Title: Prenatal exposure to sodium valproate alters androgen receptor expression in the developing cerebellum in a region and age specific manner in male and female rats

Author: Miguel Perez-Pouchoulen Marta Miquel Paul Saft Brenda Brug Rebeca Toledo Maria Elena Hernandez Jorge Manzo

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Prenatal exposure to sodium valproate alters androgen receptor expression in the developing cerebellum in a region and age specific manner in male and female rats.

List of Author Names and Affiliations

Miguel Perez-Pouchoulen*[,1,2], Marta Miquel[3], Paul Saft[2], Brenda Brug[2], Rebeca Toledo[2], Maria Elena Hernandez[2], Jorge Manzo[2].

[2]Centro de Investigaciones Cerebrales, Universidad Veracruzana, Xalapa, VER. Mexico.

[3]Area de Psicobiologia, Universidad Jaume I, Castellon de la Plana, Spain

E-mails: mpouchoulen@som.umaryland.edu, miquel@uji.es, paulsaft@uaem.edu.mx, brug_@hotmail.com, rtoledo@uv.mx, elenahernandez@uv.mx, jmanzo@uv.mx

***Corresponding author:**

Miguel Perez-Pouchoulen, Ph.D.

Centro de Investigaciones Cerebrales, Universidad Veracruzana. Medicos y Odontologos s/n, C.P. 91010 Xalapa, Ver., Mexico Tel: +52 (228) 8418900 Ext. 16308

[1]Present address: Department of Pharmacology, University of Maryland School of Medicine 655 W. Baltimore Street BRB-5014, Baltimore, Maryland 21201, USA.

Phone number: (410) 706-2654, Fax: (410) 706-8341,

E-mail: mpouchoulen@som.umaryland.edu

Conflict of Interest

The authors declare no conflict of interests. All authors have approved the final article and contributed in the research and/or article preparation.

Highlights

1. Both age and region influence androgen receptor (AR) expression in the developing rat cerebellum.
2. Prenatal exposure to valproic acid (VPA) disrupts AR expression in the developing rat cerebellum.
3. Only the posterior part of the cerebellum is affected by VPA exposure in both male and female rats.

Abstract

Valproic acid (VPA) is an anti-epileptic drug with teratogenicity activity that has been related to autism. In rodents, exposure to VPA in utero leads to brain abnormalities similar than those reported in the autistic brain. Particularly, VPA reduces the number of Purkinje neurons in the rat cerebellum parallel to cerebellar abnormalities found in autism. Thus, we injected pregnant females on embryonic day 12 either with VPA (600 mg/kg, i.p.) or 0.9 % saline solution and obtained the cerebellum from their offspring at different postnatal time points. Testosterone has been linked to autism and plays an important role during brain development. Therefore, we identified and analyzed the androgen receptor (AR) by immunohistochemistry and densitometry, respectively. We found VPA decreases AR density in the superficial Purkinje layer only in cerebellar lobule 8 at PN7, but increased it at PN14 compared to control in males. In females, VPA decreased AR density in the superficial Purkinje layer in cerebellar lobule 6 at PN14, but increased it in lobule 9 at the same time point. No differences were found in the deep Purkinje layer of any cerebellar lobule in terms of AR density neither in males nor females. We additionally found a particular AR density decreasing in both superficial and deep regions across development in the majority of cerebellar lobules in males, but in all cerebellar lobules in females. Thus, our results indicate that VPA disrupts the AR ontogeny in the developing cerebellum in an age and region specific manner in male and female rats. Future epigenetic studies including the evaluation of histone deacetylases (HDAC's) might shed light these results as HDAC's are expressed by Purkinje neurons, interact with the AR and are VPA targets. This work contributes to the understanding of the cerebellar development and it might help to understand the role of the cerebellum in neurodevelopmental disorders such as autism.

Key words

vermis, teratogen, Purkinje neuron, neurodevelopmental disorders, sex differences

1. Introduction

Valproic acid (VPA) is an anti-epileptic drug with a potent teratogenic activity capable to induce anatomical and chemical alterations in the central nervous system (CNS) of the rat (Almeida et al., 2014; Chomiak et al., 2010; Dufour-Rainfray et al., 2010; Ingram et al., 2000; Narita et al., 2002; Wagner et al., 2006). Such alterations are similar to those reported in the autistic brain (Chugani et al., 1997; Fatemi et al., 2002; Lee et al., 2002; Purcell et al., 2001), that consistently shows abnormalities in the human cerebellum in terms of volume, morphology, and number of Purkinje neurons (Bauman and Kemper, 2005; Courchesne, 1999; Murakami et al., 1989; Palmen et al., 2004). In rats, prenatal exposure to VPA results in a reduced number of Purkinje neurons in the vermis as well as a reduction in the volume of the granular layer in adult rats (Ingram et al., 2000). Thus, VPA has been used as a model for the study of autistic disorders because it has been found to induce autistic-like behavior in rats (Roullet et al., 2010; Schneider and Przewlocki, 2005; Vorhees, 1987a; Wagner et al., 2006). Nevertheless, despite these advances there are no studies focusing on the protein expression of surviving Purkinje neurons after in utero exposure to VPA.

