ABSTRACT

A Correlation Between Autism and Epilepsy: A Study of Social Behavior in PTEN Knockout Mice

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There is increasing evidence of a strong comorbidity between autism and epilepsy. One pathway that may be a significant mediator of the two conditions is the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway is a key component of mRNA translation, cell growth control, and cell proliferation. Hyperactivation of the mTOR pathway has been reported in several animal models of epilepsy and in some mouse models of autism. One of the core diagnostic criteria for autism is aberrant social behavior. The focus of this project was to observe the effect of deletion of the PTEN gene on social behavior in mice. PTEN serves as a regulatory inhibitor of the mTOR pathway, so its deletion results in hyperactive mTOR pathway. The social partition and social chamber tests were used to measure social behavior in PTEN knockout and wildtype. The outcome of both tests illustrated that the PTEN knockout mice had a significant decrease in social behavior. These results indicate that an overactive mTOR pathway may indeed result in an autistic phenotype, and comorbidity between epilepsy and autism should further be considered.

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A CORRELATION BETWEEN AUTISM AND EPILEPSY: A STUDY OF SOCIAL BEHAVIOR IN PTEN KNOCKOUT MICE

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CHAPTER ONE

Introduction

A large amount of evidence now points toward a neurobiological etiology of autism, specifically one of genetic origin. Multiple neurological disorders present with secondary autistic-like symptoms. This paper focuses on the unique relationship between autism and epilepsy, an interest to many researchers since the 1960's (Creak et. al., 1969). Early studies show high rates of seizures and EEG abnormalities in children with autism (Creak et. al., 1969), and these studies have contributed to a current area of research dedicated to determining whether or not a comorbidity of autism and epilepsy exists.

Autism

According to the Center for Disease Control's (CDC) Autism and Developmental Disabilities Monitoring Center, one in eighty-eight children are identified with an Autism Spectrum Disorder (CDC, 2012). The CDC defines autism spectrum disorders as a "group of developmental disabilities that can cause significant social, communication and behavioral challenges" (CDC, 2012). Because these disorders are spectrum disorders, they often manifest to different degrees in different children.

Symptoms of autistic disorder, or autism, specifically, include delays in language, social and communication difficulties, and unusual behaviors or interests. Autism may also be accompanied by intellectual disabilities (CDC, 2012). Because of the variability of this disorder, autism has been difficult to diagnose in the past. A child diagnosed with

autism may be able to attend school and excel in certain areas, while another may not be able to speak or interact in any sociable way. Some studies have postulated an increase in the cases of autism; however, more evidence simply points to the change in criteria for diagnosing autism.

The American Psychiatric Association's Diagnostic and Statistics Manual-IV, Text Revision (DSM-IV-TR) currently provides this standardized criteria for diagnosing autism:

A. A total of Six (or more) items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3).

1. Qualitative impairment in social interaction, as manifested by at least two of the following:

- marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction
- failure to develop peer relationships appropriate to development level
- a lack of spontaneous seeking to share enjoyment, interest, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)
- lack of social or emotional reciprocity

2. Qualitative impairments in communication as manifested by at least one of the following:

- delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mine)
- in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
- stereotyped and repetitive use of language or idiosyncratic language
- lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

3. Restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:

- encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
- apparently inflexible adherence to specific, nonfunctional routines or rituals
- stereotypes and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)
- persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play.

C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder (DSM-IV-TR).

Epilepsy

Epilepsy is one of the most common neurological disorders, fourth to Alzheimer's disease, migraines, and stroke (CDC, 2011). It is a medical condition that produces seizures which affect an individual's mental and physical functions. A person is considered to be epileptic if he or she has two or more seizures. According to the CDC about ten percent of Americans will experience a seizure during their lives, and three percent will receive a diagnosis of epilepsy by age eighty; and, because medications are suboptimal, more than one-third of medicated epileptic patients continue to experience seizures (CDC, 2011). Epilepsy can also be described as a spectrum disorder. It is often multifactorial, complicated, and varies in severity much like autism does (Jensen, 2011).

