Behavioral Effects of OrallyAdministered Valproic Acid on the Anatomy of the Superior Olive in Rats



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Behavioral Effects of Orally Administered Valproic Acid

on the Anatomy of the Superior Olive in Rats

A Senior Comprehensive Project

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Abstract

Autism Spectrum Disorder (ASD) is a pervasive disorder characterized by a variety of symptoms that include repetitive behaviors, social language development, and emotional impairments. There has been evidence that auditory deficits, specifically a hypersensitivity to sound, are also common among ASD individuals. Prenatal exposure to valproic acid (VPA) has demonstrated an increased likelihood of ASD development in humans. When VPA is administered prenatally to rats, they have shown anatomical, chemical, and behavioral abnormalities similar to those found in ASD individuals. While much of the autistic brain is still misunderstood, recent studies have shown dysmorphologies and decreased neuronal densities in the Superior Olivary Complex (SOC), the sound localization center, in rats that have been prenatally exposed to VPA.

In an effort to explore the behavioral effects of the atypical SOC shown in VPA exposed rats, the current study aimed to evaluate whether there was a difference in sound localization abilities or differences in neuronal densities or morphology of the SOC in VPA exposed and control rats. This was done via a sound localization test. The findings were significant in that no significant differences were found between the controls and VPA animals in sound localization performance. Histologically, no decrease in neuronal densities or abnormalities in neuronal morphologies were found in the VPA exposed animals as had been previously reported in the literature. Therefore, further research is needed to clarify the anatomical and behavioral effects of VPA exposure on auditory system development and performance.

Introduction

Autism Spectrum Disorder (ASD) is a developmental condition distinguished by social and communication impairments, abnormal language development, and restricted repetitive behaviors. Aside from these distinguishing behavioral characteristics, those with ASD are also more susceptible to epilepsy, infectious diseases, migraines/headaches, allergies, and intellectual and motor impairments (Rodriguez et al., 2011). In 2014, a government survey revealed that approximately one in forty-five children become diagnosed with some form of Autism in the United States (Autism Speaks, 2015). As the incidence increases, studies on ASD have become a focal point for researchers. Since the symptoms of ASD differ across affected individuals, this disorder has been thought to be a result of multiple malfunctions from several different regions of the brain. The variation of symptoms between those affected by ASD has also made the determination of an underlying cause for the disorder difficult.

Without a known cause for ASD, treatments have been targeted to addressing functional impairments for individuals with ASD. For example, a clinician would treat the epilepsy often associated with ASD, but perhaps not the language impairment (Pomerantz, 2017). Some individuals have been known to seek counseling as a treatment for language and social impairments in ASD. Francis et al., (2016) discussed promising results using oxytocin and vasopressin administration as treatments for the social impairment and repetitive behaviors of ASD. While there are many different approaches to treating ASD, the variations in severity of this disorder are so individualistic, that a particular treatment method may not work the same for all ASD individuals. However, researchers have begun to find associations between key behavioral symptoms, abnormal anatomical structures, and atypical chemical functions in the brains of individuals affected by ASD.

Structural Discrepancies in ASD

One area of the brain known to be abnormal in individuals with ASD is the cerebellum. The cerebellum is responsible for smooth movement, balance, posture, and procedural motor control, which includes speech. Wiegel and colleagues (2010) reported that the most common site for dysplasia was in the cerebellum of ASD individuals. One subject suffered severe hypodysplasia in their cerebellum with decreased Purkinje cells (Wiegel et al., 2010). Purkinje cells play an important role in motor coordination. A decrease in the number of Purkinje cells alters the output of motor effector signals and disrupts motor control. Hanaie et al., (2013) used diffusion tensor imaging to study the microstructural integrity of the cerebellum in relation to motor dysfunction in ASD individuals. They discovered that ASD subjects had lower fractional anisotropy or diffusion ability in their right side of the superior cerebellar peduncle (SCP) in comparison to controls. This was attributed to decreased axon diameter and transmission speed. This would disrupt communication in the cerebellum and produce motor and speech impairment symptoms represented in ASD.

Impaired language development and social behavior impairments are two of the main characteristics of ASD, individuals are not typically diagnosed until roughly the age of three. However, developmental behavioral abnormalities such as poor eye contact, repetitive hand movements, and/or a lack of response to verbal stimuli have been identified in babies as early as six months (Esposito, 2016). Neuroanatomical studies of ASD diagnosed children has shown focal developmental dysplasia or ectopic cells in ninety-two percent of the thirteen ASD subjects studied (Wiegel 2010). Focal dysplasias consist of abnormal cells in tissues that failed to migrate to their intended site. It was found that some ASD individuals had dysplasias within the denate gyrus, a persistent site for neurogenesis in adults. In ASD rodent models, dysplasia at this site

was represented as abnormal loops and circles, and in some cases dysplastic cells formed an additional granule layer (Wiegel et al., 2010). The hippocampus is thought to be responsible for emotion and memory. Therefore, dysfunctional cells in this area could contribute to some of the emotional or memory symptoms found in ASD.