The Purkinje neurons serve as the only output of the cerebellar cortex to communicate with the rest of the CNS (Apps and Garwicz, 2005; Ramnani, 2006; Voogd, 2003). Therefore, any alteration to the Purkinje neurons could lead to a dysfunctional cerebellar circuit. As a consequence, these altered cerebellar circuits result in abnormal connectivity between the cerebellum and any brain nuclei linked to it. Moreover, Purkinje neurons are known to express estrogen and progesterone receptors which both regulate

neuroplasticity during cerebellar development (Haraguchi et al., 2012; Sakamoto et al., 2003; Sakamoto et al., 2001; Sasahara et al., 2007; Tsutsui, 2008). Furthermore, the androgen receptor (AR) is also expressed by Purkinje neurons in the developing and adult cerebellum (Bowers et al., 2014; Perez-Pouchoulen et al., 2016; Qin et al., 2007), but its function in the neurobiology of the cerebellum remains unclear. In other brain regions, the AR regulates the expression of both cytoskeletal proteins such as tubulin (Jones and Oblinger, 1994; Matsumoto et al., 1994) and neurotrophic factors such as BDNF (brain-derived neurotrophic factor) (Yang et al., 2004) as it translocates to the cell nucleus functioning as a transcription factor (Chang et al., 1995; Lee and Chang, 2003). Thus, the study of AR function and its participation during the formation of the cerebellar circuit is highly relevant, because it is the only receptor of testosterone, a powerful androgenic hormone that plays an important role during brain development (Lombardo et al., 2012; McCarthy, 2008; McEwen, 1992) and has been linked to autism (Auyeung et al., 2009; Baron-Cohen et al., 2009; Knickmeyer and Baron-Cohen, 2006; Tordjman et al., 1997).

In this study we sought to evaluate the effect of prenatal exposure to VPA on the AR expression in the developing cerebellum using male and female rats since autism affects more boys than girls in a ratio of 4:1, respectively (Aiello and Whitaker-Azmitia, 2011; Bauman and Kemper, 2005; Moldin et al., 2006). We also included an anatomical view as an important factor for the organization and function of the cerebellum.

2. Materials and Methods

2.1 Subjects and housing

Female (Wistar, 200-250 g/bw) and male rats (Wistar, 250-350 g/bw) were used and obtained from the colony room of the Centro de Investigaciones Cerebrales, Universidad Veracruzana, Xalapa, Mexico. Animals were kept under reverse light-dark cycle (12-12 h light off at 0800 h) in standard acrylic cages containing wood-chip bedding (nu3lab). Food from Harlan Mexico (rodent chow) and water were provided *ad libitum*. All animal procedures were approved by a review committee of Universidad Veracruzana in accordance with the Official Norms in Mexico (NOM-062-ZOO-1999 and NOM-087-ECOL-SSA1-2003) and the Society for Neuroscience Policy on the use of Animals in Neuroscience Research.

2.2 Sodium valproate administration

Male and female rats were placed together for mating and when a vaginal plug was detected it was counted as embryonic day 1 (E1). Dams were randomly assigned to either control or treatment group. Treatment dams received a single intraperitoneally VPA injection on E12 using a 600 mg/kg dose as described elsewhere (Figure 1A) (Ingram et al., 2000). The VPA was purchased as sodium valproate salt (Sigma-Aldrich, Saint Louis, USA) and based on the weight of each dam it was measured and then dissolved in 0.3 ml of 0.9 % saline solution. This procedure was performed so that each dam would receive the same volume of solution containing the corresponding amount of VPA. Control dams received 0.3 ml of 0.9% saline solution the same day. Subsequently, all dams were housed individually until they gave birth, which was counted as postnatal day 0 (PN0). A total of 96 rat pups were used (8 males and 8 females for each group) and obtained from a total of 20 injected dams (5 control + 15 VPA dams). It is worth noting that we actually injected 28 dams with VPA, but only 15 dams delivered pups.

Figure 1.

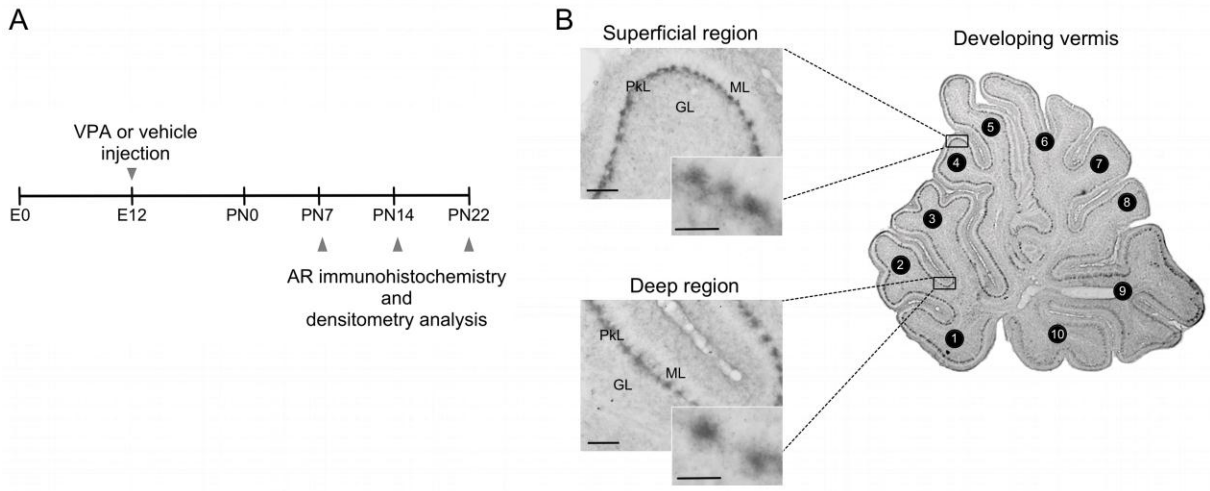

Figure 1. (A) Experimental time line of the AR analysis at three different time points during postnatal development of the cerebellum in male and female rats either treated with vehicle or VPA. (B) Sagittal view of the developing cerebellum (PN7) depicting the cerebellar lobules and the superficial (top and left) and deep (bottom and left) regions where the AR densitometry analysis was performed. Inserts show an amplification of the AR labeling in the Purkinje layer, presumably in Purkinje neurons based on their soma's morphology at this age (McKay and Turner, 2005). Scale bar = 50 µm, inserts scale bar = 12.5 µm. E = embryonic day, PN = postnatal day, VPA = valproic acid, AR = androgen receptor, ML = molecular layer, PkL = Purkinje layer, GL = granular layer.

