***N*-acetyl-cysteine prevents pyramidal cell disarray and reelin-immunoreactive neuron deficiency in CA3 after prenatal immune challenge in rats**

Aline Rideau Batista Novais^{1,2}, Janique Guiramand¹, Catherine Cohen-Solal¹, Nadine Crouzin¹, Marie Céleste de Jesus Ferreira¹, Michel Vignes¹, Gérard Barbanel¹ and Gilles Cambonie^{1,2}

BACKGROUND: Prenatal infection is a major risk factor for the occurrence of neuropsychiatric disorders. These have been associated with hippocampal neuroanatomical and functional abnormalities. In the present study, we evaluated the occurrence of pyramidal cell disarray and reelin neuronal deficit in the hippocampus, and the protective role of *N*-acetyl-cysteine (NAC) in a rodent experimental model of prenatal immune challenge.

METHODS: Sprague–Dawley rats received either 500 µg/kg of endotoxin (lipopolysaccharide, LPS) or 2 ml/kg of isotonic saline by i.p. injection on day 19 of gestation. After LPS injection, rats were or were not maintained on a preventive treatment of NAC (5 g/l in tap water), up to delivery. The pyramidal cell orientation and the number and type of reelin-expressing neurons were determined in male offspring.

RESULTS: Prenatal LPS challenge led to permanent pyramidal cell disarray and to an early and transient decreased density of reelin-immunoreactive neurons. These disorders, more pronounced in the CA3 area, were prevented by NAC.

CONCLUSION: Hippocampal cytoarchitectural alterations and reelin deficiency may be involved in the development of remote cognitive impairments in this model. The antioxidant NAC is an efficient neuroprotective drug that underlines the role of oxidative stress in prenatal infection and associated neurodevelopmental damage.

Challenging life events and stress occurring during the prenatal period induce long-term emotional and cognitive defects later in life. A growing body of data indicates that maternal infection during pregnancy represents a very serious risk factor for the emergence of neuropsychiatric diseases in children and adults, including schizophrenia and autism spectrum disorder (1,2). Exposures to viruses, such as influenza, rubella, or cytomegalovirus, during the first trimester of gestation or to bacterial pathogens during the second trimester are strongly associated with the occurrence of autism spectrum disorder in children (3).

Systemic prenatal injections of a viral mimic (poly I:C) or endotoxin (lipopolysaccharide, LPS) to rodents are widely used experimental models for understanding the mechanistic correlates resulting in neurodevelopmental deficits (4–6). Most interesting, the behavioral and cognitive deficits do not emerge until a pubertal or adult stage of development (7,8), leading to the idea of a neurodevelopmental pattern of the disease that may compare with the postpubertal onset of psychotic behavior in humans. Following prenatal LPS challenge, numerous impairments of the *N*-methyl-D-aspartate receptor functioning were evidenced as soon as the second postnatal week, both in the striatum (9) and in the hippocampus, where aberrant forms of synaptic plasticity were detected (10). As the animals matured, these were accompanied by an array of synaptic plasticity and mnemonic deficiencies (11,12).

Postmortem studies provided evidence of cytoarchitectural abnormalities in the hippocampus of patients with schizophrenia (13–15) that might correlate with abnormal functionality (16). Consistent with the disconnectivity hypothesis, an abnormal reelin expression has been repetitively reported in the brains of patients suffering from schizophrenia, bipolar disorder, or autism (17–19).

Reelin has a crucial prenatal role on the laminar organization of the cortex, cerebellum, and hippocampus (19–21). In the hippocampus, reelin also regulates the developmental switch between different subunits of *N*-methyl-D-aspartate (22,23), the phosphorylation of the *N*-methyl-D-aspartate receptor (24), and the development of the hippocampal dendritic arborization (25), and enhances long-term potentiation (26). An impairment in reelin signaling could therefore induce neuroanatomical and/or *N*-methyl-D-aspartate-regulated glutamate transmission deficits in the hippocampus.

We thus postulated that prenatal LPS would interact with reelin signaling. Considering the neurodevelopmental pattern of neuropsychiatric diseases (7,8), we decided to analyze neuronal orientation and reelin expression at several points of cerebral development corresponding to the neonatal, young,

G.B. and G.C. participated equally in this study.

