AUTISM AND THE POTENTIAL ROLE OF IMMUNE FUNCTION AND PRENATAL METHYLMERCURY EXPOSURE

A Senior Scholars Thesis

By

NIRMA DORA BUSTAMANTE

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2006

Major: Biology

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Associate Dean of Undergraduate Research: Robert C. Webb

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ABSTRACT

Autism and the Potential Role of Immune Function and Prenatal Methylmercury Exposure (April 2006)

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Methylmercury (MeHg) remains to be a major public health concern because of the presence of widespread contamination of our environment and the severe neuropathological changes that can occur with exposure to the developing central nervous system (CNS). The blood-brain barrier (BBB) represents an important target for MeHg. However, before the BBB is fully formed or if the BBB is compromised once it is formed, it can not effectively isolate the brain from circulating toxins and foreign compounds. The current project examines the hypothesis that there is a correlation between childhood vaccination and the neurotoxic effects of mercury in the form of MeHg in the pathogenesis of autism. The effects from exposure to MeHg and activation of the immune system can affect neuronal survival and development and could result in the faulty wiring that appears to occur in the CNS of autistic children. Mice were exposed to low dose (0.03 μg/kg of body weight of the pregnant mouse) MeHg during prenatal development. Lipopolysaccharide (LPS) was administered during early

postnatal development as an immunostimulant. In order to determine the optimal time in which the injection of LPS would be the most effective, mice were injected with LPS then euthanized and injected with Evans Blue dye at 4, 6, 22, and 48 hours. The brain was separated into forebrain, cerebellum, and brainstem. Using a fluorescence micro plate reader, the quantity of Evans Blue leaking into the brain through the BBB was calculated. It was determined the mice should be euthanized 6 hours after administration of LPS. LPS or the control solution, phosphate buffered saline (PBS), was administered at postnatal day 18 into mouse pups that received MeHg prenatally and then the integrity of the BBB was checked 6 hours later. No difference was observed between mice given LPS compared to the mice given PBS most likely due to too much variability. There may be an effect of LPS but it is masked by the variability that occurs when using this assay in such young mice. A radioactive assay using ^{14}C-labeled sucrose is going to be used in order to acquire better results.

DEDICATION

Para mi mami y mi cokes.

Son mi corazon, mi vida, mi alma.

Los quiero mucho.

Gracias por siempre apoyarme y quererme tanto.

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Thanks also to my friends and colleagues and the Abbott Laboratory for making my time at Texas A&M University a great experience.

Finally, thanks to my family for all their patience, love, and support.

NOMENCLATURE

ASD	Austistic Spectrum Disorder
MeHg	Methylmercury
LPS	Lipopolysaccharide
PBS	Phosphate Buffered Saline
CNS	Central Nervous System
BBB	Blood Brain Barrier
CVAA	Cold Vapor Atomic Absorption
HRP	Horseradish Peroxide
VPA	Valproic Acid
GSH	Intracellular Glutathione

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CHAPTER I

INTRODUCTION

Autism

Autism is an incapacitating, lifelong disorder whose prevalence in 2005 ranged from 1/500 to 1/2000 children in the United States from all different racial, ethnic, and social backgrounds (Murcia, 2005). It is four times more prevalent in males and currently cannot be diagnosed until the child is between the ages of 2 or 3 yrs old (Strong, 2005). Autism has recently become a topic of great interest for many researchers because there was a 556% increase in pediatric prevalence between 1991 and 1997, which was higher than spina bifida, cancer, and Down syndrome (Muhle, 2004). When diagnosed, autism is defined by a triad of deficits which include impaired social interaction, impaired communication, and restricted interests and repetitive behaviors. If speech is present, it is so unresponsive that it is unusable in normal conversation. It is often forgotten that autism is the extreme of a spectrum of abnormalities, which in addition to Asperger Syndrome falls into Autistic Spectrum Disorder (ASD) (Belmonte, 2004).

Although autism is a socio-behavioral disorder, many studies show that autistic brains tend to posses certain characteristics. There tends to be a faulty wiring problem

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This thesis follows the style of *International Journal of Developmental Neuroscience.*

associated with autistic individuals. There are some areas of the brain that are over-wired, while others are not wired enough. The over-wired areas tend to be those regions related to low level sensory; hence, autistic people tend to become overloaded with sensory import or “noise”. There is also a decreased development in long-distance, integrative circuits that are responsible for high-level skills such as language and social interaction. Not only is wiring problematic, but individuals lack the ability to integrate information together (Strong, 2005).

