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EARLY PRENATAL EXPOSURE TO LPS RESULTS IN ANXIETY- AND DEPRESSION-RELATED BEHAVIORS IN ADULTHOOD

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Abstract—Maternal immune activation can result in different behavioral abnormalities and brain dysfunction, depending on the nature of the inflammogen and the timing of the challenge. Few studies report the possible link between prenatal exposure to inflammation and mood disorders. Here we aimed to evaluate the effects of a single low lipopolysaccharide (LPS) injection to the dam at gestational day 9 on the offspring behavior and hippocampal function. We found that mice exposed to LPS show anxiety- and depression-related behaviors. Specifically, we found that animals prenatally exposed to LPS avoided the open arms of an elevated plus maze, the center of an open field and the lit side of a light/dark box, and they spent more time immobile in both the forced swimming and tail suspension tests, when compared with offspring of saline-injected dams. In addition, LPS mice had reduced serotonin and noradrenaline levels in the hippocampus and diminished Reelin immunoreactivity in the dentate gyrus, while their adult hippocampal neurogenesis was not affected. Results presented here support specific long-term effects of the response to a bacterial immunogen early in pregnancy, as opposed to different effects previously reported of viral immunogens and/or responses in late pregnancy. Our work adds to recent reports and stresses the relevance of considering prenatal exposure to a maternal immune response as a risk factor for mood disorders.

Key words: maternal immune activation, Reelin, neurogenesis, serotonin, mood disorders.

INTRODUCTION

Prenatal infection has been associated with the development of various psychiatric disorders, including autism and schizophrenia (Meyer, 2013). A developing foetus can be exposed to the maternal immune response elicited by bacteria, viruses or parasites, as well as the result of allergy or autoimmune diseases. Even stress can affect the maternal immune state (Veru et al., 2014). Although maternal and perinatal medical care has improved survival rates of newborns from mothers suffering from infections or trauma, the maternal response can still affect the foetus inflammatory status and then alter normal neurodevelopment, leaving enduring alterations in brain function.

In the recent years, different animal models have been used in an effort to understand the different effects of prenatal infection on brain physiology as well as the underlying mechanisms (Meyer, 2013). In rodents, perinatal inflammatory stimuli can result in long-lasting behavioral and physiological alterations and the effects observed depend on the timing, dose and nature of the inflammogen used (Bilbo and Schwarz, 2012).

Due to a significant link between schizophrenia and maternal infection with influenza virus, several researchers have inoculated rodents perinatally with poly riboinosinic-polyribocytidilic acid (PolyI:C) (Meyer, 2013; Reisinger et al., 2015). PolyI:C is a synthetic analog of double-stranded RNA which activates toll-like receptor (TLR) 3, thus emulating a viral infection. Mice prenatally exposed to PolyI:C show long-term alterations in behavior and the inflammatory response (Meyer et al., 2006b, 2009). Nevertheless, the effects on the offspring depend on the developmental age at which they are exposed to the maternal inflammatory response. When inflammation is elicited late in pregnancy (GD15–17), offspring shows schizophrenia-related behavior (Meyer et al., 2006b; Zhang and van Praag, 2014), reduced hippocampal volume (Piontkewitz et al., 2011), alterations in neurotransmission (Ducharme et al., 2012), reduced neurogenesis and changes in the physiology of the new neurons (Zhang and van Praag, 2014). When offspring is exposed to the PolyI:C at GD9, however, they show reduced exploratory behavior (Meyer et al., 2006b), less Reelin-positive cells in the CA1 (Meyer et al., 2006b; Harvey and Boksa, 2012), and reduced neurogenesis (Meyer et al., 2006b).

Not only viral infections, but also bacterial infections are common during pregnancy. Actually, bacterial vaginosis has a very high prevalence in women of reproductive age

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Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; A, adrenaline; BrdU, 2-bromo-5-deoxyuridine; C57, C57BL/6J strain; DCX, doublecortin; DG, dentate gyrus; EDTA, ethylenediaminetetraacetic acid; GD, gestational day; HPLC-EC, high-pressure liquid chromatography and electrochemical detection; LPS, lipopolysaccharides; MANOVA, multiple analysis of variance; NA, noradrenaline; PD, postnatal day; PFA, paraformaldehyde; PolyI:C, Polyriboinosinic–polyribocytidilic acid; Sal, saline; SGZ, subgranular zone; TLR, toll-like receptor.

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(29%), although it is commonly asymptomatic and thus not treated (Koumans et al., 2007). Therefore, the study of long-term effects of the maternal inflammatory response to bacterial infection is relevant to understanding its contribution to adult brain function. Different animal models have been developed using lipopolysaccharides (LPS) to mimic bacterial infection. LPS is a component of the outer membrane of Gram-negative bacteria recognized by TLR 4 and consequently produces a strong inflammatory effect upon injection in mammals. Again, the effects observed in the offspring after eliciting an inflammatory response in the dam depend on the developmental age and the dosage. In rats, offspring exposed to LPS at GD10.5 showed increased anxiety- and depression-related behaviors and reduced serotonin levels in the hippocampus (Wang et al., 2009; Lin et al., 2012; Lin and Wang, 2014). In mice, prenatal LPS at GD17 can result in increased anxiety and fear in adulthood (Hava et al., 2006) and affect learning (Golan et al., 2005). A high dose of LPS (0.3 mg/kg) injected subcutaneously at GD8 results in abnormal behavior in the novel object recognition task (Coyle et al., 2009).

