ABSTRACT

Rapamycin Improves Social and Stereotypic Behavior Abnormalities Induced by Neural Subset Specific *Pten* Deletion

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In both patients and rodent models, mutations to the phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) gene result in hyperactivation of the mammalian target of rapamycin (mTOR) pathway – a signaling system integral to neural growth – followed by seizures, intellectual disabilities, and autistic behaviors. Rapamycin, an inhibitor of mTOR, can reverse the epileptic phenotype of neural subset specific *Pten* knockout (NS-*Pten* KO) mice, but its impact on behavior is not known. To determine the behavioral effects of rapamycin, male and female NS-Pten KO and wildtype (WT) mice were assigned as controls or administered 10 mg/kg of rapamycin for 2 weeks followed by behavioral testing. Rapamycin improved social behavior in both genotypes, $p < .05$, and stereotypic behaviors in NS-Pten KO mice, $p < .05$. These data demonstrate the potential clinical use of mTOR inhibitors by showing its administration can reduce the production of autistic-like behaviors in NS-Pten KO mice.

Rapamycin Improves Social and Stereotypic Behavior Abnormalities Induced by Neural Subset Specific *Pten* Deletion

by

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To Adrian

CHAPTER ONE

Introduction

Neurodevelopmental disorders are conditions that are associated with impaired nervous system development and include intellectual disability (ID) and autism spectrum disorder (ASD) (American Psychiatric Association, 2013). These and other neurodevelopmental disorders are often categorized by their symptomology. The diagnostic criteria for ID are impaired intellectual performance in learning, judgement, and problem solving, as well as deficits in adaptive functioning (American Psychiatric Association, 2013). While individuals with ASD may develop some of the same symptoms as ID, ASD is characterized by restricted, repetitive behaviors and ongoing deficiencies in communication and social behavior (American Psychiatric Association, 2013).

The cognitive and behavioral manifestations of ID and ASD can be severely detrimental to an individual's daily life and often last a lifetime. Worldwide, intellectual disability and ASD were the top 3 and 4 most prevalent developmental disabilities, with intellectual disabilities being the most impactful on the number of years lived with a disability (Olusanya et al., 2018). Care for these disorders can be economically burdensome to the individual as well as both the family unit and society. ID has been associated with heightened psychological distress and caregiving costs to parents of children with ID, as well increased healthcare costs during adulthood

(Genereaux, van Karnebeek, & Birch, 2015; Lunsky, De Oliveira, Wilton, & Wodchis, 2019; Masulani-Mwale, Kauye, Gladstone, & Mathanga, 2018). In a review of nearly 50 publications on the costs of ASD in the US and abroad, Rogge and Janssen (2019) found that care often requires frequent use of healthcare specialists, pharmacotherapy and behavioral interventions; gets more expensive as those with the disorder age; and can drastically impact employment. These expenses are markedly concerning given that many neurodevelopmental disorders may disproportionately afflict low-income families (Olusanya et al., 2018; Zablotsky et al., 2019).

Despite the separate classifications within neurodevelopmental disorders, there is considerable overlap in symptomology among many of the disorders and a growing consensus that they should be viewed as occurring along a continuum rather than as discrete conditions. ASD and ID often co-occur and may sometimes confound the diagnosis of the other (Polyak, Kubina, & Girirajan, 2015). Out of a sample of nearly 1,000 autistic children in the United States, 68% demonstrated intellectual impairments (Yeargin-Allsopp et al., 2003). The etiologies of ASD and ID are often shared as well (American Psychiatric Association, 2013). While specific causes are not always known, several known and possible etiologies have been discovered. Broadly, these causative factors can be categorized as environmental or genetic. Of the known etiologies, genetic causes comprise a large portion of cases and are a unifying source of ASD and ID (American Psychiatric Association, 2013; Bélanger & Caron, 2018; Polyak et al., 2015). While the environmental contributions to the development of ASD and ID may sometimes be curtailed by awareness and prevention programs, genetic causes can be more challenging to treat and therefore represent a need in the neurodevelopmental

disorder community. programs, genetic causes can be more challenging to treat and therefore represent a need in the neurodevelopmental disorder community.

Due to the complexity of the genetic etiologies underlying ASD and ID, those involving a single gene represent a group of potentially treatable syndromes and are considered a promising target of drug development research in neurodevelopmental disorders (Enriquez-Barreto & Morales, 2016; Miles, 2011; Polyak et al., 2015; Schaaf & Zoghbi, 2011). One such example involves mutations to the phosphatase and tensin homolog on chromosome ten (*PTEN*) gene. The *PTEN* gene is vital to neural development. While complete loss of *PTEN* results in death, mutations to this gene can lead to nervous system maldevelopment (DiCristofano, Pesce, Cordon-Cardo, & Pandolfi, 1998; Stambolic et al., 1998). Recently, 87% of MRI images from a small cohort of 16 children with *PTEN* mutations were found to have a variety of morphological brain abnormalities, including excessive brain growth and white matter anomalies, as well as intellectual delays (56%) and ASD (16%) (Ciaccio et al., 2019). PTEN controls growth and proliferation during neural development by regulating the phosphoinositide 3-kinase (PI3K) signaling pathway (Stambolic et al., 1998). *PTEN* mutations, or disruptions to its associated PI3K pathway, have also been linked to altered cortical development and seizures (Jansen et al., 2015).

This thesis focuses on determining the ability of pharmacologically inhibiting the mammalian target of rapamycin (mTOR), a downstream target of PTEN signaling, to reverse the cognitive and behavioral deficits inflicted by the disruption of PTEN signaling in a mouse model of cortical dysplasia. Therefore, my aims are to (1) compare the behavioral perturbations that occur in mice absent functioning PTEN proteins to those

with functioning PTEN proteins, (2) measure the behavioral effects of administering an mTOR inhibitor, (3) and determine if sex is a factor in treatment response. To achieve these aims, (1) a cohort of untreated, neuronal subset-specific *Pten* (*Ns-Pten*) knockout (KO) mice and wildtype (WT) mice will undergo a battery of behavioral testing, (2) as well as cohort of rapamycin treated *Ns-Pten* KO and WT mice. (3) Each cohort will be comprised of both sexes to be analyzed as a separate independent variable.

CHAPTER TWO

Review of the Literature

ASD with Intellectual Impairments

Numerous major ASD dedicated research groups have been reporting a sharp upward trend in the number of reported autism cases for the past several years. In the United States (US), the CDC ran Autism and Developmental Disabilities Monitoring (ADDM) network has been focused on determining the regional and temporal characteristics of ASD for the past two decades. In 2007, the ADDM network first reported the ASD prevalence of 8-year-olds from data obtained seven years prior. Across six cities, the prevalence of ASD was reported to be 6.7 per 1,000 children (Centers for Disease Control & Prevention, 2007). In every subsequent report but one, ADDM would demonstrate a steady increase in the prevalence of ASD in the US between 2002 and 2016, culminating with a prevalence of 1 in 54 – a 175% increase (Baio et al., 2018; Centers for Disease Control & Prevention, 2012, 2014; Christensen et al., 2016; Maenner et al., 2020). In support of the ADDM data, similar trends were also discovered using parent reported surveys (Kogan et al., 2018; Xu, Strathearn, Liu, & Bao, 2018). Globally, prevalence estimates have also been observed to be rising quickly, with discoveries analogous to those in the US occurring in the Middle East, Sweden, Asia, and Australia and New Zealand (Al-Mamri et al., 2019; Idring et al., 2015; Qiu et al., 2020).

Various factors are thought to underlie the steady rise in ASD cases, such as increased awareness and expansion of the ASD criteria. However, comparisons of data

obtained from the DSM IV to that with the DSM V suggest the rise in ASD cases may be explained by the perceived decline in ID cases (King & Bearman, 2009; Polyak et al., 2015; Shattuck, 2006). While estimates on the comorbidity of ASD with ID from nearly two decades ago have been as high as 68%, more recent estimates report ASD to be associated with cognitive dysfunction in only 33% of cases (Maenner et al., 2020; Yeargin-Allsopp et al., 2003).

Recently, Polyak et al. (2015) demonstrated how education records requiring only a single diagnosis, which are used by many epidemiological surveys in the US, have caused the loss of ID to contribute to up to 59% of the increase in ASD prevalence in 8-year-olds and 97% in 15-year-olds. While the overall proportion of kids with different disabilities did not change, there was a large increase in ASD cases and a decrease in intellectual disability, emotional disturbances, and specific learning disability (Polyak et al., 2015). Furthermore, when all categories were considered together, the significant increase in prevalence rates over time disappeared (Polyak et al., 2015).

The manner in which cognitive deficits are detected in individuals with ASD may also cause a poor identification of the comorbidity. The data in the US is based off of IQ testing (Maenner et al., 2020). However, this method of testing does not consider every-day reasoning capabilities, such as adaptive reasoning, that are critical to the ASD phenotype and thus require a more thorough clinical evaluation (American Psychiatric Association, 2013). Recent studies have also demonstrated that multiple areas of ASD research are biased towards those without intellectual impairments. Jack and Pelphrey (2017) showed that, despite the differing pathological presentations between ASD with and without a comorbid intellectual impairment, neuroimaging studies are heavily

skewed towards those without cognitive deficits. Many studies fail to take this bias into account and over-generalize findings about ASD without cognitive deficits to all individuals with ASD (Russell et al., 2019). Such exclusions reduce the generalizability of ASD data and undermine efforts to developing effective diagnostic criteria and therapeutics. Therefore, despite the perceived decline in ASD with ID, it remains a much-needed area of scientific inquiry.

Etiology

Environmental Factors

The early investigations into the genetics of autism led to the belief that ASD is largely of genetic origin due to such evidence as the high concordance rates amongst monozygotic twins (around 90%) and a higher rate of non-autistic familial intellectual deficits (Folstein & Piven, 1991). The etiological origins of ASD are now thought to be much more heterogenous and consist of a variety of external causes that vary in their association with ASD along the developmental timeline. These environmental factors include a maternal pro-inflammatory environment (Jones et al., 2017); organophosphate exposure during the prenatal and infant period (von Ehrenstein et al., 2019); pre- and post-term birth (Xie et al., 2017); maternal exposure to insecticides (Brown et al., 2018); prenatal supplementation (DeVilbiss et al., 2017); and complications at birth (Schieve, Clayton, Durkin, Wingate, & Drews-Botsch, 2015).

Interestingly, the associations between some of these environmental risk factors demonstrate variability that may depend on whether the disorder co-occurs with IDs. Exposure to multiple organophosphates during pregnancy and the first year of life were

associated with ASD, but the association was 30% greater in those with a comorbid intellectual disability (von Ehrenstein et al., 2019). Higher maternal serum DDT metabolite, p, p'-dichlorodiphenyl, was associated with a greater odds of developing ASD with ID but not without ID (Brown et al., 2018). Jones et al. (2017) showed that a maternal immune response during gestation was associated with an increased risk of ASD with intellectual impairment but not ASD without the comorbidity. Furthermore, they established that mothers of children with ASD and ID display distinct expressions of inflammatory markers compared to those with children who have ASD but not ID, leading the authors to conclude that this suggests an etiological difference between the two subgroups of ASD (Jones et al., 2017).

However, not all environmental risk factors share this subgroup specificity. Others were found to be equally associated to ASD individuals regardless of an ID co-occurrence. For example, preterm birth, low Apgar scores, and low size and birth weight were associated with ASD with ID but were equally associated to ASD without ID (Schieve et al., 2015).

Many studies investigating possible environmental causes of ASD have been difficult to replicate due to variations in study design, small sample sizes, and a failure to control for confounds (Bölte, Girdler, & Marschik, 2019). Others argue that these associations represent small effects that, when compounded with other environmental or genetic factors, may increase the risk of ASD (Lyall et al., 2017). However, some researchers do not differentiate ASD by their ID comorbidity, representing another hurdle in the path towards identifying true environmental causes of either disorder or their comorbidity. Research into the environmental causes of ASD and ID demonstrate the

importance of delineating these disorders from their comorbidity. However, due to the effect of gene / environment interactions, a complete characterization of the genetic influences on ASD with and without ID is warranted.

Genetic Factors

The various genetic contributions to the development of autism with intellectual disability are vast, ranging from large scale chromosomal abnormalities, such as aneuploidies, to alterations in a single nucleotide base pair, as in missense mutations (Guissart et al., 2018; Joseph et al., 2018). Multiple smaller mutations can combine to increase the risk of the development of ASD with ID and many of these mutations can be inherited, de novo, somatic or some combination of the three (D'Gama & Walsh, 2018; Gonzalez-Mantilla, Moreno-De-Luca, Ledbetter, & Martin, 2016; Jensen, Smolen, & Girirajan, 2020). Numerous ASD risk genes also overlap genes associated with ID. When pathogenic variations (i.e., loss of function) in a single nucleotide or number of repeated DNA sequences occur in ASD susceptibility genes, these more severe and pathogenic forms have been found to be associated with reduced IQ and adaptive abilities (Jensen et al., 2020; Trost et al., 2020; Yuen et al., 2017).