Additionally, the actual structure of the brain tends to be different in autistic individuals. The cerebellum, for instance, is usually a common site of anatomic abnormality in autism. While autistic children typically have an excess volume of cerebrum or cerebral white matter that leads to an increased total brain volume; a reduction in the number of Purkinje cells also occurs (Belmonte, 2004). Purkinje cells are a site of the major histocompatibility complex (MHC) class I expression, proteins required for specific forms of developmental and functional plasticity in the brain. A decrease in MHC expression impairs reduction in inappropriate synaptic connections which may explain the increase in brain size (Belmonte, 2004). More than 100 proteins show differential expression in individuals suffering from autism; furthermore, studies shows that autism affects immunology as well. A study showing a 20% increase in B cells and 40% increase in natural killer cells leads to the conclusion that autism may be an autoimmune disorder (Davidson, 2002). Strong evidence indicating immune and protein alterations in blood samples in children with autism have lead the UC David M.I.N.D institute to announce that such data could be used in developing a blood test for

autism. Such a test could lead to an earlier detection of autism or prevent regressive autism, which occurs when a high risk individual is exposed to an environmental trigger (Strong, 2005). To this date, there is no universally-accepted theory for the cause of autism. Some researchers believe that genetics place people at high risk for autism. A probable cause is the interaction of multiple genes in addition to unknown environmental factors that contribute to the variable expression of the disorder (Belmonte, 2004; Zheng, 2003; Muhle, 2004). It is thought that twin heritability ranges from 60%-90%, while non-twin prevalence ranges from 1%-6%, which is suggestive of a significant genetic component to autism (Muhle, 2004.) However, because ASD includes such a wide range of disorders prevalence and heritability is hard to calculate (Zheng, 2003). Still, due to the greater prevalence in males, some studies suggest that it might be a X-linked disorder. Although the identity and number of genes is not yet known, there are candidate genes whose products are known to play a role in brain development or are associated with brain structures, neurotransmitters or neuromodulators. Cytogenetic abnormalities found on chromosome 15 in the q11-13 locus were reported most frequently in patients with autism. Several studies have described duplications, deletions, and inversions at this locus. Despite the abundance of investigations into the genetics of autism, there have very few significant genetic links to autism that have been identified (Muhle, 2004).

Animal Models of Autism

Because autism is strictly a socio-behavioral disorder, it is a challenge to create an animal model or animal models that will be truly useful, such an animal model or models would need to replicate a combination of behavioral, neuropathological, biochemical, and genetic components. Previous models have assigned autistic-like symptoms to mice behavior that include social interaction, communication, and patterns of repetitive behaviors. In the past there have been a number of animal models that characterize some aspects of autism in mice. In genetically-induced models, autistic-like behavioral abnormalities have been found after a mutation in a gene connected to the serotonin and oxytocin-vasopressin systems is created; still, the neuropathological abnormalities are not sufficient to explain the behavior (Mucia, 2005).

Lesion models focus on brain damage in specific regions of the brain that lead to behavioral abnormalities; yet, do not tend to reproduce genetic or developmental pathways. Such models only demonstrate the ability of defects in a particular region of the brain to produce altered behaviors of a specific nature. Some models use chemical or infectious agents to cause such lesions which are thought to have a potential role in altered CNS development (Murcia,2005). Belmonte states that maternal viral infection at midpregnancy has been called “the principle non-genetic cause of autism (Belmonte, 2004).”

Teratogenic substances are those that are believed to cause the development of abnormal cell masses during fetal growth which cause physical defects in fetuses (Oxford, 2006). Both thalidomide and valproic acid (VPA) are believed to be teratogenic.