Here, we aimed to analyze the long-lasting effects of the maternal inflammatory response at GD9 using a low dose of LPS (25 μg/kg) to mimic bacterial infection in mice. In particular, we hypothesized that an inflammatory stimulus at this age will result in alterations in behaviors related to mood disorders. Moreover, we evaluated different candidate cellular and molecular mechanisms that could underlie these behavioral alterations. We focused on the hippocampus because this region is particularly sensitive to inflammatory insults (Depino et al., 2005) and plays an integral role in the modulation of emotion.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6J (C57, The Jackson Laboratory, Bar Harbor, Maine, USA) mice were bred for several generations in the animal house at the Leloir Institute (Buenos Aires, Argentina), under non-SPF conditions. Each C57 nulliparous female was mated with a C57 male at 8–12 weeks of age during two nights, and then males were removed from the cage. The day between mating nights was considered the gestational day (GD) 0 for simplicity, and it was GD0.5 or GD-0.5 depending on the actual mating night. At GD9 pregnant mice were randomly assigned to either experimental group and injected subcutaneously with 25 μg/kg of LPS (*Escherichia coli* LPS, serotype 0111:B4, Sigma, St. Louis, MO, USA) or with sterile saline solution (Sal). The day of parturition was defined as postnatal day (PD) 0. To minimize the ''litter effect'', 9 Sal and 12 LPS litters were used and 1–2 pups per litter were randomly selected for behavioral or neurophysiological studies. We observed no effect of treatment on litter size or weight at weaning (data not shown). Animals were weaned at PD21 and housed four to five per cage. For behavior, 17 male offspring of each experimental group were studied (female mice were not included to reduce the number of independent variables evaluated, specially considering the effects of the estrous cycle on anxiety- and depression-related behaviors (Meziane et al., 2007)). To avoid the confounding effect of behavioral testing on biochemical and cellular measurements, untested littermates of tested animals were used for neurotransmitters, neurogenesis and Reelin-positive cells measurements.

All animals had water and food *ad libitum* and were housed on a 12:12 light/dark cycle with lights on at 0800 h. All animal procedures were performed according to EC Directive 86/609/EEC for animal experiments and the regulations for the use of laboratory animals of the National Institutes of Health, USA, and approved by the animal subjects review board (CICUAL) of the Leloir Institute.

Behavioral testing

Behavioral testing was performed during the light period (between 10:00 and 16:00 h) under dim light illumination. Mice were 8–10 weeks of age at the beginning of testing, and they were subjected to all tests using 1-week intervals to reduce inter-test interactions. Tests were performed in the order listed below, in the holding room. After changing illumination, mice were habituated to it for 45 min prior to the test. The investigator was blind to prenatal treatment during behavioral testing. After testing, each mouse was identified and placed in a holding cage until all animals in a cage were tested. Each apparatus was cleaned with 20% ethanol between sessions.

The elevated plus maze was performed as previously described (Depino et al., 2008, 2011; Lucchina et al., 2010). The maze consisted of two open and two closed arms (open arms: 30 × 5 cm, 105 lux, surrounded by a 0.5-cm high border; closed arms: 30 × 5 cm, 43 lux, surrounded by 19-cm high walls). The walls were made of black PVC and the floor of gray PVC. The apparatus was elevated 50 cm above the floor. Mice were placed into the central platform (5 × 5 cm, 102 lux) of the maze facing toward an open arm and allowed to explore the maze for 5 min. Locomotion data were collected by the ANY-maze system (Stoelting, IL, USA). Measured variables were: total distance, time and distance in the open and in the closed arms, and time and distance in the central platform.

The open field test was performed as previously described (Depino et al., 2008, 2011; Lucchina et al., 2010). Mice were placed in an arena (floor: 45 × 45 cm of gray PVC; walls: 30 cm high of black formic; 30 lux) for 5 min. Animals were initially placed along one side of the arena, and the center region was defined as the central 23 × 23 cm area. Locomotion data were collected by the ANY-maze system (Stoelting). Measured variables were: total distance, entries, distance and time in the center, latency to enter the center, time and distance in the periphery. We also calculated the percentage of distance in the center.

The light/dark test was performed as previously described (Depino et al., 2008; Lucchina et al., 2010; Campolongo et al., 2012). A 45-cm × 45-cm arena was divided into half with an inverted black box (lit side: 35 lux; dark side: 1 lux). Animals freely moved between

the compartments through a 12-cm × 8-cm hole in the wall. Each mouse was placed under the hole facing the lit side and observed for 5 min. The test was recorded and time spent in the lit compartment was counted using a stopwatch.

The tail suspension test was performed as previously described (Depino et al., 2011; Lucchina and Depino, 2014). Animals were suspended in the air using adhesive tape wrapped around the subject's tail (about 4/5 from the base) and fixed to a wire at 25 cm height from a wooden surface. The time spent immobile was measured during 5 min. Mice were considered immobile when they hung passively, making no movement. One Sal animal learned how to climb its own tail during the test and was removed from the analysis.

The forced swimming test was performed as previously described (Depino et al., 2011; Lucchina and Depino, 2014). Subjects were gently placed in a beaker glass (diameter, 15 cm; height, 25 cm), filled with 14 cm of water at room temperature (25 °C). The time spent immobile during 5 min was measured with a stopwatch. At the end of the test, animals were dried with a paper towel and they were placed in a holding cage with normal bedding. Mice were dry after 20 min.

