aloe et al., 1999). IL-1b is involved in the regulation of synaptic plasticity; the expression of the IL-1beta gene was specifically increased during long-term potentiation (LTP), and the blocking of LTP by NMDA receptor antagonist prevented the increase in IL-1 beta gene expression (Schneider et al., 1998). The administration of lipopolysaccharide (LPS) to pregnant rats increased the expression of TNFα and IL-1b mRNA in a fetal brain in a dose-dependent manner and modified the glia cell populations a week after the application (Cai et al., 2000).

At gestation day 17 in the mouse fetus most of the brain regions are already created. At this age in the cerebral cortex and hippocampus the neurons are already formed, however, neuronal migration, maturation and synaptogenesis still undergo (Bayer, 1980a,b; Altman and Bayer, 1990).

Based on the above data, we hypothesized that maternal inflammation at gestation day 17, will perturb offspring accurate neurogenesis and will be reflected by offspring behavioral impairment. We suggest the neurotrophic factors as possible mediators of the inflammation process.

Our results demonstrate that maternal administration of LPS altered the learning and memory performance in the adult offspring. This effect was associated with specific histopathological damage in the hippocampus region and with the increased expression of NGF and BDNF in the thalamus of the adult offspring.

2. Material and methods

2.1. Study design

Pregnant Jackson Black C-57 mice were used. All pregnant mice were treated at gestation day 17. The mice were randomly assigned to one of two groups: (1) saline injections (intraperitoneal; i.p.) – control group, fetus and newborn examinations; (2) *E. coli* LPS (Sigma Inc, St. Louis, MO, USA) injections (0.12 μg/g mouse/100 μl, i.p.) – study group, fetus and newborn examinations.

The mouse colony was maintained in a 12:12-h light/dark schedule; food and water were provided ad libitum. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and the guidelines from the Israeli Council on Animal Care and approved by the Ben-Gurion University of the Negev Animal Care and Use Committee.

2.2. Surgical procedure

On gestation day 17 (gestation day 1 was determined 24 h after mating), at 3, 6 and 9 h following treatment, six mice from each group were anesthetized with Ketamine concomitantly with Rompun administered (i.p.). After adequate anesthesia, dissections were performed on the selected mice and maternal brains and spleens and five fetal brains per mouse dissected were isolated and deep-frozen at −80 °C for later analysis. The heads of the remaining fetuses were removed and immersed in 4% PFA for histological analysis. At postnatal days 1 (P1), P7, P14, P21 and 8 months, the offspring were anesthetized and dissected. Their brains were rapidly removed into ice-cold artificial cerebrospinal fluid (ACSF) and the brain regions were separated: cortex, hippocampus, cerebellum and thalamus. Brain tissue was deep-frozen at −80 °C for later analysis. One to seven-day-old mice were anesthetized by hypothermia, while older mice were anesthetized by the i.p. administration of Ketamine and Rompun. For histological analysis, offspring at P1, P7, P14, P21 and 8 months were anesthetized and transcardially perfused with paraformaldehyde (PFA) 4%.

2.3. Phenotype aspects examined in newborn mice

Two newborns per litter were tested daily during the first month of life for their general phenotypic and morphogenic aspects, such as: body weight, hair growth, day of eyelid opening and teething.

2.4. Immunoassay

IL-6, NGF and BDNF were examined in brain homogenates, brains were homogenized in 1 ml phosphate buffer containing protease inhibitor cocktail tables 1836145 complete (Roche Diagnostics, Mannheim, Germany) using a specific ELISA (R&D Systems, Minneapolis, MN, USA). The sensitivity for IL-6 was 32 pg/ml. The sensitivity of the NGF ELISA was 4 pg/ml and 47 pg/ml for the BDNF. The data were normalized to protein concentration in the sample, measured by Bio-Rad protein assay (Bio-Rad laboratories).

2.5. Histology

Sections from paraformaldehyde-fixed (4%) paraffin-embedded tissue were used. Four-micrometer-thick

sagittal sections 0.5–0.6 mm from the midline were mounted on saline-coated slides and dried at 37 °C. Afterwards, coded sections were stained by Nissl staining and images (60 μm × 60 μm) were sampled in an Olympus IX-70 microscope equipped with a SuperCam video camera (Applitec, Israel) by experimenter, blind to the experimental groups. The analysis was performed using 'NIH Image' software (Wayne Rasband, NINDS, NIH). The following parameters were measured: cortex width and length, motor cortex cell density and size, corpus callosum width, cerebellum area, cerebellum lobe length, Purkinje cell density, molecular layer thickness, hippocampus area, granular cell-layer width, length and cell density, pyramidal cell-layer width, cell density and cell size.

2.6. Behavioral examination

2.6.1. Reflex development in newborn mice

Righting reflex was measured in seconds, as the time required for a newborn laying on its back to right itself on all four limbs (Crawley, 1999). *Rotarod* (adjusted for neonates, Golan et al., 2004; Levav et al., 2004) tests the ability to hold the rotating bar at a rate of four cycles per minute. The duration on the rotarod prior to the falls was recorded. *Locomotion on inclination*, the ability of newborn mice to climb on an inclined board, was examined at slopes of 45°, 70° and 90°.

