ABSTRACT

NS-*Pten* Knockout Mice Show Sex- and Age-Specific Differences in Ultrasonic Vocalizations

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One signaling cascade that plays a crucial role in the development of an autistic-like phenotype is the PI3K/Akt/mTOR pathway. Mouse models that illustrate this connection include *Fmr1*, *Tsc1,* and NS-*Pten* deficient mice. While numerous studies have investigated ultrasonic vocalizations in *Fmr1* knockout and *Tsc1* heterogenous mice, none have investigated USVs in NS-*Pten* knockout mice using a full spectrum recording system. In the current study, male and female knockout and wildtype NS-*Pten* pup USVs were examined. We found that knockout pups emitted fewer vocalizations for both sexes. We also found differences between genotypes and sexes for call type composition and the spectral characteristics of the calls. These findings demonstrate that deletion of NS-*Pten* results in significant decreases in vocalizations and vocalization characteristics across both sexes. Our study provides evidence of a connection between hyperactive mTOR signaling and aberrant ultrasonic vocalizations.

NS-*Pten* Knockout Mice Show Sex-and-Age Specific Differences in Ultrasonic Vocalizations

by

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A Thesis

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ATTRIBUTIONS

Chapters one, three, four, and five are all different parts of one published document. My role in this paper was to perform the experiments, run the analysis, and write up the publication. Dr. Lugo provided vital feedback, guidance, and insight throughout the project.

CHAPTER ONE

Introduction

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Autism spectrum disorder (ASD) is a neurodevelopmental condition that appears in early childhood and is characterized by impairments in social interaction, communication, and repetitive behaviors (Rapin & Tuchman, 2008). A recent survey by the Autism and Developmental Disabilities Monitoring Network (ADDM) estimated that the prevalence of ASD among eight-year-old children is 1 in 42 for boys and 1 in 189 for girls (Wingate et al., 2014). Since there is such a high concordance rate (70-90%) of monozygotic twins compared to dizygotic twins (0-10%), it appears that ASD is highly heritable (Abrahams & Geschwind, 2008). However, despite the high heritability there appears to be no single genetic cause for ASD.

The use of genome-wide association studies, whole-genome linkage studies, SNP analyses, and copy number variation screening have resulted in several candidate ASD genes (Abrahams & Geschwind, 2008). There are several single-gene mutation syndrome disorders that are associated with an increased risk of developing ASD including Fragile ^{1}X (*FMR1*), Rett Syndrome (*MECP2*), Tuberous sclerosis (either *TSC1* or *TSC2*), Timothy syndrome (*CACNA1C*), Angelman syndrome (*UBE3A*), and Cowden syndrome (*PTEN*) (Belmonte & Bourgeron, 2006; Butler et al., 2005; Nagarajan et al., 2008; Splawski et al., 2004; Wiznitzer, 2004). Despite the investigation of the underlying genes

of ASD, only 10-20% of all identified genetic risk factors for the disorder have been uncovered (Abrahams & Geschwind, 2008). However, one common factor that has been observed in many of the single-gene mutation syndrome disorders, such as Fragile X, Tuberous sclerosis, and Cowden syndrome, is hyperactivation of the PI3K/Akt/mTOR intracellular signaling pathway (Amir et al., 1999; Baker, Piven, & Sato, 1998; Hatton et al., 2006; Matsuura et al., 1997). Mutations of the suppressors of the pathway, *Tsc1*, *Tsc2*, or *Pten* in mice, produce uncontrolled activation of the mTORC1 signaling cascade that results in macrocephaly and overgrowth in hippocampal, and cortical, cellular and dendritic properties (Kwon et al., 2006; Meikle et al., 2008; Zeng, Xu, Gutmann, & Wong, 2008; Zhou et al., 2009). The Phosphoinositide 3-kinase (PI3K) signaling cascade is activated by nutrients, growth factors and hormones, and is inhibited by PTEN (phosphatase and tensin homolog on chromosome 10).

