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PRENATAL VALPROIC ACID EXPOSURE DISRUPTS TONOTOPIC c-FOS EXPRESSION IN THE RAT BRAINSTEM

A. DUBIEL [a] **AND R. J. KULESZA** [b]*

[a] *Auditory Research Center, Lake Erie College of Osteopathic Medicine, Erie, PA 16509, United States*

[b] *Auditory Research Center, Department of Anatomy, Lake Erie College of Osteopathic Medicine, Erie, PA 16509, United States*

Abstract—Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by difficulties with communication and social interactions, restricted, repetitive behaviors and sensory abnormalities. Additionally, the vast majority of subjects with ASD suffer some degree of auditory dysfunction and we have previously identified significant hypoplasia and dysmorphology in auditory brainstem centers in individuals with ASD. Prenatal exposure to the antiepileptic drug valproic acid (VPA) is associated with an increased risk of ASD. In rodents, prenatal exposure to VPA is utilized as an animal model of ASD and is associated with a number of anatomical, physiological and behavioral deficits, including hypoplasia and dysmorphology in the auditory brainstem. Based on these observations, we hypothesized that such dysmorphology in VPA-exposed animals would translate into abnormal activity in brainstem circuits and irregular tonotopic maps. Herein, we have subjected control and VPA-exposed animals to 4 or 16 kHz tones and examined neuronal activation with immunohistochemistry for c-Fos. After these sound exposures, we found significantly more c-Fos-positive neurons in the auditory brainstem of VPA-exposed animals. Further, we found a larger dispersion of c-Fos-positive neurons and shifted tonotopic bands in VPA-exposed rats. We interpret these findings to suggest hyper-responsiveness to sounds and disrupted mapping of sound frequencies after prenatal VPA exposure. Based on these findings, we suggest that such abnormal patterns of activation may play a role in auditory processing deficits in ASD.

Key words: auditory, hearing, superior olive, autism.

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by difficulties with communication and social interactions, restricted, repetitive behaviors and sensory abnormalities (Allen, 1988; Wing, 1997; American Psychiatric Association, 2013). ASD affects 1 in 68 children and 1 in 42 males (CDC.gov, 2014). Auditory dysfunction is a consistent finding in ASD, but varies from deafness to hypersensitivity and often includes difficulty in listening in noisy environments (Greenspan and Wieder, 1997; Rosenhall et al., 1999; Roper et al., 2003; Alcántara et al., 2004; Khalfa et al., 2004; Szelag et al., 2004; Kellerman et al., 2005; Lepistö et al., 2005; Teder-Sälejärvi et al., 2005; Gravel et al., 2006; Tharpe et al., 2006; Kwon et al., 2007; Tomchek and Dunn, 2007; Gomes et al., 2008; Russo et al., 2009; Bolton et al., 2012; Lukose et al., 2013). Additionally, a number of studies provide evidence that individuals with ASD have difficulties processing complex sounds, such as speech (Ceponiene et al., 2003; Boddaert et al., 2004; Bomba and Pang, 2004; Kuhl et al., 2005; Oram Cardy et al., 2005a,b; Whitehouse and Bishop, 2008). There are also a number of characteristic neuronal anomalies with ASD, including abnormal neuronal development, migration and maturation and alterations in soma size, shape and dendritic morphology (Bauman and Kemper, 1985; Ritvo et al., 1986; Gaffney et al., 1988; Arin et al., 1991; Piven et al., 1992; Hashimoto et al., 1993; Raymond et al., 1996; Fatemi et al., 2002b; Palmen et al., 2004; Schumann and Amaral, 2006; Kulesza and Mangunay, 2008; Whitney et al., 2008; Weigiel et al., 2010; Kulesza et al., 2011; Stoner et al., 2014; Wegiel et al., 2014; Lukose et al., 2015). In addition, we have identified significant hypoplasia and dysmorphology in the auditory brainstem of subjects with ASD (Kulesza and Mangunay, 2008; Kulesza et al., 2011; Lukose et al., 2015). Based on these observations, dysmorphology in the auditory brainstem, claustrum and cerebellar Purkinje cells are considered neuropathological hallmarks of ASD (Wegiel et al., 2014; Lukose et al., 2015).

The recent increase in ASD diagnoses is believed to result, at least in part from, environmental factors (Christianson et al., 1994; Moore et al., 2000; Rasalam et al., 2005). Of particular note is the observation of increased risk of neurodevelopmental disorders (most

*Corresponding author. Address: Auditory Research Center, Lake Erie College of Osteopathic Medicine, 1858 West Grandview Blvd, Erie, PA 16504, United States. Tel: +1-814-866-8423.
E-mail address: rkulesza@lecom.edu (R. J. Kulesza).

Abbreviations: ASD, autism spectrum disorder; AVCN, anterior ventral cochlear nucleus; CN, cochlear nucleus; CNIC, central nucleus of the inferior colliculus; D, dorsal; DCIC, dorsal cortex of the inferior colliculus; DCN, dorsal cochlear nucleus; E, embryonic; ECIC, external cortex of the inferior colliculus; fn, facial nerve; IC, inferior colliculus; L, lateral; LNTB, lateral nucleus of the trapezoid body; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; P, posterior; PAG, periaqueductal gray; PB, phosphate buffer; PVCN, posterior ventral cochlear nucleus; RF, reticular formation; SOC, superior olivary complex; SPON, superior paraolivary nucleus; tz, trapezoid body; VCN, ventral cochlear nucleus; VPA, valproic acid.