2.6.2. Adult mouse behavior

Open field task – performance was tested in an open field arena: a 65 cm diameter gray wall, 30 cm high, over a period of 5 min. The following variables were observed: rearing (times), grooming (times), center /perimeter time and quantity of defecation delivered (Henderson, 1967). *Hole board* – the number of holes into which the mice insert their noses during a given 5-min interval was measured (Lister, 1987). *Hind paw footprint* measures ataxia and gait abnormalities. The hind paws were dipped in nontoxic black paint. The mice were then placed at one end of a dark tunnel. The bottom surface of the tunnel was lined with white paper. The mice walked down the tunnel leaving their footprints. Stride distance and variability were measured. *Balance beam* – a beam, 8 mm in diameter and 70 cm long, was horizontal and elevated. Enclosed escape boxes were placed at the ends of the beam. The mice were placed in the center of the beam. Three trials were made. The times required to reach the box, and an upright position and duration on the beam were measured (Crawley, 1999). *Vertical pole* – the mice were placed in the center of grooved wooden poles 12 mm and 18 mm in diameter and 1 m long. The poles were held in a horizontal position, then gradually lifted to a vertical position and were held there for 1 min. The times elapsed until the mice fell off the pole were measured (Crawley, 1999). *Smell sensation* – a small amount of cheese was buried in the cage litter. The speed of detection of the buried food was measured as an index of olfactory ability (Crawley, 1999). *Hot plate* – the mice were placed on a horizontal surface that was heated to 50°, the times were measured until a rear paw was licked. Three trials were held at 30 min intervals (Crawley, 1999). *Morris water maze* – mice were trained to swim to a hidden platform in the water maze (Morris, 1984). The maze consisted of a 150 cm diameter, 40 cm high, circular pool filled with milk and maintained at 25–26 °C. The 10-cm diameter platform was 0.5–1 cm below the surface of the milk. The test was performed as described before (Golan et al., 2004). *Probe test* – after the last trial on the third day, the platform was removed from the pool. The mice were placed in the pool starting at a location opposite the platform and were allowed to swim for 60 s. The amount of time spent swimming in the quadrant where the platform had been located was recorded as another index of spatial learning. All the data was recorded on videotape and a blind analysis was done. *Visible platform training* – the spatial cues were removed and the location of the platform was made visible by the use of clear water, the marking of the platform by a blue flag and its elevation to 1 cm above the surface of the water. The remaining details were similar to those described for the hidden platform test. *Object recognition test* (Malleret et al., 2001) – during the training trial, the mice were placed in a novel environment (55 cm diameter, 20 cm high). Two (of three possible) plastic toys (between 5 and 7 cm) that vary in color, shape and texture were placed in specific locations in the environment 30 cm apart. Two different combinations of object pairs were used. The test was performed as described before (Levav et al., 2004). *Passive avoidance* (Ader et al., 1972) measures how they learn to avoid aversive stimuli. The mice were placed on a vibrating platform 9 cm in diameter. When the mice stepped off the platform, a 5 mA, 5 s, foot-shock was delivered. On the first day, the mice were trained until they achieved 120 s on the platform three times. On the second day, the mice were placed on the platform and the time until they stepped off was recorded. Conditioning was completed when the mice avoided stepping off the platform, based on this criterion, mice were determined as "learn" or "do not learn".

2.7. Statistical analysis

To evaluate the effect of maternal inflammation SPSS software, differences between the average values in the experimental groups were tested using Student *t*-test, or analysis of variance (ANOVA), dichotomy variables such as learn or not learn in the passive avoidance test, were tested by chi-square test.

3. Results

3.1. Inflammatory response in fetal brains following maternal inflammation

Induction of cytokines in fetus following maternal administration with LPS was previously demonstrated (Cai et al., 2000). In the present study we examined the pro-inflammatory cytokine, IL-6 (as a representative of pro-inflammatory cytokines), to confirm the inflammatory effect of LPS in our system. We administrated a single dose of LPS that induced cytokine production in the fetal brain without inducing pre-term delivery. When a dose of 0.3 μg/kg of LPS was injected into pregnant mice at gestation day 17, the average day of delivery was 17 ± 0.1 ($n = 6$, $P < 0.001$) and 19 ± 0.1 ($n = 26$, $p > 0.05$) following an LPS dose of 0.12 μg/kg, compared to 19 ± 0.1 ($n = 16$) in saline injection. The administration of 0.012 μg/kg LPS did not influence the levels of IL-1b in fetal brain (data not shown). The number of offspring per litter was similar in both groups; 7 ± 0.4 in the saline injected group compared to 7 ± 0.5 in the LPS (0.12 μg/kg) injected group. IL-6 in the maternal spleen was significantly elevated 3 h following an LPS injection (Fig. 1A) and gradually declined and returned to control levels after 9 h. A slight increase in the IL-6 level in the maternal brain was observed 3 h following the maternal LPS injection (Fig. 1B). In fetal brains, IL-6 levels were significantly increased 3 h after an LPS treatment. These levels declined to control levels within 6–9 h (Fig. 1C). Thus, the injection of 0.12 μg/kg LPS to pregnant mice at gestation day 17 induced a transient increase of the pro-inflammatory cytokine IL-6 in the embryos brain.

3.2. Effect of LPS administration to pregnant mice on newborn development

The morphogenic development of newborns to mice injected with LPS was comparable to that of the control group in body weight, brain weight, hair growth, teething and eyelid opening. The rates of increase in body and brain weight during the first month of newborns life was not affected by maternal LPS administration, data are presented in Table 1. No significant difference was observed between the offspring of the two examined groups. The development of motor reflexes, as examined via rotarod and the ability to climb on an inclining slope, were also similar in both groups. The development of the righting reflex showed a trend of earlier and faster development during the first postnatal week in offspring belonging to the maternal inflammation group, while

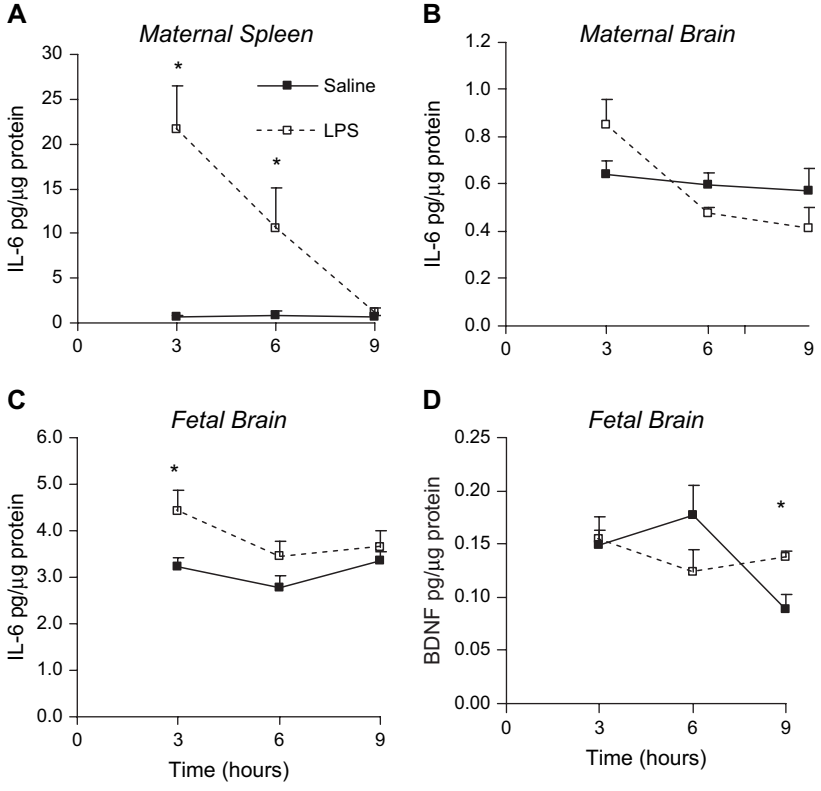

Fig. 1. Maternal injection of LPS increases maternal and fetal cytokine expression. The effect of maternal injection of 0.12 mg/kg LPS on IL-6 expression in maternal spleen (A), maternal brain (B) and fetal brain (C) was examined 3–9 h following the application. BDNF expression in fetal brains was also examined at the same time points (D). $n = 3$–6 at each point, t-test, $^*P < 0.05$.