Chemical Discrepancies in ASD

Individuals with ASD have aberrant levels of chemicals such as nitric oxide (NO). Activation of microglia releases excessive amounts of NO (Rodriguez et al., 2011). NO is an inflammatory mediator that upregulates glutamate synthesis and release and has been shown to react with other cells to create neuronal death or damage, which is thought to provoke other medical problems. People who are affected by ASD are susceptible to a variety of medical difficulties such as headaches, allergies, and epilepsy and research has suggested that excessive NO could be the cause of these complications (Rodriguez et al., 2011). Kovacs et al. (2009), proposed that NO-dependent enhancement in synaptic activity promotes epilepsy, which people with ASD are prone to. Studies on NO levels could be a key factor in understanding the medical problems which are prominent ASD individuals. However, these studies are limited to reactiveness of the disorder instead proactive preventions.

Recent studies have emphasized imbalances between excitation (E) and inhibition (I) of synaptic transmission as a cause for the disorder. Lee et al (2016) proposed that the excitation and inhibition of synaptic transmission is driven by gamma-aminobutyric acidergic (GABA) interneurons (Lee et al., 2016). GABA is known as the main inhibitory transmitter as it stimulates the switch excitatory on the fetal brain to inhibitory afterbirth (Tyzio et al., 2006). Understanding

that this switch is GABA-mediated and functions by a change in intracellular chloride (Cl^-) concentration controlled by the membrane proteins NKCCL importer and KCC2 exporter has

been key to the development of treatments to alleviate ASD symptoms (Zimmerman et al., 2014). During labor, the mother produces an oxytocin surge that creates a reduction in Cl^- concentration in the fetal brain. This Cl^- reduction aids in the GABA inhibition and ultimately serves as a neuroprotective agent (Zimmerman et al., 2014). However, the brains of ASD individuals contain higher levels of intracellular Cl^- and excitatory GABA, which creates an overall excitatory effect on the brain (Tyzio et al, 2014). Studies have shown that if oxytocin levels become disturbed when a mother is giving birth, problems in the transition of excitation to inhibition in the fetal brain could occur (Tyzio et al, 2014). The finding of this imbalance has led to advancements in research on the study of autism.

VPA Animal Models and Previous Research on ASD

Animal models have been utilized to examine symptoms and mirror anatomical features presented in ASD as a result of the discovery of abnormalities in the E/I transition in fetal brains. Rats have done this through prenatal exposure of chemicals, like Valproic Acid (VPA) administered on day 12.5 of gestation (Sinclair et al., 2016). Valproic Acid elevates intracellular chloride levels resulting in a disruption of the E/I transition shown in abnormal oxytocin levels during labor, and thus produces ASD models (Farve et al., 2013). The VPA animal models of ASD facilitates basic research on ASD and has contributed to drug treatment, development of behavioral testing, and histological analysis of neural tissues.

A current area of research in ASD focuses on the abnormalities in sensory cortexes. The majority of individuals with ASD experience hyperactive responses to sensory stimuli in addition to the typical symptoms associated with ASD (Tomcheck et al., 2007). These abnormalities have been explained through atypical sensory processing and may contribute to some of the social and communication impairments that coincide with ASD (Sinclair et al., 2016). Auditory sensitivity is one of the most common sensory abnormalities and is most frequently reported as a

hypersensitivity to sound and inability to discriminate speech sounds (Tomcheck et al., 2007). Tomcheck et al., (2007) compared sensory processes in children with and without ASD between the ages of three to six and found significant difficulties in auditory filtering in seventy-seven percent of ASD children studied. These results indicate that the sensory processing, especially auditory processing, in ASD individuals is a worthwhile exploration.

Sinclair et al. (2016) examined the sensory processing in the auditory cortex of ASD and Fragile X syndrome (FXS) in both human and animal models. They examined subjects with electroencephalographic (EEG), magnetoncephalography (MEG), and startle/EMG measurements. To calculate the brain's response to auditory stimuli the researchers used resources from the EEG, MEG, or EMG's such as, event-related potentials (ERP's) and synchrony/amplitude of neural oscillations. ERP's for ASD and FXS human and animal models created by prenatal exposure of VPA, showed increased N1 latencies (slow auditory evoked potential). Neural oscillations in animal models were not observed, but the human ASD subjects showed increased baseline gamma waves, which has been associated with auditory processing abnormalities (Cervenka et al, 2011). These observations had two major takeaways: one was that ASD and FXS individuals both have unusual or slow auditory processes and the second was that VPA animal models have functional similarities to ASD with humans and should be considered to be a valid model of ASD (Sinclair et al., 2016).

