AN ANIMAL MODEL OF AUTISM USING GSTM1 KNOCKOUT MICE AND EARLY POST-NATAL VPA-TREATMENT

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A Dissertation submitted to the Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Psychology

Written under the direction of

George C. Wagner

and approved by

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New Brunswick, New Jersey

October, 2009

ABSTRACT OF THE DISSERTATION

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Autism is a behaviorally defined developmental disorder with unknown origin. However, its etiology most likely involves a gene by toxicant by age of exposure interaction. To test this hypothesis, mouse pups with a deletion of glutathione-S-transferase M1 (a gene associated with increased risk of autism and that codes for an enzyme involved in the management of toxicant-induced oxidative stress) and wild-type controls were exposed to valproic acid (a toxicant known to cause autism-like behavior deficits following prenatal exposure and one that exerts its toxic action, in part, by inducing oxidative stress) on post-natal day 14. It was observed that VPA-treatment resulted in significant increases in the number of cells staining for TUNEL in the hippocampus and cerebellum. There was also a gene by treatment by sex interaction with VPA- treated wild-type females having increased protection against VPA-induced cell death. VPA- treatment also resulted in long-lasting deficits in social behaviors and corresponding changes in brain chemistry. Collectively, these data expand our current animal model of autism by adding a genetic component in the form of an autism susceptibility gene. In addition, these results support

the hypothesis that autism may be the result of a gene by toxicant interaction wherein both factors share a common feature of oxidative stress.

Acknowledgments

I would like to thank the members of my committee, Dr. Alexander Kusnecov, Dr. Oleg Mirochnitchenko, and Dr. Timothy Otto for their supervision and expertise throughout the completion of my dissertation. I would like to thank my parents Doreen and George Yochum for their continued love, guidance, and support throughout my life. I would like to thank my husband, Mihajlo Krsmanovic, for his understanding and love throughout my entire college and graduate career. I would like to thank the past and present members of the Wagner lab. Most importantly, I would like to thank my advisor Dr. George Wagner who has been a wonderful mentor and without whom, this dissertation would not be possible.

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INTRODUCTION

Autism Epidemiology

Autism is a neurobiological disorder involving a wide range of symptoms invariably including impaired communication, impaired social interaction, and abnormal motor behaviors. Autism typically appears before three years of age with many parents noticing abnormal development as early as one year. However, in cases of autistic regression, parents report children as having normal development up to about the age of three, but these developmental achievements are lost thereafter (Tuchman and Rapin, 1997). A recent study conducted by the Center for Disease Control combined two surveys that suggest that, in 2003-04, at least 300,000 school-aged children in the United States were diagnosed with autism spectrum disorders (ASDs). The National Health Interview Survey reported that 5.7 per 1,000 school-aged children were diagnosed with autism and the National Survey of Children's Health reported a marginally lower rate of 5.5 per 1,000 school-aged children (Schieve et al., 2006). In both surveys, prevalence was 3.7 times higher in males than in females. Fombonne's 2003 review of autism epidemiological surveys found that surveys conducted prior to 1991 reported median prevalence rates of 4.4/10,000 and surveys conducted between the years 1992-2001 reported median prevalence rates of 12.7/10,000. The correlation between prevalence rate and year of publication was statistically significant and points to an increase in prevalence estimates over the last 15 years (Fombonne, 2003). There are several possibilities why the autism rates have increased over the last several years. One possible explanation for the increase in autism prevalence is the recent change in autism diagnosis criteria. Given the high

number of autistic individuals who are co-morbidly diagnosed with other psychiatric disorders, such as mental retardation and obsessive compulsive disorder, it is possible that these new diagnostic tools enhance detection of autism in instances that might have been overlooked because of other conditions (Ming et al., 2007).

The first description of autism was in Kanner ‘s (1943) article where he described autism as an “…innate inability to form affective contact with people in the ordinary way to which the human species is biologically disposed.” Today, autism diagnosis requires patients to have several symptoms from each of three core symptom categories including: imparement in social interaction, imparment in communication, and restrictive or repetitive behaviors (DSM-IV-TR). Impairment in social interaction involves an inability for the child to maintain eye-to-eye gaze and a lack of desire to share enjoyment, joint-attention, and emotional reciprocity. Communication impairments include language deficits, and repetitive and inappropriate use of words. Finally, restrictive and repetitive behaviors include s inflexible adherence to routines and rituals, stereotypic and self-injurious behavior. The onset of these symptoms must occur prior to the age of three and cannot be accounted for by either Rett’s disorder or childhood disintegrative disorder (DSM-IV-TR).

It is important to recognize that the new criteria for diagnosing autism allows for a much broader phenotype to be categorized as autism spectrum disorder. This inclusion of a broader phenotype results in increased variation in symptoms across individuals diagnosed with autism spectrum disorder. One result of the expanded diagnostic criteria is reflected in the DSM-IV’s subtyping autism into Asperger’s

disorder, pervasive developmental disorder, and childhood disintegrative disorder. Importantly, identification of subtypes of autism may prove important in determining contributing etiologic factors. The subtyping of autism may be achieved by identifying patients who share certain phenotypes. For example, subtypes may include individuals who display more self injurious behavior, individuals who have very severe language problems, or any other combination of phenotypes on the autism spectrum. An alternative way to subtype autism would be by identifying different pathologies associated with different autistic phenotypes.

Pathology of Autism:

The first reported neuropathology of autism stated that several patients had unusually large head circumference (Kanner 1943). However, partially due to the small number of brain samples, the exact neuropathology accounting for autism symptoms has remained elusive. Bauman and Kemper (1985, 1998, 2005) identify three major neuroanatomical differences between autism and control brains: 1) changes in the normal development of the forebrain limbic system; 2) decreases in the number of Purkinje cells; and 3) age-related changes in the number and size of neurons in the diagonal band of Broca, cerebellar nuclei, and inferior olive. Of importance, the observation of a reduction in Purkinje cell number has been replicated in animal models of autism in which treated subjects manifest neurobehavioral deficits akin to those observed in autism (Ingram et al., 2000; Sobaniec-Lotowska, 2001). In mice the behavioral deficits are apparent in tasks measuring mid-air righting, negative-geotaxis, wire grip strength, Morris water maze

execution, and social behavior (Wagner et al., 2006; Yochum et al., 2008). Collectively, these studies suggest that permanent alterations in cerebellar Purkinje and granule cell density are found in adults with autism. Unfortunately, postmortem studies often suffer from lack of proper controls for symptom severity, mental retardation, long-term medication, and long-term institutionalization. Therefore, the findings from some of the human postmortem studies may represent neuroanatomical abnormalities resulting from other developmental disorders, medical treatments, or institutionalization. The limitations of these human studies highlight the importance of animal model studies for understanding human disorders.

Since the early 19th century the cerebellum has been associated with motor movement because cerebellar trauma resulted in the inability of subjects to plan and perform complex motor tasks (Fine et al., 2002; Fisher et al., 2006). Given that many autistic individuals often have difficulty performing fine and complex motor tasks, it makes sense that cerebellar dysfunction would be involved in the motor portion of the autism behavioral spectrum. A recent fMRI study found that autistic individuals had decreased cerebellar activation when asked to perform motor tasks as compared to their healthy sibling controls (Mostofsky et al., 2009). Motofsky et al. hypothesize that this decreased activation in autistic individuals may indicate poor coordination within the circuitry necessary to perform motor behavior. However, while this study indicates that the cerebellum may be involved in motor tasks, it does not account for the social and communication portion of the autism behavioral spectrum.

Recent mapping of cerebellar activity has determined that the cerebellum can be mapped into two different zones, the primary sensory motor zone and the

supramodal zone (O'Reilly et al., 2009). The sensory motor zone links to motor, somatosensory, visual and auditory cortices, while the supramodal projects to prefrontal and posterior-parietal cortex (O'Reilly et al., 2009). It is this second zone which may explain how the cerebellum is involved in cognitive, social and verbal activity. A human case study of an individual with neurological damage consisting of only cerebellar lesions had motor, visual, as well as cognitive, linguistic and behavioral problems (Marien et al., 2009). This case study indicates that proper cerebellar function is not only necessary for motor behavior, but for cognitive, social and communicative behavior as well.

Human magnetic resonance imaging (MRI) studies have demonstrated an increase in cerebral gray and white matter in autistic children compared to normally developing children (Courchesne et al., 2001; Sparks et al., 2002; Aylward et al., 2002; Hazlet et al., 2005). The magnitude of these increases is reduced between the ages of 5 and 12 depending on the study sample (Courchesne et al., 2001; Alyward et al., 2002). Recent fMRI studies attempted to associate differences in cerebellar vermis volumes and severity of social and communication deficits in autistic individuals (Webb et al., 2009; Mitchell et al. 2009). Mitchell et al. (2009) found that alterations in cerebellar volume were related to autistic phenotype severity, while Webb et al. (2009) found no specific relationship between cerebellar volume and social symptom severity or verbal IQ. However, it may not be necessary to have cerebellar volume changes for cerebellar aberrations to be involved in producing autistic behavioral symptoms. The other possibility is that there are connectivity, or activation differences which cause the social and communication differences, similar

to what is seen in the influence of the cerebellum on autistic motor deficits (Mostofsky et al., 2009). For example, fMRI studies show that attention-related cerebellar activation was lower in patients with autism and that this alteration in attention was enough to cause difficulty in performing motor tasks (Allen et al., 2004). Although the task was motor, it was not a motor deficit which made the task difficult for the autistic individuals; rather, it was their ability to attend to the directions. The combination of these results shows that both decreased cerebellar volume and altered cerebellar activity are associated with a reduction in cerebellar function. Therefore, regardless of whether or not Purkinje cells are significantly altered in size or number, the cerebellum appears to play an important role in the cognitive processing differences seen in autistic individuals.

However, when other brain regions are compared, such as the amygdala and the hippocampus, results are less conclusive. Howard et al. (2000) showed an increase in amygdala volume whereas Aylward et al., (1999) showed decreases. Hippocampal studies are just as confusing; with Saitoh et al. (1995) and Piven et al. (1998) reporting no differences in volume and Alyward et al. (1999) showing decreases. One potential reason for these conflicting results is that each of these studies used a different population of autistic subjects. Differences in mental capacity, obsessive behavior, and self-injurious behavior could all be linked to altered hippocampal or amygdala size and could account for the conflicting results of these studies. In addition, it may not be necessary to have brain region volume changes in order to produce autism phenotypes, it may just require alteration in neuronal connectivity.

Etiology of Autism:

Even though the exact etiology of autism remains unknown, several studies suggest that an interaction between environmental insults, which produce reactive oxygen species, and genetic factors may result in neuronal damage which could manifest as autism spectrum disorder (Rodier and Hyman, 1998; Kern and Jones, 2006). Human studies indicate that children with autism have higher than normal body burden of toxicants (Edelson and Cantor, 1998; Edelson and Cantor, 2000). Although the environmental stressors that have been considered in these studies are typically restricted to environmental toxicants, there are several other environmental conditions to consider that increase reactive oxygen species and contribute to neuronal damage. One environmental insult which could lead to oxidative stress is prenatal drug exposure. Drugs such as thalidomide, valproic acid, methamphetamine, and cocaine have been linked to cognitive and behavioral developmental deficits in both human subjects and animal models (Koch et al., 1996; Moore et al., 2000; Williams et al., 2001; Forrester and Merz, 2007; Morrow et al., 2007). Another environmental insult that could lead to oxidative stress is maternal immune challenge. Shi et al. (2003) demonstrated that rodent maternal immune challenge results in offspring that have abnormal behavioral responses similar to those seen in autism disorder and schizophrenia (including decreased levels of exploration and social behavior).

However, individuals are regularly exposed to illness, toxicants, and maternal immune response and these immune challenges do not manifest as autistic disorder.

Therefore, it is likely that other factors in addition to environmental insult are required for the manifestation of autism. Several genetic mutations have been implicated in increasing an individual's likelihood of developing autism (Risch et al., 1999; Pickles et al., 1995; Muhle et al., 2004). A working hypothesis is that autism is the result of early environmental insult acting upon a developing nervous system that is particularly sensitive to said insult because of genetic alterations. Autism is challenging to study and treat because these genes cover a wide range of function, ranging from cerebellar lobe formation to oxidative stress regulation.

Reactive Oxygen Species:

Reactive oxygen species contain an unpaired electron and are produced through a variety of physiologic and pathologic processes (Zoroglu et al., 2004). The brain is particularly vulnerable to reactive oxygen species and lipid peroxidation because it has a high concentration of polyunsaturated fatty acids. Neuronal oxidative stress can result in both necrosis and apoptosis when the normal balance between oxidative events and antioxidant defenses is disrupted (Bains and Shaw, 1997). Neurons contain three important antioxidant enzymes: glutathione peroxidase, catalase, and superoxide dismutases. In addition, the extracellular fluid contains antioxidant free radical scavengers and lipid peroxidation inhibitors (Oury et al., 1992). Glutathione acts as a free radical scavenger, a redox modulator of ionotropic receptor activity, and a neurotransmitter in the nervous system. When there is a localized decline in glutathione, an increase of oxidative stress occurs which can then increase the levels of excitotoxic molecules (Brains and Shaw, 1997). It is the

increase in excitotoxic molecules that leads to apoptotic and necrotic neural cell death. While oxidative stress has long been thought to be involved in neurodegenerative disorders such as Parkinson's disease, recent studies are now linking oxidative stress to neurodevelopmental diseases such as autism.

Early exposure to oxidative stress may induce neural damage that becomes functionally apparent long after the organism has experienced the insult in the form of long-latency delayed neurotoxicity (Reuhl 1991). While this theory has traditionally been used to explain neurodegerative diseases such as Parkinson's disease, the same etiology may help to explain the neuronal differences seen in autism. What differs between these two disease could be 1) time of exposure (early for autism and late for Parkinson's disease), 2) susceptibility genes, 3) and type of environmental insult i.e, pesticides versus plasticizers (Yochum and Wagner, in press). Any early developmental insult might affect either neuroanatomy and/or function but produce no immediately obvious ill-effects because of the ability of the developing brain to compensate (Rice and Barone, 2000). However, as the brain continues to mature, there is a natural reduction in brain cell number; thus, over the entire life span there may be a point after which the brain is no longer able to compensate and functional deficits become obvious. As examples, developmental exposure to methylmercury, x-ray irradiation, and triethyltin have all been shown to produce delayed neurotoxicity in both animals and humans (Rice and Barone, 2000).

Genetic Variation in Autism:

In a strict interpretation of autistic disorder, the monozygotic twin concordance rate is 65% whereas dizygotic twin concordance rate is between 6 to 8% and is similar to the rate of subsequently born siblings (Bailey, 1995). However, when milder social and cognitive deficits are combined, monozygotic concordance rates increase to 82% and the dizygotic rates increase to 10% (Folstein, 2001). Fifteen to twenty different genes have been implicated in autistic disorder. When each gene is examined individually, none account for a large enough portion of the autistic population. In order to account for the high genetic load of autistic disorder, seen in the high concordance rates, it has been hypothesized that several gene alterations must combine in an individual to result in autism. These genes code for proteins, the function of which is often identified and linked to brain neurotransmitters. Of particular importance is the serotonin system.

Genes Involved in Serotonin Levels:

Up to one third of all individuals with autism have hyperserotonimia and this increase in serotonin levels is up to 50% higher than that found in healthy individuals (McBride et al., 1998). Autistic hyperserotonimia is unusual because it occurs only in blood plasma (platelet) levels and not in the brain (cerebral-spinal fluid) (Ritvo et al., 1970; McBride et al., 1998; Lam et al., 2006). However, many autistic individuals have several of their symptoms decreased as the result of taking selective serotonin re-uptake inhibitors (SSRI's). The fact that SSRI's ameliorate autism symptoms indicates that there is a decrease in 5-HT levels or activity in the brain because SSRI's increase 5-HT levels in the synapse. In order to explain this contradiction,

Whitaker-Azamita (2005) suggested that, during early development, the blood brain barrier is only partially formed and has several places where neurotransmitters could pass through. The high levels of platelet serotonin could allow for some to enter the developing fetal brain and cause a reduction in the number of serotonin terminals through negative feedback mediated by the 5-HT1A receptor.

There are several genes which regulate serotonin levels and two of them have also been implicated in autism both separately and in the form of a gene by gene interaction (Cross et al., 2008; Ma et al., 2009). The first is SLC6A4 which encodes for the serotonin transporter which is the target for SSRI's (Shen et al., 2000). Sugie et al. (2005) examined children with autism who possessed two of either the long or short alleles of SLC6A4 to see if they responded differently to treatment with SSRI's and found that children with the long allele responded significantly better to SSRI treatment than the short allele children. In addition they found that the SLC6A4 gene variation accounted for a small percentage of overall serotonin levels in the children. The short allele for SLC6A4 has also been associated with significantly larger amounts of gray matter in all areas of the brain except for the cerebellum (Wassink et al., 2007). This finding is interesting because one of the most commonly cited neuroanatomical abnormalities in post-mortem autism studies is macrocephaly in all areas of the brain except for the cerebellum. In addition, SLC6A4 is highly expressed in all areas of the brain except for the cerebellum.

Countinho et al. (2007) also tested to see if SLC6A4 interacted with any other genes that have been implicated in autism. They found a two-way interaction between SLC6A4 and ITGB3. ITGB3 is a second gene that has been shown to regulate

serotonin (Weiss et al., 2006). ITGB3 is a β3-integrin gene which is involved in whole-blood serotonin levels in males more than in females (Weiss et al., 2005). Therefore, ITGB3 makes a good susceptibility gene target for autism because of the sex difference seen in autism with males being four times more likely to be diagnosed than females. The two-way interaction between SLC6A4 and ITGB3 accounted for the serotonin levels in autistic subjects with 67% accuracy and reinforces the hypothesis that autism is a polygenetic disorder (Countinho et al., 2007).

Genes Involved in Oxidative Stress Management:

In addition to serotonin, genetic alterations associated with the management of reactive oxygen species have been linked to autism. Glutathione is an abundant endogenous antioxidant; therefore, it is not surprising that glutathione gene mutations have been implicated in several different disease states including several forms of cancer, asthma and allergic responses (Gilland et al., 2004; Hung et al.; 2004: Yeh et al., 2005). In autism the glutathione S-transferase P1 haplotype (GSTP1) is overly transmitted in mothers who have autistic children (Williams et al., 2007). One possibility is that this haplotype may be acting in mothers during pregnancy and either contributing to or causing autism in their offspring. GSTP1 polymorphisms have also been found in mothers of children with asthma (Carroll et al. 2005). Similar to autism, markers of oxidative stress, including isoprostanes, are increased in children with asthma (Carroll et al., 2005; Zanconato et al., 2004). In addition, an allele for ALA6 GPX1 (glutathione peroxidase) is significantly under-transmitted from healthy parents to their offspring affected with autism (Ming et al., 2009). Ming

et al. (2009) suggests that these findings indicate possessing the ALA6 GPX1 allele may be protective for autistic disorder.

Another gene mutation which may be relevant to autism is glutathione-S-transferase M1 (GSTM1). GSTM1 detoxifies xenobiotics via conjugating them to glutathione (Armstrong, 1997). GSTM1 also modulates stress-mediated signals via interaction with MAP kinases which would, in turn, alter cellular signaling (Elsby et al., 2003). MAP kinases play an important role during CNS development via their regulation of the central nervous system's level of apoptosis, morphogenesis, cell survival, and response to stress. In a study looking at case-parent trios, GSTM1 deletion alleles have been associated with an increased risk for autism (Buyske et al., 2006). Although the population frequency of GSTM1 * 0 homozygotes is large, the theory is that this genetic variation may interact with either other glutathione mutations, other genetic risk factors, or environmental risk factors (such as toxicants) to result in autism (Buyske et al, 2006; Ming et al., 2009). A similar gene by environment interaction hypothesis has been used to explain the relationship of particulate matter on cardiovascular deaths. Schwartz et al., (2005) found that GSTM1 null subjects or subjects who had greater than average levels of oxidative stress (obese subjects) were particularly sensitive to the damaging effects of particulate matter.

GSTM1 is found in both mice and humans. There are 21 recognized glutathione -S-transpherase genes in mice (Board et al., 2007). The recent creation of GSTM1 knockout mice may help to determine whether or not the removal of a major component of an organism's antioxidant defense system will alter oxidative stress

levels and make the organism more susceptible to toxicants. GSTM1 removal or deletion may cause an organism to be more vulnerable to environmental insult. Thus, GSTM1 polymorphisms combined with environmental insult may result in developmental neurological and behavioral alterations that mimic those seen in individuals with autism. It is possible that other GST isoforms will be able to compensate for the GSTM1 deletion and the creation of a double knockout strain would be needed as a polygenic animal model of autism.