Table 1
Offspring brain and body weight during the first month of development

Age	1		7		14		21	
Group	Saline	LPS	Saline	LPS	Saline	LPS	Saline	LPS
Brain weight	0.1 ± 0.02	0.1 ± 0.01	0.25 ± 0.01	0.2 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01
Body weight	1.2 ± 0.1	1.3 ± 0.1	3.4 ± 0.1	3.1 ± 0.1	6.7 ± 0.2	7.2 ± 0.2	7.5 ± 0.2	8.4 ± 0.3

Maternal LPS did not influence brain and body weight in the newborns. Weight is expressed in grams. The values are presented as mean ± sem; $n = 10–34$ offspring in each time point for each group.

later, during the second postnatal week, the percentage of newborns righting themselves and the time required to do so was equal in both groups (Fig. 2).

3.3. Maternal inflammation modulated BDNF and NGF ontogeny in offspring

The short and long-term effects of maternal inflammatory response on the expression levels of BDNF were examined in fetal brains. Three and six hours following maternal LPS treatments, the levels of BDNF in fetal brain homogenate were not affected (Fig. 1D). However, an increase in BDNF levels was observed 9 h following the LPS treatment. In a different set of experiments, the brain homogenate of newborns of mice injected with LPS on the same date and with the same dosage was examined for BDNF levels. During postnatal development (P1, P7, P14, P21 and P270), BDNF levels, in all examined brain areas, increased with age, as depicted in Fig. 3. In each brain region, the data were normalized to BDNF levels in the control group at P7. In the cerebral cortex (Fig. 3A), an increase in BDNF levels was detected at P21 and P270 in both study groups. In the hippocampus (Fig. 3B), BDNF levels were stable in both groups during the first two weeks and thereafter became elevated. In both these areas, at P21 the BDNF levels were significantly lower in the LPS group, compared to the control group. However, in adult brains, no significant difference in BDNF levels was observed in those brain areas. In contrast, in the thalamus (Fig. 3C), higher levels of BDNF were observed in brains of P270 offspring from the maternal

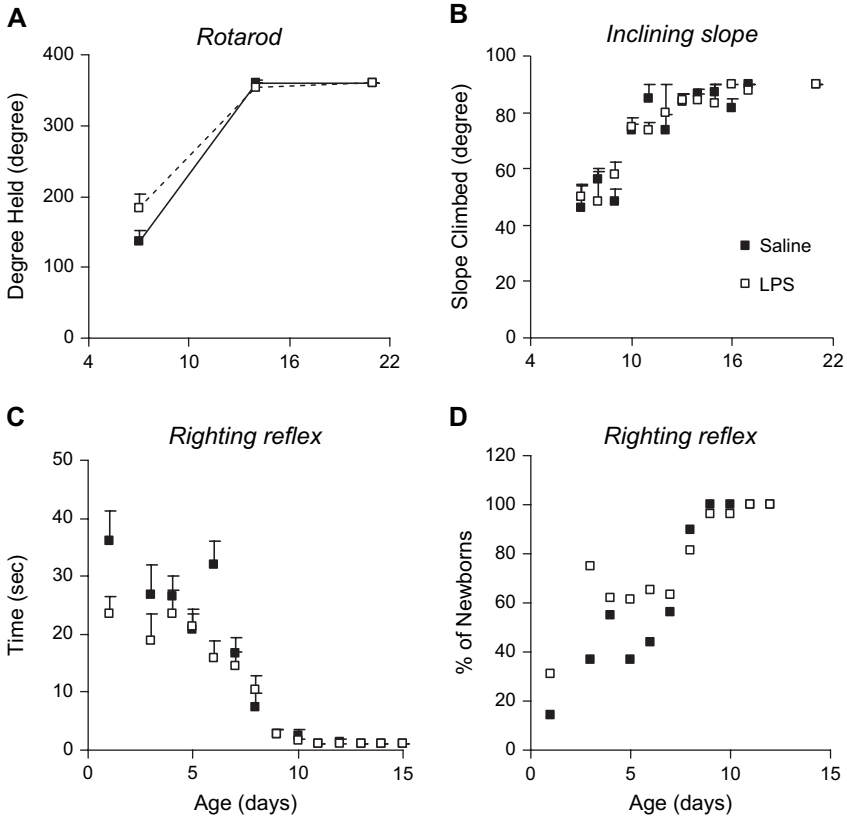

Fig. 2. The effect of maternal inflammation on newborn motor reflex development. The maternal inflammation effect on newborn performance in the rotarod (A), locomotion on inclining slope (B) or righting reflex time (C) and the percent of newborn righting (D) was tested during the first month of life: $n = 15–20$ at each point.

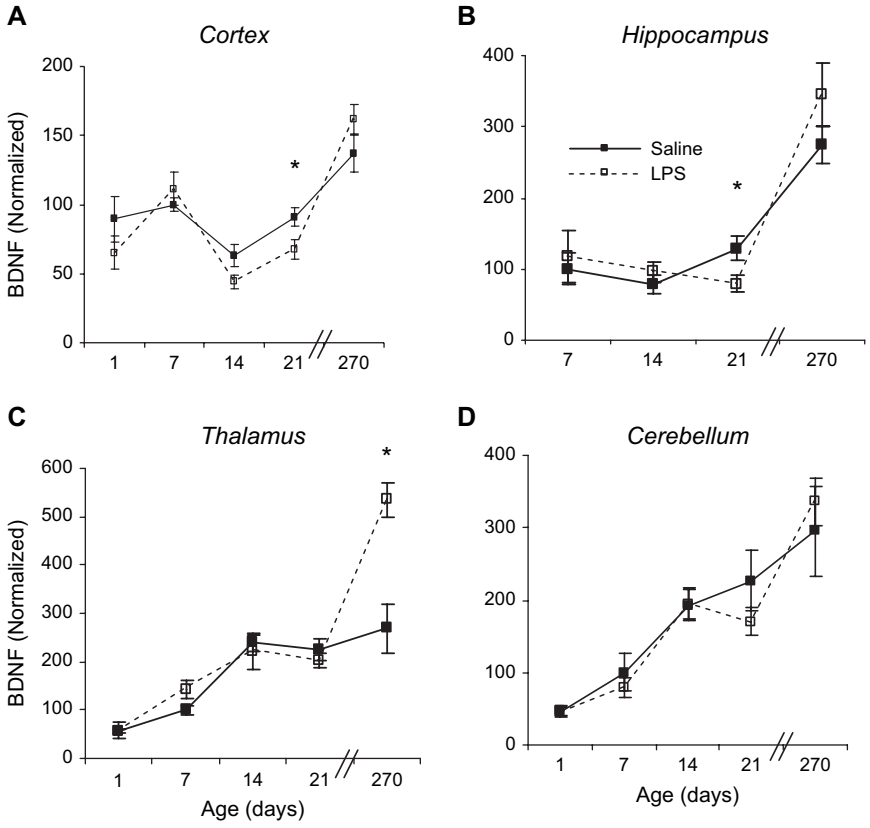

Fig. 3. The effect of maternal inflammation on BDNF levels in newborn brains. BDNF ontogeny was examined in offspring at P1, P7, P14, P21 and P270 in the cerebral cortex (A), hippocampus (B), thalamus (C) and cerebellum (D). Data are normalized to BDNF levels in control offspring at P7 in the particular brain region: $n = 4$–8 at each point, t-test, $^*P < 0.05$.