Previous investigations in animal models of Fragile X, Tuberous sclerosis, and Cowden syndrome have reported ASD-like behavioral deficits. Mice with deletion of *Fmr1* have deficits in social behavior, an increase in repetitive behavior, and communication (Frankland et al., 2004; Gauducheau et al., 2017; Hodges, Nolan, Reynolds, & Lugo, 2017; Mineur, Huynh, & Crusio, 2006; Mineur, Sluyter, de Wit, Oostra, & Crusio, 2002). Mice with deletion of *Tsc1 or Tsc2* have similar ASD-like deficits (Reith et al., 2013). For Cowden syndrome, which involves mutation of the phosphatase *PTEN*, several conditional knockouts have been created. The neuronal subset-specific KO for *Pten* (NS-*Pten*) shows deficits in social behavior and aberrant repetitive behavior (Lugo et al., 2014). No deficits in ultrasonic vocalizations (USVs) have been reported (Lugo et al., 2014).

USVs are identified as “whistle like sounds” with frequencies ranging from 30 to 90 kHz (Branchi, Santucci, & Alleva, 2001). They can be emitted when a pup is separated from its parents, during social play, and are used during courtship and mating rituals, in addition to social investigation in adult mice (D’Amato, Scalera, Sarli, & Moles, 2005; Nyby, 1983; Panksepp et al., 2007). Research also suggests that altered USVs are an important component of the phenotype for various neurodevelopmental conditions, including ASD, Fragile X, and Tuberous sclerosis complex (Reynolds, Nolan, Jefferson, & Lugo, 2016; Scattoni, Gandhy, Ricceri, & Crawley, 2008; Tsai et al., 2012).

The prior study that investigated USVs in NS-*Pten* mice examined USVs using the Ultravox recording system which provides only the broad quantitative differences in calls, focusing on the amount of calls present at four specific frequencies (Lugo et al., 2014). For our study, we will use a full spectrum analysis recording system, which can allow for the investigator to analyze both quantitative and qualitative features of ultrasonic vocalizations, providing information regarding the amount of calls over a broad range, details about the temporal characteristics of the calls, and the different types of calls used. Since the full spectrum analysis program allows for a more in-depth characterization of USVs, it is pragmatic to reassess the NS-*Pten* model for any differences that may be detected on a more sensitive system. An additional focus of this paper will be to examine whether the changes in USVs are sex-specific. In light of past evidence, we hypothesized that there would be no quantitative difference in vocalizations between WT and NS-*Pten* KO mice. However, we also hypothesized that there would be qualitative differences present between the groups. We investigated this by eliciting vocalizations from KO and WT mice, including both males and females, on postnatal

days (PD) 8 and 11 by way of the maternal isolation paradigm. Analyses of the USVs were conducted to examine both the spectral and temporal variability between WT and KO mice.

CHAPTER TWO

Review of Literature

PI3K/Akt/mTOR Pathway

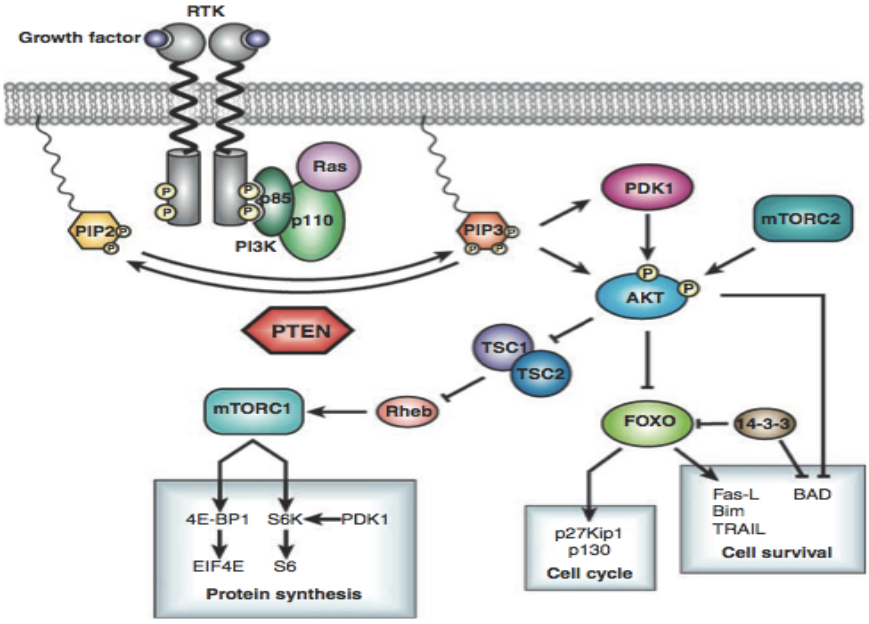

Figure 1- mTOR and PI3K/Akt signaling pathway (Endersby & Baker, 2008).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is a major regulatory pathway in the brain. As seen in Figure 1, when receptor tyrosine kinases (RTKs) are activated by various growth factors Phosphoinositide 3 Kinase (PI3K) is stimulated and phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP_2), converting it into Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Cantley, 2002; Endersby & Baker, 2008). This activity causes the colocalization of protein kinase B (Akt) and Phosphoinositide-dependent kinase-1 (PDK-1) at the cell membrane. PTEN