Thalidomide was a drug sold during the mid 1950's as an aid to pregnant women who were dealing with morning sickness while VPA inhibits the metabolism of the neurotransmitter GABA (Oxford, 2006). Studies have suggested that both drugs may be involved in the development of autism. One study points out that human mothers who took thalidomide during pregnancy to avert nausea in day 20-24th of pregnancy had a greater frequency of autistic children, while another study done by Schneider and Przew exemplifies a new rodent autistic animal model created by the exposure of rat fetuses to valproic acid (VPA) on the 12th day of gestation (Schneider, 2004; Teitelbaum, 2003). In rats, the neural tube closes on day 11. By the 12th day of gestation production of motor nuclei of the trigeminal, abducens, and hypoglossal nerves is completed. This model has striking anatomical, pathological, and etiological similarities to described symptoms of autistic individuals. The results from their study demonstrated that VPA rats exhibited lower sensitivity to pain and higher sensitivity to non-painful stimuli, diminished acoustic prepulse inhibition, locomotor and repetitive stereotypic-like hyperactivity combined with lower exploratory activity, a decreased number of social behaviors and increased latency to social behaviors. Furthermore, female rats with VPA injected on the 12th day of gestation, showed brain abnormalities resembling brain-imaging studies of autistic patients (Schneider, 2004). There have been many species that have been considered to be powerful tools for investigating the genetic, cellular, morphological, and biochemical characteristics of brain development and function of autism. Such species include zebrafish and non-human primates (Moldin, 2003).

Furthermore, the environment naturally reproduces conditions in humans that affect the brain globally (Murcia, 2005). The earlier the exposure to such toxic agents as

methylmercury (MeHg), the more generalized the damage (Zheng, 2003). Because autism is more like a “fractured mosaic then a coherent image”, it is rather difficult to create an animal model that replicates most of the characteristics of autism; therefore, a variety of animal models are needed to test the possible mechanisms that underlie autism (Murcia, 2005).

CHAPTER II

FOCUS OF RESEARCH

MeHg remains to be a major public health concern because of the presence of widespread contamination of our environment and the severe neuropathologic changes that can occur with exposure to the developing CNS (Davidson, 2004). The blood-brain barrier (BBB) represents an important target for MeHg. The intact and fully formed BBB isolates the brain and spinal cord from the systemic circulation and forms and efficient barrier against entry to toxins and foreign compounds into the brain. The BBB is formed primarily by the endothelial cells of the blood vessels in the brain and partly by the end feet of astrocytes. Tight junctions between endothelial cells regulate the selective entry of micromolecular and macromolecular solutes into the CNS from the blood. However, before the BBB is fully formed or if the BBB is compromised once it is formed, it can not effectively isolate the brain from circulating toxins and foreign compounds and takes place in two phases – brain angiogenesis is the first phase, which is initiated in the first trimester of human development, when intraneural bloods vessels are formed. In the mouse this first phase takes place from embryonic day 12 until approximately postnatal day 20 (Song, 2002). The second phase includes differentiation of the CNS blood vessel endothelium to form tight junctions and complex transport

systems for hydrophilic compounds required for CNS function and ends at approximately postnatal day 30 or in the early juvenile stage in rodents (Song, 2002). During this development phase the BBB is quite vulnerable to toxic insults as compared to the adult BBB (Eriksson, 1997). Recent evidence in the developing chick brain indicates that methylmercury may impair BBB differentiation. Recently, a connection between the increase in incidence of autism and the administration of Thimerasol (methylmercury), a preservative used in vaccines, was proposed (Parker, 2004). The incidence of autism has increased around the world in the past 25 years and concurrent with this increase has been the increase of early childhood vaccination for major childhood diseases worldwide. A number of epidemiological studies have been conducted in the past several years and the conclusion of most scientists is that there is little to no direct link between childhood vaccinations and the incidence of autism. However, the number of cases of autism are clearly on the rise and the consensus is that the disorder probably arises from multiple genes interacting together and with as yet unknown environmental factors (Belmonte, 2004).