There also appear to be distinct subsets of genes that confer a greater risk to ASD with ID rather than either disorder alone. Mutations in genes such as *PTEN* conferred a roughly equal rate of ASD and ID compared to those associated with higher rates of autism (*KDM6B*, *ANK2*, and *DSCAM*) and those which demonstrate higher rates of ID (*STXBP1*, *CTNNB1*, and *ARIB1B*) (Stessman et al., 2017). Furthermore, genes most associated with high-functioning autistics may be functionally different than genes found with both autism and an intellectual impairment. For example, those with high-

functioning ASD have a greater number of pathogenic variants in genes not found to be involved in neurodevelopment compared to those with ASD and a lower IQ (Jensen et al., 2020).

Increased brain volume has also been correlated with both high-functioning ASD and ASD with concomitant ID. For example, many studies have found macrocephaly to be a highly heritable feature associated with an ASD diagnosis but there is conflicting evidence regarding whether macrocephaly is related to the comorbidity with ID. After analyzing the genes of more than 13,000 people across the globe with neurodevelopmental disorders, Stessman et al. (2017) showed that risk genes of ASD were associated with individuals demonstrating lower rates of microcephaly but higher rates of macrocephaly when compared to those with both ASD and ID and those with genes found almost exclusively in individuals with ID. Conversely, others have found macrocephaly related genes to be associated with ASD patients displaying below average IQ scores (Bernier et al., 2014; Guo, Wang, et al., 2018; McKeague et al., 2015; Sacco, Gabriele, & Persico, 2015). In one of the largest of such studies, out of 5,225 individuals diagnosed with ASD, 15.7% were found to have macrocephaly, but the highest effect sizes were found in those with IQ scores less than 70 (Sacco et al., 2015).

Together, these data show that ASD and ID may be nosologically different from the sum of their parts. However, due to the genetic heterogeneity of this disorder, phenotypic evaluations of risk genes associated with ASD, ID, and their comorbidity have been difficult to explain and sometimes produce competing results. For instance, the emergence of ASD in genetic disorders that cause ID have led some to conclude that autistic traits observed in those with ID may be due to a lack of intellectual capacity

rather than from ASD (Richards, Jones, Groves, Moss, & Oliver, 2015). However, others have found reductions in intellectual capacity independent of the development of ASD. Jensen et al. (2020) found that pathogenic mutations in genes associated with a reduction in IQ, increased the severity of ID but had no effect on the severity of ASD. While autistics individuals with macrocephaly were more likely to develop ID compared to those without macrocephaly, increased head circumference and brain size had no effect on autism severity score (Amaral et al., 2017).

These studies demonstrate the need for further investigations into the relationship between the genetic causes of ASD with ID. However, the genetic heterogeneity underlying the comorbidity has made this task difficult. While even monogenic causes of ID demonstrate variations in ASD prevalence, they display a higher prevalence of ASD than the general population. In a meta-analysis of ASD prevalence in single gene disorders of ID, Richards et al. (2015) found the likelihood of developing ASD to be 8 to 105 times greater in those with a monogenic disorder compared to that of the general population. Therefore, single gene syndromes represent a unique opportunity to investigate the full extent of a gene's contribution to the phenotypic expression of ASD with ID.

Neuropathology

Brain Overgrowth

During early brain development, a key characteristic of autism has been shown to be early brain overgrowth. In a recent prospective neuroimaging study of infants at risk for ASD, those who displayed an increased cortical surface area and brain volume were

more likely to develop ASD (Hazlett et al., 2017). In addition to total brain volume, regional enlargements have also been found. Infants who were at risk for ASD and had enlarged cerebellar and subcortical structures at 4 to 6 months were more likely to display symptoms associated with autism (Pote et al., 2019). Moreover, both regional and total increased brain growth during infancy also predicted symptom severity. Hazlett et al. (2017) found increased cortical surface area and brain volume were positively correlated with social behavior deficits and Pote et al. (2019) showed expanded cerebellar and subcortical structures predicted stereotyped behaviors.

As individuals with autism age, however, large studies have found little to no differences in total brain volume, especially in adults. In a comparison of over 800 images from children, adolescents, and adults with autism, total brain volume was a statistically significant 1.58% higher in those with autism but was no different when they considered only the adults (Riddle, Cascio, & Woodward, 2017). While multiple smaller studies have discovered regional differences after infancy, such as in subcortical and cerebellar structures, these findings did not survive meta-analyses (Traut et al., 2018; Williams, Peyre, Toro, Beggiato, & Ramus, 2020). These data support a critical theory in autism brain development, which posits ASD may be characterized by early brain overgrowth that resolves with age but results in altered connectivity and function (Girault & Piven, 2020; Riddle et al., 2017; Traut et al., 2018; Williams et al., 2020).

Still, in a subset of adults with ASD, brain volume may continue to expand into adulthood. For instance, in a critique of the early overgrowth hypothesis, it was demonstrated that a subgroup of autistic individuals present with macrocephaly – defined by these authors as a brain size more than two standard deviations (SD) greater than

typically developing individuals – and that this growth does not normalize with age (Yankowitz et al., 2020). Persistent brain overgrowth has also been shown to impact IQ in a manner different from those with autism compared to typically developing individuals. While in controls, greater brain volumes were positively correlated with IQ, in individuals with ASD the association has been found to be either much weaker (Lefebvre, Beggiato, Bourgeron, & Toro, 2015) or not to occur at all (Amaral et al., 2017). When brain sizes reach the point of macrocephaly, growth is associated with the lowest IQ scores and a greater chance of developing ID (Amaral et al., 2017; Yankowitz et al., 2020). Therefore, this pattern of early brain growth that is alleviated by adulthood may be truer for high-functioning autistics than those with concomitant intellectual disabilities. Moreover, this suggests a continued pattern of overgrowth may be a catalyst towards the development of ID in those with ASD.

Neuronal Connectivity and Functional Activity

A variety of studies in children and adults with ASD suggest this disorder is one of altered network and neuronal microstructure connectivity, followed by disruptions in functional activity. For instance, recent post-mortem and neuroimaging data have shown cortical dyslamination at the border between grey and white matter (Andrews et al., 2017; Trutzer, García-Cabezas, & Zikopoulos, 2019), and EEG, MEG, and MRI results collectively show broad reductions in long-range activity between regions but also focal areas of increased activity (O'Reilly, Lewis, & Elsabbagh, 2017; Rane et al., 2015).

Epilepsy has been a chief form of altered functional activity found in those with ASD. Using the national patient register of children and adolescent twins from Sweden, Gillberg, Lundström, Fernell, Nilsson, and Neville (2017) found 9.7-13.7 % of those with

epilepsy had ASD. These findings were recapitulated in a recent systematic review of data from 74 studies across the globe which found the median prevalence of epilepsy in ASD between 1950 and 2016 was 12.1% (Lukmanji et al., 2019). Some evidence also seems to suggest that epilepsy could be a driver of ASD development. For example, epilepsy was found to be associated with impaired language capabilities and developmental regression in those with ASD (Viscidi et al., 2013), and the earlier a person is diagnosed with epilepsy, the more likely they may be diagnosed with ASD (Gillberg et al., 2017; van Eeghen et al., 2013).

Others have found that the likelihood of acquiring epilepsy during development may increase with age in those with ASD. In a cohort of nearly 6 thousand autistic children between the ages of 2 and 17 years old, the prevalence of epilepsy during childhood more than doubled during adolescence (Viscidi et al., 2013). This could indicate that seizures share some neuropathology with ASD, rather than contribute to ASD development *per se*. Moreover, the findings by Gillberg et al. (2017) showing that an early diagnosis of epilepsy increased the likelihood of developing ASD did not survive correction for multiple comparisons.

Epilepsy is also associated with the comorbidity of ID in those with ASD. For instance, Viscidi et al. (2013) found epilepsy was most likely to occur in autistic children with poor language function, regression, a lower average IQ score and comorbid diagnosis of ID. However, after controlling for IQ, epilepsy was no longer associated with language function and regression (Viscidi et al., 2013). This suggests the connection between epilepsy and ASD with ID may be more strongly related to cognitive dysfunction in those with ASD than the development of autistic symptoms. This finding

is supported by others who have also shown cognitive dysfunction remains a common feature of epilepsy in ASD, even in the absence of impaired language capabilities and regression (Valvo et al., 2013). Indeed, abnormal EEG patterns and epilepsy in ASD may be a significant predictor of cognitive dysfunction. In children and adolescents with autism, the likelihood of finding epileptic and paroxysmal EEG abnormalities was found to increase with the severity of comorbid ID (Unal et al., 2009). However, the most striking evidence comes from a recent investigation of the Swedish Twins cohort, which found the prevalence of epilepsy in autistic children was 36 times higher in those with learning disabilities than in those with ASD alone (Alabaf et al., 2019).

While the question of whether epilepsy is a catalyst or consequence of ASD still remains under investigation, the studies in this section suggest a link between ASD, ID, and epilepsy. If indeed the earlier one develops epilepsy the more likely they are to be diagnosed with ASD (Gillberg et al., 2017; van Eeghen et al., 2013) and/or severe ID (Unal et al., 2009; van Eeghen et al., 2013), then features that may predict epilepsy in ASD should be a high priority. While some evidence suggests the association between epilepsy, ASD, and ID is independent of MRI identified structural abnormalities (Unal et al., 2009), seizures are a prominent feature in some with ASD and macrocephaly. In a sample of 206 children with idiopathic autism, a height greater than 1.5 standard deviations above the average – interpreted by the authors to mean generalized overgrowth – predicted the presence of EEG abnormalities (Valvo et al., 2013). When these individuals also had macrocephaly, they were more likely to acquire epilepsy during early childhood development (Valvo et al., 2013). Seizures and intellectual impairments have also been found in multiple small studies of patients with macrocephaly and ASD

that were screened for mutations in genes important to cell growth and proliferation (Ciaccio et al., 2019; Hobert, Embacher, Mester, Frazier, & Eng, 2014; Orrico et al., 2009; Schwerd et al., 2016; Wu et al., 2020; Yeung et al., 2017).

The Mammalian Target of Rapamycin (mTOR)

Some of the most common developmental processes dysregulated in ASD with ID and which may contribute to the disruptions in neuronal signaling are those involving growth and migration (Andrews et al., 2017; Girault & Piven, 2020; Hazlett et al., 2017; Pote et al., 2019; Williams et al., 2020). One such signaling system is the mammalian target of rapamycin (mTOR) pathway, the major role of which is to mediate a cells response to growth signals by regulating the production of proteins needed for cellular growth in the context of available resources (i.e., AMP:ATP ratio, glucose levels) (Switon, Kotulska, Janusz-Kaminska, Zmorzynska, & Jaworski, 2017). Growth factors, as well as hormones and insulin, activate a cascade of signaling molecules that culminate in the stimulation of mTOR (Nikoletopoulou, Sidiropoulou, Kallergi, Dalezios, & Tavernarakis, 2017; Switon et al., 2017). Nervous system specific growth factors, such as brain derived neurotrophic factor (BDNF), stimulate membrane bound tyrosine receptor kinases, activating phosphoinositide 3 kinase (PI3K) (Andrews, Subramanian, & Kriegstein, 2020; Nikoletopoulou et al., 2017; Switon et al., 2017). PI3K activation results in the phosphorylation of Akt, which mediates the stimulation of mTOR complex 1 (Andrews et al., 2020; Nikoletopoulou et al., 2017; Stambolic et al., 1998). The mTOR complex then leads to the activation of eukaryotic translation initiation factor 4 proteins (eIF4) and the ribosomal protein S6; both of which regulate the translation of proteins

required for axonal migration, dendritic arborization, synaptic plasticity and neuronal excitability regulation (Switon et al., 2017).