counters PI3K activity by dephosphorylating PIP_3 back into PIP_2, serving as a negative regulator of the pathway (Wu, Senechal, Neshat, Whang, & Sawyers, 1998).

Akt is an essential component of numerous intracellular signaling pathways that control cell growth and survival (Bodine et al., 2001). Functionally, Akt phosphorylates Tuberous Sclerosis complex (TSC)2 which in turn inhibits the TSC1/TSC2 complex, resulting in a downregulation of GTPase-activating protein (GAP) (Huang & Manning, 2009; Luo, Manning, & Cantley, 2003). The reduction in GAP allows for the buildup of GTP-bound ras homolog enriched in brain (Rheb), resulting in an upregulation of the mammalian target of rapamycin complex 1 (mTORC1), as seen in Figure 1 (Endersby & Baker, 2008; Garami et al., 2003; Inoki, Li, Zhu, Wu, & Kun-Liang, 2002; Zhou et al., 2009). Upon activation, mTORC1 controls the synthesis of ribosome protein S6 kinase-1 (S6K1) and eukaryotic initiation 4E-binding protein 1 (4E-BP1), which in turn leads to additional protein synthesis (Zhou & Parada, 2012).

Functionally, this increase in mTORC1 activity has been linked to a multitude of responses including: the control of local protein synthesis in dendrites, synaptic plasticity, neuronal cell size, cell proliferation, and the regulation of dendritic arborization (Cammalleri et al., 2003; Hou & Klann, 2004; Jaworski, Spangler, Seeburg, Hoogenraad, & Sheng, 2005; Kwon, Zhu, Zhang, & Baker, 2003; Takei et al., 2004; Tang et al., 2002). Thus, altogether, the mTOR signaling cascade comprises a vital pathway in the brain that is integral for maintaining a broad diversity of functions that allow for basic functioning.

PTEN

PTEN, or phosphatase and tensin homolog (PTEN) is a protein encoded by the *PTEN* gene. It is a negative regulator of the mTOR pathway and is widely expressed throughout the body and cytoskeleton, appearing in a multitude of different cell types and locations (Brenner et al., 2002; Depowski, Rosenthal, & Ross, 2001). PTEN is also expressed in the brain starting at birth, with a higher level of expression in neurons and a lower level of expression in glial cells (Lachyankar et al., 2000). Overall, the lowest levels of PTEN can be found in the brainstem and greater levels can be found in the cortex, cerebellum, and hippocampus (Lachyankar et al., 2000).

In terms of function, *PTEN* has been shown to modulate extrasynaptic signaling by mediating protein-protein coupling (Ning et al., 2004). When this is paired with its wide expression in the brain, and the numerous interactions with neurotransmitter receptors such as NMDARs, it becomes clear that PTEN regulates a large variety of brain functions and has differential effects which are dependent upon where it is expressed (Jurado et al., 2010; Lachyankar et al., 2000; Ning et al., 2004).

One of the most commonly observed cellular effects of *PTEN* deletion is hypertrophy (Kwon et al., 2006). The hypertrophy manifests itself in the form of increased dendritic arborization, growth, abnormal axon morphology, abnormal synaptic morphology, and an increase in pre-synaptic vesicles (Backman et al., 2001; Fraser, Bayazitov, Zakharenko, & Baker, 2008; Jaworski et al., 2005).