Vaccinations given in order to produce the desired immunity to disease organisms, will generate an immune response in the vaccinated infant. The immune response to a vaccine will not effect brain development nor will a significant number of immune cells and or immune-related molecules enter the CNS. However, if the infant has been exposed to MeHg during prenatal development then brain development can be compromised (Zheng, 2003). As previously stated, changes in serotonin levels during development can contribute to structural brain abnormalities and core behavioral characteristics of autism. An increase of 30-50% of platelet serotonin levels in

individuals with autism has be recorded (Muhle, 2004). Results in a study indicate that mammalian astrocyte serotonin and D-aspartate uptake is very sensitive to low concentrations of MeHg. A mechanism of methylmercury (MeHg) neurotoxicity associates it with oxygen radical formation and a decrease of cellular levels of intracellular glutathione (GSH). Astrocytes play an important role in providing GSH precursors to neurons. If the GSH pathway is altered, it may lead to the destruction astrocytic handling of glutamate and neuronal energy metabolism (Shanker, 2001). CNS blood vessels develop more slowly than normal and the endothelial cells differentiate poorly (Zheng, 2003). If delayed and/or poor development of the BBB occurs because of MeHg exposure and then the infant is vaccinated, the infant may have increased penetration of immune cells from the blood vascular system that may result in abnormal CNS development leading to autism.

The current project examines the hypothesis that there is a correlation between childhood vaccination and the neurotoxic effects of mercury in the form of MeHg in the pathogenesis of autism. Mice will be exposed to methylmercury (MeHg) during neonatal development and then lipopolysaccharide (LPS) will be administered as an immunostimulant. The effects from exposure to MeHg and activation of the immune system can affect neuronal survival and development and could result in the “faulty wiring” that appears to occur in the CNS of autistic children.

CHAPTER III

MATERIALS AND METHODS

Prenatal methylmercury (MeHg) exposure and measurement

Pregnant mice were given moistened chow containing vehicle only (deionized water – control mice) or vehicle plus MeHg at a dose of 0.03µg/Kg/day (experimental mice) during days 8-18 of gestation. This dose of MeHg produces no signs of toxicity in the pregnant mice or in the offspring once they are born. To sample the brain for levels of mercury the mice were anesthetized with isoflurane and then decapitated. Brain tissue samples were then analyzed for mercury by cold-vapor atomic absorption (CVAA) in the Trace Element Research Laboratory of Dr. Robert Taylor, Department of Veterinary Biosciences in the College of Veterinary Medicine. Each group of brain tissues samples that were analyzed were analyzed in duplicate and included: the samples, a "blank" sample (a sample tube with only vehicle in it and no mercury at all), a spiked blank (a blank sample with a known concentration of mercury added), a certified reference material, and a spiked sample.

MeHg is a hazardous chemical and great care was taken to ensure no exposure to any of the laboratory personnel working with the mice. Only the faculty advisor (Dr.

Louise C. Abbott) dosed the mice and handles the cage bedding. Four pregnant control mice and three pregnant experimental mice were used in this study.

Integrity of the Blood Brain Barrier

The integrity of the blood brain barrier was determined by degree to which molecules that are not suppose to pass the BBB are found in brain tissue, indicating that they had moved from the lumen of blood vessels located in the brain into the brain tissue.

- HRP Assay - Used to determine effects of lipopolysaccaride (LPS) on the blood brain barrier (BBB).
- Evans Blue Assay - Used to determine the optimal time in which the injection of LPS would be the most effective.

HRP (Horseradish Peroxidase) Assay

HRP (0.3mg/g) is a large molecule dye that was injected into the young mice after the LPS injection (0.05µg). After the euthanizing the juvenile mice and collecting the brains, brain sections were stained using diaminobenzidine as the chromagen. Densitometry was used to calculate if there was any difference between the amount of HRP located in the treated versus control brain tissues, which show up as a brown color.

Evans Blue Assay

Evans Blue is a dye with smaller molecular size than HRP, which is able to get through leakage sites that the HRP dye may not have been able to enter. Using a fluorescence plate reader, the quantity of Evans Blue leaking into the brain tissue was determined. First, the optimal time of LPS injection was determined, then MeHg treated mice went through the same Evans Blue assay. Mice were injected with LPS then euthanized with Ketamine/Xylazine (ip). Evans blue was injected at 4, 6, 22, or 48 hours

(15mg/Kg bwt) through the left ventricle. After three minutes, mice were perfused using PBS (12cc) Forebrain, cerebellum, and brainstem were dissected out and weights were recorded. Tissues were dried at 55^{o} overnight. Tissues were crushed in 50% ice cold trichloroacetic acid (TCA) (400µl for forebrain, 300µl for cerebellum and brainstem). Mixtures were centrifuged at 3000 rpm for 10 minutes and the supernatant collected. Supernatant was diluted 1:1 with diluent (1 part TCA: 3 parts 95% ethanol). Samples and standards were loaded in a black-bottomed 96 well microplate, and read in fluorescent plate reader at 620nm excitation, and 680 nm emission. Concentration of Evans Blue was expressed as ng/mg wet wt.