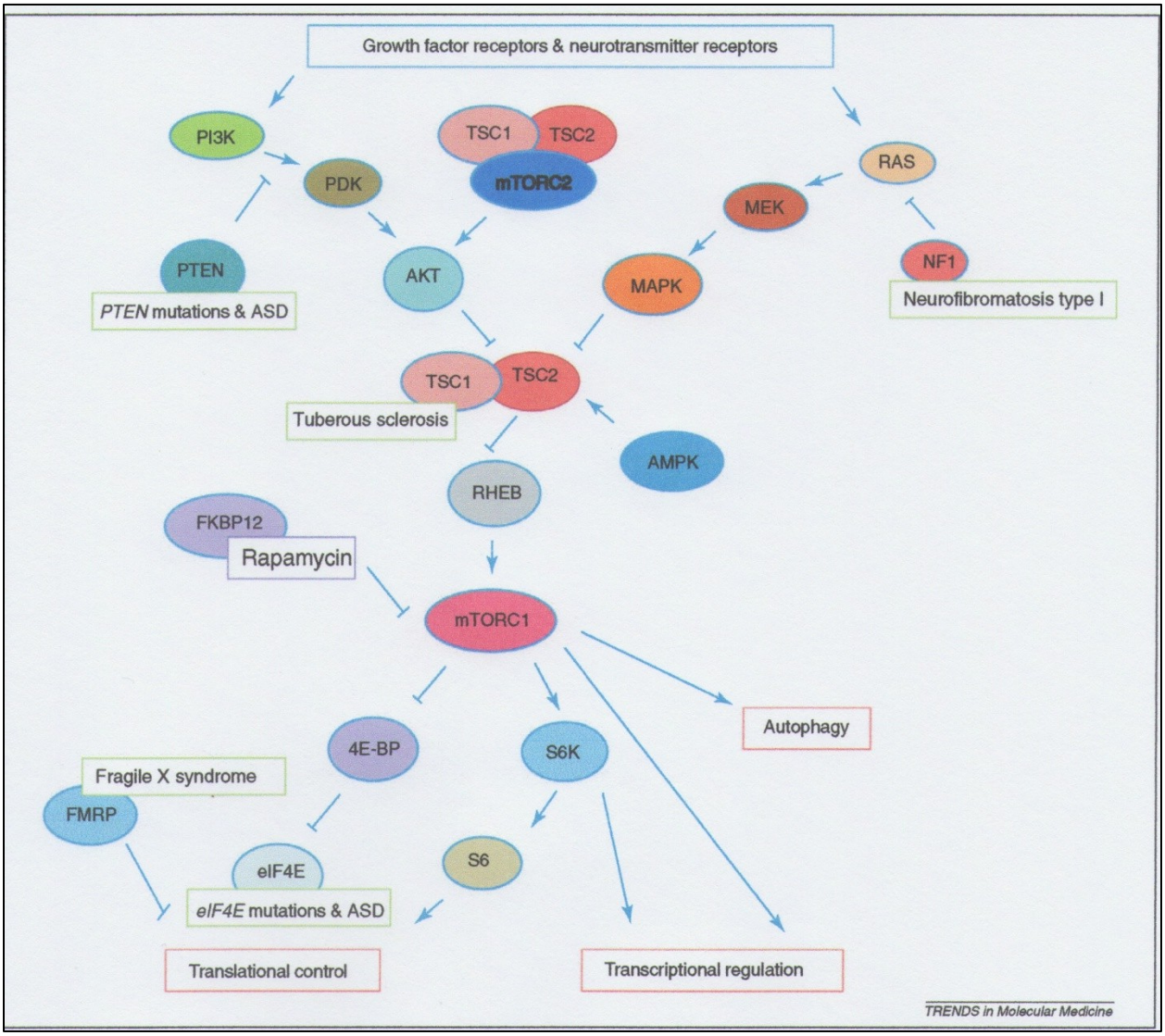
Epilepsy and Autism

Children who experience epilepsy at younger ages are more likely to develop other complications such as mental retardation and learning disabilities (CDC, 2011). Research from as early as the 1960s suggests that comorbidity exists between epilepsy and autism. Rates of epilepsy are high among children with autism (Fombonne, 1999). Ten to thirty percent of children with autism also experience epilepsy (Canitano, 2007). Furthermore, EEG abnormalities and epilepsy in autistic children presented the first evidence of the neurobiological etiology of autism (Levisohn, 2007). A question that needs to further be addressed is whether seizures may cause autism or whether they are comorbid with autism.

The mTOR Pathway

The mammalian target of rapamycin (mTOR) is a key element in the regulation of mRNA translation, cell growth control, proliferation; and, it is involved in the regulation of cellular and dendritic plasticity in both cortical and hippocampal neurons (Wong, 2010).

Figure 1: From Ehninger, 2010, p. 4



The PI3K/AKT signaling pathway plays an important role in activating the mTOR pathway. Transmembrane receptors send signals that activate PI3K, which is a lipid kinase. This then leads to the production of inositol phospholipids from membrane inositols. These phospholipids then signal PDK-1, a membrane threonine kinase, and Akt, a cytosolic protein kinase. PDK-1 and Akt form a complex that phosphorylates many important substrates in various downstream pathways. Akt is also known to indirectly activate the mTOR pathway. An interruption at any phosphorylation or dephosphorylation site of the pathway can inactivate the mTOR pathway. The pathway is hyperactivated with deletion of the phosphatase and tensin homolog (PTEN). PTEN removes phosphate groups from the inositol phospholipids. Without PTEN there is a prolonged response to inositol phospholipid signals in response to growth factor stimulation. This overstimulation may lead to tumor growth, and thus, PTEN is identified as a tumor suppressor gene (Ali et. al., 1999).

PTEN Knockout as a Model

PTEN knockout mice experience seizures, an enlarged brain, memory and learning deficits, and aberrant dendrite growth (Kwon et. al., 2006). Because of these observations, the PTEN knockout mouse serves as an appropriate model for observing the presence of both seizures and behavioral deficits. Mutations in the mTOR signaling pathway are associated with an increased rate of epilepsy, autism, and other mental impairments (Gipson & Johnston, 2012). PTEN negatively regulates the mTOR pathway, and mice that lack PTEN have deficits in learning, memory and social behavior (Kwon et al., 2006). Because a deficit in social behavior is a key symptom of autistic

disorder, this study may lead to conclusions about the relationship between epilepsy and autism.