Due to the role of *Pten* in tumor suppression, cancer, and autism, numerous murine analogues have been utilized. However, a homozygous deletion of *Pten* results in embryonic lethality (Yin & Shen, 2008). In light of this, various subset specific *Pten*

knockouts have become the standard. These transgenic mice allow researchers to investigate the effect of *Pten* in a specific region or organ. In order to assess the role *Pten* plays in the brain, a neuronal subset specific (NS) *Pten* KO mouse was created. The knockout of *Pten* in the NS-*Pten* model results in the deletion of *Pten* in mature neurons of the cerebellum, hippocampus, and throughout the cortex, with disruptions in granule cells accounting for a significant portion of the observed phenotype (Backman et al., 2001). On a behavioral level, NS-*Pten* KO mice display impaired prepulse inhibition, increased initial startle responses, increased anxiety in open field tests and light/dark apparatus, but decreased anxiety in the elevated plus maze, reduced spatial learning in the Morris water maze, hyperactivity under stressful conditions, decreased social behavior, and cerebellar-derived ataxia (Backman et al., 2001; Kwon et al., 2006; Lugo et al., 2014). In addition to these more specific effects, there are also some systemic ramifications following deletion of *Pten* in the brain such as spontaneous epilepsy and premature death (Ljungberg, Sunnen, Lugo, Anderson, & D'Arcangelo, 2009; Sunnen et al., 2011).

Perhaps the greatest utility in garnering a better understanding of PTEN in both humans and animals is its relationship with ASD. Research in humans has shown that a subset of individuals with *PTEN* hamartoma syndromes also meet criteria for autism and intellectual disability, often displaying pronounced social deficits (Herman et al., 2007; Marsh et al., 1999; Waite & Eng, 2002). Additionally, mutations in *PTEN* have been found in 7.1 % of a subset of autistic individuals who have macrocephaly (McBride et al., 2010). Meanwhile in mice, the NS-*Pten* model mimics the cellular changes seen in ASD, such as: neuronal hypertrophy and increased synaptic density, as well as the behavioral

changes seen, such as: social anxiety, a co-morbidity with seizures, and learning deficits. Thus, the NS-*Pten* model embodies a wide range of characteristics that are frequently associated with the autistic phenotype illustrating the effectiveness, as well as the potential, of the model (Kwon et al., 2006; Ljungberg et al., 2009; Lugo et al., 2014).

Early Life Communicating Behavior and Autism

Early life communicating behaviors, such as crying, have been shown to have value in assessing the development, as well as the future health, of a child or infant (Farsaie Alaie, Abou-Abbas, & Tadj, 2016; Furlow, 1997; McDuffie, Yoder, & Stone, 2005; Soltis, 2005). For instance, one study found that the rate at which bouts of early life crying occurred in neurotypical children all positively correlated with later expressive vocabulary (McCathren, Yoder, & Warren, 1999).

In terms of neurodevelopmental conditions, the implications for early life communicating, or prelingual, behaviors can be seen in children with ASD. A study using the communication and symbolic behavior scales (CSBS), a measure of non-verbal, early life communication, found that children diagnosed with ASD demonstrated a pattern of slowed communication and social development between 14 and 24 months of age, when compared to typically developing children (Landa, Holman, & Garrett-Mayer, 2007). Therefore, the study indicates that abnormal early life communicating behaviors may be indicative of an autistic, or an autistic-like, phenotype.

Furthermore, a longitudinal study by McDuffie et al. (2005) demonstrated the predictive validity of prelinguistic abilities within an autistic cohort (mean age of 32 months). The study examined prelinguistic abilities through measuring four behaviors: attention following, motor imitation, commenting, and requesting behaviors (McDuffie et

al., 2005). Attention following is the child's ability to change the direction of their head and eyes in response to a change in the direction of adult focus (Scaife & Bruner, 1975). Children who can reliably follow these adult attentional cues should learn more words and demonstrate greater vocabulary comprehension (Baldwin, 1991). Motor imitation refers to prelinguistic children's ability to coordinate attention between social partners and objects during interactions (McDuffie et al., 2005). Commenting and requesting behaviors are a commonly used measure of intentional communication and encompass any gesture or vocalization that the child uses in either a "conventional or symbolic form or in combination with a behavior that demonstrates coordinated attention to an object and a person" (McDuffie et al., 2005). Behaviors such as waving, pointing, or nodding would fall under this category (Bates, 2014). Upon measuring the behaviors that comprise prelinguistic abilities, the above study found that, after controlling for cognitive delay, better performance on commenting behavior was indicative of increased vocabulary comprehension and production in the autistic children, whereas a better score for motor imitation resulted in solely an increase in vocabulary production (McDuffie et al., 2005). Therefore, even within the autistic population, prelingual behaviors demonstrate a predictive validity for certain aspects of language.