¹Institut des Biomolécules Max Mousseron (IBMM), Unité Mixte de Recherches 5247 Centre National de la Recherche Scientifique–Université Montpellier 1–Université Montpellier 2, Montpellier, France; ²Neonatal Intensive Care Unit, Arnaud de Villeneuve Hospital, University Hospital Centre of Montpellier, Montpellier, France.

Correspondence: Gérard Barbanel (gerard.barbanel@univ-montp2.fr)

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and adult period. In addition, we evaluated the putative neuro-protective action of *N*-acetyl-cysteine (NAC). Indeed, we previously reported that exposure of pregnant rats to LPS induced an early oxidative stress in the fetal hippocampus and that NAC, an antioxidant, given before or after the injection of LPS, provided efficient protection with respect to synaptic plasticity and memory impairment in male offspring (11,12).

The present study showed that a late gestational LPS-induced immune challenge led to permanent local distortion of the cytoarchitecture of pyramidal neurons and to early deficiencies in hippocampal reelin-immunoreactive (Rln⁺) neurons. Both were prevented by a prenatal administration of NAC. As such, they may contribute to the deficits observed in prenatally LPS-challenged male offspring (7,11,12,27).

RESULTS

Prenatal Administration of NAC Prevented the LPS-Related Permanent Defect in the Spatial Organization of the CA3 Area

The orientation of the dendrites could not be defined clearly in postnatal day (P)10 animals. In older rats, NeuN-labeled neurons aligned all over the hippocampus in a densely packed

layer. The proximal segment of their apical dendrites arranged in a network of parallel processes, which were roughly orthogonal to the cellular layer, as illustrated in [Figure 1a,b](#).

Prenatal LPS induced a pronounced disorganization in the CA3 area ([Figure 1c,d](#)). As compared with that of control animals, the percentage of cells with nonaligned apical dendrites in the CA3 area decreased with age in both controls and LPS offspring, but LPS offspring had a higher percentage of cells with nonaligned apical dendrites on P30–40 ($P < 0.001$) ([Figure 1e](#)). The orientation of pyramidal neurons was mainly preserved in the CA1 area (data not shown). NAC treatment between the LPS challenge and delivery fully prevented the LPS-induced disorganization observed in NeuN-labeled processes in P30–40 animals ([Figure 1f](#)).

Prenatal Administration of NAC Prevented the Adverse Effects of Prenatal LPS on the Developmental Profile of Reelin-Expressing Neurons in the Hippocampus

In the whole hippocampus of control animals, the density of Rln⁺ neurons (i.e., the sum of CA1, CA3, and dentate gyrus densities) decreased with age both in control and LPS animals

Figure 1. Cytoarchitecture of NeuN-labeled neurons in the CA3 area. In (a) P30–40 and (b) P140–160 SAL male offspring, note the uniform organization of the pyramidal neurons, with the apical most apparent (one prominent proximal dendrite being darkly stained) dendrites oriented toward the stratum radiatum, all along the CA3 area. This cytoarchitectural organization was disturbed in both (c) P30–40 and (d) P140–160 LPS rats. The rectangular area is the region of interest selected for the quantification of the CA3. Bar = 100 μ m. The percentage of nonaligned neurons in the pyramidal layer of the CA3 area in P30–40 and P140–160 adult male control (SAL, white bar) and LPS (black bar) offspring (e), was determined as described in Material and Methods, on three to four bilateral slices of the hippocampus. Values are means $\pm$ SEM from three animals in each group. ** $P < 0.01$ P140–160 vs. treatment-matched P30–40 rats; † $P < 0.01$ LPS vs. age-matched control (SAL) rats. The protective effect of NAC is illustrated in f. ** $P < 0.01$ LPS (black bar) vs. age-matched control (SAL) rats (white bar). † $P < 0.01$ LPS/NAC (gray bar) vs. age-matched LPS rats. LPS, lipopolysaccharide; NAC, *N*-acetyl-cysteine; P, postnatal day; SAL, saline-injected.

($F_{2,58} = 104.1$; $P < 0.001$) (Figure 2). The density of Rln^+ neurons was lower in P10 and P30–40 LPS animals ($P < 0.01$, LPS vs. saline-injected (SAL)). No deficit was observed in older LPS animals (Figure 2). The most prominent deficit was observed in the CA3 of P10 animals (Figure 3).