2.3 Tissue collection and immunohistochemistry

On PN7, PN14 and PN22 rat pups were deeply anesthetized with an overdose of sodium pentobarbital (i.p., 30 mg/kg) and transcardially perfused with phosphate buffered saline (PBS) and 4 % paraformaldehyde (pH = 7.4). The entire cerebellum was removed and post-fixed for 24 h in 4 % paraformaldehyde at 4 °C. Subsequently, tissue was placed in 30 %

sucrose at 4 °C until they were saturated (approximately 72 h) before sectioning on a Leica cryostat. Forty micrometers sagittal sections were obtained from the mid-vermis and processed for immunohistochemistry as described elsewhere (Perez-Pouchoulen et al., 2016). Briefly, cerebellar sections were incubated with a polyclonal antibody against the AR (1:1500, rabbit anti-AR, Santa Cruz Biotechnology sc-816) in 0.4 % PBS with Triton X-100 (PBS-T) for 48 h at 4 °C. This antibody has been validated to localize the AR in Purkinje neurons (Bowers et al., 2014; Perez-Pouchoulen et al., 2016; Qin et al., 2007), and in other neurons of the CNS (Wood and Keller-Wood, 2008). Subsequently, sections were incubated with biotinylated anti-rabbit secondary (1:500, Vector Laboratories) in 0.4 % PBS-T for 90 min at room temperature with constant agitation. Sections were then incubated with avidin-biotin complex (1:500, Vectastain® Elite ABC-Peroxidase kit Standard, Vector Laboratories) in 0.4 % PBS-T for 90 min at room temperature with constant agitation. AR positive staining (Figure 1B) was visualized using diaminobenzidine as chromogen in the presence of nickel sulfate for 5-6 min (Vector Laboratories kit). Finally, sections were exhaustively rinsed with PBS, mounted on gelatin-coated slides, cleared with ascending alcohol concentrations, defatted with xylene, and coverslipped with Permount (Fisher Scientific). All cerebellar sections were processed under similar conditions. Additionally, negative and positive controls for AR immunoreactivity were run to rule out false AR immunostaining.

2.4 Densitometry

Digital images of cerebellar sections were obtained with an Olympus Provis AX70 microscope (Tokio, Japan) interfaced with a camera sending photomicrographs to a computer system containing the Image-Pro Plus software (Media Cybernetics). Two images

were taken in both superficial and deep regions in all cerebellar lobules under 40x magnification and converted to 16-bits gray scale before analysis. The absolute values obtained from those two images were averaged to use a single value per region for each lobule. It is important to note that densitometric values from sub-lobules of cerebellar lobules 6 and 9 were combined to generate a single result. A total of ten cerebellar lobules were studied here. A grid of 60 squares of 25 x 25 μm each (total area of 37,500 μm^2) was superimposed on the Purkinje layer for densitometry analysis using the Photoimpact program (Corel). **Only the AR-positive cell of the Purkinje layer within the grid was analyzed using a standardized circular tool of 176.715 μm^2. In every image, we analyzed and combined the background staining from the granular and molecular layer to normalize the AR density (from the Purkinje layer) value, which was expressed as "relative optical density". We used 3 cerebellar sections from the mid-vermis per animal, they were grouped and then averaged for each animal.** This method has been used in our lab (Perez-Pouchoulen et al., 2016) and with modifications by others (Finesmith and Favero, 2014; Hamson et al., 2009).

2.5 Statistical analysis

All data are expressed as mean ± S.E.M. In both control males and females, a one-way ANOVA with age as fixed factor was performed for each cerebellar lobule (1-10) in the superficial and deep regions separately. A two-way ANOVA with age and treatment as fixed factors was used to analyze AR expression in every cerebellar lobule in both males and females considering both superficial and deep regions. The Tukey's post hoc test or pairwise comparison was used when significant differences were seen at $p \leq 0.05$. Additionally, we calculated either eta squared (η^2) or Cohen's d effect size estimates where

appropriate. All statistical tests were computed in SPSS 22 and graphed using GraphPad Prism 6.

3. Results

3.1 AR expression in Purkinje layer decreases during the second postnatal week in the majority of cerebellar lobules in both superficial and deep regions in males.

We first determined the AR pattern expression in the Purkinje layer in two anatomical regions during the postnatal development of the male cerebellum at three different time points. The one-way ANOVA detected significant differences among ages for the AR density in the superficial region of cerebellar lobules (full statistical details in Figure 2A). Post hoc analysis revealed the expression of AR decreased in the Purkinje layer at PN14 compared to PN7 in: **lobule 2 (PN7, 0.39 ± 0.17 vs. PN14, 0.00 ± 0.00; *p* = 0.05); lobule 3 (PN7, 0.48 ± 0.19 vs. PN14, 0.00 ± 0.00; *p* = 0.034); lobule 4 (PN7, 0.54 ± 0.20 vs. PN14, 0.00 ± 0.00; *p* = 0.026); lobule 5 (PN7, 0.56 ± 0.16 vs. PN14, 0.02 ± 0.00; *p* = 0.02); lobule 6 (PN7, 0.70 ± 0.17 vs. PN14, 0.00 ± 0.00; *p* = 0.001); lobule 7 (PN7, 0.63 ± 0.19 vs. PN14, 0.02 ± 0.02; *p* = 0.004), and lobule 8 (PN7, 0.58 ± 0.20 vs. PN14, 0.11 ± 0.05; *p* = 0.032)**. However, the AR density also decreased at PN22 compared to PN7, but only in **lobule** 7 **(PN7, 0.63 ± 0.19 vs. PN22 0.10 ± 0.07; *p* = 0.013),** and **lobule** 8 **(PN7, 0.58 ± 0.20 vs. PN22 0.00 ± 0.00; *p* = 0.008)** (Figure 2A). No significant differences were detected for **lobule** 1 ($F_{(2,21)} = 1.431$, $p > 0.05$), **lobule** 9 ($F_{(2,21)} = 2.567$, $p > 0.05$), and **lobule** 10 ($F_{(2,21)} = 0.977$, $p > 0.05$).