inflammation group, compared to the control group. In adult offspring from the maternal inflammation group, the level of BDNF was 4 ± 0.2 pg/mg protein ($n = 6$), compared to 2 ± 0.3 ($n = 4$) in the control group ($P < 0.01$, t-test). The levels of BDNF in the cerebellum (Fig. 3D) increased with age in a similar manner in both study groups.

The expression of NGF was examined in offspring's brain homogenates. We present data of postnatal offspring, since the NGF levels in the embryos' brains were below the detection sensitivity of the kit used. In the cerebral cortex region, the highest levels of NGF were measured at P7 (Fig. 4A, data normalized to P7 in the control group). At P14, newborns from the maternal inflammation group showed significantly lower levels of NGF, compared to the control group, but this difference disappeared at the later ages. No difference in NGF levels in the hippocampus (Fig. 4B) or cerebellum (Fig. 4D) was observed between the LPS and control groups. In the thalamus region, significantly elevated levels of NGF were observed at P7 and in the adult offspring (P270, Fig. 4C) from the maternal inflammation group (1 ± 0.1, $n = 6$, $P < 0.01$, t-test), compared to offspring from the control group (0.6 ± 0.1, $n = 4$).

Exposure to inflammatory agents at gestation day 17 caused mild long-term consequences to the levels of BDNF and NGF in the cerebral cortex, hippocampus and cerebellum regions. A significant increase in the levels of BDNF and NGF was measured in the thalamus of adult mice offspring from the maternal inflammation group, compared to the control mice.

3.4. Maternal inflammation modified offspring brain morphology

Sagittal brain sections of newborns from the maternal inflammation group at E17 were examined for the developmental consequences of the treatment. Gross measurements of different brain areas were analyzed at P7, P14 and in the adult offspring brains (P270). Results depicted in Table 2 demonstrate that the left ventricle area, CC width, cerebral cortex length, width and layer 1 width were similar in both experimental groups, during development and in the adult offspring (except for a transient difference in cerebral cortex length at P14). The area of the cerebellum region, as well as the length of cerebellar lobules 2–10, were similar in both groups (for details see Supplementary data). Moreover, we measured the thickness of the molecular layer and

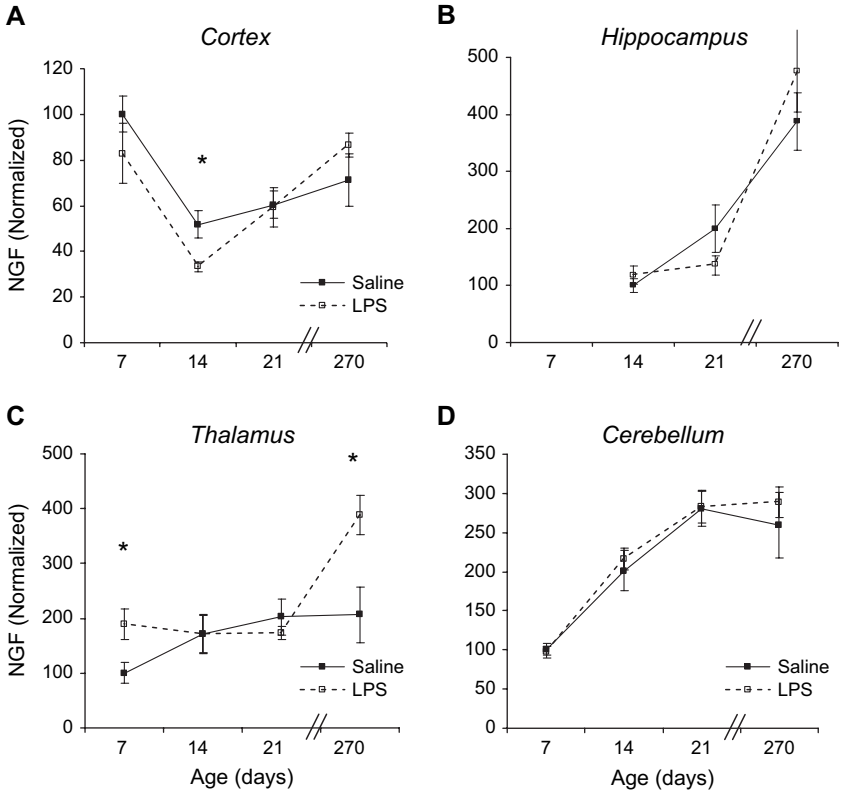

Fig. 4. The effect of maternal inflammation on NGF levels in newborn brains. The NGF ontogeny was examined in offspring at P7, P14, P21 and P270 in the cerebral cortex (A), hippocampus (B), thalamus (C) and cerebellum (D). Data are normalized to NGF levels at the homogenate of control offspring at P7 in the particular brain region, except for the levels in the hippocampus, which were normalized to control P14 levels: $n = 4–8$ at each point, t-test, $^*P < 0.05$.

Purkinje cell density in cerebellum lobules 3 and 10 and found no difference between the experimental groups in both parameters at P14 and in the adult mouse brain. In contrast, developmental differences were observed in the dentate gyrus beginning at P14. Specifically, the length of granular cell layer (inner and outer blades, Table 2) was longer and thicker in the adult offspring of the maternal inflammation group, compared to the control group. In the CA1 region of the hippocampus, developmental consequences of the treatment on pyramidal layer thickness were observed at early ages, P7 and P14, but not in the adult offspring. To further explore the source of the difference observed in cell-layer thickness of the main excitatory cells of the hippocampus, granular cells and pyramidal cells, we have examined cell density and size in both the dentate gyrus and the CA1 region. Pyramidal cell density was higher in the adult offspring of the maternal inflammation group, compared to the control group, in all three subfields examined. In addition, pyramidal cell size was smaller, as depicted in Fig. 5. In the dentate gyrus, although a thicker granular cell layer was measured, no difference in cell density or size was detected (Fig. 5C,D). This may indicate that the change in layer thickness was a result of the increase in the amount of cells, rather than a change in single cell parameters.