To further explore the source auditory differences in ASD, Lukose and colleagues (2011) examined the anatomy of the superior olivary complex (SOC), an area responsible for sound localization, located in the auditory brainstem nuclei of rats. They studied the morphology and neuron densities in the SOC of VPA and control rats. They discovered that VPA models exhibited dysmorphologies such as abnormally directed dendrites, inadequately innervated

neurons, and disrupted tonotopic axises, and significantly reduced neuronal densities in this area. These findings are consistent with anatomical abnormalities found in children with ASD.

In a similar study, Dubiel et al. (2016) used VPA rat models and control animals to assess anatomical differences in neuron density and morphology in relation to auditory brainstem centers, specifically in areas such as the SOC, cochlear nucleus (CN), and inferior colliculus (IC) . They placed VPA models and controls in a sound booth for thirty minutes in silence and then presented them with a sound stimulation either of 4-kHz, 16-kHz, or no stimulus for sixty minutes. Directly after, the animals were sacrificed and their brainstems were sectioned. Sections were processed for c-Fos immunohistochemistry, an immediate early gene that codes for a transcription factor and is thought to regulate long-term changes in neuronal activity. The prolonged auditory stimulation resulted in increased activation of c-Fos positive neurons, but the found a decreased density of c-Fos positive neurons in the VPA rats exposed to 4-kHz and 16-kHz of stimulation. They also detected c-Fos positive neurons, which were displaced beyond the tonotopic bands in the VPA models. Their studies indicated problems with frequency coding in the CN , SOC, and IC (Dubiel et al., 2016).

Bumetanide, an FDA-approved diuretic drug, has been shown to reverse effects of prenatal exposure to VPA when administered one day prior to delivery in rats. Bumetanide functions by facilitating normal E/I transformation of GABA through the promotion of chloride transporters (NKCCL and KCC2), and therefore can counteract the teratogenic actions of VPA (Lee et al., 2016). Tyzio et al. (2014) orally administered Bumetanide to one group of pregnant rats and oxytocin receptor antagonists group of pregnant rats to another group of pregnant rats one day prior to birth in VPA treated and control females. They discovered that prenatal delivery of Bumetanide to VPA rats enhanced the conversion of GABA from excitatory to inhibitory, as

seen in normal development. Tyzio et al. (2014) showed that prenatal administration of the oxytocin receptor blocker, SSR126678A to normal pregnant rats creates autistic like characteristics in their pups. This demonstrated that Bumetanide reverses ASD symptoms in the VPA autism model. It also showed that Bumetanide could regulate intracellular Cl^- levels as oxytocin does normally in the transition of GABA-mediated excitation to inhibition during birth.

Oral administration of Bumetanide was used as a treatment in a randomized controlled trial in children diagnosed with ASD with ages varying from three to eleven years old (Lemonnier et al., 2012). Of the fifty-four subjects in this study, half of the subjects were given 0.5 mg of Bumetanide and the other half were given a placebo twice a day for three months. Throughout the three months, the subjects were tested using Childhood Autism Rating Scale (CARS) a behavioral rating scale used to evaluate ASD symptoms, Clinical Global Impressions (CGI) which is an evaluation by a clinician to determine severity of illness and change over time, and Autism Diagnostic Observation Schedule (ADOS) an assessment of social interaction and communication. The CARS test indicated a significant decrease in severity of ASD symptoms in Bumetanide-treated subjects, and the CGI test revealed a seventy-seven percent improvement in social interaction and communication demonstrated by the individuals who took Bumetanide. Results from the ADOS test indicated that bumetanide was most effective in individuals who had less severe symptoms of ASD. Although the drug's reported that side effects were minimal, one of the fifty-four subjects were removed from the study due to low blood levels of potassium (hypokalema). Overall, the study concluded that Bumetanide was successful in decreasing the social, communication, and behavioral effects of ASD (Lemonnier et al., 2012).

VPA animal models that are also prenatally treated with Bumetanide have shown promise as a way of preventing ASD. However, clinical studies with Bumetanide have only employed

postnatal administration. There are a number of factors that contribute to the risk of ASD development in offspring such as: infection during pregnancy, birth complications (e.g. low oxygen), and parental age of conception (Autism Speaks, 2015). The minimal adverse side-effects of Bumetanide reported by Lemonnier et al. (2012) suggest that Bumetanide not only could act as a suppressant of ASD symptoms, but if administered prenatally to mothers, it could potentially be a preventive treatment for ASD. However, in order for drugs like Bumetanide to be prescribed as a preventative measure, the oxytocin E/I theory as a cause for ASD must have concrete experimental evidence.

