An Animal Model of Autism: Remediation with Tactile Stimulation

SONJA RICHARDS

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Department of Neuroscience

University of Lethbridge

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ABSTRACT

This thesis examines both behavioral and anatomical effects of prenatal exposure of Valproic Acid (VPA) on Long Evans rats. Tactile stimulation (TS) is then used to investigate its' effect on remediating any abnormalities VPA may produce. Several behavioral tests were done to assess the behavioral effects of VPA and TS. It was found that VPA had an effect of many of the tasks, whereas, TS had almost none with the exception of an effect on females in the elevated plus maze. However, anatomical data showed that TS had a profound effect on neuronal branch order, cell complexity, and spine density in pyramidal neurons in the medial prefrontal cortex, the orbitofrontal cortex and the amygdala. Where VPA decreased the above in all of these areas, TS increased neuronal complexity in the aforementioned structures. This study demonstrates that prenatal exposure to VPA is a viable model of autism in rats and that TS has significant anatomical effects in these animals as well as in control animals.

DEDICATION

To my children, Hannah, Jakob, and Maxwell Richards.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, R. Gibb and B. Kolb for the opportunity to learn and for their support these past three years. I would like to thank my committee members S. Pellis and L. Brown. I have a great deal of gratitude to those in the Kolb lab for their time and expertise. I would like to thank A. Nakahashi for her time and assistance. I would like to thank A. Muhammad and R. Mychasiuk for their patience and for answering my numerous questions. I would also like to thank B. Himmler for his help. Finally, I would like to thank my family for their support and patience.

AN ANIMAL MODEL OF AUTISM

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List of Abbreviations

ANOVA – Analysis of variance

ASD – Autism spectrum disorder

EPM – Elevated plus maze

FFA – Fusiform face area

I.P – intraperitoneal

NTS – Non-tactile stimulation

OFC – Orbitofrontal cortex

mPFC – medial prefrontal cortex

P – postnatal day

STS - superior temporal sulcus

TS – tactile stimulation

VPA – Valproic acid

CHAPTER ONE

General Introduction

Autism Spectrum Disorder (ASD)

In 1943, Kanner first described a subset of children with three common behavioral abnormalities: first, was a social delay, awkwardness or self-isolation, second, was language delay, and third, was repetitive and stereotypical behavior and obsessive interests (Kanner, 1943). This behavioural profile was introduced as *autism*. Most researchers agree upon the three fundamental components of the disorder, all in line with Kanner's introductory paper. Since that time, the rate of autism has steadily increased; much has been discussed and debated on possible causes and treatments. According to a report in 2010, the rate of autism in children has risen to 1 in 100 children (Zwaigenbaum, Scherer, Szatmari et al., 2011). There is currently much debate on whether this increase in the rate of autism reflects a true increase in incidence or simply reflects better methods of detection. The increased incidence is suspected by some to be caused by the expansion of criteria in the DSM-IV and by an increase in public awareness (Wing, Gould, & Gillberg, 2011; Gernsbacher, Dawson, & Goldsmith, 2005).

Many studies cite neurodevelopmental abnormalities as the cause of ASD (Bachevalier & Loveland, 2006; Bauman & Kemper, 2005; Critchley, Daly, Bullmore et al., 2000). Some areas of concern are the prefrontal cortex, the limbic system and cerebellum (Bachevalier & Loveland, 2006; Critchley et al., 2000; Girgis, Minshew, Melhem et al., 2007). Specifically, the orbital frontal cortex (OFC) has been found to

be involved in the social deficits associated with ASD (Goursaud & Bachevalier, 2007). A study done by Girgis (2007) found decreased grey matter volume in the 0right lateral OFC of ASD patients. Additionally, Bachevalier and Loveland (2006) posit that ASD is caused by a "developmental dysfunction" of the OFC-amygdala circuit. Current research has also identified brain overgrowth in the first to second year of life in children with ASD (Carper & Courchesne, 2005; Courchesne, 2005). This is suggested by some to be an early indicator of ASD [specifically overgrowth in the PFC (Carper & Courchesne, 2005; Courchesne, 2005)]. On the strength of research implicating the involvement of the OFC in ASD, this brain region will be investigated in the current study.

There are some inconsistencies in autism research in that at different ages, brain regions and methodology may demonstrate different results, both abnormal, but may be opposite in direction which makes the findings difficult to interpret. A case in point is the Girgis study in which there was a decrease in OFC whereas in Carper and Courchesne (2005) there was an increase in brain growth. There are also inconsistent results in relation to amygdala size in fMRI and other studies. Some reseachers find smaller amygdala size while others report larger amygdala and yet others find no size difference from controls (Ball et al., 2009; Baron-Cohen et al., 2000; Critchley et al., 2000; Hall et al., 2007). Autism is a complex disorder in which different behavioral abnormalities are manifest in affected individuals, and behavioral differences occur even within families wherein more than one child is diagnosed with the disorder. Such behaviors as communication issues, social adaptability, repetitive interests, or sleep issues may all vary between children with the same diagnosis.

In autism, the amygdala is thought to be hypoactive leading to difficulty in facial processing (Baron-Cohen, Ring, Bullmore et al., 1999). Baumen (2005) found that the amygdala in individuals with ASD contains smaller neurons than that of controls. Another implication for the amygdala's involvement in autism is the lack of regional activation during functional imaging studies when viewing facial expressions (Baron-Cohen et al., 1999, Critchley et al., 2000). The apparent involvement of the amygdala in ASD predicates the study of this area in the current research project.

Early detection is a key component for enhancing success of many intervention strategies. There is an abundance of literature demonstrating that earlier is better (see for example: Rogers 1996; Ramey & Ramey, 1998; Dawson, 2008). Intervention strategies that educate and involve parents and family, people who have an existing relationship with the child, are deemed helpful and empower caregivers (Koegal, Koegal, & Dunlap, 1996). Involving caregivers with intervention strategies that can be used during typical daily routines and interactions are more effective for ASD children than static table methods of interventions (Koegal & LaZebnik, 2004). These methods include sitting at a table teaching communication skills and social interactions in a setting that does not reflect true daily routine schedules. This makes it difficult for the child to transfer acquired knowledge to other routine settings in daily living. Programs such as the Pivotal Response Training (Koegal et al., 1996) and the Early Start Denver model (Vismara & Rogers, 2008) are examples of these parent friendly tools. Experience, good or bad, has the ability to change the organization of the brain (Kolb, Pellis, & Robinson, 2004) and changes in brain organization may lead to changes in behavior. A neuroimaging study done by Rolls,

Kringelbach, O'Doherty, et al. (2001) found that pleasant touch activated the OFC (Rolls, 2010). Research has also shown that touch/tactile stimulation has significant remedial powers (Field, 2001) Based on these findings we hypothesize that touch may have the ability to remediate some abnormalities in the ASD brain. Infant massage may be a useful early intervention that can be early implemented by all families in any environment.

Valproic Acid (VPA) has been used to create an animal model of autism by many researchers (Ingram, Peckham, Tisdale et al., 2000; Rodier, 1996). This model was based on the finding that there was an increased rate of autism in human offspring exposed to VPA *in utero* (Ornoy, 2009). Researchers found that pregnant rats given VPA during gestation produce pups that have similar behavioral characteristics as those of humans with ASD (Klauck & Poustka, 2000; Ornoy, 2009; Schneider & Przewlocki, 2005). Rodier (1996) points out that an animal model must "exhibit characteristics that resemble those of the condition of study." It has been shown that the abnormalities in the ASD brain were also found in rats with prenatal exposure to VPA. These abnormalities include changes in the prefrontal cortex, amygdala, and cerebellum (Ingram et al., 2000; Ornoy, 2009; Rodier, 1996; Rodier, Ingram, Tisdale et al., 1997).

Other animal models of autism are also currently in use. Prenatal exposure to thalidomide causes similar abnormalities in behavior and brain anatomy to VPA (Miyazaki, Narita, & Narita, 2005; Narita, Oyabu, Imura et al., 2010; Yochum, Dowling, Reuhl et al., 2008). Lesion studies damage specific areas of the brain or several structures thought to be involved in autism (Klauck & Poustka, 2006).

Additionally, genetic models involve some of the genes suspected to be involved in ASD. There are several genes under investigation. These include the engrailed gene, neuroligin genes (3 and 4), Pten gene, and GSTM1 gene (Klauck & Poustka, 2000; Yochum et al., 2010). Mice models may provide a means to manipulate genes but are difficult to use in the assessment of behavioral changes. Mice lack some of the behavioral flexibility observed in rats and tend to show a high degree of variance on most standard behavioral tests. The study of the PFC and the role that it plays in ASD is particularly difficult in mice (Kolb, Calder, & Gibb, 2010). There is a strong sex difference in ASD; males outnumber girls 4:1. The reason for this is, as yet, unknown. (Giarelli, Wiggins, Rice et al., 2010). There is speculation that it may be due to genetics. Others believe it is the diagnosis process itself and that girls are often overlooked. There is a scarcity of studies aimed at answering this question and research that has been done, has been hindered by a small subject pool (Rivet & Matson, 2011).

Valproic Acid (VPA)

Valproic acid (VPA) has most commonly been prescribed as anti-seizure medication (Ikonomidou & Turski, 2009; Ornoy, 2006). However, it is also used as an anti-depressant and as bipolar medication (Umka, Mustafa, Elbeltagy et al., 2010). VPA's principal mode of action is on the γ amino butyric acid (GABA) neurotransmitter system. It increases GABA availability by preventing its breakdown. When taken during pregnancy mothers were found to have a 10% increased risk of having a child with autism (Ornoy, 2009). The effects of VPA are not fully understood, however it is known that VPA inhibits histone deacetylase, prevents cell proliferation, increases

apoptosis, and causes cerebellar anomalies (Ikonomidou & Turski, 2009; Ingram et al., 2000; Klauck & Poustka, 2006).

In the rat model of autism, VPA is given to pregnant dams on day 12 of gestation, which results in brain abnormalities in offspring consistent with those observed in humans with autism (Ornoy, 2006; Schneider & Przewlocki, 2005). Animal models of ASD are necessary to test various hypotheses of drug effects on cognitive, motor, and emotional behaviors (Belzung, Leman, Vourc'h et al., 2005). Ornoy (2009) also found that VPA administered to pregnant dams produced a set of behavioral changes in the offspring similar to those observed in humans with ASD. Some autism related behavioral changes include a lower sensitivity to pain, decreased number of social behaviors, increased repetitive-like behaviors, reduced exploratory behavior, and cognitive rigidity (Belzung et al., 2005; Ornoy, 2006; Rodier, Ingram, Tisdale et al., 1996; Schneider, 2005). The anatomical pathology observed after VPA administration also mimics that of humans with ASD (Ornoy, 2006; Rodier, et al., 1996; Schneider, 2005) With this and other research on VPA exposed animals, many investigators have found that prenatal VPA administration to rats serves as a useful model for autism (Ikonomidau, 2009; Ingram et al., 2000; Ornoy, 2009; Rodier et al., 1996).

Tactile stimulation (TS)

Tactile stimulation (TS) has been shown to improve behavioral and physiological outcomes for preterm infants (Field, 2010; Blackwell, 2000). TS has also been shown to improve behavioral outcomes in rats that were given medial prefrontal cortex removals on postnatal day two (Kolb and Gibb, 2010). Research on rats has shown

that tactile stimulation activates the nucleus accumbens, pons, thalamus, and orbitofrontal cortex (OFC) (Ho, Higuchi, Roberts et al., 2009; Gallace & Spence, 2010). Field (Field 2010) reports that massage in humans increases blood flow to several brain areas including the amygdala and hypothalamus, which may aid in emotion management.

In animal studies using TS as an intervention, rats showed lower anxiety behaviors in elevated plus maze and demonstrated higher activity and exploration in an open-field task (Narita et al., 2010). It has been proposed that TS acts as a protective agent against brain damage during neonatal manipulation (Imanako, Morinobu, Toki et al., 2008, Rodrigues, Arteni, Abel et al., 2004).

In children with ASD, massage has decreased stereotypic behavior, and increased on-task and social-relatedness behaviors (Escalona, Field, Singer-Strunck et al., 2001). Field (1997) also demonstrated that with tactile stimulation in children with ASD, attentiveness increased in the classroom and touch aversion decreased, due in part to the predictable nature of touch during massage. Infant massage can begin from day one, encourages face-to-face interaction, communication (rhymes and talking), and positive repetitive interaction between infant and caregiver. These are common deficits identified in ASD individuals. Practicing these interactions may serve as an initial intervention for families with a heightened risk of ASD.

Neuroanatomy and Behavior

The brain is plastic, meaning that it can change in relation to experiences and environments (Kolb, Gibb, Forgie, et al., 1998). The brain influences behavioral

output and behavior can in turn influence the brain. What we do impacts how our brain works. In autism it is suspected that several brain areas are not organized in a typical manner. It is thought that through certain interventions or experiences the brain can change allowing the affected individual to behave in a more typical fashion. In other words "change the brain, change the behavior". The areas to be investigated in this study are the OFC, medial prefrontal cortex (mPFC) and the amygdala. Other areas of concern in ASD are the cerebellum, superior temporal sulcus (STS), and fusiform face area (FFA) but these areas are beyond the scope of this study.

Prefrontal Cortex (PFC) and Autism

The prefrontal cortex includes the orbital frontal cortex, the medial prefrontal cortex, and the dorsolateral prefrontal cortex (Happaney, Zelazo, & Stuss, 2004).

Orbital Frontal Cortex and Autism

The OFC is involved in executive functions that include planning, interaction, adaptability, and the ability to adjust behavioral responses to changing conditions (Girgis et al., 2007; Kolb et al., 2004; Loveland, Bachevalier, Pearson et al., 2008). The OFC has a role in social cognition, theory of mind, social appropriateness, and emotional regulation (Bachevalier & Loveland, 2006; Burruss, Hurley, Taber et al., 2000; Parsons, Young, Murray et al., 2010; Sabbagh, 2004). In addition, Pellis et al. (2006) state that the OFC has a "part in the perception and production of species-typical social signals." Rats with OFC lesions show abnormalities in social behaviors (Kolb et al., 2004; Pellis, Hastings, Shimizu et al., 2006). The OFC connects with the amygdala and other medial temporal cortical areas (Happaney et al., 2004, Rempel-

Clower, 2007). The OFC may be involved in the production of the repetitive behaviors of ASD (Amaral, Schumann, & Nordahl, 2008). Bachevalier and Loveland (2006; 2008) believe that a dysfunction of the OFC – amygdala circuit is the main cause of ASD symptoms. This damaged circuit leads to a dysfunction of self-regulation and socio-emotional functions such as theory of mind (Sabbagh, 2004; Bachevalier & Loveland, 2006; Loveland et al., 2008). A study done by Ashwin et al. (2007) found a decrease in functioning in the OFC in individuals with ASD. The OFC is also involved in deciphering tactile information and fMRI studies have shown that touch activates the OFC (Field, 2010).

Medial Prefrontal Cortex and Autism

The mPFC has reciprocal connections to the OFC (Bell, Pellis, & Kolb, 2010). It also connects with the superior temporal sulcus and hypothalamus (Nelson, de Haan, & Thomas, 2006; Price, 2006). The main role of the mPFC is to help modulate social behaviors such as theory of mind, empathy, and understanding emotional expression (Neuhaus, Beauchaine, & Bernier, 2010). Damage to this area is thought to cause difficulties with theory of mind tasks (Nelson et al., 2006). Damage to the mPFC is also thought to produce difficulties in a rat's ability to shift attention, a common trait seen in persons with ASD (Sutherland, 2005). Neuhaus (2010) reported that the mPFC has diminished activation in ASD individuals compared to controls. The mPFC has also been found to be hyper-reactive in the VPA animal model of ASD, which may affect amygdala functioning (Markram, Rinaldi, La Mendola, et al., 2007). A recent study that involves testing multitasking components of executive function in the mPFC, showed deficits in individuals with ASD (Gilbert, Bird,

Brindley et al., 2008). Furthermore, there appears to be a reciprocity between the OFC and the mPFC; increases in cell morphology in one results in a decrease in the other (Bell et al., 2010).

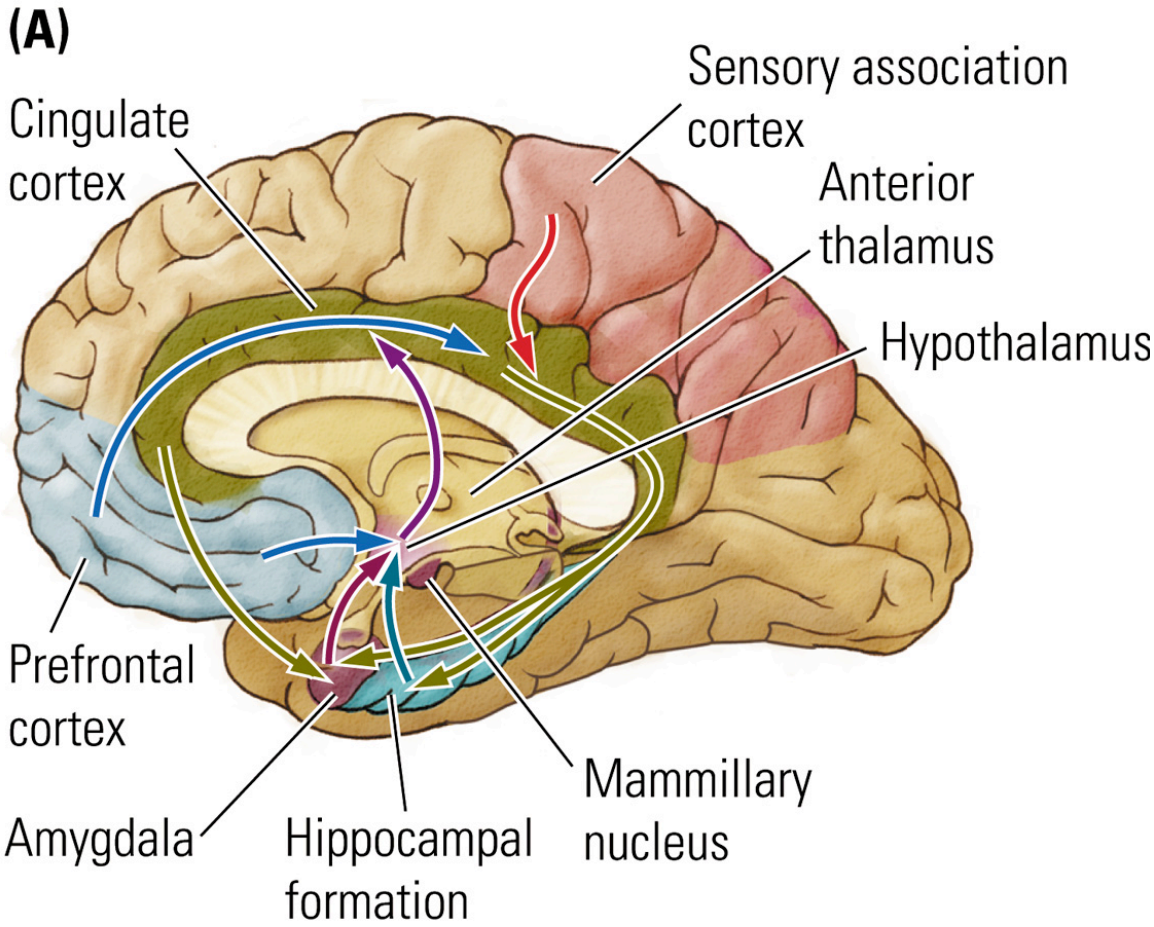

Figure 1.1. This diagram shows the location of the Amygdala and the PFC

Kolb and Whishaw 2010 Brain and Behavior. (The figure is used with permission of the authors.)

