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# Postnatal behavioral and inflammatory alterations in female pups prenatally exposed to valproic acid

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ABSTRACT

In Autism Spectrum Disorders (ASD), a bias to a higher incidence in boys than in girls has been reported. With the aim to identify biological mechanisms acting in female animals that could underlie this bias, we used an extensively validated mouse model of ASD: the prenatal exposure to valproic acid (VPA). We found postnatal behavioral alterations in female VPA pups: a longer latency in righting reflex at postnatal day (P) 3, and a delay in the acquisition of the acoustic startle response. We also analyzed the density of glial cells in the prefrontal cortex, hippocampus and cerebellum, in VPA and control animals. Female VPA pups showed alterations in the density of astrocytes and microglial cells between P21 and P42, with specific dynamics in each brain region. We also found a decrease in histone 3 acetylation in the cerebellum of female VPA pups at P14, suggesting that the changes in glial cell density could be due to alterations in the epigenetic developmental program. Finally, no differences in maternal behavior were found. Our results show that female VPA pups exhibit behavioral and inflammatory alterations postnatally, although they have been reported to have normal levels of sociability in adulthood. With our work, we contribute to the understanding of biological mechanisms underlying different effects of VPA on male and female rodents, and we hope to help elucidate whether there are factors increasing susceptibility to ASD in boys and/or resilience in girls.



## 1. Introduction

Autism spectrum disorders (ASD) are a group of developmental disorders of the nervous system, characterized by impairment in social interaction and communication, accompanied by stereotyped repetitive behaviors (American Psychiatric Association, 2013). The number of diagnosed children has increased in recent years, reaching values as high as 1 in 38 (Kim et al., 2011; Zablotsky et al., 2015). Remarkably, these reports show incidence is three times higher in boys than in girls. This gender bias can be partially explained by specific genetic differences between men and women (Verma et al., 2014), but other sexual differences [e.g. testosterone levels (Auyeung et al., 2009)] could play a role both in increased male susceptibility and in female resilience. Moreover, differences in the type and strength of symptoms in girls and boys have been reported (Mandy et al., 2012).

Most animal model research in the field of ASD has been performed in male animals. This bias is usually justified by the higher incidence of autism in boys and by the difficulties arising from the estral cycles in female subjects, especially in behavioral analyses. However, we consider that studying female subjects in these animal models could, on the one hand, help understanding the particular symptoms observed in autistic girls, and, on the other hand, give evidence on possible mechanisms of resiliency in the female population.

Among animal models of ASD, prenatal exposure to 600 mg/kg VPA at gestational day 12.5 has been widely used and validated. VPA animals show reduced sociability (Lucchina and Depino, 2014), increased stereotyped behaviors (Schneider et al., 2008) and several cellular and molecular alterations also observed in autistic individuals (de Theije et al., 2014; Lucchina and Depino, 2014). Previous reports show that only male rats prenatally exposed to VPA express

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a reduction on sociability, while female rats exhibit normal levels on social interaction (Kim et al., 2013; Schneider et al., 2008 Schneider et al., 2008). A similar result was obtained in mice (Kataoka et al., 2013). Repetitive and anxiety-related behaviors, along with immunological alterations, were observed in adult male VPA rats, but not in female rats (Schneider et al., 2008).

As ASD symptoms typically appear during the first years of childhood, we hypothesize that there is a critical period during postnatal development in which maturation and consolidation of the neural systems responsible for these symptoms occur. Identifying this critical period could help elucidate the biological mechanisms that alter development and result in the behavioral and physiological alterations observed in ASD, and in ASD animal models.

We (Lucchina and Depino, 2014) and others (Schneider et al., 2008) have found immunological alterations in the VPA model of ASD. Specifically, we found an increased density of glial cells in the cerebellum and an exacerbated peripheral inflammatory response. In addition, we showed that cerebellar inflammation can alter social behavior in adult male mice. In the VPA model, adult neuroimmune alterations could be determined early in life. Identifying the developmental age in which these changes occur could help reveal the role of glial cells in determining social behavior.

Here we aimed to study the postnatal consequences of prenatal exposure to VPA, particularly in female pups. We evaluated different postnatal behaviors, the hypothalamus-pituitary-adrenal response, neuroinflammation and Purkinje cells development, every postnatal week until postnatal day (P) 42. Moreover, we analyzed histone acetylation as a possible epigenetic mechanism underlying the observed alterations.

## 2. Material and methods

### 2.1. Animals

Outbred CrlFcen:CF1 female and male adult mice were obtained from the animal house at the Faculty of Exact and Natural Sciences, University of Buenos Aires (Buenos Aires, Argentina). We chose this outbred stock because these animals showed better breeding performance than inbred strains and reliable postnatal behavior. 8–10 weeks old male mice were mated with nulliparous female mice. Female mice were controlled every morning to detect the presence of a vaginal plug, and this day was considered the gestational day (GD) 0.5.

On GD12.5, pregnant mice were injected subcutaneously with 600 mg/kg of valproic acid sodium salt (VPA; Sigma, St. Louis, MO, USA) in saline solution or with saline solution, and housed individually. The parturition day was registered as postnatal day 0 (P0), and the cage bedding was not changed during the first postnatal week to avoid nest alterations. On P21, litters were weaned in cages containing 4–5 animals of the same sex and treatment. Only female mice were studied here. As all testing was performed before animals reached sexual maturity (typically around P60), we did not need to control for estrous cycle stage. Offspring belonging to the same treatment group were mixed at weaning to reduce the litter + cage effect.

Three cohorts of animals were used for the experiments. The first and second cohorts were used in postnatal behavioral tests. To avoid the effect of previous testing on other variables, we used untested animals from the first two cohorts and animals from a third cohort for histological analysis (immunofluorescence and Purkinje cell quantification), LPS challenges and histone acetylation analysis.

All animals were housed in the animal house on a 12:12 light:dark cycle and 18–22 °C temperature, with food and water ad libitum. All animal procedures were performed according to the regulations for the use of laboratory animals of the National Institute of Health, Washington, DC, USA and approved by the institutional animal care and use committee of the Faculty of Exact and Natural Sciences, University of Buenos Aires (CICUAL Protocol Nr. 6/2012).

### 2.2. Postnatal behavioral testing

We analyzed 11 Sal and 9 VPA litters from two cohorts, and a total of 72 female pups, 41 Sal and 31 VPA, from P2 to P21. All animals were evaluated in Section 2.2.2, but to maintain a balanced n in both groups, not every pup in each litter was included in behavioral analyses, and those used were randomly chosen. In addition, to fulfill the requirements of the repeated measures ANOVA, we removed from the analysis every animal in which a particular test could not be completed (e.g. animals that jumped from the grid in the grip strength test at older ages).

#### 2.2.1. Maternal care

During the first postnatal week, typical maternal behaviors were quantified: arched back nursing, grooming the pups, sleeping in or outside the nest, eating, drinking, nest building, self-grooming and carrying the pups. We performed 60 daily observations in the homecage, every minute during one hour (9:30–10:30) as previously described (Lucchina et al., 2010). The cage tag was removed to prevent the experimenter from identifying treatment and to allow a clear view of the dam and litter. Observations were made live, without disturbing the cage, by a single experimenter (NK).

#### 2.2.2. Early developmental markers

To keep track of their development, we registered the postnatal day when 1) pups opened their eyes, 2) their ears separated from their head, and 3) hair appeared on their back. Also, the pup's weight was measured from P2 to P21. This evaluation was performed for the 72 pups.

#### 2.2.3. Righting reflex

The animal was placed onto its back above a flat surface, and the time necessary to go back to its rectified position was measured. It was considered a total righting only when the 4 paws were placed in the surface, with a cutoff of 60 s. This reflex was evaluated between P4 and P8 in 15 Sal and 12 VPA animals. Also, we used an independent group (from the second cohort) of 9 Sal and 12 VPA animals to measure this reflex at P3.