Several other genes whose alteration may contribute to increases in oxidative stress have been implicated in autism. The reduced folate carrier (RFC) and methylenetetrahydrofolate (MTHFR) are both part of the methionine cycle. The G allele of the RFC has been associated with increased risk for several birth defects (De Marco et al., 2003). James et al. (2006) found that children with the RFC-1 GA or GG genotypes were two-times more likely to be autistic. In addition, there was an interaction between the RFC-1 GG genotype and the MTHFR 677CT genotypes which showed a three-fold increase in autism susceptibility (James et al., 2006). Individuals with interactions between several COMT (methylates dopamine) alleles and TCN2 (required for the cellular uptake of B12) alleles have also been shown to have a seven-fold risk for developing autism (James et al., 2006). In conclusion, alterations in folate-dependent methionine metabolism and glutathione metabolism have both been associated with gastrointestinal, immunologic and CNS dysfunction and may be associated with increased risk for developing autism via neuronal damage due to oxidative stress (Bains and Shaw, 1997; James et al., 2006).

Evidence for Oxidative Stress in Autism:

Zoroglu et al. (2004) compared oxidant and antioxidant levels in autistic and healthy control children. They found increased thiobarbituric acid-reactive substances (TBARS), superoxide dismutase, and xanthine oxidase in autistic children compared to controls. In addition, they found decreases in catalase in autistic children. The TBARS levels (a measure of lipid peroxidation) in autistic patients were increased by up to three-

fold in some of the autistic patients even though superoxide dismutase was also increased in these patients. Chauhan et al. (2004) compared different lipid peroxidation markers of autistic children to their non-autistic and behaviorally normal siblings. They found that autistic children had significantly increased levels of lipid peroxidation, again indicating that oxidative stress is increased in autistic children. In addition, they found decreased levels of several proteins that reduce oxidative damage and there was a correlation between the reduction in these proteins and the loss of previously acquired language skills in the autistic children. James et al. (2004) observed an imbalance in methionine and homocysteine metabolism in autistic children compared to healthy controls. They state that the autistic children's lower baseline levels of S-adenosylmethionine, cystathionine, cysteine, and glutathione combined with their increased levels of S-adenosylhomocystine, adenosine, and oxidized glutathione indicate an impaired capacity for methylation, increased oxidative stress, and increased oxidative stress vulnerability. Ming et al. (2005) compared urinary excretions of 8-isoprostane, a lipid peroxidation biomarker, in 33 children with autism and 29 aged matched controls. They found that autistic children

had significantly increased levels of 8-isoprostane, with a small portion of the autistic children having between 25 to 46 fold increases above the mean. Collectively these and other observations led Kern and Jones (2006) to hypothesize that autistic children are selectively vulnerable to environmental factors which cause oxidative stress and that this oxidative stress, in turn, causes the neuronal insult seen in autism.

Immune System Alterations in Autism:

Individuals diagnosed with autism have also been shown to have abnormal immune system activity when compared to healthy controls. Immune changes include alterations in macrophage, T-cell, B-cell and natural killer cell activity (Gupta et al. 1996, Warren et al., 1987; Vojdani et al., 2008). Gupta et al. (1988) found decreases in IL-2 interferon-gamma, but increases in IL-4 in autistic individuals. Other studies show increases in several proinflammatory cytokines, such as TNF-alpha, IL-6, and GM-CSF are increased in the brain of autistic individuals as compared to healthy controls (Croonesburg et al., 2002; Li et al., 2009). Jyonouchi et al. (2001) suggests that autistic individuals who have symptoms of behavioral regression tend to have major immune system abnormalities. Children with autistic regression had parental reports of behavioral regression after early childhood illness, formula intolerance, and adverse vaccine reaction. In addition, immune studies have also found lower levels of glutathione, similar to those seen in oxidative stress studies (Vojdani et al., 2008). Lowered levels of glutathione may be able to at least partially account for the lowered natural killer cell and T-cell activity. Although these studies suggest that autistic individuals have immune system dysfunction, very little is understood about what

mechanism is responsible for the dysfunction and if the dysfunction can be reversed. In addition, the oxidative stress system, the immune system, and the central nervous system are so intertwined it is difficult to determine which of the three systems, if not all, is creating the behavior abnormalities seen in autism. Animal models may help to identify the behavioral abnormalities which result from damage to or alteration of any one of these three systems.

Animal Models of Autism:

Sodium valproate (VPA) has a variety of mechanisms of action, including increasing GABA-ergic transmission and reducing release and or effects of excitatory amino acids (Perucca, 2002). VPA is used widely as an antiepileptic drug because it reduces seizure frequency and because of its relatively few side effects in adults (Perucca, 2002). However, pre-natal exposure to VPA appears to produce a pattern of behavioral and neuroanatomical abnormalities in children similar to those found in children with autism. When compared to other antiepileptic drugs, VPA causes the greatest risk to fetus when taken by the mother *in utero* (Meadot et al., 2006). Clinical studies of children exposed to VPA *in utero* have characterized a fetal valproate syndrome with symptoms similar to autism including deficits in language and communication, stereotypic behavior, hyperexcitability, and global delays in behavioral development (Ardinger et al., 1988; Koch et al., 1996; Mawer et al., 2002; Moore et al., 2000; Williams et al., 2001). In addition, clinical case studies of infants with fetal valproate syndrome report physical abnormalities including low myelomeningocele lesion, minor abnormalities of the face and ear, and microphaly

(Ardinger et al., 1988; Moore et al., 2000). These findings have led to the suggestion that VPA exposure at the time of neural tube closure causes changes in Purkinje cell number and cerebellar cell volume similar to what is observed in patients with autism (Rodier et al., 1997; Ingram et al., 2000; Sobaniec-Lotowska, 2001). Schneider and Przewtocki (2004) found that rats pre-natally injected with VPA exhibit lowered sensitivity to pain, repetitive/stereotypic-like behavior combined with lower exploratory activity, and decreased numbers of social behaviors and increased latency to engage in social interactions. Previous studies demonstrated that injections of VPA at crucial developmental time points can produce a functioning animal model of autism (Wagner et al., 2006). By using a battery of behavioral tests, Wagner et al. (2006) has demonstrated that animals injected with VPA, both pre and post-natally, display a retardation and/or regression of certain critical behaviors such as surface righting, mid-air righting and negative geotaxis.

Even though motor and cognitive deficits are often seen in autistic individuals, social deficits are the core symptoms of autism. Therefore, it was imperative that social behavior also be assessed following early post-natal VPA exposure. P14 VPA exposure was found to cause a significant decrease in several social behaviors in comparison to control animals (Yochum et al., 2008). The social behavioral deficits include both investigative and play behaviors with decreases in ano-genital sniffing, crawl-under/over behaviors, and allogrooming. Under social conditions, VPA-treated pups had significantly decreased motor movement. However, in non-social conditions, VPA-treated mice had significantly increased motor movement, indicating that the lack of social interaction seen in the paired movement test was not the result

of a motor deficit. Early post-natal administration of VPA to mouse pups also causes a 30-fold increase in the number of cells staining for apoptosis in the cerebellum and a 10-fold increase in cells staining for apoptosis in the hippocampus (Yochum et al., 2008).

Sodium Valproate and Oxidative Stress:

As stated above, although VPA appears to be neuroprotective in adults, it has a teratogenic effect in developing organisms. Despite extensive study, very little is known about how VPA causes this damage. One possibility is that VPA affects developing cells in a similar fashion to other teratogenic compounds, such as thalidomide or phenytoin, which causes an imbalance in the intracellular redox state, which in turn causes oxidative stress and possible DNA damage (Winn and Wells, 1995; Sauer et al., 2000). Two of VPA's metabolites may also cause increases in reactive oxygen species (ROS).

Na et al. (2003) found that therapeutic levels of VPA elevate intracellular ROS levels which cause an inhibition of cardiomyogenesis in *in vitro* murine embryonic stem cells. Na et al. (2003) also found the inhibition of cardiomyogenesis can be reversed using the free radical scavenger vitamin E. In addition, Kawai and Arinze (2006) found that VPA-treatment increases gene transcription in three reporter gene constructs (two human and one mouse) which are involved in antioxidant response elements and that this gene transcription is reactive oxygen species mediated.

Sodium Valproate and the Immune System:

VPA has also been shown to alter cytokine levels in both *in vivo* animal studies and *in vitro* human cell studies. Ichiyama et al. (2000) found that VPA inhibits the production of certain pro-inflammatory cytokines. While this would appear to indicate that VPA is protective against cell death, decreasing pro-inflammatory cytokines leaves the organism vulnerable to outside immune challenges such as bacteria and viruses. On the opposite end of the spectrum, Schneider et al (2008) found alterations in immune system components (T cells, INF-γ, IL-10) which they suggest allowed for increased susceptibility of stress in male rats. In addition, they found behavioral alterations in the VPA-treated rats which may be attributed to these immune changes. These studies indicate that VPA causes an imbalance in the immune system by altering both pro and anti- inflammatory cytokine levels. Several cytokines such as TNF-alpha, IL-1 and others may be involved in healthy brain development, both directly and indirectly, by affecting neural cell migration, proliferation and differentiation as well as death (Merril ,1992) Therefore, any alteration in cytokine levels during early development is potentially harmful because it could disrupt the delicate balance of brain organization.

For reasons mentioned above, VPA appears to be a logical drug for use in an animal model of autism. However, there are still several limitations to using VPA in our animal model, the largest of which is trying to figure out which mechanism of action is causing the cell death we have observed in pervious VPA rodent studies (Yochum et al., 2008). Through the use of knockout mice which have selective genetic vulnerabilities, this and future studies will help to determine which one of

VPA's mechanisms of action is toxic to developing organisms and not to the adult system.

Study Objectives:

Current research involving human subjects with autism have numerous obstacles to overcome when trying to determine the etiology of autism. These obstacles include subject controls for mental retardation and seizure disorder, medication history, and environmental conditions. It is through translational research and the creation of useful animal models of human diseases where well controlled studies can added to the understanding of autism's etiology. That being said, it is important to define what makes up a good working animal model of autism. A good working animal model of autism must be able to test three different factors at the same time, 1) genetic factors, 2) environmental factors and 3) age of exposure. In addition, a some combination of these three factors must result in a gender difference which is similar to what is seen in autism spectrum disorder; with male subjects being more likely to develop autism-like symptoms than their female counterparts. Whereas previous studies using VPA have incorporated environment (VPA administration) and have determined one of the optimal time-points for age of exposure (post-natal day 14), the current group of studies improves the model through the addition of a genetic component (GSTM1 knockout mice).

One important initial step for using the newly created strain is to determine the extent of phenotypic and neurochemical differences between the GSTM1 knockout and wild-type mice. Therefore, the first objective of this study was to

characterize adolescent GSTM1 knockout and wild-type exploratory behavior as well as, self-maintenance, social, and motor behaviors. In addition, this study established the baseline neurochemistry of dopamine and serotonin and their metabolites in several brain regions. The second objective was to determine if the GSTM1 knockout and wild-type mice had similar responses to VPA-treatment as those seen in BALB/c mice treated with the same toxicant. Specifically, the second objective was to determine if VPA-exposure altered any of these behaviors and if there was a genotype effect with knockout pups having greater behavioral deficits as compared to the saline-treated and wild-type controls. The third objective was to determine if the GSTM1 wild-type and knockout pups showed greater TUNEL staining in the hippocampus and cerebellum when treated with VPA as compared to saline controls. Finally, the fourth objective was to determine if VPA-treatment on P14 resulted in any long lasting alterations in regional monoamine neurochemistry as compared to saline treated controls.

MATERIALS AND METHODS

Animals:

Breeding colonies of *GSTM1*$^{+/+}$ and *GSTM1*$^{-/-}$ mice on a C57Bl6J x 129 SvEv background were established. All animals were maintained under standard vivarium conditions, with ad libitum food and water and a 12:12 hour light:dark cycle. All procedures have been approved by the Animal Care Committee and are in accordance with AAALAC guidelines. Non-sibling mice of the same confirmed genotype were housed together in pan cages (3 females and 1 male per cage) and allowed to breed. Mice were monitored and the day pups were born was recorded as postnatal day 0 (P0). Mice were injected with injected with either VPA or saline control on P14. Pups were then separated into one of 3 studies; the apoptosis study, the play behavior study, and the biochemistry study. Pups in the apoptosis study were sacrificed 24 hours following VPA-injection. Pups in the play behavior study were then weaned on P21 and housed in single mouse cages with free access to food and water until their behavior analysis on post-natal day 29 (P29) and post-natal day 30 (P30). Pups in the biochemistry study where weaned on P21 and housed in single mouse cages with free access to food and water until day of sacrifice on P30.

GSTM1 Knockout

Dr. Oleg Microchnitchenko of the University of Medicine and Dentistry of New Jersey (UMDNJ) and Dr. Guo C. (Gene Targeting & Transgenic Facility, Dept. Genetics & Developmental Biology, University of Connecticut) created the GSTM1

knockout mouse. A murine *GSTM1* genomic clone was obtained from BAC/PAC Genomics Resource Center (CHORI). Eleven kb fragment containing all 8 exons of GSTM1 was amplified and subcloned in MC1TK-containing vector (Figure 1). A loxP-neo-Lox P cassette was inserted to replace exons 1-5, and final construct was obtained in Bluescript vector (Figure 1). Genomic organization of mouse *GSTM1* gene. 1-5 exons were replaced with loxP-neo-loxP cassette. Confirmation of targeted ES clones, selected for expansion and aggregation by PCR analysis. Two sets of primers (one from neo gene and another one from GSTM1 sequence for each set) were used for PCR analysis. Clones 1, 2 and 6 were used for blastocysts injection. The vector was linearized and electroporated into 129SVEv x C57BL/6 F1 hybrid ES cells. ES cell clones, which had undergone homologous recombination with the *GSTM1* target vector, were selected on G418 (250 μg/ml) and gancyclovir (2 μM) -containing medium. If required the neo cassette can be excised in the future by crossing animals with *Cre* mouse line. Eight positive clones were identified (Figure 1). Gene-targeted mouse ES cells were injected into blastocysts and transferred to pseudopregnant females. Chimeric pups were born (Figure 2), germline transmission was confirmed for at least 3 animals and we are now establishing colony of the homozygous mutant animals. Our results indicate that homozygous GSTM1 mice appeared to be normal and fertile.

Groups Design:

Mice were injected subcutaneously with either saline or VPA 400 mg/kg (Sigma) on postnatal day 14 of life. Three groups of mice were required for this

study. The first group of mice pups [consisting of 8 knockout (KO) saline-treated female, 10 KO VPA-treated female, 8 KO saline-treated male, 11 KO VPA-treated male, 8 wild-type (WT) saline-treated female, 10 WT VPA-treated female, 8 WT saline-treated male, and 11 WT VPA-treated male pups] was used in the apoptosis portion of this study. Mice in this group were injected subcutaneously with either saline or VPA 400 mg/kg (Sigma) on postnatal day 14 of life and sacrificed 24 hours later. The 24 hour time point was where significant TUNEL staining was found in previous VPA studies (Yochum et al. 2008). After sacrifice whole brains were taken and placed in formalin for later TUNEL staining.

A second group of pups [consisting of 7 knockout (KO) saline-treated female, 7 KO VPA-treated female, 7 KO saline-treated male, 6 KO VPA-treated male, 7 wild-type (WT) saline-treated female, 7 WT VPA-treated female, 7 WT saline-treated male, and 6 WT VPA-treated male pup pairs for a total of 108 total pups tested] was assigned to the play behavior study and was placed in a reversed light cycle room on P21, which was their first day of weaning. Next, they were assessed for behavioral tasks comprised of a social interaction and play study on post-natal day 30 (P30). P30 was chosen for play behavior testing because it is the only time during development where the animals are fully weaned, but are not yet sexually mature. When the mice start entering adolescence, after P40, the males become aggressive towards novel males and fighting behaviors replace play behaviors.

The third group of mice [consisting of 15 knockout (KO) saline-treated female, 16 KO VPA-treated female, 14 KO saline-treated male, 15 KO VPA-treated male, 15 wild-type (WT) saline-treated female, 13 WT VPA-treated female, 13 WT

saline-treated male, and 16 WT VPA-treated male pups] was injected subcutaneously with either saline or VPA 400 mg/kg (Sigma) on postnatal day 14 of life and then sacrificed on post natal day 30 for brain chemistry analysis. The time-point of 30 days was chosen for several reasons. First, it was chosen because it occurs when the blood brain barrier is fully formed and we did not want platelet monoamine levels interfering with our brain region neurochemistry (Witaker-Azimita, 2005). Second, at this time-point the brain regions are fully formed and more accurate dissection can be performed. For this portion of the study 4 brain sections, the hippocampus, cerebellum, frontal cortex and striatum, were dissected and then flash frozen in liquid nitrogen.

TUNEL Assay:

The apoptosis identification technique used in this study was the ApopTag kit which uses a TUNEL assay (Chemicon manual). Pups were sacrificed 24 hours following the P14 injection and whole brains were placed in 10% neutral buffered formalin for 24 hours, rinsed in PBS, and stored in a 70% ethanol solution until the day of paraffin embedding. One VPA brain and one Saline brain of the same sex and sacrifice time point were embedded side by side in a single block. Brains were marked before embedding for later differentiation. Seven-micron sagittal sections were collected with each subsequent slice taken 56 microns from the previous section which allowed for the sampling of an average of 504 microns of tissue.

Cell Counting:

Nine sections were taken from each brain starting from midline outward (brains were embedded parasagittaly). Photomicrographs were taken at 400X for each of these brain slices. All cerebellar photomicrographs were taken from the external granule cell layer of anterior lobe three of the cerebellum in order to standardize sections used for cell counts. All hippocampal photomicrographs were taken from the dentate gyrus. These similar regions were then compared using the Scion Image (NIH) program with particle size and density index set at a constant. Particle size and density settings were determined by matching Scion counts with manual counting under microscope.

Motor Activity:

Motor activity was assessed twice in this study in order to determine if VPA-treated pups of either genotype had motor deficits that would interfere with social behavior. First, motor activity was assessed for individual mice of both genotypes and sexes previously injected on P14 with either VPA or saline control, placed into individual housing on P21 and then tested for motor behavior on P29. P29 is the day prior to social interaction and play behavior trials occurring on P30. The motor activity chamber consisted of a plexiglass box 42 x 22 x 14 cm. Six infrared sensors were placed approximately 7 cm apart and 2.5 cm above the floor, and were used to count the number of horizontal movements made by an individual mouse over a 30 minute time period. A crossing of the beam represented a unit of horizontal movement. The same plexiglass box and infrared sensors were used during the social interaction and play behavior trials to assess the motor activity of the mouse pairs.

Social Interaction and Play:

Naïve mice that were injected with either VPA or saline on P14 were individually housed on P21 for four-five days prior to the test session in order to increase social interaction during the time of behavioral testing. Age, sex, treatment and genotype matched pairs of non-sibling GSTM1 knockout and wild-type mice were then observed for social interactions for one 30 minute session and scored by two trained observers. All observers were trained on mouse pairs not used in this study until there was 98% agreement in the observation scores. These observers then scored pairs of animals blind to treatment condition. Testing was conducted at the start of the dark cycle and the testing room was illuminated by red light only. During testing, the behaviors they observed were: ano-genital sniffs, face sniffs, crawl-under/over behaviors (crawl-under/over behaviors are defined as one mouse crawling under or over another mouse) self-grooming, and allogrooming (allogroom behaviors are defined as one mouse rising up on its hind legs to touch paws and snout to the other mouse to perform grooming motions). Again, paired motor movement was assessed during this 30 minute trial using the six infrared sensors described in the Motor Activity section.

Brain Chemistry:

Male and female GSTM1 knockout and wild-type mice were injected on P14 with either VPA or saline and then allowed to mature until P30. On P30 mice were sacrificed and cerebellum, hippocampus, striatum and frontal cortex regions were

dissected, snap frozen in liquid nitrogen, and stored until homogenization. All tissue was then homogenized in 0.3 ml of 0.4 N perchloric acid with 0.1 mM EDTA. Homogenized samples were centrifuged at 20,000x*g* for 20 min at 4°C and the supernatant was flash frozen in liquid nitrogen until analyzed. Supernatant was assayed for dopamine serotonin and their metabolites using HPLC-electrochemical detection (Bioanalytical System, West Lafyayette, Indiana). The mobile phase consisted of 0.1375 M sodium phosphate (dibasic), 0.0625 M citric acid, 5.0mg EDTA, and 14% methanol with a flow rate of 0.7ml/min.

Statistical Analysis:

All data was analyzed using SPSS statistics package. ANOVAs including genotype, treatment and sex as main factors were performed for all behavioral tests. For TUNEL cell counting and HPLC analysis, ANOVAs including treatment, genotype and sex as main factors was used with Tukey's HSD as a posthoc test. P values <0.05 were considered statistically significant.

RESULTS

TUNEL Staining:

Separate groups of GSTM1 knockout and wild-type pups injected on P14 (saline or VPA 400mg/kg) were sacrificed 24 hours later, and their brain tissue was analyzed using a TUNEL stain for apoptosis. Cerebellum and hippocampus showed substantially high levels of cells staining for TUNEL following treatment (see Figures 5, 6, 7 and 8). This level of staining was not observed in other regions. The cerebellum and hippocampus were analyzed using the Scion Image program (NIH) in conjunction with photomicrographs.