Amygdala and Autism

The amygdala plays a primary role in emotion, including the recognition of emotion in faces (Hall, West, & Szatmari, 2007) and the fear response. The amygdala is

located beneath the temporal lobe close to the hippocampus. It is composed of three groups of nuclei: first the basolateral nucleus which is responsible for responses to faces and actions of others. This nucleus has its main connections with the OFC and mPFC. Second, the centromedial nucleus, which is involved in respiratory and cardiovascular control, has its main connections with the olfactory bulb and cortex. Third, the central and anterior group of nuclei, which are involved in selective attentional processing and responses to faces (Ball, Derix, Wentlandt et al., 2009; Pessoa, 2010). The third grouping of nuclei has connections to the brainstem and hypothalamus (Baron-Cohen, 1999). The amygdala connects to the OFC, thalamus, hippocampus, insular cortex, fusiform face area (FFA) and nucleus accumbens (Baron-Cohen et al., 2000; Hall et al., 2007). Damage to the amygdala in rhesus monkeys causes varied fear responses (Amaral, Capitanio, Jourdain, et al., 2003). There is some debate to the amygdala's exact role in autism. However, it is thought to play a role in the social impairments related to fear and anxiety that are common co-morbid features in ASD (Amaral et al., 2003). The amygdala develops abnormally in children with ASD. Research has demonstrated an enlargement in amygdala volume in toddlers with ASD (Schumann, Barnes, Lord et al., 2009).

Repetitive training has been shown to strengthen the weakened region of the fusiform face area (FFA) connected to the amygdala for facial recognition in autistic children (Shultz, 2005). Shultz (2005) also hypothesizes that affective involvement is a key factor to success in gaining expertise in facial perceptions. Infant massage is an early repetitive intervention that may aid in this line of training.

The studies that comprise this thesis used the Valproic Acid model of Autism in Rats (VPA administered on E12.5) and postnatal tactile stimulation in an attempt to remediate autistic like behaviors in the VPA exposed animals. We used a cross-litter design with an equal number of animals from each litter and of each sex assigned to the TS or NTS groups. Behavioral analyses were done starting on P30 for play. Adult behavioral data was collected starting on P60.

Organization of the Thesis

Chapter One is the General Introduction to this Thesis. Chapter Two describes the methods used and Chapter Three contains the results. The final chapter, Chapter 4, is a General Discussion of the Thesis

Research Questions Addressed in this Thesis

Three research questions were addressed in this thesis:

1.How does prenatal exposure to VPA affect animal behavior and brain anatomy?

2.Will TS remediate the effects of VPA exposure?

CHAPTER TWO

Methods

Subjects

Male and female Long-Evans rats were used for this project. Animals were divided, into control and VPA exposed, and TS and NTS, and male/female groupings. There were 23 animals in the female VPA-NTS group and 23 in the female VPA-TS group. The female control-NTS group had 16 subjects and the female control-TS group had 21. In the male groupings, the VPA-NTS had 17subjects and the VPA-TS group had 19. In the control-NTS group there was 14 animals and in the control-TS group there was 18. There were a total of 151 animals used in this study. All subjects were from the University of Lethbridge vivarium. The animal room was maintained on a 12/12 hr. light dark cycle (7:30am-7:30 pm). All testing was done during the light cycle and all subjects completed all behavioral tests. Pups were weaned on postnatal (P) 21 and put in a cage with their same sex siblings. On P30 play behavior testing began. Food and water was delivered *ad libitum* with the exception of the duration of the tray-reaching task when food was restricted to 20g/rat/day. Following behavioral testing all animals had their brains removed for Golgi-Cox processing. The University of Lethbridge animal welfare committee approved all animal protocols.

VPA Administration

Administration of VPA is normally by either an intraperitoneal (I.P) injection or by a feeding tube with a dosage ranging from 500mg/kg to 800mg/kg (Ingram et al., 2000; Narita, Kato, Tazoe, et al., 2002; Snow, Hartle, & Ivanco, 2008). Owing to an adverse

reaction to the I.P. injection by the rats we used in our initial studies (ataxia, apparent abdominal discomfort, etc), we chose to administer VPA at 800mg/kg in peanut butter and fed it to pregnant dams in this manner. Physiological reactions to VPA administration with this method were less severe than the previously described methods (Kolozsi, Mackenzie, Roullet et al., 2009).

Behavior

A range of behaviors was analyzed to obtain a global perspective of the VPA animal model of autism. These included maternal care of VPA exposed dams, and offspring behavior including: play behavior, Whishaw tray reaching task, activity box, T-maze, novel object recognition, and elevated plus maze. Belzung (2005) suggested at least three behavioral tests that should be administered in an animal model of autism. These are social play, t-maze for cognitive rigidity, and Elevated plus maze for anxiety (Belzung et al., 2005). All are included in this research project.

Maternal Care

To assess maternal care requires examination of several components of maternal behavior. These include; arch back nursing, passive nursing, licking/grooming, contact, no contact, nesting, and pups out of the nest. Calculating the amount of time dams spent in each of these activities gauges parental care (Wei, David, Duman et al., 2010). This care is important to pups because maternal care and tactile stimulation from the mother stimulates the release of growth hormone (Diorio & Meaney, 2006). A lack of maternal care leads to increased anxiety-like behavior (Wei et al., 2010). Because it was possible that the VPA administered during gestation could affect

maternal care we needed to analyze this behavior. Observing the care dams give to their pups will determine if maternal care was different from control mothers.

Figure 2.1. Passive nursing of rat pups.

Play Behavior

Play behavior in rats is thought to aid in the development of the social brain and behavior (Pellis, Pellis, & Bell, 2010; Siviy & Panksepp, 2011). Play in rats is focused on nuzzling the nape of the play partner. This is the goal of play interactions (Bell et al., 2010; Pellis, Field, Smith, 1997; Pellis & Pellis, 2009). With this goal ever present, animals will attack either the nape directly or other areas such as head, upper back, lower back and anogenital region. In defending the nape, the partner will use a defensive tactic such as complete rotation, partial rotation, evasion, nothing, (ignore the attacker) or other less common tactics of jumping or rearing (Pellis &

Pellis, 2009). In juveniles the most common defensive technique is the complete rotation also known as rolling to supine (Pellis, Pellis, & Bell, 2010).

The OFC and mPFC are both thought to be involved in play behavior. The OFC is thought to play a role in distinguishing play partners (e.g., adult vs. juvenile partner) and creating appropriate interactions with a variety of play partners (Bell et al., 2010). Damage to the OFC impedes a rat's ability to change their play behavior with regard to the partner's identity (Pellis et al., 2010; Siviy & Panksepp, 2011). The mPFC is thought to execute proper sequences of movements in the play encounter (Bell et al., 2010). Damage to the mPFC leads to difficulties with sequences of movements (Pellis et al., 2010; Siviy & Panksepp, 2011).

Rats that have been raised in isolation have been found to have long-term deficits in areas of social and cognitive behaviors (Pellis et al., 2010). These rats become socially incompetent as adults, are hyper-defensive, and fail to show appropriate submissive behaviors when in contact with dominant males (Pellis & Pellis, 2009). Pellis & Pellis (1999) also found that these rats have difficulty coordinating movements with their partners, perhaps a sign of mPFC damage.

The peak of juvenile play behavior is between postnatal (P) day 30 – 40 (Pellis et al., 2010). In the following study juvenile play was filmed during this time and adult play was filmed at P60.

The questions we asked were: 1) Will VPA treatment alter play behavior in juvenile or adults rats? 2) Will VPA-TS animals have similar play behavior to that of controls?

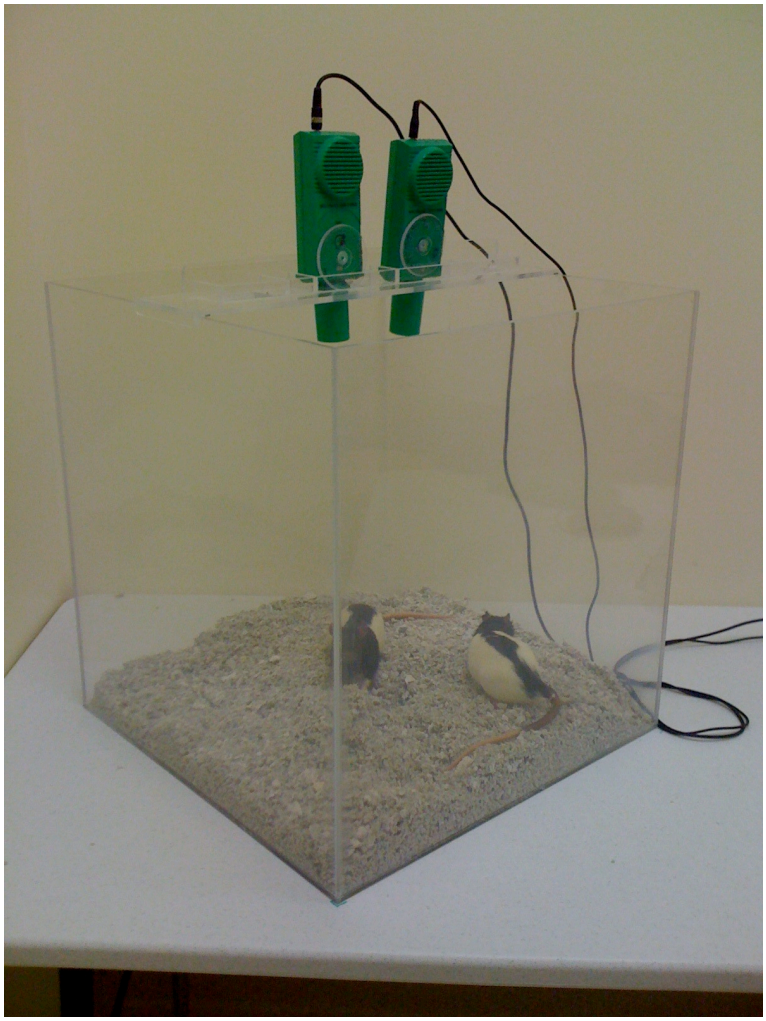

Figure 2.2. Image of the play box with bat monitors above to record vocalizations.

Whishaw Tray Reaching Task

The Whishaw tray-reaching task provides and endpoint measure of skilled reaching movement (Kolb, Cioe, & Comeau, 2008, Whishaw, 1996). It is often used to assess motor lesions and prefrontal cortex damage (Gibb, Gonzalez, Wegenast, et al., 2010).

The reaching cage is built of Plexiglas® with 2mm bars placed 9mm apart in the front. The floor is made of a wire grid so chicken feed can fall through. In front of the cage is a tray, designed to hold the chicken feed (Kolb et al., 2008). During this task

animals are on a food-restricted diet but kept at 95% of original body weight. In order for the animal to grab food they are required to reach through the bars and grab feed from the tray. Rats are trained for 20 days before being filmed. The number of reaches are counted as well as the number of hits (animal obtained food and was able to eat it) Hit percent = (number of hits/number of reaches) X 100 (Gharbawie, Gonzalez, & Whishaw, 2005). Damage to PFC or motor areas cause abnormal limb movements resulting in a greater number of misses.

The question asked was: How accurate are VPA animals at the reaching task in comparison to controls?

Figure 2.3. Whishaw Tray Reaching Apparatus.

Activity Box

The activity box measures both exploratory and mobility behaviors in rats. The apparatus tracks overall activity as well as vertical and horizontal activity (Koob, Cirillo, & Babbs, 2006; Narita et al., 2010). The box uses infrared beams to follow the movement of the rat calculating activity based on the number of beam breaks. Narita (2010) has found that in the initial trial exposure to a novel activity box VPA

animals show hyperactivity due to what may be an increased level of anxiety. It is thought that this behavior may be linked to frontal cortex abnormalities (Uylings, Groenewegen, & Kolb, 2003).

The question asked was: Will VPA animals have a decreased activity response in a novel environment? Will this activity return to normal levels following TS?

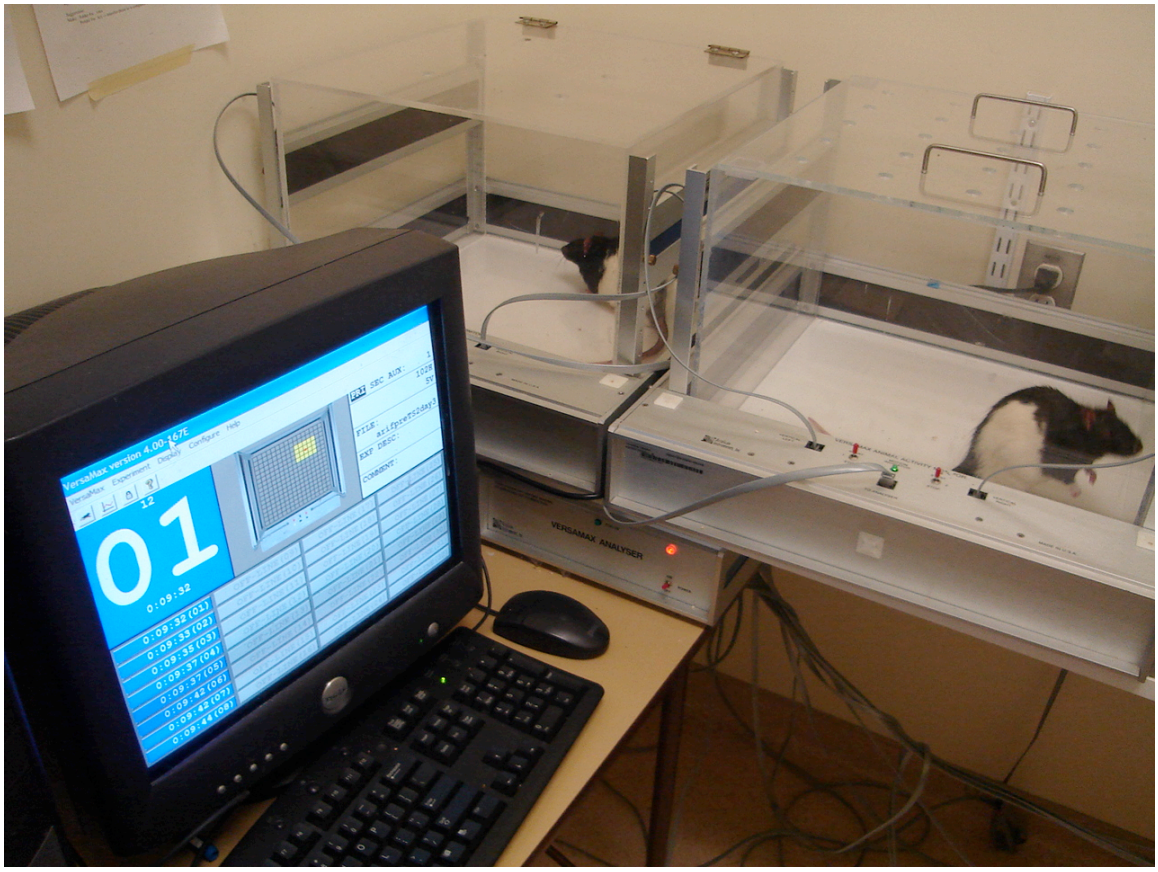

Figure 2.4. Image of the Activity box.

T-Maze

The Non-match-to-sample T-maze is used as a measure of working memory and prefrontal cortex functioning (Porter, Burk, & Mair, 2000). Lesions to the prefrontal

cortex create working memory deficits (Zironi, Iacovelli, Aicardi et al., 2001). Animals with OFC damage will often perseverate on items or direction taken in maze tasks (Happaney et al., 2004).

The questions investigated were: Will VPA animals take longer and make more errors than the control group and will TS demonstrate benefits to this task?

Figure 2.5. Rat in the T-maze apparatus with one arm blocked.

Novel Object Box

The Novel object recognition task tests temporal order memory, which is memory for the order in which items are experienced. This type of memory is believed to take

place in the prefrontal cortex, specifically the mPFC (Dudchenko, 2004, Hannesson, Howland, & Phillips, 2004, Mitchell & Laiacona, 1998).

Previous studies by Ennaceur (2010) and Mitchell & Laiacona (1998) indicate that rats spend more time exploring objects that are novel compared to familiar objects. This finding extends to recency of exposure to the object, in that the familiar object is the most recent object explored. Rats with mPFC lesions show a lack of discrimination between old and new objects with both being explored comparably (Mitchell & Laiacona, 1998).

The question asked was: Will VPA animals spend more time with the old familiar object or with the new unfamiliar object?

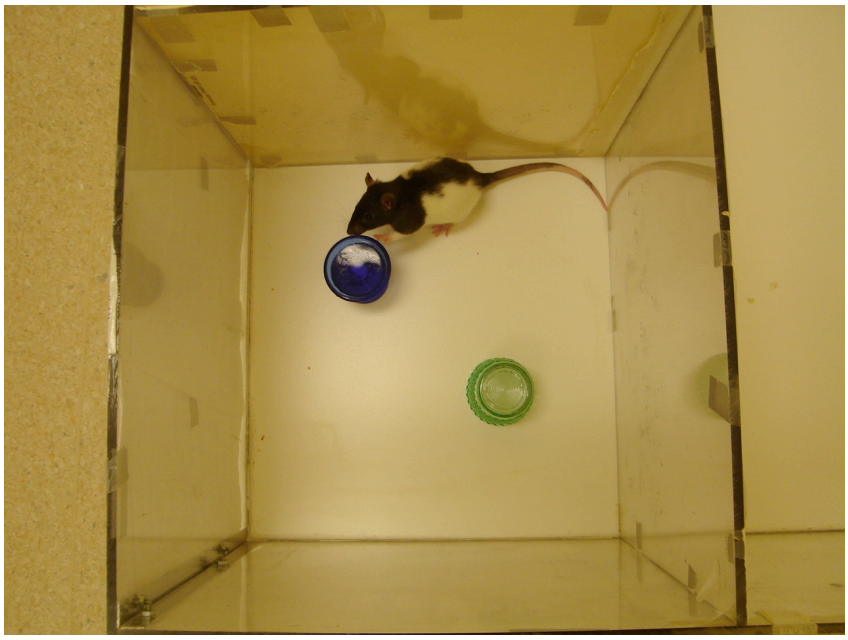

Figure 2.6. Rat in the Novel object box during the third trial.

Elevated Plus Maze

The elevated plus maze is a test to measure anxiety. It consists of four elevated arms; two open and two closed. Anxiety is measured by the exploration activity and time spent in either the open or closed arms of the maze. (Pellow, Chopin, File et al., 1985; Rodgers & Dalvi, 1997). Rats prefer the closed arms (Viana, Tomaz, & Graeff,1994). Levels of plasma corticosterone are increased if rats are forced to stay in the open arm (Pellow et al., 1985).

Individuals with autism often have higher levels of anxiety (Reaven, 2011; Dickerson-Mayes, Calhoun, Murray et al., 2011). Testing the level of anxiety in the VPA animal model is consistent with behavioral issues common in humans with ASD.

The question asked was: Will VPA animals spend more time in the closed arms than controls?