Monoamine quantification by high-pressure liquid chromatography and electrochemical detection (HPLC-EC)

Animals were deeply anesthetized (intramuscular 80 mg/kg ketamine chlorhydrate and 8 mg/kg xylazine) and sacrificed by decapitation. Brain was rapidly dissected in ice, hippocampus, cortex (enriched in prefrontal and motor cortex) and cerebellum removed and quickly frozen in liquid nitrogen. Tissue concentrations of the monoamines noradrenaline (NA), adrenaline (A), and serotonin (5-HT) and of the metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined using HPLC-EC. Tissue was sonicated in 0.5 ml of 0.1 M perchloric acid with 1 mM EDTA. Samples were then centrifuged twice at 4 °C for 25 min at 14,000 RPM and the supernatant was transferred to new tubes. Samples were injected by means of an automatic injector (Gilson 234) onto a Ultrasphere C18 250 × 4.6-mm, 5-μm particle analytical column (Alltech Beckman) at a flow rate of 1 mL/min and 49 °C, and detection was carried out electrochemically (Gilson 142). The mobile phase consisted of 75 mM sodium phosphate, 0.1 mM EDTA, 7.5% methanol and 0.3 mM 1-octanesulfonic acid sodium salt, adjusted to pH 3.1. The position of the peak for each analyte was compared to two external standard solutions which contained 25 ng/mL each of NA, DA, 5-HT and 5-HIAA or A, DOPAC and homovanillic acid (HVA). Chromatographic peak analysis was accomplished by identification of unknown peaks in a sample matched according to retention times. Analyte levels were expressed as ng/mg tissue.

Analysis of hippocampal neurogenesis

Hippocampal neurogenesis was assessed as previously described (Graciarena et al., 2010, 2013). Animals received daily intraperitoneal (ip) injections of 2-bromo-5-deoxyuridine (BrdU, 50 mg/kg in saline; Sigma) during 7 days and they were transcardially perfused with 4% paraformaldehyde (PFA) 24 h after the last injection. Brains were then post-fixed for 4 h in 4% PFA and then cryopreserved in 30% sucrose. 35-μm-coronal sections were prepared on a cryostat (Leica Biosystems, Nussloch, Germany). Every sixth section was processed as previously described (Campolongo et al., 2012) using rat anti-BrdU (1:200, Abcam, Cambridge, MA, USA) and rabbit anti-doublecortin (DCX; 1:500, Abcam) as primary antibodies, and Cy2-conjugated donkey anti-rat and biotin-SP-conjugated donkey anti-rabbit (1:200, Jackson Laboratories, West Grove, PA, USA) as secondary antibodies, followed by Cy3-conjugated streptavidin (1:200, Jackson Laboratories). Single- and double-stained cells in the subgranular zone (SGZ) were quantified using z-scan confocal microscopy (Olympus FV300) at 400× magnification. Densities of BrdU and DCX-positive cells were estimated by counting the number of positive cells and dividing it by the volume of the hilus.

Quantification of Reelin-positive cells in the hippocampus

Brains were prepared as specified in the previous section and immunohistochemistry for Reelin performed as previously described (Depino et al., 2011). Briefly, every 12th section was incubated with mouse anti-Reelin primary antibody (1:700, Millipore, Temecula, CA, USA) over night at room temperature and then with a biotinylated secondary antibody followed by the ABC kit (Vector Laboratories, Burlingame, CA, USA). Reelin-positive cells were counted under 400× magnification and the volume measured in 40× photographs using ImageJ (Rasband, 1997–2009).

Statistical analysis

Statistica 7 (StatSoft Inc., Tulsa, OK, USA) and Prism 5 (Graphpad Software Inc., La Jolla, CA, USA) were used for all statistical analyses. Uncorrelated variables ($|r| < 0.7$) within a test were analyzed by multiple analysis of variance (MANOVA). Group comparisons were then done using unpaired Student's *t*-test for normally distributed data. When variances were unequal, unpaired *t* test was performed with Welch's correction. When data showed no normal distribution, the non parametric Mann Whitney test was performed. For all tests, statistical significance was assumed where $p < 0.05$.

RESULTS

Prenatal immune activation at GD9 results in increased anxiety-related behavior in adult offspring

Offspring of dams exposed to the maternal inflammatory response to LPS at GD9 were compared in adulthood with offspring prenatally exposed to saline. At 8 weeks of age, mice were tested for anxiety-related behaviors. In the elevated plus maze, we selected four variables that were not highly correlated ($|r| < 0.7$): total distance,

time in the central platform, time in the open arms and percentage of distance in the open arms. MANOVA showed an effect of prenatal treatment [F (4, 29) = 2.861, p = 0.041], with mice prenatally exposed to LPS showing a decrease in the time spent in the open arms (Fig. 1A) and in the percentage of distance walked in the open arms (Fig. 1B). The treatment had no effect on total locomotion (Fig. 1C) or time spent in the central platform (Fig. 1D).

A week later, mice exposed to LPS also showed an anxiety-related behavior in the open field test. MANOVA on uncorrelated variables (total distance, time in the center, latency to enter the center and percentage of distance walked in the center) showed a significant effect of treatment [F (4, 29) = 2.880, p = 0.040]. Animals exposed to LPS spent less time in the center (Fig. 1E), showed a longer latency to enter the center (Fig. 1F), explored less the field (Fig. 1G), and walked less in the center (Fig. 1H).