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commonly ASD) in children exposed to the antiepileptic valproic acid (VPA), compared to unexposed children (Bescoby-Chamber et al., 2001; Williams et al., 2001; Rasalam et al., 2005; Koren et al., 2006; Bromley et al., 2013). The prevalence of ASD in children exposed prenatally to antiepileptic drugs is estimated to be 8–18 times higher than unexposed children (Rasalam et al., 2005) and VPA produces malformations in a dose-dependent manner (Tomson et al., 2011). There is also evidence, from a number of studies, that timed prenatal exposure to VPA (in rodents) produces consistent anatomical, functional and behavioral abnormalities (Vorhees, 1987; Binkerd et al., 1988; Ingram et al., 2000; Koren et al., 2006; Rinaldi et al., 2007; Snow et al., 2008; Gandal et al., 2010; Tashiro et al., 2011; Mychasiuk et al., 2012; Reynolds et al., 2012; Banerjee et al., 2014; Engineer et al., 2014b). Thus, prenatal VPA exposure is widely considered an animal model of ASD (Rodier et al., 1996; Ingram et al., 2000; Kolozsi et al., 2009; Kim et al., 2011). Accordingly, we have identified significant hypoplasia and dysmorphology in the superior olivary complex (SOC) of rats exposed to VPA on embryonic day 12.5 (E12.5; Lukose et al., 2011). Specifically, we found significantly fewer neurons in the SOC while surviving neurons were generally smaller, with abnormal neuronal cell body shape and orientation compared to control animals. We interpret the irregular orientation of neurons in the lateral superior olive (LSO) and medial nucleus of the trapezoid body (MNTB) to implicate abnormal dendritic arbors and disorganized tonotopic axes, diminished sound localization capabilities and diminished encoding of temporal features of complex sounds (i.e. vocalizations; Lukose et al., 2011).

Recent studies have shown significant dysfunction in the auditory cortex of rodents exposed to VPA on E12.5 (Gandal et al., 2010; Engineer et al., 2014a,b). Specifically, in the cerebral cortex of VPA-exposed animals there is evidence for abnormal tonotopic maps, elevated thresholds, delayed responses, decreased phase locking and abnormal temporal responses to speech sounds (Engineer et al., 2014b). Additionally, there is evidence for altered levels of glutamic acid decarboxylase (GAD; the rate limiting enzyme in the synthesis of GABA) and GABA receptors in ASD (Fatemi et al., 2002a,b, 2009; Yip et al., 2007, 2008, 2009; Oblak et al., 2010) and following VPA exposure (Fukuchi et al., 2009). Indeed, we have proposed previously that disrupted GABA signaling is involved in the auditory difficulties so common in ASD (Kulesza et al., 2011; Lukose et al., 2015). Despite our previous observations of dysplasia in the SOC, the functional organization (i.e. tonotopy) of these brainstem centers has not been examined. Moreover, it is unclear to what degree the observed cortical dysfunction is inherited from brainstem centers such as the cochlear nuclei (CN), SOC and inferior colliculus (IC). Based on aforementioned observations, we hypothesize abnormal functional organization of auditory brainstem centers. Since exposure to pure tone stimuli has been shown to result in expression of the immediate early gene product c-Fos in clear topographic bands that vary according to the tonal frequency of the sound (Ehret and Fischer, 1991; Friauf, 1992, 1995; Rouiller et al., 1992), we choose to use c-Fos expression as a marker of neuronal activation in the auditory brainstem. To examine our hypothesis, we exposed rats to VPA on E12.5 and subjected control and VPA animals to 4-kHz or 16-kHz tones on postnatal day 28 (P28). We then used immunohistochemistry to indirectly quantify neuronal activity patterns in the CN, SOC and IC by identifying neurons expressing c-Fos.

EXPERIMENTAL PROCEDURES

Animals

Timed-pregnant Sprague–Dawley rats were injected intraperitoneally on embryonic day 12.5 (E12.5) with 600 mg/kg VPA or 0.9% saline. On postnatal day 10 (P10), litters were culled to 4–6 male pups and then weaned on P21. All experimental procedures were performed on male pups at P28 (Fig. 1A). Animals were maintained on a 12-h light/dark cycle with free access to food and water. The VPA-exposed group includes 11 animals. The control group includes 6 saline-exposed animals and 8 non-injection animals (see below). All procedures were approved by the LECOM IACUC.

Sound exposure

On P28, animals were placed in a sound-attenuated booth for 30 min without sound stimulation (Fig. 1B). The booth was lined on all six walls with Auralex Acoustic Foam which created a 20-dB sound attenuation from ambient noise. After the 30-min acclimatization period in silence, the animals were presented either a 4-kHz (68 dB; 7 control and 6 VPA) or 16-kHz (62 dB; 5 control and 4 VPA) tone for 60 min through Altec Lansing ACS410 speakers (Fig. 1B). The stimuli had 5-ms onset and offset ramps and were 60 min in duration. Animals were awake and permitted free movement in the booth for both the isolation and exposure periods. Sound intensities within the booth were calibrated with a sound level meter (Greenlee Textron, 93–20). Analysis of stimulus waveforms revealed energy maxima at 4 and 16 kHz, respectively. Additionally, two controls and one VPA-exposed animal served as no-sound controls (i.e. they remained in the sound-attenuated chamber for 90 min).

Immunohistochemistry

Immediately after the sound exposure, animal were anesthetized with 80 mg/kg of pentobarbital. When animals were unresponsive, they were perfused through the ascending aorta with normal saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2; fixative). Brains were then dissected from the skull and stored in fixative for at least 24 h. Brainstems were dissected and placed in a solution of 30% sucrose in fixative until they were saturated. Brainstems were sectioned on a freezing microtome in the coronal plane at a thickness of 40 μm. Free-floating sections spanning from the dorsal cochlear nucleus (DCN) through the inferior colliculus (IC) were collected in 0.1 M PB. Every third tissue section was processed

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