Cerebellum: Similar to the BALB/c VPA study (Yochum et al., 2008) there was very little cell staining in the cerebellum for apoptosis in the 6 and 48 hour sacrifice time points regardless of sex, genotype or treatment (data not shown). Therefore, the statistical analysis performed included brains sacrificed 24 hours after either saline or VPA injection. There was a significant effect of treatment [$F(1,66) = 36.46$, $p<.0001$], with VPA-treated pups of both sexes and genotypes having increased staining compared to the saline treated animals of both sexes and genotypes (Figure 3) . In addition, there was a gene by treatment by sex interaction [$F(1,66)= 4.21$, $p=.0442$] with VPA-treated wild-type females having significantly less cells staining for apoptosis than VPA-treated knockout and wild-type males, and knockout females (Figure 3). The VPA-treated wild-type females had staining that was similar to the saline treated knockout and wild-type pups.

Hippocampus: TUNEL staining in the hippocampus did not significantly appear in the 6 and 48 hour sacrifice time points regardless of sex, genotype or treatment (data not shown). Therefore, the statistical analysis performed included brains sacrificed 24 hours after either saline or VPA injection. There was a significant effect of treatment [$F(1,66)=52.26$, $p<.0001$], with VPA-treated pups of both sexes and genotypes having increased TUNEL stained cells as compared to saline treated pups of both sexes and genotypes (Figure 4). In addition, there was a gene by environment by sex interaction [$F(1,66)=4.20$, $p=.0441$] with VPA-treated wild-type females having significantly less cells staining for apoptosis than VPA-treated knockout and wild-type males and knockout females (Figure 4).

GSTM1 Play Behavior Analysis:

Eight treatment groups were compared during this play study 1) knockout males treated with saline, 2) knockout males treated with VPA, 3) knockout females treated with saline, 4) knockout females treated with VPA, 5) wild-type males treated with saline, 6) wild-type males treated with VPA, 7) wild-type females treated with saline and 8) wild-type females treated with VPA. There were four different types of behavior observed during play behavior trials. The first was self-maintenance behavior measured by the number of self-grooms the pair of pups make (these are non-social behaviors). In this study there was no difference for VPA-treatment, genotype, and sex (data not shown). The second type of behavior was exploratory behavior, characterized by the number of pair-performed face-sniffs and ano-genital

sniffs. For both face sniffs and ano-genital sniffs there was no significant difference for VPA-treament, genotype or sex (data not shown). The third was social behaviors characterized by alo-grooming, counted as one member of the pup pair grooming the other member of the pair. There was a significant difference for genotype [F (1,46)= 9.94, p=.0028], with knockout pup pairs performing fewer alo-grooms than wild-type pup pairs (Figure 10). In addition, there was a trend towards significance for both treatment (p=.08) and genotype by sex interaction (p=.06) with knockout VPA-treated males performing the fewest alo-grooms (data not shown). The fourth and final measure was play behavior (a social behavior measure) characterized by one mouse crawling either over or under the other mouse. There was a significant difference for the treatment by genotype interaction [F(1,46)= 4.28, p=.04] with VPA-treated knockout mice performing fewer crawl-under behaviors than the saline treated knockout and wild-types receiving both treatments (figure 9). There was a trend toward significance for a gene by treatment by sex interaction with VPA-treated knockout males performing the fewest crawl-under behaviors (data not shown). Saline-treated knockout animals performed significantly more crawl-under behaviors than VPA-treated knockouts and wild-type mice receiving either VPA or saline treatments (figure 9). One possible explanation for this result is that the saline-treated knockout males were prone to fighting by the end of their play behavior trials. This level of fighting behavior in males between the ages of 30-40 days was not seen in the wild-type animals or in the knockout females. It is possible that observers misinterpreted early fighting behavior with crawl-under play behavior until it became so violent that the observer had to separate the animal pairs.

Motor Movement:

In addition to social behavior, motor activity of the pair of mice was monitored during the play behavior trial. There was a significant genotype by treatment interaction [$F(1,50) = 14.25$, $p = .0004$] with saline-treated wiltype mice performing the most horizontal movements in a social environment and VPA-treated wild-type mice performing the fewest (figure 11). In order to determine if the decrease in motor activity in wild-type VPA-treated mice was due to either the social environment or global motor impairment, a second motor test was performed with an additional group of mice. Each mouse had a horizontal movement analysis which consisted of a total activity count for 30-minute period that was separated into three 10-minute bins (equal to the amount of movement time analyzed during play behavior). There was no significant difference for treatment genotype or sex in the non-paired movement motor assessment. These results suggest that the decreased activity displayed by the VPA-treated wild-type mice in a social context was not the result of motor impairment.

Neurochemistry:

GSTM1 knockout and wild-type male and female mice were injected with either 400mg/kg of VPA or saline control on P14 and then sacrificed between 12 and 24 hours post injection. Previous studies have shown that between 12 and 24 hours is the peak time-point for TUNEL staining following VPA-injection on P14 (Yochum et

al. 2008). The cerebellum, hippocampus, striatum and frontal cortex were analyzed for dopamine, serotonin, their metabolites, and their turnover ratios.

Cerebellum: Serotonin (5-HT) was significantly increased in GSTM1 knockout male and female control mice when compared to control wild-type mice [F (1,107) = 22.46, p <.0001] (Figure 12). There was a genotype by treatment interaction with the VPA-treated knockout mice of both sexes having decreases in 5-HT when compared to their saline controls [F(1,107) = 14.48, p= .0002] (figure 12). There were no significant differences in 5HIAA across all genotypes, sexes and treatments (data not shown). However, there was an increase in 5HT-turnover in VPA-treated animals across all sexes and genotypes [F(1,107) = 4.04, p=.0471] (Figure 13). Dopamine (DA) was significantly increased in the GSTM1 knockout of both sexes compared to the wild-type mice of both sexes [F(1,107) = 8.36, p= .0046] (Figure 14). Both sexes of both genotypes receiving VPA had significant increases in DA compared to saline controls [F(1,107) = 13.34, p=.0004] (Figure 14). There was a treatment effect for DOPAC levels [F(1,107) = 92.56, p<.0001] with VPA-treated mice of both sexes and genotypes having significantly reduced amounts of DOPAC (Figure 15). DOPAC/DA turnover ratios were significantly larger in wild-type mice of both sexes [F(1,107) = 18.96, p<.0001] and in saline-treated males compared to saline-treated females of both genotypes[F(1,107) = 7.37, p=.0077] (Figure 16). In addition, DOPAC/DA turnover ratios were also significantly decreased in VPA-treated mice of both sexes and genotypes [F(1,107) = 220.67, p <.0001] (Figure 16). There were no significant differences in HVA levels or HVA turnover ratios in the cerebellum (data not shown).

Hippocampus: There was a significant 30% increase in 5-HT in all VPA-treated mice [$F(1,107) = 27.27$, $p<.0001$] (Figure 17). There was also a significant increase in 5HIAA in all VPA-treated mice (Figure 18). However, there were no significant differences in 5-HT turnover (data not shown). There were also no differences in DA across genotype, sex and treatment (data not shown). However, there was a significant decrease in DOPAC in mice who received VPA-treatment for both genotypes and sexes [$F(1,107) = 48.80$, $p<.0001$] (Figure 19). There was also a decrease in DOPAC/DA turnover in VPA-treated mice compared to saline-treated mice [$F(1,107) = 35.32$, $p<.0001$] (Figure 20). There was a gene by treatment by sex interaction with VPA-treated wild-type females having increased HVA compared to all other treatment, sex and genotype groups [$F(1,107) = 5.82$, $p= .0175$] (Figure 21). In addition, VPA-treated mice of both genotypes and sexes had an increase in HVA/DA turnover [$F(1,107) = 29.12$, $p<.0001$] (Figure 22).

Striatum: There were no significant differences in 5-HT or 5HIAA across genotype, treatment or sex (data not shown). However, there was a significant difference in 5-HT turnover between saline and VPA-treated mice [$F(1,107) = 7.86$, $p=.0060$], with VPA-treated mice of both sexes and genotypes having increase compared to controls (Figure 23). There were also no significant differences in DA across genotype, sex and treatment (data not shown). There was a significant increase in DOPAC in VPA-treated mice across all genotypes and sexes [$F(1,107) = 3.96$, $p=.0491$] compared to saline-treated mice (Figure 24). There were no significant differences in DOPAC/DA

turnover across all genotype, sex and treatment groups (data not shown). There were also no significant differences in HVA and HVA/DA turnover across all genotype, sex and treatment groups (data not shown).

Frontal Cortex: There is significant genotype effect for 5-HT in the frontal cortex [$F(1,107) = 10.73$, $p=.0014$], with knockout mice of both sexes and treatments having more 5-HT than wild-type mice (Figure 25). There were no significant differences in 5HIAA across treatment, genotype, and sex (data not shown). There was a sex by treatment interaction for 5-HT turnover [$F(1,107) = 4.34$, $p=.0396$] with VPA-treated males of both genotypes having significant decreases in 5-HT turnover (Figure 26). VPA-treated mice of both genotypes and sexes had a significant increase in DA compared to saline-treated mice [$F(1,107) = 3.95$, $p=.0499$] (Figure 27). Knockout males, regardless of treatment, had significantly more DA than the wild-type males and females and the knockout females [$F(1,107) = 6.31$, $p=.0135$]. There were no significant differences in DOPAC or DOPAC/DA turnover (data not shown). There was a genotype by treatment interaction for HVA [$F(1,107) = 5.61$, $p=.0196$] with wild-type saline animals having the less HVA than the other treatment groups (Figure 28).

DISCUSSION

While the etiology of autism remains unknown, it is proposed to include a gene by environment by age of exposure interaction. This study furthered our previous animal model of autism, which only included an age and environmental stressor component, by adding a genetic component in the form of GSTM1 knockout mice. While our previous studies mainly focused on the differences between toxicant-exposed and non-exposed mice, this study had to also evaluate the differences between the two genotypes both prior to toxicant exposure and after toxicant exposure. Prior to exposure, GSTM1 knockout mice showed significant differences in paired social behaviors and in neuro-chemistry in several brain regions compared to wild-type mice. After VPA-treatment, significant differences were seen in paired social behavior, neuro-chemistry and TUNEL staining. These findings indicate that both gene manipulation and toxicant exposure can result in changes to both behavioral phenotype (in the form of social deficits) and neurochemistry (in the form of altered serotonin levels) which are relevant to autism spectrum disorder.

TUNEL Staining

Apoptosis in the brain is a normal part of development and is still occurring during our injection time-point of P14. However, the large waves of apoptosis occurring in the cerebellum and hippocampus are typically over by post-natal day 6.5 (Bessis et al., 2007; Sandau and Handa, 2006). Previous studies, using VPA-treatment on P14 in BALB/c mice resulted in a 30-fold increase in TUNEL staining in the cerebellum and a 10-fold increase in the hippocampus (Yochum et al. 2008). Given

that the treatment time-point occurs after the typical apoptosis waves seen in these regions, we can attribute the large increases in cell death to the teratogenic effects of VPA. In this study, we sought to reproduce these results in GSTM1 wild-type (C57/129) and knockout mice. From the results of these two studies it can be concluded that a 400mg/kg VPA administration at P14 causes significant TUNEL staining of granule cells in the cerebellum and hippocampus, as compared to saline treatment, regardless of strain.

VPA-induced Treatment Effect:

With only saline treatment there are no genotypic differences in staining for apoptosis in any brain regions. However, 24 hours after VPA-exposure both GSTM1 knockout and wild-type animals have an increased number of cells staining for TUNEL in the cerebellum and hippocampus. These data are consistent with the TUNEL stain results seen in the Yochum et al. (2008) study which used BALB/c mice instead of GSTM1 animals. BALB/c mice are sensitive to toxicants, have serotonin abnormalities and behavioral abnormalities and have been recommended as a good strain to use as an autism mouse model (Brodkin, 2007). The GSTM1 knockout and wild-type mice, which only have one genetic difference, had the same brain regions stain for TUNEL (the cerebellum and the hippocampus) and the same cell types appear vulnerable to toxicant exposure (migrating granule cells). These results further reinforce that P14 is an age where the mouse cerebellum and hippocampus are sensitive to damage by toxicants regardless of strain. The same dose of VPA given to mice over the age of post- natal day 60 does not produce any

increases in TUNEL staining, which points to a critical time window where developing cells are vulnerable to VPA's toxic effects. Therefore, a 400mg/kg dose of VPA given in the early post-natal developmental period is strong enough to cause increases in cells staining for TUNEL regardless of genotype or gender.

The time-point of P14, chosen for this study appears to be a particularly vulnerable time for cerebellar and hippocampal granule cells because they are still migrating (Altman, 1966). This time-point is specifically vulnerable for the dentate gyrus section of the hippocampus because the granule cells are very densely packed and generated late (mostly during the post-natal period) as compared to other neural cells (Altman, 1966). In addition, the granule cells of the dentate gyrus are primarily controlled by Reelin, which is a trophic factor which is known to be altered by VPA (Frotscher et al., 2003). Altering Reelin activity is just one of the many mechanisms of action for VPA, which also alter GABA levels and causes increases in oxidative stress (Perucca, 2002; Winn and Wells, 1995; Sauer et al., 2000). Alcohol is another substance which affects GABA levels and which is also known to cause alterations in cerebellar granule cell migration in the fetus when taken during pregnancy (Jiang et al., 2008). Therefore, given that VPA acts upon the developing fetal brain in a similar way to alcohol, VPA treatment may also change neural cell migration which was not examined in this study. In addition, if VPA-treatment is given at an earlier time-point (such as gestational day 10) a different group of cells may potentially stain for TUNEL and/or have their migration disrupted because a different group of cells would be migrating and differentiating at that time.

Treatment by Genotype by Sex Interaction:

Of interest, there was a gene by treatment by sex interaction seen in our TUNEL stain results. VPA-treated female GSTM1 wild-type animals had reduced cells staining for TUNEL in both the cerebellum and hippocampus as compared wild-type males and GSTM1 knockout animals of both sexes. These results indicate that female mice with an intact GSTM1 gene may have reduced sensitivity to the damaging effects of VPA. In addition, GSTM1 knockout females appear to be just as sensitive to the damaging effects of VPA as the male wild-type and knockout mice making this current study a valuable mouse model of autism because it reflects both a sex and genetic difference to toxicant exposure. Similarly, autism in humans has a sex difference with males having an increased likelihood of diagnosis. By this we mean that just being male confers susceptibility to being diagnosed with autism, similar to how male mice seem to be especially susceptible to VPA-induced cellular damage. In addition, in our model, the female mice require both a genetic susceptibility and toxicant exposure to show neuronal damage equivalent to what is seen in male mice regardless of genotype.

Sex differences in individuals with the GSTM1 polymorphism have been seen in human studies of other diseases (Imboden, 2007; Pisani et al., 2006; Srivastava et al., 2004). The GSTM1 null haplotype is more often found in females and it has been suggested that the GSTM1 detoxification pathway is gender dependent (Stavropoulou et al., 2007). Females express higher levels of GSH-enzymes as they grow older than males and may be important in determining colon cancer outcomes; however it is not yet known whether this difference is also seen at a younger age (Hoensch et al.,

2006). More testing would need to be done to determine how the GSTM1 null polymorphism may be relevant to the gender difference see in autism.

GSTM1 Play Behavior Analysis

In a previous study using BALB/c mice, it was observed that VPA-treated animals performed fewer crawl-under behaviors than their saline treated counterparts (Yochum et al, 2008). These results indicated that VPA-treatment at P14 can alter social behavior. Based on these results, a deficit in the social behaviors of VPA-treated wild-type mice and enhanced deficits in GSTM1 knockout mice was expected for all social behaviors. However, reductions in social behaviors were not seen across all of the social behavior measures.

Behavior Differences by Genotype:

Allogrooms: Wild-type female mice performed significantly more allogroom behaviors than male wild-types or knockout animals of both sexes. There was no treatment effect, only a gene by sex interaction. Previous studies have shown that female mice perform more same-sex paired social behaviors than male mice (Terranova et al., 1993). While the wild-type females in our study demonstrated behavior consistent with these findings, the knockout females did not. The knockout female's alo-groom behavior, regardless of treatment, was statistically similar to that of the male knockout mice. These results indicate that the sex difference, typically seen in mouse social behavior, is no longer present in GSTM1 knockout animals.

Crawl-unders: Of note, the saline-treated GSTM1 Knockout animals had significantly more crawl-under behaviors than wild-type animals of both treatments. While crawl-under behaviors are typically associated with play behavior, there is the potential for aggressive contacts to interfere with accurately assessing positive social behavior. The peak time for social play in rodents starts to decline after P40 and is gradually replaced by adult sexual and aggressive behaviors (Panksepp, 1981). As this study progressed the behavior scorers blind to treatment conditions noticed that certain pup pairs were fighting 10 minutes or so into the 30 minute behavior trial, which is unusual if the pair being assessed is 30 days old. The fighting became so intense for some pairs that the trial had to be stopped and the pups had to be separated. Later analysis showed that the pups that were separated were all GSTM1 knockout animals. These findings lead to speculation that the GSTM1 knockout animals may either be more prone to aggressive behavior or may start aggressive behavior earlier than other strains we have tested. In addition, female pup pairs, which normally do not fight at any age, also engaged in aggressive behavior during the behavior trial. Therefore, given that the crawl-unders were accompanied by such aggressive behavior, it is difficult to determine if the increase in the crawl-under behaviors is a measure of positive social behavior for this study.

Behavior Differences by Treatment:

Crawl-unders: Although GSTM1 knockout animals were expected to be more vulnerable to VPA treatment, wild-type animals were still expected to show some deficits in social behavior to a VPA dose of this magnitude. However, following VPA

exposure, only GSTM1 knockout animals had significant decreases in crawl-under behaviors in comparison to wild-type animals of both sexes given both treatments. These results are interesting because in previous studies, BALB/c mice given VPA-treatment showed significant decreases in crawl-under behaviors (Yochum et al., 2008). A potential explanation for this finding is that BALB/c mice respond to toxicants and or social interaction more like GSTM1 knockout animals than wild-type. There may be neurochemical changes during development in different brain regions which could account for behavioral differences later in life. Future studies should compare the developmental neurochemistry of GSTM1 knockout and wild-type pups and BALB/c pups and determine if the BALB/c animals are more similar to the knockout or wild-type animals.

Motor Movement

Un-paired and paired motor movement: Prior to the social behavioral study, a motor movement assessment was performed on individual mice of both sexes, genotypes and treatments in order to determine if there were motor deficits in any of the study groups. There was no difference in un-paired horizontal movement between genotypes, treatment or sex indicating that there were no motor deficits which could potentially confound the study. However, in the paired motor condition, the saline treated wild-type animals performed significantly more horizontal movements than knockout animals of both treatments and their wild-type VPA-treated counterparts. Due to the fact that there was no difference in horizontal movements in the unpaired

condition, these data indicate that the decrease in pair movement observed in the VPA-treated wild-type pairs is due to a decrease in social behavior.

In addition, we are seeing two different effects in the knockout animals. First, saline-treated knockout pairs performed fewer motor movements than their wild-type counterparts. Second, the VPA-treated knockout pairs did not show a deficit in paired motor movement (a typical measure of social measure). Both of these results may be explained by the potential confound of the increased aggressive behavior observed in the saline treated knockout animals of both sexes. For example, instead of knockout animal pairs interacting playfully or exploring the environment of the test chamber, they were fighting with each other in one corner of the test chamber, thus reducing their overall motor movement and preventing social deficits from being observed.

Neurochemisry

Neurochemistry Differences by Genotype*:*

Certain neurotransmitters, their metabolites, and turnover rates were different between the GSTM1 knockouts and the wild-type controls in the saline condition. In the cerebellum, GSTM1 knockout animals had significantly higher levels of serotonin and dopamine than wild-type controls. In addition, they had lower DOPAC/DA turnover rates. In the frontal cortex, saline treated GSTM1 knockout animals had higher serotonin and HVA (dopamine metabolite) levels. There were no significant genotypic differences in the striatum and hippocampus of saline treated mice. These results are interesting because they show that there is regional hyperserotonemia in GSTM1 knockout animals prior to any toxicant treatment in the cerebellum and

frontal cortex. While a large portion of autistic patients have platelet level hyperserotonemia, studies which have examined cerebral spinal fluid do not show increases in overall brain serotonin levels compared to healthy sibling controls (McBride et al., 1998). However, Chugani et al. (1997 and 1999) showed that brain serotonin synthesis capacity was altered in autistic children. In addition, the cerebral spinal fluid studies were not able explore the differences in regional levels of serotonin. Given the results of our study it may be interesting to see if autistic patients with the GSTM1 mutations respond differently to SSRI therapy than those who do not.