Finally, at 10 weeks of age mice exposed to LPS spent less time in the lit compartment of the light/dark test [t (32) = 2.417, p = 0.022, Fig. 1I].

These results show that prenatal exposure at GD9 to 25 μg/kg LPS results in increased anxiety-related behavior in the adult offspring.

Prenatal immune activation at GD9 results in increased depression-related behavior in adult offspring

We next evaluated depression-related behavior using the forced swimming test and the tail suspension test. In both tests, mice prenatally exposed to LPS showed an increase in the time spent immobile [forced swimming: Welch corrected t (22) = 3.336, p = 0.003, Fig. 2A; tail suspension: t (31) = 4.494, p < 0.0001, Fig. 2B]. The time spent immobile has been interpreted as 'behavioral despair', and it can be modulated by antidepressants. It can then be interpreted that animals exposed to LPS at GD9 show an increase on depression-related behavior later in life.

Prenatal exposure to LPS results in long-term alterations in neurotransmitter content in the hippocampus

Rats prenatally exposed to LPS show changes in neurotransmitter brain content (Wang et al., 2009). To evaluate the long-term effects of prenatal immune activation on neurotransmitter content in the mouse brain, we performed HPLC analysis of NA, A, 5-HT and the serotonin metabolite 5HIAA (Table 1). We evaluated neurotransmitter content in the hippocampus (a region involved in both anxiety- and depression-related behavior). LPS-exposed animals showed a decrease in NA and 5-HT, while other neurotransmitters were not affected. Interestingly, the levels of 5-HIAA and the 5-HIAA/5-HT ratio were not altered, suggesting that these animals have higher levels of 5-HT in the hippocampus but normal serotoninergic function.

To evaluate the specificity of these alterations to the hippocampus, we also measured neurotransmitter concentration in a related region (cortex) and in an unrelated region (cerebellum). No differences between groups were observed in the cortex. In the cerebellum, we found a decrease in A, and an increase in 5-HT along with a decrease in the 5-HIAA/5-HT ratio.

Prenatal exposure to LPS does not alter adult hippocampal neurogenesis

Prenatal exposure to PolyI:C results in reduced adult neurogenesis (Meyer et al., 2006b). To determine whether LPS exposure at GD9 also resulted in long-lasting alterations in neurogenesis, we quantified the density of proliferating cells in the SGZ of the dentate gyrus (DG) (Fig. 3A, B). LPS-exposed mice showed similar levels of proliferation in the hippocampus when compared with saline-exposed mice (Fig. 3C; t (10) = 0.029, p = 0.978). Moreover, they showed normal density of DCX-positive cells in the DG (Fig. 3D; t (6) = 1.109, p = 0.310). To estimate the level of neurogenesis, we calculated the percentage of BrdU-positive cells that were also immunoreactive for DCX, and we found no differences between the groups (Fig. 3E; t (7) = 0.055, p = 0.958). In conclusion, we found no effect of LPS-exposure at GD9 on adult hippocampal neurogenesis.

Prenatal exposure to LPS results in less Reelin-positive cells in the adult DG

It was previously shown that prenatal exposure to PolyI:C results in reduced expression of Reelin in the hippocampus (Meyer et al., 2006b). To evaluate whether prenatal LPS has a similar effect, we quantified the number of Reelin-positive neurons in the hilus and molecular layer of the DG (hilus and Mol) and in the *stratum oriens* of the CA1 (Sto) (Fig. 4A, B). We found a significant decrease in the number of Reelin-positive cells in the hilus of offspring prenatally exposed to LPS [t (14) = 2.552; Fig. 4C–E]. However, LPS-exposed animals showed a similar number of Reelin-positive cells in the other regions analyzed [Mol, Saline 1351 ± 197 cells/mm^3, LPS 1101 ± 374 cells/mm^3, t (14) = 0.591, p = 0.564; Sto, Saline 3324 ± 243 cells/mm^3, LPS 2724 ± 361 cells/mm^3, t (14) = 1.378, p = 0.189].

DISCUSSION

Here we show that the offspring of dams injected with 25 μg/kg of LPS at GD9 exhibit anxiety- and depression-related behaviors in adulthood. Moreover, these animals have altered levels of serotonin and NA in the hippocampus and a reduced number of Reelin-positive cells in the DG. To our knowledge, this is the first report on the long-term effects of LPS exposure in early gestational period on these behaviors, and the candidate cellular and molecular mechanisms underlying them in mice.

The effect of inflammogen nature: LPS vs PolyI:C

A previous report showed that maternal immune activation at GD9 using PolyI:C results in offspring who