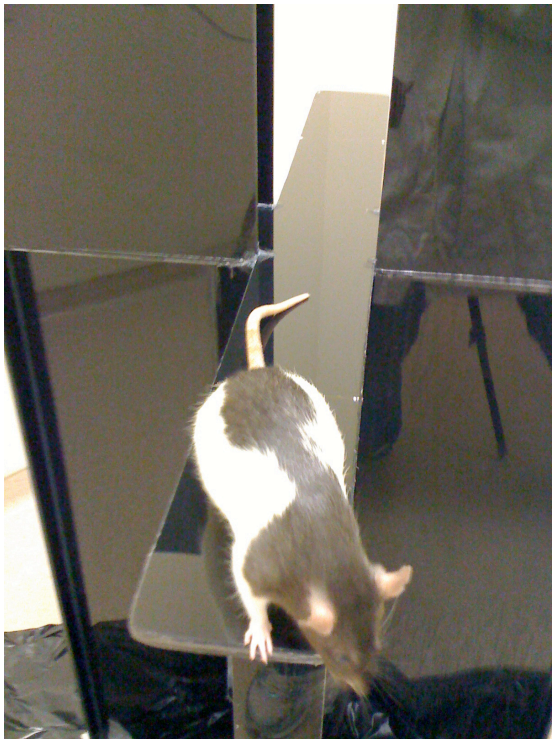

Figure 2.7. Rat in the elevated plus maze on the open arm.

Anatomy

Cortical Thickness

Cortical thickness measurements were obtained from Golgi Cox stained coronal sections projected on a Zeiss-Jena MF2 projector at a magnification of 17.5X (following the method described by Stewart & Kolb, 1988). Briefly, three cortical measures were made at points medial, central and lateral on five sections of tissue identified by the following landmarks; Plane 1: first caudate-putamen visible, Plane 2: anterior commissure, Plane 3: first hippocampal section, Plane 4: posterior commissure, Plane 5: last hippocampal section. A plastic metric ruler was used to measure from the edge of the cortex to the edge of the white matter (figure 2.8). An

average for each plane and for each animal was calculated and used for statistical comparison.

Thalamic Area Measurements

Thalamic cross-sectional area was measured from two coronal sections stained with Golgi Cox stain using a Kodak digital camera to capture the image and the Scion Image program to measure thalamic area. One measure was taken of the anterior thalamus (approximately –1.80mm from the Bregma). The second measure was made in posterior thalamus at approximately –4.30mm from the Bregma [as described in a study by Kolb and Whishaw (1981)].

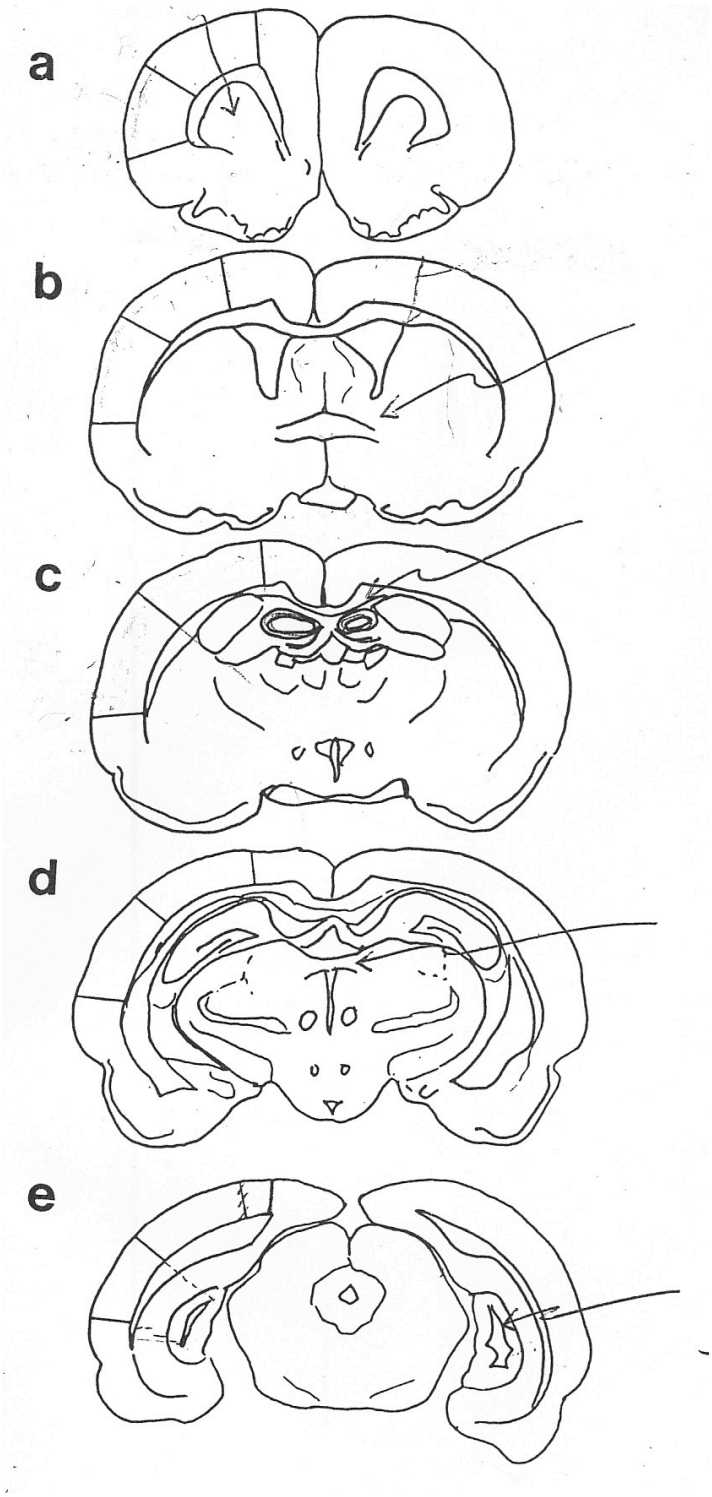

Figure 2.8 Representation of the coronal planes used to make cortical thickness measurements.

Golgi-Cox Analysis

The Golgi-Cox staining method was used to help identify cells within the brain. Cells were drawn using a camera lucida and analyzed in three different ways. The first analysis was Branch Order, which looks at the complexity of the apical and basilar branches as they proceed from the cell body. The second analysis was the Sholl analysis, used to estimate dendritic length in μm. The third analysis was dendritic spine density, which counts the number of spines on a particular branch and provides an estimation of synaptic contact. (Gibb, Gonzalez, & Wegenast, 2010). We sought to determine if there were changes in neuroanatomy of Cg3, AID and amygdala of VPA animals.

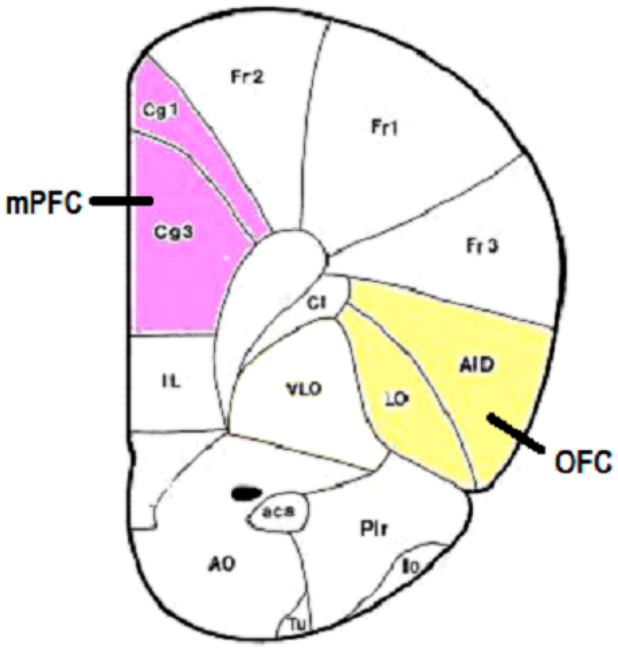

Figure 2.9. Areas that cells were drawn from, the OFC and the mPFC.

Prenatal and Postnatal Timelines

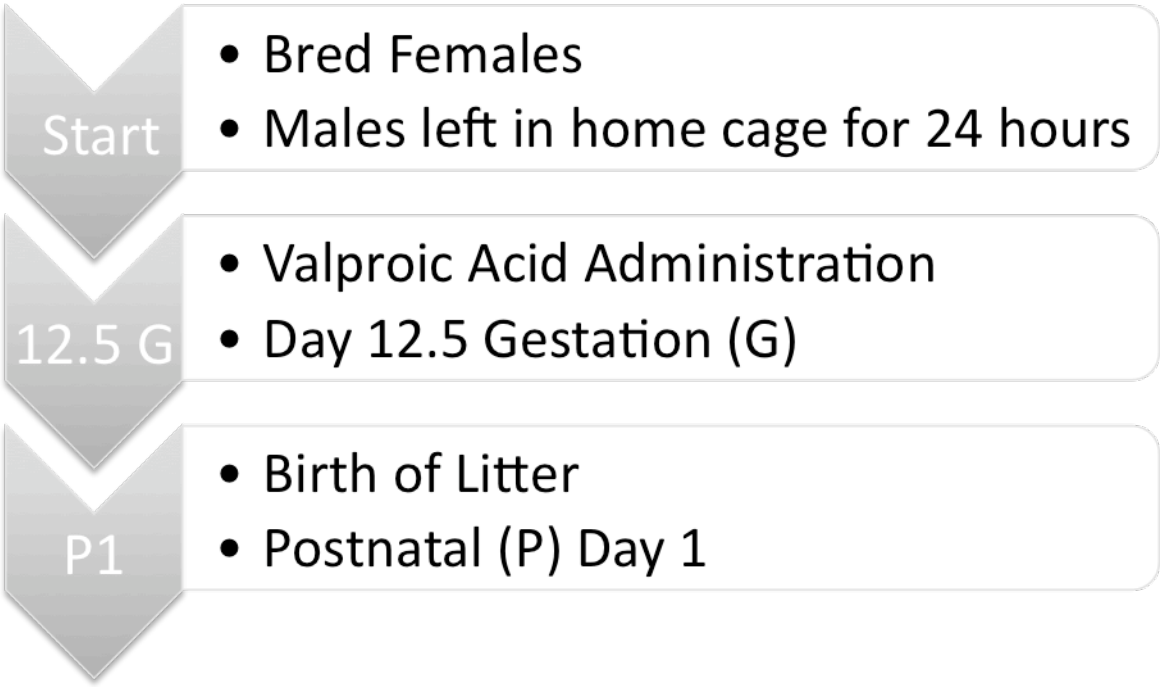

Figure 2.10 Timeline showing breeding and administration of VPA.

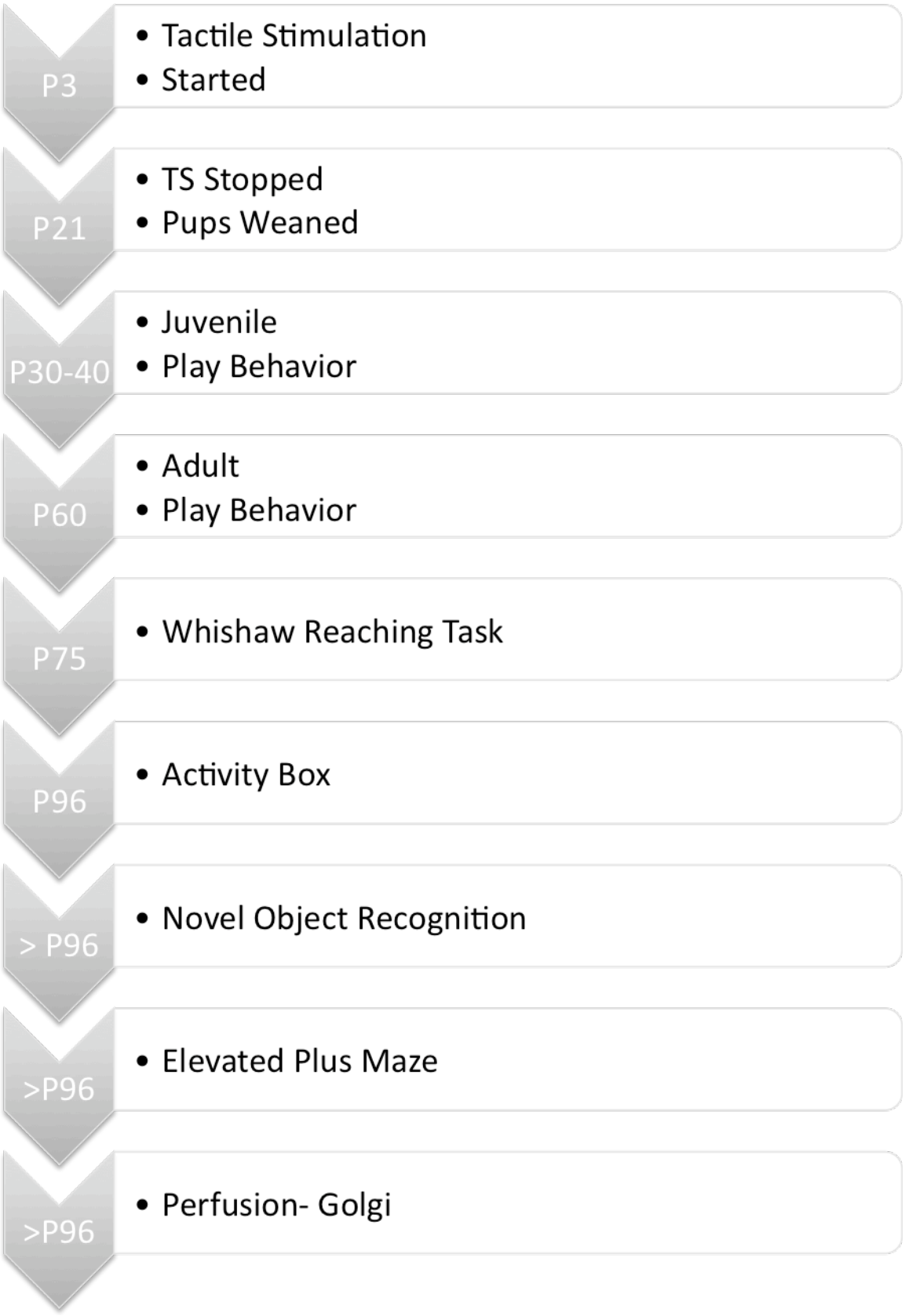
P3
• Tactile Stimulation
• Started
P21
• TS Stopped
• Pups Weaned
P30-40
• Juvenile
• Play Behavior
P60
• Adult
• Play Behavior
P75
• Whishaw Reaching Task
P96
• Activity Box
> P96
• Novel Object Recognition
>P96
• Elevated Plus Maze
>P96
• Perfusion- Golgi

Figure 2.11. Postnatal timeline.

Individuals Scoring Behavior

Due to the extensive behavioral measures and the large number of subjects, undergraduate students were recruited to assist in behavioral testing and scoring of behaviors. Undergraduate students were trained in one behavioral task until proficient in scoring behaviors. Where possible one student was assigned to score one behavior to keep scoring consistent. Random samples of behavior scored were checked for reliability and accuracy.

Breeding

Male rats were placed with females in a cage and observed for 20 minutes. If breeding took place within that time males were left with the females for 24 hours. If no breeding behaviors were observed the male was taken out and the process was repeated the next morning.

VPA administration

Three days prior to administration of VPA all females received 1.5 grams of peanut butter. On day 12 of gestation half of the females were given 800mg/kg VPA mixed with peanut butter and the controls received 1.5g of peanut butter. Peanut butter was fed to individual rats with a scoopula.

Figure 2.12 Rat being fed peanut butter.

Treatment

Tactile stimulation

On postnatal (P) day 3, dams were removed from the home cage and placed in a cage with food. Rat pups were taken in the home cage to a separate room for tactile stimulation (TS). The cage was placed on a heating pad set on medium heat (24°C). A Swiffer® duster was used to brush the rats for 15 minutes three times a day: 9:00 AM, 1:00 PM, and 4:00 PM. Male and female rat pups within each litter were

randomly assigned to tactile stimulation and non-tactile stimulation groups. A laminated board was used to keep the two groups separated during tactile stimulation. Rat pups were returned to mother at the end of each session. TS continued until P21 when pups were weaned.

Picture 2.13. Rat pups being tactilely stimulated by Swiffer® duster.

Behavioral Methods

Play behavior

Play behavior was filmed at both juvenile (P30 – P40) and adult (P60 +) stages. Rats were placed in 50 X 50 X 50 cm Plexiglas® box. Care-Fresh® bedding was used in the bottom of the box. Animals were habituated to the room and box starting three

days prior to filming. Habituation entailed placing animals in the box for 10 minutes each day with their play partner and the lights off.

Play partners were assigned using a quadrad (Pellis et al., 2006), the four groups were; Control tactile stimulation, Control non-tactile stimulation, VPA tactile stimulation, and VPA non-tactile stimulation. Filming occurred on three different days with different pairings for each day. A 24-hour period between testing was given and animals were isolated for 24 hours before testing.

Play behavior was scored as described in a study by Pellis (1998). Attacks were scored as occurring on the head, nape, upper back, lower back and anogenital region. Defense behaviors are partial rotation, complete rotation, nothing, evasion, other (facing, rearing, jumping). Individuals blind to groups and treatment scored the play behavior.

Whishaw Reaching Task

From day 1 to day 7 of initiation of this task rats were food restricted (animals given 25g of rat chow each per day). Animals were weighed each day to ensure no animal lost more than 10% of its pre-test body weight. On days 8 to 21 food restriction was continued (animals given 20 grams each per day). Starting on day 8, animals were placed in reaching cages for 30 minutes each day. On the final day each rat was filmed individually for 5 minutes in the reaching cage. Data was collected for attempted reaches, hits (when food was obtained and eaten) and misses. A percent hit score (# hits/total # of attempts) was calculated for each rat.

Activity Box

Rats were placed in an Accuscan® activity monitoring system consisting of electronically fitted Plexiglas® boxes measuring 41 cm by 41 cm by 30.5 cm (height) that recorded movements of each individual rat. Rats were placed in the box for 10 minutes and their exploratory behavior was recorded in five two-minute intervals. Data was recorded on a computer with the VersaMax™ program. This was then converted to a file using VersaDat™ software. The key measures obtained were overall activity, vertical, and horizontal activity.

T-Maze

Non-match-to-sample T-maze consisted of two trials given 10 times. To begin the trial the rat was placed at the stem of the maze with the door blocking entry into the maze arm. Trial one was a forced run where one arm was blocked and the other left open so the rat was forced to enter one arm to receive a food reward. Trial two was a choice run wherein the rat could choose which arm to go down but was only rewarded if it chose the arm opposite to the arm selected in the initial trial. Trial two was started after a ten-second delay on completion of the first trial. If the rat went down the incorrect arm there was no reward and the rat was removed from the maze. When the rat went down the correct arm and found a food reward it was allowed to eat the food before being removed from the maze. The open arm was varied between trials in a semi-random schedule.

Day 1	RLRRLRLLRL
Day 2	LRLLRLRRLR

Table 2.1. Random assignment of the blocked arm.

The task was scored by how many times the animal enters the non-match-to-sample arm (correct arm). When the rat reached three days in a row of 80% or greater proficiency it was deemed to have met criterion. A cumulative error score was also calculated for each animal. The task was run for 10 days, starting with a habituation day. Habituation entailed placing the rat in the maze for 5 minutes with fruit-loops at either end of the T-maze arms. The maze was cleaned with Virkon® between each trial.

Novel Object Recognition

Novel object recognition for temporal order memory was run in three trials starting one hour apart on filming day. The rats were placed in a white plastic container 48cm X 48 cm X 52 cm (height) for 5 minutes three days prior to filming. On filming day the initial trial had two identical objects in the base of the tub and the animals were left in for 4 minutes. The second trial began one hour later and different identical objects were placed in the same location as on the initial trial. The third trial used one object from the first and one from the second trial. Each trial last for four minutes and the container and objects were washed with Virkon® between each trial and animal.

The time spent with each object was calculated. An animal was deemed to be in contact with an object if the animal's nose was within two centimeters of that object.

Time spent with the initial trial object versus the second trial object was compared. Scoring was done by an individual blind to group and treatment.