Purpose of this Study

This paper will examine social behavior in PTEN knockout mice through several social behavior tests. PTEN knockout mice exhibit seizures. Identifying social behavior deficits in them can confirm that this model can successfully be used to observe both symptoms in the same subject. The discussion explains the possible correlation between autism and epilepsy, and how such a correlation can target a new direction for autism research.

CHAPTER TWO

Review of Literature

This section discusses the literature in support of comorbidity between epilepsy and autism as a result of a genetically altered mTOR pathway.

mTOR Pathway

The mammalian target of rapamycin is a serine/threonine kinase that plays a key role in controlling cell growth and cell differentiation (Schmelzle & Hall, 2000).

mTOR Implication in Autism

There are several studies that indicate an overactive mTOR pathway can lead to autism. The mTOR pathway is regulated by multiple proteins. Two known regulatory inhibitors of the pathway are TSC1/TSC2 and PTEN. Mutations in either of these result in a hyperactive mTOR/PI3K pathway and can lead to an autism spectrum disorder (ASD) with tuberous sclerosis or macrocephaly (Bourgeron, 2009). Because this pathway is involved in cell growth, its hyperactive state results in abnormal cellular and synaptic growth. Due to recent studies that convey a ten to thirty percent correlation between ASD and macrocephaly, it is this abnormal growth that is suggested to contribute to ASD (Bourgeron, 2009).

mTOR Implication in Epilepsy

Temporal lobe epilepsy is one of the more common types of epilepsy, and its pathogenesis is not clearly identified (Engel et. al., 1997). Many patients with temporal

lobe epilepsy present with aberrant sprouting of granule cell axons, or mossy fibers (de Lanerolle et. al., 1989). Inhibition of the mTOR pathway suppresses development of mossy fiber sprouting (Buckmaster, 2009). This suggests that an overactive mTOR pathway may very well result in epilepsy. Previous evidence has shown that many individuals with ASD have a peak incidence of seizures in early childhood (Saemundsen et. al., 2007).

PTEN as a model for mTOR Hyperactivation

Some studies show that PTEN mutations have been discovered in individuals with autism spectrum disorders (Kwon et. al., 2006). PTEN is a protein that serves as an inhibitory regulator of the mTOR pathway. Geneticists can easily knock out the gene that codes for PTEN in various strains of mice, and both heterozygous and homozygous PTEN knockout mice can be purchased for scientific experiments. These mice present with seizures and several social behavior deficits, creating a good model for observing a model with both behaviors.

Social behavior deficits have been discovered in mice with germ-line PTEN mutations (Kwon et. al., 2006). Social behavior is the most common characteristic used in autism diagnosis, and thus, it is a good variable to observe in behavioral studies in mice (Kwon et. al., 2006). Heterozygous PTEN mutations have been discovered in individuals with autism and macrocephaly (Page et. al., 2009), which further points to a genetic basis for autism.

Prevalence of Autism and Epilepsy Comorbidity

Several studies document that children with autistic spectrum disorders have an increased prevalence of seizures (Clarke et. al., 2005). There are not as many studies focused on the prevalence of ASD in individuals with epilepsy. Research identifies that one in three individuals exhibit symptoms associated with autism (Clarke et. al., 2005).

Tuberous Sclerosis Complex: example of comorbidity

Tuberous sclerosis, a genetic disease that results in the growth of multiple non-malignant tumors in the brain and other systems, is frequently associated with mental retardation, autism and epilepsy (Ehninger et. al., 2008). Tuberous sclerosis results from mutations in TSC1 or TSC2, and causes brain lesions, subependymal giant cell astrocytomas, cortical tubers, intractable epilepsy in 60-90%, autism in up to 61%, and intellectual disability in 45% (Gipson et. al., 2012). Mutations in TSC1 and TSC2 are associated with overexpression of mTOR. This condition also explains mTOR's involvement in both epilepsy and autism, further illustrating the coexistence of the two symptoms and pointing to a genetic mutation as the root cause.

Fragile X Syndrome: example of comorbidity

Fragile X Syndrome (FXS) is the most common cause of mental retardation. It is a monogenetic neurodevelopmental disorder that results from an abnormal expansion of a CGG trinucleotide repeat within the promoter region of the gene FMR1 on the X chromosome (Coghlan et al., 2012). Symptoms of FXS are very similar to those seen in ASD, including intellectual disability, a distinct physical phenotype, social and communication impairment, and repetitive behaviors. In addition to symptoms of ASD,

twenty to twenty five percent of FXS cases present with epilepsy (Coghlan et. al., 2012). Thus, FXS is a disorder that is commonly studied when investigating the coexistence of seizures and autistic symptoms.

Sociability in Mice

One of the major characteristics in diagnosing any autistic spectrum disorder is degree of social interaction or behavior. Mice, along with most rodents, usually exhibit strong social communities, and social interactions between mice are easily quantified (Nadler, 2004). Because of this, many social preference tests are used in a laboratory setting to study social tendencies in different groups of mice (Nadler, 2004).