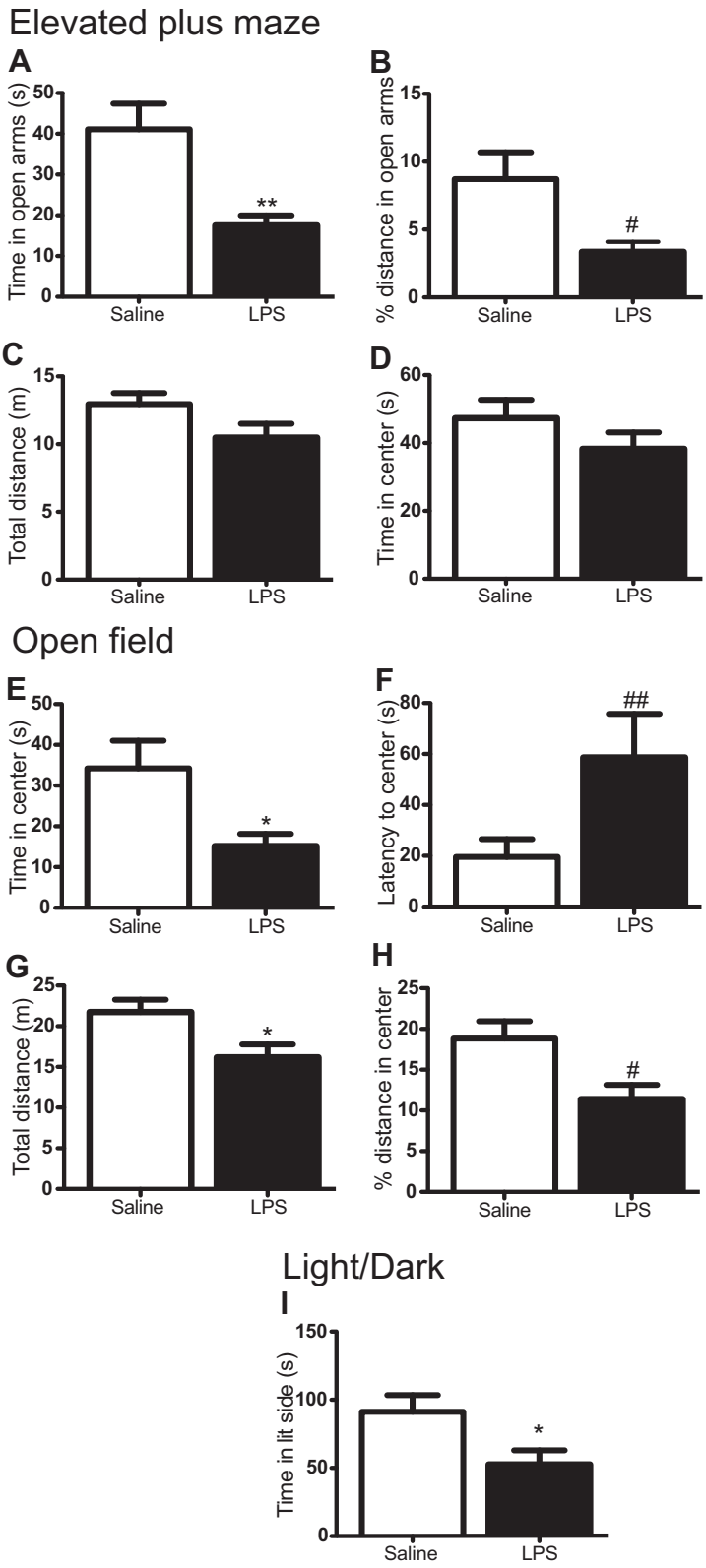

Fig. 1. Increased anxiety-related behavior in adult animals prenatally exposed to LPS. (A)–(D) LPS-exposed animals showed an increased anxiety-related behavior in the EPM, spending less time (A) and reducing locomotion (B) in the open arms. All animals showed similar levels of exploration (C) and spent a similar amount of time in the central platform (D). (E)–(H) LPS-exposed offspring showed an increased anxiety-related behavior in the OF. LPS animals spent less time in the center (E), showed a higher latency to go to the center (F), reduced exploration (G), and less locomotor activity in the center (H). (I) LPS-exposed animals spent less time in the lit compartment in the light/dark test. $N = 17$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t test; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, Mann Whitney test.

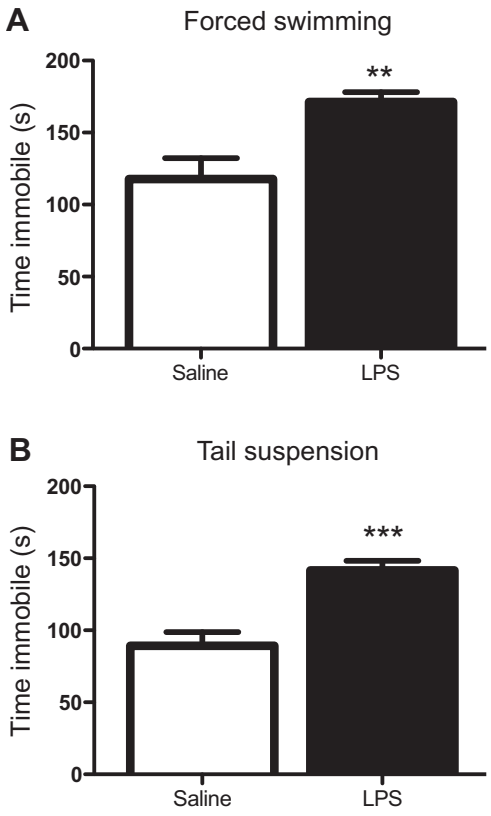

Fig. 2. Increased depression-related behavior in adult mice exposed to LPS at GD9. LPS-exposed animals spent more time immobile both in the forced swimming test (A) and in the tail suspension test (B). $N = 16–17$ per group. $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t test.

entered less frequently to the center of an open field (Meyer et al., 2006b, 2008a). We found a similar anxiety-related behavior in mice prenatally exposed to LPS, showing that this long-lasting effect of the maternal inflammatory response is independent of the inflammogen used. We further characterized the effect, showing that mice also present anxiety-related behavior in the elevated plus maze and the light/dark box. However, Meyer et al. (2006b, 2008a) found that PolyI:C-exposed animals had normal locomotor activity in the open field, whereas we found a reduction in locomotion in this test (Fig. 1G). Given that we did not find a reduction in exploration in the EPM (Fig. 1C), we suggest that these differences could be due to experimental conditions (e.g. illumination could have been more aversive in our conditions, i.e. 30 lux, than in the other experimental setups, where illumination is not reported) or a sex effect which could mask this difference (we used 17 males per group, Meyer et al. used mixed-sex groups of 10 or 16 animals). Nevertheless, we cannot rule out that prenatal LPS exposure at GD9 leads to a subtle reduction in locomotor activity that is observed in adverse conditions, or that this treatment may affect motor coordination.