Neurochemistry Differences by Treatment*:*

Cerebellum: Following VPA- treatment there were several changes in brain neurochemistry in all four brain regions for both the GSTM1 knockouts and wild-type animals. In the cerebellum there were significant decreases in serotonin in the knockout animals but not the wild-types. In addition, there were increases in serotonin turnover in knockout males and females and wild type females but not in wild-type males. In addition, DOPAC and DOPAC/ DA turnover rates were decreased in both genotypes of both sexes following VPA-treatment. Of these results, the serotonin findings are the most interesting for several reasons. First, as stated above, serotonin differences have been found in individuals diagnosed with autism whereas, dopamine's involvement in autism, if any, is not well understood. Second, traditional understanding of the cerebellum's make-up and function does not include a large role for dopamine. However, Melchitzky and Lewis (2000) have found

staining for dopamine axons in the granule cell layer of certain lobes of the cerebellum. These results indicate that dopamine may play a larger role in cerebellar functioning than originally thought and may also need to be revisited for its role in autism.

Hippocampus: In the hippocampus there were increases in serotonin and its metabolite 5HIAA in both genotypes and sexes following VPA-treatment. These results indicate potential hypersotonemia in the hippocampus in both genotypes following VPA-treatment. However, there were DOPAC and DOPAC/DA turnover rate decreases in both genotypes and sexes. In addition, HVA/DA turnover rates were increased in both genotypes and sexes following VPA-treatment. Wild-type females had significant increases in HVA following VPA-treatment compared to knockout animals and wild-type males. Serotonin, Dopamine, and their metabolite changes in this region may be due to the heavy TUNEL staining seen in the hippocampus following VPA-treatment on P14. Changes to the dopamine system have been indicated in animal models of age related psychosis which mirror behavioral abnormalities seen in humans with age related psychological diseases such as Alzheimer's disease and dementia (Francesco et al., 2001).

Dopamine and Autism: Dopamine disruption throughout the brain has also been implicated in developmental disorders such as Lesh-Nyhan disease which has a behavioral phenotype similar to that of autistic individuals. Lesh-Nyhan is caused by a deficit in the function of the hypoxanthine-guanine phosphoribosyltransferase enzyme which alters dopamine receptor activity and results in dysfunction of the dopamine system (Lesch and Nyhan, 1964; Saito and Takashima, 2000). In addition,

drugs exerting dopamine antagonist action, such as haloperidol and risperdone, have been the most successful for reducing the restricted, repetitive, and stereotypic behaviors and interests and self-injurious behaviors in autism (Luchins, 1990; Gualtieri et al., 1990; McDougle et al., 2005). Therefore, regional dopamine disruption may play a larger role in cause of autistic behaviors than currently understood, especially individuals who tend toward the self-injurious side of the autism behavioral spectrum.

Animal Models of Autism

Children with autism have been shown to have increased body burdens of environmental toxicants (Edelson and Cantor 1998; Edelson and Cantor 2000), to have increased levels of biomarkers of oxidative stress (Sogut et al., 2003; Zoroglu et al., 2003, Zoroglu et al., 2004; Chauhan et al. 2004; Ming, 2005), and to have gene alterations that compromise the ability to manage toxicant-induced oxidative stress (Buyske et al., 2006; Williams et al., 2007; Ming et al., 2009). In order for the autism spectrum of behaviors to occur, it appears that there must be a gene by toxicant exposure interaction along a complex continuum. For some individuals, toxicant exposure (in terms of dose and timing) may be severe enough to result in autism with little or no genetic alteration. For example, Bromley et al. (2008) showed that autism symptoms resulted from early valproic acid exposure in individuals who had no other relatives with autism. On this end of the continuum, toxicant burden and timing fully account for the appearance of autism symptoms. Likewise, for some individuals,

genetic alteration may be severe enough to result in autism with no statistically increased body burden of toxicants.

For the majority of cases, both toxicant exposure and genetic sensitivity are likely involved to varying degrees. Our previous animal model of autism, using BALB/c mice, tested the effect of toxicant treatment during a sensitive period of neural and behavioral development (Wagner et al., 2006; Yochum et al., 2008). Although significant differences were found in behavior and TUNEL staining between VPA- and saline-treated animals, there was no specific genetic component to this model. In order to assess the effects of a specific genetic sensitivity in combination with a toxicant exposure, this study incorporated a GSTM1 knockout.

While the animal model used in this study is innovative, there are several ways in which it can be improved upon to make it more applicable to human autism. Firstly, the type of environmental challenge employed, VPA, is a prescribed medicine which the human population is not generally exposed to. In addition, the offspring of that population has a high likelihood of genetic sensitivity given that their mothers are most likely taking VPA to treat a seizure disorder. Second, while our study uses a single acute dose of VPA, the human subject exposure is more likely to be chronic low doses. These two challenges to our animal model may be remedied by using a toxicant which humans are more regularly exposed to at chronic, low doses. One such toxicant is Bisphenal-A, which is regularly found in soil, air, water and food and is currently being studied in relation to childhood developmental disorders (Tsai, 2006; Kang et al., 2006). However, in order to comply with our animal model of autism, it

would have to produce a behavioral phenotype similar to what is seen in human autism.

That being said, VPA was chosen as our environmental stressor because it produces behavioral phenotypes in children with fetal valproate syndrome similar to those seen in human autism. In addition, rodent studies using VPA also produce behavioral deficits similar to those seen in human autism. However, the mechanism for how VPA causes these behavioral changes is unknown. Several *in vitro* and *in vivo* studies indicate that VPA increases reactive oxygen species and these increases may have damaging effects on both neural cells and behavior acquisition in developing organisms (Na et al., 2003; Kawai and Arinze 2006; Wagner et al., 2006; Yochum et al., 2008; Cheh et al., in press). If in a future study, a different environmental stressor is used in lieu of VPA, it should be investigated for its role in the oxidant/anti-oxidant system.

Future studies using VPA should also include antioxidant treatment during the time of exposure, to determine if the neural damage and social behavior deficits seen in these experiments and the experiments performed in Yochum et al. (2008) can be either attenuated or completely eliminated. While antioxidant treatment would not be able to correct any genetic sensitivity to insults which increase oxidative stress, it would potentially be able to protect against the damage caused by insults occurring during the antioxidant treatment. If promising results are seen from this proposed study, human autism treatment with antioxidants may be beneficial for either reducing autism phenotypes or preventing their occurrence.

In summary, the etiology of autism most likely includes an environmental component, a genetic component and an age component. Currently, the only understood etiological component of autism is the age of exposure. Given that autism is typically diagnosed on or before the age of three, the environmental insults the organism experiences must occur during early development. Since the sensitive time-point for exposure is known, the other two etiological components that need further testing are genetic sensitivities and types of environmental stressors.

The current study reproduces the damaging effects of VPA exposure to a developing organism in the form of behavioral changes and increases in cells staining for TUNEL. In addition, it includes a genetic component in the form of GSTM1 knockout mice. By manipulating the GSTM1 gene, our study produced a sex difference, which is a key feature seen in autism epidemiology with four times as many males being diagnosed as females. In terms of genotypic differences, our behavior and TUNEL staining studies lead us to believe that while the GSTM1 knockout male mice may be similar to wild-type males, the knockout of the GSTM1 gene confers susceptibility in female mice that the wild-type females do not have. We propose that male wild-type animals may be initially susceptible, whereas wild-type females are somehow protected from toxicant induced damage. Furthermore, from our previous TUNEL stain findings and the findings from this study, it can be concluded that the 400mg/kg dose of VPA given at P14 will cause increased staining for TUNEL in the cerebellum and hippocampus of mice, regardless of strain, genotype and sex.

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FIGURE LEGENDS

Figure 1: Eleven kb fragment containing all 8 exons of GSTM1 was amplified and subcloned in MC1TK-containing vector (Part A). A loxP-neo-Lox P cassette was inserted to replace exons 1-5 (Part B), and final construct was obtained in Bluescript vector (Part C).

Figure 2: A. GSTM1 knockout chimeras with agouti coat color. B. Genotyping of the F1 progeny of *GstM1(+/-)* mice; PCR was performed using one common forward primer and two reverse primers to obtain a 390-bp product (a) for wild type allele and a 216-bp product (b) for mutant allele. Animals 1, 5 are wild type; 3, 4 - are heterozygous and 2, 6 are homozygous GSTM1 knockout mice.

Figure 3: Average number of granule cells positively stained for apoptosis taken from three sections of randomly chosen sections of seven micron thick sagittal section of the granule cell layer of lobe 3 of the cerebellum (taken at 400x magnification) 24 hours following either sodium valproate injection (VPA, 400mg/kg s.c.) or saline control at P14 in male and female GSTM1 wild-type and knockout pups.

* indicates significantly different from saline treated mice.

** indicates significantly different from saline treated mice and from VPA treated knockout mice of both sexes and VPA treated wild-type male mice.

Figure 4: Average number of granule cells positively stained for apoptosis taken from three sections of randomly chosen sections of seven micron thick sagittal sections of

the dentate gyrus area of the hipopcampus (taken at 400x magnification) 24 hours following either sodium valproate injection (VPA, 400mg/kg s.c.) or saline control at P14 in male and female GSTM1 wild-type and knockout pups.

* indicates significantly different from saline treated mice.

** indicates significantly different from saline treated mice and from VPA treated knockout mice of both sexes and VPA treated wild-type male mice.

Figure 5: Photomicrographs of seven micron thick sagittial sections of the external granule cell layer of lobe three of the cerebellum (taken at 400x magnification) taken 24 hours following either sodium valproate injection (VPA, 400mg/kg s.c.) or saline control on P14 in female and male GSTM1 knockout mouse pups. Red cells indicate TUNEL staining (a marker of apotosis).

Figure 6: Photomicrographs of seven micron thick sagittial sections of the external granule cell layer of lobe three of the cerebellum (taken at 400x magnification) taken 24 hours following either sodium valproate injection (VPA, 400mg/kg s.c.) or saline control on P14 in female and male GSTM1 wild-type mouse pups. Red cells indicate TUNEL staining (a marker of apotosis).

Figure 7: Photomicrographs of seven micron thick sagittial sections of the dentate gyrus of the hippocampus (taken at 400x magnification) taken between 12 and 24 hours following either sodium valproate injection (VPA, 400mg/kg s.c.) or saline

control on P14 in female and male GSTM1 knockout mouse pups. Red cells indicate TUNEL staining (a marker of apotosis).

Figure 8: Photomicrographs of seven micron thick sagittial sections of the dentate gyrus of the hippocampus (taken at 400x magnification) taken between 12 and 24 hours following either sodium valproate injection (VPA, 400mg/kg s.c.) or saline control on P14 in female and male GSTM1 wild-type mouse pups. Red cells indicate TUNEL staining (a marker of apotosis).

Figure 9: Number of crawl-under behaviors completed by sex, genotype and treatment-matched pairs of GSTM1 wild-type and knockout mice over a 30 minute open field trial (run between post-natal days 30-40) following P14 sodium valproate (VPA, 400 mg/kg s.c.) or saline treatment.

* indicates significantly different from saline and VPA treated wildtype mice and VPA treated GSTM1 knockout mice..

** indicates significantly different from saline treated knockout mice.

Figure 10: Number of alo-groom behaviors completed by sex, genotype and treatment-matched pairs of GSTM1 wild-type and knockout mice over a 30 minute open field trial (run between post-natal days 30-40) following P14 sodium valproate (VPA, 400 mg/kg s.c.) or saline treatment.

* indicates significantly different from wild-type male pups and GSTM1 knockout male and female pups of both treatments.

Figure 11: Number horizontal movements completed by sex, genotype and treatment-matched pairs of GSTM1 wild-type and knockout mice over a 30 minute open field trial (run between post-natal days 30-40) following P14 sodium valproate (VPA, 400 mg/kg s.c.) or saline treatment.

* indicates significantly different from GSTM1 knockout pup pairs of both sexes and treatments and VPA-treated wild-type pup pairs.

**indicates significantly different from GSTM1 pup pairs of both sexes and treatments and saline-treated wild-type pup pairs.

Figure 12: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Cerebellum serotonin levels were calculated using HPLC.

* indicates a genotype effect with saline treated GSTM1 knockouts having increased levels of serotonin in the cerebellum compared to wild-type pups of both treatments.

** indicates a genotype by treatment effect with VPA treated GSTM1 knockout pups having significantly reduced levels of serotonin compared to their saline counterparts.

Figure 13: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Cerebellum serotonin turnover levels (5-HIAA/5-HT) were calculated using HPLC.

* indicates a treatment effect with VPA treated pups having significantly more serotonin turnover.

Figure 14: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Cerebellum dopamine levels were calculated using HPLC.

* indicates a genotype effect with GSTM1 knockout pups having significantly more dopamine than wild-type controls.

** indicates a treatment effect with VPA causing increases in dopamine levels as compared to saline treated pups.

Figure 15: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Cerebellum DOPAC levels were calculated using HPLC.

* indicates a treatment effect with VPA causing significant decreases in DOPAC.

Figure 16: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Cerebellum DOPAC turnover levels (DOPAC/DA) were calculated using HPLC.

* indicates a genotype effect with GSTM1 knockout pups having significantly reduced levels of DOPAC/DA turnover than wild-type pups.

** indicates a treatment effect with VPA causing significant decreases in DOPAC/DA turnover for both genotypes.

Figure 17: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Hippocampus serotonin levels were calculated using HPLC.

* indicates a treatment effect with VPA treated animals having significantly greater levels of serotonin in the hippocampus.

Figure 18: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Hippocampus 5-HIAA levels were calculated using HPLC.

* indicates a treatment effect with VPA treated animals having significantly greater levels of 5-HIAA in the hippocampus.

Figure 19: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Hippocampus DOPAC levels were calculated using HPLC.

* indicates a treatment effect VPA treated animals having significantly lower levels of DOPAC in the hippocampus.

Figure 20: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Hippocampus DOPAC turnover levels (DOPAC/DA) were calculated using HPLC.

* indicates a treatment effect VPA treated animals having significantly lower levels of DOPAC/DA turnover in the hippocampus

Figure 21: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Hippocampus HVA levels were calculated using HPLC.

* indicates a genotype by treatment by sex effect with wild-type females receiving VPA having significant increases in HVA.

Figure 22: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Hippocampus HVA turnover levels (HVA/DA) were calculated using HPLC.

* indicates a treatment effect with VPA causing significant increases in HVA/DA turnover in the hippocampus in both genotypes.

Figure 23: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Striatum serotonin turnover levels (5-HIAA/5-HT) were calculated using HPLC.

* indicates a treatment effect with VPA causing significant increases in serotonin turnover in the hippocampus in both genotypes.

Figure 24: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Striatum DOPAC levels were calculated using HPLC.

* indicates a treatment effect with VPA causing significant increases in DOPAC levels in the hippocampus in both genotypes.

Figure 25: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Frontal cortex serotonin levels were calculated using HPLC.
* indicates a genotype effect with saline treated GSTM1 knockout pups having significant increases in serotonin compared to saline treated wild-type pups.

Figure 26: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Frontal cortex serotonin turnover levels (5-HIAA/5-HT) were calculated using HPLC.
* indicates a sex by treatment by sex effect with VPA treated wild-type females having increased levels of serotonin turnover as compared to all GSTM1 knockout animals, saline treated wild-type females, and wild-type males of both treatment groups.

Figure 27: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Frontal cortex dopamine levels were calculated using HPLC.
* indicates a treatment effect with VPA treated pups of both genotypes having significant increases in dopamine levels.

Figure 28: Wild-type and knockout mice were injected with 400 mg/kg of either VPA or saline on P14 and sacrificed on P30. Frontal cortex HVA levels were calculated using HPLC.

*indicates a genotype effect with saline treated GSTM1 knockout pups having significant increases in HVA compared to saline treated wild-type pups.

** indicates a genotype by treatment effect with VPA treated wild-type pups having significant increases in HVA levels as compared to saline treated wild-type pups.

Figure 29: Balb/c mice were injected at either P14 or P60 with either 400 mg/kg of VPA or saline control and then sacrificed 12 hours later. Whole brains were paraffin embedded, sliced, and TUNEL stained. Photomicrographs were taken of lobe 3 of the cerebellum. Red dots indicate cells staining for TUNEL.

