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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

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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.

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

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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, 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 (Fig. 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.

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 (Fig. 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 40× 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 sublobules 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 × 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