Mabunga and colleagues (2015) reviewed the evidence that demonstrated that the VPA model has a high validity construct to ASD individuals as studies have consistently shown the neuro-chemical, anatomical, and behavioral similarities between the model and ASD. However, there are still elements of ASD that need to be explored more thoroughly. Lukose et al., (2011) reported anatomical abnormalities in the olivary complex in VPA treated rats. There have been no demonstrations of the functional consequences of these anatomical abnormalities. One important function of the olivary nuclei is sound localization. The present study sought to examine sound localization discrimination in VPA and control rats.

Hypothesis and Experimental Aim

Dubiel et al., (2016) discovered functional discrepancies shown by significantly more F-cos positive neurons in the brainstem of VPA rats when exposed to different sound frequencies, but did not explore how these differences affected the rats behaviorally. Similarly, Lukose and colleagues (2011) identified significant dysmorphologies and decreased neuron densities in the SOC of VPA models, but did not examine how this would contribute to their ability to localize sound. The present study focused on the behavioral effects of prenatal exposure on performance with a sound localization test. I predicted that the dysmorphologies and lower number of neurons

within the SOC and brainstem in VPA rats would impair the ability to discriminate the source of a tone. To test this hypothesis hungry rats were trained to run toward the source of a tone in a four-arm apparatus shaped like a plus (+) sign from a central start chamber. Food was available in a baited well in the floor at the end of each arm. The location of the tone source predicted the location of available food. VPA and control rats were tested on a series of tone localization trials to assess whether functional differences in sound localization existed between VPA and control rats.

Methods and Materials

Subjects

Three female Sprague-Dawley rats were bred to males at Allegheny College Carnegie Animal Facility. They were examined hourly until a sperm plug was evident, this event was defined gestational Day One. On day 12.5 of gestation (GD 12.5), the progeny of these dams were the source of VPA subjects and given 600 mg/kg body wt. of VPA mixed in Nutella was orally administered to two of the pregnant dams. The remaining pregnant dam was given an equal volume of Nutella on GD 12.5 and the progeny of this dam provided control subjects. Once the pups were delivered, the mothers nursed their respective litters until weaning on postnatal day 21. Three VPA and three control rats randomly selected from the two VPA litters and the control litter were used as subjects for this study. The rats were group housed in dimensions cages that were in a temperature-controlled room with a 12 hr light/dark schedule. The rats received ad libation and water except during behavioral by training and testing periods During training and testing periods, rats were kept at 85% of their ad lib. weight by food deprivation. Animals were provided with additional food after daily training and training sessions to maintain this weight. In addition to training and testing, animals were handled and examined at least once a week for 10 minutes for exercise and to reduce the stress of handling by the experimenter. All animals were

treated according to ethical guidelines for the care and use of animals as approved by the Allegheny College Animal Care and Use Committee (ACARC).

Apparatus

The behavioral apparatus in this experiment was a cross-shaped runway with 36 inch arms extending from a central square area, which was designated as the Start Box (Figure 1). A speaker was attached to the end wall of each runway. Food wells were placed at the end of each arm by drilling a ½ inch deep x 1 inch wide hole in the floor of the runway. The food wells were baited with Honey Nut Cheerios which were not visible until the subject was within 2 inches of the food well. The Start Box was constructed of hardware cloth. At the start of the trial, a tone of 400 Hz and 70 dB a time period that varied by training and testing periods. As the tone ended, the Start Box was lifted, which permitted the subject to enter any of the four arms of the runway. Petri dishes full of Honey Nut Cheerios were placed on the lids of each arm to reduce smell association.

Behavioral Training and Testing

1. On post-natal day 120 (P120) the rats were weighed and then food deprived in order to reduce them to 85% of their ad lib. weight. When this weight was achieved, they began their first period of runway training. The purpose of the first period of training was to familiarize the animals for with the apparatus. To do this, animals were placed inside the Start Box, an enclosed tube of ¼" thick hardware cloth. The start box was then raised, which permitted the subject to explore the arms of the runway to find the well with Honey Nut Cheerios and tone stimulus. For first testing period, the animals participated in four trials daily and were exposed to each arm of the apparatus. A trial consisted of placing the animal in the Start Box, turning on the stimulus, and permitting the subject to find the food. They were timed from the point of entrance in to the apparatus until the time of food consumption during this period. The stimulus was turned off

once the subject located the food source. Once the subject consumed the food, the animal was placed back inside the transportation cage and the next rat was placed inside the Start Box to begin a new trial. Once all VPA and control animals finished their first trials, a new arm was baited and the next set of trials began. This continued until all 4 arms were baited and all subjects completed the 4 trials.