Elevated Plus Maze

An elevated plus maze made of black Plexiglas® was used. The base measured 94 cm high with two open arms measuring 10 cm by 40 cm and two closed arms measuring 40 (height) by 39 cm (long). The maze was placed in an empty room and lights were on during filming. All trials were filmed with a camera placed in front of an open arm and raised above the maze.

Rats were placed with their front paws in the center square of the maze facing a closed arm. Each rat was filmed for five minutes. The maze was cleaned between each animal with Virkon® solution.

Scoring was based on time spent in the open arms, closed arms, centre of the apparatus and past the halfway mark (past the halfway on the open arm). The number of entries into each area was also scored. Animals were considered in an arm when the first half of their body was within the arm or center square. Individuals scoring the tapes were blind to group and treatment conditions.

Anatomical Methods

Golgi Method

After completion of behavioral task a subset of animals were given an overdose of sodium pentobarbital and perfused with 0.9% saline solution. Brains were removed and weighed before being placed a Golgi-Cox solution. Brains were left in this

solution for two weeks and then the Golgi solution was replaced with a 30% sucrose solution. Brains were left in this solution for at least three days after which they were sliced on a vibratome at 200um following a procedure described by Gibb and Kolb (1998).

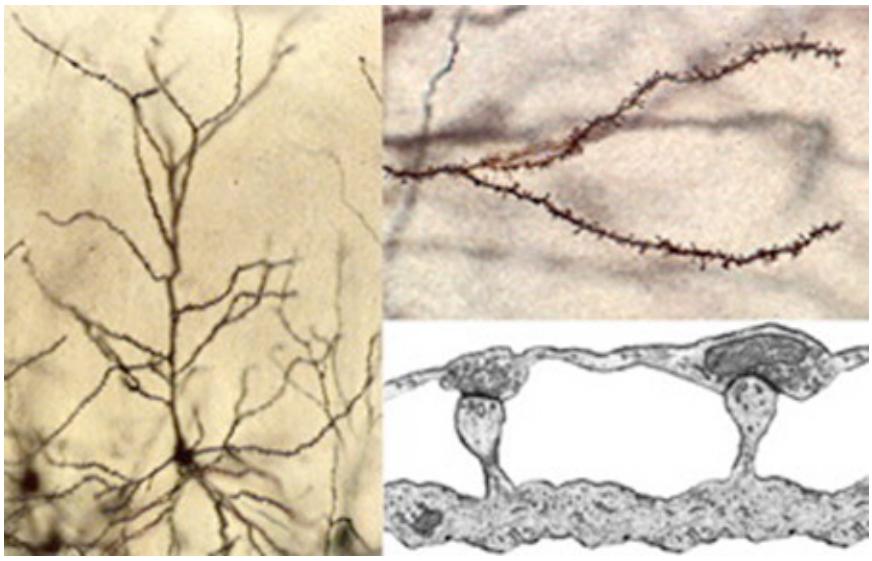

Figure 2.14 Cell stained with Golgi-Cox solution. Spines are evident in the upper right panel. The lower right panel is an enlargement showing dendritic spines making synaptic contacts.

CHAPTER THREE

RESULTS

Statistics

For all of the behavioural tests analyzed a three-way ANOVA was run with sex, group (VPA or control), and treatment (TS or NTS) as variables. To simplify the data analyses in cases where no sex effect was observed and there was no interaction of Sex with Group or Treatment the data were collapsed across sex. In cases where no treatment effect was observed and there was no interaction of Treatment with Sex or Group the data were collapsed across treatment.

Behavioral Results

Maternal Care

No behavioral differences, such as nest building, nursing or contact, were observed in the preliminary data analyzed for maternal care (n = six). At the time of the writing of this thesis, tapes of several litters remained to be analyzed. Once all of the behavior has been scored maternal care will be re-assessed.

Play Behavior

Juvenile Play

Control NTS animals were paired with VPA-TS, VPA-NTS and control-TS. It was these pairings that were analyzed. There was no significant difference for attack behaviors between VPA treated animals and controls [$F(1,28)=0.159$, $p=0.69$].

However, juvenile VPA treated animals engaged in significantly more overall defenses than controls [$F(1,28)=8.84$, $p<.01$]. VPA animals used evasion maneuvers significantly more often than controls [$F(1,28)=12.86$, $p<.005$]. At the juvenile stage, VPA animals did not differ significantly from controls in their probability of engaging in other forms of defense behaviors (facing, rearing & jumping) although there was a trend towards a significant increase in these behaviors [$F(1,28)=3.22$,$p=.08$].

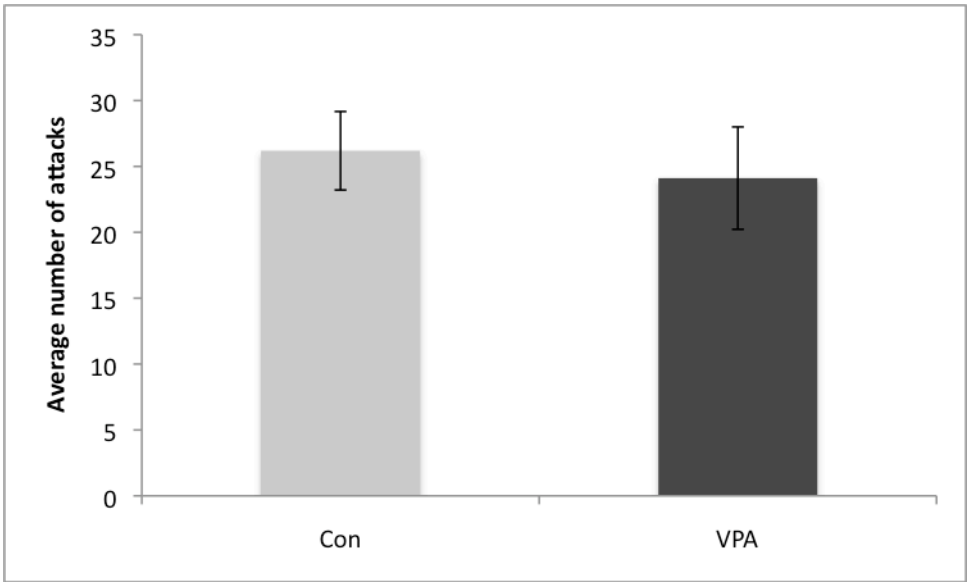

Figure 3.1 Juvenile play attacks. Attacks on head, nape, upper back, lower back and anogenital were areas that were scored. No significant differences were observed.

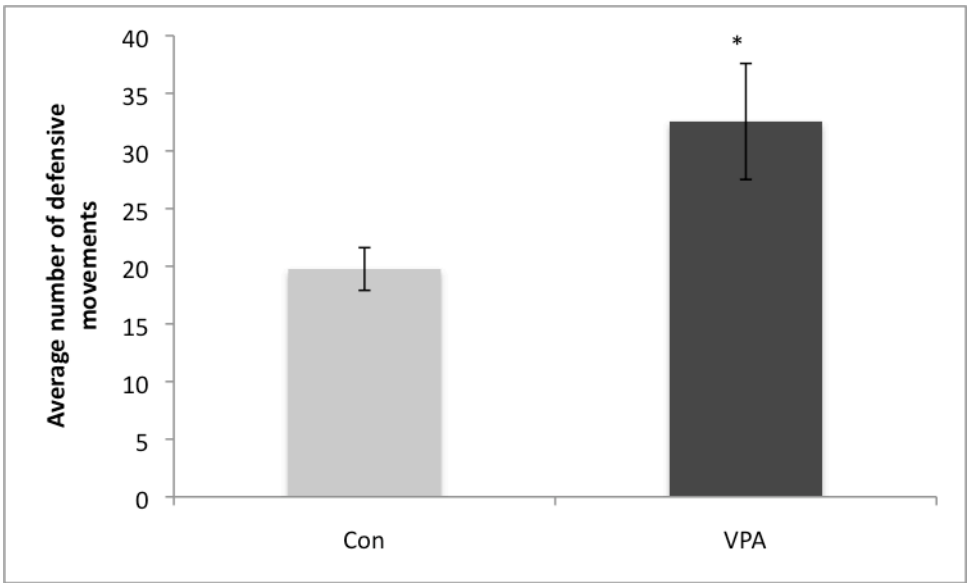

Figure 3.2. Juvenile play behavior, overall defensive moves, such as complete or partial rotation, evasion, ignoring and other (rearing, facing and jumping), were scored. VPA animals used significantly more defensive maneuvers (* = p<.05).

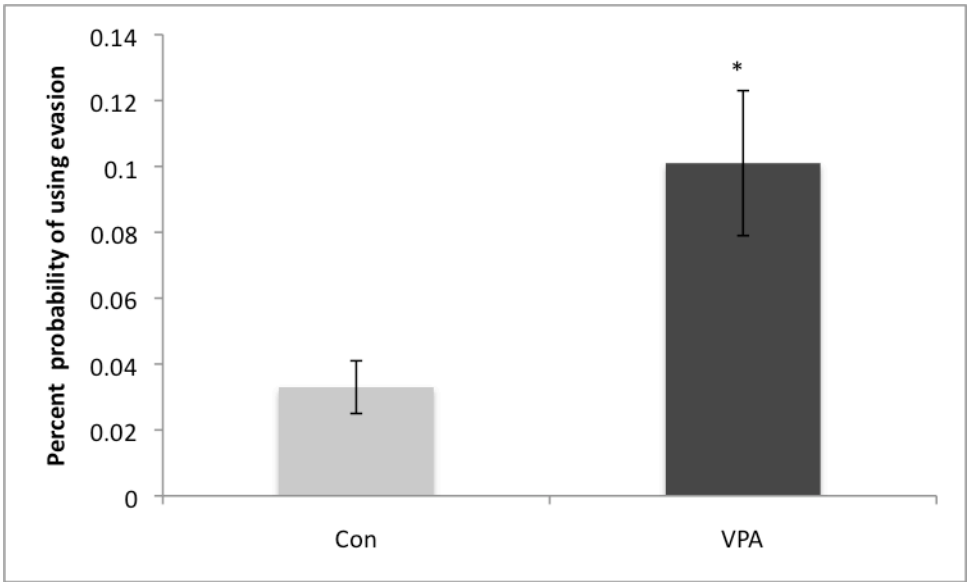

Figure 3.3. Juvenile play behavior, probability of defense using evasion. The VPA animals are significantly more likely to use evasion compared to controls (* = p<.05).

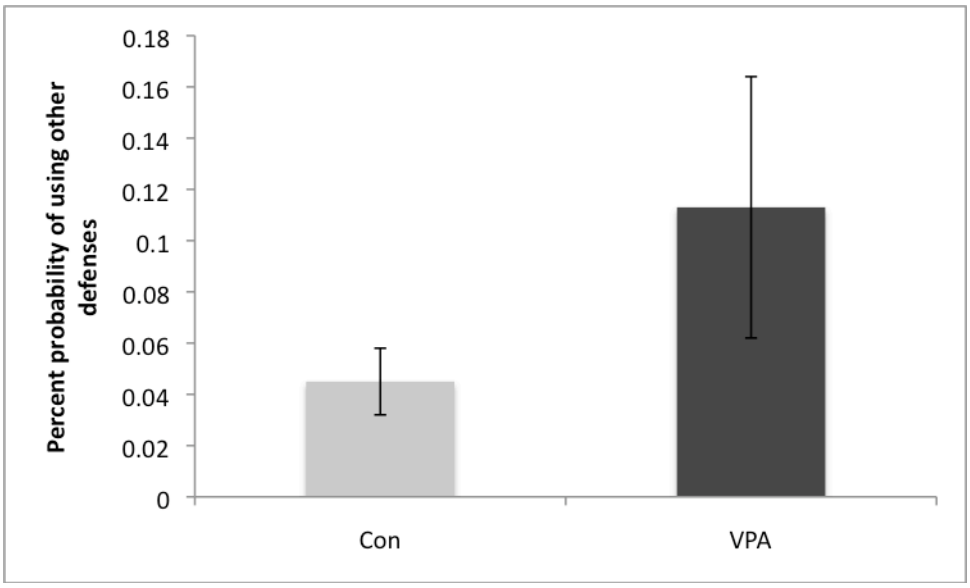

Figure 3.4. Juvenile play behavior, probability of defense using other (facing, jumping, rearing).

Adult Play

There were no significant effects for sex, group, or treatment in adult play behavior for attack behaviors [F's <0.19, p's.>67] or defense behaviors [F's<1.1, p>.3] and none of the interactions were significant.

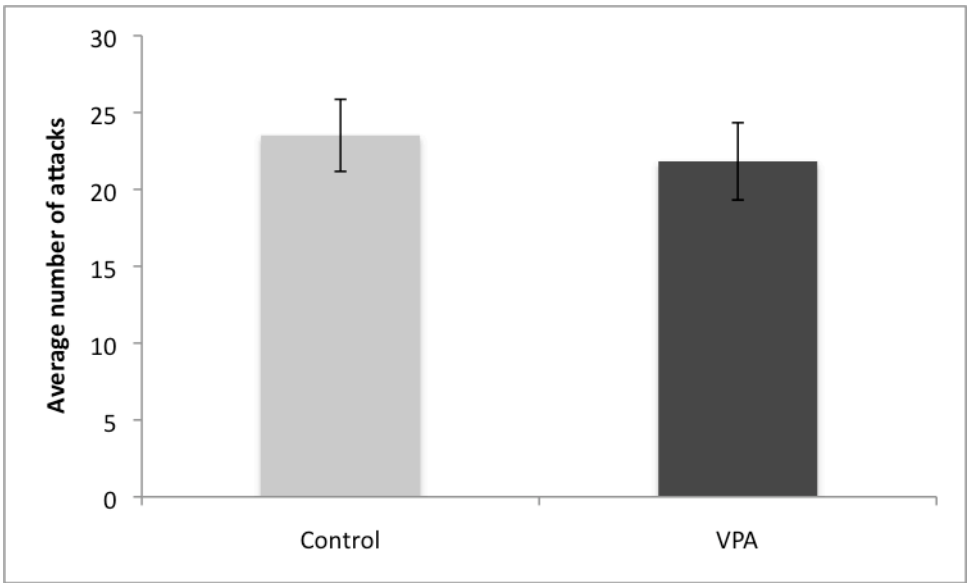

Figure 3.5. Adult play behavior, attack behaviors. There was no significant difference between the two experimental groups.

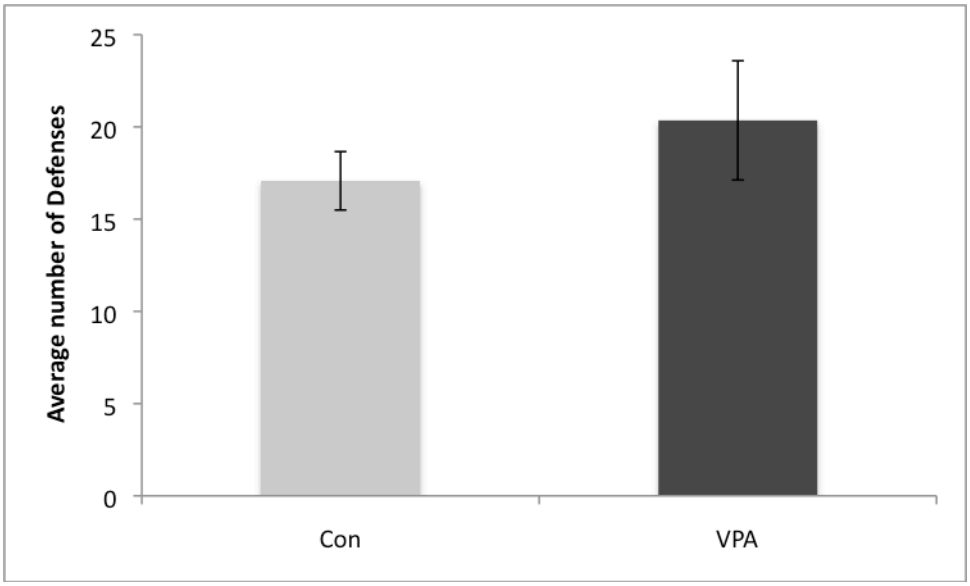

Figure 3.6. Adult play behavior, defensive maneuvers. There was no significant difference between control and VPA animals.

Discussion

VPA animals in adulthood showed no significant differences in their play behavior compared to control animals. This may be due to the fact that by the time they have reached adulthood they have learned social expectations or social rules and guidelines. By adulthood animals and humans may have learned what behaviors are needed to get along in their social circumstances. Rats become increasingly mellow with age compared to juveniles who tend to be more hyperactive (Pellis & Pellis, 2009). This was not the case for the juvenile animals. Many significant differences were observed in defense behavior during play in the juvenile period. This may reflect their inability to understand social expectations. Bell et al. (2010) state that "juvenile play enhances play performance later in adulthood. The reciprocal nature of play trains juvenile for more complex adult social play." Schneider et al. (2005) also found a difference in play in VPA juvenile rats. In their study VPA rats showed a decrease in frequency of pinning.

Whishaw Tray Reaching Task

There was a significant main effect of group on reaching, with VPA animals making more errors than controls [$F(1, 136)=5.44$, $p=.02$]. There was no effect of TS [$F(1, 136)=.35$, $p=.57$] nor sex (p's$>.05$). There were no significant interactions (p's$>.05$).

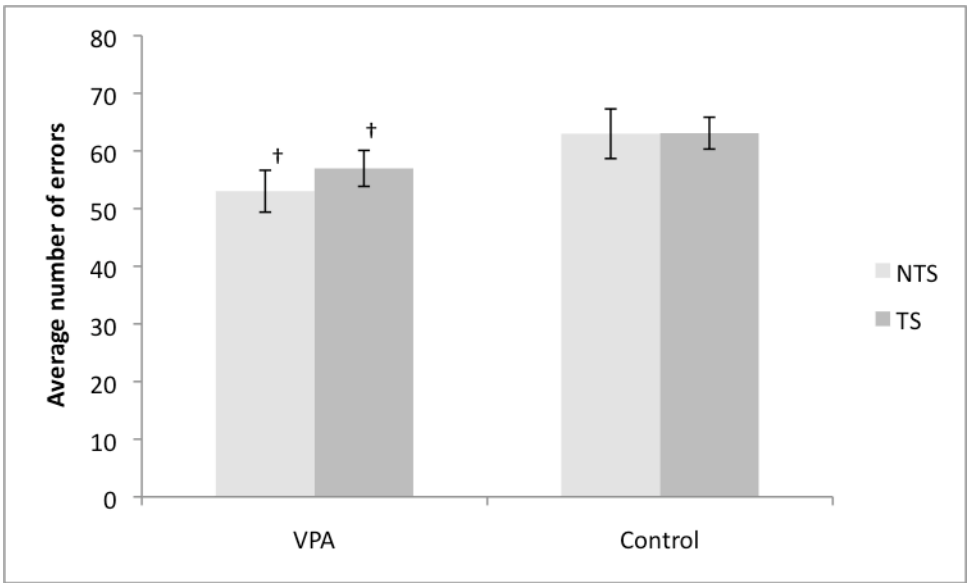

Figure 3.7. Whishaw tray reaching scores shows a significant deficit in VPA animals († = p<.05- VPA compared to control animals).

Discussion

VPA animals were significantly impaired in the reaching task. TS did not significantly improve the impairment. The motor impairment falls in line with human studies in motor delays in children with ASD (Papadopoulos, McGinley, Tonge, et al. 2011).

Activity Box

A two-way ANOVA (sex and group) was performed on the total distance travelled in the activity box. The results demonstrate a main effect of group [$F(1,129)=12.00$, $p<0.01$] and a main effect of sex [$F(1,129)=8.23$, $p<0.01$]. VPA treated animals covered more distance than controls and females covered more distance than males.