CHAPTER IV

RESULTS

Once it was determined that low dose exposure to MeHg resulted in consistent brain levels of mercury in mice exposed prenatally, which is shown in Figure 1, it also had to be proven that the BBB could, in fact, be compromised by the injection of LPS.

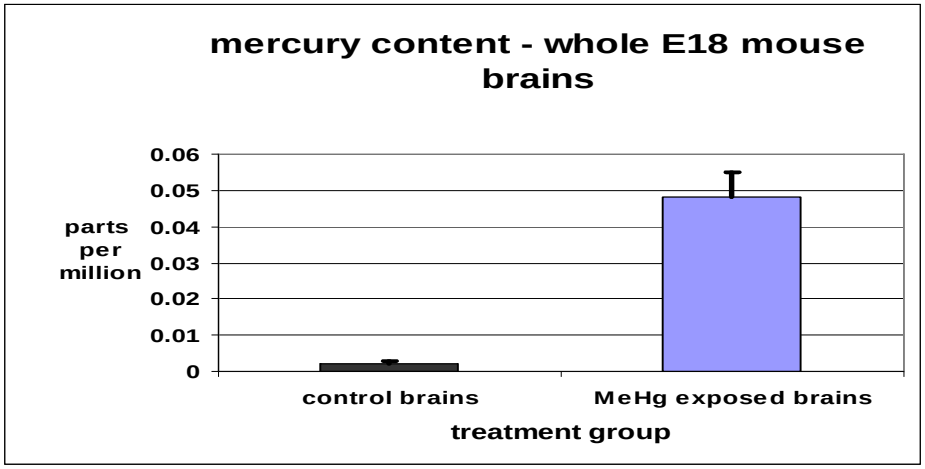

Figure 1. It was determined that the low dose exposure of MeHg used in this model resulted in consistent brain levels of mercury in mice exposed prenatally.

In order to do this the HPR assay was first used. Results, shown in Figure 2, did not indicate a difference between the experimental mice that received LPS and control mice that were injected with PBS. It was determined that because HPR is a large

molecule dye, the assay was not sensitive enough to address the question at hand. If the leaks in the BBB that was compromised by LPS were small, HRP would not have been able to pass through the BBB because the molecules were too large. It was determined that a smaller molecule dye, Evans Blue, should be used to repeat the assay.

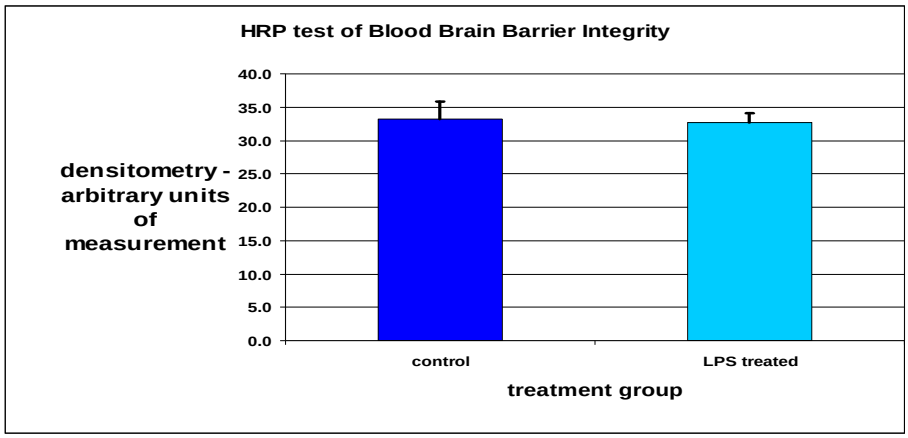

Figure 2. It was determined that HRP was not a sensitive assay to use to address this question