With regard to the deep region of lobules, the one-way ANOVA also detected significant differences among ages for the AR density (full statistical details in Figure 2B).

The post hoc analysis indicated that the AR density was lower at PN14 and PN22 compared to PN7 in **lobule 3 (PN7 0.38 ± 0.19, PN14 0.00 ± 0.00, PN22 0.00 ± 0.00; $p = 0.05$); lobule 4 (PN7 0.45 ± 0.19, PN14 0.00 ± 0.00, PN22 0.00 ± 0.00; $p = 0.021$); lobule 5 (PN7 0.35 ± 0.17, PN14 0.00 ± 0.00, PN22 0.00 ± 0.00; $p = 0.058$); lobule 6 (PN7 0.51 ± 0.16, PN14 0.00 ± 0.00, PN22 0.00 ± 0.00; $p = 0.002$); lobule 7 (PN7 0.35 ± 0.12, PN14 0.00 ± 0.00, PN22 0.00 ± 0.00; $p = 0.005$), and lobule 8 (PN7 0.19 ± 0.08, PN14 0.00 ± 0.00, PN22 0.00 ± 0.00; $p = 0.035$)**. No significant differences were found for cerebellar lobules 1 ($F_{(2,21)} = 2.323$, $p > 0.05$), and 10 ($F_{(2,21)} = 1.000$, $p > 0.05$) (Figure 2B).

Figure 2.

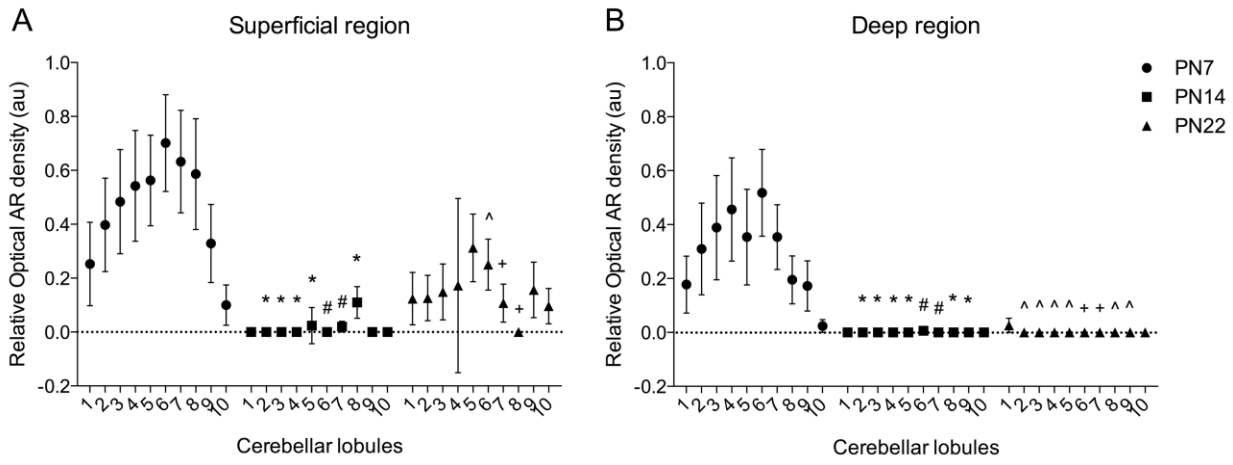

Figure 2. Postnatal AR expression in the developing vermis in male rats. (A) Superficial Purkinje layers expressing the AR across ten cerebellar lobules in the mid-vermis. Statistical significant differences among ages for the AR density in the superficial region of **lob 2** ($F_{(2,21)} = 3.316$, $p = 0.05$, $\eta^2 = 0.240$); **lob 3** ($F_{(2,21)} = 3.830$, $p = 0.038$, $\eta^2 = 0.267$); **lob 4** ($F_{(2,21)} = 4.161$, $p = 0.030$, $\eta^2 = 0.284$); **lob 5** ($F_{(2,21)} = 4.890$, $p = 0.018$, $\eta^2 = 0.318$); **lob 6** ($F_{(2,21)} = 9.198$, $p = 0.001$, $\eta^2 = 0.467$); **lob 7** ($F_{(2,21)} = 7.935$, $p = 0.003$, $\eta^2 = 0.430$), and **lob 8** ($F_{(2,21)} = 6.366$, $p = 0.007$, $\eta^2 = 0.377$). (B) Deep Purkinje layers expressing the AR across ten cerebellar lobules in the mid-vermis. Statistical significant differences among ages for the AR density in the deep region of **lob 3** ($F_{(2,21)} = 4.042$, $p = 0.033$, $\eta^2 = 0.278$); **lob 4** ($F_{(2,21)} = 5.677$, $p = 0.011$, $\eta^2 = 0.351$); **lob 5** ($F_{(2,21)} = 3.984$, $p = 0.034$, $\eta^2 = 0.275$); **lob 6** ($F_{(2,21)} = 10.203$, $p = 0.001$, $\eta^2 = 0.493$); **lob 7** ($F_{(2,21)} = 8.682$, $p = 0.002$, $\eta^2 = 0.453$), and **lob 8** ($F_{(2,21)} = 4.822$, $p = 0.019$, $\eta^2 = 0.315$). Data are expressed as mean ± S.E.M.; n = 8 males per group. **Symbols indicate significant differences: *$p < 0.05$ and**

$^{\#}p < 0.01$ when PN14 is compared to PN7; ^$p < 0.05$ and $^{+}p < 0.01$ when PN22 is compared to PN7. PN = postnatal day, Lob = cerebellar lobule.