#### 2.2.4. Negative geotaxis

Pups were placed in a metallic grid at a 45° angle with their head pointing down. The latency to move and turn their head to the opposite position was measured. The test ended when the animal turned 90° or after 60 s. This test was performed between P7 and P17 in 25 Sal and 25 VPA animals. We also performed this test at P5 in an independent group of 9 Sal and 10 VPA animals.

#### 2.2.5. Grip strength

A metallic grid was placed over a cage and the mouse was positioned on top of it. The grid was then inverted on top of the cage so that the animal had to grasp the grid to avoid falling down. The latency to fall was measured, with a test cutoff of 60 s. Grip strength was evaluated from P13 to P21 in 28 Sal and 22 VPA animals.

#### 2.2.6. Acoustic startle

The auditory capacity of mice was measured from P9 to P21 in 30 Sal and 25 VPA animals. The pups were placed over a flat surface and a finger snap was executed 10 cm on top of the animal's head.

The presence or absence of a startle response was registered by a single experimenter (NK).

### 2.3. Immunofluorescence

Animals were deeply anesthetized (intramuscular 80 mg/kg ketamine chlorhydrate and 8 mg/kg xylazine) between P14 and P42, and transcardially perfused with heparinized saline followed by cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH = 7.2. The brains were removed and placed in PFA for 4 h at 4 °C and then cryopreserved in a 30% sucrose solution in PB at 4 °C. P7 mice were quickly decapitated and had their brains extracted and then fixed in PFA for 24 h, before storing them in 30% sucrose. All brains were frozen with isopentane and 20 μm sagittal sections were obtained with a cryostat (Leica Biosystems, Nussloch, Germany) and stored at −20 °C. Every 6th section was processed for immunofluorescence.

For astroglial analysis, each slide with 10–12 sections were processed as previously described using the primary antibody rabbit anti-glial fibrillary protein (GFAP; 1:700, DAKO, Glostrup, Denmark) and the secondary antibody Alexa Fluor 488 anti-rabbit (1:200, Jackson, Baltimore, USA).

For microglial analysis, an antigen retrieval protocol was required. Sections were incubated with citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and heated in the microwave in three different stages with a 5 min separation, replacing the buffer for a new one each time (1 min at maximum power and 2 × 2 min at medium power). Blockage was performed with 1% normal donkey serum in PBS containing 0.1% triton for 1 h. Sections were incubated with rabbit anti-IBA1 primary antibody (WAKO, Osaka, Japan) for 2 nights, washed and then incubated with the Alexa Fluor 488 anti-rabbit secondary antibody (1:200, Jackson) for 2 h in the dark.

Confocal microscopy photographs were obtained with a Confocal Olympus FV300/BX61 microscope under 400× magnification. Z stack images were taken 1 μm apart and analyzed with Olympus Fluoview 2.0 viewer.

For each marker, between 3 and 5 sections per animal were analyzed to obtain the cellular density in each brain structure. 4–5 animals per treatment were used.

### 2.4. LPS challenge

VPA and Sal mice were injected intraperitoneally with 25 μg/kg lipopolysaccharides (LPS) (*Escherichia coli* LPS, serotype 0111:B4, Sigma-Aldrich, St. Louis, USA) or with sterile saline solution (Sal). Animals were challenged between 9:00 and 10:00 am, to avoid the effect of normal corticosterone circadian variations. Two hours after injection animals were deeply anesthetized and sacrificed, as this time has been shown to be the peak of corticosterone levels in blood after a LPS injection (Pitossi et al., 1997). The blood from P7 mice was collected by decapitation, whereas in P14 to P42 animal's trunk blood was collected from the heart. Blood was collected in heparinized tubes. N was 4–6 with the exception of P7 Sal-Sal and P42 VPA-Sal for which we analyzed 3 pups.

### 2.5. Corticosterone radioimmunoassay (RIA)

Corticosterone plasma levels were measured by means of RIA as previously described (Lucchina et al., 2010). Two blood samples of P7 mice from the same treatment were pooled together to obtain the necessary plasma volume to carry out the analysis. The assay was performed following the RIA protocol provided by the anti-corticosterone antibody manufacturer (C8784, Sigma-Aldrich, St. Louis, USA) using $^{3}$H-corticosterone (1,2,6,7-3H(N)-corticosterone, Perkin-Elmer, Waltham, MA, USA).

### 2.6. Quantification of linear Purkinje cell density

Sagittal sections were analyzed from each animal after Nissl staining. Cerebellar photomicrographs of lobules V, VI and VII were captured using an Olympus CX31 microscope equipped with an Infinity1 camera, and analyzed with the aid of the Infinity Capture software (Lumera Corporation, Ottawa, ON, Canada). The linear density of Purkinje cells was determined as the number of neurons counted along a line drawn on the Purkinke cell layer. The length of the line was measured using the ImageJ software (Rasband, 1997–2016). The density per lobule in each animal was calculated averaging the linear density of three sections. 3–5 animals per age and treatment were analyzed.

### 2.7. Histone-enriched extracts and western blotting

Histone-enriched nuclear protein extracts were obtained as previously described (Federman et al., 2012). Animals were deeply anesthetized and, after decapitation, the cerebellum and the hippocampus were extracted and processed.

For western blotting, samples were incubated at 100 °C for 5 min and then immediately placed on ice. Proteins were resolved on 15% SDS-PAGE and electrotransferred to a nitrocellulose membrane for detection by specific antibody binding.

Primary antibodies rabbit anti-acetyl histone H3 (1:5000; Millipore, Temecula, USA) and goat anti-histone H3 (1:2000; Abcam, Boston, USA) were used following the manufacturer's protocol. The detection was performed using the secondary antibodies IRDye 680RD donkey anti-rabbit and IRDye 800CW donkey anti-goat (1:20000; LI-COR Biosciences, Lincoln, USA), and the Odyssey CLx (LI-COR) infrared imaging system. The relative optical density was estimated with ImageJ 1.44p software. Each membrane was analyzed with both antibodies to determine the acetylated H3/total H3 ratio and normalized to the same external sample that was loaded in each gel. 4–5 animals per group were analyzed.

### 2.8. Statistical analysis

For the postnatal behavioral tests described in section 2.2, where animals from the same litter were evaluated, nested repeated measures analysis of variance (ANOVA) was used with litter as a subgroup, using InfoStat software (version 2016, InfoStat Group, Córdoba National University, Córdoba, Argentina). If a main effect or interaction was detected, then a one-way nested ANOVA was performed. A chi-square test was used for dichotomic variables analysis.

In all other analyses, only one animal per litter was used, so one-way ANOVA was performed using the Statistica software (version 8, StatSoft Inc., Tulsa, OK, USA). For RIA analysis, a two-way ANOVA followed by Fisher's LSD post-hoc test was used, as not all pairwise comparisons were made.

In all cases, statistical significance was assumed where $p < 0.05$.

## 3. Results

### 3.1. Injection of VPA at gestational day (GD) 12.5 does not alter maternal behavior but results in low body weight of female pups at weaning

Given that maternal care received by the pups during the first postnatal weeks can affect their behavior in adulthood (Lucchina et al., 2010; Weaver et al., 2006), we evaluated the levels of arched