The effect of a prenatal treatment with NAC, given between LPS injection and delivery, was evaluated in a separate experiment, using immunofluorescence detection. This treatment fully prevented the occurrence of the Rln^+ deficits in LPS P10 male rats (Figure 4).

Prenatal LPS Affected Both Populations of Reelin-Secreting Cells in CA3 at P10

Two distinct Rln^+ neuron populations clearly emerged in both the 3,3-diaminobenzidine immunohistochemical study and the double immunofluorescence study, distinguished by their different morphological characteristics and different distribution during postnatal development (Figure 5). In younger animals, most of the Rln^+ neurons were small, bipolar, and ovoid-shaped, giving rise to long dendritic processes that ran axially, one prominent proximal dendrite being darkly stained (Figure 5a). These cells did not express GAD_{67} (Figure 5b). In the whole hippocampus, their density progressively declined in adults ($F_{2,39} = 58.0$, $P < 0.0001$). By contrast,

the morphological characteristics and the distribution of Rln^+ / GAD_{67}^+ cells (Figures 5c,e) corresponded to a subpopulation of γ -aminobutyric acid (GABA)-ergic interneurons. The density of these neurons increased progressively up to adulthood ($F_{2,39} = 5.8$, $P < 0.01$), and represented a large majority of Rln^+ cells in adults. Prenatal LPS did not affect this developmental profile but reduced both populations in CA3 at P10 (Table 1).

DISCUSSION

Prenatal LPS challenge induced a long-lasting pyramidal cell disarray and a decrease of hippocampal Rln^+ neurons most pronounced in the CA3 of young rats. Of note, all these effects could be prevented by a post-LPS (still prenatal) oral administration of NAC.

Cerebral dysplasia and local distortion of the cytoarchitecture of the hippocampus are common features of schizophrenia, autism, and mood disorders. Increased variability of neuronal orientation has furthermore been reported in patients with schizophrenia (13–15). However, these postmortem studies led to conflicting results, probably due to the region considered, the staining method used, the way the cellular disarray was measured, and the influence of perimortem factors on cerebral cytoarchitecture.

Prenatal LPS challenge is linked to cognitive and behavioral phenotypes in the offspring, reminiscent of that of neuropsychiatric diseases (4,5). We observed early and long-lasting neuronal disarray in the pyramidal layer in these offspring, which makes this experimental model very relevant to the context of neuropsychiatric disorders and raises the question of the mechanisms involved. In patients with schizophrenia, the number of nonaligned neurons was negatively correlated with the number of pyramidal cells in the Ammon's horn, suggesting either a migration and/or a neurogenesis disorder (14). Prenatal LPS may affect both mechanisms. Indeed, a decreased expression of transcription factors regulating neuronal migration was observed 4 h after LPS injection to pregnant dams, supporting the hypothesis of a migration disorder (28). On the other hand, prenatal immune challenge may also alter neurogenesis in the fetal and young offspring's brain (29).

Reelin is an extracellular matrix protein that has a crucial prenatal role in multiple processes such as neurogenesis and migration, but also organization and targeting of hippocampal

Figure 2. Developmental profiles of reelin-immunoreactive (IR) neuron density in the whole hippocampus of control (SAL) and of LPS-treated male offspring. The density of reelin-immunolabeled neurons was obtained using DAB-immunostaining in control (SAL, white bar) and LPS (black bar) offspring. Values are means $\pm$ SEM (from three animals). ** $P < 0.01$ LPS vs. age-matched control (SAL) rats. DAB, 3,3-diaminobenzidine; LPS, lipopolysaccharide; SAL, saline-injected.

Figure 3. Age and structure specificities of the effects of prenatal LPS on the density of reelin-immunoreactive (IR) cells. The density of reelin-labeled neurons in the CA3 and CA1 areas and the dentate areas of (a) P10, (b) P30–40, (c) and P140–160 animals were obtained using DAB-immunostaining in control (SAL, white bar) and LPS (black bar) offspring. Values are means $\pm$ SEM (from three individuals). * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$, LPS vs. age-matched control (SAL) rats in the same area. DAB, 3,3-diaminobenzidine; LPS, lipopolysaccharide; P, postnatal day; SAL, saline-injected.