A detailed analysis of the neurons in three sub-regions of the motor cortex (M2; layers 2–3, layer 4 and layers 5–6) was performed. A reduced number of neurons was observed in all layers in the young P14 offspring belonging to the maternal inflammation group, compared to the controls. However, this difference did not reach significance. In the adult offspring of both groups, a remarkable reduction in cell number was observed, as a consequence of developmental selection. Nevertheless, a tendency to increased cell number in offspring belonging to the maternal inflammation group was observed. A significantly higher number of pyknotic cells (type 3) was counted in layers 2–3, 5–6, but not in layer 4, as demonstrated in Fig. 6C–E. Cells in offspring from the maternal inflammation group had a tendency to swell, as reflected in cell size. In layers 2–3, the cell area was 251 ± 128 μm in offspring of the control group, compared to 269 ± 168 in offspring from the maternal inflammation group ($P = 0.09$, t-test). In layer 4, the cell area was 264 ± 145 and 290 ± 166 μm, respectively ($P = 0.02$, t-test). While the cell areas in layers 5–6, 242 ± 130 and 247 ± 118 μm in the control and

Table 2
Maternal inflammation effect on the measures of different brain regions

Age		P7		P14		Adult	
Region	**Treatment**	**Mean**	**Sem**	**Mean**	**Sem**	**Mean**	**Sem**
Ventricle	Saline	-	-	3205	1346	22193	1995
	LPS	-	-	2098	1097	19821	2492
CC	Saline	760	50	492	58	707	5
	LPS	631	101	556	51	745	51
Ctx length	Saline	8731	566	9651	492	10254	297
	LPS	8526	294	11386	403	10502.1	214
Ctx width	Saline	2437	127	2630	188	2735	82
	LPS	2286	77	3039	92	2717	77
Ctx Layer 1	Saline	194	16	278	39	257	15
	LPS	167	20	289	14	259	11
DG - in - length	Saline	1399	91.2	1930.3	100.4	1802.3	125
	LPS	1362.2	80	1328 **	74	2155	121
DG-out-length	Saline	1484	114	1885	292	2682	134
	LPS	1393	115	2151	17	3058	127
DG-width	Saline	359	47	246	31	165	11
	LPS	294	29	345	34	211*	4
CA1-width	Saline	205	12	161	9	162	9
	LPS	161*		197**	9	155	6

CC, corpus callosum; Ctx, cerebral cortex; DG, dentate gyrus. $n = 3–4$ animals for each group and age. The numbers in micrometer presented are mean $\pm$ sem, statistical significance was tested by Student t-test for two populations with different variance at significance levels of $*P < 0.01$, $**P < 0.05$.

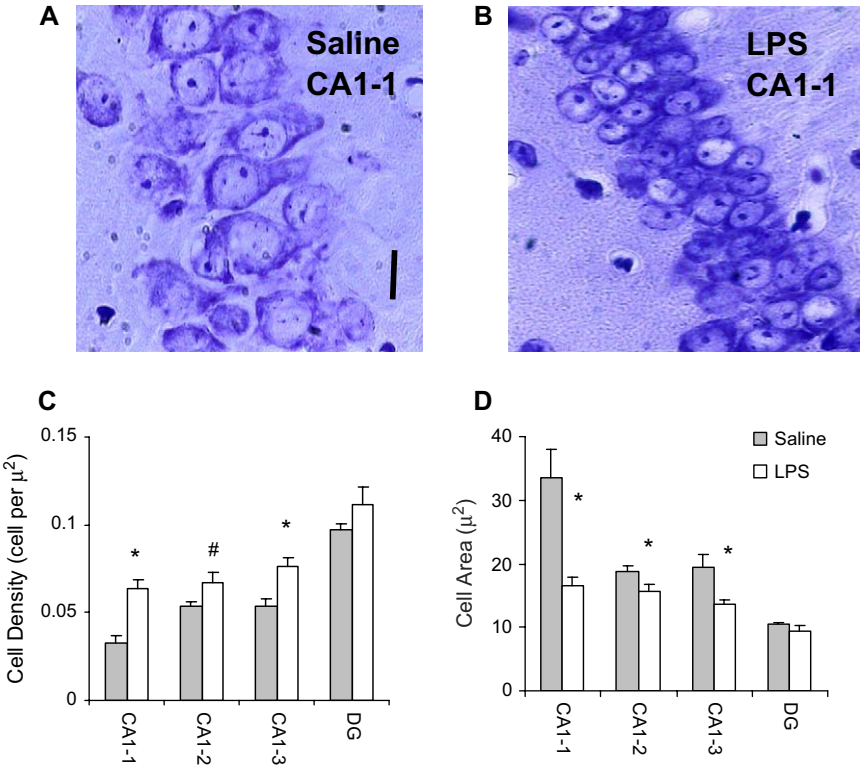

Fig. 5. The effect of maternal inflammation on cell number in the hippocampus. Pyramidal cells in the CA1 region of the hippocampus of adult offspring of saline (A) and maternal inflammation groups (B) were analyzed. Cell density, as measured in three sub-fields of the CA1 region and one field of the DG, are presented (C) and the cell area in the same fields (D): $n = 3$–4 in each group, at each point, $^*P < 0.05$, $\#P < 0.08$, t-test. Calibration bar −10 μm.

maternal inflammation groups, respectively, were similar. Thus, we detected changes in cell size and an increase in pyknotic cells in the motor cortex following maternal inflammation.

3.5. Behavioral consequences of maternal inflammation in adult offspring

3.5.1. Exploration

Behavior was examined in an open field arena in both experimental groups, in order to search for general behavioral consequences in adult offspring. The time mice spent in the arena perimeter vs. time spent in the center of the fields was similar in offspring from the maternal inflammation and control groups, 3.44 ± 0.38, $n = 21$ and 3.44 ± 0.38, $n = 24$, respectively (Fig. 7A). A comparable number of moves in the maternal inflammation and control groups, 57 ± 3 and 54 ± 3, respectively, were measured. In addition, the number of rearing and grooming activities in a time period of 5 min was observed. These results indicate no general difference in offspring behavior, exploration or mobility between both groups. To further explore possible differences in mouse exploration following maternal inflammation, offspring were tested on a hole-board. Offspring from the maternal inflammation group explored 31 ± 2 holes, compared to 32 ± 2 holes explored by the control group, indicating no difference in exploratory behavior (Fig. 7B).

3.5.2. Motor function

An examination of motor function, coordination and motor strength was performed. In the 'balance beam' test, all the mice from both groups increased their ability to hold on to the beam and arrived at the escape boxes with training (Fig. 7D) in a similar manner. An additional test used for the study of motor function and strength was the 'vertical pole'. On poles, 12 mm and 18 mm in diameter, mice from both groups performed equally in endurance on the pole and the percent of mice that succeeded in holding on to the pole for 60 s (Fig. 7C). A careful analysis of hind paw footprints showed a similar stride length in offspring from the maternal inflammation and control groups (681 mm and 651 mm, respectively). However, smaller variance in the stride length was observed in the maternal inflammation offspring, 8 mm, compared to 13 mm in the control offspring ($P < 0.001$, F test).