Recent studies have also demonstrated that infants at high risk for ASD, due to having an older sibling with the condition, have an increased fundamental frequency in their cries (the pitch), a shorter cry duration, increased variance in the crying amplitude (the volume), shorter pauses between crying bouts, and increased dysphonation (Esposito, del Carmen Rostagno, Venuti, Haltigan, & Messinger, 2014; Esposito & Venuti, 2010; Sheinkopf, Iverson, Rinaldi, & Lester, 2012; Unwin et al., 2017). Thus, in

conjunction with the predictive properties of these early life prelingual vocalizations, there are accompanying changes to the structure of the infant's cries.

Comorbid Autistic Conditions and Early Life Communication Deficits

Some developmental neurological disorders that display early life communication deficits and symptoms associated with an autistic phenotype (social impairment, stereotyped behavior, and communication insufficiencies) are: Fragile X syndrome and Tuberous sclerosis complex (TSC). FXS occurs in 1:4000 males and 1:8000 females when there is a genetic mutation in the *FMR1* gene, which encodes for the Fragile X mental retardation protein (FMRP). Specifically, this occurs when there are more than 200 trinucleotide (CGG) repeats in the *FMR1* gene, this hypermethylates the gene and functionally silences the production of FMRP (Verkerk et al., 1991). FMRP has been shown to repress protein synthesis at dendritic and synaptic locations, functioning as a negative regulator (Sethna, Moon, & Wang, 2014). One effect caused by a lack of FMRP, is an increase in mGluR1/5, in addition to increased PI3K signaling (Dölen & Bear, 2008; Sharma et al., 2010). Both of these increases have been associated with a hyperactivation of mTOR, which is thought to be an underlying mechanism responsible for much of the conditions' phenotype.

There are a diverse array of symptoms associated with FXS including: learning problems, hyperactivity, stereotyped behaviors, intellectual disability, and an autistic-like phenotype (Hagerman et al., 2009; Kidd et al., 2014; Zink et al., 2014). There are also early life communication deficits present. Children with FXS are less persistent in social engagements, have increased anxiety in social situations, avoid direct eye contact, can lack social reciprocity, have a tendency to be more withdrawing, and display decreased

prelingual social babbling (Bailey, Hatton, Mesibov, Ament, & Skinner, 2000; Belardi et al., 2017; Rogers, Wehner, & Hagerman, 2001). Therefore, due to the phenotypic similarities between FXS and ASD, there is a high comorbidity between the two conditions, with some estimates of the comorbidity being as high as 50% (Demark, Feldman, & Holden, 2003).

Tuberous sclerosis complex (TSC) is a disorder that results from mutations in either the TSC1 or TSC2 genes. In 30% of TSC cases the condition is autosomally dominantly inherited, whereas 70% of cases arise from spontaneous mutations (Jones et al., 1997). It is estimated that 1:6000 infants are born with the disorder, however, there is a large amount of variability in its severity, attributable to the type of gene, TSC1 or TSC2, involved (O'Callaghan, 1999). In general, TSC2 mutations lead to a more severe phenotype (Dabora et al., 2001; Lewis, Thomas, Murphy, & Sampson, 2004). On a molecular level, the result of a TSC1 or a TSC2 mutation leads to an increase in Rheb that in turns causes hyperactivation of mTORC1. The increase in mTORC1 is thought to drive the anatomical, cellular, and behavioral phenotype (Tee, Manning, Roux, Cantley, & Blenis, 2003).

The defining feature of TSC is the presence of hamartomas that are in multiple organ systems. However, the hamartomas are most common in the derma, renal, and nervous systems (Curatolo, Bombardieri, & Jozwiak, 2008). TSC's neurological manifestations include infantile spasms, intractable epilepsy, cognitive disabilities, and autism (Orlova & Crino, 2010). Of these, epilepsy is the most common, occurring in 60-90% of clinically diagnosed patients (Holmes & Stafstrom, 2007).

The behavioral features of children with TSC include: angry outbursts, restlessness, and language deficits (de Vries, Hunt, & Bolton, 2007; Jeste, Sahin, Bolton, Ploubidis, & Humphrey, 2008). They also display early life communication impairments such as: avoiding eye contact, decreased social interest, deficits in social referencing (when an infant uses affective displays of adults to regulate their behavior), and decreases in prelingual social babbling (McDonald et al., 2017). Therefore, because of the high degree of phenotypic overlap between TSC and ASD, the two conditions are highly co-morbid with one another. It is estimated that 50-60% of infants diagnosed with TSC meet the criteria for ASD by 3 years of age (McDonald et al., 2017).