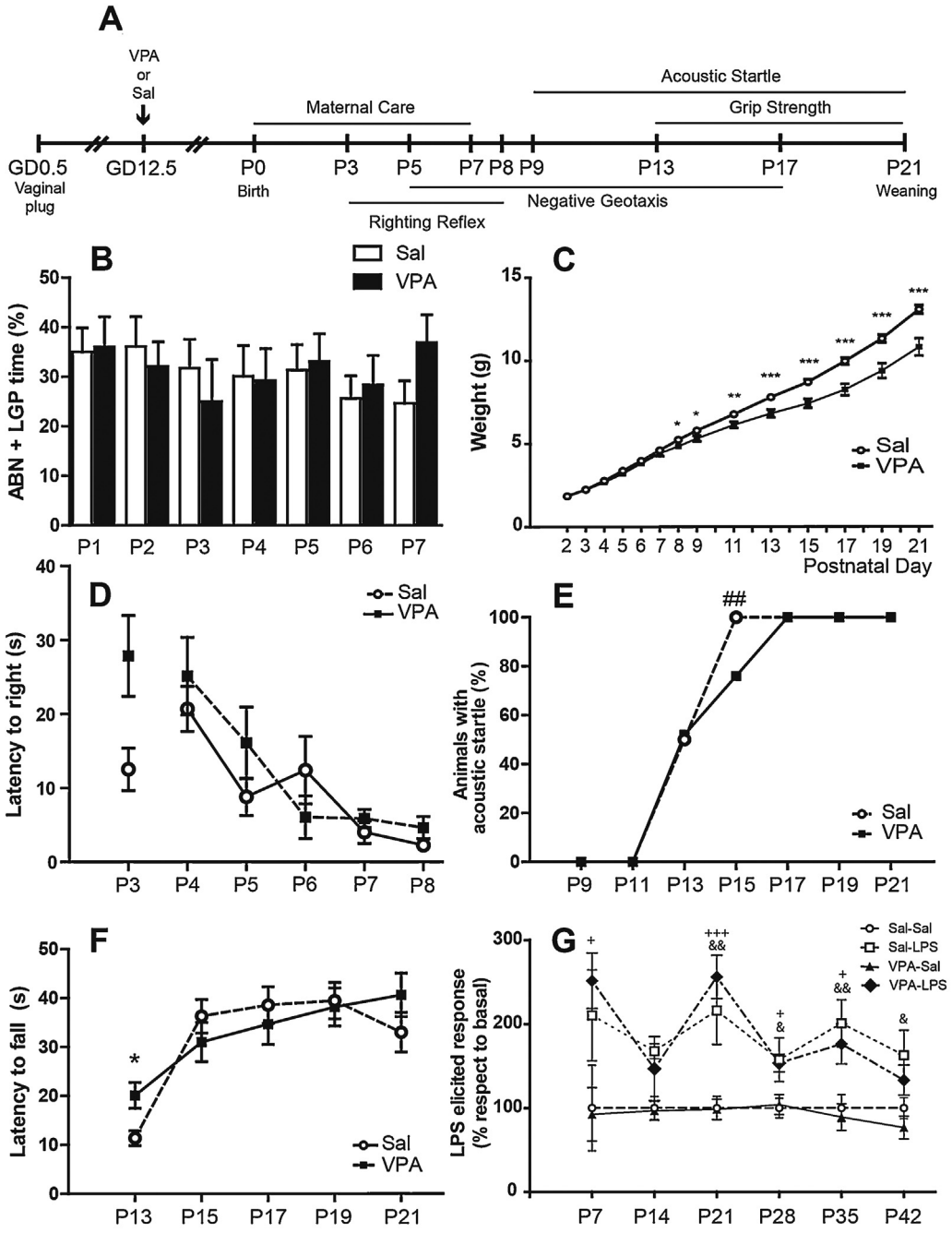


**Fig. 1.** Prenatal exposure to VPA alters postnatal behavior and weight gain, but not the peripheral inflammatory state on female pups. (A) Scheme of the experimental design used to evaluate postnatal behavior. (B) VPA does not alter maternal behavior, measured as the percentage of time that mothers spent arched back nursing (ABN) or licking and grooming the pups (LGP). (C) Prenatal VPA exposure leads to low body weight from P8 until weaning. VPA animals show a longer latency to right at P3 (D), delayed acquisition of the acoustic startle reflex (E) and bigger grip strength at P13 (F). There are no differences in corticosterone levels between Sal and VPA animals injected with saline (Sal-Sal vs VPA-Sal), and a LPS challenge activated HPA axis in both groups (Sal-LPS and VPA-LPS), at all postnatal ages (G). One way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Chi square test, ##$p < 0.01$. Mean ± SEM. Fisher's LSD test, & $p < 0.05$, && $p < 0.01$, Sal-Sal vs Sal-LPS; + $p < 0.05$, +++$p < 0.001$, VPA-Sal vs VPA-LPS. Mean + SEM (B) or Mean ± SEM (C-G).

back nursing, and licking and grooming of the pups in all the litters. We found no effect of treatment [$F(1, 18) = 0.033$; $p = 0.858$], time [$F(6, 108) = 0.732$; $p = 0.625$] or interaction effect [$F(6, 108) = 0.709$; $p = 0.643$], showing that maternal care remains constant along the first postnatal week and prenatal VPA treatment does not affect maternal behavior (Fig. 1B).

To assess for possible VPA effects on neonatal development, we evaluated three physical parameters that occur in particular developmental moments in the mouse's lifespan. In female pups, ear detachment occurred between postnatal day (P) 3 and P4 [P3: $\chi^2(1) = 1.178$; $p = 0.278$], hair appearance between P5 and P6 [P5: $\chi^2(1) = 0.192$; $p = 0.662$] and eye opening between P13 and P15 [P13: $\chi^2(1) = 0.521$; $p = 0.470$], and we found no differences between the groups. Coordination in the appearance of these physical markers in VPA and control mice suggests a normal neonatal development.

We also measured the weight of female pups from P2 until P21. We found a VPA effect [$F(1, 70) = 10.662$; $p = 0.002$], a time effect [$F(13, 910) = 1407.172$; $p < 0.001$] and an interaction effect [$F(13, 910) = 21.291$; $p < 0.001$]. From P8, VPA animals showed a decrease in their weight gain that remains until weaning (Fig. 1C). As these differences could not be due to a maternal care effect nor to an abnormal neonatal development, we can conclude that there is a specific effect of VPA treatment on body weight gain in pups.

### 3.2. Prenatal exposure to VPA alters postnatal behavior in female pups

As our aim was to characterize VPA effects in early developmental stages, we evaluated typical behaviors and reflexes that appear on predictable days of the lifespan of the mouse.

We tested the righting reflex from P4 to P8 and found a time effect [$F(4, 119) = 12.12$; $p < 0.001$] but no effect of treatment [$F(1, 119) = 0.79$; $p = 0.408$] nor interaction [$F(4, 119) = 1.32$; $p = 0.265$]. However, as the reflex could have already been acquired at P4, we performed an independent experiment testing the righting reflex at P3 but we found no significant differences between both groups [$F(1, 15) = 1.78$; $p = 0.253$; Fig. 1D].

All animals showed similar latencies in the negative geotaxis test from P7 to P17, we found no treatment [$F(1, 276) = 0.05$; $p = 0.828$], time [$F(5, 276) = 1.61$; $p = 0.157$] or interaction effects [$F(5, 276) = 1.43$; $p = 0.213$]. We evaluated whether the reflex is acquired before P7 and performed an independent experiment at P5, but we found no VPA effect either [$F(1, 13) = 0.36$; $p = 0.583$]. This could mean that VPA does not affect the negative geotaxis reflex in female pups, or that the reflex is acquired at even earlier stages.

Given that mice are born without being able to hear, the reflex to startle in response to an auditory stimulus is acquired postnatally. At P15, fewer VPA mice presented the reflex when compared to offspring from saline-injected dams (Sal mice) [$\chi^2(1) = 8.08$; $p = 0.005$; Fig. 1E]. However, these differences disappeared at P17, when all pups showed the startle response. This shows a delay in the acquisition of the acoustic startle reflex for female pups as a consequence of prenatal VPA exposure.

When testing mice grip strength in a hanging wire net, we found a time effect [$F(4, 228) = 15.66$; $p < 0.001$] but no prenatal treatment effect [$F(1, 228) = 0.1$; $p = 0.758$] or interaction between time and treatment [$F(4, 228) = 1.77$; $p = 0.136$]. VPA mice showed an increased latency to fall from the net at P13 than Sal mice [$F(1, 36) = 4.67$; $p = 0.051$; Fig. 1F]. However, as VPA animals are lighter than Sal animals at this age, our results could be influenced by this body weight difference and animals could have similar grip strength. Further studies could distinguish between these hypotheses.

In summary, our results show that the prenatal administration of VPA causes effects in female pups that can be detected at early ages, affecting the development of the acoustic startle reflex.