Figure 1: GSTM1 Genetics Part I

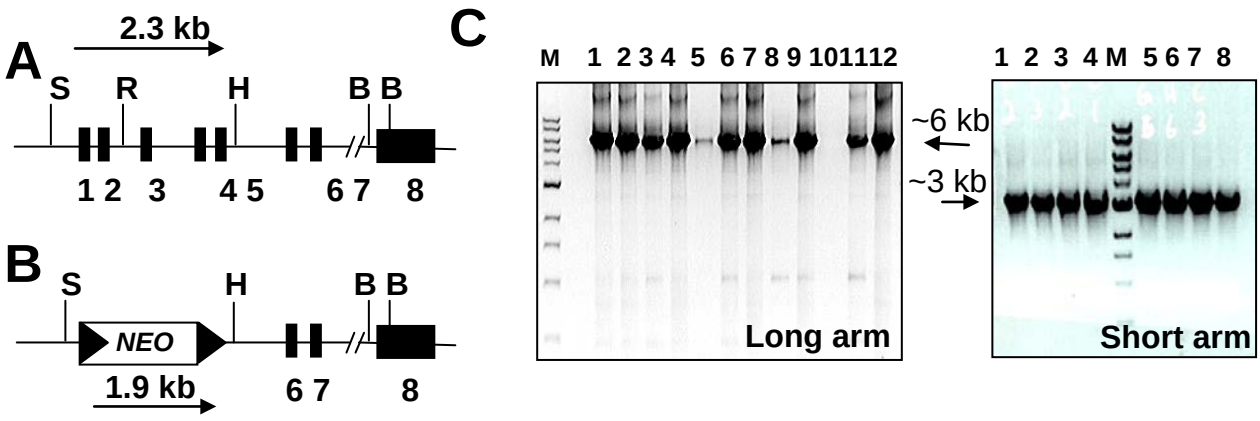


Figure 2: GSTM1 Genetics Part II

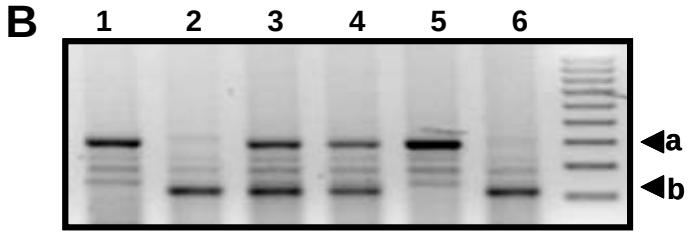


Figure 3: TUNEL Staining Results

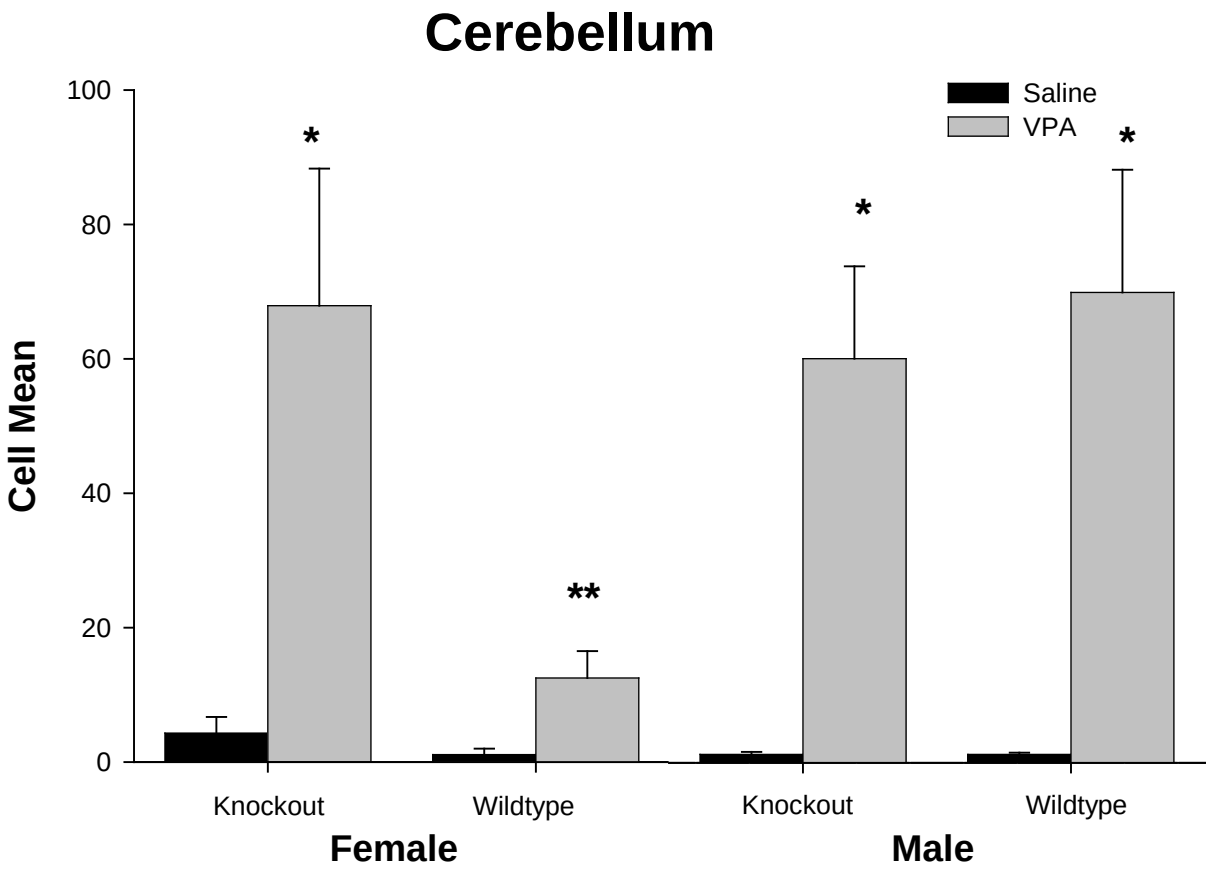


Figure 4: TUNEL Staining Results

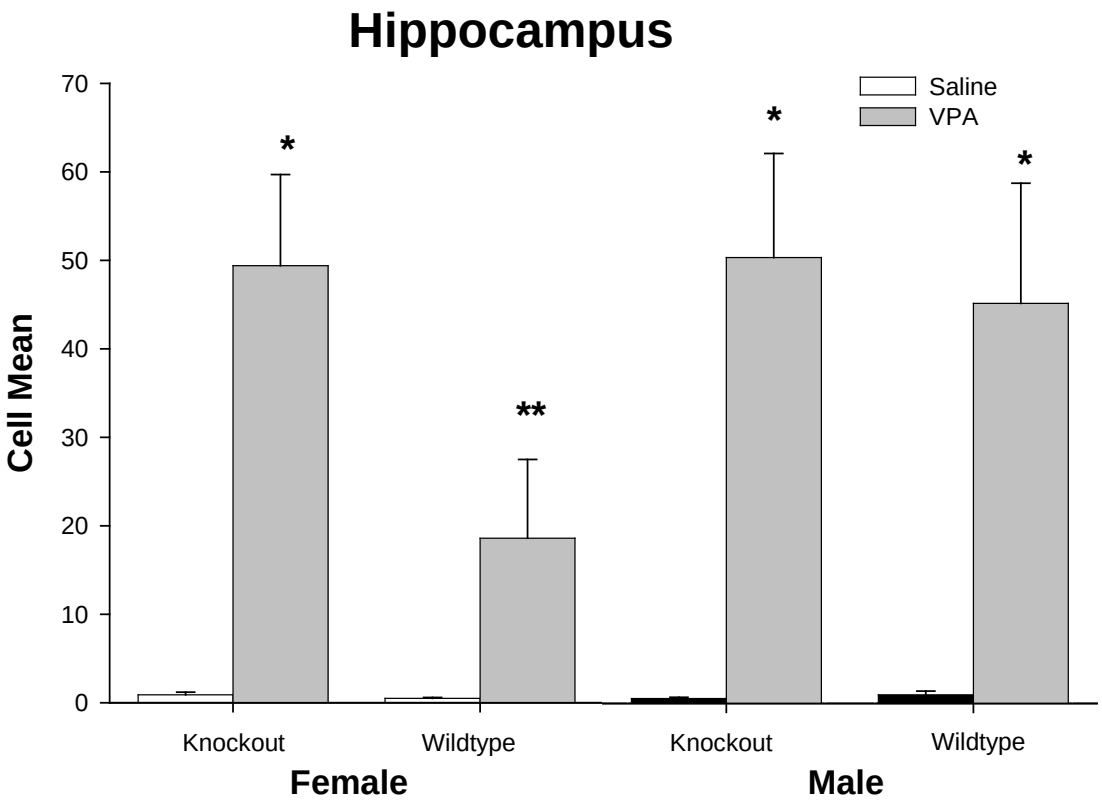


Figure 5: Photomicrographs of TUNEL Staining

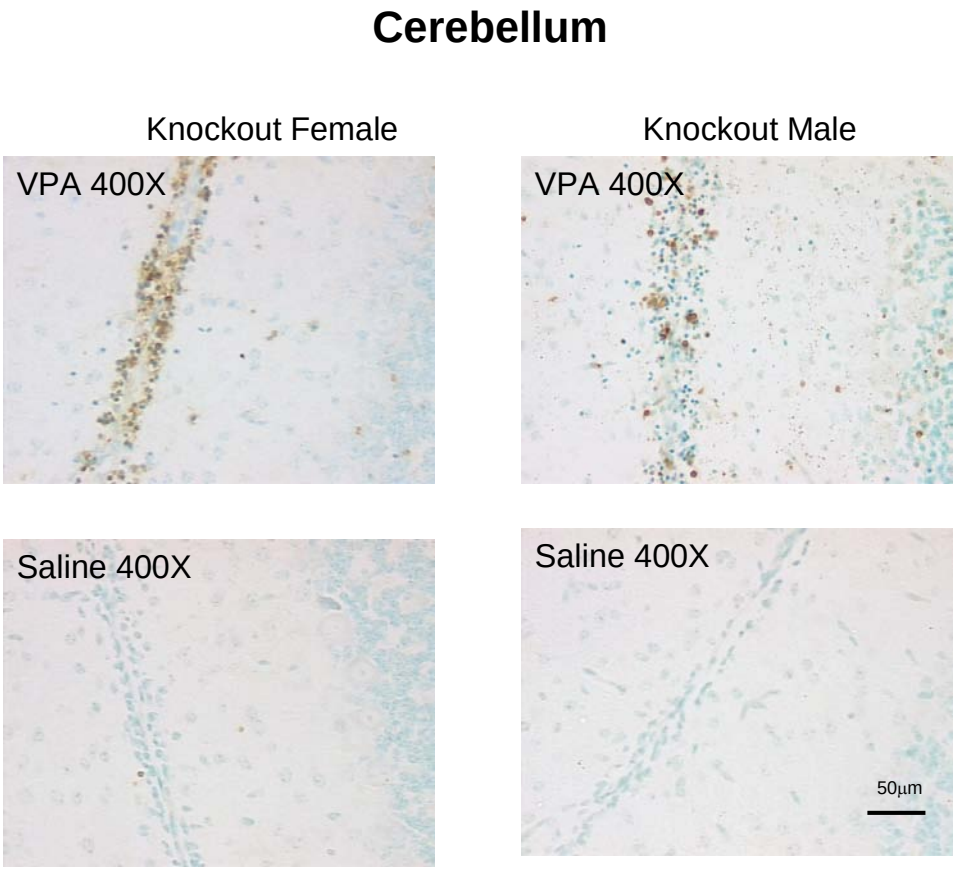


Figure 6: Photomicrographs of TUNEL Staining

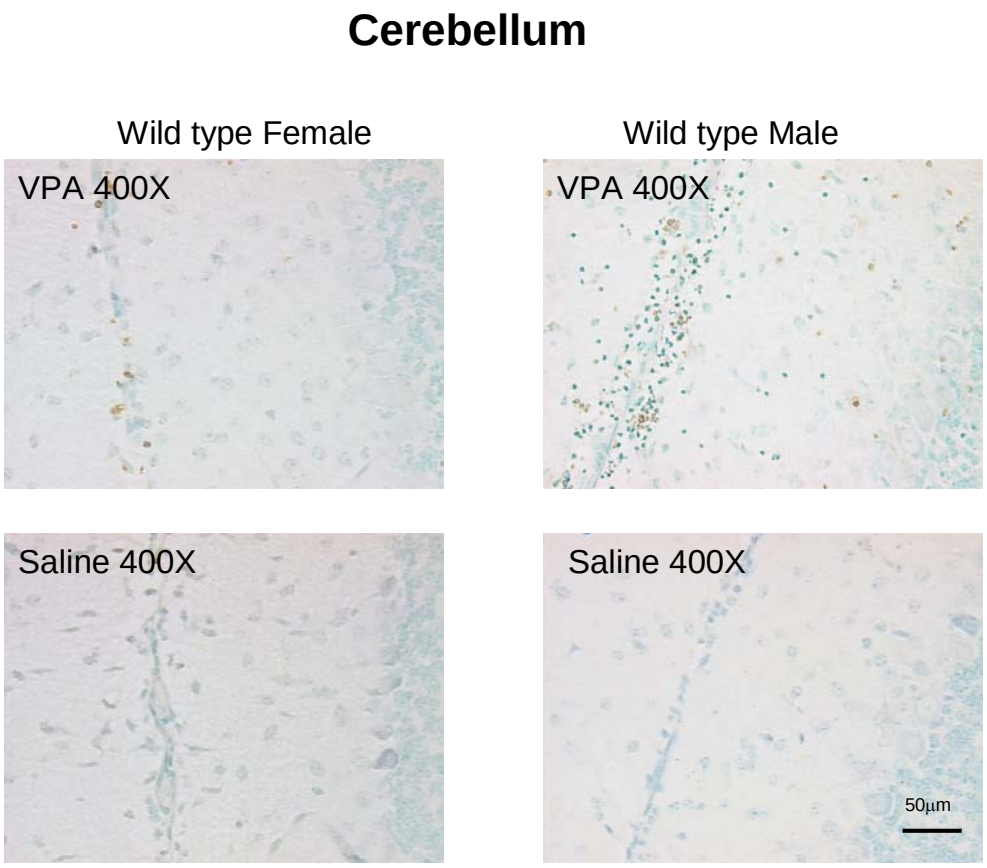


Figure 7: Photomicrographs of TUNEL Staining

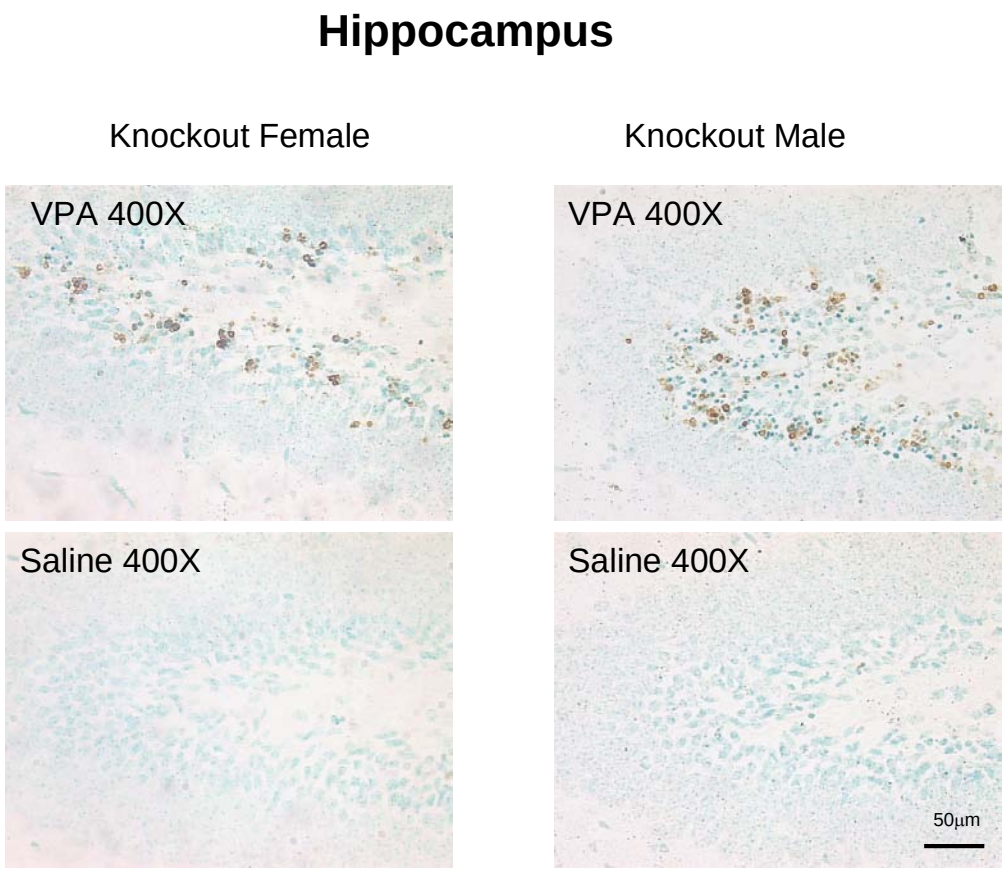


Figure 8: Photomicrographs of TUNEL Staining

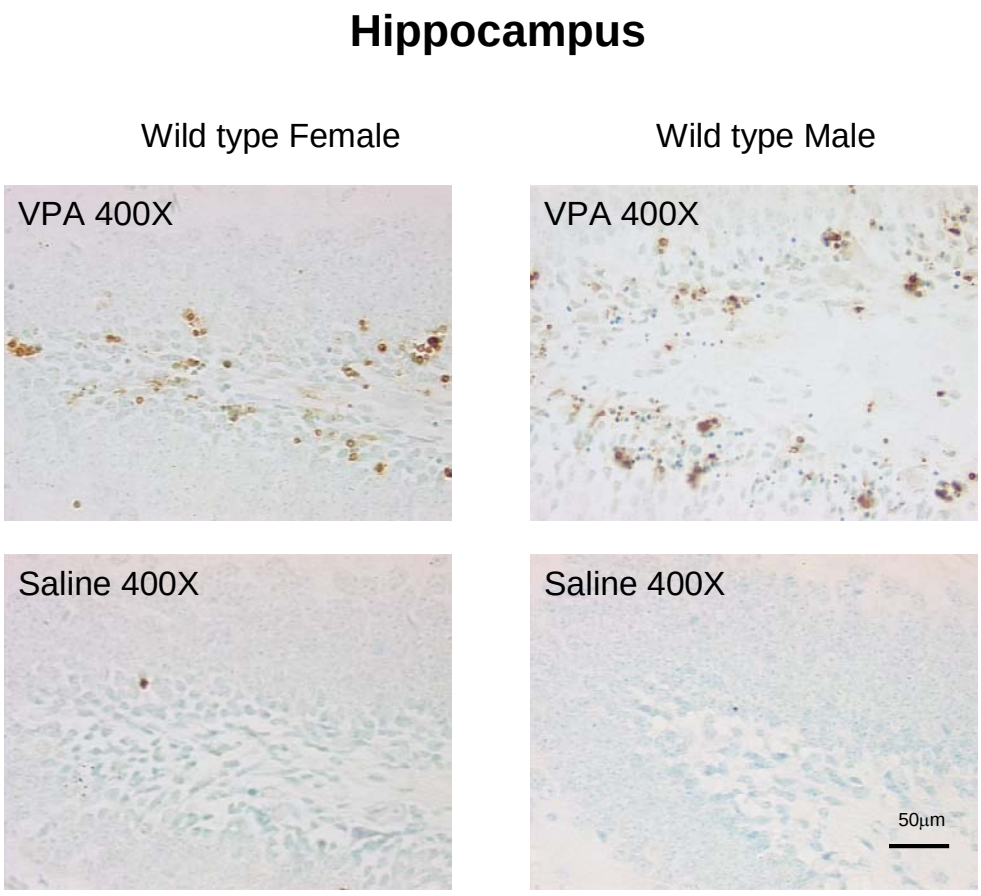
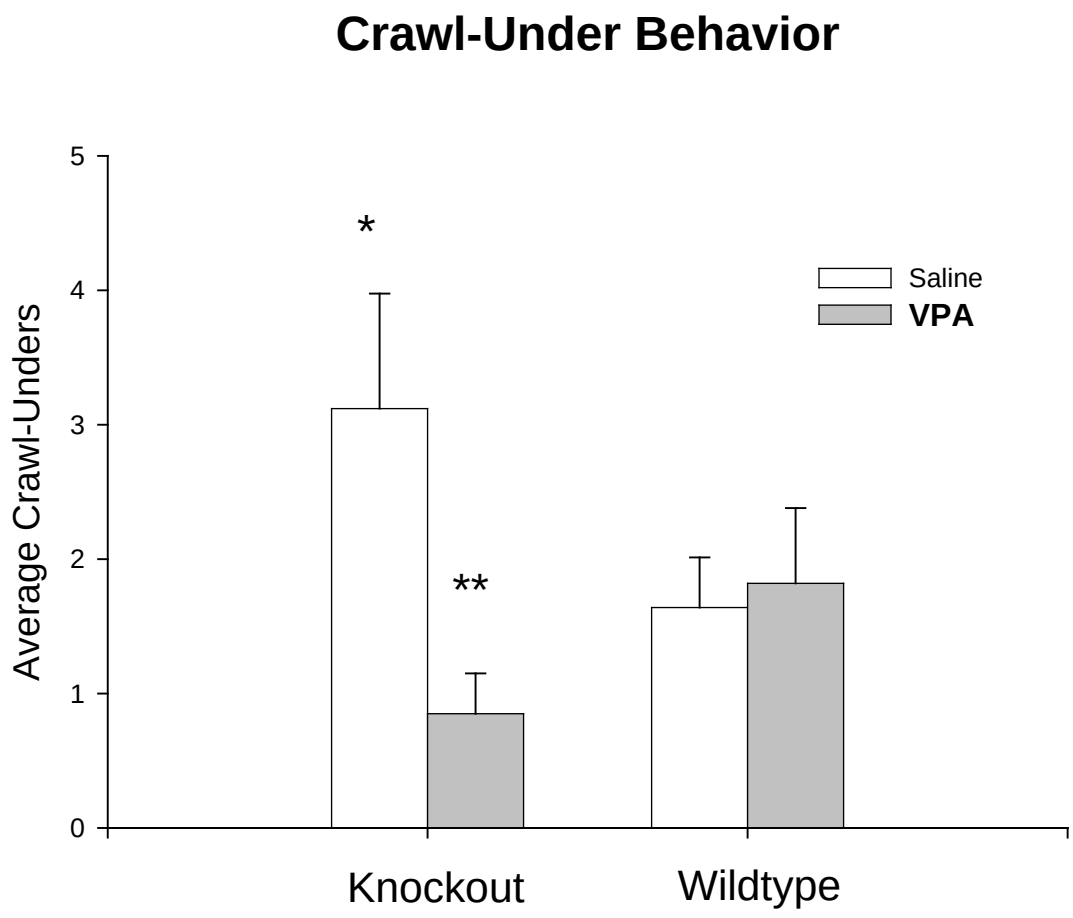


Figure 9: Play Behavior



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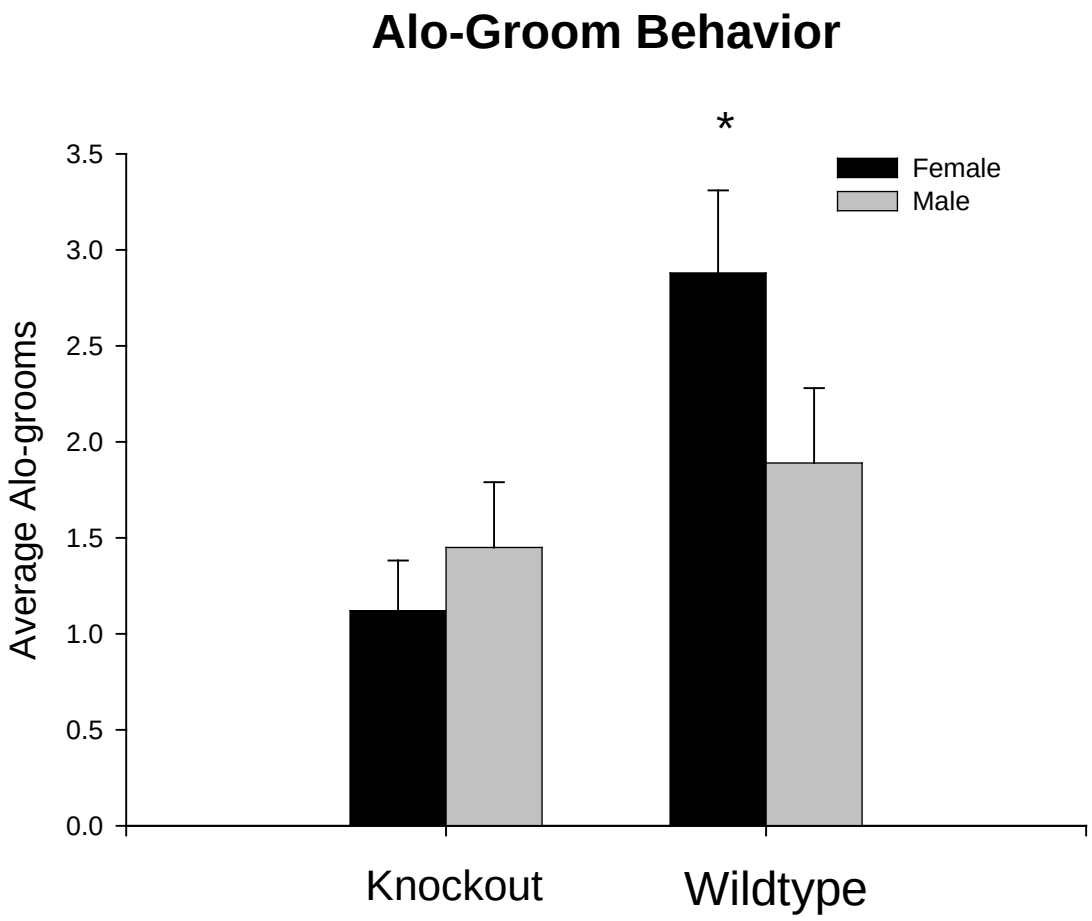