There was no interaction of Sex and Group [F(1,129)=.56,p=.46] for total distance. There was no effect of TS (p>.05).

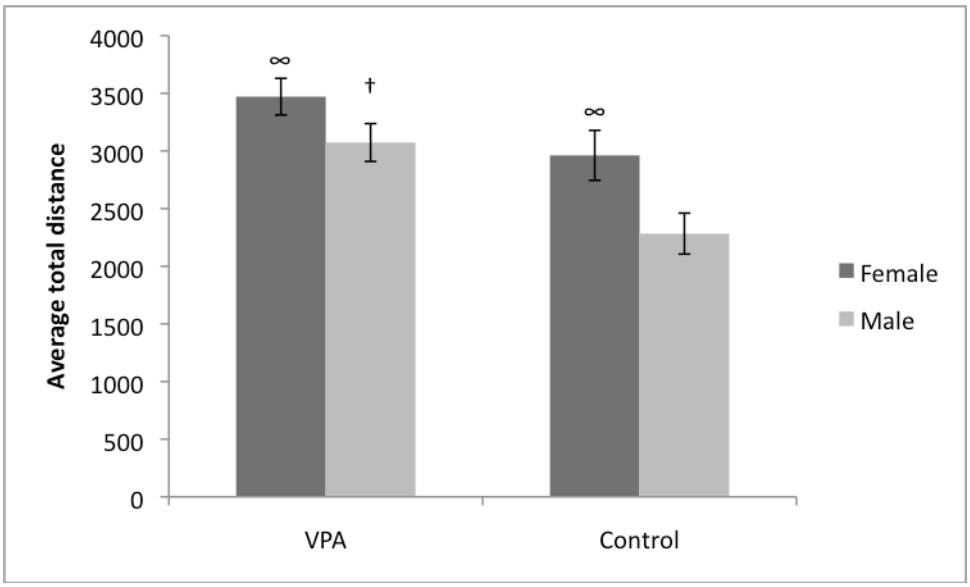

Figure 3.8. Total Distance. The VPA group was significantly more active than the control group and females were significantly more active than males in the activity box (†=p<.05-VPA compared to controls) (∞=P<.05-females compared to males).

Discussion

The VPA group showed significantly greater exploratory activity in the activity box demonstrating that in a novel environment this group was more likely to explore their surroundings. Females were also significantly more active in total distance covered. This was opposite of what was expected. Being in a novel environment it was thought that the VPA animals would have a decreased activity level compared to controls. Narita (2010) also found hyperactivity in their research on VPA treated animals although their findings were obtained using an open-field test and younger rats than those used in this study. The observation of hyperactivity in VPA animals falls in line

with the theory that in new environments ASD individuals are initially hyperactive perhaps due to the need to explore their new surroundings. TS had no effect on this behavioral task.

T-Maze

We examined the errors made to reach criterion, the days to reach criterion and the cumulative errors to reach criterion (total errors made over the days required to reach criterion). ANOVAs with sex, treatment, and group as variables were run on all three measures. No sex differences were observed. Overall VPA animals were impaired in this behavioral task when compared to controls. VPA animals made significantly more errors to reach criterion than control animals. The ANOVA of errors to criterion showed a significant effect of VPA [$F(1,38)=6.39$, $p=0.02$] (figure 3.9) . VPA animals also took significantly longer to reach criterion than controls, [$F(1,38)=6.61$, $p=0.02$] (figure 3.10). VPA animals also made significantly more errors overall [$F(1,38)=10.59$, $p<0.01$] (figure 3.11). TS did not have a significant effect on performance for either the experimental or control group. None of the interactions were significant (p's$>.05$).

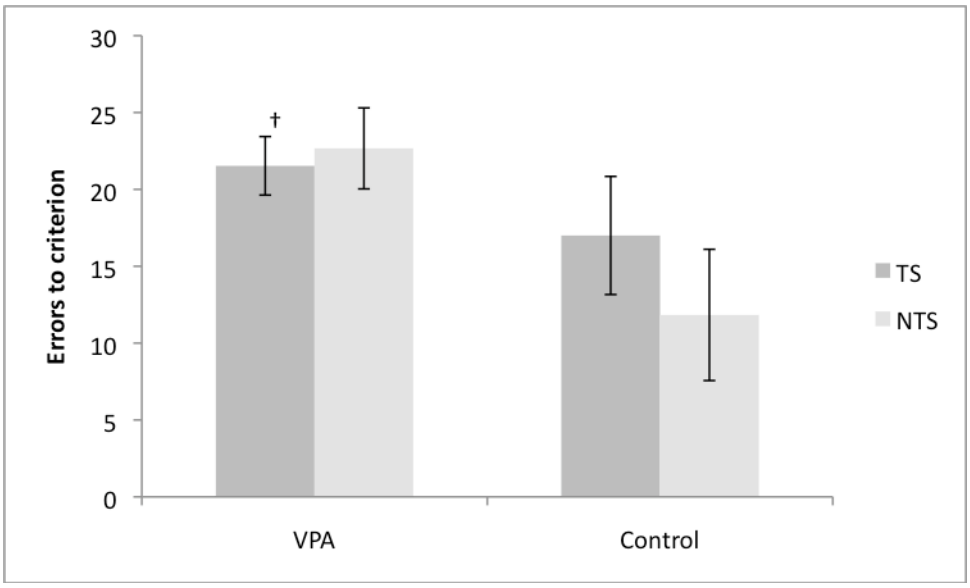

Figure 3.9. Errors to Criterion. VPA animals made more errors to reach criterion (†=p<.05-VPA compared to controls).

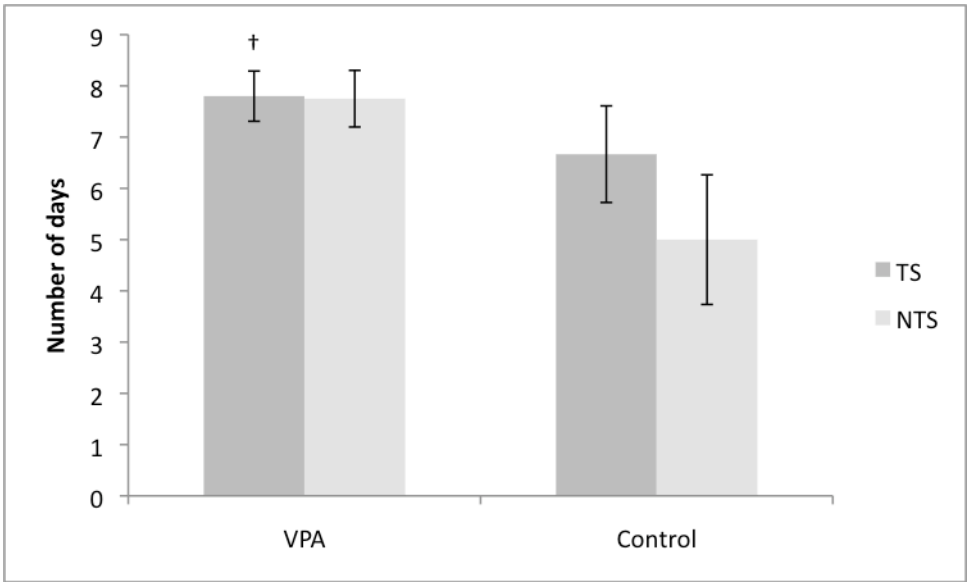

Figure 3.10. Number of days to reach criterion. VPA animals took significantly longer to reach criterion than controls (†=p<.05-VPA compared to controls).

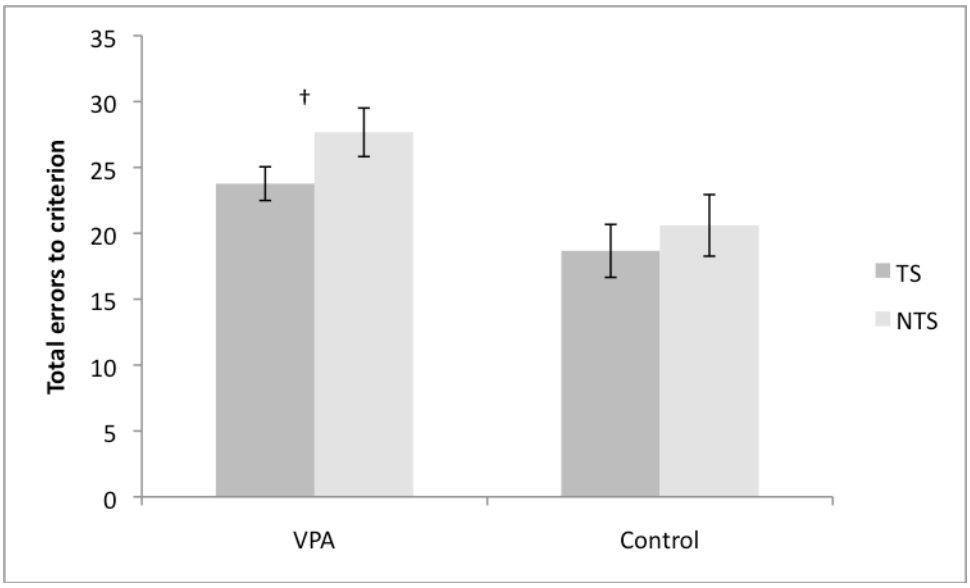

Figure 3.11. Total errors made over the 9-day period. VPA rats made significantly more errors (†=p<.05-VPA compared to controls).

Discussion

VPA animals showed deficits in non-match-to-sample in the T-maze confirming the potential of prenatal VPA administration as a model for autism (ASD). The results show that VPA animals took longer to reach criterion and made significantly more errors in the process. TS did not remediate this. Individuals with ASD often perseverate on situations and are reluctant to change even with reward. The reluctance of VPA animals to the change to rewarded arm demonstrates a tendency for perseveration (Sander et al., 2009). Damage to the PFC often leads to individuals demonstrating perseveration and an inability to change thinking (Burruss et al., 2000).

Novel Object Recognition

There were no significant effects for group, treatment, or sex, on time spent with old or new objects (p's > 0.25). There were also no significant effects of group, treatment, or sex on touches of the old familiar object (p's>0.1). The VPA animals touched the new object over the old object significantly more than control animals [F(1,138)=6.31, p=.01] (figure 3.12).

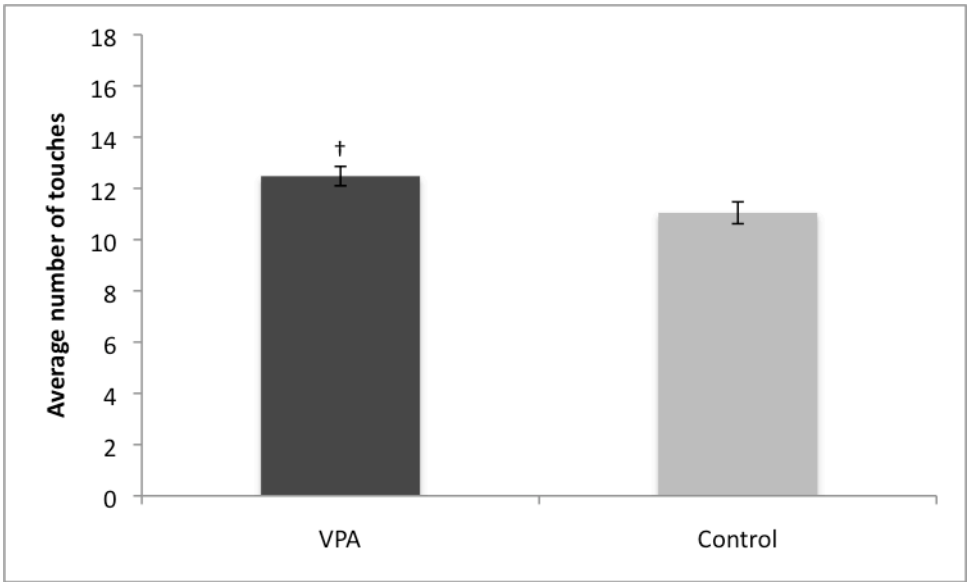

Figure 3.12. Touches for the new object. VPA animals touched the new object significantly more than controls (†=p<.05-VPA compared to controls).

Discussion

Although the time spent with the old or new object was not found to be significantly different between the VPA and control groups, the VPA group touched the new object significantly more. This may be due to being more familiar with the object and

therefore touching it more often. This may be a perseverating behavior on the newer object, because it is more recent in recall for the VPA animal.

Elevated Plus Maze

An ANOVA showed that the VPA group spent less time in the closed arm [$F(1,139)=4.80$, $p=.03$] (Figure 3.13). There was no significant effect of treatment or sex (and no significant interaction; p's>0.09) on time spent in the closed arm. For time spent past halfway on the open arm of the maze, VPA-NTS animals spent marginally more time than VPA-TS animals [$F(1,139)=3.28$,$p=.07$]. (Figure 3.14) (The VPA-TS animals performance mimicked the control animals). This may indicate that TS remediated the VPA influence on behavior in these females. There was a significant sex effect for time spent in the centre. [$F(1,139)=16.65$, $p<.01$] (Figure 3.15). For all of the other significant effects observed in the EPM, sex was the mediating variable. Females were significantly higher in all measures reported below. There was a significant sex effect on the numbers of entries into the closed arm, open arm, center arm, and past the half-way mark. ANOVAs for closed entry [$F(1, 139)=11.53$, $p<.01$] (Figure 3.16), for open entry [$F(1, 139)=20.47$, $p<.01$] (Figure 3.17), for center entry [$F(1, 139)=29.23$, $p<.01$] (3.18), and for past half way [$F(1, 139)=13.85$, $p<.01$] (Figure 3.19), were all significant.

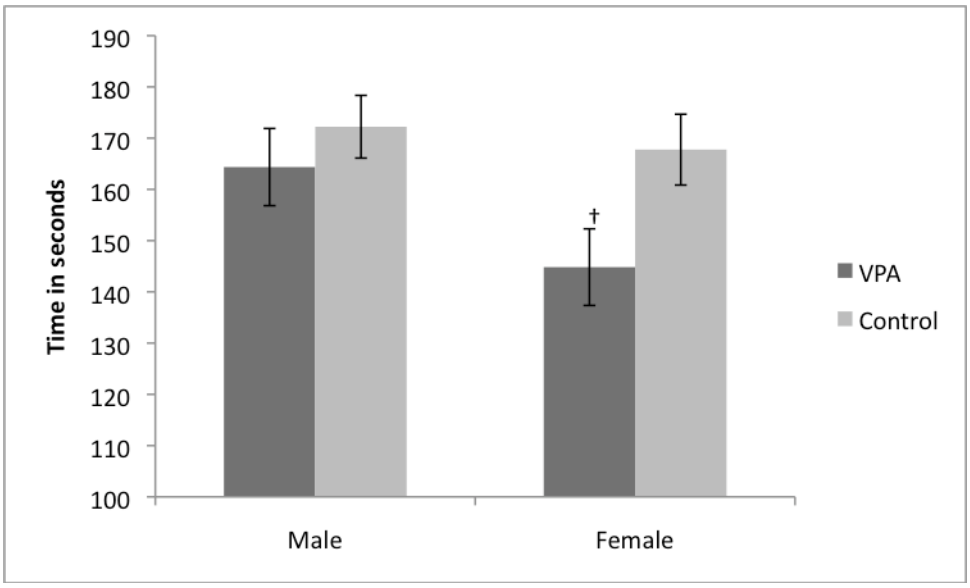

Figure 3.13. Time spent in closed arm. The VPA animals spent less time in the closed arm (†=p<.05-VPA compared to controls).

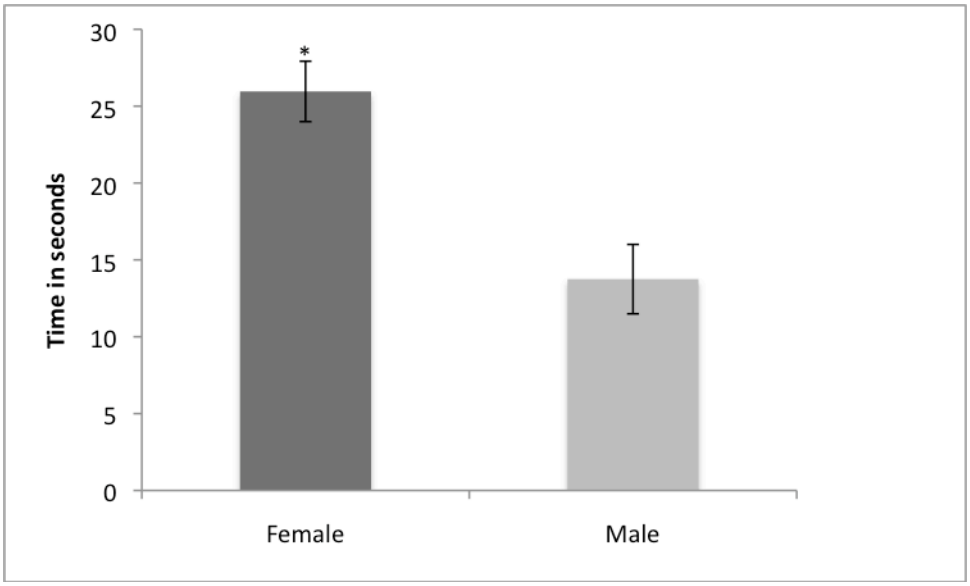

Figure 3.14. Time spent past the half way mark. There was a main effect for sex, females spent more time past the half way mark (* = p<.05).

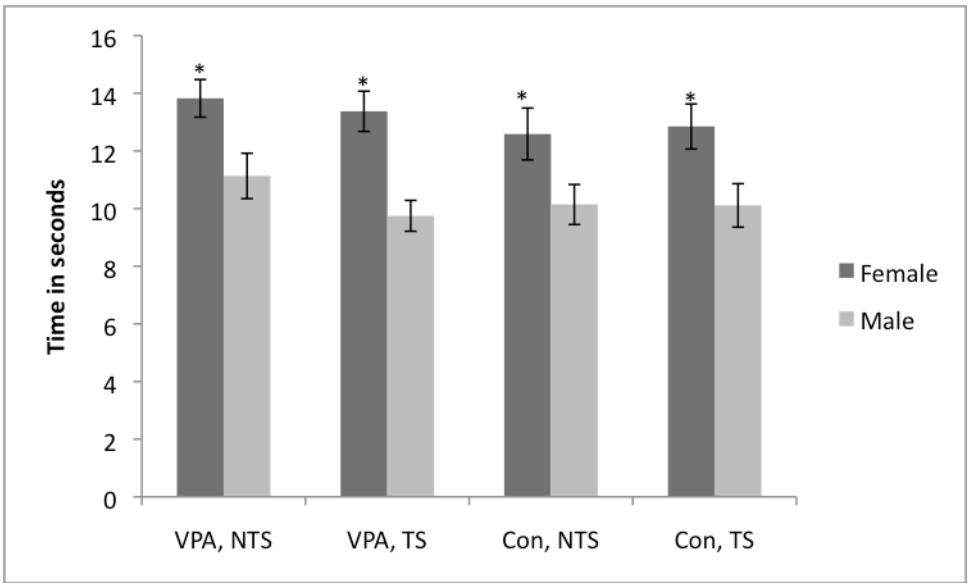

Figure 3.15. Time spent in the center; there is a significant sex effect (* = p<.05).