3.2 AR expression in Purkinje layer decreases during the second and third postnatal week in all cerebellar lobules in both superficial and deep regions in females.

We also determined the AR pattern expression in the developing cerebellum of females and the one-way ANOVA showed a significant main effect for age with respect to AR density in the superficial region of all cerebellar lobules studied (full statistical details in Figure 3A). The post hoc analysis revealed the expression of AR decreased in the Purkinje layer similarly at PN14 and PN22 when compared to PN7 in all ten cerebellar lobules: **lobule 1 (PN7 1.71 ± 0.02, PN14 0.42 ± 0.17 $p < 0.000$, PN22 0.45 ± 0.11 $p < 0.000$); lobule 2 (PN7 1.81 ± 0.04, PN14 0.77 ± 0.21 $p < 0.000$, PN22 0.79 ± 0.11 $p < 0.000$); lobule 3 (PN7 1.73 ± 0.04, PN14 0.70 ± 0.19 $p < 0.000$, PN22 0.86 ± 0.05 $p < 0.000$); lobule 4 (PN7 1.77 ± 0.04, PN14 0.50 ± 0.18 $p < 0.000$, PN22 0.52 ± 0.10 $p < 0.000$); lobule 5 (PN7 1.79 ± 0.05, PN14 0.48 ± 0.15 $p < 0.000$, PN22 0.78 ± 0.14 $p < 0.000$); lobule 6 (PN7 1.61 ± 0.07, PN14 0.65 ± 0.13 $p < 0.000$, PN22 0.82 ± 0.13 $p < 0.000$); lobule 7 (PN7 1.57 ± 0.07, PN14 0.58 ± 0.22 $p = 0.003$, PN22 0.63 ± 0.21 $p = 0.005$); lobule 8 (PN7 1.72 ± 0.03, PN14 0.52 ± 0.16 $p < 0.000$, PN22 0.90 ± 0.20 $p = 0.003$); lobule 9 (PN7 1.62 ± 0.03, PN14 0.12 ± 0.07 $p < 0.000$, PN22 0.75 ± 0.10 $p < 0.000$), and lobule 10 (PN7 1.32 ± 0.06, PN14 0.38 ± 0.11 $p < 0.000$, PN22 0.86 ± 0.16 $p = 0.04$)** (Figure 3A).

Furthermore, the statistical analysis detected similar differences in the deep region with respect to AR density in the Purkinje layer for all cerebellar lobules analyzed (full statistical details in Figure 3B). Likewise, post hoc analysis revealed the expression of AR

decreased in the Purkinje layer similarly at PN14 and PN22 when compared to PN7 in all ten cerebellar lobules: **lobule 1 (PN7 1.24 ± 0.20, PN14 0.16 ± 0.06 $p < 0.000$, PN22 0.15 ± 0.05 $p < 0.000$); lobule 2 (PN7 1.81 ± 0.03, PN14 0.24 ± 0.14 $p < 0.000$, PN22 0.10 ± 0.10 $p < 0.000$); lobule 3 (PN7 1.87 ± 0.03, PN14 0.24 ± 0.11 $p < 0.000$, PN22 0.05 ± 0.05 $p < 0.000$); lobule 4 (PN7 1.85 ± 0.04, PN14 0.07 ± 0.04 $p < 0.000$, PN22 0.02 ± 0.02 $p < 0.000$); lobule 5 (PN7 1.80 ± 0.04, PN14 0.02 ± 0.02 $p < 0.000$, PN22 0.02 ± 0.02 $p < 0.000$); lobule 6 (PN7 1.65 ± 0.03, PN14 0.40 ± 0.14 $p < 0.000$, PN22 0.04 ± 0.02 $p < 0.000$); lobule 7 (PN7 1.48 ± 0.08, PN14 0.07 ± 0.04 $p < 0.000$, PN22 0.08 ± 0.04 $p < 0.000$); lobule 8 (PN7 1.77 ± 0.04, PN14 0.00 ± 0.00 $p < 0.000$, PN22 0.08 ± 0.08 $p < 0.000$); lobule 9 (PN7 1.63 ± 0.03, PN14 0.01 ± 0.01 $p < 0.000$, PN22 0.01 ± 0.01 $p < 0.000$), and lobule 10 (PN7 1.33 ± 0.06, PN14 0.08 ± 0.03 $p < 0.000$, PN22 0.09 ± 0.05 $p < 0.000$)** (Figure 3B).

Figure 3.