Figure 11: Play Behavior

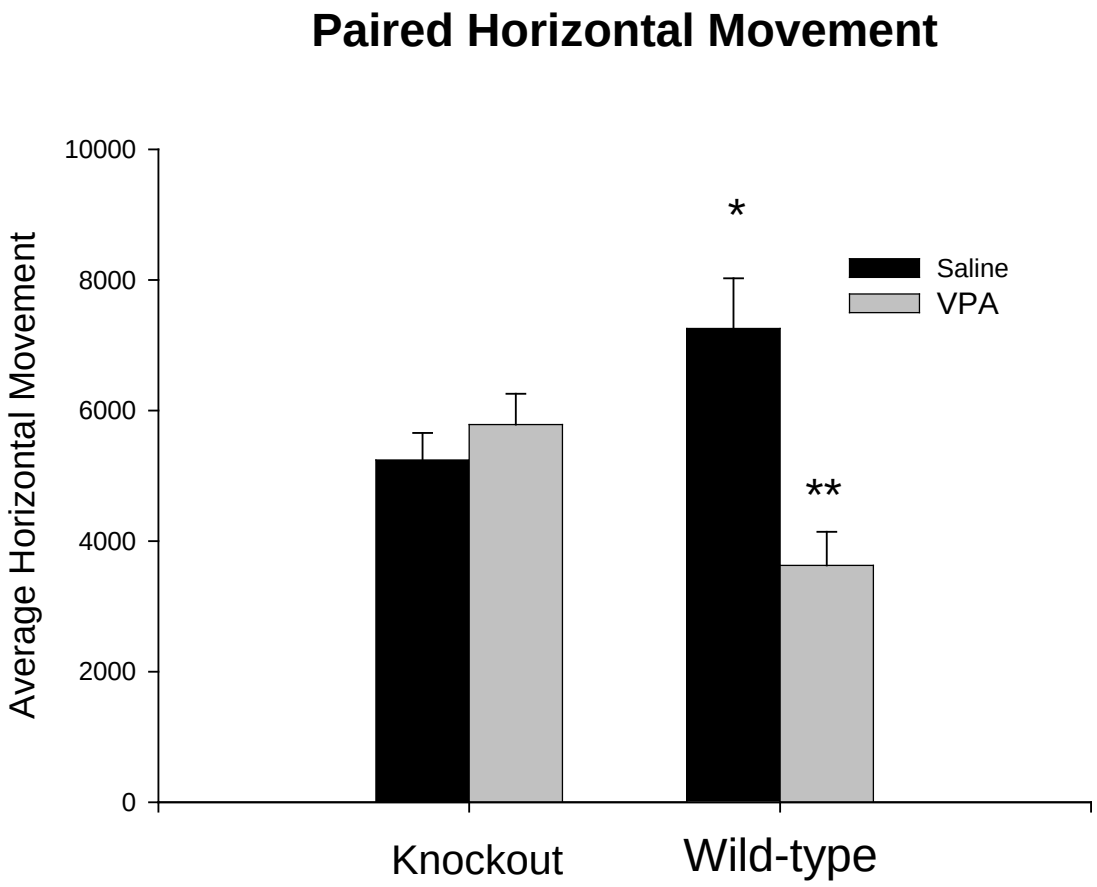
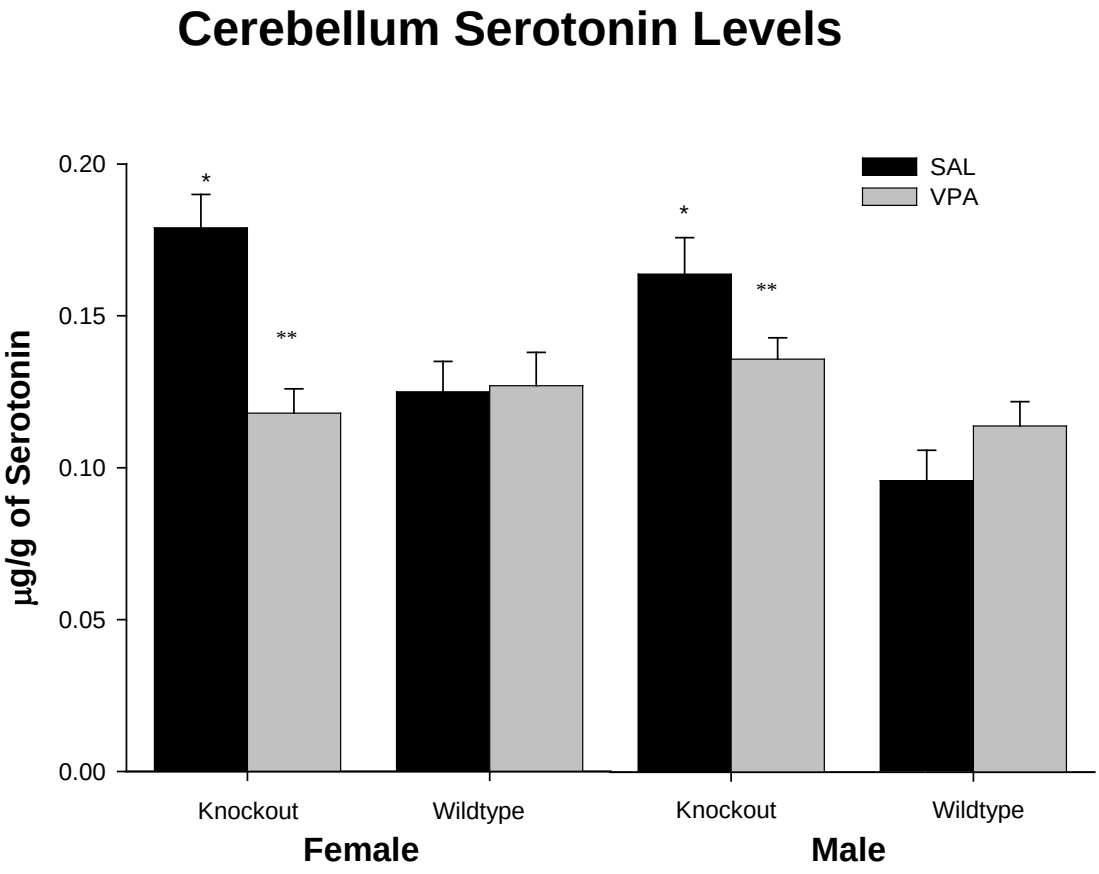


Figure 12: Neurochemistry



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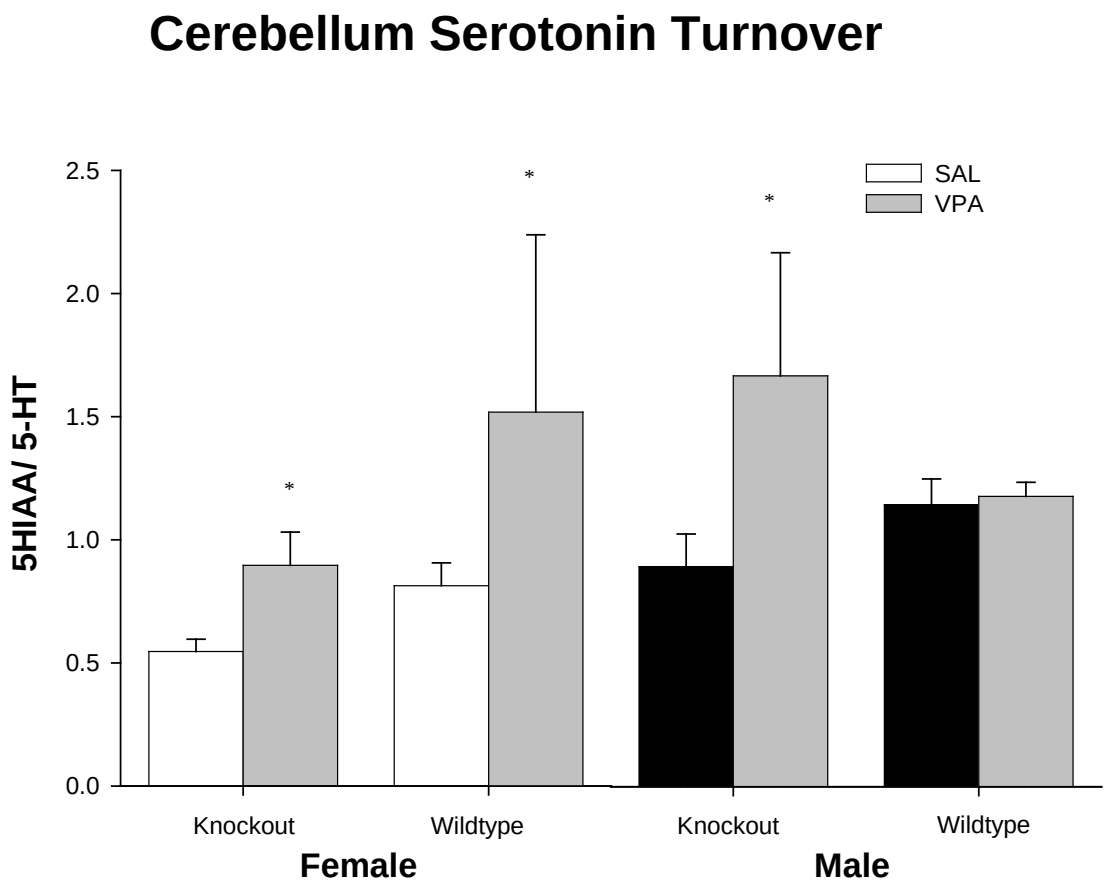

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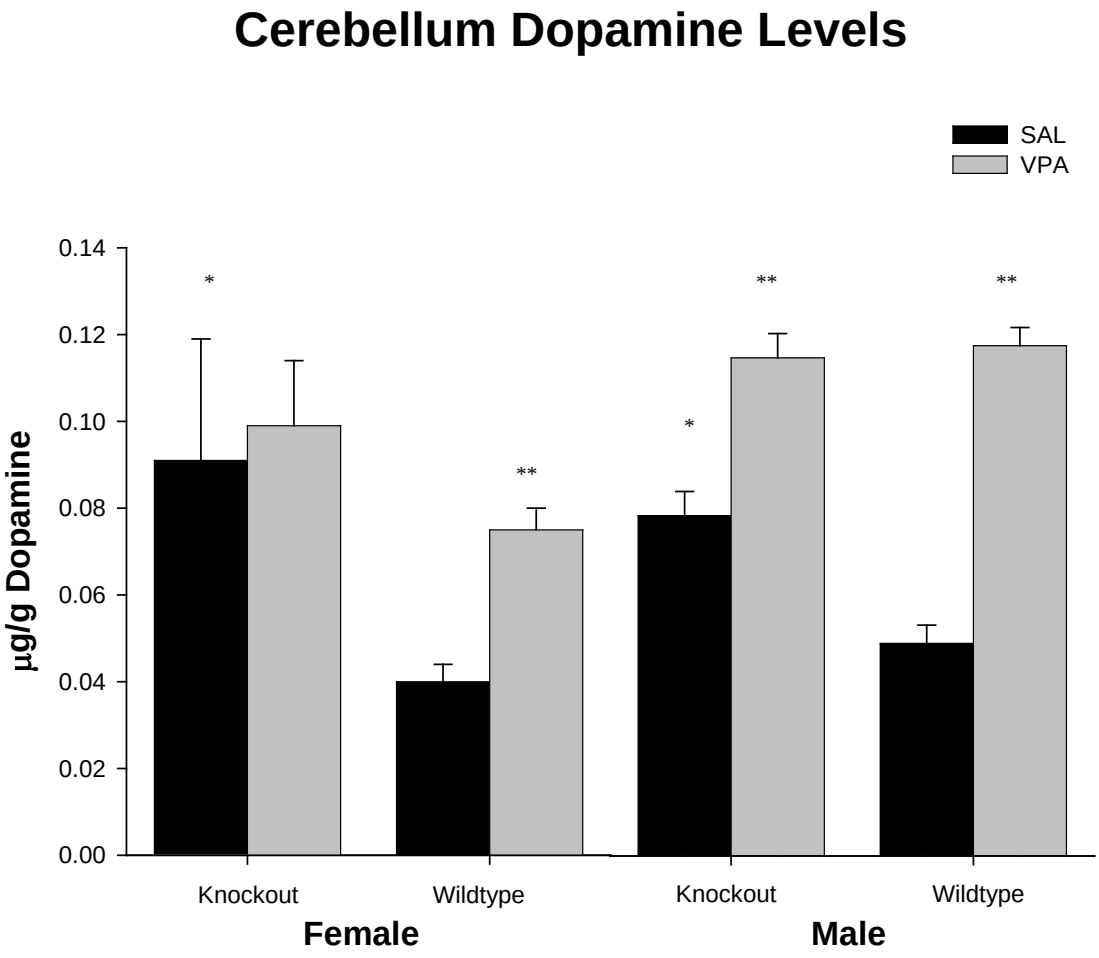

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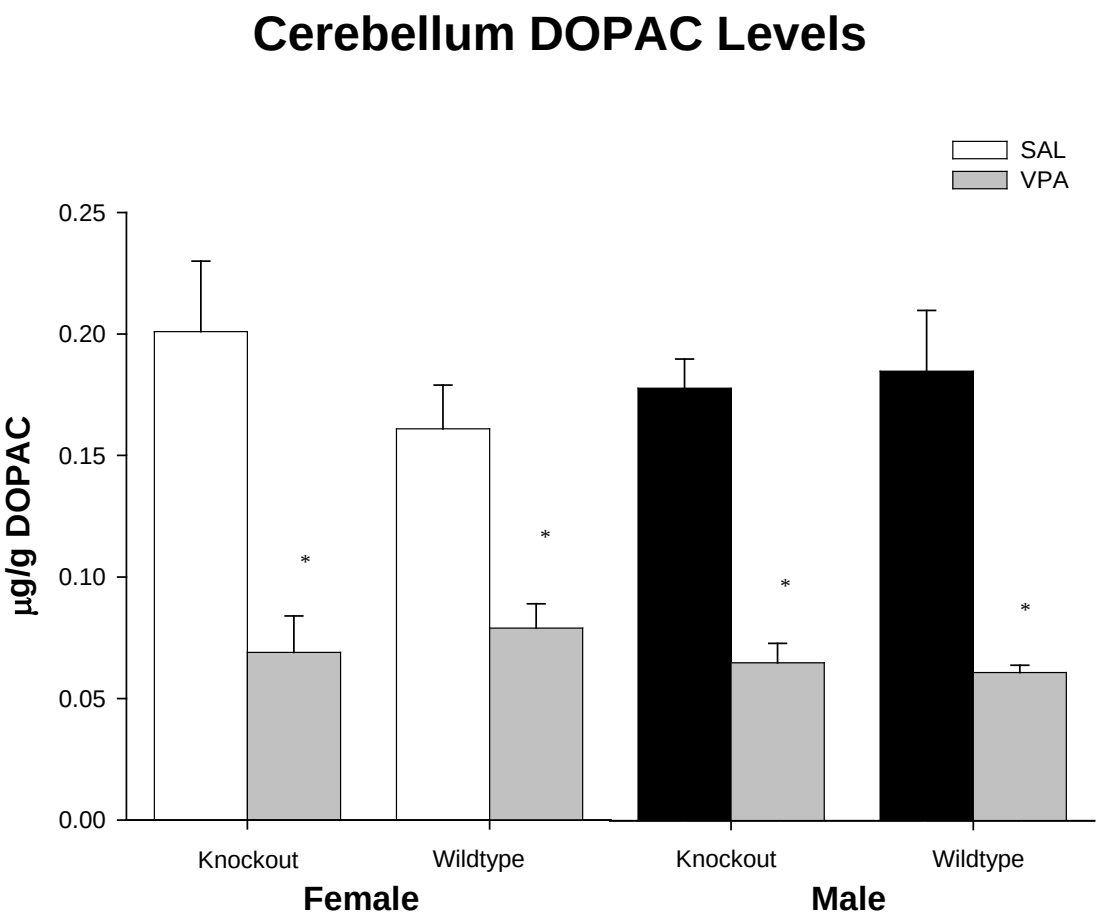

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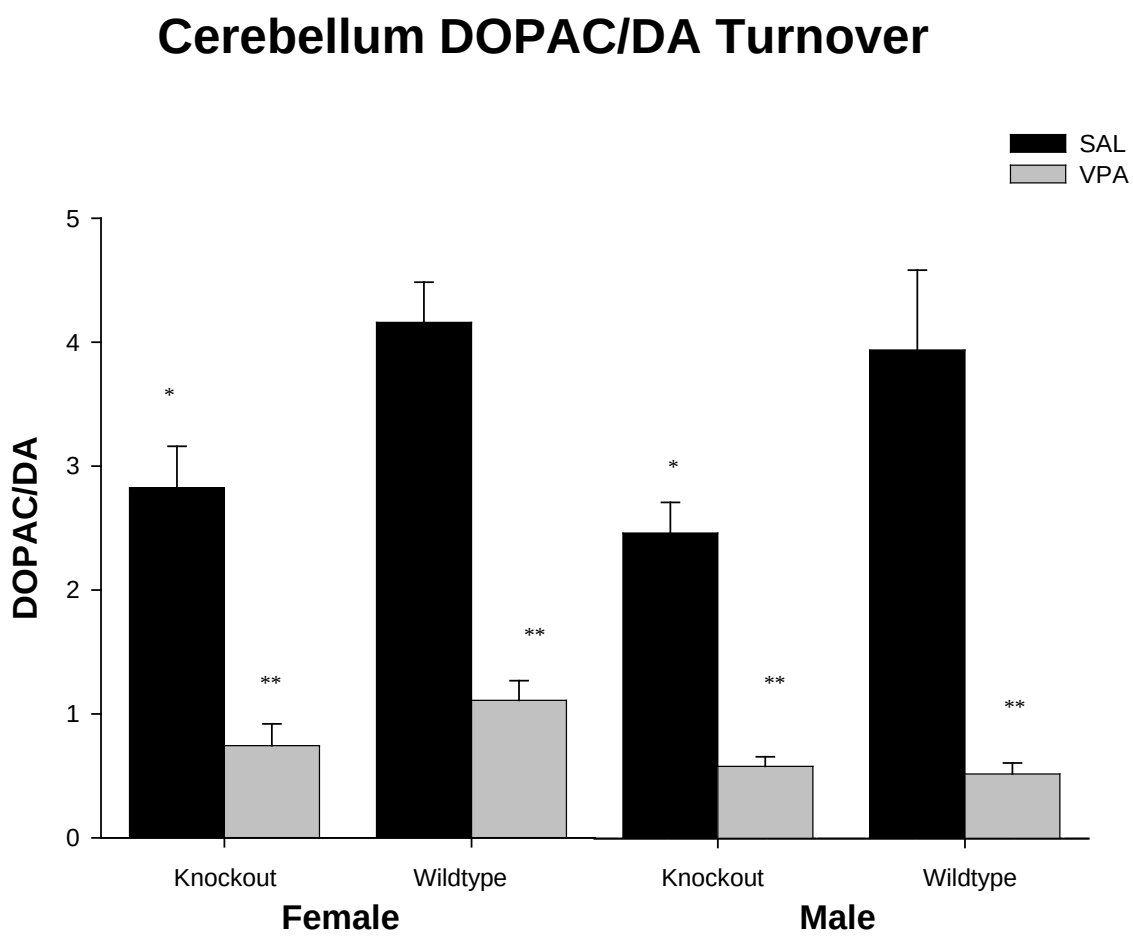