Figure 4. NAC fully prevented reelin-immunoreactive (IR) neuronal deficiency in the CA3 area in P10 LPS male offspring. The density of reelin-IR neurons was obtained using immunofluorescence labeling. It was evaluated in the CA3 area of P10 male offspring of dams injected with saline or LPS and given a postinjection treatment with NAC (LPS/NAC). Values are means $\pm$ SEM (from three animals). ** $P < 0.001$, LPS (black bar) vs. age-matched control (SAL) rats (white bar); † $P < 0.001$, LPS/NAC (gray bar) vs. age-matched LPS rats. LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; P, postnatal day; SAL, saline-injected.

Figure 5. Immunohistochemical characteristics of the reelin-immunoreactive neuronal populations in the CA3 area. (a) DAB labeling of reelin in a representative SAL P10 offspring. Note the specific Cajal–Retzius-like (thin arrow) morphology of some small Rln^+ neurons as compared with other large neurons (large arrows). (b) Double immunofluorescence of GAD_{67} (green) and reelin (red) in the CA3 area of the hippocampus of a representative P10 SAL animal. Note the coexistence of both GAD_{67}^+ and Rln^+ single-labeled cells. (c–e) Double immunofluorescence of GAD_{67} (green) and reelin (red) in the CA3 area of the hippocampus of a representative P30–40 SAL animal. (c) GAD_{67} immunoreactivity; (d) reelin immunoreactivity; and (e) merged image. Note the coexistence of GAD_{67}^+ interneurons and reelin-immunopositive GABA-ergic interneurons (arrows). Bar = 25 μm . DAB, 3,3-diaminobenzidine; GABA, γ -aminobutyric acid; P, postnatal day; Rln^+ , reelin-immunoreactive; SAL, saline-injected.

afferents, and glutamate transmission (21–23,26). Deficiencies of reelin expression could also be responsible for a large range of neurodevelopmental issues (17), and the heterozygous ($Reel^{+/-}$) mouse (30), exhibiting a permanent disruption of reelin expression, is considered to be a model of schizophrenia. Previous studies reported that early or mid-gestation prenatal challenges with poly I:C or LPS induced a deficit in Rln^+ hippocampal neurons in young rodents, most particularly in the stratum oriens of CA1 and the dentate gyrus (31–33). In the present study, we found an early (P10) deficit in reelin-expressing neurons after prenatal LPS, most prominent in the CA3 area. This deficit was transient and might support the hypothesis of migration disorders (28).

There is a general consensus for a progressive decrease of the number of reelin-producing cells during postnatal development (34,35). Starting from the first postnatal week, reelin is secreted by Cajal–Retzius cells and by GABA-ergic

Table 1. Effect of prenatal LPS on the density of Cajal–Retzius-like neurons and of reelin-containing GABA interneurons in the CA3 area of the hippocampus

	$Rln^+/GAD67^-$ (/mm ²)		$Rln^+/GAD67^+$ (/mm ²)	
	SAL	LPS	SAL	LPS
P10	33 $\pm$ 4	18 $\pm$ 2**	12 $\pm$ 2	6 $\pm$ 2*
P30–40	25 $\pm$ 2	29 $\pm$ 5	10 $\pm$ 1	11 $\pm$ 2
P140–160	9 $\pm$ 1	12 $\pm$ 2	22 $\pm$ 3	18 $\pm$ 1

Quantitative evaluation of $Rln^+/GAD67^-$ and of $Rln^+/GAD67^+$ neurons was obtained from the double immunofluorescence study. Values are means $\pm$ SEM ($n = 3$ –4 individuals).

GABA, γ -aminobutyric acid; LPS, lipopolysaccharide; P, postnatal day; Rln^+ , reelin-immunoreactive; SAL, saline-injected.

* $P < 0.05$ vs. age-matched control (SAL) rats; ** $P < 0.01$ vs. age-matched control (SAL) rats.

interneurons. Cajal–Retzius cells and reelin expression show a marked reduction from P21 onward, so that reelin is mostly secreted by GABA-ergic interneurons in adults (36). Consistent with these data, we found a global decrease of the density of reelin-producing cells, together with a progressive augmentation of Rln^+ GABA-ergic interneurons, these cells representing the major part of reelin-expressing neurons in adult rats. Excitotoxic mechanisms, which are increased during postnatal maturation (37), may account for the age-dependent lessening of Cajal–Retzius cells. Prenatal LPS, potentiating the glutamate-induced free radical production (9), may further enhance the excitotoxic mechanisms, resulting in the observed LPS-induced deficit in reelin-positive neurons.