Recent studies using human cortex tissue have demonstrated that proper mTOR signaling is critical to brain development. In primary human cortex tissue, enhancing mTOR signaling by BDNF administration increased the sprouting of extra processes, shortened preexisting processes, reduced migration and altered the orientation of some neural precursors cells during cortical development (Andrews et al., 2020). Somatic mutations to mTOR that enhance its phosphorylating capabilities lead to aberrant neuronal migration, disrupted patterns of cortical layering, and enlarged neuronal soma size (Park et al., 2018). Others have shown dysregulation of mTOR signaling may disrupt synaptic pruning during development in brain regions correlated with autistic behaviors. In a postmortem examination of brain tissue from children and adolescent patients with ASD, Tang et al. (2014) examined layer V pyramidal neurons of the temporal lobe, due to its association with social behavior, and found hyperactive mTOR signaling was associated with increased synaptic densities.

Mutations to genes regulating mTOR signaling have also been discovered in individuals with ASD and ID. This was first identified in case studies of patients with macrocephaly and led to the recommendation of genotype testing in individuals that presented with macrocephaly accompanied by ASD and/or ID (Goffin, Hoefsloot, Bosgoed, Swillen, & Fryns, 2001; Zori, Marsh, Graham, Marliss, & Eng, 1998). While recent studies are small, they too demonstrate an association between hyperactive mTOR signaling and ASD with ID. Yeung et al. (2017) genotyped 21 patients with macrocephaly and found 10 had mutations along the mTOR pathway. While all 10

individuals with mTOR mutations had either a developmental delay or intellectual disability, half of them were also diagnosed or suspected to have ASD (Yeung et al., 2017).

Hyperactive mTOR signaling in brain development may also be a cause of macrocephaly and epilepsy, both of which are associated with the ASD/ID comorbidity. Sections of cortical tissue surgically resected from patients with an enlarged and focally disorganized cortex, as a procedure to alleviate seizures, demonstrated enhanced mTOR signaling (Park et al., 2018). Mutations to genes responsible for inhibiting mTOR have also been identified in patients with macrocephaly and epilepsy (Ciaccio et al., 2019; Hobert et al., 2014; Orrico et al., 2009; Schwerd et al., 2016; Wu et al., 2020). Animal studies involving mice have recapitulated these findings. Mice with gain of function mutations to mTOR develop EEG abnormalities, spontaneous seizures, and macrocephaly (Park et al., 2018). These studies suggest hyperactive mTOR signaling may play a critical role in the development of ASD with ID. Identifying a common contributor to this hyperactivation may provide a target for future therapeutic development.

PTEN Mutations

A major function of PTEN, which has lipid phosphatase activity, is dephosphorylating PIP3, thereby reducing the downstream activation of Akt by PI3K (Maehama and Dixon, 1998; Stambolic et al., 1998; Weng et al. 1999). When PTEN's phosphatase activity is impaired, unfettered Akt signaling leads to enhanced mTOR activity and causes the intractability of multiple cellular process underlying development. Early *in vitro* studies of murine embryos and cultured cell lines showed homozygous

Pten disruptions resulted in enhanced cell proliferation, overgrowth of the cephalic region, asynchronous germ cell layer development, and apoptotic resistance to several endogenous and exogenous compounds known to induce cell death, but was lethal to the embryo (Stambolic et al., 1998; Di Cristofano et al., 1998).

In addition to overall embryonic development, *PTEN* plays a major role in regulating the development of the central nervous system. During embryonic development, *PTEN* is expressed in humans in various tissues, including those of the nervous system (Gimm et al., 2000). Using murine models, specific cell types and brain regions were identified as expressing *PTEN* and include the pyramidal neurons of the cortex, the Purkinje and pyramidal cells of the cerebellum, and in neurons from across every region of the hippocampus (Lachyankar et al., 2000). In the absence of *PTEN*, brain wide alterations in growth, cell number, and organization occur. Using the Cre/loxP system, Groszer and colleagues (2001) demonstrated that CNS wide deletion of *PTEN* in the embryo during gestation caused macrocephaly and increased cell proliferation as early as embryonic day 14 (E14), with brain weight and cell number twice that of controls at birth, as well as a dramatic dyslamination of the cortex, cerebellum, and hippocampus.

The first case studies reporting the nervous system effects of mutations to the phosphatase and tensin homolog gene on chromosome 10 (PTEN), which can occur via inherited or germline mutations, only mentioned the gliomas of Cowden disease (Nelen et al., 1996; Li et al., 1997). Research then quickly began to include other nervous system and behavioral characteristics of individuals with *PTEN* mutations, such as intellectual impairments (Arch et al., 1997; Liaw et al., 1997), autism (Goffin et al., 2001; Zori et al.,

1998), and seizures (Parisi et al., 2001). Every patient notably had a comorbid diagnosis of macrocephaly, leading some of the authors to suggest screening for *PTEN* mutations in patients with macrocephaly and developmental disorders (Arch et al., 1997; Goffin et al., 2001; Liaw et al., 1997; Parisi et al., 2001; Zori et al., 1998). These early studies indicated that excessive head growth associated with mutations to *PTEN* could impair cognition, promote the development of ASD, and lead to epilepsy.

It had already been demonstrated that system wide deletion of Pten was lethal in mice (DiCristofano et al., 1998; Stambolic et al., 1998) and that even isolating the deletion of *Pten* to the central nervous system resulted in death soon after birth (Groszer et al., 2001). In order to analyze the role of PTEN in brain development and behavior, cell and region-specific models were needed. Using Cre/loxP with a modified glial fibrillary acidic protein (GFAP) promoter, *Pten* expression was successfully ablated primarily in the excitatory granule cells of the cerebellum and dentate gyrus during postnatal brain development, as well as some excitatory pyramidal neurons of the cortex (Backman et al., 2001; Kwon et al., 2001; Ljungberg, Sunnen, Lugo, Anderson, & D'Arcangelo, 2009). As a result, these neuronal subset specific (NS) *Pten* KO mice acquired macrocephaly, dysplasia, and seizures that progress with age (Backman et al., 2001; Kwon et al., 2001; Ljungberg et al., 2009; Nguyen et al., 2015; Sunnen et al., 2011). A similar model was later produced using a neuron-specific enolase (NSe) promoter to drive *cre* expression that resulted in post-mitotic *Pten* deletion of neurons in the cortex, hippocampus, and cerebellum during development (Kwon, Zhou, et al., 2006). NSe-*Pten* KO mice also demonstrated seizure activity, macrocephaly, and dysplasia (Kwon, Luikart, et al., 2006).

These early models collectively showed the importance of *Pten* in regulating brain growth and structural organization *in vivo* and showed that the neurological consequences of neuronal subset *Pten* loss in mice mimic what is found in many patients with germline *PTEN* mutations, namely seizure activity, macrocephaly, and dysplasia (Ciaccio et al., 2019; Hobert et al., 2014; Mester, Tilot, Rybicki, Frazier, & Eng, 2011; Parisi et al., 2001). Further characterization of these *Pten* KO mice showed they exhibited a variety of behavioral impairments analogous to those seen in autistic and cognitively impaired patients with *PTEN* mutations.

Both models have been shown to demonstrate impaired social behavior. In the social partition and three-chamber social preference tests, NS-*Pten* KO mice spend less time interacting with novel conspecifics and more time interacting with a novel object compared to a novel mouse (Lugo et al., 2014). The enolase promoter driven KO demonstrates less interaction with other mice as juveniles and do not prefer to interact with a novel mouse over a novel object (Kwon, Luikart, et al., 2006; Zhou et al., 2009). NS-*Pten* KO pups emit fewer and shorter ultrasonic vocalizations when separated from the dam compared to pups with typical *Pten* expression (Binder & Lugo, 2017). As adults, NSe-*Pten* KO males display reduced mounting behavior and pups born to NSe-*Pten* KO females (sired by wildtype males) have an increased mortality by PD5, indicative of poor maternal care (Kwon, Luikart, et al., 2006).

The expression of other behaviors associated with ASD varies between these two models. Increased repetitive behaviors have been found in the NS-*Pten* KO, such as increased circling behavior in the open field, that have not been demonstrated in the enolase model (Kwon, Luikart, et al., 2006; Lugo et al., 2014). In addition to repetitive

behaviors, motor coordination problems are also found in ASD (Pote et al., 2019). While NS-*Pten* KO mice demonstrate ataxia and poor motor coordination on a variety of tests, including the rotarod and sticker removal test, the enolase model demonstrate increased motor coordination on the rotarod test compared to wildtype mice (Backman et al., 2001; Kwon, Luikart, et al., 2006; Kwon et al., 2001; Lugo et al., 2014; Nolan et al., 2019).

Epileptiform activity has been identified in both the enolase model and GFAP driven models. In the NSe-*Pten* KO mice, epileptiform activity appears to be low in rate and duration (Kwon, Luikart, et al., 2006) with a progression that occurs over 33 weeks (Ogawa et al., 2007) while NS-*Pten* KO mice demonstrate an early progression of high abnormal EEG activity and seizures (Backman et al., 2001; Kwon et al., 2001; Ljungberg et al., 2009; Sunnen et al., 2011) with epileptiform activity occurring at a near continuous rate during EEG traces by 9 weeks of age (Nguyen et al., 2015). Timm staining of mossy fiber sprouting, which often follows seizure like activity, is recorded in NS-*Pten* KO mice as early as 4 weeks of age (Sunnen et al., 2011) whereas NSe-*Pten* KO mice show abnormal Timm staining at 7 months (Kwon, Luikart, et al., 2006).

There may also be an anxiety-like behavioral phenotype in these models. Kwon, Luikart, et al. (2006) reported that NSe-*Pten* KO mice demonstrate hyperactivity during tasks that place mice in compromising and stressful situations, such as the open field test and during handling. Although both *Pten* mutant models display increased locomotion and anxiety-like behavior in the open field, and Kwon, Luikart, et al. (2006) found increased anxiety-like behavior in the light/dark test, these data are difficult to reconcile with reduced anxiety-like behavior shown in the elevated plus maze also found in both models (Kwon, Luikart, et al., 2006; Lugo et al., 2014).

The divergence in symptoms and epilepsy severity between these two models may be the result of the differences in manner and timing of *Pten* deletion. The deletion of *Pten* via the GFAP promoter occurs prior to migration, and potentially neuronal differentiation, and deletion using the enolase-based model occurs after post-mitotic growth and migration (Backman et al., 2001; Kwon, Luikart, et al., 2006; Kwon et al., 2001). This may indicate that the behavioral impact and development of autistic-like behavior in *Pten* mutant mice may be dependent on the time in development the deletion occurs, and indicate that the earlier the mutation, the more severe the deficits. Another, and not necessarily mutually exclusive possibility, is that the increase in epilepsy in the NS-*Pten* KO mice may be contributing to the more severe autistic-like phenotype and cognitive deficits in these mice. NS-*Pten* KO mice, which show high degrees of epileptiform activity, demonstrate memory impairments and impaired stereotyped behavior that are absent in the NSe-*Pten* KO mice (Hodges et al., 2018; Kwon, Luikart, et al., 2006; Lugo, Smith, Morrison, & White, 2013).

These data show how a spectrum of deficits can occur as a function of minor changes in the timing of *Pten* deletion and the development of seizure activity. NS-*Pten* KO mice provide the opportunity to evaluate the effects of brain overgrowth and epilepsy in the development of ASD with ID. Future studies are needed to determine the behavioral impact of reducing mTOR and epileptiform activity in the NS-*Pten* KO model.

Rapamycin

In neurons with hyperactive mTOR signaling secondary to *Pten* deletion, Akt becomes hyperphosphorylated, causing mTOR hyperactivity as measured by a number of

targets downstream from mTOR (Nguyen et al., 2015), such as translation initiation factor eIF4G and ribosomal protein S6 (Ljungberg et al., 2009; Nguyen et al., 2015). Rapamycin is an anti-fungal compound that has immunosuppressive activity and impairs the phosphorylating capabilities of mTOR (Kang et al., 2013; Lu et al., 2015). After administration, rapamycin combines with the FK506 binding proteins before allosterically reducing the affinity of mTOR to its target substrates (Kang et al., 2013). Previous studies have demonstrated that rapamycin administration can retard mTOR hyperactivity in the *Pten* null neurons of NS-*Pten* KO mice. Two weeks of rapamycin treatment reduced the activation eIF4G and S6 in the pyramidal neurons of cortical layer V (Ljungberg et al., 2009) and the activation of Akt and S6 in the granule cells of the hippocampus (Nguyen et al., 2015).

NS-*Pten* KO mice administered rapamycin for two weeks also reduces a majority of the neurological abnormalities observed in these mice. The cortical pyramidal neurons of NS-*Pten* KO mice are hypertrophic by postnatal day 12 (Ljungberg et al., 2009) and the granule cells of the hippocampus are two times larger compared to controls by 4 weeks of age (Backman et al., 2001; Nguyen et al., 2015). Although the hippocampus has not been directly evaluated, rapamycin reduced the hypertrophy of cortical pyramidal neurons (Ljungberg et al., 2009).