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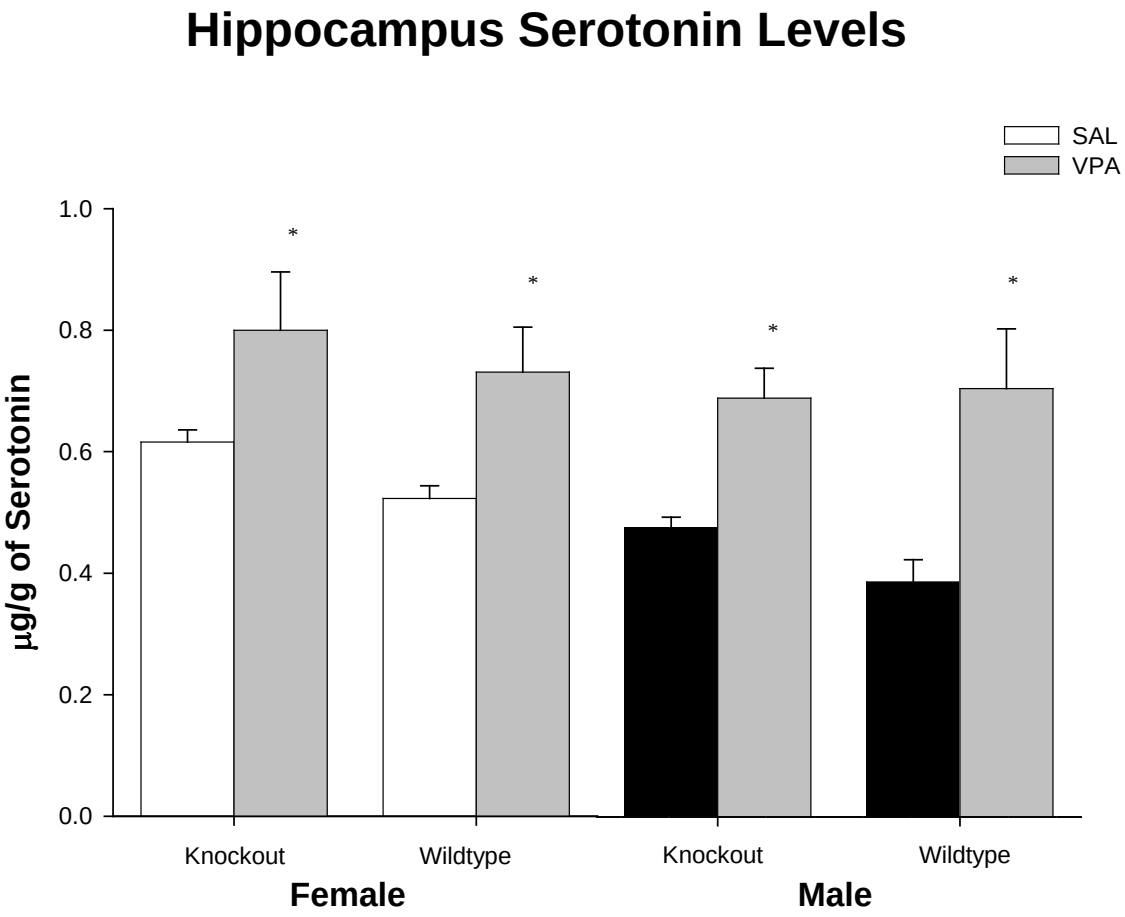
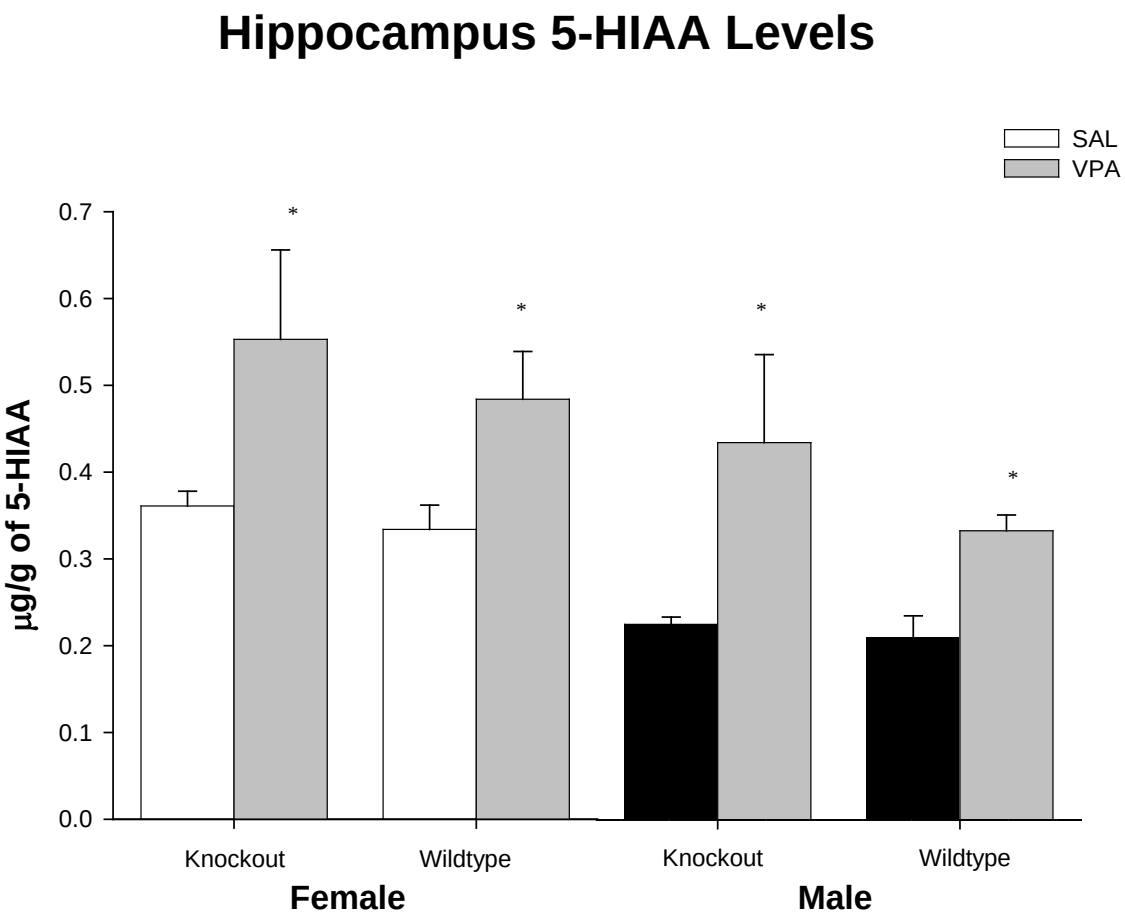


Figure 18: **Neurochemistry**



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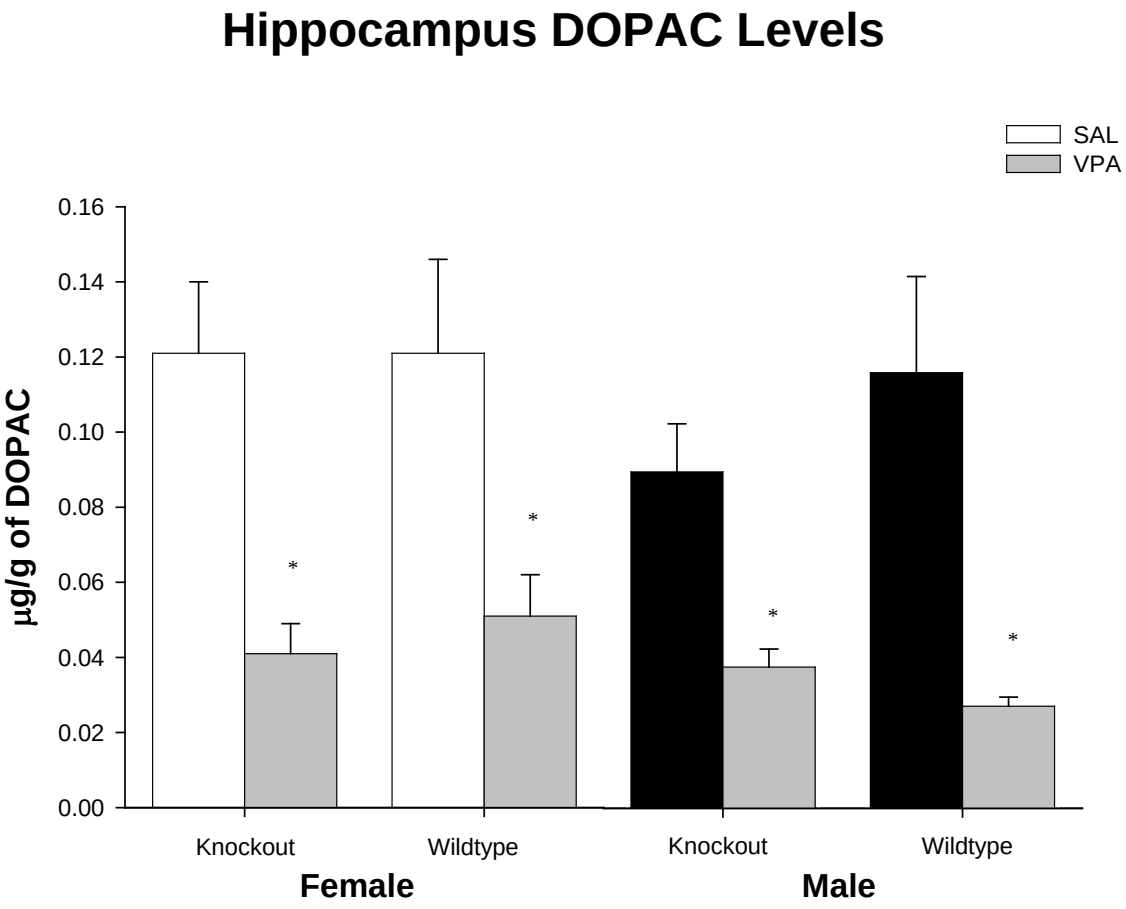

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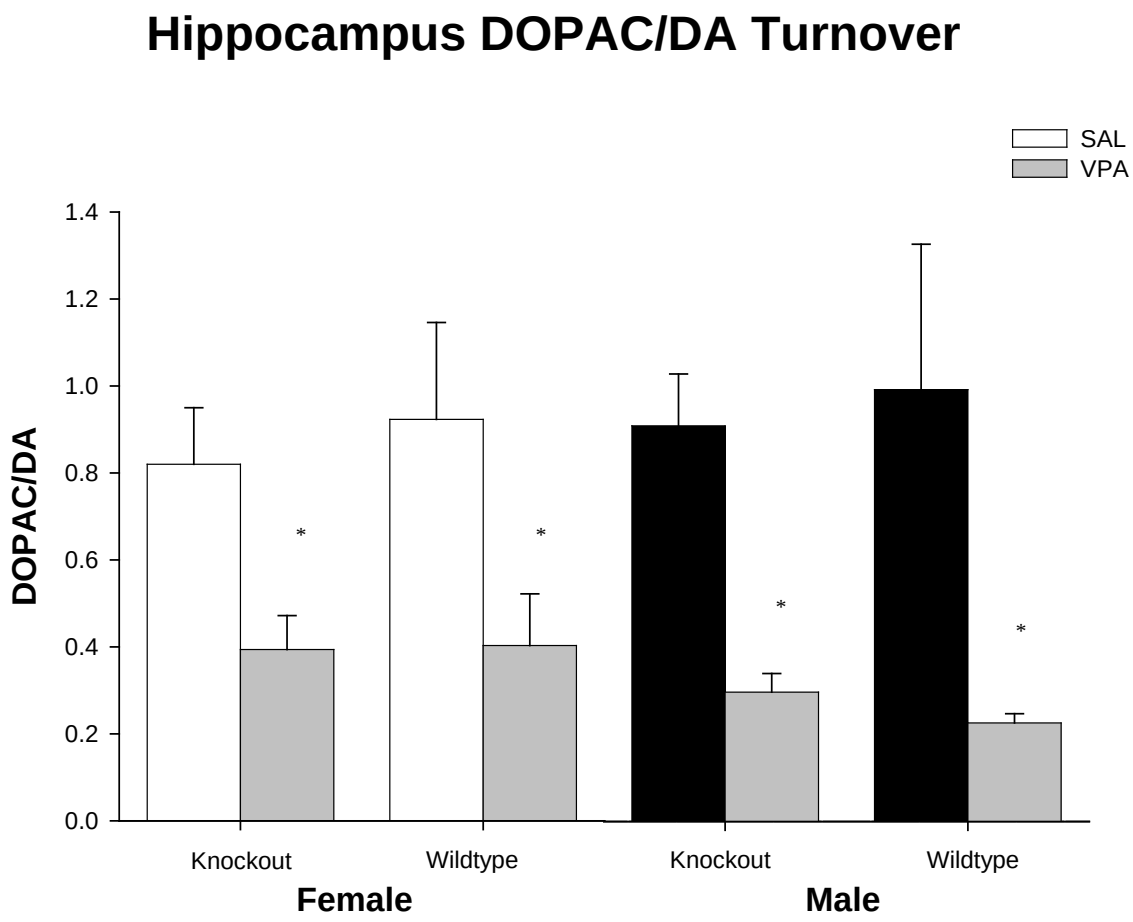

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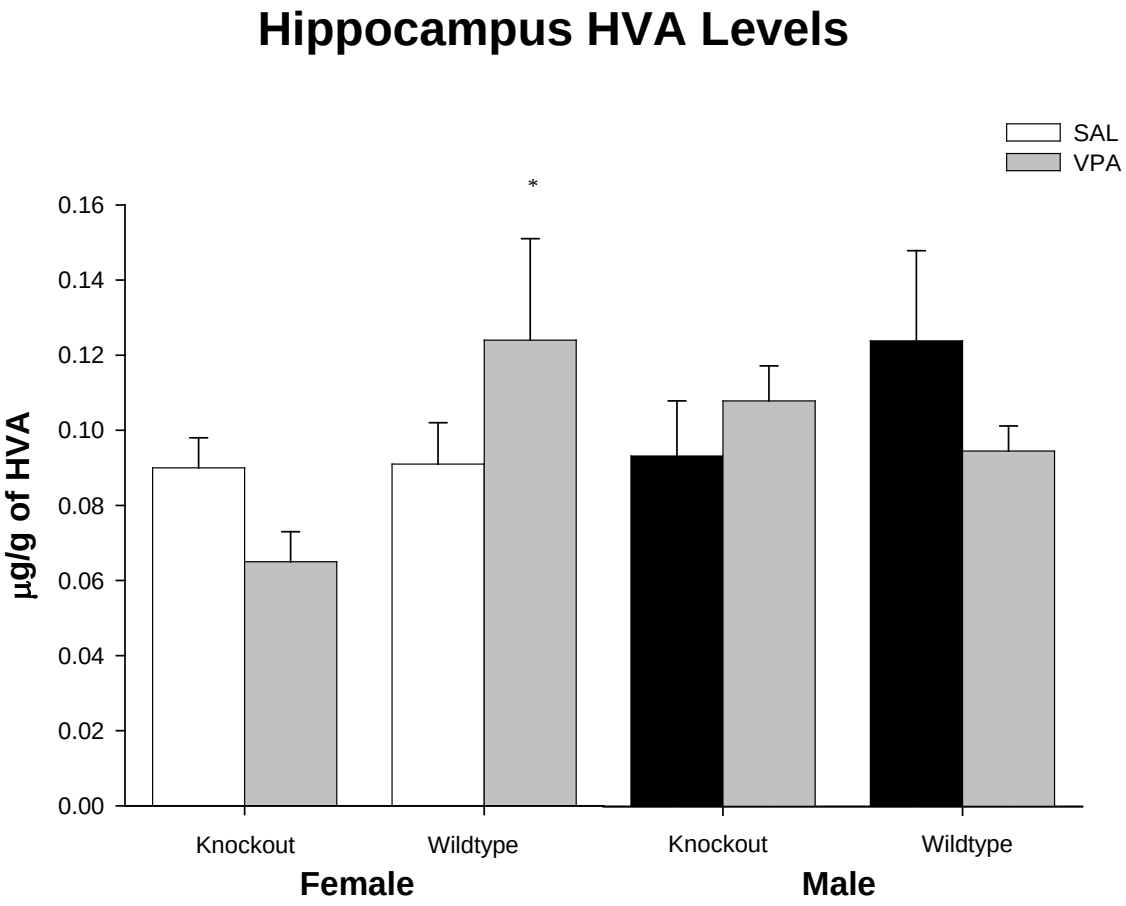

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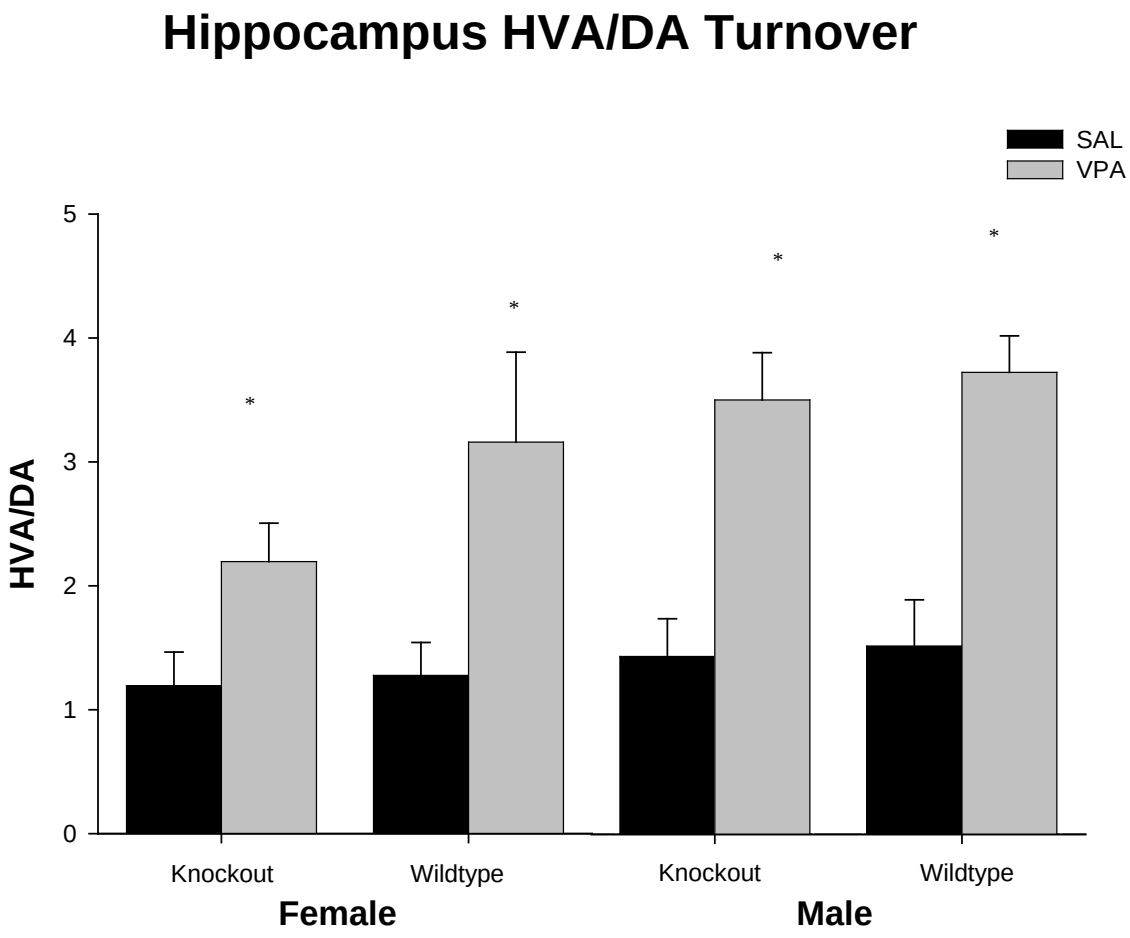

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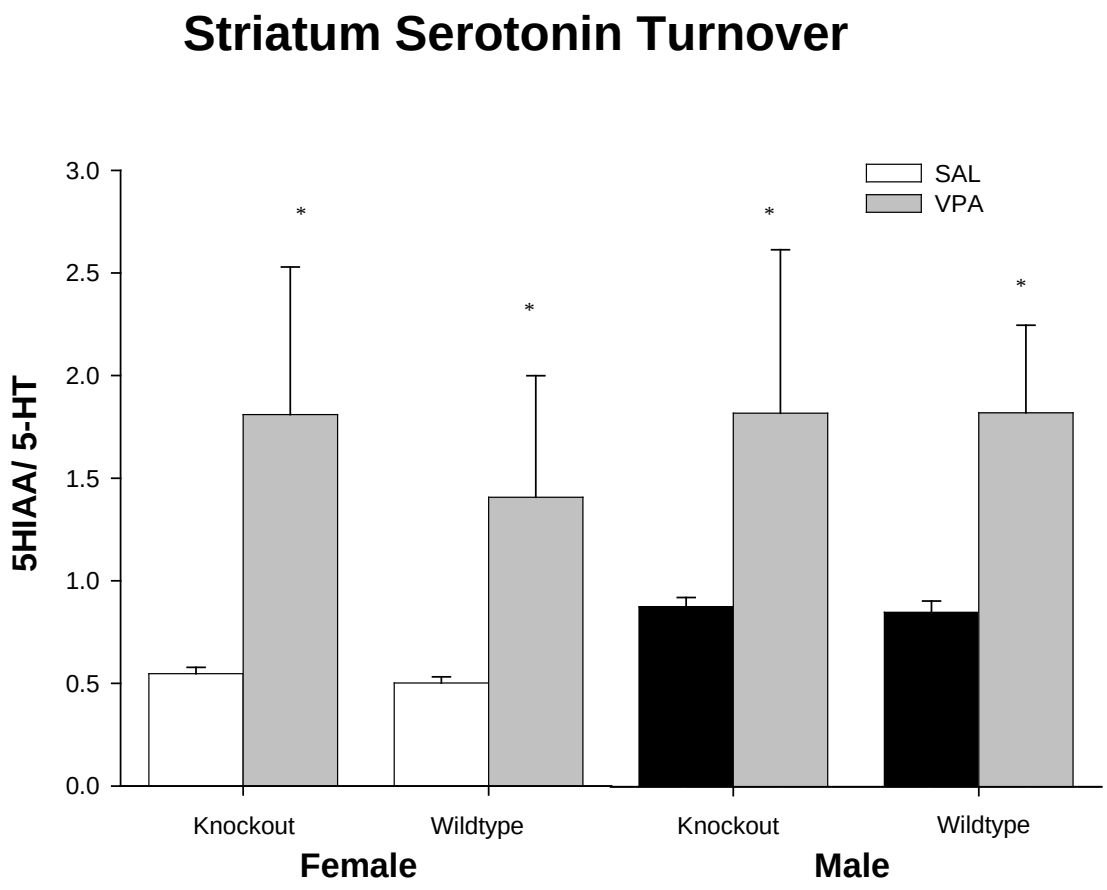