3.5.3. Sensory function

The accurate development of sensory function in both study groups was similar for the senses of smell and pain. In the control group, 71% found the hidden piece of

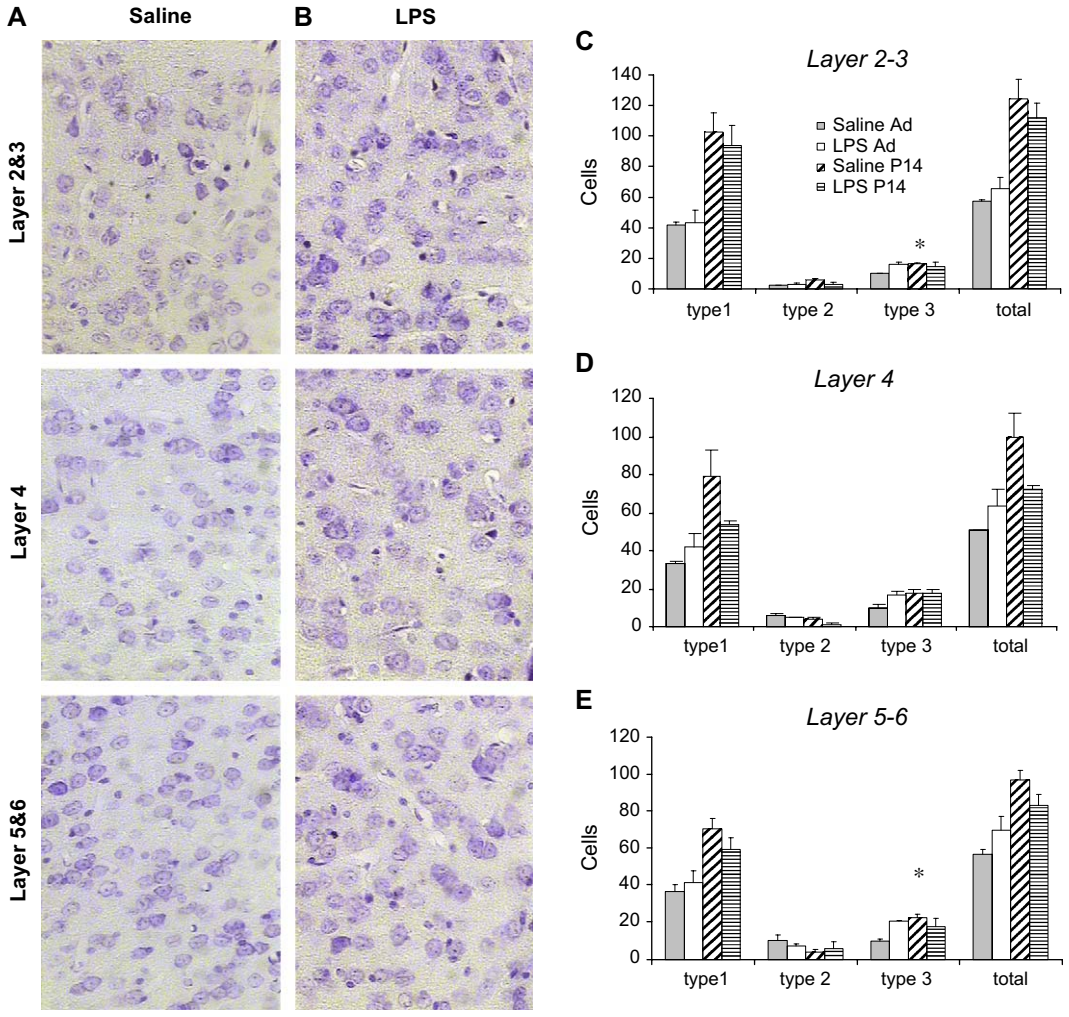

Fig. 6. The effect of maternal inflammation on motor cortex (M2) histology. A detailed analysis of cell number in the M2 motor cortex in the brains of P14 and adult (Ad) offspring of control (A) and the maternal inflammation group (B) are demonstrated. The cells in layers 2–3 (C), layers 4 (D) and layers 5–6 (E) were categorized as neurons (Type 1), epithelia cells (type 2) and pyknotic cells (type 3): $n = 4$ animals in each day and group, three sections from each animal were analyzed. $^{*}P < 0.01$, t-test.

cheese, compared to 83% in the maternal inflammation group. Among the offspring that found the cheese, the time required for offspring of the maternal inflammation group was 38 ± 6 s, compared to 38 ± 6 s for the control group. Pain sensation was tested by means of a 'hot plate' apparatus. Similar reaction times were recorded for both groups, 25 ± 1 s and 24 ± 1 s, for the control and maternal inflammation groups, respectively.

3.5.4. Learning and memory

Offspring were tested by several tasks that examine aspects of learning and memory. Spatial learning in the 'Morris water maze' demonstrated a similar learning profile for both groups, during the training episodes and in the retention test, as depicted in Fig. 8A,B. However, in the cued version of this test, learning the association between a local cue (flag) and the platform was slower in the maternal inflammation offspring during the first two training days, compared to the controls. In the retention test, maternal inflammation offspring spent a longer time in the quarter where the platform used to be in previous days, although the associative cue was not present. This may indicate that offspring from the maternal inflammation group did not memorize the association between the local cue and the location of the platform (Fig. 8C,D). In an additional test of memory, 'Object recognition', mice learn to distinguish two objects different in size, shape and color. The memory of the familiar object was examined 24 h later. Offspring from the maternal inflammation group had more interactions with the new object on the second day, compared to the control group (11 ± 1 vs. 9 ± 1, LPS, and 8 ± 1 vs. 8 ± 1, control). This may indicate that offspring from the maternal inflammation group prefer the new object, compared to the controls. Fig. 8E shows the ratio between the time mice spent with the familiar vs. the new object on each test day.