### 3.3. Prenatal VPA exposure does not alter the postnatal hypothalamus-pituitary-adrenal axis response to lipopolysaccharides (LPS)

We have previously demonstrated that male adult VPA mice show an exacerbated peripheral inflammatory response to an intraperitoneal LPS challenge, evidenced by increased plasma corticosterone levels 2 h after injection (Lucchina and Depino, 2014). To evaluate whether female mice also show this exacerbated response in early postnatal ages, we injected animals with LPS or saline (Sal) intraperitoneally at several postnatal ages, and measured their corticosterone levels 2 h later. LPS injection has been widely used to mimic a bacterial infection, triggering an acute inflammatory response.

We found no differences in corticosterone basal levels between VPA and Sal groups at any postnatal age (Fig. 1G). However, LPS injection activated the hypothalamus-pituitary-adrenal (HPA) axis in all animals [LPS challenge effect: $F(1, 12) = 9.943$; $p = 0.008$ for P7; $F(1, 19) = 6.886$; $p = 0.017$ for P14; $F(1, 16) = 28.54$; $p < 0.001$ for P21; $F(1, 20) = 10.72$; $p = 0.004$ for P28; $F(1, 16) = 18.96$; $p < 0.001$ for P35 and $F(1, 11) = 8.223$; $p = 0.015$ for P42] as expected. We found no interaction between prenatal treatment and LPS challenge from P7 to P42, showing that there is no exacerbated peripheral inflammatory response at these ages. Therefore, female mice have a normal inflammatory state in their periphery at early ages, independently of their prenatal treatment. These results suggest that alterations in the inflammatory response could arise at later ages or may not be present at all in female mice.

### 3.4. Prenatal VPA exposure alters postnatal microglia and astrocyte cell density in the hippocampus and the cerebellum

In a previous study, we analyzed microglial and astroglial activation in the hippocampus and the cerebellum, as these regions are linked with autism-related behaviors in the mouse. Our results indicate that prenatal exposure to VPA leads to an activated glial state in adult male animals (Lucchina and Depino, 2014). This altered neuroinflammatory state in adulthood could be due to a chronic glial activation starting in early developmental stages, but could also arise later on the lifespan of the animals. To elucidate this, we aimed to characterize the neuroinflammatory state from P7 to P42 in animals prenatally exposed to VPA or Sal, studying astrocytes and microglial cells by immunofluorescence in the hippocampus, the cerebellum and the prefrontal cortex.

#### 3.4.1. Prefrontal cortex

Astrocytes (Fig. S1A) and microglial cells (Fig. S1B) in the prefrontal cortex showed a homogeneous distribution throughout postnatal development and also between Sal and VPA mice, as no prenatal treatment effect was found.

#### 3.4.2. Hippocampus

We analyzed both the dentate gyrus (DG) and the CA1 regions of the hippocampus, estimating the density of astroglial cells and the density and level of activation of microglial cells. To estimate the level of activation of microglia, we classified cells into type I, type II–III and type IV according to their morphology (Kreutzberg, 1996; Lucchina and Depino, 2014).

We divided the dentate gyrus into three areas: the molecular and granular cell layers and the hilus. We found that VPA animals present an increased GFAP-positive cell density in the molecular and granular cell layers at P21, but no differences were observed in the hilus (Fig. 2A–E). We found no differences between experimental groups for type I microglial cells or total microglial cell density in any of the three regions. Type I cells comprised the majority of the cells in both animal groups; whereas, type IV cells were hardly seen. However, for type II–III cells, an activated form of microglia, VPA mice showed an increased cell density in the molecular layer at P21, and also in the hilus at P21 and P28, but we found no differences in the granular cell layer (Fig. 2F–J).

CA1 region was also divided into three areas: the *stratum oriens*, the pyramidal cell layer and the *stratum radiatum*. VPA treatment increased GFAP-positive cell density at P21 in the *stratum oriens* and the pyramidal cell layer, and had a similar effect in the *stratum radiatum* at P21, P28 and P42 (Fig. 3A–E). VPA mice also presented higher total microglial cell density in the *stratum oriens* and the pyramidal cell layer at P35, while no differences were observed in the *stratum radiatum* (Fig. 3F–J). In the pyramidal cell layer, type I cell density was particularly increased in VPA mice at P35 [$F(1, 8) = 6.142$; $p = 0.038$].

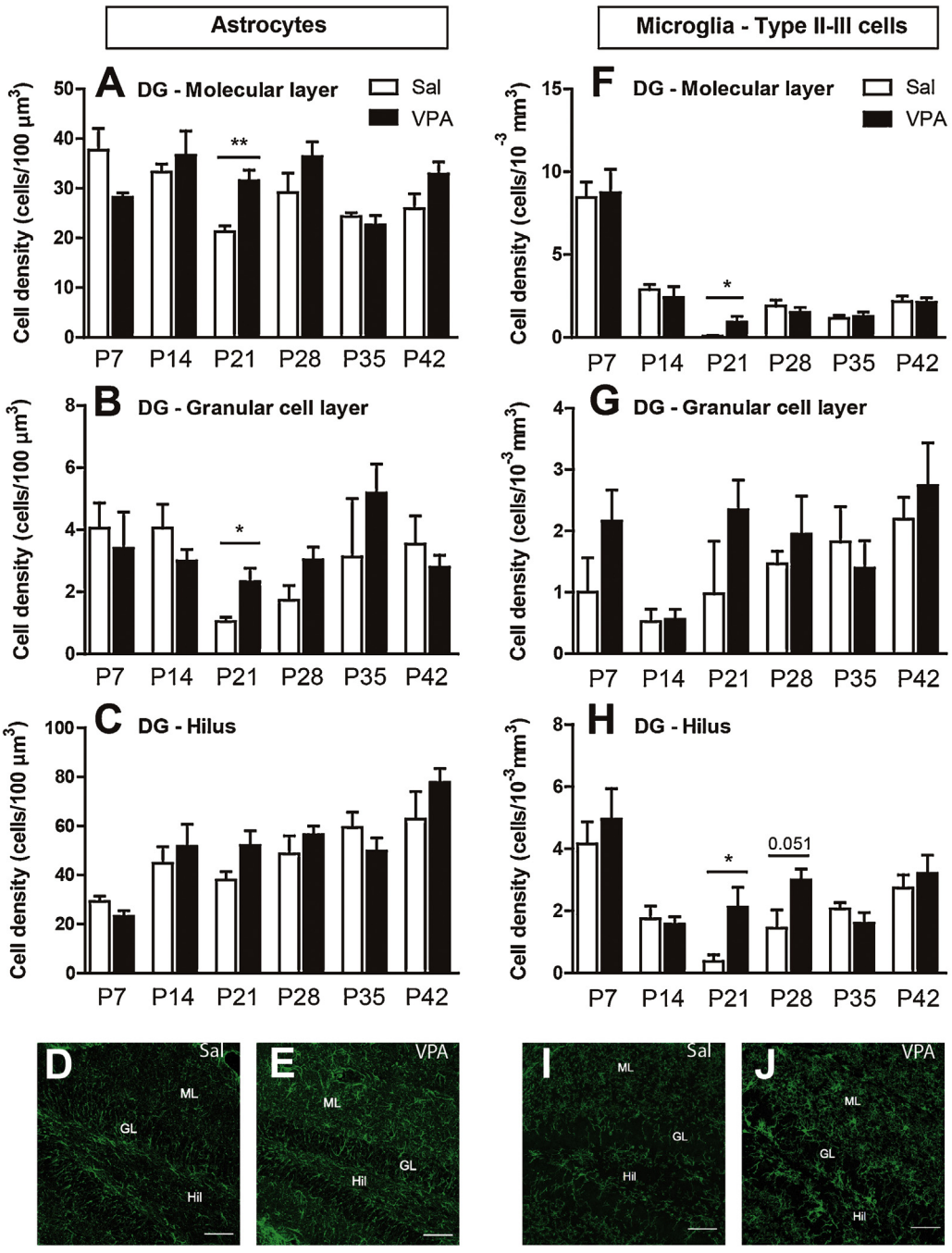


**Fig. 2.** VPA exposure alters postnatal astrocyte and microglia cell density in the dentate gyrus (DG). VPA mice show increased GFAP-positive cell density in the molecular (A) and granular cell (B) layers at P21, but no differences in the hilus (C). Representative confocal microscopy photographs of anti-GFAP immunofluorescence performed on P21 Sal (D) and VPA (E) mice. VPA treatment increases type II–III microglial cell density at P21 in the molecular layer (F) and hilus (H), but has no effects in the granular cell layer (G). Representative confocal microscopy photographs of anti-IBA1 immunofluorescence performed on P21 Sal (I) and VPA (J) mice. One way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, vs Sal. Mean + SEM. Scale bar, 50 µm. ML, molecular layer; GL, granular cell layer; Hil, hilus.