In order to maximize results, the Evans Blue dye was initially used to determine the optimal time at which the mice should be euthanized after the administration of LPS. A fluorescence microplate reader was used to assay the amount of Evans Blue in the brain tissue and whether it was optimal to euthanize the mice at 4, 6, 22, or 48 hours after the LPS injection. Because uniform results were anticipated, having leakage throughout the brain, the forebrain, brainstem, and cerebellum were extracted in order to determine the integrity of the BBB. It was determined that the mice should be euthanized at 6 hours after the administration of LPS. Results shown in Figure 3a-c

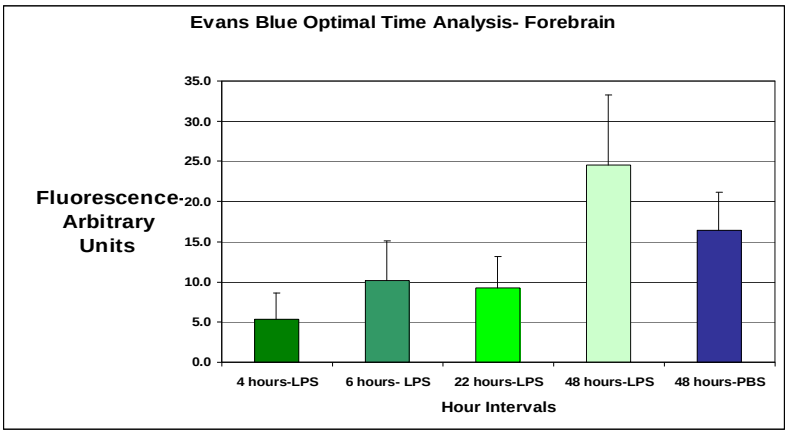

Figure 3a. It was determined the mice should be euthanized 6 hours after the administration of LPS.

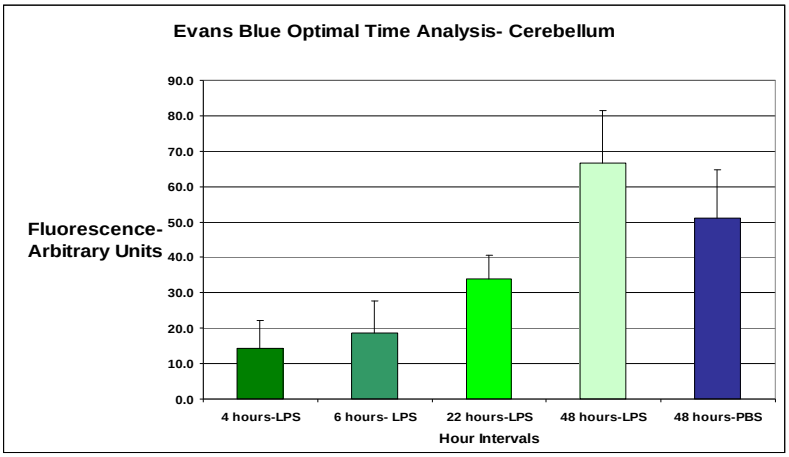

Figure 3b. It was determined the mice should be euthanized 6 hours after the administration of LPS.

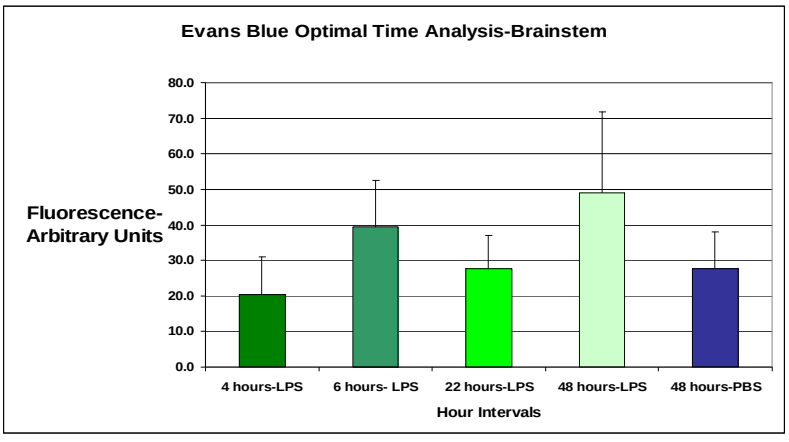

Figure 3c. It was determined the mice should be euthanized 6 hours after the administration of LPS.