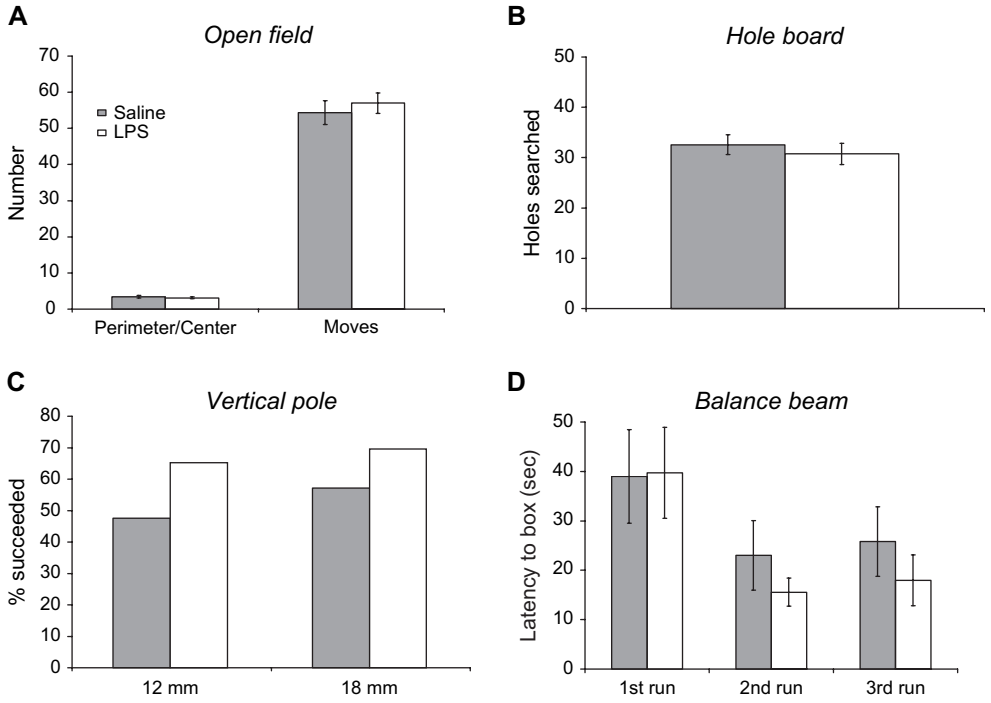

Fig. 7. The effect of maternal inflammation on the exploration and motor function of offspring. The ratio of exploration time on the perimeter vs. the center, the number of moves in the 'Open field' arena (A) and the number of holes explored in the 'Hole-board' (B) are shown. Motor strength, balance and coordination were examined via the 'Vertical pole' (C) and 'Balance beam' (D) tests. The percentages of success in holding vertical poles (12 and 18 mm wide) and the times required to arrive to the escape box in the 'Balance beam' test are shown: $n = 21$ and 24, for saline and maternal inflammation groups respectively.

Learning to avoid aversive stimuli was examined in the 'Passive avoidance' test. Twenty-four hours following training, offspring were tested on their memories of foot-shock avoidance. Fifty-two percent of the offspring in the control group learned to avoid stepping off the platform, compared to 25% of the maternal inflammation offspring (Fig. 8F). To confirm that these differences were not affected by a variation in the sensitivity to pain, all the offspring were tested using the 'hot plate', as described above.

Altogether, the results of these behavioral tests demonstrate that maternal inflammation causes a specific impairment of distinct categories of learning and memory.

4. Discussion

The short, systemic maternal inflammation by the i.p. administration of LPS at E17 produced particular long-term effects in the adult offspring, as detected by specific alterations in hippocampus histology and the impairment of distinct aspects of learning and memory. These findings may result from a variation in the sensitivity of different regions in the fetal brain to products of the inflammatory response. Among the proteins produced by the inflammatory response are the pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6. In addition to the stimulation of a cell-death program in the neurons, both TNFα and IL-1 were shown to affect neuron survival (Yang et al., 2002), growth (Neumann et al., 2002) and AMPA receptor expression (Beattie et al., 2002), as well as synaptic plasticity (Cunningham et al., 1996; Tancredi et al., 1992, 2000; Schneider et al., 1998). Moreover, we have previously demonstrated the involvement of TNFα in morphological aspects of hippocampal development in TNFα-KO mice (Golan et al., 2004). In the present study, exposure of the developing brain to increased levels of the pro-inflammatory factors IL-6 was limited in time (Fig. 1). Although the inflammatory response may last for additional hours or days, its intensity declined in the absence of continuous stimulation of the immune response.

The inflammatory response may induce cell death in the developing brain (Cai et al., 2000; Dammann et al., 2002). Neurotrophic factors, such as NGF and BDNF, are known for their protective action against cell death and their expression is up-regulated in response to injury (Van Eden and Rinkens, 1994; Sofroniew et al., 2001; Zhou and Shine, 2003). The increase in BDNF levels, observed 6 h following the elevation of IL-6 in fetal brains, may be part of the regenerative response stimulated by the cell-death signals (Chaisuksunt et al., 2003) or a result of a direct regulation by cytokines