2. For the second training period, animals received to 10 trials per day for eight days over a period of two weeks. Animals were timed from Start Box opening until food was located and recorded for accuracy. Accuracy was measured by whether or not the animal selected the correct runway arm with the tone and food on the first attempt. Animals were allowed to roam the maze until food was found. Animals were returned to the Start Box after the food was eaten and not placed back into the transportation cage until all 10 trials were completed. A different arm was baited for each trial.

3. The third stage of behavioral examination was the testing period which consisted of a total of 40 total trials. The trials occured in blocks of 10 per day, and testing was done every other day. The order of reward arm was randomized. A new Start Box of the same dimensions to the one used previously that was made from ¼" hardware cloth, was employed to reduce any interference with the identification of the source of discriminative stimulus (tone) that identified the baited arm of the apparatus (Figure 1). To start a trial, the discriminative stimulus (tone) of 400 Hz and 70 dB was presented for 10 seconds, the Start Box was lifted and the subject made its' response. Animals were measured for accuracy. The trial was recorded as correct if the subject went to the food well on the correct arm. The subject was permitted to consume the food in the well. After the food was consumed, the animal was removed from the apparatus and placed in a transportation cage. A new arm was baited and the animal was placed back inside the

Start Box. A new trial began 30 seconds later. If the subject traveled past the error line (a red line drawn at 12 in. from the start box on each runway), on an unbaited arm, the Start Box was returned, which closed the arms of the apparatus and the subject was removed from the unbaited arm then placed in a transportation cage. This was considered an incorrect response. A new arm was baited. The animal was then placed back inside the Start Box. The next trial began 30 seconds later.

Histology

Immediately following the behavioral testing, the animals were sacrificed through with an intraperitoneal injection of Euthasol. When respiratory arrest occurred and the animals were unresponsive to a foot pinch, transcardial perfusions were performed with 10% formal saline and the brains were removed from the skull with rongeurs and an elevator. The brains were stored in formal saline for a minimum of 24 hours. Prior to sectioning, the brainstems were with a Leica cryostat at 50 um. Sections were freeze mounted on subbed slides and then stained with buffered thionine, a Nissl stain. The Nissl stain was used to highlight neuronal cell body density in the superior olivary complex. The stained brain slices were examined using a Leica microscope. Neurons were counted manually with the assistance of the Leica software tagging program.

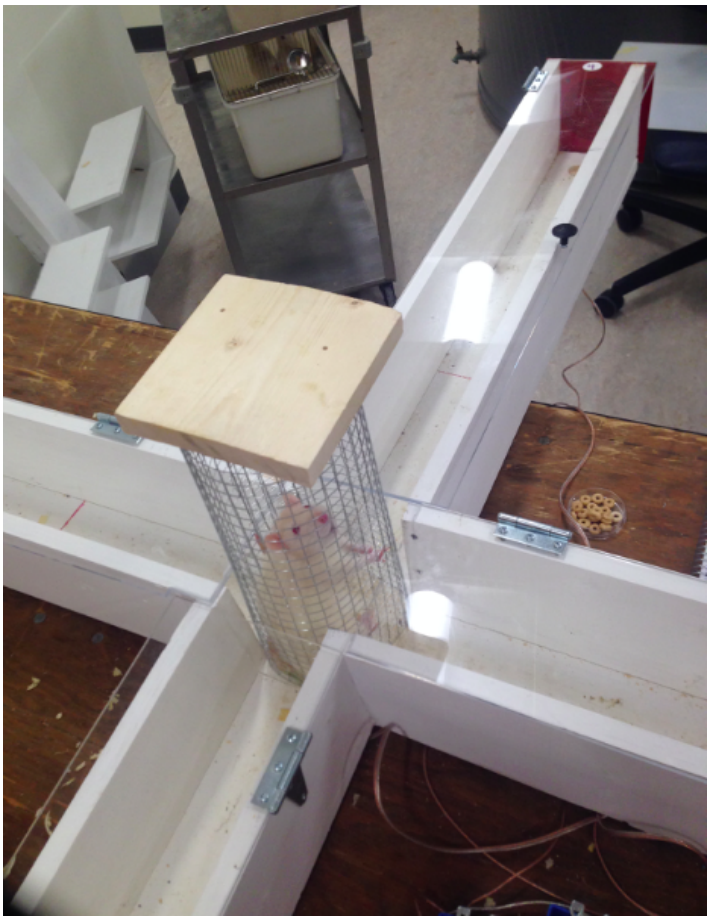


Figure 1. The behavioral apparatus with speakers attached to each arm. The start box in the center where the rat was contained was used in the Stage Three testing where the animals could hear the stimulus prior to entering the maze.