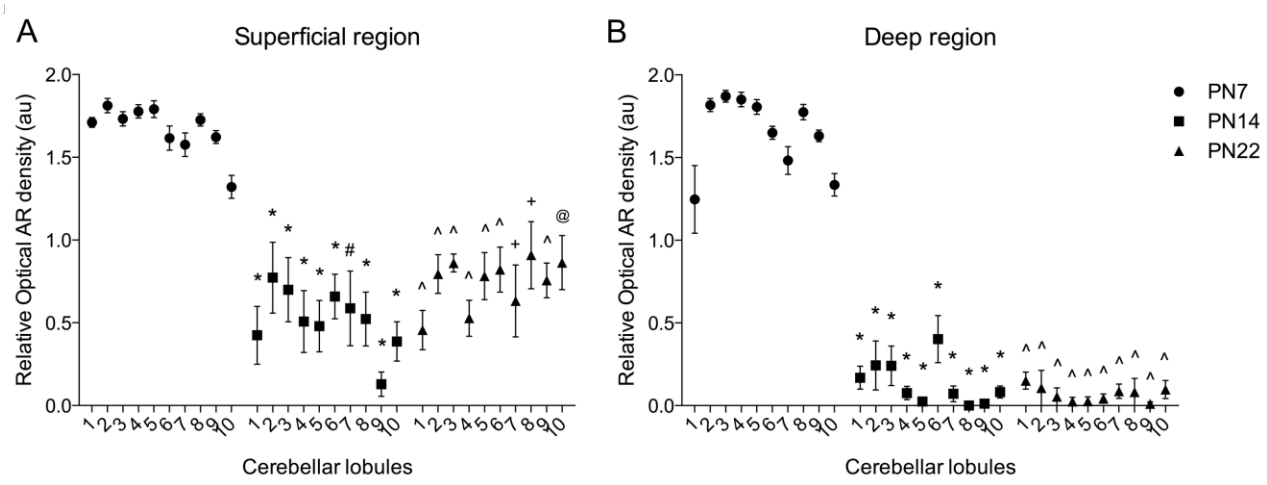

Figure 3. Postnatal AR expression in the developing vermis of female rats. (A) Superficial Purkinje layers expressing the AR across ten cerebellar lobules in the mid-vermis.

Statistical significant differences among ages for the AR density in the superficial region of **lob** 1 ($F_{(2,21)} = 35.387$, $p < 0.000$, $\eta^2 = 0.771$); **lob** 2 ($F_{(2,21)} = 19.731$, $p < 0.000$, $\eta^2 = 0.664$); **lob** 3 ($F_{(2,21)} = 21.853$, $p < 0.000$, $\eta^2 = 0.675$); **lob** 4 ($F_{(2,21)} = 32.730$, $p < 0.000$, $\eta^2 = 0.757$); **lob** 5 ($F_{(2,21)} = 30.037$, $p < 0.000$, $\eta^2 = 0.741$); **lob** 6 ($F_{(2,21)} = 18.731$, $p < 0.000$, $\eta^2 = 0.641$); **lob** 7 ($F_{(2,21)} = 9.054$, $p = 0.001$, $\eta^2 = 0.463$); **lob** 8 ($F_{(2,21)} = 16.429$, $p < 0.000$, $\eta^2 = 0.610$); **lob** 9 ($F_{(2,21)} = 94.604$, $p < 0.000$, $\eta^2 = 0.900$), and **lob** 10 ($F_{(2,21)} = 14.375$, $p < 0.000$, $\eta^2 = 0.578$). (B) Deep Purkinje layers expressing the AR across ten cerebellar lobules in the mid-vermis of **lob** 1 ($F_{(2,21)} = 23.956$, $p < 0.000$, $\eta^2 = 0.695$); **lob** 2 ($F_{(2,21)} = 86.454$, $p < 0.000$, $\eta^2 = 0.896$); **lob** 3 ($F_{(2,21)} = 163.082$, $p < 0.000$, $\eta^2 = 0.940$); **lob** 4 ($F_{(2,21)} = 768.269$, $p < 0.000$, $\eta^2 = 0.987$); **lob** 5 ($F_{(2,21)} = 955.753$, $p < 0.000$, $\eta^2 = 0.989$); **lob** 6 ($F_{(2,21)} = 94.118$, $p < 0.000$, $\eta^2 = 0.900$); **lob** 7 ($F_{(2,21)} = 176.648$, $p < 0.000$, $\eta^2 = 0.944$); **lob** 8 ($F_{(2,21)} = 337.130$, $p < 0.000$, $\eta^2 = 0.970$); **lob** 9 ($F_{(2,21)} = 1721.103$, $p < 0.000$, $\eta^2 = 0.994$), and **lob** 10 ($F_{(2,21)} = 173.310$, $p < 0.000$, $\eta^2 = 0.943$). Data are expressed as mean ± S.E.M.; n = 8 females per group. **Symbols indicate significant differences: *$p < 0.05$, $^{\#}p < 0.01$ when PN14 is compared to PN7; ^$p < 0.05$, $^{+}p < 0.01$, $^{@}p < 0.000$ when PN22 is compared to PN7**. PN = postnatal day, Lob = cerebellar lobule.

3.3 VPA decreases and increases the AR expression in the Purkinje layer of males and females in a region and age dependent manner.

In order to elucidate the effect of prenatal exposure to VPA on the AR expression pattern in the cerebellum of males and female rats, we only analyzed only those cerebellar lobules that showed an important AR pattern expression in the developing cerebellum (see Figure 2 and 3). In males, the 3 X 2 ANOVA detected a significant interaction for age X treatment

for the AR density in the superficial Purkinje layer only in lobule 8 ($F_{(2,48)} = 13.184$, $p < 0.000$, $\eta^2 = 0.386$). **Pairwise comparison** revealed that VPA decreases the AR density at PN7 **(Ctrl 0.58 ± 0.20, VPA 0.00 ± 0.00, $p = 0.013$, d = 1.52)**, but it increases the AR at PN14 **(Ctrl 0.11 ± 0.05, VPA 0.67 ± 0.17, $p = 0.008$, d = 1.66)** compared to control levels (Figure 4). No significant differences were found in any other cerebellar lobule neither in the superficial nor the deep region ($p > 0.05$, data not shown).