Clinical studies found no clear link between prenatal infection and depression later in life, showing no association (Pang et al., 2009), decreased risk (Mino et al., 2000), or increased risk for mood disorders (Machon et al., 1997). However, this and other animal reports suggest that an underlying link could be in place (Reisinger et al., 2015). In mice, this effect on increasing behavioral despair also appears to be independent on the nature of the inflammogen. Mice exposed to PolyI:C at GD12.5 show increased depression-related behavior in adulthood (Khan et al., 2014), similar to the abnormal behavior characterized here in mice exposed to LPS at GD9. Unfortunately, we found no reports on depression-related behaviors after the injection of PolyI:C at GD9. Further studies should be carried out to confirm that this effect is independent on the nature of the inflammogen.

The hippocampal serotonergic system modulates anxiety behavior (File et al., 2000) and depression (Luo et al., 2008). Rats exposed to LPS at GD10.5 show reductions on DA and 5-HT content in various regions of the adult brain, including the frontal cortex, amygdala, hippocampus and hypothalamus (Wang et al., 2009); while rats injected with PolyI:C at GD9 also show a reduction on serotonin levels in the hippocampus but no changes in 5-HIAA at PD50 (Ohkawara et al., 2015). C57BL/6J offspring exposed to PolyI:C at GD9 showed reduced 5-HT and 5-HIAA levels in the hippocampus, but normal 5-HIAA/5-HT ratios at 12 weeks of age (Winter et al., 2009). These results are similar to those presented here, and our results extend the effects on serotonergic levels to adult mice exposed prenatally to a bacterial endotoxin, suggesting that the alteration of hippocampal serotonin is independent to the nature of the inflammatory factor used.

Hippocampal neurogenesis is related to both anxiety and depression (Santarelli et al., 2003; Goshen et al., 2008; Revest et al., 2009; Fuss et al., 2010a,b), and it has been reported that it is altered in some rodent models of maternal immune activation (Green and Nolan, 2014).

Table 1. Animals prenatally exposed to LPS have reduced noradrenaline and serotonin levels in the hippocampus

Area	Treatment	[NA] ng/g tissue	[A] ng/g tissue	[5-HT] ng/g tissue	[5-HIAA] ng/g tissue	5-HIAA/5HT ratio
Hippocampus	Saline	336 ± 35	192 ± 22	7452 ± 717	464 ± 53	0.062 ± 0.003
	LPS	197 ± 11**	166 ± 8	5255 ± 303*	361 ± 23	0.069 ± 0.004
Cortex	Saline	333 ± 35	193 ± 11	5737 ± 595	155 ± 15	0.028 ± 0.001
	LPS	393 ± 41	213 ± 24	4729 ± 612	147 ± 16	0.032 ± 0.002
Cerebellum	Saline	395 ± 35	198 ± 17	776 ± 98	91 ± 7	0.14 ± 0.02
	LPS	363 ± 51	140 ± 15*	1250 ± 198*	67 ± 10	0.06 ± 0.01**

*$p < 0.05$, **$p < 0.01$, Student's t test.
NA, noradrenaline; A, adrenaline; 5-HT, serotonin; 5-HIAA, 5-hidroxyindoleacetic acid.