Results

Behavioral Tests

Stages One and Two of behavioral training were used to familiarize the animals with the maze as well as to condition them to associate the sound stimulus with the reward of food. Stage Three was the final testing period in which the VPA and control subjects tested for the correct number or responses per day (Figure 2). Data were examined using a two-way between subjects Analysis of Variance (ANOVA). The analysis revealed no main effect across days $F(3,12) = 1.283$, $p >$

0.05. There was no main effect across groups $F(1,4) = 0.029$, $p > 0.05$. No main effect was found between days and groups $F(3,12) = 0.196$, $p > 0.05$. Data were analyzed for split-half effect, but no effect was shown. VPA animals showed improvement or no change from Day 1 to Day 4. Control animals 2 and 3 showed improvement from Day 1 to Day 4. Control animal 1 showed a slight decrease in correct number of responses across the four trial days.

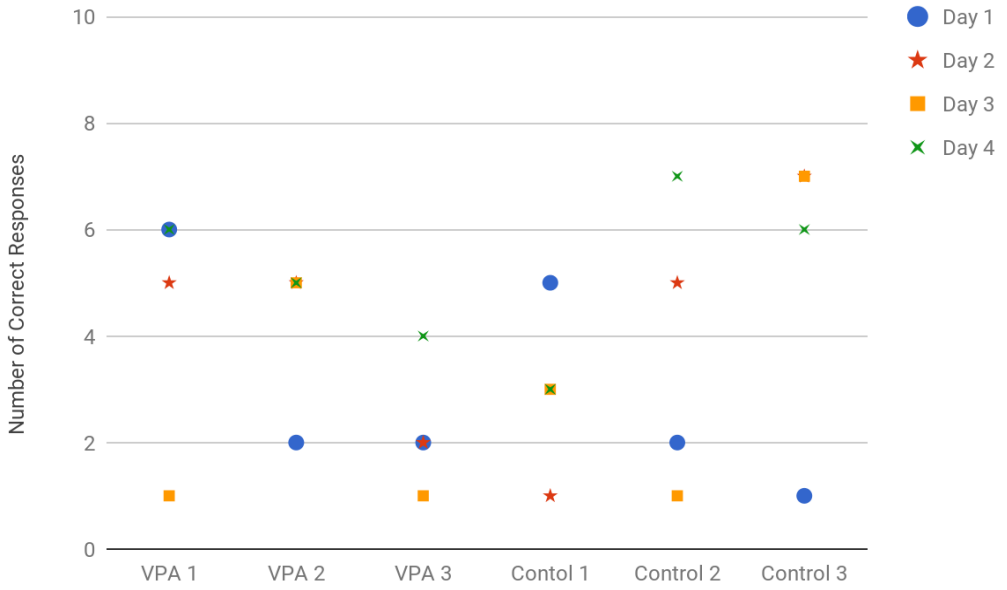


Figure 2. Number of correct responses to stimuli out of ten trials per day. Total number of accuracy recorded was: VPA 1: 45%, VPA 2: 42.5%, VPA 3: 22.5%, Control 1: 30%, Control 2: 37.5%, and Control 3: 52.5%.

Histology of the Superior Olivary Complex (SOC)

Overall, prenatal exposure to VPA did not result in a decrease of neuronal density in the SOC when compared to controls (Figure 3) . The highest average of neurons in the SOC structure was VPA 2 with 203 estimated neurons and the lowest was Control 3 with 169. Number of neurons in the SOC was not represented with a higher number of correct responses on the behavioral trial as VPA 2 did not have the highest total number of accuracy recorded. No differences in structure of

the SOC were seen in VPA and control animals on either hemispheres (Figure 4) . No discrepancies were evident in the morphology of neurons in VPA specimens (Figure 5).