In summary, both FXS and TSC are conditions that encompass many of the features of autism, illustrating their respective comorbidities with the disorder, with early life communication deficits being particularly prominent in each condition. While there are numerous differences between FXS and TSC, another underlying communality is the hyperactivity of mTOR. In FXS this is mediated via an increase in mGluR and increased PI3K activity, whereas in TSC, this is mediated via increased Rheb activation.

Murine Models of FXS and TSC

Due to the prevalence and severity of both FXS and TSC, in addition to their high comorbidity with ASD, numerous mouse models have been created to mimic the conditions. FXS murine models most commonly utilize a genetic knockout of *Fmr1*, which results in suppression of FMRP. On a behavioral level, the *Fmr1* KO mouse exhibits: increased activity levels, disruptions in social behaviors, repetitive and stereotyped behaviors, and neonatal communication deficits (Mines, Yuskaitis, King, Beurel, & Jope, 2010; Moy et al., 2008; Pietropaolo, Guilleminot, Martin, D'Amato, &

Crusio, 2011; Reynolds et al., 2016; Veeraragavan et al., 2011). In addition to the numerous genetic and behavioral similarities in this model, there is an increased phosphorylation of ribosomal protein S6 kinase (S6K1) and eukaryotic translation initiation factor 4E (EIF4E), both of which are downstream effectors of mTOR, indicating aberrant mTOR activity (Hoeffer et al., 2012). Thus, due to the large amount of overlap in the core symptomology of FXS, the murine model is thought to be a valid representation of the disorder.

The *Tsc* murine model was originally created through gene targeting in embryonic stem cells, resulting in a KO of *Tsc1* or *Tsc2*. However, the deletion resulted in embryonic lethality (Kobayashi et al., 1999; Onda, Lueck, Marks, Warren, & Kwiatkowski, 1999). To overcome this problem, heterozygote *Tsc* mice and organ specific transgenic Cre mice were created. *Tsc* heterozygote mice have a similar behavioral profile to those reported in studies with humans. Specifically, these mice display social impairments, learning deficits, repetitive behavior, and early life communication deficits (Goorden, van Woerden, van der Weerd, Cheadle, & Elgersma, 2007). However, the *Tsc* +/- model does not present with brain lesions or spontaneous epilepsy, two important characteristics of the disorder that are seen in humans (Goorden et al., 2007). Therefore, while *Tsc* HT mice may have a similar phenotype to humans, there are underlying differences in the accompanying brain changes (Goorden et al., 2007).

Organ or area-specific transgenic *Tsc* mice are another type of *Tsc* model that is commonly used. Some of these models, such as the NEX-*Tsc*, which suppresses *Tsc* specifically in the forebrain, presents with both spontaneous epilepsy and brain lesions,

matching up well with the brain changes seen in humans (Crowell, Hwa Lee, Nikolaeva, Dal Pozzo, & D'Arcangelo, 2015). Other models, such as when *Tsc* is suppressed in Purkinje cells, do not present with brain lesions or epilepsy but do display behavioral patterns similar to humans (Tsai et al., 2012). Therefore, the degree to which the *Tsc* models overlap with the primary brain and behavioral changes are dependent upon the specific region of the brain that *Tsc* is being suppressed in (Crowell et al., 2015; Tsai et al., 2012). While these transgenic mice are helpful in determining the particular effects that occur in a given region of the brain, their utility is lessened when examining the changes that take place in the brain as a whole when *Tsc* is suppressed. Towards this end, neuronal *Tsc* transgenic mice were created. Neuronal *Tsc* mice present with brain changes reminiscent of those seen in humans (Meikle et al., 2007). However, these mice have significantly reduced life spans, which may contribute to the lack of behavioral assessment present in this model (Goto et al., 2011; Meikle et al., 2007). While there are many differences between the various *Tsc* models, one communality is hyperactivity of mTORC1. This hyperactivity is due to an increase in Rheb which in turn upregulates mTORC1 and is thought to be responsible for many of the brain and behavioral changes associated with *Tsc* dysfunction.