In summary, VPA mice showed signs of glial activation at weaning both in the dentate gyrus and in the CA1 region, persisting until later stages in some sub-areas but not in others. Despite these local differences, P21 seems to be the most critical stage for hippocampal neuroinflammation.

### 3.4.3. Cerebellum

In a previous work, we analyzed adult male cerebellum globally and found that microglial cell density was increased in VPA mice. Moreover, we found that inflammation in the cerebellar lobule VII was particularly linked to social behavior abnormalities (Lucchina and Depino, 2014). In addition, we found an increased GFAP-positive area in the granular cell layer of the lobule VII in male adult VPA mice. Therefore, here we focused on the analysis of this particular lobule in female pups at early postnatal ages.

Astroglial morphology in the cerebellum precluded us from distinguishing individual cells as we did in the other brain regions. Thus, we measured the GFAP-positive area in the molecular and granular cell layers of the lobule VII of the cerebellum. We found that GFAP-positive area was decreased in both layers at P35 in VPA mice, but increased at P42, when comparing with control mice (Fig. 4A–F).

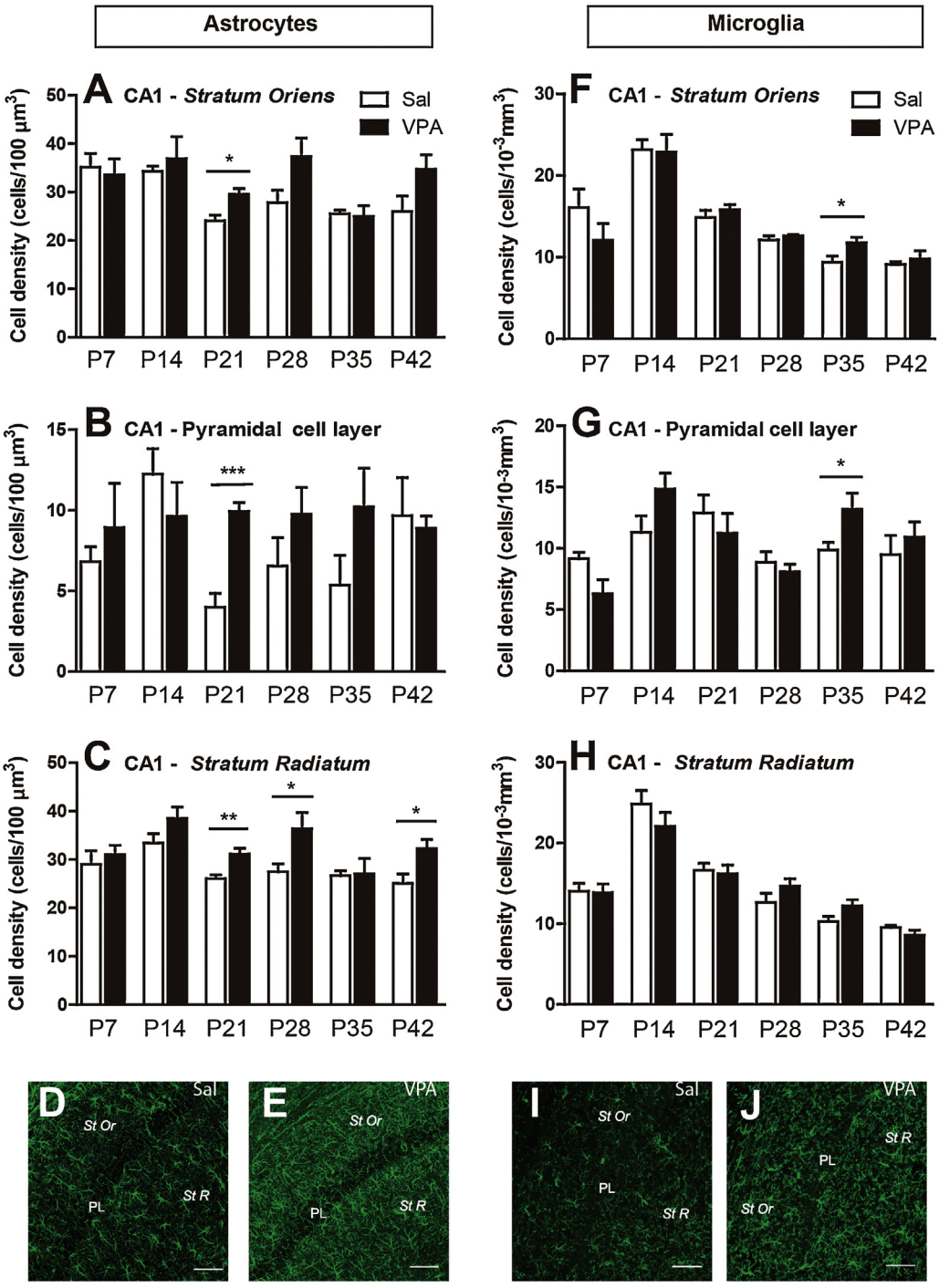


**Fig. 3.** VPA exposure alters postnatal astrocyte and microglia cell density in the CA1. VPA prenatal treatment increased GFAP-positive cell density at P21 in the *stratum oriens* (A) and pyramidal cell layer (B), and at P21, P28 and P42 in the *stratum radiatum* (C). Representative confocal microscopy photographs of anti-GFAP immunofluorescence performed on P21 Sal (D) and VPA (E) mice. VPA animals also show increased microglial cell density at P35 in the *stratum oriens* (F) and in the pyramidal cell layer (G), but no alterations in the *stratum radiatum* (H). Representative confocal microscopy photographs of anti-IBA1 immunofluorescence performed on P35 Sal (I) and VPA (J) mice. One way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Mean + SEM. Scale bar, 50 µm. *St Or, stratum oriens;* PL, pyramidal cell layer; *St R, stratum radiatum.*

Regarding the microglial analysis, results were similar to those obtained in the DG for type I and total microglial cell density, where no differences between the experimental groups were found. However, VPA mice showed a decreased type II–III cell density in the granular cell layer at P28 and P35, which was not observed in the molecular layer (Fig. 4G–L).

These results show that the neuroinflammatory alterations observed in the lobule VII of the cerebellum of VPA mice starts at a particular time period during development. Although astro and microgliosis appear to follow different temporal lines, they both show the same profile: an initial reduction followed by an increase in cell density and cell area. Given this phenomenon, the activated neuroinflammatory state that we observe in the cerebellum of adult