The three-chamber social test used in this study is similar to one commonly used to observe social behavior in mice. Hamilton (2011) employed this behavioral test among many others when observing autism-like behaviors in a novel transgenic mouse model. Sociability is usually defined as the "subject mice spending more time in the chamber containing the novel target mouse than in the chamber containing the inanimate novel object" (Silverman et. al., 2010).

CHAPTER THREE

Materials and Methods

Odor Discrimination Test

Social behavior in mice is observed through their interaction with other objects or living things; thus, the ability to detect different odors is important when measuring social behavior (Yang, 2009). One possible bias for tests aimed at assessing social behavior may be the presence of an olfactory deficit, which could result in the inability of the mouse to detect another mouse. The odor discrimination test was performed to identify if the PTEN knockout mice had odor discrimination deficits. A normal olfactory response is important when determining the social behavior in PTEN knockouts. For this project, the test was completed on 18 mice.

Set Up

The non-social odors used for this test were water, almond, and banana. A fresh sample of each of these odors was prepared on each test day. A volume of 100 ul of each odor was pipetted into a test tube holding 10 mL of distilled water to create a 1:100 dilution. Two social odors were used for each mouse tested. The social odors were prepared by swiping a cotton swab in a zigzag fashion across the bottom of a three-day old cage. Any bedding or substance that stuck to the swab was shaken off. The two cages used for obtaining the social odors each previously housed the same number of mice, which were of the same sex as the mice being tested with those odors. The odor source cages were always outside the experimental testing room. After swabbing the

cages, the swabs were kept in a beaker covered with parafilm. Social odors were prepared each morning before the test was performed (Yang, 2009).

Procedure

Each tested mouse was first weighed and labeled. A clean testing cage was prepared for each mouse, and the mouse was placed in the testing cage and allowed to acclimate for 45 minutes in a room that was not the testing room. A second mouse was usually prepared and allowed to acclimate in the same room right before a previous mouse was about to be tested (Yang, 2009).

To begin the test, the mouse was taken in its testing cage to the testing room. The tip of a cotton swab was dipped into the scent to be tested, while avoiding touching the tip to anything, including fingers, the cage, or any other substance. The end of the swab was then taped to the side of the cage in such a way that about 1 inch of the end with the cotton tip hung down into the cage. The cumulative time spent sniffing the tip during a 2 minute trial was recorded using the Ethom program, an automated computer scoring program. Sniffing refers to when the animal is oriented toward the cotton tip of the swab within the testing cage with its nose 2 cm or closer to the tip (Yang, 2009).

After each trial, the wire lid was raised, and the cotton swab was removed without touching the tip to anything in the cage. The swab was then placed and sealed in a 500 mL bottle. The computer was set up for the next trial, and the procedure was repeated with the next swab. A total of three non-social (water, almond, banana) and two social odors were tested. Each odor was tested for three trials for a total of 15 trials per mouse.

Analysis

The data was analyzed for each group using repeated measures ANOVA followed by post hoc tests to determine significant habituation, i.e. less time sniffing successive same smells, and dishabituation, i.e. more time sniffing a new smell.

Social Partition Test

The social partition test is used to evaluate social behaviors of mice.

Set-up

The experimental animals were individually housed for 24 hours. Each mouse was placed into one side of a cage that was divided by clear perforations. (Spencer et al., 2011) A partner C57BL/6J mouse with identical sex, similar age and similar weight was placed in the other half of the partition. Each animal had a source of water and food.

Procedure

The next day the approaches and time spent at the partition by the experimental mice were measured for five minutes using the Ethom program. Ethom is an automated scoring program on a computer that can be used to quantify certain variables such as time and frequency. The first observation, the first familiar condition, was with the partner that the mouse had been housed with overnight. The partner mouse was then removed, and a novel C57BL/6J mouse with the same sex, similar age and similar weight was added and the behaviors recorded. This served as the unfamiliar condition. The unfamiliar mouse was then removed, and the previously housed mouse was reintroduced. This served as the second familiar condition. Thus each mouse went through five minutes of testing for three different conditions.