NS-Pten KO mice exhibit epileptiform EEG activity as early as 3-4 weeks after birth (Nguyen et al., 2015) that increase in severity and frequency with age (Backman et al., 2001; Ljungberg et al., 2009; Nguyen et al., 2015; Sunnen et al., 2011). Several studies have shown that rapamycin reduces the amount of time NS-*Pten* KO mice spend producing epileptiform EEG activity in the hippocampus and cortex (Ljungberg et al.,

2009; Nguyen et al., 2015; Sunnen et al., 2011), even when administered during the later stages of seizure progression when mice are producing near constant epileptiform activity (Nguyen et al., 2015). Mossy fiber sprouting in the hippocampus, a consequence of seizures, is found by 4 weeks of age in NS-*Pten* KO mice and also increases in severity with age (Sunnen et al., 2011). Sunnen et al. (2011) showed that, in addition to reducing seizure activity in the brain, rapamycin also prevented seizure induced aberrant mossy fiber sprouting in the hippocampus.

Although these data collectively demonstrate mTOR inhibition by rapamycin treatment reverses the morphological and signaling abnormalities that arise in NS-*Pten* KO mice, to date, no studies have investigated the impact of rapamycin administration on the development of the autistic-like behavioral phenotype in these mice. To determine if seizure suppression and the reversal of neuronal hypertrophy produce or exacerbate the behavioral deficits in NS-*Pten* KO mice, future studies should explore the effect of mTOR inhibition in this model.

Conclusion

Early brain overgrowth has been shown to be associated with a diagnosis of epilepsy during early childhood (Valvo et al., 2013), both of which increases the likelihood of developing cognitive impairments and autistic behaviors (Gillberg et al., 2017; Girault & Piven, 2020; Hazlett et al., 2017; Pote et al., 2019; Riddle et al., 2017; van Eeghen et al., 2013; Viscidi et al., 2013). Both mice and humans with neuronal hyperactive mTOR signaling, such as that caused by PTEN mutations, display macrocephaly, intellectual impairments, and autistic behaviors (Ciaccio et al., 2019; Hobert et al., 2014; Lugo et al., 2014; Lugo et al., 2013; Mester et al., 2011; Parisi et al.,

2001). Studies using NS-Pten KO mice have shown that rapamycin administration reverses neuronal hypertrophy and suppresses seizure activity (Ljungberg et al., 2009; Nguyen et al., 2015; Sunnen et al., 2011). Since the effect of rapamycin on behavior in NS-*Pten* KO mice has yet to be examined, these data pave the way to determine the impact of preventing brain overgrowth and seizure progression on the development of cognitive impairments and autistic-like behaviors in this model.

CHAPTER THREE

Methods

Animals

Mice were neuron subset-specific *Pten* (*NS-Pten)* conditional knockout (KO) mice generated using the Cre-Lox recombination system (GFAP-Cre; $Pten^{loxP/loxP}$) as previously described (Backman et al., 2001; Kwon et al., 2011). $Ns\text{-}Pten^{loxP/+}$ heterozygote mice were bred to produce $Ns\text{-}Pten^{+/+}$ wildtype (WT), $Ns\text{-}Pten^{loxP/+}$ heterozygous (HT), and $Ns\text{-}Pten^{loxP/loxP}$ KO mice. All mice were bred, group housed, and evaluated according to the guidelines set forth by Baylor University's Institutional Care and Use Committee and the National Institute of Health's *Guide for the Care and Use of Laboratory Animals*. Humidity was controlled, the temperature maintained at 22°C, and the lighting was set to provide a 12h:12h diurnal cycle with lights on at 0700 and off at 1900. Access to food and water was provided *ad libitum*. Mice were weaned at approximately three weeks of age. Prior to each behavioral test, all mice were weighed, visually inspected to ensure the health of all participants, and then allowed to habituate for 30 minutes before testing. To reduce carryover effects no less than 24 hours occurred between tests, no more than two tests occurred per week, and behavioral testing occurred in order of least invasive to most invasive: open field, elevated plus maze, marble burying, and social preference, trace fear conditioning (McIlwain et al., 2001). Behavioral testing was conducted during the light phase between 8am and 5pm. After testing, mice from the same home cage were kept separate from naïve mice until all mice

completed testing. Mice were returned to their original home cage with their littermates. All testing areas were cleaned with 30% isopropyl alcohol between each testing session.

Treatment

Rapamycin (LC Laboratories, Woburn, MA, USA) was dissolved in a vehicle solution containing 4% ethanol, 5% polyethylene glycol 400 (Sigma, St. Louis, MO, USA) and 5% Tween 80 (Sigma, St. Louis, MO, USA) (Eshleman et al., 2002). To the examine the effects of suppressing the hyperactive mTOR activity in NS-*Pten* KO mice, subjects were administered either rapamycin dissolved in the vehicle solution, the vehicle alone, or were naïve to both the vehicle and treatment (Sunnen et al., 2011). Rapamycin was administered 10 mg/kg intraperitoneally for 10 days at approximately 4 weeks old. Vehicle and naïve treated mice were collapsed to form the control group after an initial analysis determined a lack of differences between these groups across each test.

Behavioral Testing

Open Field

As an ethologically relevant general measure of locomotion and anxiety-like behaviors, all subjects underwent the open field test (Bailey & Crawley, 2009). Mice naïve to the test innately display increased thigmotaxis in response to a brightly lit, unprotected, open space. The testing environment consisted of a Fusion Node acrylic arena (40 cm x 40 cm x 30 cm) in an isolated room (Omnitech Electronics, Columbus, OH, USA). Individual mice were placed in the arena for 30 minutes. Photobeams at the edge of each zone detected activity within the arena using Fusion Software (Omnitech Electronics, Columbus, OH, USA). Light levels, temperature, and background noise were

kept constant for all mice. Total distance traveled, rearing frequency and rearing time were analyzed as measures of exploratory activity and locomotive behavior. Circling behavior and time spent grooming (stereotypy time) determined differences in stereotyped behavior. Both the distance traveled, and time spent in the center (inner 50%, 20cm x 20cm region) of the open field were compared between groups to evaluate the possible presence of anxiety-like and exploratory behavior.

Elevated Plus Maze

Using the natural tendency of mice to avoid elevated, open, and brightly lit spaces mouse behavior was analyzed in an elevated plus maze as an additional measure of anxiety-like behavior (Lister, 1987; Walf & Frye, 2007). A plus-shaped maze was suspended 40 cm above the ground and consisted of 2 arms enclosed within acrylic walls and 2 unenclosed arms. Each arm was 30 cm x 5 cm and stemmed from a 5 cm x 5 cm platform. The maze was isolated in a temperature, light, and noise-controlled room. Each subject was placed in the center of the maze and allowed to freely explore the maze for 10 minutes without an experimenter present. Ethovision XT video tracking software (Noldus, Netherlands) was used to record the time each subject spent in the open and closed arms, as well as track the number of entries into the arms.

Marble Burying

Mice display an innate tendency to bury objects in bedding that does not habituate, is stereotypic, and performed excessively (Gyertyan, 1995). This suggests that burying behavior is compulsive. As a measure of stereotypic and compulsive behaviors, mice underwent a marble burying task used previously (Lugo et al., 2014). Mice were

placed in a plastic cage containing a grid shaped pattern of twenty black marbles on 3 cm of Sani-Chip bedding. The number of marbles at least 75% buried in bedding after 30 min were compared between groups.

Social Preference

Mice were measured for their preference of social interaction between a novel object using the three-chambered social preference task modified to include a novel object (Moy et al., 2004; Lugo et al., 2014). The testing apparatus was a clear acrylic box (24.5 in x 16.75 in x 8.75 in) with three chambers separated by a wall with removable partitions. Testing occurred across two phases. During the baseline phase, an empty wire cup was inverted in each of the of the side chambers (8 in x 16.75 in x 8.75 in) with a tall plastic cylinder placed above the cup to prevent mice from climbing on the cups. Subjects were placed in the center chamber (7.25 in x 16.75 in x 8.75 in) with the partitions removed and allowed to freely explore all three chambers for 10 min. The testing phase consisted of an age-, sex-, and weight matched novel mouse housed within the inverted cup in one side chamber, and a novel object (Lego® block) within another inverted cup in the opposite side chamber. The novel mouse was placed in the cup for 1 h for 2 days prior to testing to be habituated to the cup. During each phase, test mice were placed in the center chamber with the partitions removed and allowed to explore the entire testing apparatus freely for 10 min. The novel mouse and object were placed on different sides for each test subject as a counter-balance measure to prevent side-bias. The time spent in each chamber was recorded in each phase, as well as the time spent at the cups housing the novel mouse and novel object.

Trace Fear Conditioning

Both mice and humans can learn to connect benign stimuli and environmental cues (conditioned stimulus [CS]) with a painful stimulus (unconditioned stimulus [US]), a Pavlovian association that relies on both the hippocampus and amygdala (Wehner & Radcliffe, 2004). When a short period of time without any stimuli (a trace interval) separates the US from the CS, fear conditioning becomes highly reliant on the frontal cortex and hippocampus and less so on areas of the amygdala important to traditional fear conditioning paradigms (Kochli et al., 2015). To determine differences in fear memory, contextual and cued fear-based learning was analyzed using a trace fear conditioning task and Freeze Frame software (Coulbourn, Ohio, USA) (Wiltgen et al., 2005; Smith et al., 2016). Testing occurred over 4 days, with 24 hours between each session. On day 1, to habituate mice to the testing apparatus and determine baseline responses, mice were placed in the chamber for 12 minutes without either a CS or US. Day 2 was the training day and consisted of a 260 s baseline period followed by 6 CS (white noise) / US (foot shock) pairings. The white nose (70 dB) was presented for 20 s, followed by an 18 second trace interval separating the CS from the US. At the end of the trace interval a 0.5 mA foot shock was given for 2 s. On day 3, cued memory was examined. The testing chamber was altered to present an unfamiliar background. The smell, sight, layout, sound, and feel of the chamber was changed by adding vanilla extract (Adams Extract, Gonzales, TX, USA) in the tray below the floor, altering the lighting, adding a plexiglass section diagonally inside the chamber, activating a fan in the chamber, and placing an opaque section of plexiglass over the grid floor. The bedding in the transfer cage was comprised of torn paper towels instead of Sani-chip bedding. Mice were placed in this

new context for 580 s. For the first 200 s, neither the CS nor US were presented to determine baseline activity within the chamber. Mice were then presented with 4 iterations of the CS for 20 s followed by an 80 s inter-trial interval (ITI). The percentage of time spent freezing during the CS, considered as no movement other than breathing, was used as a measure of fear memory with higher rates of freezing indicating greater fear memory. On day 4, contextual fear memory was examined. Mice were placed in the original context for 3 min and measured for percent of time spent freezing.

Statistical Analysis

Data were analyzed using GraphPad Prism for Windows, version 7 (GraphPad Software, La Jolla, California, USA) and SPSS for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). Data are mean ± standard error, unless otherwise stated. A three-way analysis of variance (ANOVA) was used to determine the effects of three dichotomous factors - treatment (control, rapamycin), genotype (WT, KO), and sex (male, female) on the model-specific, *a priori* determined behavioral variables. Interactions and main effects were investigated using pairwise comparisons with a Bonferroni adjustment or by Mann-Whitney U tests when data violated Levene's test of homogeneity of variance ($p < .05$) and were not normally distributed as assessed by boxplot. For trace fear conditioning, day 1 used a three-way ANOVA, while days 2 and 3, used a repeated-measures, four-way mixed ANOVA with 3 between-subjects factors (treatment, genotype, and sex) and 1 within-subjects factor "trial". These are further discussed at the beginning of each results section. Repeated measures data complied with Mauchly's test of sphericity ($p > .05$).

CHAPTER FOUR

Results

Open Field

Total Distance

Utilizing a three-way ANOVA, the total distance traveled in the center of the open field was analyzed and revealed a main effect of treatment, $F(1, 109) = 8.83$, $p < .01$, and sex, $F(1, 109) = 10.15$, $p < .01$. Pairwise comparisons with a Bonferroni adjustment showed rapamycin treated mice traveled less than control mice, $p < .01$ (Figure 4.1A), and males traveled less than females, $p < .01$ (Figure 4.1A). There were no other significant outcomes for total distance traveled, including no main effect of genotype, $F(1, 109) = .40$, $p = .53$, two-way interaction between treatment and genotype, $F(1, 109) = 1.17$, $p = .28$, sex and genotype, $F(1, 109) = .074$, $p = .79$, sex and treatment, $F(1, 109) = 1.90$, $p = .17$, or three-way interaction, $F(1, 109) = .14$, $p = .71$.