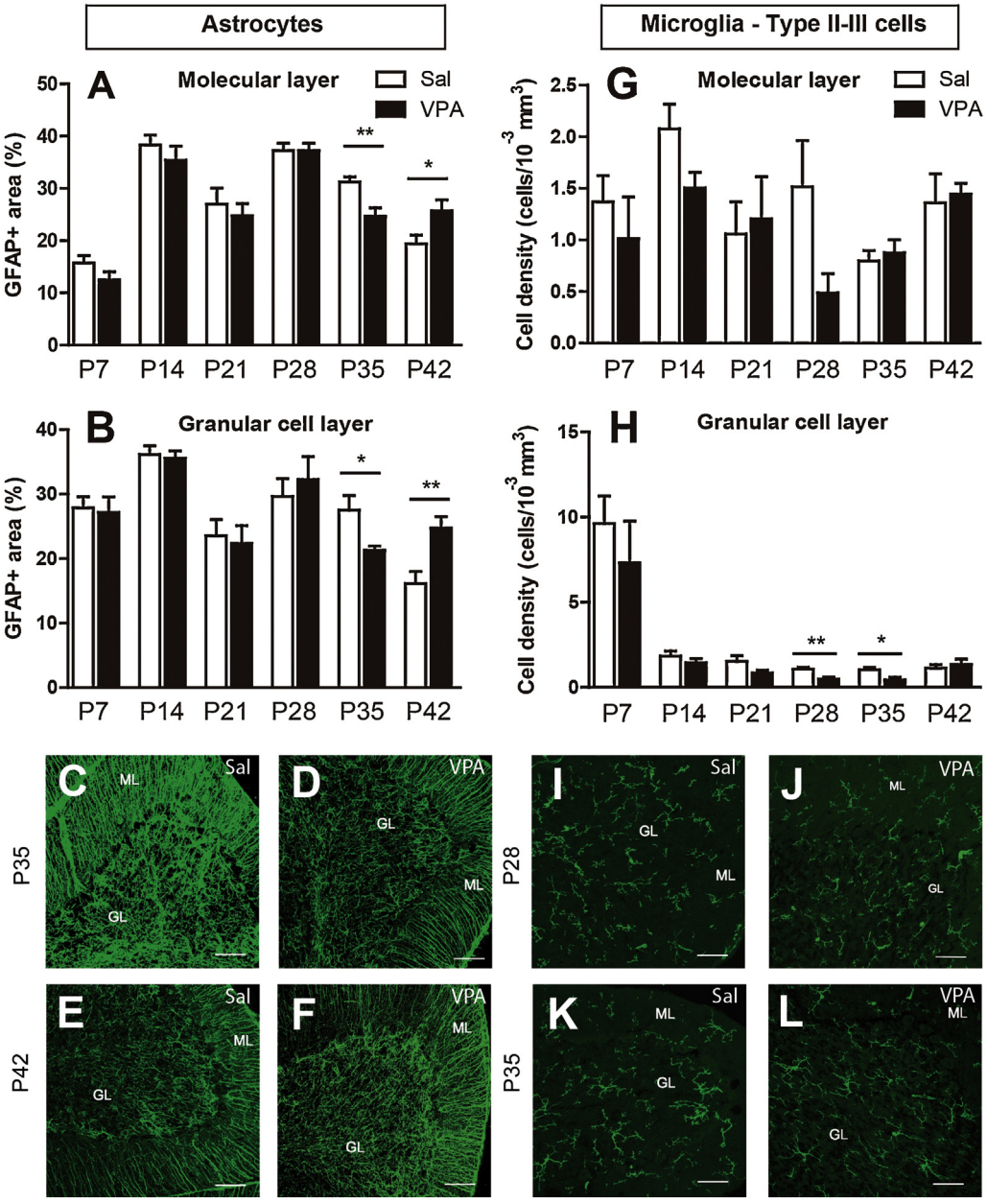


**Fig. 4.** Prenatal VPA exposure alters postnatal microglia and astrocyte cell density in the lobule VII of the cerebellum. GFAP-positive cell density was decreased at P35 in the molecular (A) and granular cell (B) layers of the lobule VII of the cerebellum, but increased at P42, in VPA-exposed animals. Representative confocal microscopy photographs of anti-GFAP immunofluorescence performed on P35 Sal (C) and VPA (D) mice, and P42 Sal (E) and VPA (F) mice. VPA treatment also leads to decreased type II–III microglial cell density in the granular cell layer (H) but has no effects on the molecular layer (G). Representative confocal microscopy photographs of anti-IBA1 immunofluorescence performed on P28 Sal (I) and VPA (J) mice, and P35 Sal (K) and VPA (L) mice. One way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$. Mean + SEM. Scale bar, 50 μm. ML, molecular layer; GL, granular cell layer.

animals could be a consequence of an overcompensatory mechanism.

### 3.5. Prenatal exposure to VPA does not alter Purkinje cell postnatal development

To evaluate whether the altered glial activation levels observed in VPA female pups could affect postnatal Purkinje cell development, we measured Purkinje cell density in lobules V, VI and VII. We found no differences between experimental groups at any of the evaluated ages (Fig. S2). This shows that neither cerebellar reduction in astrocyte and microglia density before P35, nor the increase in astrocyte density observed later, affect Purkinje cell proliferation, migration and survival.

### 3.6. Prenatal VPA exposure alters postnatal histone 3 (H3) acetylation levels

Although in our model VPA administration is a unique event during the gestational period, its effects can be detected through the early postnatal developmental stages and also remain present in adulthood. As, among other demonstrated effects, VPA functions as a histone deacetylase (HDAC) inhibitor, we aimed to study acetylation levels of histone H3 in the hippocampus and cerebellum. No VPA effect was found for the hippocampal analysis, as both groups

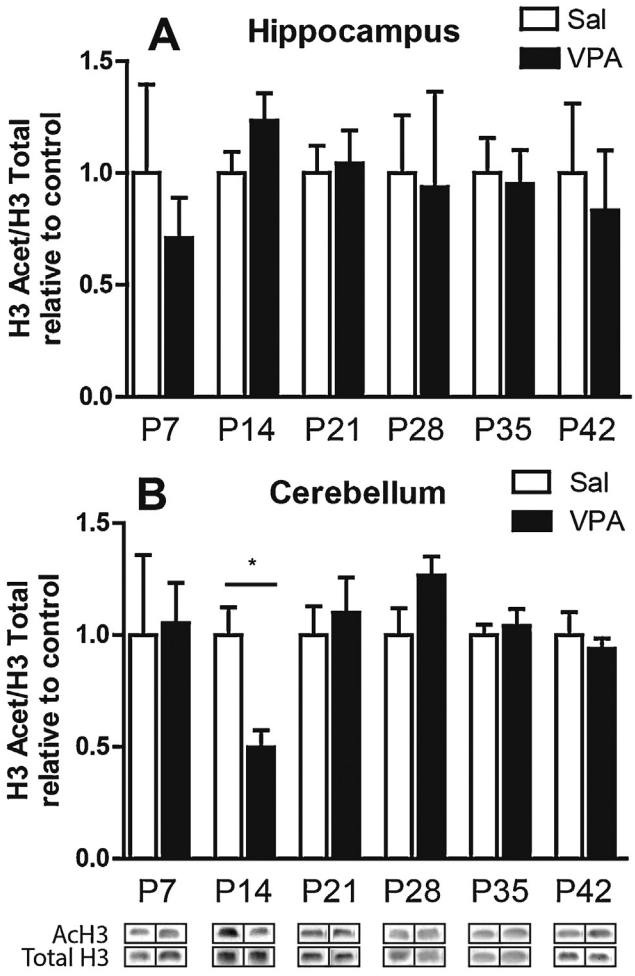


**Fig. 5.** Prenatal VPA exposure alters postnatal histone 3 acetylation levels. VPA mice show no differences in histone 3 acetylation levels in the hippocampus at any of the evaluated ages (A), but present lower histone 3 acetylation levels in the cerebellum at P14 (B). Representative results of acetylated histone 3 (AcH3, channel 700) and total H3 (channel 800) in the cerebellum at the different ages are shown. One way ANOVA, $^{*}p < 0.05$. Mean + SEM. N, 4–5 per group.

show similar H3 acetylation levels from P7 to P42 (Fig. 5A). However, in the cerebellum, VPA mice showed lower H3 acetylation levels at P14 than Sal mice (Fig. 5B). Given that this epigenetic change occurs prior to any of the observed neuroinflammatory alterations, it is possible that these may be guided by an epigenetic mechanism.

## 4. Discussion

### *4.1. Early neuroimmunological alterations: possible role in the behavioral effects of prenatal VPA exposure*

Since the original finding of neuroinflammation in autistic brains (Vargas et al., 2005), we and many other groups have searched for the functional significance of glial activation in ASD. On the one hand, clinical research has shown immunological alterations in young autistic individuals (Gupta et al., 1998; Jyonouchi et al., 2001; Molloy et al., 2006). On the other hand, different mouse models have added evidence on a role of neuroinflammation both in the development and in the manifestation of autism-related phenotypes (Depino et al., 2011; Hsiao et al., 2012; Lucchina and Depino, 2014; Malkova et al., 2012).