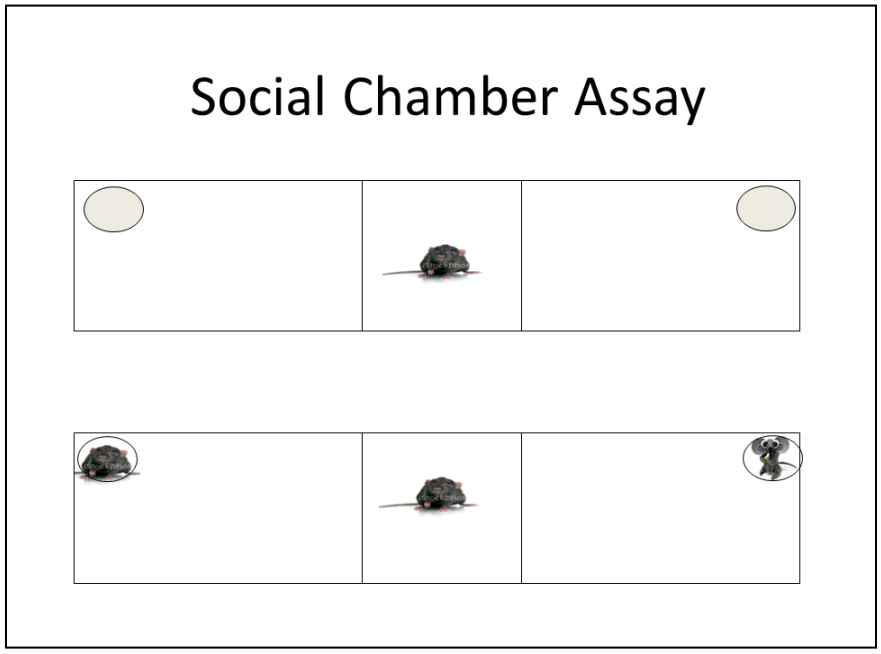
Social Chamber Test

The social chamber test is used to examine social behavior in mice. Mice are observed to determine whether they spend more time interacting with a novel object or with another mouse.

Set-up and Procedure

Each tested mouse was placed in a clear acrylic box with removable partitions and three chambers (Moy et al., 2008). Mice went through two phases of testing. During the first, a mouse was placed in the center chamber. The doors that led through the partitions separating this chamber from the left and right were removed, and the animal was allowed to explore the entire box. The corners of the two side chambers housed empty black wire-mesh cylinders. A tall plastic cylinder was placed on top of each wire-mesh cylinder so that the animal could not climb on top of the cylinder. The time the mouse spent at each cup and the number of entries into each chamber by the mouse were quantified using the Ethom program. The mouse was then placed back in the center chamber after 10 minutes for the second phase. During this phase an unfamiliar C57BL/6J mouse of identical sex, similar age and similar weight was placed in one cylinder, and a similar sized black Lego® block object was placed in the other cylinder. The partner mice were initially habituated to being housed in the cylinder for one hour for two days prior to testing. The side where the novel partner mouse was placed was alternated to correct for possible side-preference. The mouse was then removed after a total of 10 minutes of testing, and the box was cleaned with 30% isopropyl alcohol. The time the mouse spent at each cup and the number of entries into each chamber by the mouse were quantified using the Ethom program.

Figure 4: Set-up of Social Chamber Test



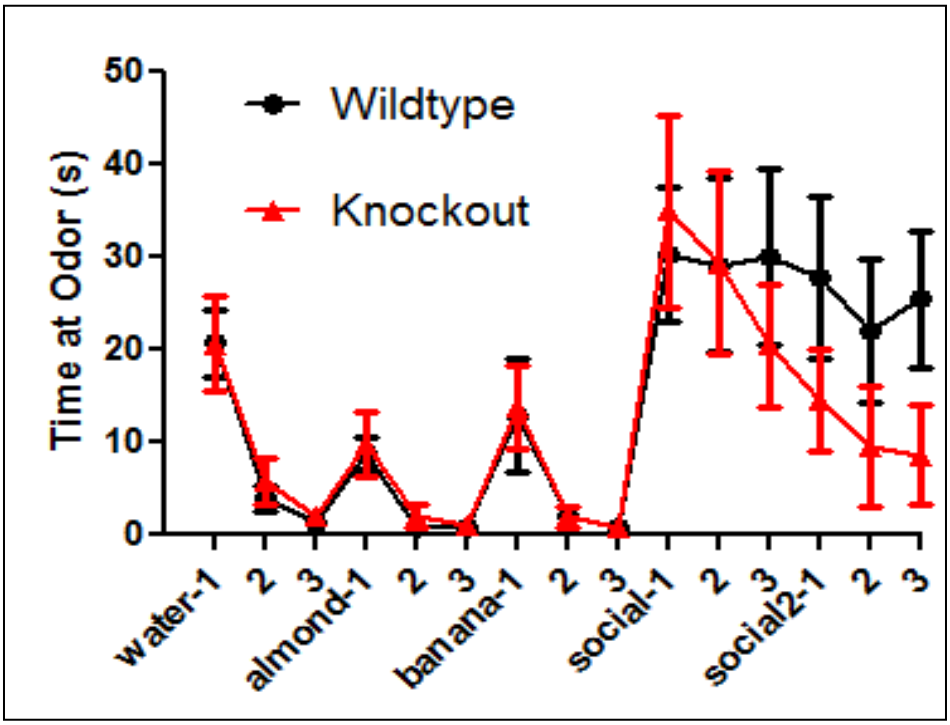
CHAPTER FOUR

Results and Conclusion

Odor Discrimination Test

The odor discrimination test was performed to confirm that the NS-PTEN wildtype and knockout mice do not have alterations in their ability to detect a novel odor. This test also verifies the ability of the mice to habituate to a novel odor over time. The odors used for this test were water, almond, banana, social 1 and social 2. The two social odors are obtained from cages of different mice of the same strain.

Figure 2: Results of Odor Discrimination/Habituation Test



The wildtype and knockout mice showed an increase in time with a novel odor across the different odor presentations. The WT and KO mice also habituated to the repeated

presentations of the odors. However, the KO mice did not habituate to the social odor $F(2,60) = 0.72$, $p = 0.5$ but did not respond differently than the WT mice. In the second social odor there was a marginal group effect $F(1,30) = 3.0$, $p = 0.093$. This test had a sample size of 17, and the bars represent the mean with Standard Error of the Mean. Because the knockout mice present with a similar discrimination/habituation pattern to the wildtype mice, one can conclude that their olfactory senses are intact and can be used as a social behavior indicator.