It is tempting to speculate that the early and CA3-preminent deficit in reelin neurons could be responsible for the later, long-lasting region-selective impairment of the cytoarchitecture of the same area of the hippocampus. A full demonstration of such an instrumental role of reelin would require specifically regenerating the reelin production in the CA3 of LPS animals, which is out of the scope of the present study. We chose instead to explore whether a prenatal treatment with NAC could prevent either the reelin failure or the cytoarchitectural distortion of the hippocampus, or both.

NAC is an anti-inflammatory and antioxidative drug. It is able to inhibit the activation of nuclear factor- κB (38), a transcription factor involved in the upregulation of many cytokines. NAC's antioxidant properties are related to its ability to scavenge free radicals and its role as precursor of glutathione (39).

NAC was able to prevent both the LPS-induced synaptic impairment and the spatial memory deficit (11,12). The present study shows that NAC is also able to prevent both LPS-induced cytoarchitectural impairment and reelin deficiency. Future studies are required to determine whether all deficits are independently linked to a single prenatal perturbation or if they are associated with complex interactions.

Summing up, the present study showed that a prenatal LPS challenge induced early reelin deficit, and early and long-lasting cytoarchitectural alterations in the hippocampus. Both deficits might be involved in the impaired cognitive performance observed later in mature male offspring. The efficacy of

NAC prenatal treatment underlines the putative role of inflammation and/or oxidative stress. The protective action of NAC should be confirmed in other experimental models of maternal inflammation in the quest for efficient drugs for the prevention of human brain injuries.

METHODS

Animals

Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC) and with agreement of the Directory Committee of the Université Montpellier 2.

Pregnant Sprague–Dawley rats (Centre d'Élevage Dépré, St Doulchard, France) were used throughout this study. Experimental groups included (i) a group of control animals born from saline-injected dams (2 ml/kg i.p. at day 19 of gestation) (SAL animals), (ii) a group of animals born from LPS-treated dams (500 µg/kg i.p. at day 19 of gestation) (LPS animals), (iii) a group of rats born from SAL-injected dams given NAC in their drinking water (corresponding to a estimated daily oral intake of 500 mg/kg/d) after the injection and up to delivery (NAC/LPS animals), and (iv) a group of rats born from LPS-injected dams given the same NAC dosage in their drinking water. This drug delivery procedure was preferred to intragastric injection to avoid any stress during the prenatal stage. A further requirement was to find a NAC concentration that did not induce any difference in drinking, another potent source of stress, due to a pronounced taste of the drug. Extensive preliminary studies performed before our initial publication (12) showed that pregnant rats at this stage of gestation drank a mean of 60 (±10%) ml/d of tap water, and that this consumption was not affected by the incorporation of 5 g NAC/l. The dams' circulating NAC concentration was not evaluated because it would necessitate a blood collection, i.e., a stressful surgical procedure just before delivery. In all subsequent studies, the dams were thus allowed to drink *ad libitum* a 5g/l NAC solution. Due to the limited stability of NAC in water, this solution was replaced every day up to delivery and changed to normal tap water thereafter. After birth, the size of the litters was restricted to 10 pups. LPS (from *Escherichia coli*, serotype O55:B5) and NAC were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All procedures were done on male offspring at P10, P30–40, and P140–160. For each age group, three independent litters were considered to avoid possible litter-specific effects.

Immunohistochemistry

After lethal anesthesia, animals were perfused with 0.9% saline, followed by 4% paraformaldehyde. Brains were removed and postfixed for 2 h in the same fixative.

Vibratome 30 µm sections were collected and stored at –20 °C in cryoprotectant until further processing. Free-floating sections were processed for 3 min with 3% hydrogen peroxide to block endogenous peroxides, rinsed in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (PBS/BSA-0.2%) at room temperature, and blocked for 1 h in PBS/BSA-0.2% containing 20% normal goat serum and 0.2% Triton X-100 (Sigma-Aldrich). Sections were incubated overnight at 4 °C with mouse-anti-NeuN (Chemicon, Merck Millipore, Molsheim, France; diluted 1:2,000), mouse-anti-Reelin (G10; diluted 1:1,000) generously provided by A. Goffinet (Leuven, Belgium), or mouse-anti-GAD₆₇ (Chemicon, Merck Millipore; diluted 1:1,000). All primary antisera were diluted in PBS/BSA-0.2% containing 2% normal goat serum and 0.02% Triton X-100.