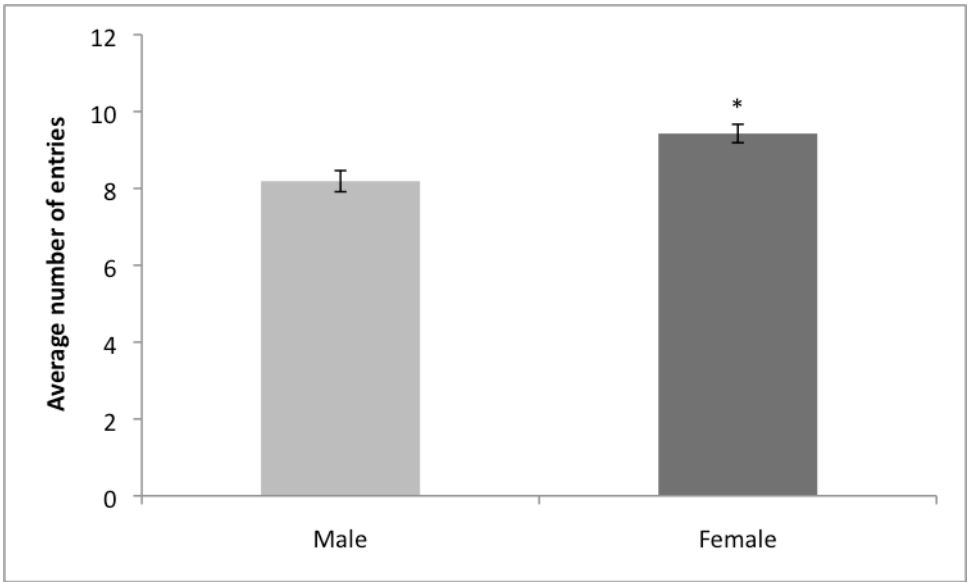

Figure 3.16. Mean number of entries into the closed arm. Females made significantly more entries into the closed arm (* = p<.05).

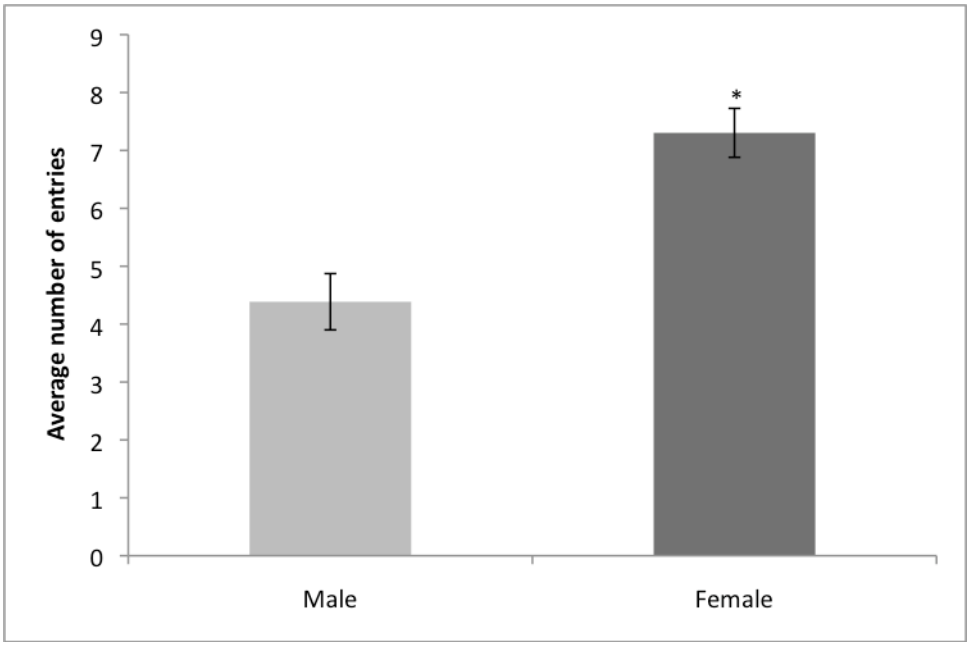

Figure 3.17. Mean number of entries into the open arm. Females made a significant more entries into the open arms (* = p<.05).

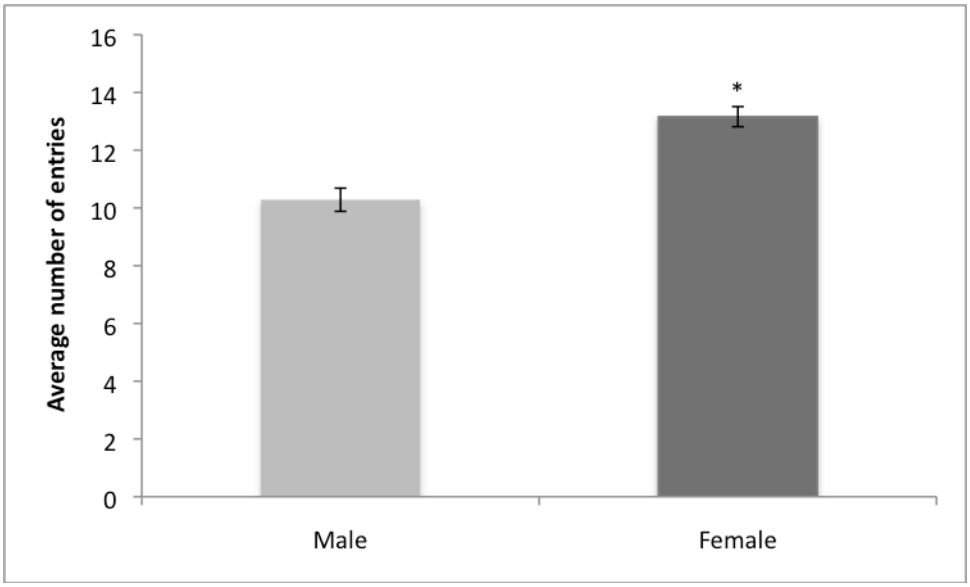

Figure 3.18. Mean Entries into centre. Females made signifcantly more entries into the centre of the maze (* = p<.05).

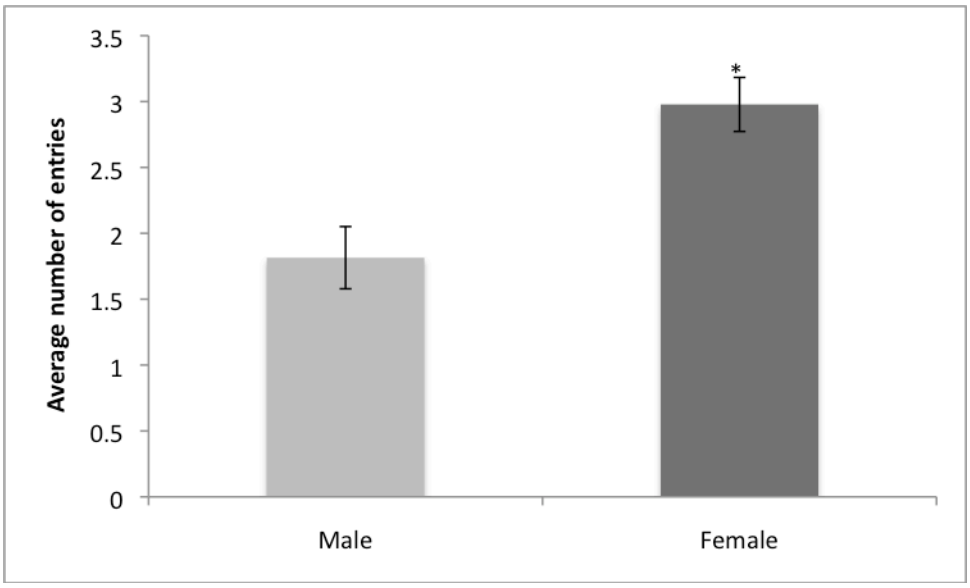

Figure 3.19. Mean number of entries past the halfway mark. Females made significantly more entries past the halfway mark on the open arm than males (* = $p<.05$).

Discussion

VPA animals did not spend more time in the closed arms, but rather spent more time in the open arm, the opposite of what was expected. This is similar to findings by Schneider (2008) who found that VPA females spend more time in the open arm than control females. This also follows the female's higher activity level in the novel environment of the activity box. Entries into the closed, open, center, and past the half way mark were also significantly higher in females than males, though the effects of VPA and TS were not significant.

Anatomical Results

Brain and Body Weights

A three-way ANOVA with group, treatment, and sex as variables showed a main effect of group [F(1, 140)=6.87, p=0.01]. VPA rats had significantly smaller brain weights than controls. The effect of TS was not significant (p>.05). There was also a significant difference between the sexes [F(1,140)=114.53, p=.0001] (Figure 3.20). None of the interactions were significant (p>0.05). There was the usual effect of sex on body weight; males were heavier than females [F(1, 140)=729.92, p=.0001]. There was no effect for group [F(1, 140)=2.04,p=.156] nor treatment [F(1,140)=.64,p=.426] on body weight (results not shown).

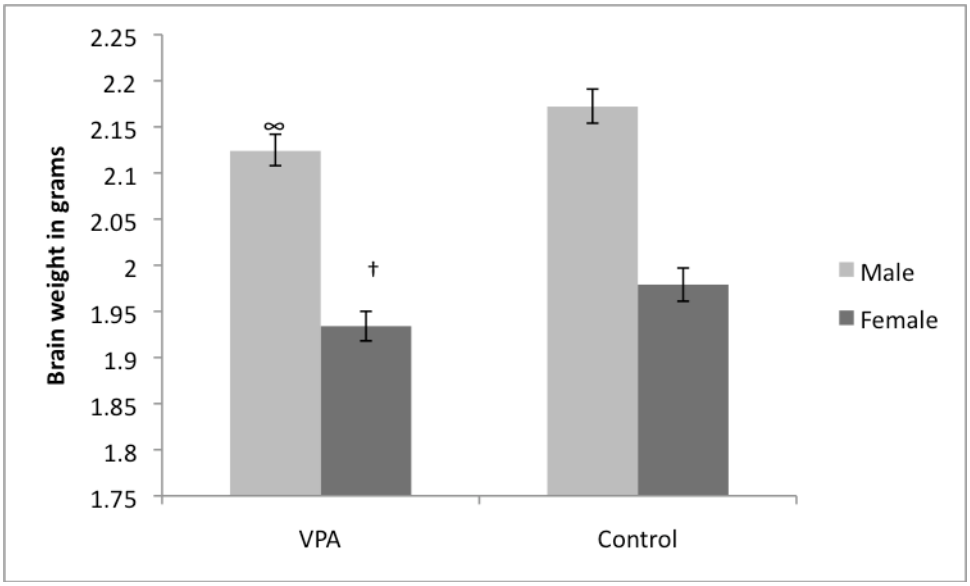

Figure 3.20. Average brain weights (in grams) of VPA group versus control group. The VPA group had significantly smaller brains. Brain weight were different for males and females, with males having significantly heavier brain weights than females (∞ = p<.05- male compared to female) (†=p<.05- VPA compared to control).

Discussion

On average VPA animals had a 2.5% reduction in brain weight compared to control animals. This is similar to findings by Ingram et al. (2000). The male animals in the NTS condition showed a reduction in brain weight of 2.5% and the TS males had a similar reduction in brain weight (2%). The NTS females showed the greatest changes with VPA exposure (4.5% reduction) whereas the TS females showed the least reduction in brain weight (1%). This may be an indication that the TS prevented cell loss in the female animals only. Differences in male and female brains are a common finding (e.g., Kolb, Gibb & Gorny, 2003).

Cortical Thickness

VPA animals had significantly thinner cortices than control animals. A three-way ANOVA with group, treatment, and sex as variables showed a main effect of group, on both right [$F(1,85)=23.24$, $p<.01$] (figure 3.21) and left cortical thickness [$F(1,85)=30.14$, $p<.01$] (figure 3.22). All planes showed a significant reduction in thickness following VPA exposure. Plane 2 is the only plane where a sex effect was observed [$F(1,84)=12.79$,$p<.01$] (figure 3.23). The effect of TS was not significant ($p>.05$). None of the interactions were significant ($p>.05$).

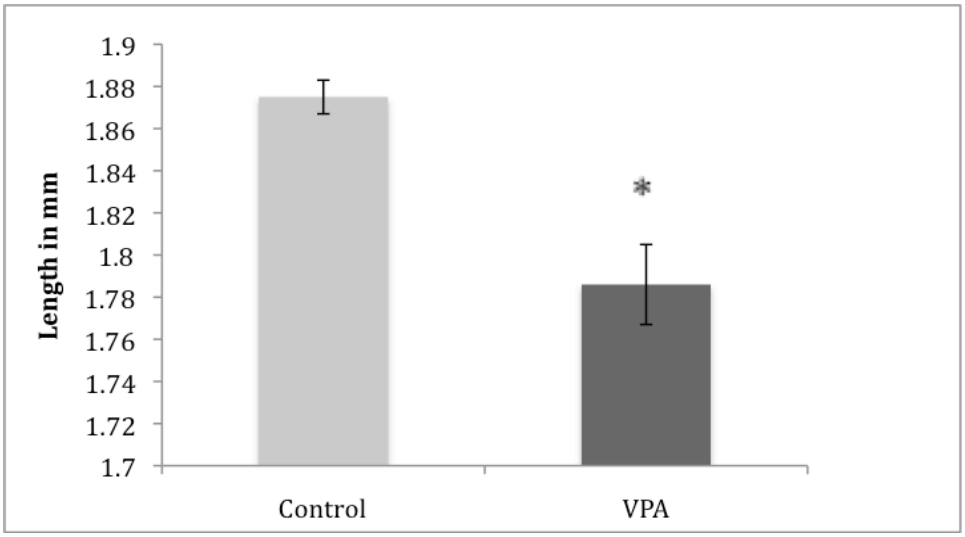

Figure 3.21. Total right hemisphere cortical average. VPA animals have significantly thinner right cortices (* = p<.05).

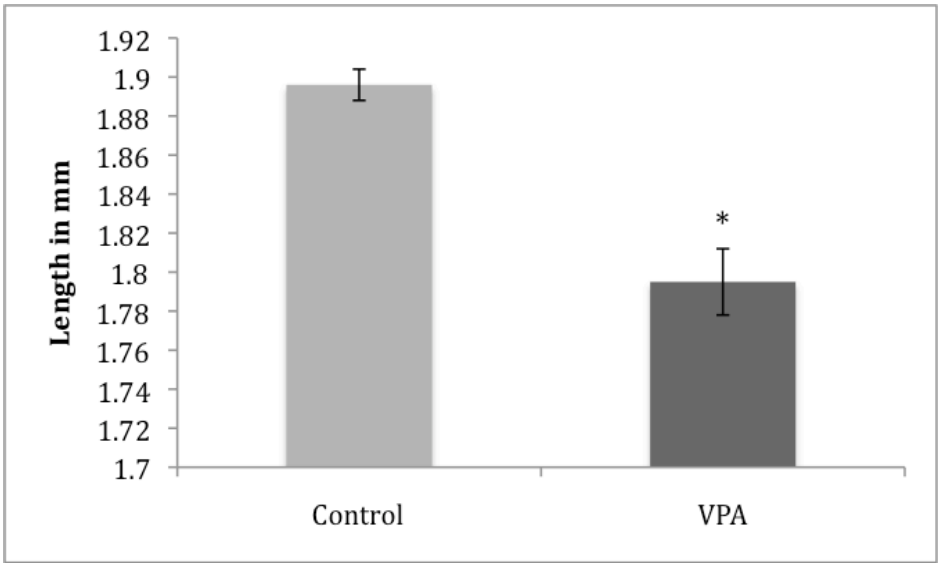

Figure 3.22. Total left hemisphere cortical average. VPA animals have significantly thinner left cortices (* = p<.05).

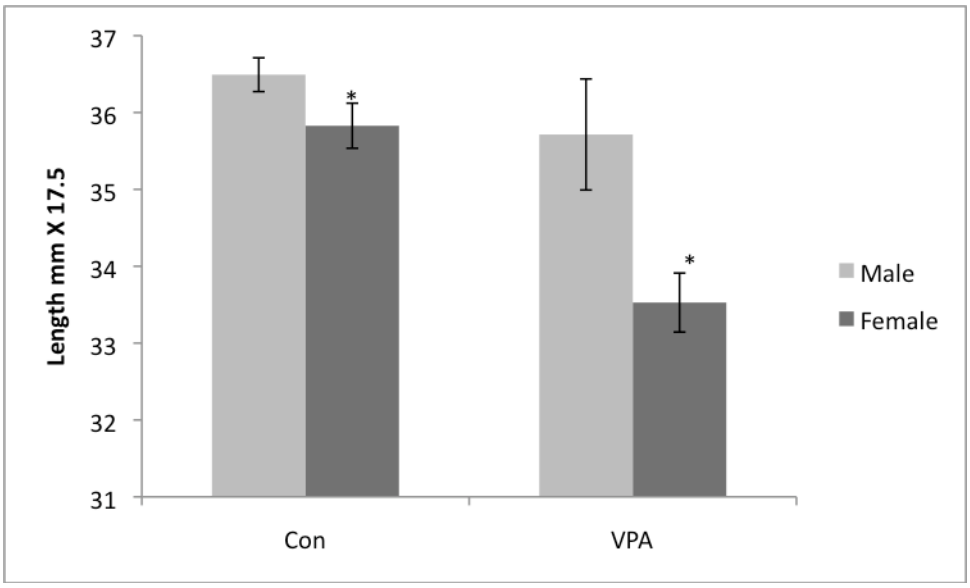

Figure 3.23. Cortical thickness on Plane 2. Females had significantly thinner cortices than males (*=p<.05).

Discussion

Overall, the thickness of the cortex is reduced by 5% in VPA animals. Plane 2 is the only area that showed an effect of sex. Females had thinner cortices at this plane than did males. This may reflect the presence of gonadal hormone receptors in the cortex in this particular area.

Thalamic Volume

Anterior Thalamus

Although there was no main effect of group on thalamic volume, there was significant effect of sex as females had a smaller thalamic volume [$F(1,36)=8.98, p<.05$] (figure 3.24). There was also a significant effect of treatment with TS animals showing a decrease in anterior thalamic volume [$F(1,36)=4.47, p=.04$].

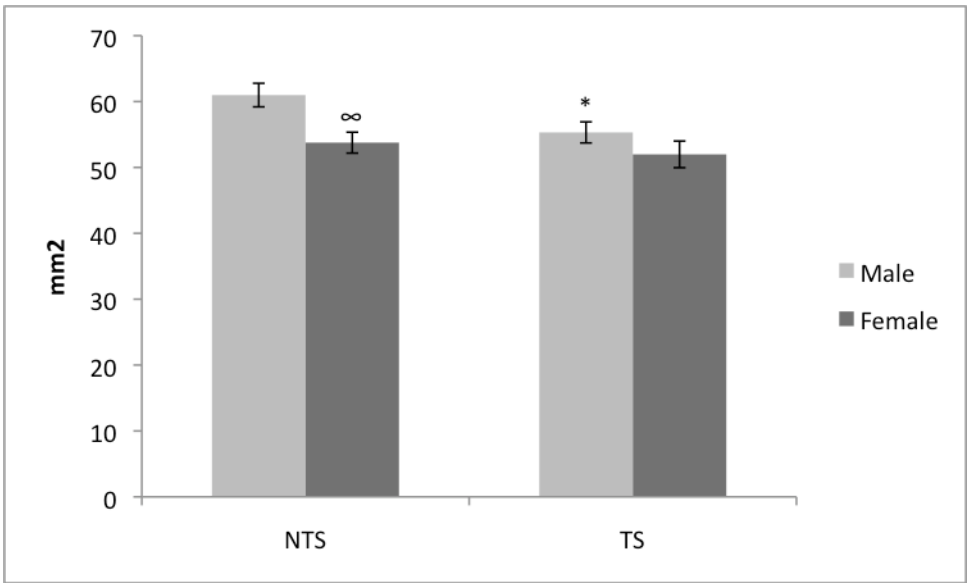

Figure 3.24. Anterior thalamic area showed a significant reduction following TS. Females had smaller anterior thalamic area than did males (*=p<.05)(∞=p<.05).