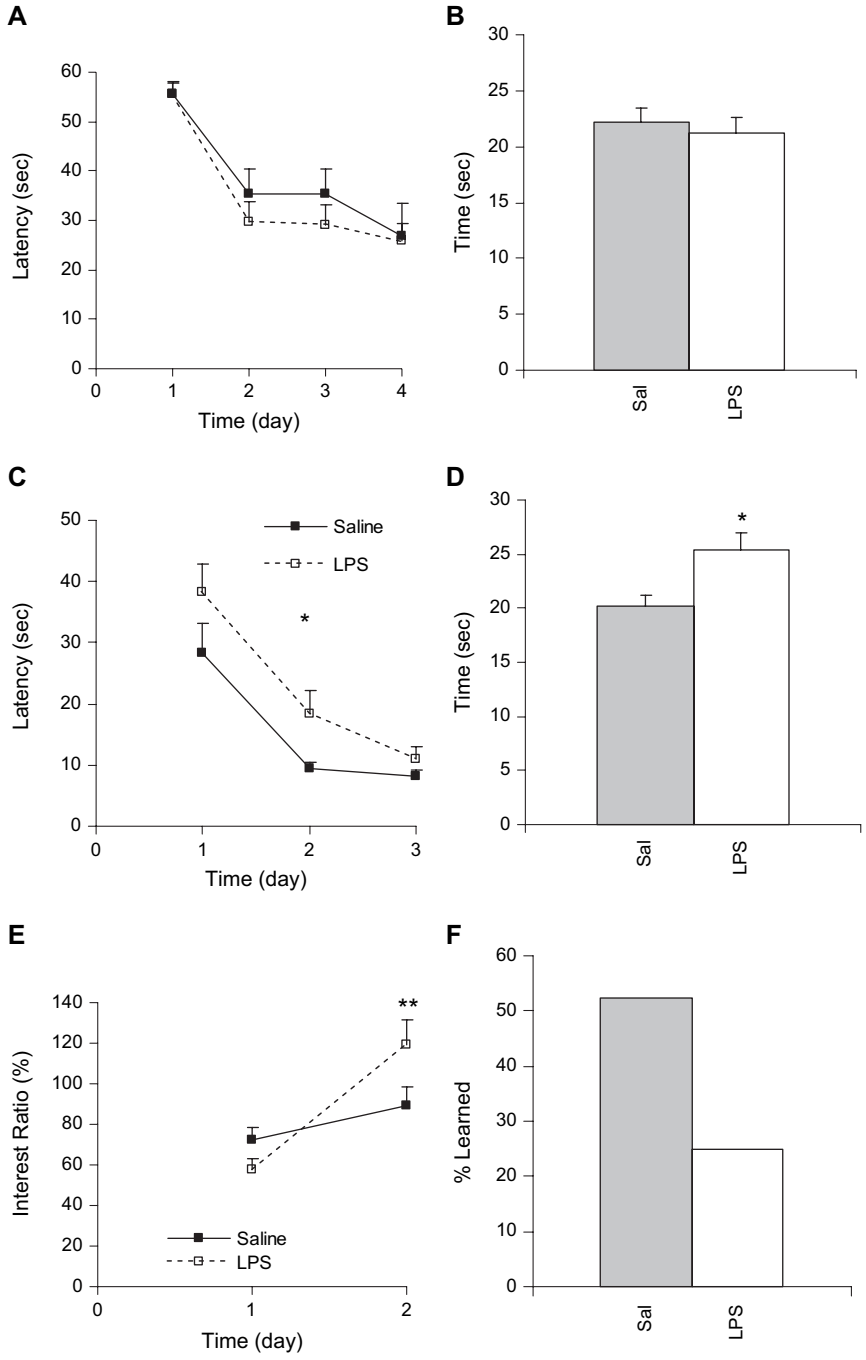

Fig. 8. Maternal inflammation modifies specific forms of learning and memory. Spatial learning in the 'Morris water maze' is defined as the time taken to find the platform (A) and the time spent in the quarter where the platform was located in the probe test (B). Associative learning in the 'Morris water maze' is defined as the time taken to find the platform during the days of training (C) and the time the mice searched in the quadrant where the platform was located in absence of the local cue in the probe test (D). Offspring performance in the 'Object recognition' test is given as the percent of interactions with the novel vs. novel objects in the first day and the percent of interactions with the new vs. the familiar object in the second day (E). (F) The percent of offspring that learned to avoid stepping off the platform in the 'Passive avoidance' test: $n = 21$ and 24, in saline and maternal inflammation groups, respectively. *$p < 0.05$ and **$p < 0.001$, t-test, #$P < 0.06$, chi-square test.

(Rothwell and Hopkins, 1996; Juric and Carman-Krzan, 2001; Villoslada and Genain, 2004). However, three days following the inflammatory response (in P1), we could not detect any effects on BDNF levels in any of the examined brain regions. It is possible that the elevated levels of NGF, in the thalamus region of maternal inflammation offspring at P7, are also a regenerative response of the tissue. Overall, maternal inflammation did not affect the developmental profile of BDNF and NGF in newborn brains. Long-term

modulation of these factors following maternal inflammation was restricted to the thalamus region.

Although the examined neurotrophic factors in the hippocampus region of the offspring were not affected by maternal inflammation, consistent impairment of the morphological parameters in the hippocampus was observed throughout development and clearly in the adult hippocampus. At the age of E17, the CA1 region had already accomplished cell proliferation and the neurons had undergone extensive maturation, dendrite formation, synaptogenesis (Bayer, 1980a,b; Altman and Bayer, 1990; Tyzio et al., 1999) and spinogenesis (Sorra and Harris, 2000). LPS and the induced pro-inflammatory cytokines were expected to trigger programmed cell death in the fetal brain (Bell and Hallenbeck, 2002; Cai et al., 2000). However, we observed an increase in the number of cells in both CA1 and dentate gyrus in the treated group. This may reflect a stimulation of the regeneration process in the injured tissue, resulting in enhanced cell proliferation or reduced developmental apoptosis, which may be regulated by increased levels of BDNF (Cheng et al., 1997; Han et al., 2000). The enhancement of astroglia proliferation, but not microglia, was demonstrated 8 days following a higher dose of maternal (E18) LPS administrated to rats (Cai et al., 2000). Whereas we do not have a quantification of direct neuronal staining, we assume, based on cell structure and the layer organization, that the vast majority of cells counted in the CA1 pyramidal cell layer were neurons. Moreover, differences in pyramidal cell size in the CA1 region may indicate differences in the microenvironment surrounding the cells. In the DG, the addition of new granular cells is a continuing process in the developing and adult hippocampus (Gould et al., 1998; Van Praagh et al., 2002). We found an expanded number of granular cells that may be either an immediate or a long-term response to maternal inflammation. A twofold increase in the number of granular cells in the DG were observed in mice overexpressing the anti-apoptotic human gene Bcl-2 (Rondi-Reig et al., 2001). This finding supports the possibility that the increased number of granular cells emerge from the stimulation of the regeneration program. In contrast to the CA1 region, the cell size in the DG was not affected. A different sort of change was observed in the motor cortex; the increased number of pyknotic cells in layers 2, 3 and 4 of the maternal inflammation group may be a result of the long-term modulation of death signals in these cells. The lack of difference in the levels of the neurotrophic factors may indicate no involvement of their signaling activity.

In accordance with our morphological analysis in the secondary motor cortex, CC and cerebellum, maternal inflammation did not affect motor reflex development, exploratory behavior, sensory gain and motor function. Specific behavioral deficiencies were observed in learning and memory tasks, depending on the neuronal circuitry of the hippocampus in association with the related brain region, such as the hippocampus and amygdala in the passive avoidance task and the hippocampus and frontal cortex regions in the cued version of Morris water maze (Morris et al., 1982; Morris, 1984, Phillips and LeDoux, 1992). We were surprised to find that learning and memory, which predominantly depend on the hippocampal neuronal circuitry, such as spatial learning in the 'Morris water maze' and 'object recognition' (Morris et al., 1982; Sutherland et al., 1983; Morris, 1984; Eichenbaum et al., 1989; Steckler et al., 1998; Giese et al., 2001; D'Hooge and De Deyn, 2001), were not disturbed or even improved in the maternal inflammation offspring, compared to the control offspring. Distinct effects of different learning tasks were also observed in the Bcl-2 transgenic mice, which have numerous cells in the DG, but not in the CA1 region (Rondi-Reig et al., 2001). They found impairment to be correlated with the difficulty of the tests, which is not true in our study.

In conclusion, the systemic maternal inflammation by the i.p. administration of LPS at E17, specifically impairs distinct forms of learning and memory in mice offspring, due to the dependence on the association between the hippocampus and additional brain circuits, that may be a result of perturbed morphogenesis in the hippocampus region.

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Appendix A. Supplementary information (data)

Supplementary information(data) for this manuscript can be downloaded at doi: 10.1016/j.neuropharm.2004.12.023.

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