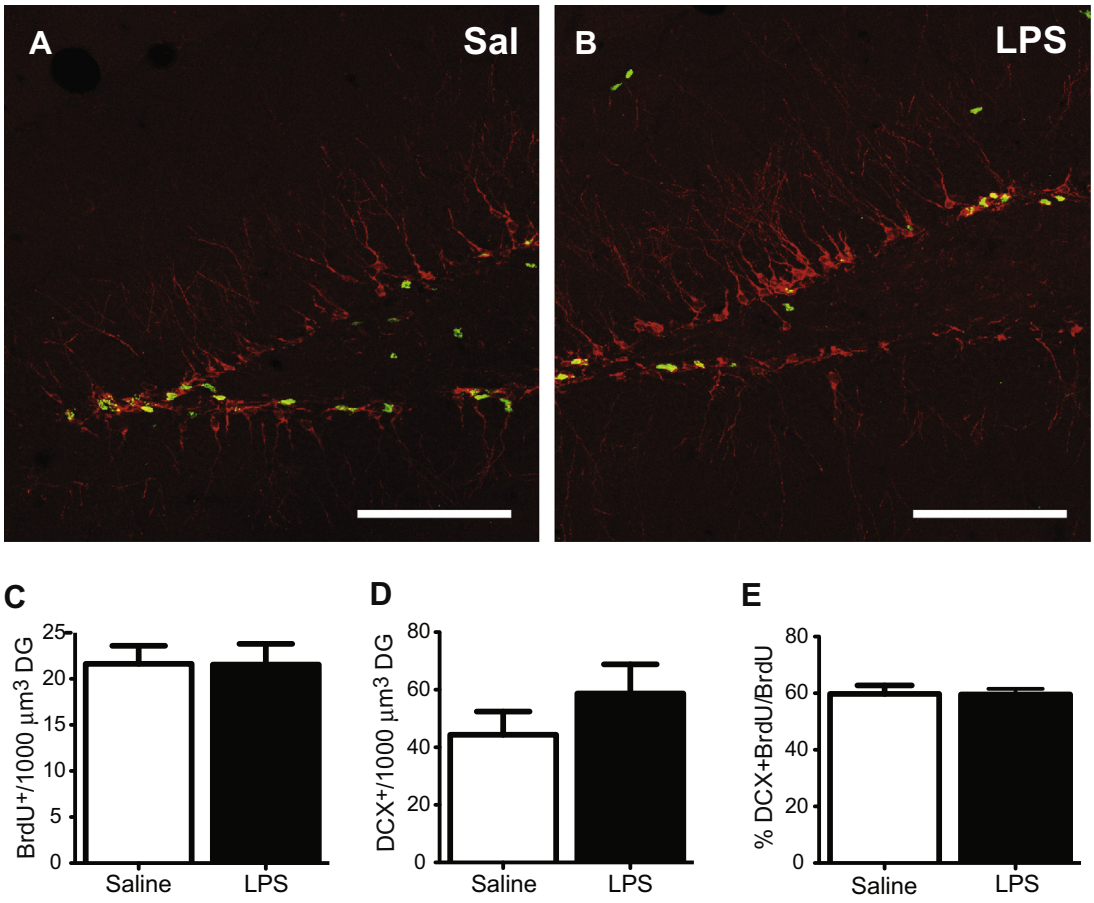

Fig. 3. Neurogenesis in the adult dentate gyrus is unaltered in mice exposed to LPS at GD9. (A) and (B) Representative confocal image of a DCX (red)/BrdU (green) immunofluorescence in control (A) and LPS-exposed (B) mice. Images are z-project of 18 confocal 2-µm-stacks. Both groups show a similar density of DCX-positive cells (C), BrdU-positive cells (D) and percentage of BrdU-positive cells that are also DCX-positive (E). Scale bar = 0.1 mm.

However, we found that density of newborn neurons did not differ between the groups. The timing of prenatal immune challenge and/or the nature of the inflammogen could differently affect neurogenesis in the SGZ of the DG, explaining the different results obtained. Indeed, exposure to PolyI:C at GD9 caused a reduction of DCX immunoreactivity in the DG (Meyer et al., 2006b), that was not observed after exposure to LPS (Fig. 3E). In addition, another report showed that maternal immune activation using PolyI:C at GD12.5 also results in reduced proliferation in the DG (Khan et al., 2014), supporting the concept that prenatal PolyI:C exposure results in long-lasting effects on hippocampal neurogenesis. To explain how different inflammogens can have similar effects on behavior but different effects on neurogenesis needs further research. However, the contribution of neurogenesis to behavior has been recently challenged, stressing that studies usually underestimate the effects of other mechanisms (Lazic et al., 2014).

Reelin regulates neuronal migration during early development and it plays a critical role in the formation of the glial scaffold in the DG (Forster et al., 2002). So, reduced hippocampal Reelin due to prenatal infection could affect hippocampal development. Previous reports showed that mice prenatally exposed to PolyI:C (at GD9) have reduced numbers of Reelin-positive cells in the dorsal *stratum oriens* of CA1 at PD28, but mice exposed to LPS do not (Harvey and Boksa, 2012). This is in agreement with our results, as we did not observe differences in the densities of Reelin-positive cells in the *stratum oriens*. However, we did find a reduction in the density of these cells in the hilus of the DG of adult mice exposed to LPS at GD9. In adult hippocampus, Reelin is produced by a subset of GABAergic neurons and it modulates LTP (Weeber et al., 2002). Whether the reduction of Reelin-positive cells in the DG can result in the behavioral deficits observed in animals exposed to LPS at GD9 needs further analysis.

The effect of inflammation timing

In the context of previous results, the data presented here add to the notion that the long-term behavioral effects of maternal immune activation depend on the developmental age of the embryos, when genetic contribution is ruled out. Indeed, a comparison of strains showed that NMRI mice but not C57 mice exhibit an increase in anxiety- and depression-related behaviors after the exposure to the maternal response to LPS at GD17 (Babri et al., 2014), confirming previous results in