Posterior Thalamus

There was no main effect of group or treatment on posterior thalamic size (p's>0.38). There was a significant effect of sex on posterior thalamic size, however , [F(1,36)=6.445, p,0.05] (figure 3.25) with females having a smaller thalamic size than males.

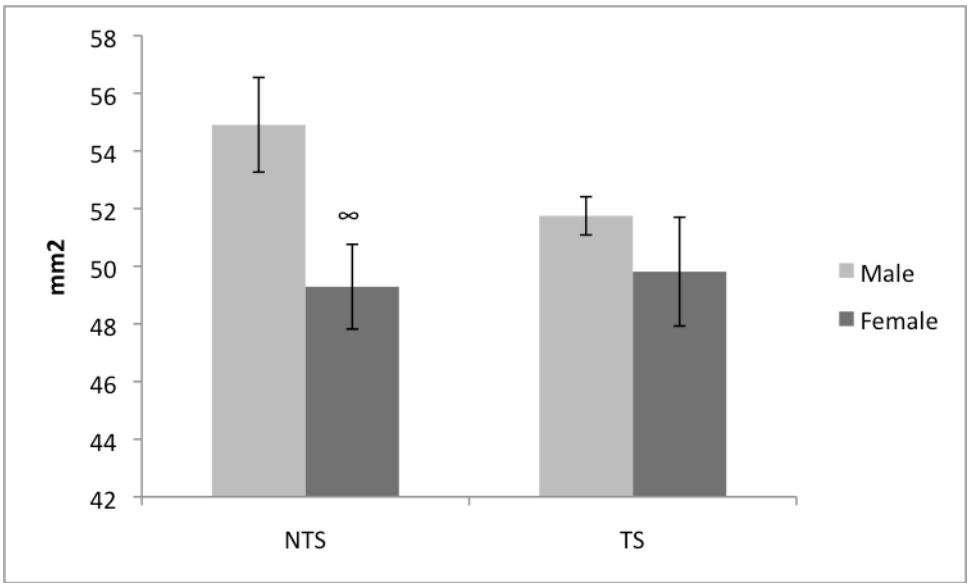

Figure 3.25. Posterior thalamic area. Females had smaller posterior thalamic area than did males (∞=p<.05)

Golgi Results

AID

Cells from AID were drawn and quantified in the basilar field only.

Branch Order

There was no main effect of group [F(1,178)=1.683,p=0.196] on cell complexity in AID but there was a highly significant effect of TS treatment on cells in this area [F(1, 178) = 30.163, p<0.0001]. The TS treated animals had more complex cells. There was no main effect of sex on branch order [F(1,178)=0.002, p=0.98]. There was a significant interaction of Group X Treatment [F(1,178)=8.98, p<0.005]. This reflected a greater influence of TS on VPA treated animals over controls. TS increased cell complexity to a higher degree in VPA animals than in controls. There

was also a significant interaction of Group X Treatment X Sex [F(1,178)=4.636, p<0.05]. VPA treated males showed the greatest response to tactile stimulation compared to all other groups (Figure 3.26).

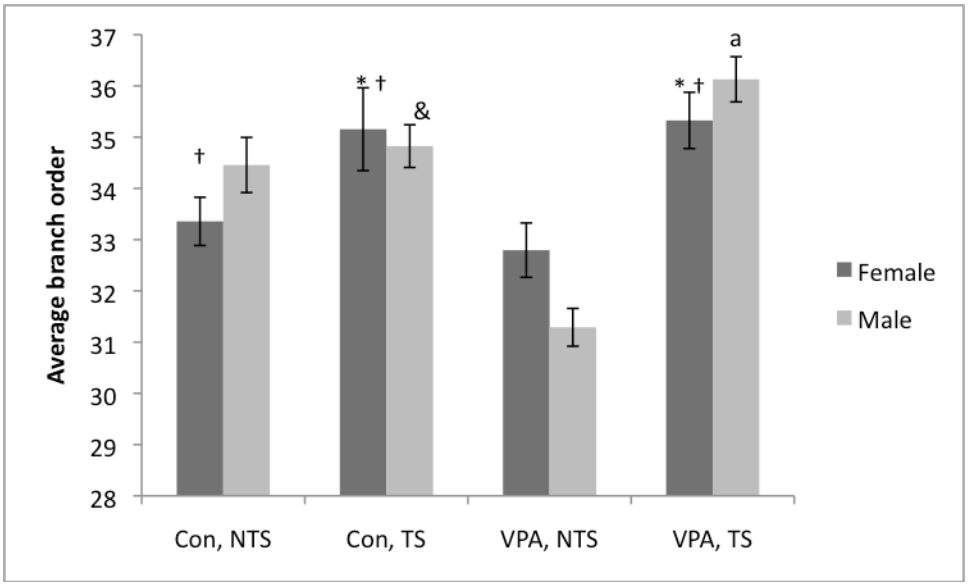

Figure 3.26. AID branch order. TS had a significant effect on cells in AID by increasing cell complexity. VPA treated animals responded more to TS than did controls and VPA males showed the greatest changes in anatomical organization following TS (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS) (∞=p<.05- Males compared to controls) (&=p<.05 Interaction for Group and Treatment) (a=p<.05 Interaction for Group X Treatment X Sex).

Sholl Analysis

Although the effect of group did not reach significance, there was a strong trend for VPA to reduce dendritic length on cells in AID [F(1,200)=3.405, p=0.0665]. There was a highly significant main effect of TS treatment on dendritic length [F(1,200)=96.056,p<0.0001]. TS increased dendritic length. There was also a highly

significant interaction of Group X Treatment [F(1,200)=23.71,p<0.0001] reflecting a greater enhancement in dendritic length on VPA exposed TS animals over controls (Figure 3.27).

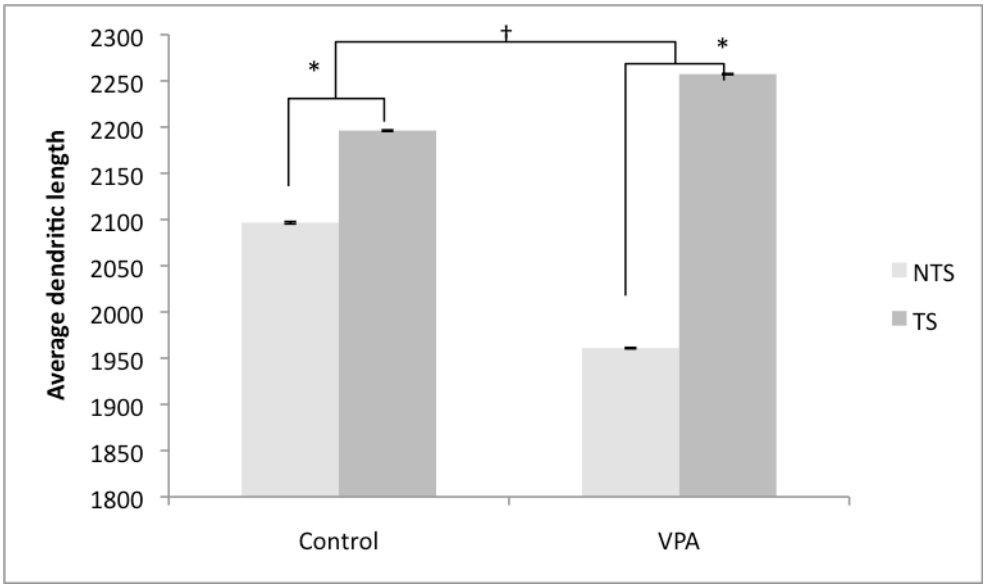

Figure 3.27. AID Sholl analysis. VPA showed a trend to reduce dendritic length in exposed animals. TS increased dendritic length in both control and VPA treated animals but the effect was larger for the VPA animals (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS) .

Spines

There was a main effect of group on spine density and this effect was a highly significant reduction in spines with exposure to VPA [F(1,182)=20.867,p<0.0001] Neither TS treatment [F(1,182)=0.004,p=0.9499] nor sex [F(1,182)=0.002,p=0.9632] showed main effects on spine density. Several interactions were significant in this analysis. The Group X Treatment interaction was highly significant [F(1,182)=25.442,p<0.0001] and reflected the finding that while TS increased spine

density in VPA animals, it decreased spine density in controls. The Group X Sex interaction was also significant [F(1,182)=11.20,p=0.001]. Males showed a greater reduction of spines than did females in response to VPA exposure. The Treatment X Sex interaction [F(1,182)=8.556, p<0.005] reflected the finding that females showed a greater loss of spines and males a greater increase in spines after TS. The Group X Treatment X Sex interaction was not significant [F(1,182)=0.1,p=0.75](Figure 3.28).

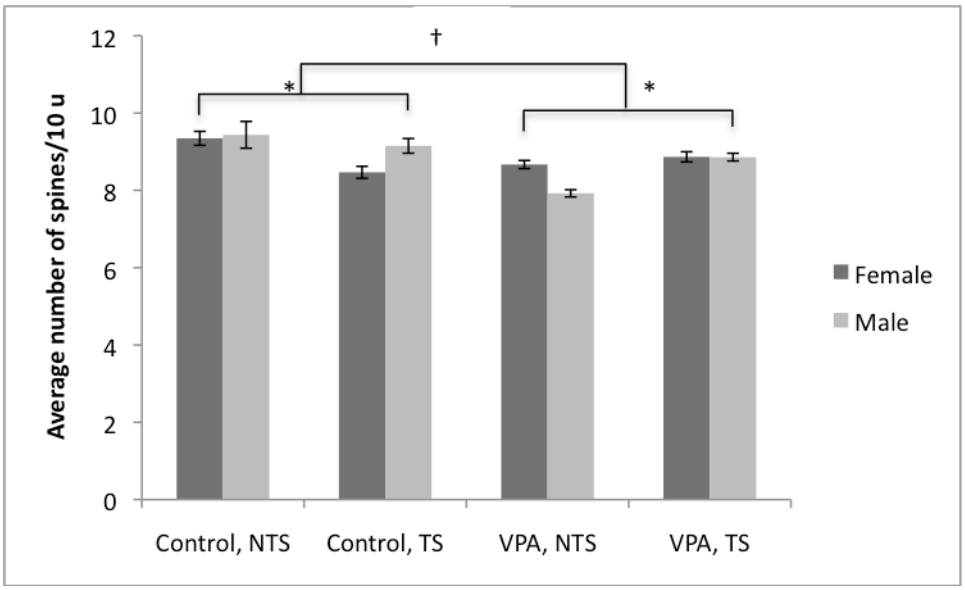

Figure 3.28. AID spines. VPA treatment resulted in a significant reduction in spine density in AID. VPA animals with TS increased synaptic contacts in contrast to controls with TS who showed a decrease in synaptic contact. Males were more sensitive to VPA exposure than were females (greater loss of spine density). TS increased spine density in males and decreased it in females (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

CG3

Cells in the CG3 area were drawn and analyzed in both the apical and basilar domains. The apical and basilar trees were analyzed separately for branch order, Sholl and spine density.

Apical Branch Order

There was a main effect of group [$F(1,125)=6.741$, $p=0.01$] on branch order. VPA exposure reduced cell complexity. Tactile stimulation had a highly significant effect on branch order [$F(1,125)=46.66$, $p<0.0001$]. Animals exposed to TS showed increased cell complexity. There was no main effect of sex [$F(1,125)=0.49$, $p=0.485$]. The Treatment X Sex interaction was significant [$F(1,125)=4.868$,$p<0.05$] and reflected the tendency for females to show greater cell complexity following TS than males. The Group X Treatment X Sex interaction was also significant [$F(1,125)=12.56$,$p=0.0006$]. The females in the control group showed a response to TS whereas the males did not. In the VPA group both males and females showed a benefit of TS (Figure 3.29).

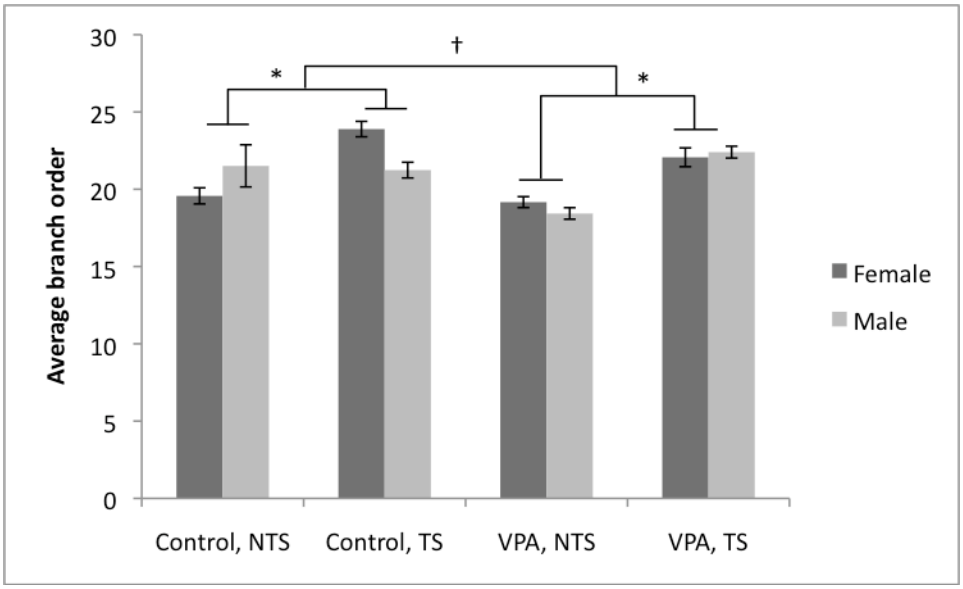

Figure 3.29. CG3 Apical Branch order. VPA exposure reduced cell complexity and treatment with TS increased it. Females showed a greater response to TS. The control males showed no benefit of TS but all other groups showed an increase in cell complexity in response to TS (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

Basilar Branch Order

In the basilar tree, there was no main effect of group [$F(1,128)=2.047, p=0.155$] but there was a highly significant effect of treatment [$F(1,128)=44.61, p<0.0001$]. TS increased cell complexity in the basilar tree. There was also a highly significant effect of side [$F(1,128)=17.667, p<0.0001$] as it was discovered that cells from the right hemisphere were more complex than cells from the left (Figure 3.30). None of the interactions were significant (F's<1, p's>0.4).

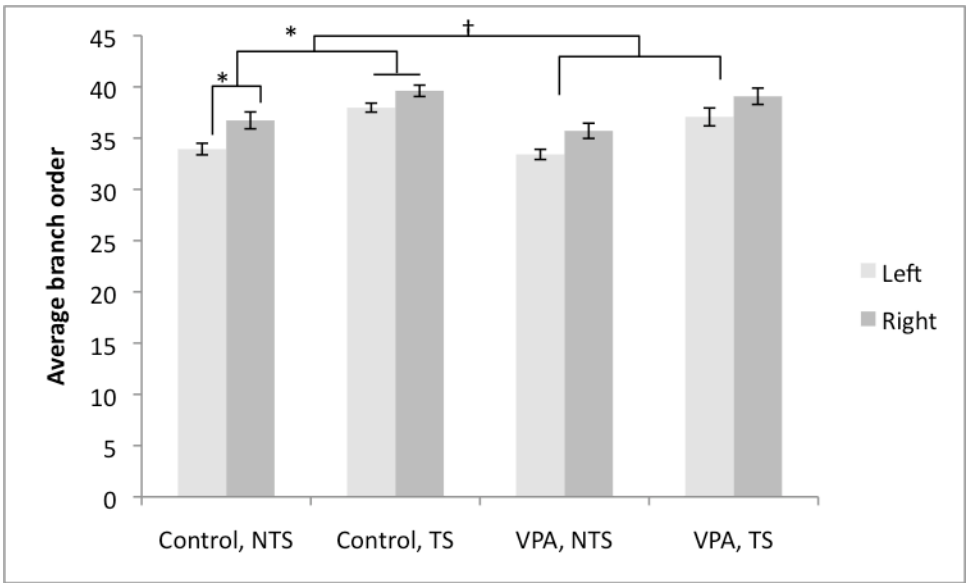

Figure 3.30. CG3 Basilar branch order. Tactile stimulation increased cell complexity in the basilar field. Cells drawn from the right were more complex than cells drawn from the left (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

Apical Sholl Analysis

There was a main effect of group on dendritic length in the apical field of cells in CG3 [$F(1,198)=6.613$, $p<0.05$]. VPA treatment reduced dendritic length as compared to control animals. There was also a highly significant main effect of TS treatment [$F(1,198)=149.7$, $p<0.0001$] on dendritic length. TS increased dendritic length. The Group X Treatment interaction was not significant [$F(1,198)=3.05$, $p>0.08$] (Figure 3.31).

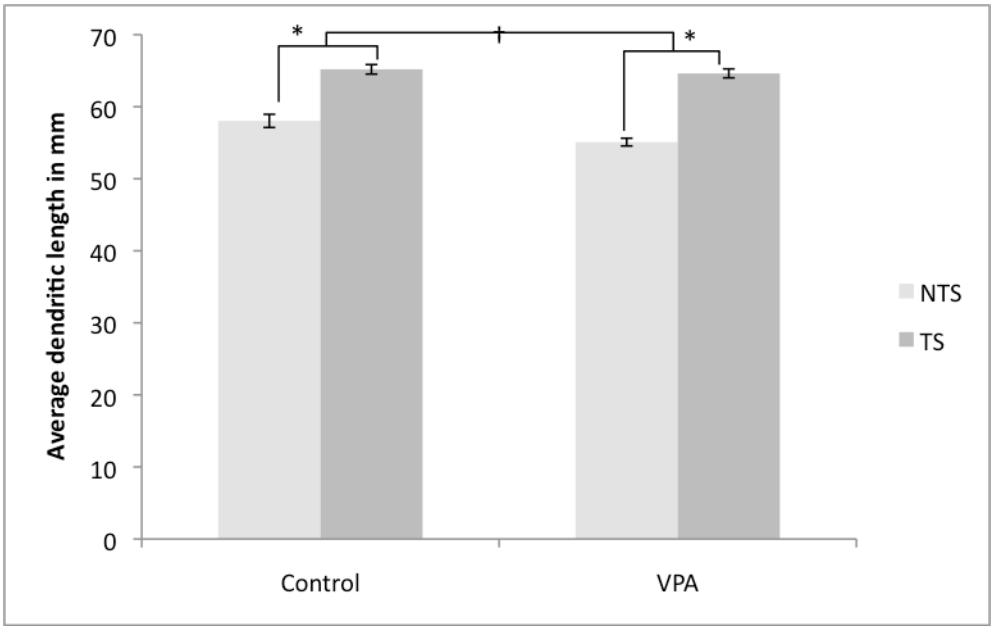

Figure 3.31. CG3 Apical sholl. VPA reduced dendritic length and TS increased it in the apical field of cells in area CG3 (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

Basilar Sholl Analysis

There was a main effect of group [$F(1,190)=7.942$, $p<0.01$], treatment [$F(1,190)=90.8$, $p<0.0001$] and side [$F(1,190)=20.904$, $p<0.0001$] on dendritic length in the basilar field of cells in CG3. VPA reduced dendritic length, TS increased dendritic length, and cells drawn from the right hemisphere had longer dendrites than those drawn from the left hemisphere (Figure 3.32). None of the interactions reached significance (F's<2, p's >0.16).