Center Distance

The distance traveled demonstrated a main effect of genotype, $F(1, 109) = 6.35$, $p < .05$, treatment, $F(1, 109) = 8.13$, $p < .01$, and sex, $F(1, 109) = 4.72$, $p < .05$, (Figure 4.1B). Post hoc analysis showed KO mice traveled further in the center than WT mice, $p < .05$; control mice traveled further than treated mice, $p < .01$; and females traveled further than males, $p < .05$. There was also a two-way interaction between treatment and genotype, $F(1, 109) = 5.69$, $p < .05$. In the control group, KO mice travel less distance in

center compared to WT mice, $p < .001$. After treatment with rapamycin, there was no difference between WT mice and KO mice in center distance, $p = .93$. The effect appears to be primarily on WT mice, as those treated with rapamycin were found to travel less in the center than controls, $p < .001$. In KO mice, there was no difference between those treated with or without rapamycin, $p = .76$. There was no two-way interaction involving sex with either genotype, $F(1, 109) = 3.05$, $p = .083$, or treatment, $F(1, 109) = .27$, $p = .60$. No three-way interaction was found, $F(1, 109) = .54$, $p = .47$.

Surround Distance

The distance traveled in the surround was investigated and revealed a main effect of both treatment, $F(1, 109) = 5.47$, $p < .05$, and sex, $F(1, 109) = 8.0$, $p < .01$, but not genotype, $F(1, 109) = .10$, $p = .76$ (Figure 4.1C). Control mice were found to travel further than treated mice, $p < .05$, and females traveled further than males, $p < .01$. There was no two-way interaction between sex and treatment, $F(1, 109) = 2.69$, $p = .10$, sex and genotype, $F(1, 109) = .001$, $p = .97$, treatment and genotype, $F(1, 109) = .38$, $p = .54$, as well as no three-way interaction between sex, treatment, and genotype, $F(1, 109) = .31$, $p = .58$.

Stereotypy Time

Stereotypy time was analyzed and showed a main effect of genotype, $F(1, 109) = 5.78$, $p < .05$, sex, $F(1, 109) = 4.09$, $p < .05$, and treatment, $F(1, 109) = 12.95$, $p < .001$ (Figure 4.1D). KO mice spent more time performing stereotypies compared to WT mice, $p < .05$, as did males when compared to females, $p < .05$. Treatment with rapamycin induced an overall reduction in stereotypy time, $p < .001$. There was no two-way

interactions between treatment and genotype, $F(1, 109) = 1.14$, $p = .29$, sex and genotype, $F(1, 109) = .50$, $p = .48$, or sex and treatment, $F(1, 109) = 2.03$, $p = .16$. No three-way interaction was found, $F(1, 109) = .88$, $p = .35$.

Vertical Episodes

The frequency of rearing events (vertical episodes) was also analyzed, revealing a main effect of treatment, $F(1, 109) = 22.04$, $p < .001$ (Figure 4.1E). Mice administered rapamycin performed fewer vertical episodes than control mice, $p < .001$. There were no main effects for genotype, $F(1, 109) = .001$, $p = .98$, or sex, $F(1, 109) = .77$, $p = .38$, and no interactions were found. There were no two-way interactions between treatment and genotype, $F(1, 109) = .12$, $p = .73$, sex and genotype, $F(1, 109) = .22$, $p = .64$, or sex and treatment, $F(1, 109) = .00$, $p = .98$. There was also no three-way interaction, $F(1, 110) = .40$, $p = .53$.

Circling Behavior

Analysis of circling behavior also showed a main effect of treatment, $F(1, 109) = 10.05$, $p < .01$, as well as a main effect of sex, $F(1, 109) = 10.31$, $p < .01$ (Figure 4.1F). Rapamycin reduced circling behavior compared to control mice, $p < .01$, and females had higher counts of circling than males, $p < .01$. There was no main effect of genotype, $F(1, 109) = 1.32$, $p = .25$, and no interactions were found. There were no two-way interactions between treatment and genotype, $F(1, 109) = 1.40$, $p = .24$, sex and genotype, $F(1, 109) = .005$, $p = .94$, or sex and treatment, $F(1, 109) = .53$, $p = .47$. There was no three-way interaction, $F(1, 109) = .11$, $p = .74$.

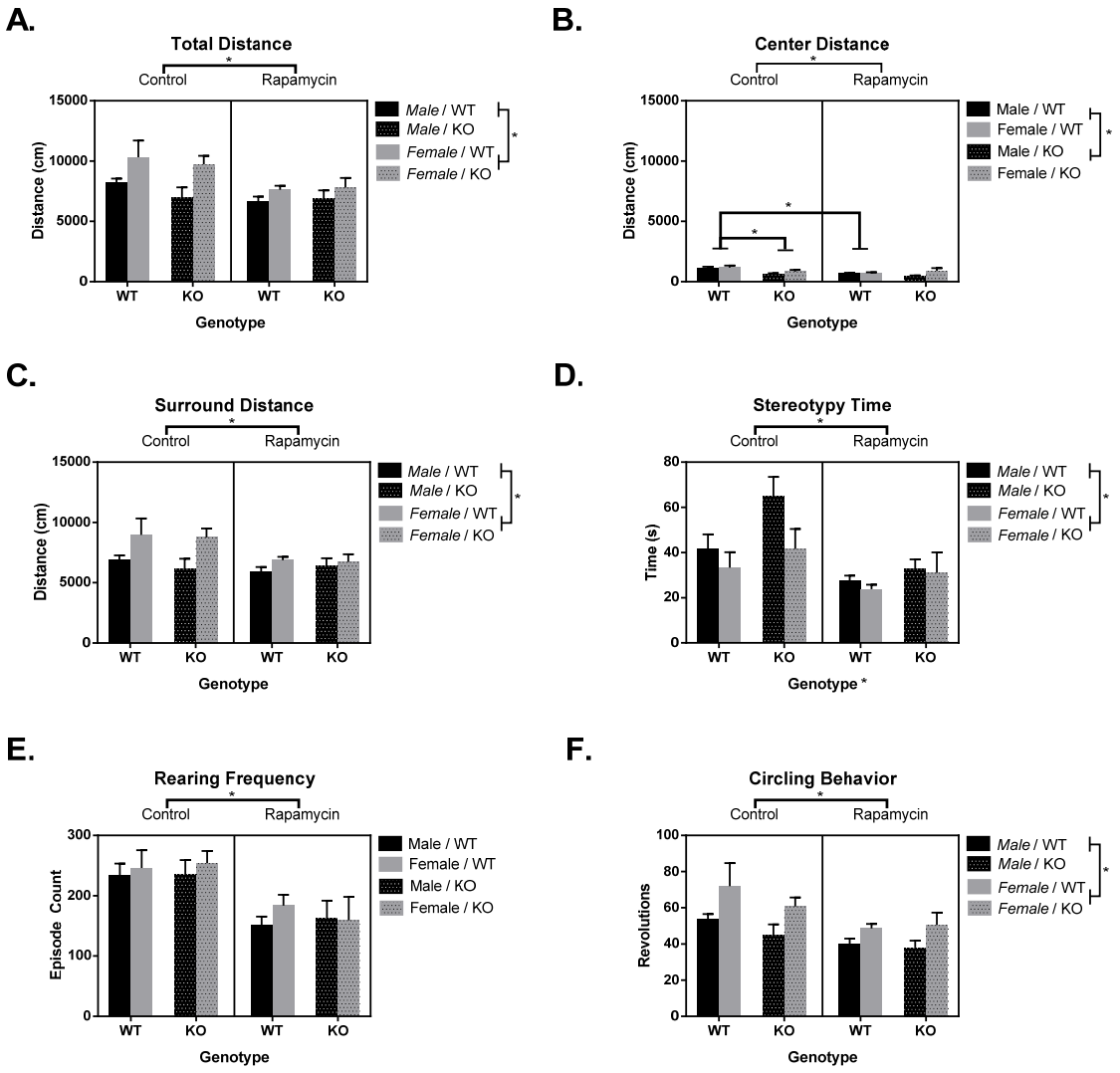

Figure 4.1. *Open Field*. Rapamycin reduced the total distance traveled (A), distance traveled in the center (B) and surround (C), stereotypy time (D), rearing frequency (E), and circling behavior (F) when compared to untreated mice. KO mice traveled less in the center compared to controls (B), and spent more time performing stereotypies (D). WT control mice traveled more in the center compared to KO controls and WT mice treated with rapamycin (B). Males traveled less distance overall (A) and in the surround (C) and performed less circling behavior (F) compared to females. Data are expressed as means ± SEM (* $p < .05$).

Elevated Plus Maze

Open Arm Duration

To measure differences in anxiety-like behavior, we analyzed the frequency of entries and time spent in the open and closed arms of the EPM. Using a three-way ANOVA we found a significant main effect of genotype on the duration of time spent in the open arms, $F(1, 94) = 4.55$, $p < .05$, with KO mice spending more time in the open arms compared to WT mice, $p < .05$ (Figure 4.2A). There were no other main effects or interactions. There was no main effect of treatment, $F(1, 94) = 1.79$, $p = .18$, or sex, $F(1, 94) = .33$, $p = .57$. There was no two-way interactions between genotype and treatment, $F(1, 94) = 2.4$, $p = .13$, sex and treatment, $F(1, 94) = .22$, $p = .64$, or sex and genotype, $F(1, 94) = .03$, $p = .87$, as well as no three-way interactions between sex, treatment, and genotype, $F(1, 94) = .07$, $p = .80$.

Closed Arm Duration

No differences were found in the amount of time spent in the closed arms (Figure 4.2B). There were no main effects of either treatment, $F(1, 94) = .68$, $p = .41$, genotype, $F(1, 94) = 2.48$, $p = .12$, or sex, $F(1, 94) = 2.35$, $p = .13$. There was also no two-way interaction between genotype and treatment, $F(1, 94) = .37$, $p = .55$, sex and treatment, $F(1, 94) = .05$, $p = .83$, or sex and genotype, $F(1, 94) = .56$, $p = .46$, as well as no three-way interaction, $F(1, 94) = .48$, $p = .49$.

Open Arm Frequency

The number of entries into the open arms were also analyzed with no differences discovered (Figure 4.2C). There were no main effects of treatment, $F(1, 94) = 3.55$, $p =$

.06, genotype, $F(1, 94) = .098$, $p = .76$, or sex, $F(1, 94) = .005$, $p = .94$. No two-way interaction between genotype and treatment, $F(1, 94) = .44$, $p = .51$, sex and treatment, $F(1, 94) = 1.99$, $p = .16$, or sex and genotype, $F(1, 94) = .38$, $p = .54$, was discovered, as well as no three-way interaction, $F(1, 94) = .038$, $p = .85$.

Closed Arm Frequency

Assessment of the frequency of entries into the open arms was also not significant (Figure 4.2D). There were no main effects of either treatment, $F(1, 94) = .19$, $p = .66$, genotype, $F(1, 94) = 2.12$, $p = .15$, or sex, $F(1, 94) = 2.00$, $p = .16$. There were no two-way interactions between genotype and treatment, $F(1, 94) = .00$, $p = 1.0$, sex and treatment, $F(1, 94) = .89$, $p = .35$, or sex and genotype, $F(1, 94) = .14$, $p = .71$. There was also no three-way interaction, $F(1, 94) = .57$, $p = .45$.

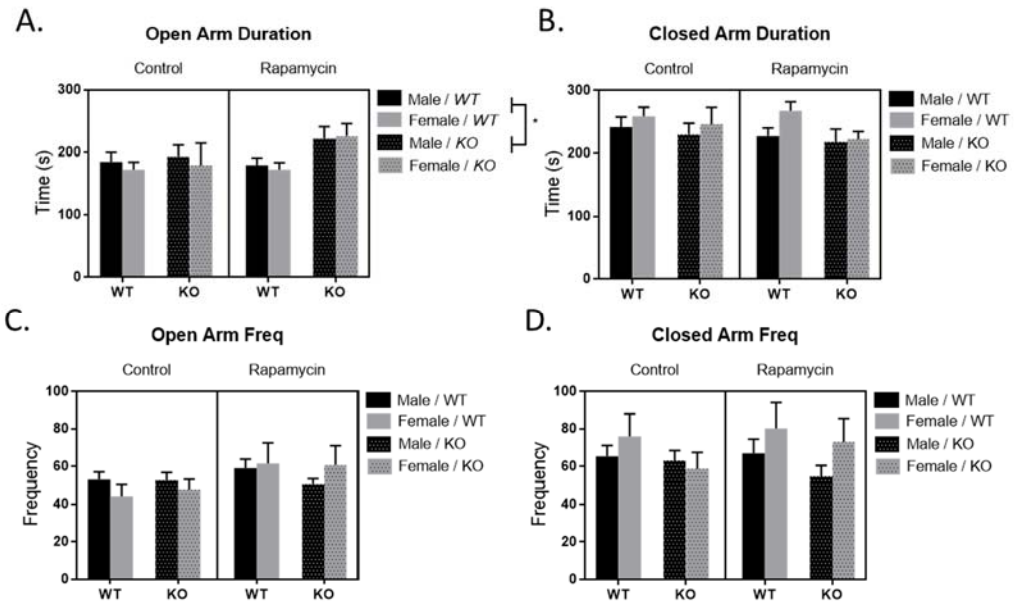

Figure 4.2. *Elevated Plus Maze*. KO mice spent more time in the open arms of the maze compared to WT mice (A). No differences were observed in the time spent in the closed arms (B), or the frequency of open arm (C) or closed arm entries (D). Data are expressed as means ± SEM (* $p < .05$).