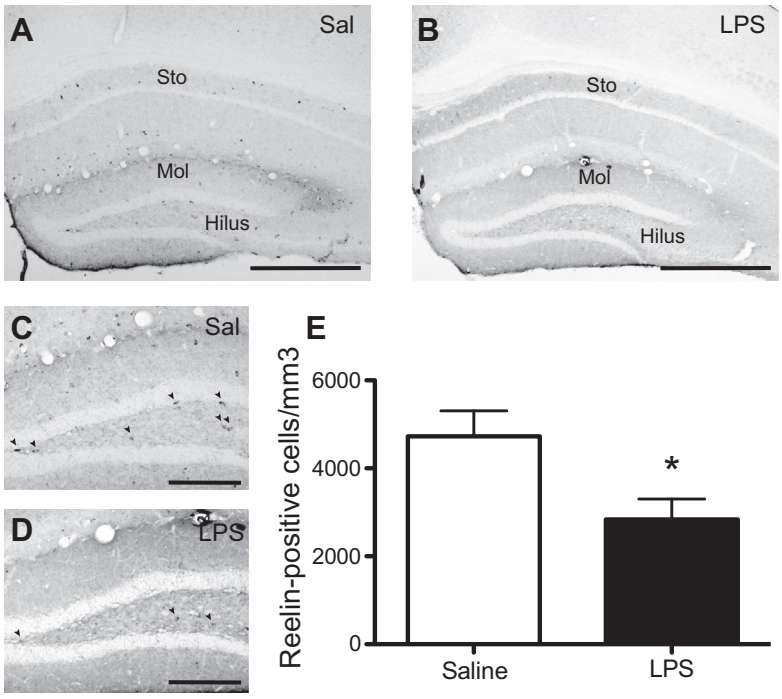

Fig. 4. Reduced Reelin expression in the adult hippocampus of offspring after GD9 immune challenge. (A) and (B) Distribution of Reelin-positive cells in the hippocampus of control animals (A) and LPS-exposed animals (B). (C) and (D) A reduction was observed specifically in the hilus of the dentate gyrus of LPS-exposed animals (D) compared with Saline-exposed animals (C). Reelin-positive neurons are indicated by arrowheads. (E) LPS-exposed animals showed a significant reduction in the density of Reelin-positive cells in the Hilus of the DG. Scale bars = A and B, 0.5 mm; C and D, 0.2 mm. $N = 8$ per group. $^{*}p < 0.05$, Student's t test. Mol, molecular layer of the dentate gyrus; Sto, *stratum oriens*.

the NMRI strain (Enayati et al., 2012). Others reported that C57 mice exposed to LPS at GD17 did not avoid the center of an open field (Golan et al., 2005). In contrast, we show here that C57 mice exposed to LPS at GD9 show both anxiety- and depression-related behaviors. Similar differences in the behavioral effects of early (GD9) or late (GD17) immune activation were described when PolyI:C was used as an inflammogen (Meyer et al., 2006b) (Meyer et al., 2008b). In summary, various reports show that maternal immune activation during the last week of pregnancy results in alterations in schizophrenia-related behavior and cognitive abnormalities, along with diminished neurogenesis and other cellular and molecular dysfunctions (Meyer et al., 2006b, 2010; Graciarena et al., 2010). In contrast, exposure to the inflammatory response during the second week of pregnancy results in anxiety- and depression-related behaviors, altered Reelin immunoreactivity and altered serotonin levels in the hippocampus (Meyer et al., 2006a).

The noradrenergic system modulates the response to stress and it is associated with anxiety (Bremner et al., 1996). Different serotonin-norepinephrine reuptake inhibitors (SNRIs), which raise the extracellular levels of NA, act as antidepressants. To our knowledge this is the first report that shows a long-lasting alteration in hippocampal NA in animals exposed to maternal immune activation. On the same line, another study showed that exposure to influenza virus at GD9 results in upregulated expression of the norepinephrine transporter in the neonatal brain (Fatemi et al., 2005). Future work could shed light on whether NA can mediate some of the long-lasting effects of maternal immune activation on behaviors, and whether this effect on noradrenergic transmission depends on the timing of maternal infection.

Based on temporal patterns of development of the mouse brain (Rodier, 1980), it should not be surprising that the timing of the immune challenge can have very different effects on brain function later in life. Specifically, the late development of the hippocampus, and especially of the DG, could make it more sensitive to late pregnancy immune activation than to earlier challenges. However, our finding of long-lasting effects on Reelin immunoreactivity in the hilus suggests that these effects can indeed take place and could underlie the behavioral alterations observed. Further studies are needed to elucidate the different cellular and molecular targets of early and late inflammation in the developing brain.

Toward a model of early bacterial infection effects on behavior

Our work was aimed to characterize some of the behavioral effects of exposing mice to LPS at GD9, and to identify cellular and molecular pathways that were also affected. However, caution should be taken on linking these alterations until further experiments specifically test their contribution. For example, although we consider that the long-term effects of maternal immune activation should be stable during adulthood, we cannot rule out that some of the cellular and molecular alterations that were observed in animals of 9 weeks of age could be absent at later times, when some of the behavioral tests were performed.

Based on our results and considering the reports cited in the previous sections, we could elaborate a model to be tested experimentally. Injection of LPS can elicit an immune response in the mother, upregulating the expression of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Meyer et al., 2006a,b). These cytokines can in turn upregulate the expression of similar cytokines in the fetus brain (Meyer et al., 2006b). Cytokines can affect brain development through various mechanisms and their effects would depend on the timing of the stimulus (Deverman and Patterson, 2009). At GD9, we hypothesize that cytokines affect the development of serotoninergic, noradrenergic and GABAergic neurons that modulate hippocampal function. As a result, we observe reduced 5-HT content, reduced NA content and reduced Reelin-positive cells numbers. This reduction in modulation could underly the behavioral effects we observe: increased anxiety- and depression-related behaviors.

CONCLUSIONS

We show here that the maternal response to bacterial LPS at GD9 can have profound behavioral effects on the offspring, resulting in increased anxiety- and depression-related behaviors, and molecular and cellular alterations in the hippocampus. Although some clinical studies have failed to find a link between maternal infection and the development of mood disorders, our results add to others suggesting that those reports can be confusing, mixing different inflammatory factors and their effects on different developmental ages. Further studies dissecting the different effects of specific inflammogens on specific developmental ages could then contribute to understanding the role of maternal infection on mood disorders.

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