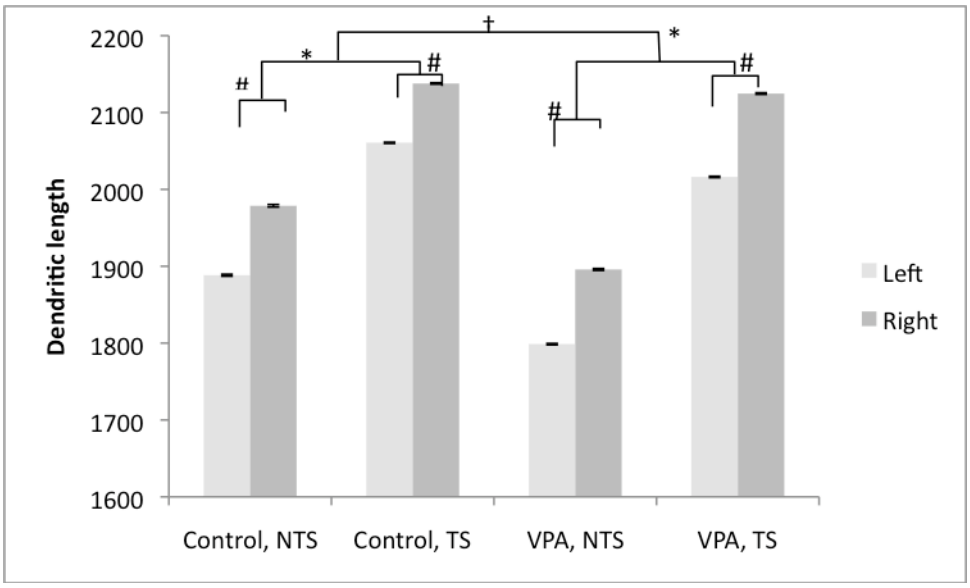

Figure 3.32. CG3 Basilar sholl. VPA reduced dendritic length. TS increased dendritic length. Cells drawn from the right hemisphere had longer dendrites than did cells drawn from the left hemisphere (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS) (#=p<.05- Left compared to Right).

Apical Spine Density

There was a main effect of group on spine density in the apical field [$F(1,196)=4.733$, $p<0.05$]. VPA reduced the density of spines in this area. There was a highly significant effect of TS treatment on spine density [$F(1,196)=53.302$, $p<0.0001$] with TS increasing the density of dendritic spines. The Group X Treatment interaction was significant [$F(1,196)=10.321$, $p<0.005$] and reflected the tendency for the VPA treated animals to show a higher response to TS than the controls. The Group X Sex interaction was also significant [$F(1,196)=5.822$, $p<0.05$] and reflected the finding that males were more sensitive to the VPA treatment than the females (Figure 3.33).

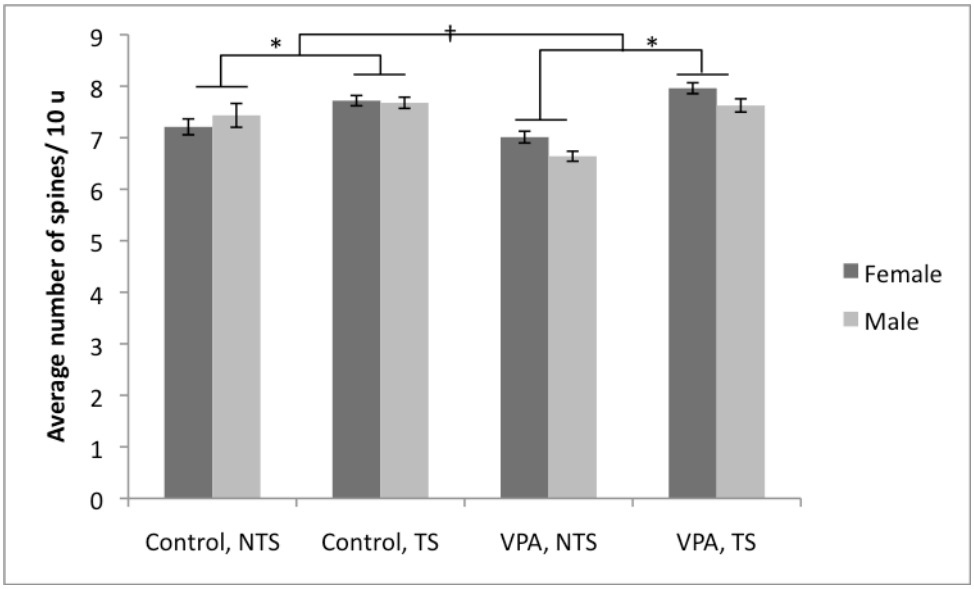

Figure 3.33. CG3 Apical spines. VPA exposure decreased spine density and TS increased it. The VPA treated animals were more sensitive to TS than the control animals. Males were more sensitive to VPA exposure than were the females (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

Basilar Spine Density

There was a main effect of group on spine density in the basilar field of CG3 [$F(1,196)=13.84$, $p<0.0005$]. VPA exposure reduced the density of spines in this area. There was also a main effect of treatment on spine density [$F(1,196)=106.083$, $p<0.0001$] with TS increasing the density of spines. There was a significant interaction of Group X Treatment [$F(1,196)=35.9$, $p<0.0001$]. VPA exposed animals were more sensitive to TS than controls. The Group X Sex interaction was also significant [$F(1,196)=6.591$, $p<0.05$] and reflected the finding that males were more sensitive to VPA treatment than were females. The Treatment X Sex interaction did

not reach significance [F(1,196)=3.276, p=0.072] but a trend was noted for females to benefit more from TS than males (Figure 3.34)

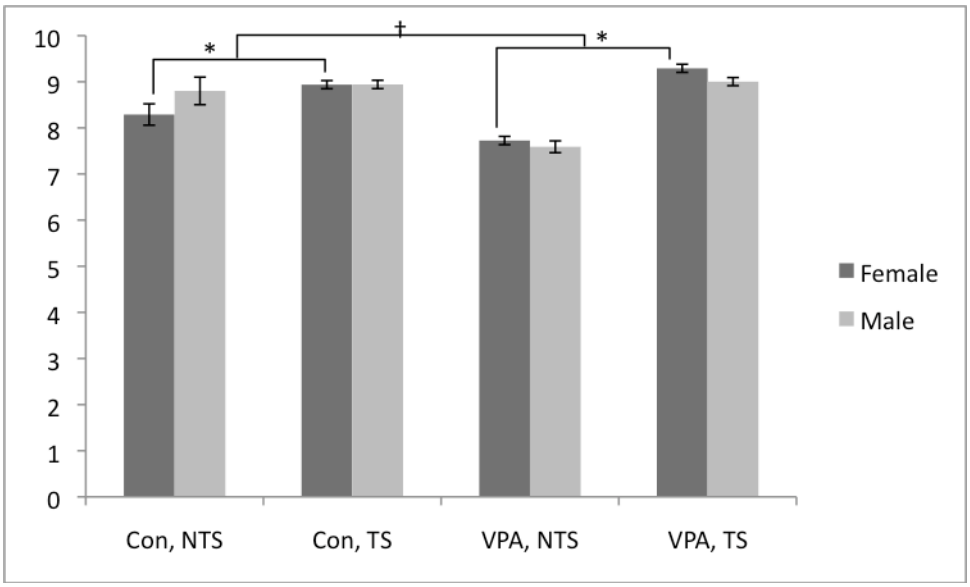

Figure 3.34. CG3 Basilar Spines. VPA reduced spine density and TS increased in the basilar field of CG3 cells. VPA treated animals were more sensitive to TS than controls and males were more sensitive to VPA exposure than were females. There was a tendency for females to show an enhanced response to TS over males (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

Amygdala

Spines

There was no main effect of group [F(1, 180)=0.942, p>0.33] or sex [F(1,180)=1.714, p>0.19] on spine density in amygdala but there was a main effect of TS treatment and it was highly significant [F(1,180)=109.17, p<0.0001]. TS increased the density of spines in amygdala. The Group X Sex interaction was also highly significant

[F(1,180)=16.35, p<0.0001] and reflected the finding that males showed a greater reduction in spine density in response to VPA exposure than did females. The Treatment X Sex interaction was also significant [F(1, 180)=6.562, p<0.05]. TS had a more robust affect on females than on males (Figure 3.35).

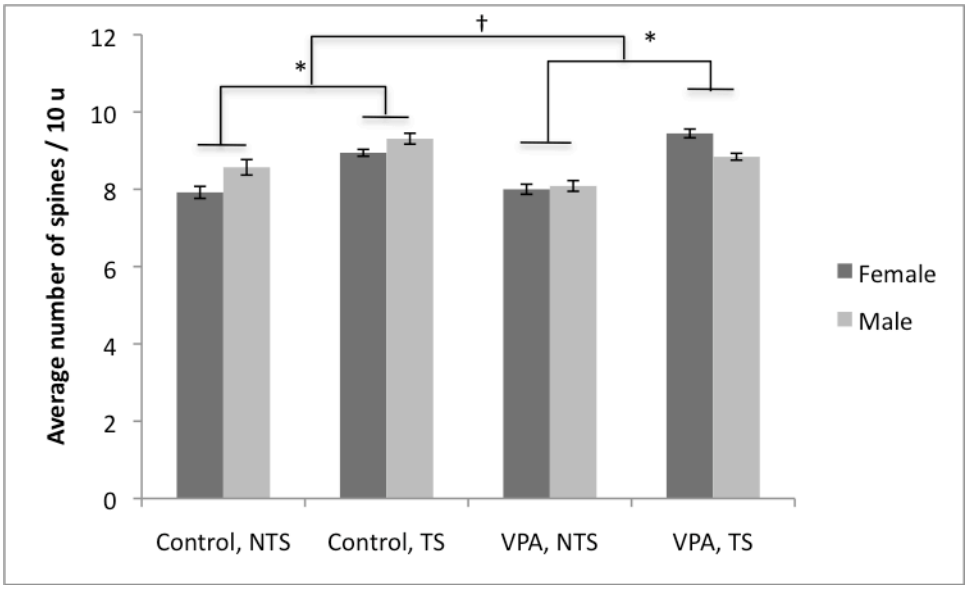

Figure 3.35. Amygdala Spines. TS increased spine density in the amygdala. VPA treatment affected the males more than females. Females showed a greater response to TS than did males (†=p<.05-VPA compared to controls) (*=p<.05 NTS compared to TS).

DISCUSSION

Overall the anatomical results show that the VPA decreases cell complexity, branch order and spine density in most areas. TS has the opposite effect in that it increases cell complexity, branch order and spine density in VPA animals. TS also increases these measures in control animals. Though it does this equally in both the mPFC and the OFC, which is contrary to previous studies that find an opposite effect. Studies

have shown that when there is an increase in volume in the mPFC there is a decrease in the OFC (Bell et al., 2010; Stigler, McDonald, Anand et al., 2011).

CHAPTER FOUR

General Discussion

Overall the VPA animals show abnormal behaviors in most behavioral tasks used in this study. In play behavior there were only significant results in juvenile play and not in adult play behavior. Juveniles VPA animals used evasion and other defense behaviors that included jumping, rearing and facing, significantly more than controls. Perhaps adult animals, who tend to play less in adulthood anyway, were more adapt at navigating the social behavior than at the juvenile state. Significant deficits in the Whishaw tray reaching and the T-maze were observed. VPA animals also showed increased movements in the activity box and open arm in the elevated plus maze. VPA animals varied from controls in the novel object recognition task in that they touched the new object more than controls. There were also significant sex effects, with females more active in the activity box and the elevated plus maze. An interesting finding is that while TS had no main effects on behavior, it was often seen as remediating in female animals.

Anatomically, VPA animals had smaller brains. Smaller brain weights were also reported in other VPA animal studies (Schneider, Roman, Basta-Kaim, 2008). Cortical thickness was decreased on average by 5% in VPA animals.

Thalamic area was unaffected by VPA but tactile stimulation reduced thalamic size. This finding was unexpected. Thalamic area was not examined after TS in previous studies done in the lab, but we would have predicted an increase rather than a decrease in thalamic area after this treatment. How reduced thalamic size contributes

to brain function will require further investigation. VPA's widespread effect on cortex seems to be limited to that structure. The volume of subcortical structures such as thalamus and amygdala appeared to be unaffected by exposure to VPA

The results of the Golgi analyses were surprising. VPA administration had a consistent negative effect on cell morphology in both PFC areas studied but no effect of VPA was seen in amygdala. Males were more sensitive to the negative effects of VPA than were the females. Although TS had limited behavioral effects, the anatomical effects were impressive. In every area examined TS had a positive anatomical effect (increased complexity, dendritic length, and synaptic contact.

Analysis	**VPA**	**TS**
AID-Complexity	=	↑↑
-Dendritic Length	↓	↑↑
-Spines	↓↓	=
CG3 -Apical Complexity	↓	↑↑
-Basilar Complexity	=	↑↑
-Apical Dendritic Length	↓	↑↑
-Basilar Dendritic Length	↓↓	↑↑
-Apical Spines	↓	↑↑
-Basilar Spines	↓↓	↑↑
Amygdala Spines	=	↑↑

Table 4.1 Summary of the main effects of group and treatment on cell morphology in PFC and amygdala.

Expectations were that VPA would create similar behavioral and anatomical tendencies in an animal model as in humans with ASD. This study was a thorough examination of the VPA model of autism including behavioral and anatomical components. Deficits were found in many of the behavioral tasks and decreases in brain weights and cortical thickness are similar to findings in human ASD research. TS was expected to remediate the behavioral and anatomical abnormalities as it has

been shown to do in past research (Gibb, Gonzalez, Wegenast et al., 2010; Kolb et al., 2010). In previous research by Kolb and Gibb (2010) TS was shown to be effective in remediating prefrontal lesions in postnatal day 3 rat pups. However, TS did not have a strong remediating effect on behavioral tendencies of the VPA animals, although there was a trend for females to benefit more from this experience than the males. Male pups receive more licking and grooming from their mothers (to stimulate urination) than do their female siblings and this may account for why females show an enhanced response to TS over the males. VPA also seems to have had a more pronounced effect on the male pups (as seen in the anatomical findings) and this may account for why TS failed to remediate their behavior. Research has also shown an increase in basic fibroblast growth factor (FGF-2) expression in skin when TS was performed (Gibb et al., 2010). Perhaps FGF-2 expression is reduced in the VPA animals thus preventing the effects of TS. Although TS showed marginal effectiveness at remediating VPA induced behaviors in rats, humans seem to respond very well to TS. Studies by Field et al. (1997) have shown massage to have beneficial behavioral effects on children with ASD however. TS reduced repetitive behavior, improved sleep and increased attentiveness in the classroom. ASD individuals are at increased risk of developing other psychological disorders such as depression and anxiety (Davis, Fodstad, Jenkins et al. 2010). Davis et al. (2010) found that infants and toddlers at risk or diagnosed with ASD had greater anxiety levels than controls. Massage is a reliable method of reducing stress and anxiety as it has been found to reduce cortisol levels in children and infants (Field, Morrow, Valdeon et al., 1992; Field, 2010). Escalona (2001) has shown that massage aids ASD

children in improving sleep, increased social attentiveness and decreased stereotypic behaviors. With daily massage interactions between caregiver and infant may increase joint attention, communication and engagement that may help remediate some of the characteristic symptoms of ASD. These aforementioned behaviors have been found to be decreased in infant siblings of ASD individuals (Elsabbagh, Holmboe, Gliga et al., 2011; Osterling, Dawson, & Munson, 2002).

Infants who where later diagnosed with autism showed aversion to social touch (Osterling et al., 2002). Infant massage may be a method of intervention that would give touch in a predicable manner to children. Infant siblings were found to have differing social attention behaviors than controls. These behaviors included unusual eye contact, lack of orientation to name and reduced changes in attention redirection (Elsabbagh et al., 2011; Zwaigenbaum, Bryson, Rogers et al., 2005). Massage may be an early intervention method that encourages joint attention and communication with caregivers. With the increased use of fMRI in autism studies, future research to view anatomical changes that may occur due to TS or massage may be a next step in human research.

One theory proposes that ASD individuals are slower in processing the world around them. If speech and other sensory cues were slowed down the ASD individual would have time to process the information more accurately and thus respond more appropriately (Gepner & Feron, 2009). Programs such as Fast For word produced with the help of Michael Merzenich have produced improvements in speech and language skills after the implementation of this computer program. A limitation of this approach is the prohibitive cost of the program. Massage is often done when the

setting is calmer and speech, touch and contact are slowed, encouraging face-to-face interactions. The predictable touch of the massage routine is slow and methodical; this encourages slowing sensory processing (Field, 2001).

It is interesting that the TS had a large effect of the prefrontal pyramidal neurons, especially in the VPA animals, but failed to significantly reverse the behavioral deficits. This may be due to the testing measures that were used. Perhaps they were not sensitive enough to detect the behavioral effects of TS in the VPA animals. Selecting tests that are more dependent on the PFC, such as working memory may be a more accurate. Another reason may be the effect of VPA on neurons in other cerebral regions, including the neocortex or hippocampus, which were not affected by TS.

Future Studies

The VPA animal model of autism appears to be a promising avenue for future behavioral and anatomical research. Future directions would be to look at the impact of prenatal TS and on pup behavior and brain anatomy. Comeau et al. (2007) has shown that prenatal treatment of FGF-2 improved later recovery of postnatal day 3 mPFC lesions in rats. FGF-2 injections to VPA rat pups instead of TS could be a follow up to this research. Another aspect would be to repeat the postnatal TS but measure the FGF-2 in skin samples after weaning. Vocalizations of pups during maternal care and later during social play would be helpful additions to the compilation of research. This approach would ascertain if communication of VPA animals vary from controls. This is a significant deficit in ASD individuals.

Anatomical observations of the cerebellum would be another area of expansion as it is consistently mentioned in research to be an area of concern with ASD. Another direction would be the impact of exercise on VPA animals, with the use of a running wheel. Research by Lang et al. (2010) has found that exercise has beneficial effects for ASD individuals. These benefits include increase attention, on-task behavior and decreased repetitive behaviors, and aggression.

With respect to the first question, how does prenatal exposure to VPA affect animal behavior and brain anatomy? We found that overall both behavior and anatomy were impaired in prenatally exposed VPA animals. The behavioral impairments we identified were: 1) an increase in the use of evasion and other (jumping, rearing, and facing) defensive behaviors in the play behavior task, 2) reaching exhibited an decrease in successful hits on the Whishaw tray reaching task, 3) VPA animals were more active than controls in the activity box, 4) VPA animals took longer to reach criterion and made more errors in the T-maze, 5) VPA animals touched the newer object more often than control animals on the novel object recognition task, and 6) in the elevated plus maze, VPA animals spent more time in the open arms exhibiting less anxiety. Anatomically, we saw a general decrease in connectivity in the OFC, mPFC, and amygdala induced by prenatal VPA exposure.

The second question asked if TS would remediate the effects of prenatal VPA exposure. TS did remediate the effects of prenatal VPA exposure and increased overall synaptic contact. TS did not remediate any of the behavioral effects, however its impact on anatomy was significant. TS generally increased branch order, sholl analysis and spine density in the OFC, mPFC and amygdala, whereas VPA decreased

these measurements. From this conclusion the use of infant massage may be a first step intervention for those infants that may be a risk for developing autism.

The above findings add to the previous research indicating prenatal exposure to VPA is a reasonable animal model of autism. Prenatal exposure to VPA created behavioral and anatomical deficits in areas similar to those affected in humans with ASD. It adds to the already robust research indicating these same results.

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APPENDIX

Female	VPA	NTS	23
		TS	23
	Control	NTS	16
		TS	21
Male	VPA	NTS	17
		TS	19
	Control	NTS	14
		TS	18

Table 4.2. 12 litters were used for the behavioural studies. Animal groups showing division of litters and numbers within each grouping for behavioural testing.