Social Partition Test

Mutation in PTEN gene results in social behavior interaction deficits. PTEN wildtype (WT), heterozygous (HT), and knockout (KO) mice were tested in a social partition test. Figures 3A and 3B illustrate social behavior in the partition test for PTEN wildtype (black bars), heterozygous (white bars), and knockout littermates (grey bars). Bars represent means ±SEM for time in seconds at the partition during the first test with a familiar overnight partner, time in seconds with an unfamiliar partner, and time in seconds with the reintroduction of the overnight partner. Asterisks indicate a significant difference ($p < 0.05$) between PTEN-KO and WT and HT littermates at the time point.

Figure 3A illustrates that the KO mice spent significantly less time at the partition with the partner animal compared to the WT and HT animals in the first familiar condition: $F(2,41) = 4.7$, $p < 0.05$; unfamiliar condition: $F(2, 41) = 4.2$, $p < 0.05$; and in the second familiar condition: $F(2,41) = 5.6$, $p < 0.01$.

Figure 3A: Results for Social Partition Test

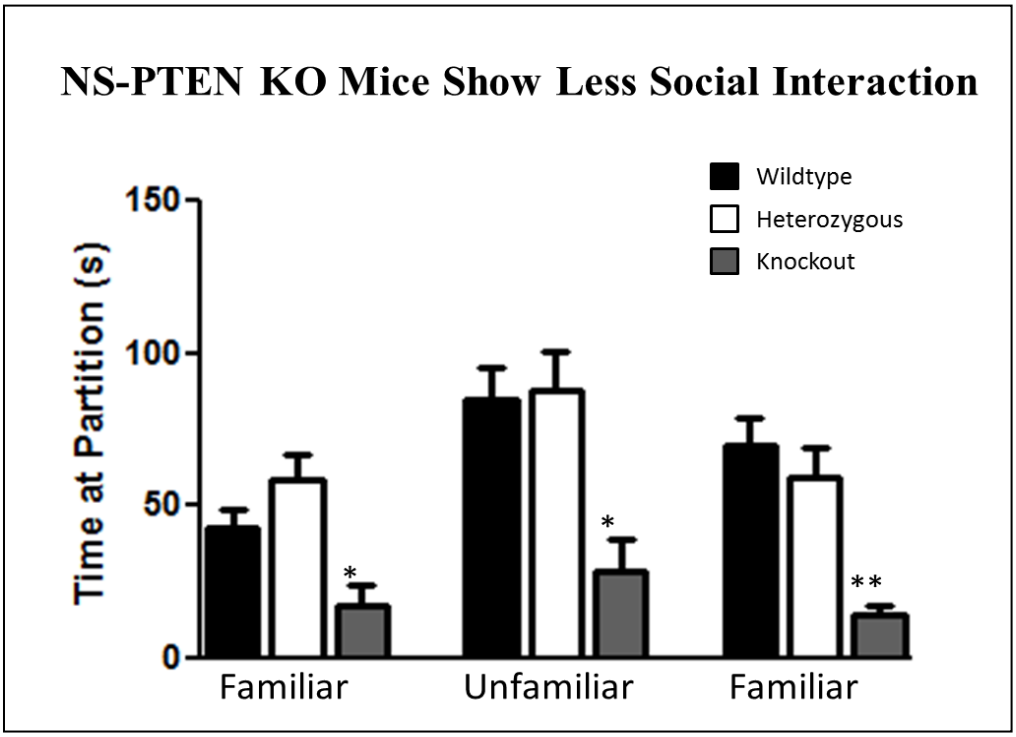


Figure 3B: Frequency at Each Partition

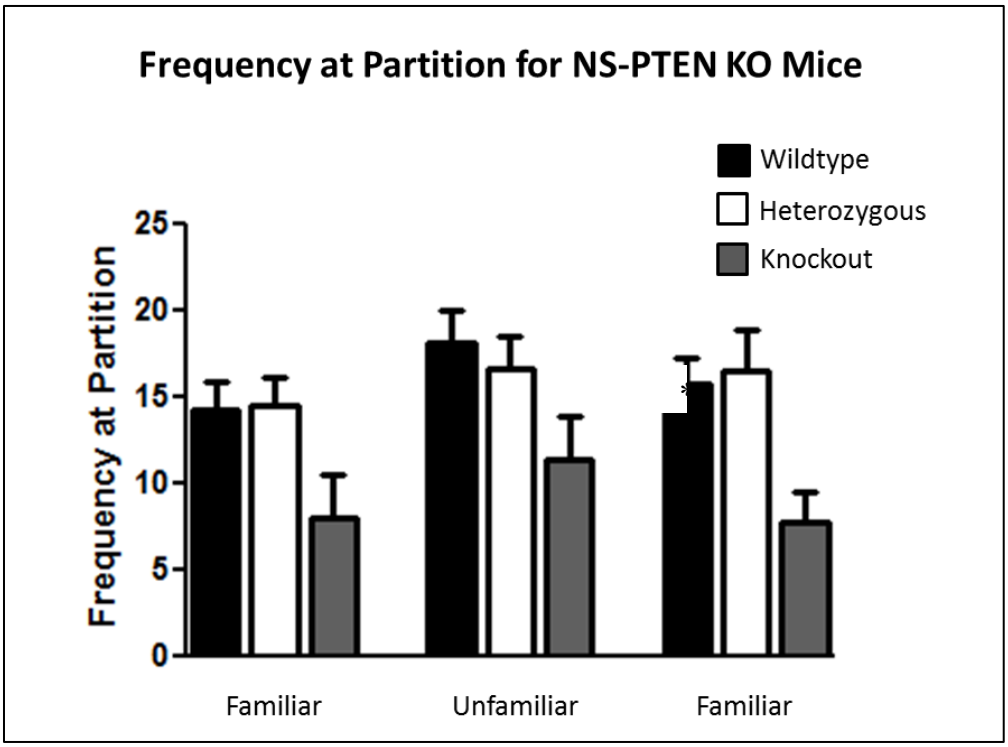


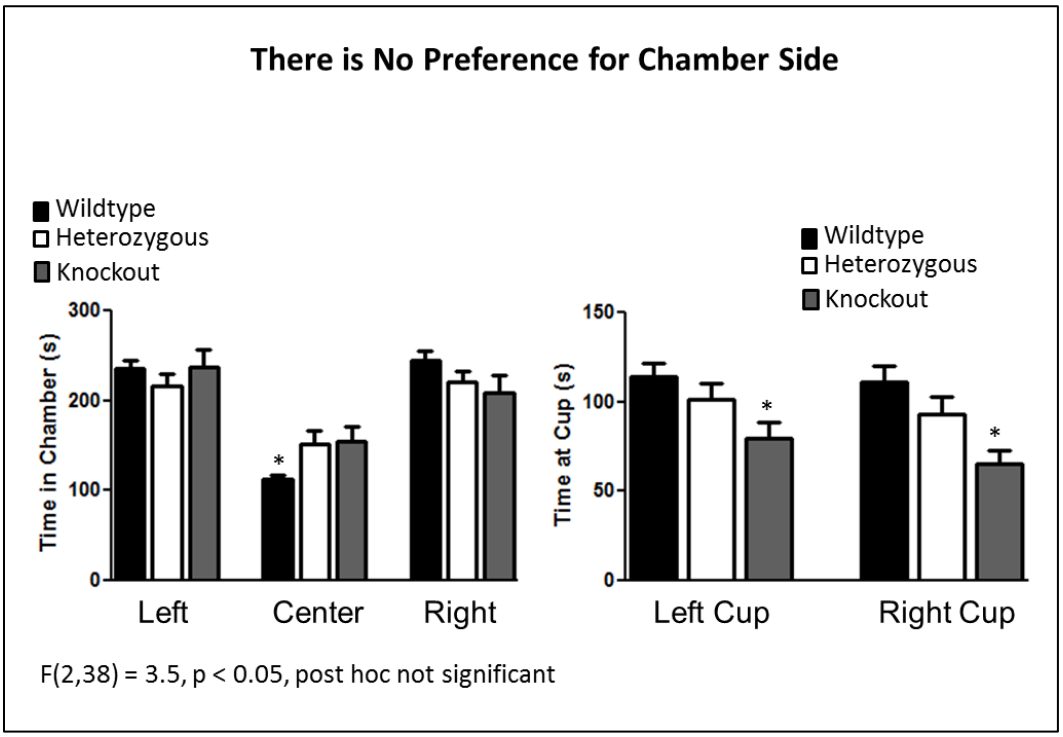
Figure 3B indicates that a mutation in the PTEN gene results in less frequent social behavior interaction. PTEN wildtype (WT), heterozygous (HT), and knockout (KO) mice were tested in a social partition test. The KO mice visited the partition less frequently in the second familiar condition: $F(2,41) = 3.4$, $p < 0.05$. Bars represent the mean and error bars represent the standard error of the mean. ** = $p < 0.01$; * = $p < 0.05$.

Social Chamber Test

The social chamber test was used to test for social behavior in mice. There are three chambers in the apparatus used. The left and right chambers house either a live mouse or an inanimate object, and the mouse being tested is placed in the middle chamber. An indicator of social behavior deficits is if the mouse spends more time with the inanimate object versus the live mouse.