We previously showed that male mice prenatally exposed to VPA display signs of neuroinflammation and exacerbated inflammatory response in adulthood (Lucchina and Depino, 2014). In particular, we found evidence of chronic neuroinflammation in the cerebellum of VPA mice. Here, we found that female VPA pups already show alterations in microglia and astrocytes early in life. Although we cannot rule out gender differences (discussed below), these results suggest that adult glial alterations are determined early and remain during the whole life of the animal.

In the cerebellum, we observed a reduction in the density of astrocytes at P35, followed by an increase in P42, while less type II–III microglial cells were counted in the granular cell layer at P28 and P35. This apparent discrepancy with the increased microglia and astrocyte cell density that we found in adult VPA mice could actually reveal a compensatory/homeostatic response in adult animals to a decrease in glial cells in the developing cerebellum. These alterations in the presence of glial cells at each age could have different consequences in the development and function of neurons.

In a maternal immune activation model (prenatal exposure to PolyI:C) that shows impaired social behavior (Malkova et al., 2012), a reduction in the linear density of Purkinje cells was observed in the lobule VII of the cerebellum (Shi et al., 2009). To evaluate whether prenatal VPA can have a similar effect on these cells, we quantified Purkinje cells at different postnatal ages. However, we found no differences in their density, showing that migration, maturation and survival of Purkinje cells is normal in female VPA pups. Nevertheless, we need to perform physiological studies to verify whether their function and connectivity is unaffected. Alternatively, cerebellar glial cells could affect other neuronal populations and this, in turn, could alter behavior.

Worth to mention here, not all brain regions are affected at the same developmental ages. For example, changes in hippocampal astrocyte density appear earlier in life (at P21) than in the cerebellum (P35). In addition, microglial cell density is increased at P21 in the DG, but only at P35 in the CA1. Given the different neuronal developmental dynamics of these regions, these alterations in glial density could have different effects on neuron migration, maturation and connectivity. For example, synaptic pruning in the CA1 is mediated by microglia and can affect sociability (Paolicelli et al., 2011; Zhan et al., 2014). The authors showed that, in the CA1, microglia appears to be essential between P14 and P28, when pruning is necessary. In other regions dynamics could be different.

In addition, we found no alterations in the HPA axis response to an inflammatory stimulus at any of the evaluated ages, an unexpected result given our previous finding of an exacerbated response in adult VPA male mice (Lucchina and Depino, 2014). Again, we cannot rule out sex-differences, but these results could also suggest that this phenotype requires maturation through adolescence and is only observed in adult animals.

How can prenatal VPA exposure result in changes in glial cell density? We hypothesized that VPA having HDACs inhibitory effects, epigenetic mechanisms could underlie its long-lasting consequences. A previous work has shown a rapid increase in histone acetylation in the mouse embryonic brain, 2 and 4 h after VPA exposure (Kataoka et al., 2013). However, the same group showed that histone acetylation returns to normal 12 h after exposure. Our results add to these previous evidences showing that histone acetylation can be affected later, probably following an altered developmental pathway, and suggesting a role of HDACs in the changes observed later in life. Further work will help elucidate whether the decrease in acetylated H3 at P14 is responsible for the following changes in glial cell density and, later, for the changes in behavior in VPA mice. Alternatively, they could represent a mechanism of resiliency in female pups.

### 4.2. Biological bases for the sex bias in susceptibility observed in ASD

Various studies have looked at the effects of diverse prenatal treatments on male and female offspring on autism-related phenotypes. Some of these studies have found no sex differences, while others found that certain phenotypes seem to be affected differently in male and female offspring. Prenatal LPS, propionic acid (PPA) and polyinosinic-polycytidylic acid (PolyI:C) have similar effects on both sexes in some behaviors (Foley et al., 2014; Howland et al., 2012), but sex-specific effects in others (Foley et al., 2015; Zhang et al., 2012 Zhang et al., 2012).

Here we performed an exhaustive postnatal characterization of female pups prenatally exposed to VPA, analyzing changes in behavior, inflammatory response, glia, Purkinje cells and histone acetylation. We consider that this characterization complements previous characterizations of male VPA animals (Schneider and Przewlocki, 2005; Schneider et al., 2006). Moreover, these results add to previous reports on differences in postnatal development in VPA male and female mice (Wagner et al., 2006), along with reports where no sex differences were observed (Roullet et al., 2010).

Similar to what was found in male VPA rats (Schneider and Przewlocki, 2005), our female VPA mice showed lower body weight. We found statistical differences only after P8, no differences in maternal behavior and a bigger difference in P21 (when animals are independently feeding). These results suggest that differences are due to metabolism or food consumption rather than to maternal care and feeding. Moreover, VPA effects on body weight appear to be independent of sex (unpublished data) or species.

Schneider and Przewlocki (2005) also found a delay in eye opening in male VPA rats that we did not find in female VPA mice. Nevertheless, we found a higher latency to right at P3 and a delay in the acquisition of the acoustic startle response. These results suggest that VPA also affects postnatal development in female mice. The righting reflex suggests a slight delay in maturation of locomotor abilities that is rapidly compensated given that no differences were observed later. In turn, the delay in the acquisition of the acoustic startle response could suggest that VPA affects the development of the auditory system. Indeed, a previous report shows that adult VPA mice present a delayed auditory evoked response similar to what is observed in autistic individuals (Gandal et al., 2010). As no sex effect was evaluated in that report, further work is needed to establish whether VPA affects in a similar way male and female auditory system development. Worth to mention here, we found the same postnatal effects on behavior in male offspring (unpublished data).

The HPA hyperactivity observed in male VPA mice (Lucchina and Depino, 2014; Schneider et al., 2008), was not observed in female VPA mice neither during postnatal development (reported here) nor in adulthood (Schneider et al., 2008). Similarly, some of the effects on glial cell density reported here in female VPA mice could be absent in male VPA mice. These differences in the postnatal effects of prenatal VPA in male and female animals could underlie the behavioral differences observed later in life, when the reduction in sociability is observed in male but not in female animals (Kataoka et al., 2013; Kim et al., 2013).

In summary, postnatal behavior is affected similarly in female and male VPA offspring [Fig. 1 vs unpublished results, and (Wagner et al., 2006)], while social behavior is reduced in adult male VPA mice (Kim et al., 2013; Lucchina and Depino, 2014; Schneider et al., 2008 Lucchina and Depino, 2014; Schneider et al., 2008) but not in adult female VPA mice (Kim et al., 2013; Schneider et al., 2008 Schneider et al., 2008). We show here that female VPA offspring have altered glial activation at P21 and P35 (Figs. 2–4). Future research should evaluate whether these effects are only observed in female mice, and whether they could underlie the sex differences observed in adulthood. Indeed, preliminary, unpublished data shows that male VPA offspring do not have alterations in glial cells at P21 in the regions were we found differences in female VPA mice. We hope that these results could guide future research on key factors affecting social behavior in male VPA mice and those contributing to resilience in VPA females.

## 5. Conclusions

Here, we show that female pups exposed to VPA at GD12.5 manifest behavioral and glial alterations during the postnatal period. These alterations are not explained by changes in maternal behavior, but could be partially due to epigenetic changes. Our results contribute to understanding the role of early immune alterations in the development of behavioral alterations in animals prenatally exposed to VPA and they suggest possible differences between male and female offspring in the VPA model.

## Conflict of interest

None.

## Role of funding source

The funding source approved the study design proposed in the grant application, but had no role in the collection, analysis and interpretation of data, or in writing the report and the decision to submit the article for publication.

## Contributors

AMD has conceived and designed the work, participated in the interpretation of results and drafted and revised the manuscript. NK has acquired data, participated in the interpretation of results and drafted and revised the manuscript. MC, LL and CZ have acquired data and participated in the interpretation of results.