In females, the two-way ANOVA analysis also detected a significant interaction for age X treatment for the AR density in the superficial Purkinje layer in cerebellar lobules 6 ($F_{(2,48)} = 4.908$, $p = 0.012$, $\eta^2 = 0.189$), and 9 ($F_{(2,48)} = 3.363$, $p = 0.044$, $\eta^2 = 0.138$). **Pairwise** comparisons showed that VPA reduces AR density in the superficial Purkinje layer in lobule 6 (**Ctrl 0.65 ± 0.13, VPA 0.08 ± 0.04,** $p = 0.001$, d = 2.13), but it increases AR density in lobule 9 (**Ctrl 0.12 ± 0.07, VPA 0.44 ± 0.10,** $p = 0.027$, d = 1.31) at PN14 compared to control (Figure 5). No significant differences were found in any other cerebellar lobule neither in the superficial nor the deep region ($p > 0.05$, data not shown).

Figure 4.

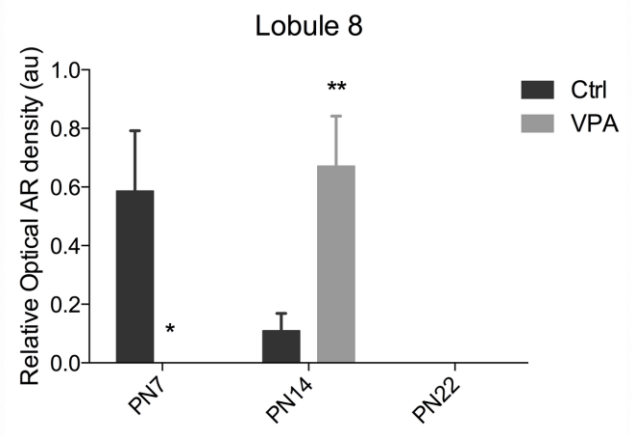

Figure 4. Effect of prenatal VPA exposure on the AR in the developing vermis of male rats. Prenatal exposure to VPA significantly decreased and increased AR density in the superficial Purkinje layer in cerebellar lobule 8 at PN7 and PN14, respectively. Data are expressed as mean ± S.E.M.; n = 8 males per group. Asterisks indicate significant differences: *$p < 0.05$, **$p < 0.01$ when compared to control. PN = postnatal day, Ctrl = control, VPA = valproic acid.

Figure 5.

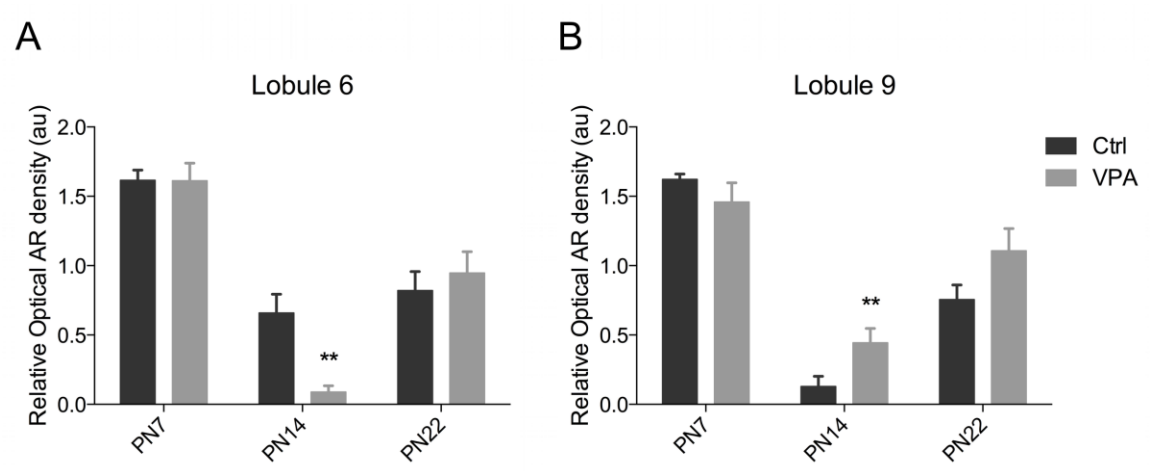

Figure 5. Effect of prenatal VPA exposure on the AR in the developing vermis of female rats. Prenatal exposure to VPA significantly reduced AR density in the superficial Purkinje layer in lobule 6, but it increased AR expression in lobule 9 at PN14. Data are expressed as mean ± S.E.M.; n = 8 females per group. Asterisks indicate significant differences: *$p <$ 0.05, **$p < 0.01$ when compared to control. PN = postnatal day, Ctrl = control, VPA = valproic acid.

4. Discussion

The VPA model has been used for the study of autistic-like features in terms of anatomical, neurochemical, and behavioral changes in rodents due to its teratogenicity activity (Almeida et al., 2014; Cusmano and Mong, 2014; Ingram et al., 2000; Kolozsi et al., 2009; Narita et al., 2002; Rodier et al., 1997; Roullet et al., 2010; Vorhees, 1987a, b). VPA has also been found to impact the cerebellum by decreasing the number of Purkinje neurons, the only output of the cerebellar cortex, in the vermis (Ingram et al., 2000). Here, we report prenatal exposure to VPA alters AR expression in the postnatal developing cerebellum in an age and region specific manner in both male and female rats. Additionally, prenatal exposure to VPA affected the AR density in the Purkinje layer, but only in the posterior vermis in both sexes, demonstrating a specific impact of VPA to the developing cerebellum. This particular action of VPA on Purkinje neuron's protein levels might be influenced by developmental timing or chemical properties among Purkinje neurons as they are born during a period of three days (E11-13) (Carletti and Rossi, 2008). Furthermore, Purkinje neurons also exhibit differences in protein, enzyme and amino acid expression according to anatomical location (Chan-Palay et al., 1981; Hawkes and Herrup, 1995; Leclerc et al., 1992; Scott, 1963). Nonetheless, further investigations are required to specifically test those factors determining VPA's effect on Purkinje neurons. Interestingly, a similar anatomical difference between the anterior and posterior vermis has been noted in the autistic cerebellum, where anatomical and morphological alterations are concentrated in the posterior part of the vermis (Amaral et al., 2008; Bauman and Kemper, 2005; Hampson and Blatt, 2015; Murakami et al., 1989). This pathological feature is of high relevance since the posterior vermis has connections with regions of the limbic system as well as the prefrontal cortex (Bostan et al., 2013; Hampson and Blatt, 2015), and these have been

found with anatomical abnormalities in the autistic brain (Amaral et al., 2008; Courchesne et al., 2007; Palmen et al., 2004). Nevertheless, whether cerebellar alterations underlie such cortical and subcortical abnormalities and therefore, the autistic behavior, remains unknown.