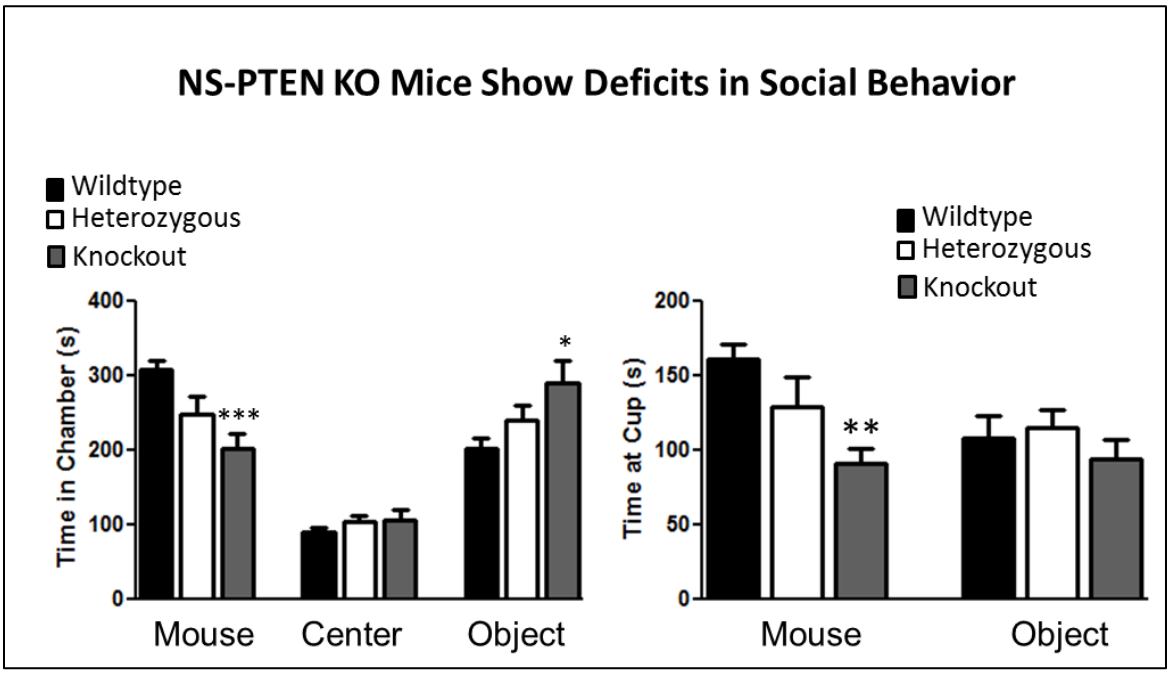
Figure 4A: From Nadler, 2004, p.3

Figure 5A: Results for Chamber Preference



One bias that must be accounted for with the social chamber test is whether or not the mice have a preference for a particular chamber side. We observed that a mutation in the PTEN gene does not produce a chamber bias in the social chamber test. This is shown in Figure 5A. PTEN wildtype (WT), heterozygous (HT), and knockout (KO) mice were tested in a social chamber test. They did not show any side preference for the chambers on the first trial of the test. Bars represent the mean, and error bars represent the standard error of the mean.

Figure 5B,C: Social Chamber Test Results



Mutation in the PTEN gene results in a decrease in social behavior in the social chamber test. Figure 5B (left) shows the time the tested mouse spent in each chamber. PTEN wildtype (WT), heterozygous (HT), and knockout (KO) mice were tested in the second trial of the social chamber test. The KO mice spent less time investigating the mouse than the WT mice $F(2,38) = 9.10$, $p < 0.001$. The KO mice spent more time investigating the novel object than the WT mice $F(2,38) = 4.06$, $p < 0.05$. Bars represent the mean and error bars represent the standard error of the mean. *** = $p < 0.001$; * = $p < 0.05$.

Figure 5C (right) shows that the PTEN KO spent less time at the cup with a mouse compared to WT mice $F(2,38) = 6.74$, $p < 0.01$. However, all groups spent equal time investigating the novel object $F(2,38) = 0.54$, $p = 0.59$. Bars represent the mean and error bars represent the standard error of the mean. ** = $p < 0.01$.

Discussion

This study successfully illustrated that a PTEN knockout gene results in social behavior deficits. When tested for odor discrimination and habituation, the knockout mice responded similarly to the wildtype mice, demonstrating intact olfaction and the ability to habituate to a novel odor. In the social partition test, the knockouts spent significantly less time at the partition for all conditions compared to the heterozygotes and wildtypes. For the social chamber test, the knockout mice spent more time at the cup with the inanimate objects, which is indicative of decreased sociability. To eliminate bias the mice were tested for chamber preference, and the results show no specific preference. Overall, the PTEN knockout mice respond as expected for being socially deficient.

Autism predominantly results in social behavior deficits in patients. Children with the disorder are commonly observed to engage in little social interaction, and the degree of sociability is one factor used in the DSM-IV to diagnose the specific level of autism in a patient. Because mice are an accepted model for examining expected neurological phenotypes, this project assumes that a social behavior deficit in mice can represent a likely deficit in humans with similar genetic mutations.

The mTOR pathway is useful in observing genetic mutations because it can be easily altered in mouse models. It is highly regulated by genes such as PTEN, and alterations of these genes can quickly elicit phenotypic changes that can be easily quantified. Because PTEN mice present with seizures and social behavior deficits, it can be concluded that both seizures and social behavior deficits can coexist in individuals. These results further add to the evidence that epilepsy and autism present as comorbid in specific genetic neurological disorders.

For further research it would be important to research the possible causality of epilepsy in autism. Epilepsy may itself contribute to the etiology of autism, although evidence does not yet support this. Future experiments can possibly introduce antiepileptic drugs in the PTEN knockout mice before seizure onset to solely examine the effect of mTOR hyper-activation on social behavior.

The significant results of the social behavior tests in this project indicate that epilepsy and autism present as comorbid during mTOR hyper-activation, a genetically caused state. This points to the need for further research to be done in this area, which will hopefully illumine a contributor to the etiology of autism.

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