After two 10-min washes with PBS/BSA-0.2%, sections were incubated with the biotinylated secondary goat antimouse antibody (Eurobio-Abcys, Les Ulis, France), diluted 1:500 in PBS/BSA-0.2% for 1 h at room temperature. Sections were washed twice for 10 min in PBS/BSA-0.2% and incubated with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) diluted in PBS/BSA-0.2% for 30 min. After two rinses in PBS, sections were stained with 1.25% 3,3'-diaminobenzidine, Tris-NaCl 1 mol/l pH 7.6, and 0.08% H₂O₂; rinsed in PBS; dehydrated; and cover slipped with Mountex (Eurobio-Abcys).

For reelin/GAD₆₇ double immunofluorescence staining, sections were incubated for 1 h at room temperature with the corresponding secondary antibodies: Cy3-conjugated AffiniPure Goat antimouse IgG_{2a} specific for G10 antibody, diluted 1:400; or Cy2-conjugated AffiniPure Goat antimouse IgG₁ specific for anti-GAD₆₇ antibody, diluted 1:200 (Fluoroprobe, Interchim, Montluçon, France). They were rinsed three times in PBS/BSA-0.2% and cover slipped with FluorSave (Calbiochem, Merck Millipore, Molsheim, France).

Pyramidal Cell Apical Dendrite Orientation

Four to six slices from each animal, spanning across the whole antero-posterior dimension of the dorsal hippocampus, were stained with anti-NeuN antibody to visualize all neurons. Two rectangular areas (1,000 µm × 335 µm) were selected, one each within the hilar and genu zones of the CA3 area. Pyramidal cell orientation was evaluated following a method previously described (40). A line was defined from the center of the soma through the proximal segment of the adjacent apical dendrite. The angle made by this line with the tangent to the hippocampal stratus was expressed in degrees. Apical dendrites of pyramidal neurons from P140–160 SAL rats were analyzed and found to have an angle of 93° ± 34° (mean ± SD). Nonaligned apical dendrites were defined as those whose main process formed an angle <45° or >135° (±1.5 SD from the mean) with the tangent. An experimenter blind to the group/pretreatment of the slice performed the assignment. The total number of neurons was estimated and the percentage of “nonaligned” neurons vs. the total number of neurons was calculated for each slice and averaged for each animal, giving rise to a mean ± SEM percentage of nonaligned neurons across the frontal to caudal parts of the dorsal hippocampus.

Quantification of Immunoreactive Cells' Density

To determine possible effects of the prenatal treatment on the amount of neuronal populations in the hippocampus, slices of SAL or LPS age-matched animals were processed simultaneously. Handling of the different age-pairs was chosen at random to prevent any processing bias. 3,3'-Diaminobenzidine sections were scanned with a Hamamatsu Nanozoomer (Montpellier RIO Imaging facility of Institut des Neurosciences de Montpellier). Rln⁺ cells were counted using Image J cell (US National Institutes of Health, Bethesda, MD). Three regions in the hippocampal formation were defined: CA1, CA3, and the dentate gyrus. The surface of each region was measured using Image J software (US National Institutes of Health). The number of neurons was then reported for the surface of each region.

Reelin/GAD₆₇ double-stained sections were visualized by fluorescence microscopy (Olympus MVX10) (Montpellier RIO Imaging facility of DBS, UM2) allowing the quantification of reelin-positive/GAD₆₇-negative (Rln⁺/GAD₆₇[–]) and of GABA-ergic interneurons, which were both Reelin- and GAD₆₇-positive (Rln⁺/GAD₆₇⁺).

Statistical Analysis

After a first F test for equality of variance, a two-way ANOVA was used to discriminate between a treatment factor (SAL or LPS) and an age factor (P10, P30–40, or P140–160) for the percentage of non-aligned neurons and neuronal densities. All analyses were followed by *post hoc* comparisons (Bonferroni) as required. A Mann–Whitney rank sum test was used for comparison between SAL and LPS age-matched animals and between LPS and LPS/NAC age-matched animals. Probabilities of *P* < 0.05 were considered significant (StatView package, SAS).

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