The Purkinje layer contains both Bergmann glia and Purkinje neurons (Bellamy, 2006; Voogd and Glickstein, 1998), and even though we did not specifically identify the AR within Purkinje neurons in this study, the phenotype of cells expressing the AR resembles one week old Purkinje neurons, and not Bergmann glia. This premise is supported by previous reports showing that Purkinje neurons express the AR during postnatal development (Bowers et al., 2014; Qin et al., 2007). Purkinje neurons are the only output of the cerebellar cortex that communicates the cerebellum with the rest of the CNS (Apps and Garwicz, 2005; Ramnani, 2006). They synthesize *de novo* sexual hormones and their receptors, which are involved in neuroplasticity for the establishment of the cerebellar circuit (Tsutsui, 2008; Ukena et al., 1998). Since the AR is expressed by Purkinje neurons during postnatal development (Bowers et al., 2014; Qin et al., 2007), the regulation of tubulin and BDNF synthesis by the AR (Jones and Oblinger, 1994; Matsumoto et al., 1994; Yang et al., 2004) might be altered in surviving Purkinje neurons of both male and female developing rats that were prenatally exposed to VPA. This could lead to an abnormal function of Purkinje neurons and therefore, to a dysfunctional cerebellar circuit.

Purkinje neurons also express histone deacetylases (HDAC's) 1 and 2 during the pre- and postnatal development (Yoo et al., 2013), and it has been shown that HDAC-1 interacts with the AR (Bennett et al., 2010; Gaughan et al., 2002). More importantly, HDAC's are targets of VPA (Gurvich et al., 2004; Hsieh et al., 2004; Ornoy, 2009; Phiel et

al., 2001). Thus, **the study of the effect of VPA on the HDAC's might be an important route to elucidate AR alterations on Purkinje neurons by VPA**.

Interestingly, the majority of cerebellar lobules exhibited a decreasing in AR density across development in both males and females. This decline was more consistent in females **than in** males. Furthermore, the AR expression appeared to differ between the superficial and deep regions within the majority of cerebellar lobules in both sexes suggesting the location of AR-expressing neurons in the Purkinje layer as an important anatomical factor regulating AR activity. This peculiar pattern expression of AR in Purkinje neurons resemblances the one observed in the adult cerebellum at least in male rats (Perez-Pouchoulen et al., 2016). **The AR has been found in some, but not all, mature Purkinje neuron's dendrites (Perez-Pouchoulen et al., 2016). In our present study, we did not find any evidence indicating that the AR is also expressed in the immature Purkinje neuron's dendrites. Therefore, the specific localization of the AR in Purkinje neurons might depend on level of their maturation. Purkinje neuron's dendrites interact during development with parallel fibers from granule cells as well as with basket and stellate interneurons to form connections; although, whether these interactions modulate AR expression in Purkinje neurons remains poorly understood.**

Although the functional significance of the developmental AR changes in Purkinje neurons **of the vermis** remains unclear, they might **be part of neuroplasticity processes involved in** the establishment of connections between the cerebellum **and other brain regions such as the frontal lobe**, which is also highly abnormal in the autistic brain.

The implications of AR in the development of the cerebellum go beyond the motor system as it interacts with Foxp2, a transcription factor highly involved in human language

(Enard et al., 2002; Lai et al., 2003) and animal communication (Bowers et al., 2013; Wohlgemuth et al., 2014), within Purkinje neuron (Bowers et al., 2014). Foxp2 has been shown to regulate genes altered in autism (Mukamel et al., 2011; Vernes et al., 2008), a disorder characterized by language and communication impairments (Amaral et al., 2008; Hampson and Blatt, 2015). However, how AR/Foxp2 interactions shape the developing cerebellum is still unknown and warrant further investigation.

Finally, our results showed that VPA disrupts AR expression in the developing cerebellum in male and female rats. However, our data also showed that more cerebellar lobules exhibited AR alterations in females than males. These data do not correlate with the autism sex ratio reported, but they establish the basis to further investigate the **VPA rat model for the study of autism where usually only males are used** (Almeida et al., 2014; Arndt et al., 2005; Chomiak et al., 2010; Cusmano and Mong, 2014; Foley et al., 2012; Hara et al., 2012; Murcia et al., 2005; Rodier et al., 1997; Schneider and Przewlocki, 2005; Schneider et al., 2008; Schneider et al., 2007). Additional studies including protein and mRNA analysis as well as motor behavior are needed to have a more complete picture of the VPA effect on the cerebellum. Thus, we strongly believe the inclusion of both sexes when exploring the VPA animal model for autism is a more optimal way to study autism-like features from the molecular to behavioral level.

5. Conclusions

Our present findings show that prenatal exposure to VPA disrupts AR expression in the Purkinje layer in an age and region dependent manner. Furthermore, our results demonstrate that prenatal exposure to VPA results not only in anatomical abnormalities of

the cerebellum (Ingram et al., 2000), but also in chemical alterations of Purkinje neurons. Overall, this work contributes to the understanding of cerebellar development under normal and abnormal conditions that might help to understand the role of the cerebellum in neurodevelopmental disorders such as autism.

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