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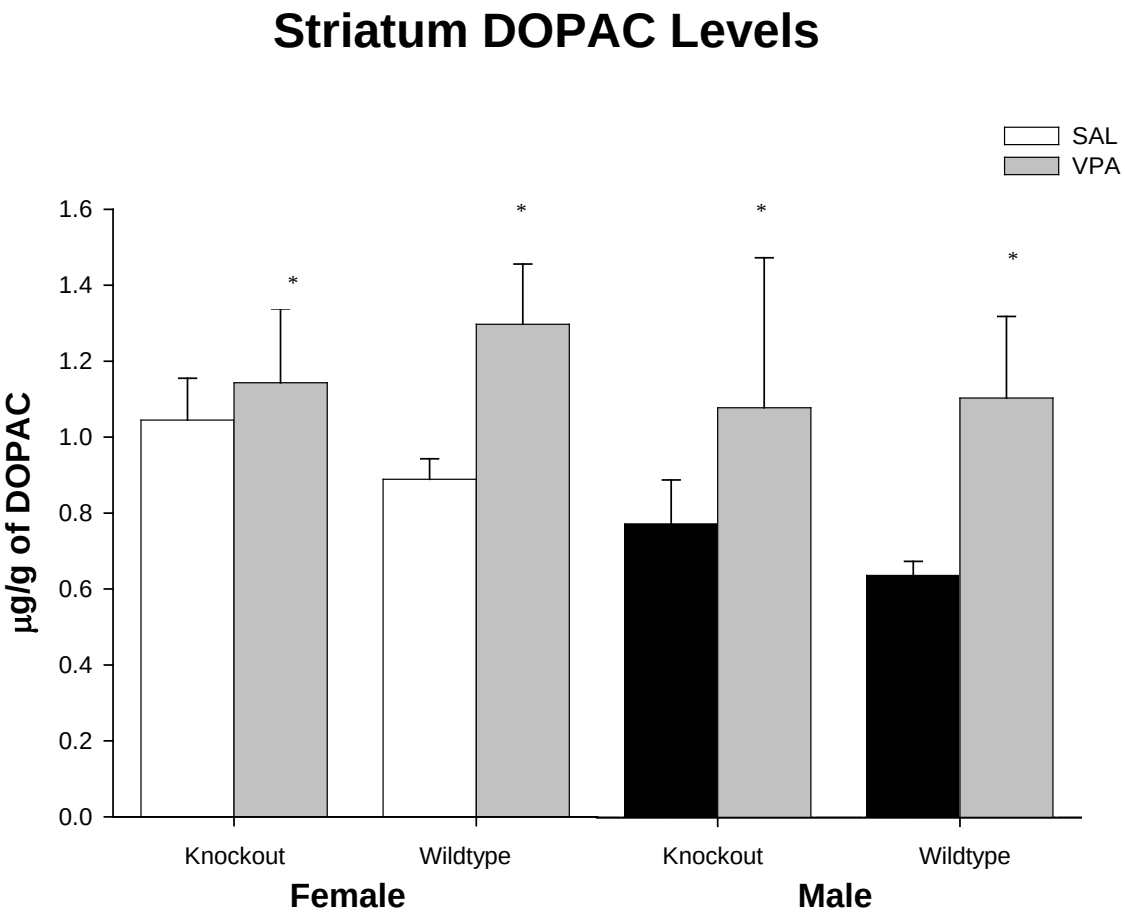

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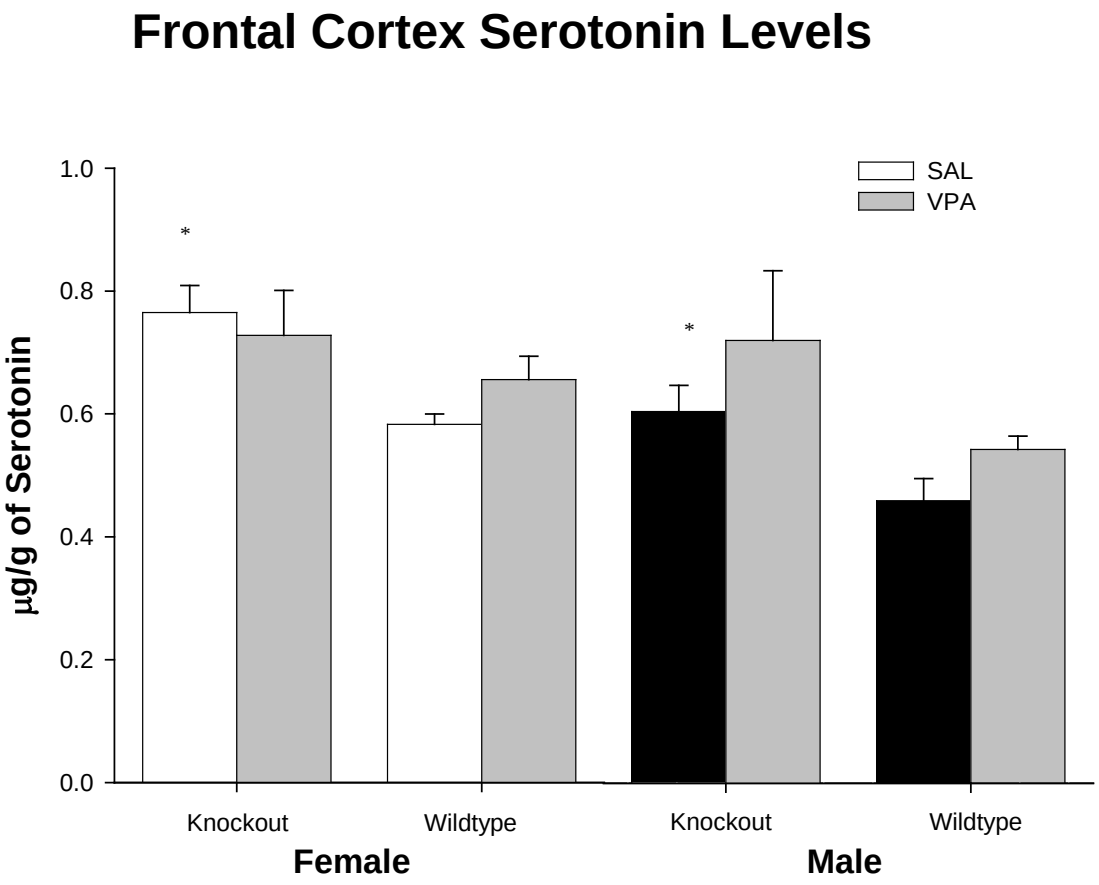

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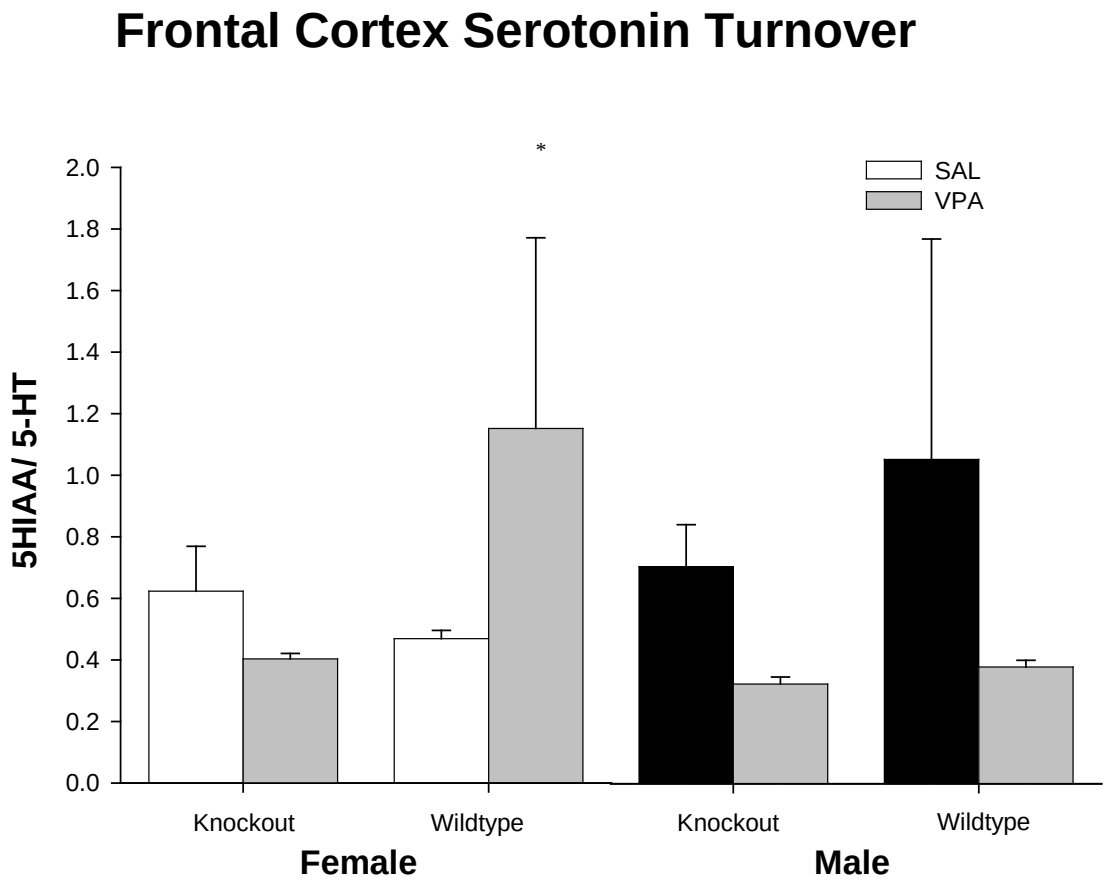

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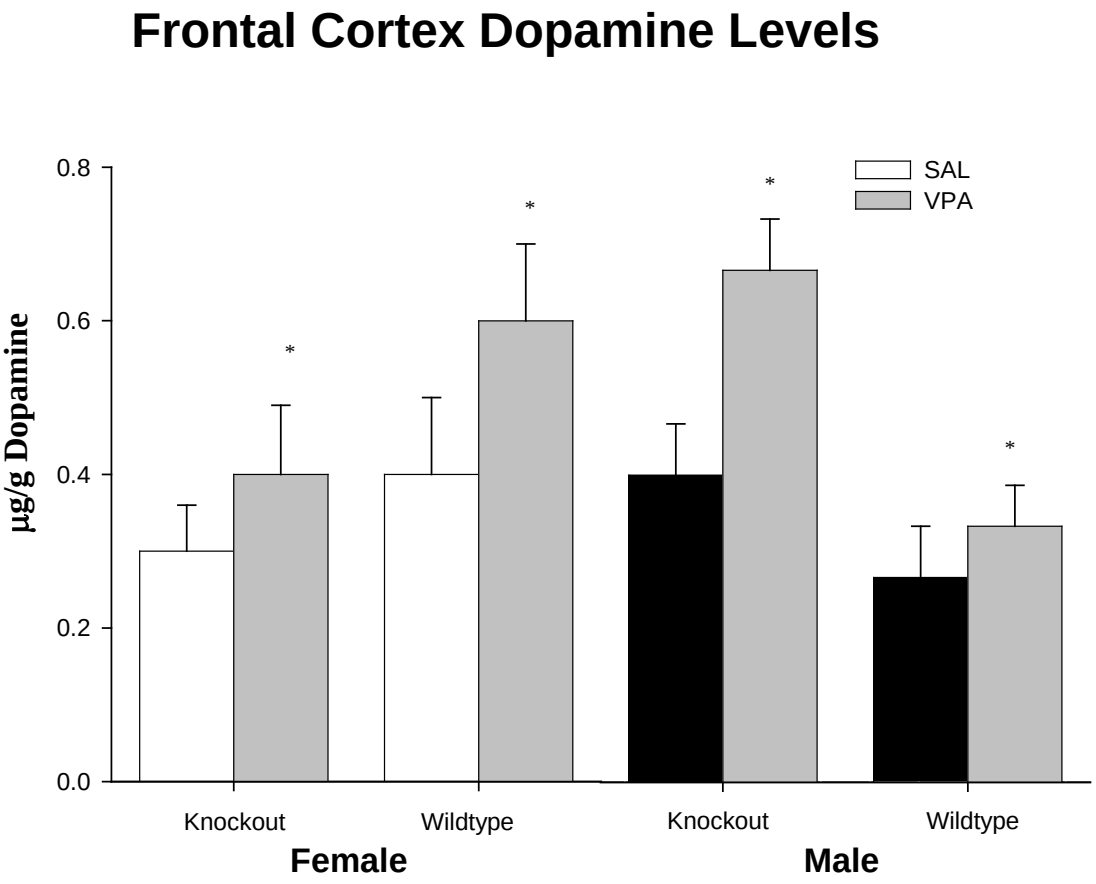

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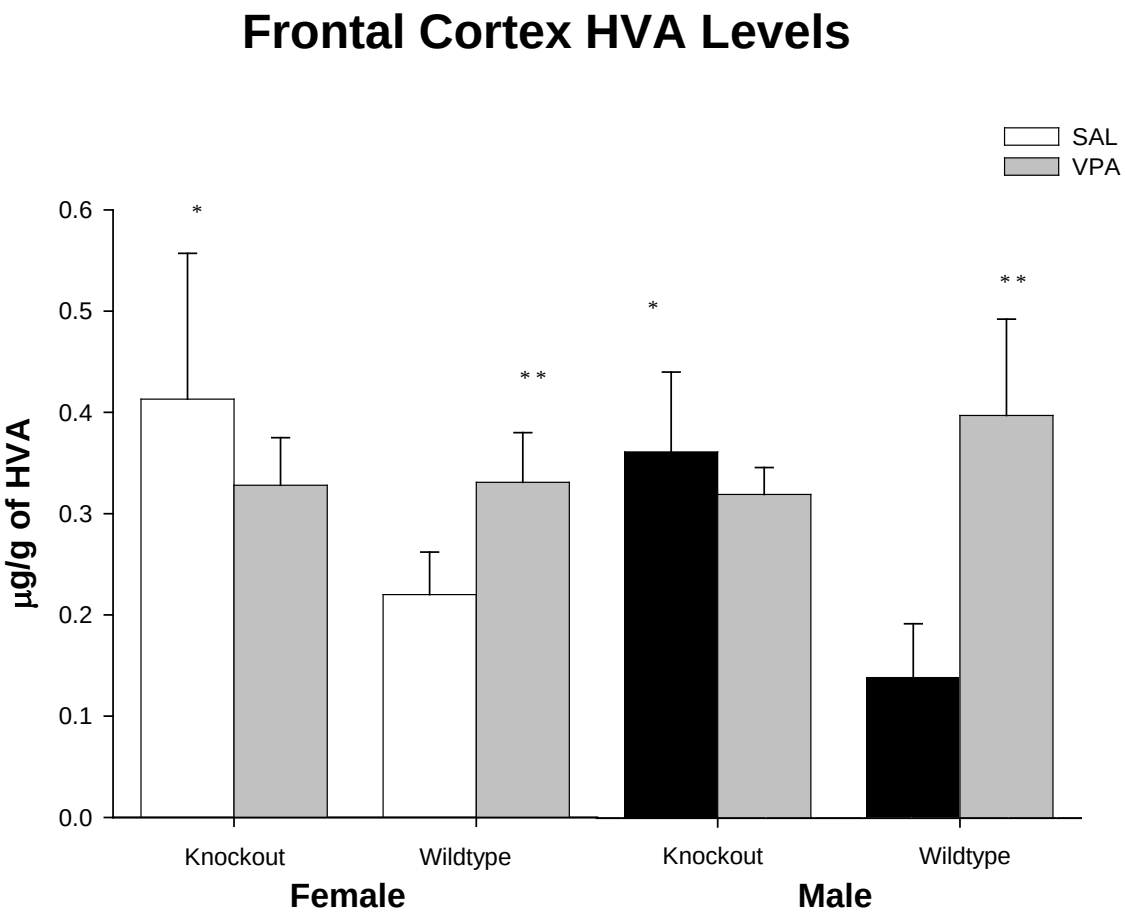
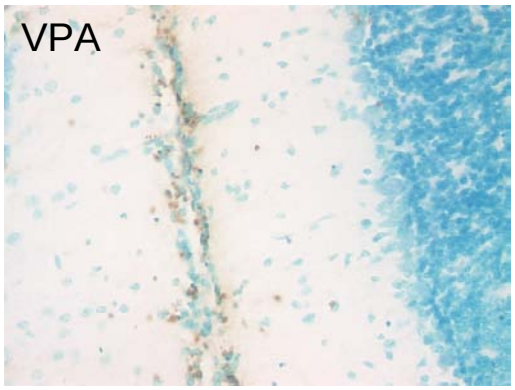
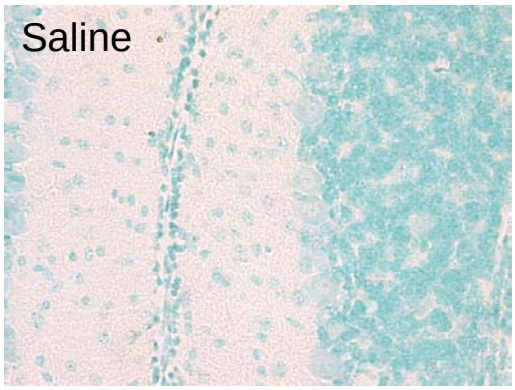


Figure 29:TUNEL Stain Age of Exposure Study

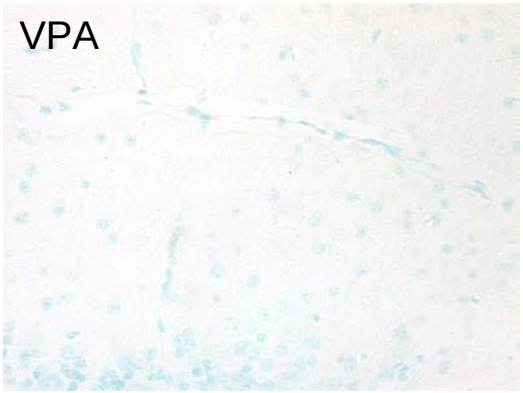
Cerebellar Regions 12 hours after

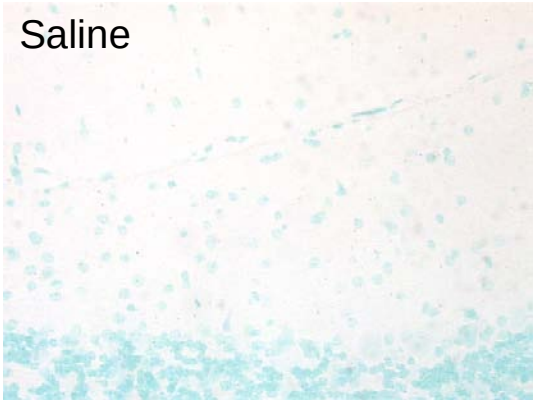
14 Day Old Pups





90 Day Old Adult





Curriculum Vitae

Carrie Leigh Yochum

EDUCATION:
2006- 2009*, Ph.D., Psychology, Rutgers University, New Brunswick, NJ
2004-2006, M.S. Behavioral Neuroscience, Rutgers University, New Brunswick, NJ
1998-2002, B.A. Neuroscience, Franklin and Marshall College, Lancaster, PA
(* expected date)

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2004-Present: Graduate Student, Psychology Dept., Rutgers University, New Brunswick, NJ
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2001-2003: Corporate Paralegal, PalmSource, Inc., San Jose, CA
2000-2001: Research Assistant, Dept. of Psychology, Franklin and Marshall College, Lancaster, PA

PUBLICATIONS:
1) Ming X., Cheh MA, Yochum CL, Halladay AK and Wagner GC. Evidence of oxidative stress in autism derived from animal models. American Journal of Biochemistry and Biotechnology 2008; 4: 218-225

2) Yochum CL, Dowling P, Reuhl KR, Wagner GC and Ming X. VPA-induced apoptosis and behavioral deficits in neonatal mice. Brain Research 2008; 1203: 126-132.

3) Cheh, M.A., Halladay, A.K., Yochum, C.L., Reuhl, K.R., Polunas, M., Ming, X. and Wagner, G.C. Autism and Oxidative Stress: Evidence from an Animal Model. In: Autism: Oxidative Stress, Inflammation and Immune Abnormalities, Eds: Chauhan, A., Chauhan, V. and Brown, T. Taylor & Francis/CRC Press, New York 2009 (in press).

4) Yochum C.L., Wagner G.C. Autism and Parkinson's Disease: Animal Models Suggest a Common Etiological Mechanism. Chinese Journal of Physiology, (in press).