Marble Burying

Using a three-way ANOVA, the number of marbles buried up to 75% in bedding were analyzed and showed a main effect of both treatment, $F(1, 105) = 5.61$, $p < .05$, and genotype, $F(1, 105) = 101.92$, $p < .001$ (Figure 4.3). WT mice buried more marbles than KO mice, $U = 2740.5$, $z = 6.96$, $p < .001$, but burying behavior was not found to be significantly different between controls and rapamycin, $U = 1839.5$, $z = 1.42$, $p = .16$. There was no main effect of sex, $F(1, 105) = 3.35$, $p = .07$. There was a two-way interaction between genotype and treatment, $F(1, 105) = 4.5$, $p < .05$. Rapamycin treatment increased the number of marbles buried in KO mice compared to KO control mice, $U = 518$, $z = -.34$, $p < .05$, and had no effect on marble burying in WT mice, $U = 37$, $z = 2.10$, $p = .74$. WT mice buried more marbles than KO mice treated with rapamycin, $U = 57$, $z = 4.16$, $p < .001$, or were controls, $U = 788$, $z = 5.44$, $p < .001$. There were no two-way interactions between sex and treatment, $F(1, 105) = .99$, $p = .32$, or sex and genotype, $F(1, 105) = .035$, $p = .85$. There was also no three-way interaction, $F(1, 105) = 1.52$, $p = .22$.

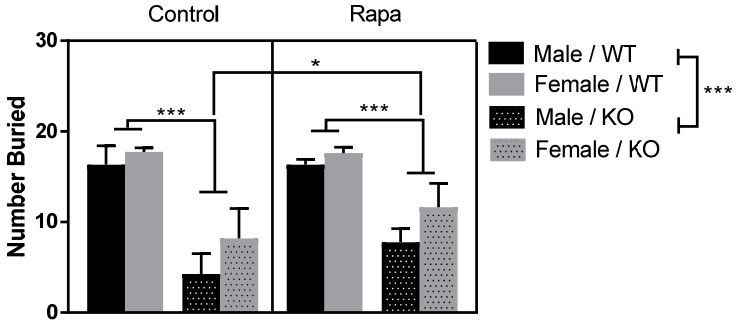

Figure 4.3. *Marble Burying*. KO mice administered rapamycin buried more marbles than control KO mice. WT control mice buried more marbles than KO control mice and WT rapamycin treated mice buried more marbles than KO rapamycin mice. Data are expressed as means ± SEM (* $p < .05$; *** $p < .001$).

Social Preference

Left Chamber

To analyze differences in sociability, mice underwent a social preference test consisting of two trials. Five mice were not used due to an equipment malfunction. Two mice were removed for not entering all three chambers, which prevents the ability to determine preference. A three-way ANOVA was used to determine differences among the three between-subjects factors (treatment, genotype, and sex) on chamber duration. In the first trial of testing, mice were analyzed for differences in time spent in the left and right chambers, each containing an empty wire mesh cup. There were no significant differences in the time spent in the left chamber (Figure 4.4A). There were no main effects of sex, $F(1, 98) = 1.04$, $p = .31$, genotype, $F(1, 98) = .21$, $p = .65$, or treatment, $F(1, 98) = 2.37$, $p = .13$. There were no two-way interactions between sex and genotype, $F(1, 98) = .10$, $p = .75$, sex and treatment, $F(1, 98) = .10$, $p = .75$, or genotype and treatment, $F(1, 98) = .39$, $p = .53$, as well as no three-way interaction, $F(1, 98) = .36$, $p = .55$.

Right Chamber

In the first trial, the time spent in the right chamber showed a significant main effect of sex, $F(1, 98) = 4.98$, $p < .05$. Females spent more time in the right chamber compared to males, $p < .05$ (Figure 4.4B). There were no other significant main effects or interactions. There were no main effects of genotype, $F(1, 98) = .41$, $p = .52$, or treatment, $F(1, 98) = 3.05$, $p = .08$. There were no two-way interactions between sex and genotype, $F(1, 98) = .02$, $p = .88$, sex and treatment, $F(1, 98) = .00$, $p = 1.0$, or genotype

and treatment, $F(1, 98) = .27$, $p = .61$. There was also no three-way interaction, $F(1, 98) = .00$, $p = 1.0$.

Novel Mouse Chamber

On the second trial, we placed a novel mouse or novel object under a wire mesh cup in either the left or right chamber. The chambers containing the novel object or the novel mouse were randomly assigned to counteract side bias. In the time spent in the chamber housing the novel mouse, a three-way ANOVA demonstrated a main effect of genotype, $F(1, 98) = 7.85$, $p < .01$. KO mice spent less time in the chamber with the novel mouse compared to WT mice, $p < .01$ (Figure 4.4C). There was also a main effect of treatment, $F(1, 98) = 6.08$, $p < .05$, with mice administered rapamycin spending more time in the chamber with the novel mouse than control mice, $p < .05$ (Figure 4.4C). There was no main effect of sex, $F(1, 98) = .05$, $p = .82$, or two-way interactions between sex and genotype, $F(1, 98) = 1.45$, $p = .23$, sex and treatment, $F(1, 98) = .11$, $p = .74$, genotype and treatment, $F(1, 98) = .73$, $p = .40$, as well as no three-way interaction, $F(1, 98) = 2.53$, $p = .12$.

Novel Object Chamber

We found no differences in the time spent in the chamber housing the novel object (Figure 4.4D). There were no main effects of sex, $F(1, 98) = 1.08$, $p = .30$, genotype, $F(1, 98) = 2.73$, $p = .10$, or treatment, $F(1, 98) = 2.44$, $p = .12$. There were no two-way interactions between sex and genotype, $F(1, 98) = .06$, $p = .80$, sex and treatment, $F(1, 98) = .25$, $p = .62$, or genotype and treatment, $F(1, 98) = .004$, $p = .95$. There was also no three-way interaction, $F(1, 98) = 1.58$, $p = .21$.

Novel Mouse Cup

As a further measure of novel mouse interaction, we also assessed the time spent at the cup housing the novel mouse to determine if mice interacted with the novel mouse or merely preferred this chamber. Our analysis revealed a main effect of genotype, $F(1, 98) = 10.85$, $p < .01$. KO mice spent less time at the cup with the novel mouse than WT mice, $p < .01$ (Figure 4.4E). There were no other main effects of sex, $F(1, 98) = .603$, $p = .44$, or treatment, $F(1, 98) = .17$, $p = .68$, as well as no two-way interactions between sex and genotype, $F(1, 98) = .17$, $p = .68$, sex and treatment, $F(1, 98) = .12$, $p = .73$, or genotype and treatment, $F(1, 98) = .11$, $p = .74$. There was also no three-way interaction, $F(1, 98) = .43$, $p = .52$.

Novel Object Cup

Analysis of the time spent at the cup housing the novel object showed no differences between any of the groups (Figure 4.4F). There were no main effects of sex, $F(1, 98) = 1.56$, $p = .22$, genotype, $F(1, 98) = .23$, $p = .64$, or treatment, $F(1, 98) = .21$, $p = .65$. We found no two-way interactions between sex and genotype, $F(1, 98) = 1.04$, $p = .31$, sex and treatment, $F(1, 98) = .049$, $p = .83$, or genotype and treatment, $F(1, 98) = .12$, $p = .74$. There was also no three-way interaction, $F(1, 98) = .25$, $p = .62$.

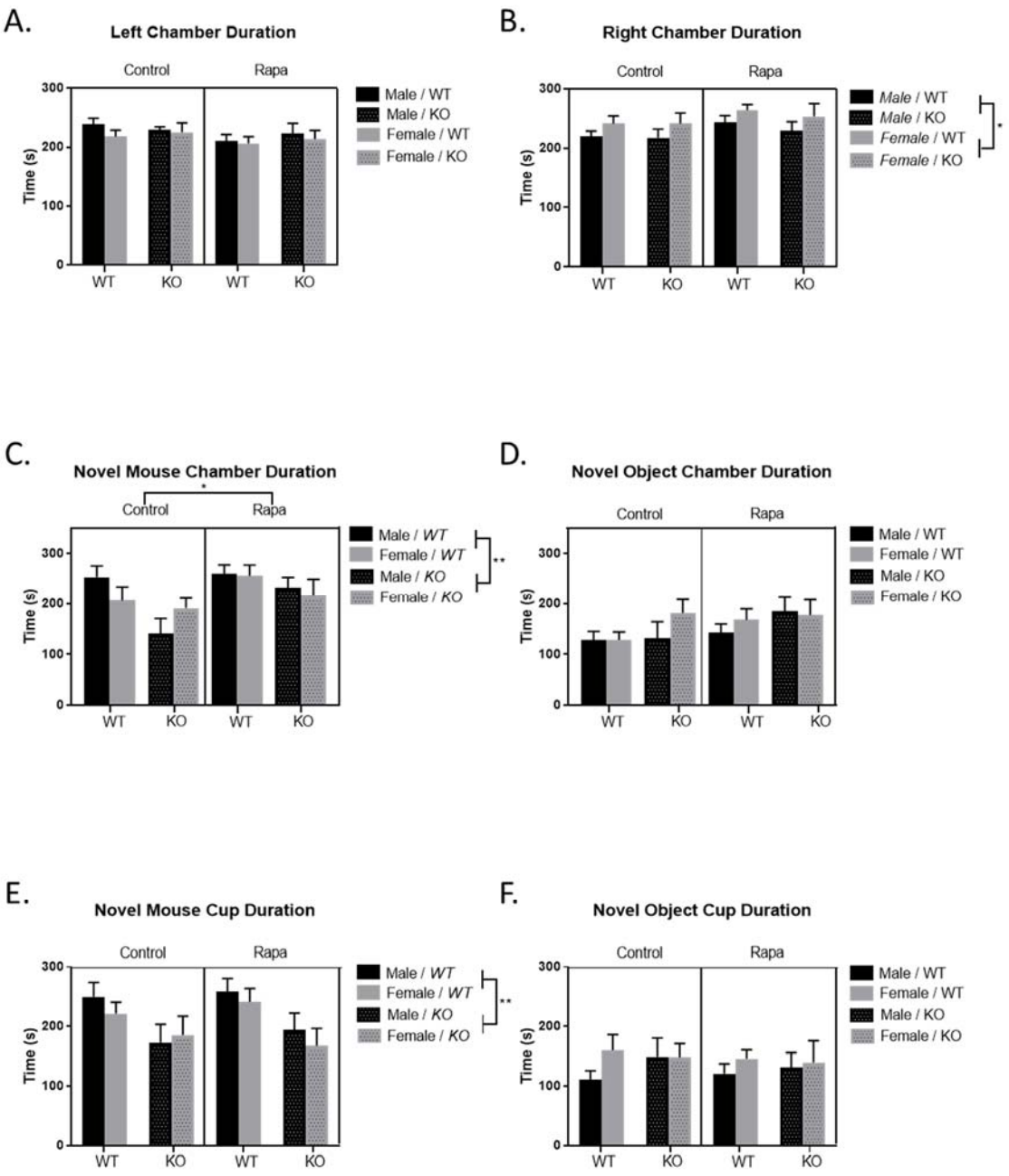

Figure 4.4. *Social Preference*. Females spent more time in the right chamber compared to males (B). Rapamycin treated mice spent more time in the novel mouse chamber than control mice (C). WT mice spent more time in the novel mouse chamber (C) and at the cup housing the novel mouse (E) compared to KO mice. No differences were observed in the time spent at the cup housing the novel object (F). Data are expressed as means ± SEM (* $p < .05$; ** $p < .01$).