Once the 6 hours time interval was selected, the assay was repeated using mice that had been exposed to MeHg. In the results obtained using mice exposed to MeHg, there appeared to be no difference between the mice given LPS compared to the mice given PBS.

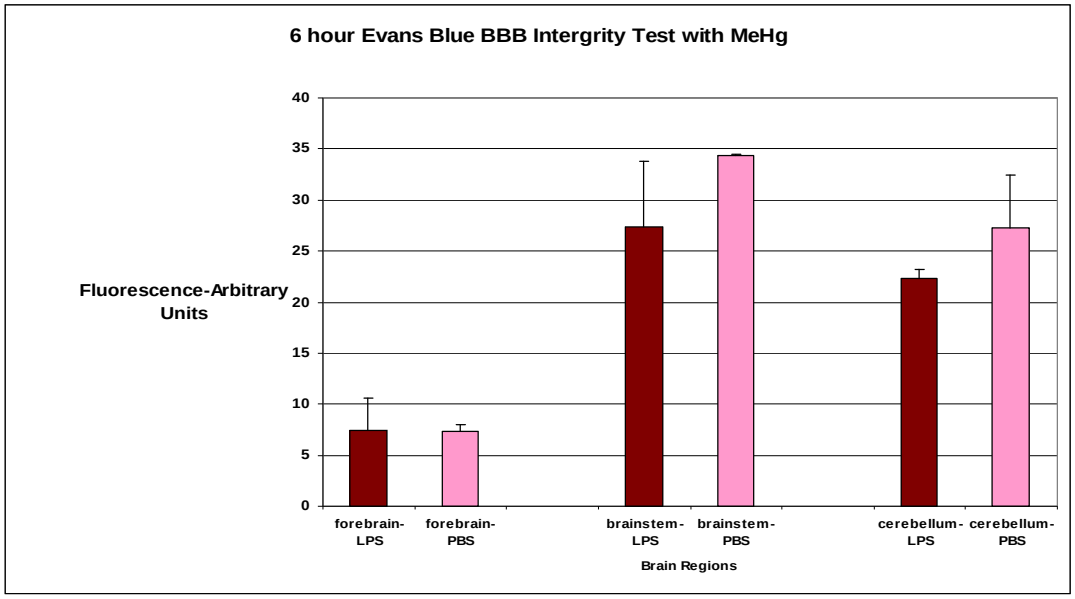

Figure 4. MeHg treated mice that were also given either LPS or PBS on day 18; the forebrain, cerebellum, and brain were extracted 6 hours later.

CHAPTER V

SUMMARY AND CONCLUSION

It would appear that we were getting too much variability in the mice injected with Evan's blue dye directly into the heart to see a difference between treated and control mouse brains. There may be an effect of LPS but it is masked by the variability that we get with using this assay in such young mouse pups. The variability could also be due to the short times that elapsed between the time of injection of Evans Blue dye and the time of death of the mice. The fact that the diaphragm needed to be cut in order to access the heart also reduced blood flow to the brain, again making delivery of Evans Blue dye to the brain. Another reason for the variability was that due to the fact that the euthanization of the mice was initially done by Dr. Abbott in order to demonstrate the procedure. Because the administration of the Evans Blue assay through the heart is such a sensitive procedure, any variability in the methods could produce a large difference in the results that may not have been due to those factors under question.

In the future, a radioactive assay using ^{14}C-labeled sucrose will be used in order to acquire more uniform results. We also have devised a different method of delivering the labeling compound (Evans Blue or ^{14}C-sucrose) that appears to be more reliable. We

open the abdominal body wall in anesthetized mice and use a 33 gauge needle attached to the cannula filled with Evans Blue dye. The dye is injected into the caudal vena cava and the diaphragm remains intact. This allows the mice to live up to 20 minutes after the labeling compound is administered.

Because the research is sailing on uncharted waters, all the methods presented were continuously altered in order to acquire better results. The research presented demonstrates a new take on autism and its causative factors. A lot of the aspects of the research are still being developed. Such novel research not only needs time to develop and the opportunity to experience additional trial and error.

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