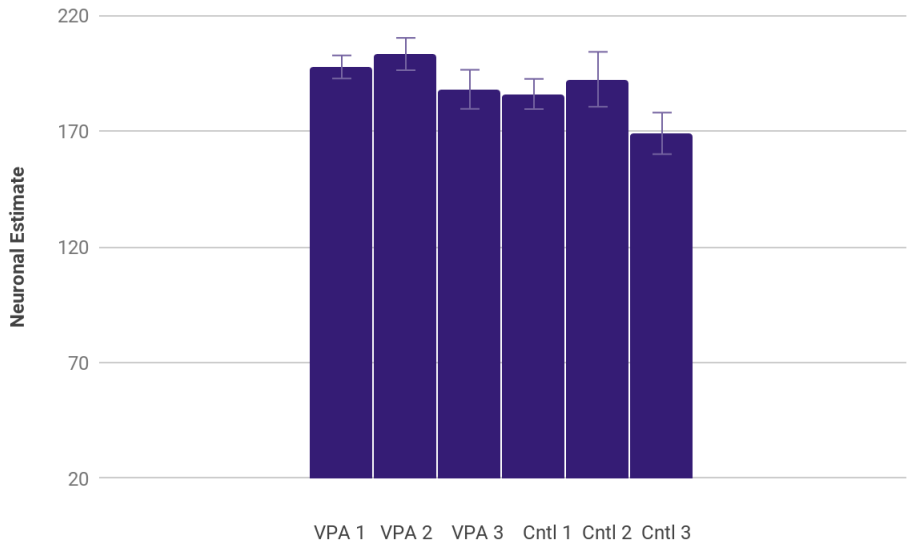


Figure 3: Average number of neurons from the total SOC in both hemispheres of controls and VPA animals. Prenatal exposure to VPA did not have an effect on the neuronal density in the SOC. Cntl = Control animals. Error bars represent ± 1 standard errors of the mean. VPA 1 (M= 197.6 SD=8.62), VPA 2 (M=203.25 SD=13.93), VPA 3 (M=188 SD=6.36), Cntl 1(M=186 SD=9.19), Cntl 2 (M=192.33 SD=20.53), Cntl 3 (M=169 SD=20.04).

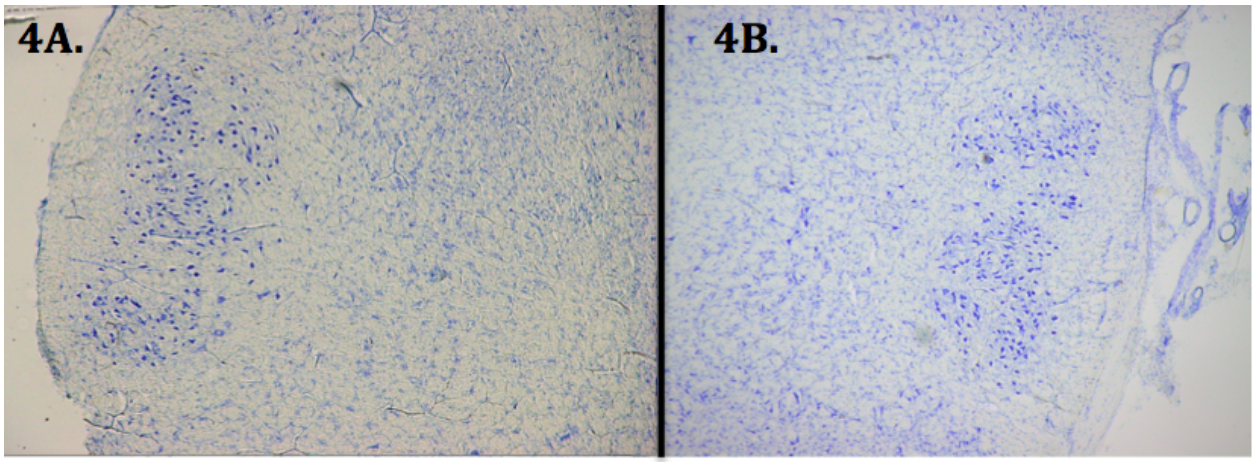


Figure 4. Nissl stain of the Superior Olivary Complex in (A) control 2 and (B) VPA 1 specimens.

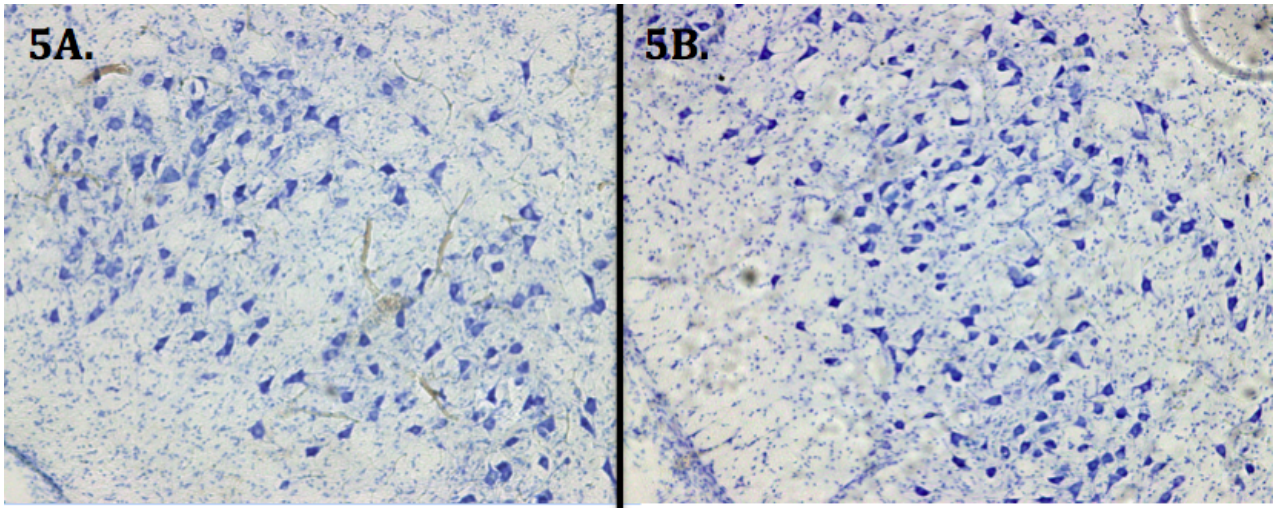


Figure 5. Morphology and density of neurons in the SOC of (A) Control 2 and (B) VPA 1.