Trace Fear Conditioning

Habituation

Five mice were removed from analysis due to death, seizures during training, or equipment malfunction. On day 1, mice were placed in the testing chamber without the CS (white noise) or US (foot shock) to determine baseline activity levels and habituate the mice to the chamber. A three-way ANOVA was used to compare the percent of time spent freezing in the chamber and revealed no significant differences between groups after post hoc tests (Figure 4.5A). There were no significant main effects of treatment, $F(1, 101) = .06$, $p = .81$, sex, $F(1, 101) = 1.25$, $p = .27$, or genotype, $F(1, 101) = .19$, $p = .66$. While there was a two-way interaction between genotype and treatment, $F(1, 101) = 4.38$, $p < .05$, after pairwise comparisons with a Bonferroni adjustment there were no significant differences. We observed no two-way interaction between sex and either genotype, $F(1, 101) = .55$, $p = .46$, or treatment, $F(1, 101) = 1.07$, $p = .30$; and there was no three-way interaction between sex, treatment, and genotype, $F(1, 101) = 3.12$, $p = .08$.

Cued Freezing

Mice were analyzed for cued memory using 4 presentations of the CS in an altered testing chamber 24 hours after training. The within-subjects factor, “trial”, consisted of the average percent freezing behavior during the baseline, tone and intertrial intervals for each level of the between-subjects factors (sex, genotype, and treatment). We analyzed these factors using a repeated measures ANOVA, and analysis of the within-subjects effects showed an interaction between genotype and trial, $F(2, 198) = 3.77$, $p < .05$. Despite the altered context of the testing chamber, pairwise comparisons of

percent freezing at each trial across genotype showed that KO mice froze more than WT mice at baseline, $p < .01$ (Figure 4.5B), but not during the tone, $p = .49$ (Figure 4.5C), or intertrial interval, $p = .90$ (Figure 4.5D). There were no other significant findings on day 3. We found no interactions between trial and treatment, $F(2, 198) = 1.77$, $p = .17$, or trial and sex, $F(2, 198) = 1.98$, $p = .14$. There were also no interactions between the trials with genotype and treatment, , $F(2, 198) = .51$, $p = .60$, sex and treatment, , $F(2, 198) = .36$, $p = .70$, or sex and genotype, $F(2, 198) = 2.45$, $p = .09$. Trial also did not interact with sex, genotype, and treatment, $F(2, 198) = 2.11$, $p = .12$, There were also no overall differences in freezing as calculated in the between-subjects effects. We found no main effects of treatment, $F(1, 99) = 1.88$, $p = .17$, genotype, $F(1, 99) = .35$, $p = .56$, or sex, $F(1, 99) = .15$, $p = .70$. There were no two-way interactions between genotype and treatment, $F(1, 99) = 1.0$, $p = .32$, sex and treatment, $F(1, 99) = .42$, $p = .52$, or sex and genotype, $F(1, 99) = 1.33$, $p = .25$, as well as no three-way interaction between sex, treatment, and genotype, $F(1, 99) = .56$, $p = .46$.

Contextual Freezing

On day 4 of trace fear conditioning, we placed mice in the original testing chamber to determine contextual fear memory. A three-way ANOVA analyzing the differences in percent freezing during the time each mouse was in the chamber revealed a main effect of treatment, $F(1, 98) = 4.26$, $p < .05$ but not sex, $F(1, 98) = .19$, $p = .66$. Control mice froze more than rapamycin treated mice, $p < .05$ (Figure 4.5E). Although there was no main effect of genotype, $F(1, 98) = 3.72$, $p = .057$, KO mice demonstrated a marginal increase in freezing compared to WT mice, $p = .057$ (Figure 4.5E). There was no two-way interaction between genotype and treatment, $F(1, 98) = .74$, $p = .39$, sex and

treatment, $F(1, 98) = .17$, $p = .68$, or sex and genotype, $F(1, 98) = 2.04$, $p = .16$. We found no three-way interaction between sex, genotype, and treatment, $F(1, 98) = .002$, $p = .96$.

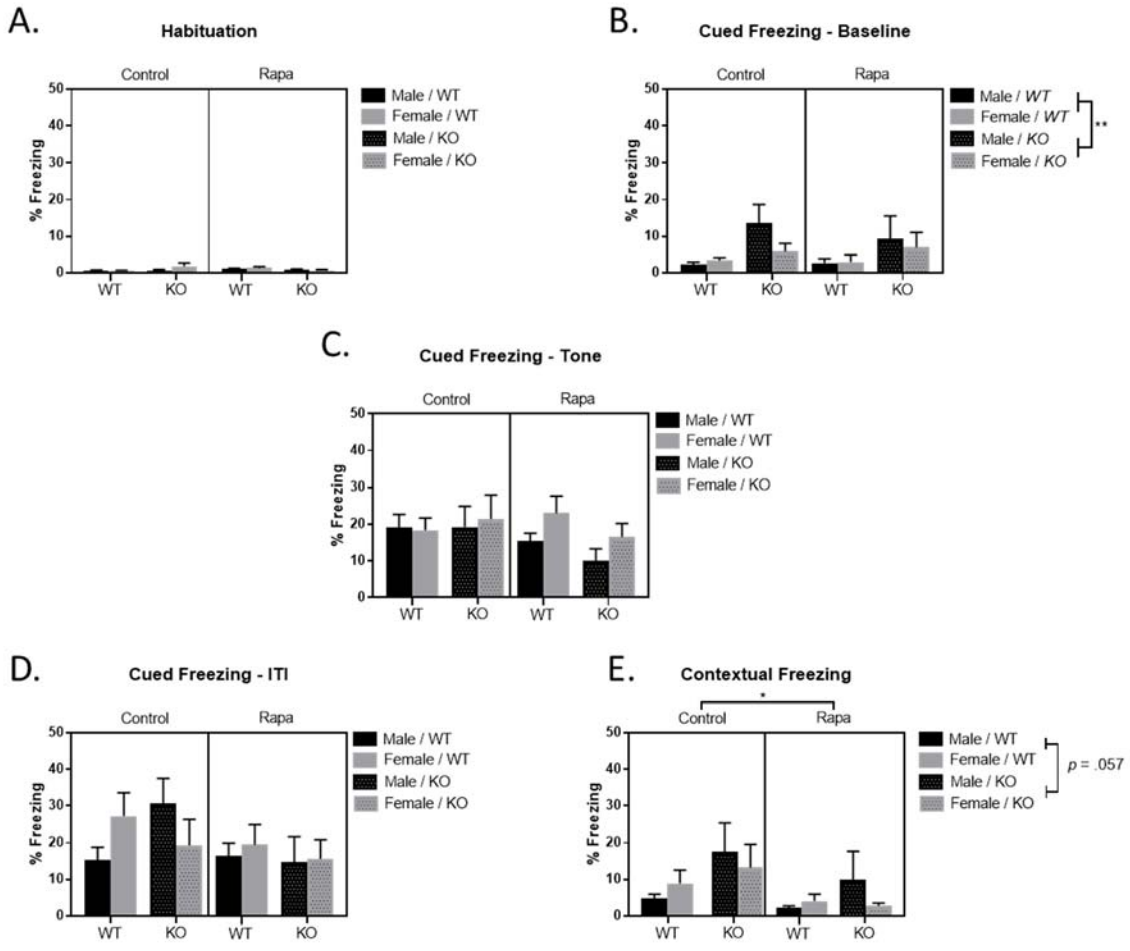

Figure 4.5. *Delay Fear Conditioning*. There were no differences in activity levels during habituation (A). KO mice spent more time freezing to the novel context compared to WT mice (B) but not during the tone (C) or intertrial-interval (D). In the trained context, mice administered rapamycin froze less than control mice and KO mice spent marginally more time freezing compared to WT mice (E). Data are expressed as means ± SEM (* $p < .05$; ** $p < .01$).

CHAPTER FIVE

Discussion

Many patients with *PTEN* mutations develop macrocephaly and seizures and are at a higher risk of being diagnosed with cognitive deficits and autistic behaviors (Ciaccio et al., 2019; Hobert et al., 2014; Mester et al., 2011; Parisi et al., 2001). Neuron subset specific *Pten* knockout (NS-*Pten* KO) mice, with *Pten* deleted from pyramidal cortical cells and granule cells of the hippocampus and cerebellum, exhibit hyperactive mTOR signaling, seizures, and macrocephaly (Backman et al., 2001), and develop autistic-like behaviors and cognitive impairments (Binder & Lugo, 2017; Hodges et al., 2018; Lugo et al., 2014; Lugo et al., 2013; Nolan et al., 2019). Using a 2-week treatment regimen of rapamycin previously demonstrated to reduce hypertrophy and seizures in NS-*Pten* KO mice (Ljungberg et al., 2009; Nguyen et al., 2015; Sunnen et al., 2011), we found that while rapamycin suppressed activity in the open field and contextual fear memories, it corrected stereotypies and increased sociability. Sex did not impact the development of autistic-like behaviors and cognition, nor the attenuation of these behaviors by rapamycin. We also reveal that NS-*Pten* KO mice demonstrate an aberrant enhancement in the generalization of fear memory not diminished by rapamycin.

Rapamycin Suppresses Activity in the Open Field

After rapamycin treatment, both KO and WT mice traveled a shorter distance overall, reared and performed circling behaviors less often, and spent less time grooming. Others have shown rapamycin induced reductions in activity in the open field were

associated with reductions in exploratory behavior and increases in measures of anxiety-like behavior. Healthy rats administered chronic rapamycin show reduced activity and increases in anxiety-related measures in an open field, such as increased freezing behavior, increased fecal production, and less time spent in the center (Lu et al., 2015), as well as less time and distance traveled in the open arms of an elevated plus maze (Hadamitzky et al., 2018).

Although we found rapamycin had no effect on anxiety-like behavior in the elevated plus maze suppression of locomotive behavior was accompanied by a reduction in exploratory behavior in the open field of treated WT but not treated KO mice Since the rapamycin induced reduction in activity is associated with less exploratory behavior in treated WT but not treated KO mice, this side effect may only be found in those with typical, rather than hyperactive, mTOR activity. Together these data suggest that rapamycin induced suppression of mTOR activity may correct the typically observed hyperactivity in KO mice without inducing anxiety-like behavior. Additional testing is needed, however, to fully determine the impact of rapamycin on affect-like behaviors in KO mice.

Rapamycin Corrects Stereotypic Behavior in KO Mice

Stereotypies are compulsive behaviors that are important to the survival and reproductive success of an animal. Mice bury foreign objects that are potentially harmful, investigate small holes that may contain food, and groom themselves to keep clean. In cases of disease or stress, an aberrant enhancement or suppression of these behaviors can be observed (de Brouwer, Fick, Harvey, & Wolmarans, 2019). NS-*Pten* KO mice were previously shown to demonstrate an overall reduction in stereotypic behavior compared

to WT mice across a variety of tasks, including the hole-board and marble burying test, as well as grooming behavior in an open field (Lugo et al., 2014). In the current study, we similarly found KO mice exhibit a reduction in marble burying behavior and an increase in grooming behavior in the open field These data show that KO mice display impairments in the regulation of stereotypic behaviors. In both cases, rapamycin corrected the performance of these behaviors, reducing the enhanced grooming behavior in the open field and increasing marble burying performance

It is important to consider that rapamycin may increase anxiety like behavior (Hadamitzky et al., 2018; Lu et al., 2015) and that marble burying is enhanced when mice are under duress (de Brouwer et al., 2019). While untreated KO mice were less active and demonstrated increased grooming behavior in the open field in the current study, rapamycin did not cause anxiety-like behavior in the elevated plus maze or suppress exploratory behavior in the open field task in KO mice Therefore, it is unlikely that rapamycin-induced anxiety is the catalyst to the increases in marble burying observed here.

KO mice have been previously shown to demonstrate impaired motor capabilities (Backman et al., 2001; Nolan et al., 2019). While marble burying may be a measure of stereotypic behavior, mice with altered motor activity can confound the outcome of this task (de Brouwer et al., 2019). Since the stereotypic behavior produced in treated KO mice is suppressed by rapamycin in the open field, motor capabilities may be improved and allow for increased performance during the marble burying test. Future studies should further examine the possibility that rapamycin treatment may improve the motor capabilities of KO mice.

Rapamycin Increases Sociability

Previous studies have revealed NS-*Pten* KO mice exhibit a reduction in social behavior in both the three-chamber social task and the social partition task, wherein KO mice spent less time than WT mice investigating and interacting with a novel mouse (Lugo et al., 2014). We were able to recapitulate this social impairment also using the three-chamber social interaction task and show that KO mice spend less time in the chamber and at the cup of a novel conspecific. Although the effect of rapamycin on social behavior was not genotype specific, treatment did improve the social deficits in KO mice.