All authors have approved the final article.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2016.06.001.

## References

American Psychiatric Association, 2013. Diagnostic and statistical manual of mental disorders, Fifth ed. American Psychiatric Publishing.

Auyeung, B., Baron-Cohen, S., Ashwin, E., Knickmeyer, R., Taylor, K., Hackett, G., 2009. Fetal testosterone and autistic traits. Br. J. Psychol. 100, 1–22.

Depino, A.M., Lucchina, L., Pitossi, F., 2011. Early and adult hippocampal TGF-beta1 overexpression have opposite effects on behavior. Brain Behav. Immun. 25, 1582–1591.

Federman, N., Fustinana, M.S., Romano, A., 2012. Reconsolidation involves histone acetylation depending on the strength of the memory. Neuroscience 219, 145–156.

Foley, K.A., Ossenkopp, K.P., Kavaliers, M., Macfabe, D.F., 2014. Pre- and neonatal exposure to lipopolysaccharide or the enteric metabolite, propionic Acid, alters development and behavior in adolescent rats in a sexually dimorphic manner. PLoS One 9, e87072.

Foley, K.A., MacFabe, D.F., Kavaliers, M., Ossenkopp, K.P., 2015. Sexually dimorphic effects of prenatal exposure to lipopolysaccharide, and prenatal and postnatal exposure to propionic acid, on acoustic startle response and prepulse inhibition in adolescent rats: relevance to autism spectrum disorders. Behav. Brain Res. 278, 244–256.

Gandal, M.J., Edgar, J.C., Ehrlichman, R.S., Mehta, M., Roberts, T.P., Siegel, S.J., 2010. Validating gamma oscillations and delayed auditory responses as translational biomarkers of autism. Biol. Psychiatry 68, 1100–1106.

Gupta, S., Aggarwal, S., Rashanravan, B., Lee, T., 1998. Th1- and Th2-like cytokines in CD4+ and CD8+ T cells in autism. J. Neuroimmunol. 85, 106–109.

Howland, J.G., Cazakoff, B.N., Zhang, Y., 2012. Altered object-in-place recognition memory, prepulse inhibition, and locomotor activity in the offspring of rats exposed to a viral mimetic during pregnancy. Neuroscience 201, 184–198.

Hsiao, E.Y., McBride, S.W., Chow, J., Mazmanian, S.K., Patterson, P.H., 2012. Modeling an autism risk factor in mice leads to permanent immune dysregulation. Proc. Natl. Acad. Sci. U. S. A. 109, 12776–12781.

Jyonouchi, H., Sun, S., Le, H., 2001. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. J. Neuroimmunol. 120, 170–179.

Kataoka, S., Takuma, K., Hara, Y., Maeda, Y., Ago, Y., Matsuda, T., 2013. Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid. Int. J. Neuropsychopharmacol. 16, 91–103.

Kim, Y.S., Leventhal, B.L., Koh, Y.J., Fombonne, E., Laska, E., Lim, E.C., Cheon, K.A., Kim, S.J., Kim, Y.K., Lee, H., Song, D.H., Grinker, R.R., 2011. Prevalence of autism spectrum disorders in a total population sample. Am. J. Psychiatry 168, 904–912.

Kim, K.C., Kim, P., Go, H.S., Choi, C.S., Park, J.H., Kim, H.J., Jeon, S.J., Dela Pena, I.C., Han, S.H., Cheong, J.H., Ryu, J.H., Shin, C.Y., 2013. Male-specific alteration in excitatory post-synaptic development and social interaction in pre-natal valproic acid exposure model of autism spectrum disorder. J. Neurochem. 124, 832–843.

Kreutzberg, G.W., 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 19, 312–318.

Lucchina, L., Depino, A.M., 2014. Altered peripheral and central inflammatory responses in a mouse model of autism. Autism Res. 7, 273–289.

Lucchina, L., Carola, V., Pitossi, F., Depino, A.M., 2010. Evaluating the interaction between early postnatal inflammation and maternal care in the programming of adult anxiety and depression-related behaviors. Behav. Brain Res. 213, 56–65.

Malkova, N.V., Yu, C.Z., Hsiao, E.Y., Moore, M.J., Patterson, P.H., 2012. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. Brain Behav. Immun. 26, 607–616.

Mandy, W., Chilvers, R., Chowdhury, U., Salter, G., Seigal, A., Skuse, D., 2012. Sex differences in autism spectrum disorder: evidence from a large sample of children and adolescents. J. Autism Dev. Disord. 42, 1304–1313.

Molloy, C.A., Morrow, A.L., Meinzen-Derr, J., Schleifer, K., Dienger, K., Manning-Courtney, P., Altaye, M., Wills-Karp, M., 2006. Elevated cytokine levels in children with autism spectrum disorder. J. Neuroimmunol. 172, 198–205.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., Ragozzino, D., Gross, C.T., 2011. Synaptic pruning by microglia is necessary for normal brain development. Science 333, 1456–1458.

Pitossi, F., del Rey, A., Kabiersch, A., Besedovsky, H., 1997. Induction of cytokine transcripts in the central nervous system and pituitary following peripheral administration of endotoxin to mice. J. Neurosci. Res. 48, 287–298.

Rasband, W.S. ImageJ, U.S. National Institutes of Health Bethesda, Maryland, USA 1997–2016; http://imagej.nih.gov/ij/.

Roullet, F.I., Wollaston, L., Decatanzaro, D., Foster, J.A., 2010. Behavioral and molecular changes in the mouse in response to prenatal exposure to the anti-epileptic drug valproic acid. Neuroscience 170, 514–522.

Schneider, T., Przewlocki, R., 2005. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30, 80–89.

Schneider, T., Turczak, J., Przewlocki, R., 2006. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. Neuropsychopharmacology 31, 36–46.

Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., Przewlocki, R., 2008. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 33, 728–740.

Shi, L., Smith, S.E., Malkova, N., Tse, D., Su, Y., Patterson, P.H., 2009. Activation of the maternal immune system alters cerebellar development in the offspring. Brain Behav. Immun. 23, 116–123.

Vargas, D.L., Nascimbene, C., Krishnan, C., Zimmerman, A.W., Pardo, C.A., 2005. Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann. Neurol. 57, 67–81.

Verma, D., Chakraborti, B., Karmakar, A., Bandyopadhyay, T., Singh, A.S., Sinha, S., Chatterjee, A., Ghosh, S., Mohanakumar, K.P., Mukhopadhyay, K., Rajamma, U., 2014. Sexual dimorphic effect in the genetic association of monoamine oxidase A (MAOA) markers with autism spectrum disorder. Prog. Neuropsychopharmacol. Biol. Psychiatry 50, 11–20.

Wagner, G.C., Reuhl, K.R., Cheh, M., McRae, P., Halladay, A.K., 2006. A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate. J. Autism Dev. Disord. 36, 779–793.

Weaver, I.C., Meaney, M.J., Szyf, M., 2006. Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. Proc. Natl. Acad. Sci. U. S. A. 103, 3480–3485.

Zablotsky, B., Black, L.I., Maenner, M.J., Schieve, L.A., Blumberg, S.J., 2015. Estimated prevalence of autism and other developmental disabilities following questionnaire changes in the 2014 National Health Interview Survey. In: N.C.f.H (Ed.), Statistics. Hyattsville, MD.

Zhan, Y., Paolicelli, R.C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., Vyssotski, A.L., Bifone, A., Gozzi, A., Ragozzino, D., Gross, C.T., 2014. Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. Nat. Neurosci. 17, 400–406.

Zhang, Y., Cazakoff, B.N., Thai, C.A., Howland, J.G., 2012. Prenatal exposure to a viral mimetic alters behavioural flexibility in male, but not female, rats. Neuropharmacology 62, 1299–1307.

de Theije, C.G., Wopereis, H., Ramadan, M., van Eijndthoven, T., Lambert, J., Knol, J., Garssen, J., Kraneveld, A.D., Oozeer, R., 2014. Altered gut microbiota and activity in a murine model of autism spectrum disorders. Brain Behav. Immun. 37, 197–206.