Discussion

General Comments

This work constitutes the first known sound localization testing in VPA animals. Although Lukose and colleagues (2011) have demonstrated discrepancies in neuron densities and morphologies within the SOC of VPA animals, no significant differences were found in the animals of this study. The ability to detect where the stimulus was coming from was predicted to be flawed in VPA animals, but instead VPA history had effects when compared to control animals. The hypotheses of this study were not supported by either functional or anatomical findings. Dysmorphology and decreased density in the SOC was predicted to relate to an inability or decreased ability to localize sound. The animals in this study did not display abnormal neuronal densities or morphologies and were able to localize sound with comparable accuracy to controls, which suggests that further anatomical exploration of the SOC and behavioral testing in the VPA animal model is needed.

Limitations and Future Directions

The experimental protocol could be adjusted to allow for more training sessions as well as final testing periods. If the training sessions were extended, the animals could potentially develop a stronger association with the stimulus and reward. The behavioral outcome may show differences in accuracy in the final testing stage across an extended period of time. Another consideration for change is the sample size. Schönbrodt and Perugini (2013) showed that for stable estimates in data, a large sample size is needed. A larger sample size would benefit this study in that it would allow for elimination of outliers and could account for variability across subjects from different impregnated dams. Additionally, Dubiel and Kulesza (2016) showed significantly more c-Fos-positive neurons in VPA animals than controls at 4 kHz, but when 16 kHz was used, the VPA animals had four times the amount of c-Fos-positive neurons than those

found at 400 Hz indicating that the frequency of stimulus had an effect on the VPA animals. This study had the stimulus set at 400 Hz across all testing periods, but if it was higher it may have affected their behavior more intensely.

There are additional possibilities that should to be considered for a change in protocol. The highest total accuracy recorded from stage three of the behavioral tests was Day 4 from Control 3 (Figure 2). With this number being slightly above half in control animals, adjustments in the apparatus itself may be needed to increase accuracy. Rajan and colleagues (2006) examined the olfactory system in rats through the use of an odor localization test and found that rats could detect where a smell was coming from in one or two sniffs, indicating that rats have an exceptional sense of smell. Although food was placed on each arm of the apparatus to enhance smell in all directions, the rats may have been able to detect which arm the stimulus was coming from due to the stronger odor in the well of the correct arm in the training trials. This may have disrupted the pairing of the stimulus and food if their sense of smell was localizing the food source instead of the stimulus. The use of water or pleasure could be used to condition animals to the stimulus instead. In an operant chamber with a speaker attached to both sides, one could used water deprived animals or rats that have had an electrode inserted into their nucleus accumbens to create a more suitable association between stimulus and reward.

Another aspect of this study to note is that the ability of rats to localize sound may have been affected by an emotional response to the apparatus or presented stimuli. The data was analyzed for a split-half effect, but there was no indication of a startle response. Because ASD individuals express fear, anxiety, and social withdrawal in certain environments, Farve and colleagues (2015) observed VPA rats in predictable non-surprising environments versus non-predictable surprising environments and found that an unpredictable environment can produce

negative effects in their sociability, nociception (response to harm), stereotypy (repetition), fear conditioning and anxiety. If the heart rate of animals was observed throughout trials and a shock was administered when a mistrial occured, one may see a notable difference between the change in the VPA animal's heart rate in comparison to controls. This finding could adhere to the fear and anxiety that ASD individuals feel in a change of environment and thus affect behaviors in response to certain stimuli.

An important result of this study to investigate is that the VPA animals expressed no significant differences in the SOC of VPA animals when previous research from Lukose et. al., (2011) has shown otherwise. The DSM-5 defines autism as being a spectrum, meaning that there is a variability of symptoms across subjects (Autism Spectrum Disorder, DSM-5; American Psychiatric Association, 2013). It is possible that this variability would apply to rats affected by VPA as well. A study could examine the penetration of VPA and distribution across pups to see if placement inutero or the reproductive system of the dam has an effect. It may also be a worthwhile exploration to determine what percentage of ASD individuals have sound localization problems. Lukose et al. (2011) showed that people with ASD display abnormalities in the SOC as well as sound localization problems, but it is uncertain how common that is or how many people display symptoms in difficulty localizing sound.

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