Genetic mutations that lead to neuronal hyperactive mTOR signaling has been reported in individuals with macrocephaly and seizures and may increase the chances of developing autistic behaviors (Ciaccio et al., 2019; Jansen et al., 2015; Tang et al., 2014; Yeung et al., 2017). Tang et al. (2014) observed enhanced mTOR signaling and increased synaptic densities within a portion of the temporal lobe associated with social behavior in patients who had ASD, 30% of which also had seizures.

In addition to NSe-*Pten* KO mice (Zhou et al., 2009), animals models of genetic mutations that increase mTOR signaling and result in seizures, such as *Tsc* (Tang et al., 2014) and *Cntnap2* (Xing et al., 2019), lead to impairments in social behavior that are corrected by retarding mTOR signaling with rapamycin. Our data support the mounting evidence that suppression of mTOR hyperactivity via rapamycin may improve social behavior deficits that occur secondary to enhanced mTOR signaling. We add to the literature by demonstrating that the social deficits acquired as a result of pre-mitotic deletion of *Pten* can also be reversed by rapamycin.

Rapamycin Impairs Contextual Fear Memory

Analyses of the hippocampus in NS-*Pten* KO mice have revealed the hippocampus is dysmorphic and enlarged (Backman et al., 2001; Ljungberg et al., 2009; Nguyen et al., 2015), and as seizures increase in frequency, develops gliosis, and becomes sclerotic (Nguyen et al., 2015; Sunnen et al., 2011). Since rapamycin was previously shown to be effective in reducing mTOR signaling, as well as the hypertrophy and seizure related damage within the hippocampus (Nguyen et al., 2015; Sunnen et al., 2011), we sought to determine if rapamycin could improve the memory of NS-*Pten* KO mice in a hippocampal dependent trace fear conditioning task.

We found rapamycin had no impact on freezing in a novel context or to the auditory cue but reduced freezing in both WT and KO mice in the trained context (Figure 4.5). Contextual fear memories have been shown to rely on proper mTOR signaling in the dorsal hippocampus. After the learning and recall of contextual fear memories, increased mTOR signaling is observed in the dorsal hippocampus (Gafford, Parsons, & Helmstetter, 2011). Inhibiting mTOR immediately before or after learning, by either intrahippocampal injections directly onto the dorsal hippocampus or systemic administration via intraperitoneal injection of rapamycin, reduces freezing to fear conditioned contextual cues in a dose dependent manner (Blundell, Kouser, & Powell, 2008; Gafford et al., 2011; MacCallum & Blundell, 2020). The effects can also be long lasting, as a single intraperitoneal injection of rapamycin can impair contextual memory up to 3 weeks later (Blundell et al., 2008; MacCallum & Blundell, 2020).

Although rapamycin reduced freezing in the trained context, in the current study we found trace fear conditioning still resulted in the acquisition of contextual fear

memories. Previous literature detailing the memory impairments of systemic rapamycin utilized acute doses 2-4 times that administered here and focused their administration immediately around the time of learning and during consolidation (Blundell et al., 2008; MacCallum & Blundell, 2020). When rapamycin is administered over repeated sessions, memory may be less affected when the doses are given outside of time periods critical to learning. Sub-chronic doses of 40 mg/kg administered at a time other than just before or after learning for 3 days had no effect on contextual fear memory (Blundell et al., 2008). Moreover, while cued recall during trace fear conditioning requires mTOR activity within the rodent frontal cortex and is impaired by direct injection of rapa into the frontal cortex (Sui, Wang, & Li, 2008), we found systemic administration of rapamycin had no impact on freezing to the auditory cue. These data collectively suggest the memory deficits associated with rapamycin are minor at the 10 mg/kg dose used here.

Other medications used to treat epilepsy also impair memory but are considered worth the risk given their ability to save the lives of patients. Since rapamycin also increases the lifespan of NS-*Pten* KO mice (Nguyen et al., 2015; Sunnen et al., 2011), the limited reduction in memory performance demonstrated here may also be considered a minor side effect. Together these data advocate for the use of rapamycin as a treatment option worthy of further investigation for individuals with neuronal hyperactive mTOR signaling.

NS-Pten KO Impacts Males and Females Equally

Like ASD, males are more susceptible to developing ASD with cognitive deficits compared to females (Xie et al., 2017). We have previously shown that NS-*Pten* KO mice demonstrate early communicative differences in ultrasonic vocalization spectral

properties compared to WT mice, and that, in addition to these differences being sex specific, the USVs also differed between male and female KO mice (Binder & Lugo, 2017). Since both autistic-like behaviors (Lugo et al., 2014) and memory impairments (Hodges et al., 2018; Lugo et al., 2013) were previously identified in NS-*Pten* KO mice, we sought to determine the impact of sex on the performance of NS-*Pten* KO mice in this test battery and how these behaviors may be impacted by rapamycin.

In the current study we found no interactions between sex with either genotype or treatment. Due to the neural specificity and timing of *Pten* deletion in this model, it is possible that this mutational model does not confer sex specific deficits in the behaviors measured here. Sex specific differences have been reported in some Pten mutation phenotypes, but they differ depending on the way *Pten* is targeted. Regarding social behavior, for example, in mice with reduced nuclear *Pten*, females demonstrate no differences compared to WT mice but males exhibit increased social behavior (Tilot et al., 2014) while those with reduced cytoplasmic *Pten* demonstrate social behavior deficits in both males and females (Sarn, Thacker, Lee, & Eng, 2021). These differences reflect the heterogeneity of ASD which, despite the core symptomology, can manifest differently between individuals, including in the development of comorbid disorders such as ID (American Psychiatric Association, 2013).

The lack of a sex-specific effect in this model may also be due to the type of behaviors analyzed. Other tests may be more apt to identify differences. For example, aggressive behavior (Clipperton-Allen & Page, 2015) and mating or care behaviors (Kwon, Luikart, et al., 2006), which are already sexually dimorphic, may provide a more sensitive measure of analyzing how the NS-*Pten* KO phenotype impacts different sexes.

Since both ASD and ID are known to impact males and females differently (Xie et al., 2017), it is of critical importance that pre-clinical models identify and examine how sex-specific symptoms develop if appropriate therapeutics are to be produced. Although we found no sex differences in NS-*Pten* KO mice, it is difficult to compare our results to that of other models because many of the Pten models developed to date have only analyzed the behavioral phenotype of males. We add to the growing literature demonstrating Pten mutations lead to autistic like behaviors in mice (Rademacher & Eickholt, 2019; Tilot, Frazier, & Eng, 2015) and by presenting how the NS-*Pten* KO model impacts males and female for others to compare.

KO Mice Demonstrate an Enhanced Generalization of Fear Memory

The primary difference between this study and our previous study, is an increase in handling due to medication administration and multiple testing. Behavioral test batteries, which increases handling and subjects mice to multiple novel test environments, are widely known to alter performance on subsequent behavioral tests and, as a result, are often structured in a manner that limits the amount of stress imposed on the mice (McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001). Although we ordered our behaviors in a way that reduces the impact of multiple testing, it may have still negatively impacted KO mice and effected the outcome of tests associated with fear- and anxiety-like behavior.

NS-*Pten* KO mice have been shown to exhibit a robust deficit in cognition, demonstrating memory impairments across four different tasks. In the novel object recognition task and Lashley maze, KO mice displayed deficits in acute and procedural memory, respectively (Hodges et al., 2018). In delay and trace fear conditioning, KO

mice demonstrated intact amygdala-based fear memories but a general deficit in hippocampal-based memory (Lugo et al., 2013). Here we found KOs not only exhibited an equal capacity for fear memory but also an increase in generalized fear memory, wherein KO mice exhibited an increase in freezing in the untrained novel context (Figure 4.5B).

While NS-*Pten* KO mice in our previous analyses were hyperactive, less anxious-like, and exhibited hippocampal based fear memory impairments (Lugo et al., 2014; Lugo et al., 2013), this phenotype was not exhibited here. In the open field, KO mice were previously found to travel a greater distance overall and in the periphery, show a higher frequency and time of rearing, and spend more time in the open arms of the elevated plus maze (Lugo et al., 2014). Contrary to these findings, in the current study we found KO control mice were equally as active but traveled less distance in the center of an open field and did not spend more time in the open arms of the elevated plus maze compared to WT control mice.

As reported with NSe-*Pten* KO mice, who are easily agitated and aggressive to handling and medication administration (Kwon, Luikart, et al., 2006; Zhou et al., 2009), our KO mice may exhibit a lower threshold to handling and multiple testing. In our previous analysis on the effects of NS-*Pten* deletion on fear memory, KO mice were only tested once, they did not undergo an entire behavioral battery and were not handled for medication administration (Lugo et al., 2013). It is possible that an enhanced sensitivity to stress may have potentiated the fear response in KO mice after fear training. Stress enhanced fear learning has been shown to increase the fear response (Perusini et al., 2016; Poulos et al., 2016) and lead to generalized fear responses (Poulos et al., 2016).

The neurobiology of NS-*Pten* KO mice may also underlie this over generalization. Increased mTOR activity in granule cells of the dentate gyrus reduces their threshold for activation and may contribute to the development of seizures in mice lacking *Pten* (Santos et al., 2017). Mice with dentate gyrus granule cells with lower thresholds for activation also promote the generalization of fear memories (Guo, Soden, et al., 2018). Although further analysis would be needed to empirically determine this hypothesis, these studies indicate that an enhanced sensitivity to the stress induced by multiple testing and handling (McIlwain et al., 2001) may explain the near reversal in the fear- and anxiety-like specific behaviors previously demonstrated in NS-Pten KO mice.

Anxiety disorders are a commonly found comorbidity in individuals diagnosed with ASD or ID (American Psychiatric Association, 2013), and occur in an estimated 20% of those with ASD and ID (Hollocks, Lerh, Magiati, Meiser-Stedman, & Brugha, 2019). Individuals with ASD, who also often demonstrate an insistence for sameness and exhibit an inability to adapt to change, can become agitated when their routines are disturbed and result in an increase in the severity of symptoms (American Psychiatric Association, 2013). Due to the possible heightened sensitivity to handling and testing in NS-*Pten* KO mice observed here, future analyses should determine the behavioral consequences of this hyperarousal state within the context of perseverative behavior by examining the impact of stress on the production of ASD-like behaviors. Our results show NS-Pten KO mice exhibit the potential to generate an over generalization of fear memories and support the literature suggesting *Pten* deletion in mice may demonstrate an increased sensitivity to stress (Kwon, Luikart, et al., 2006; Sarn et al., 2021; Zhou et al., 2009).

Conclusion

NS-*Pten* KO mice suffer from epilepsy and macrocephaly, as well as cognitive and behavioral deficits. Epilepsy and macrocephaly are both frequently found in those with intellectual disabilities and/or ASD. While epilepsy and macrocephaly have been postulated as possible mechanisms underlying the development of cognitive and behavioral impairments, it has not been empirically determined. Rapamycin has been previously demonstrated to reduce the impact of hyperactive mTOR on the production of enlarged neurons and the progression of epilepsy in NS-*Pten* KO mice (Ljungberg et al., 2009; Nguyen et al., 2015; Sunnen et al., 2011). Here we show that mTOR pathway inhibition with rapamycin increases sociability and corrects stereotypic behavior. Our results are congruent with findings from NSe-*Pten* KO mice, who demonstrate increased sociability with rapamycin treatment (Zhou et al., 2009). Since previous studies have shown that rapamycin reverses neuronal hypertrophy and impedes the progression of seizures but cannot reverse the aberrant migration of *Pten* null neurons (Getz, DeSpenza, Li, & Luikart, 2016), these data provide evidence suggesting that hypertrophy and seizures may contribute to the development of autistic-like behaviors in NS-*Pten* KO mice.

Studies in pre-clinical models are coming under an increasing amount of scrutiny for not examining the impact of sex on genetic deletions or treatments. Here we analyzed the impact of both in NS-*Pten* KO mice and show this model demonstrates no sex-specific differences. We also outline evidence suggesting NS-*Pten* KO mice are sensitive to stress. Cognitive rigidity and preservative behaviors are hallmarks of individuals with ASD who also demonstrate increased anxiety when presented with changing or

challenging circumstances (American Psychiatric Association, 2013). Our findings, therefore, expand our understanding of the ASD-like behavioral phenotype expressed in NS-*Pten* KO mice and open new avenues for future behavioral research.

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