Thus, both *Fmr1* and *Tsc* models mimic many of the core features of their associated disorders. Since these mice also demonstrate decreased sociability, stereotyped behaviors, and communication deficits, they seem to reliably encompass the comorbidity with autism that both Fragile X syndrome and Tuberous sclerosis complex share. Ultimately, these models provide a window into the molecular underpinnings of their respective disorders. The animals also provide a means to obtain an understanding of

FXS, TSC and, by proxy, ASD, due to the manipulations that can performed in these mice, that would otherwise not be possible in humans. Furthermore, the murine models provide additional evidence that an underlying communality between FXS and an autistic-like phenotype, as well as between TSC and an autistic-like phenotype, is hyperactive mTOR signaling (Waung & Huber, 2009; Zhang et al., 2003). This connection between the mTOR signaling pathway and an ASD-like phenotype is perhaps most clearly seen in the communication deficits present in both *Frm1* KO mice and *Tsc* heterogeneous mice.

Early Life Communication in Mice

One way to assess communication behavior in rodents is through ultrasonic vocalizations (USVs). USVs in murine models are an innate form of communication (Bowers, Perez-Pouchoulen, Edwards, & McCarthy, 2013). They are classified as "whistle like sounds" with frequencies ranging from 30 to 90 kHz, typically increasing around day 5, reaching their peak around day 7 and decreasing around day 14 (Branchi et al., 2001; Elwood & Keeling, 1982). They can be emitted when a pup is separated from its parents, during social play, and are used during courtship and mating rituals, in addition to social investigation in adult mice (D'Amato et al., 2005; Nyby, 1983; Panksepp et al., 2007).

Aberrant mTOR activity is one factor that has been shown to impact these USVs in *Fmr1* KO mice, *Tsc1* HT mice, and *Tsc2* HT mice. Particularly, when *Fmr1* pups are separated from their mother by being removed from the home cage and placed into another cage with a recording system (the maternal isolation paradigm), the KO pups emit significantly fewer vocalizations than their WT counterparts (Reynolds et al., 2016).

When the same paradigm is utilized for *Tsc*1 and *Tsc*2 HT mice, aberrant vocalizations are also observed. Specifically, in the *Tsc*1 model, heterogeneous pups emit significantly more cries than wild type mice (Tsai et al., 2012). However, in the *Tsc*2 model, heterogeneous pups emit significantly fewer vocalizations when compared to WT mice (Greene-Colozzi, Sadowski, Chadwick, Tsai, & Sahin, 2014). Therefore, while the particular quantity of vocalizations emitted is not constant across different hyperactive mTOR models, the underlying aberrant vocalizing patterns are still observed, indicating a commonality.

FXS, TSC and the Pten Model

While the murine models of FXS and TSC have been integral to examining USVs, they do have some caveats that should be considered in each model. Principally, *Fmr1* KO mice are a systemic gene knockout model. This means that there are a wide variety of changes that take place throughout the organism, resulting in a complex and diverse phenotype (Loesch & Hagerman, 2012). The full KO, in essence, creates numerous ripples throughout the body which make it more difficult to distinguish the specific effects the KO has in any one particular area, such as the brain. Therefore, while global KO models may be invaluable for understanding the systemic effects a particular mutation or KO produces, they make it more difficult to determine the effects in a specific area. Similarly, the heterozygote *Tsc* model is also a global knockout model, and suffers the same pitfall (Goorden et al., 2007).

Tsc transgenic mice present with different shortcomings than the systemic models. Principally, the neuronal suppression of *Tsc* results in early death and the behavioral profile has not been well established (Goto et al., 2011; Meikle et al., 2007).

The brain region specific suppression of *Tsc*, such as in the forebrain or the cerebellum, is integral in understanding the effects of *Tsc* in their associated regions but are less helpful in understanding how a *Tsc* suppression impacts the brain as a whole (Crowell et al., 2015; Tsai et al., 2012). Additionally, the wide variety of observable brain changes present in different transgenic mice may indicate the presence of an altered underlying mechanism when compared to humans with the condition (Crowell et al., 2015; Curatolo, Bombardieri, & Jozwiak, 2008; Orlova & Crino, 2010; Tsai et al., 2012). Due to these drawbacks, there is a need for a neuronal-subset specific model, which has been behaviorally profiled, that allows for the observation of hyperactive mTOR in the brain. Only then can the connection between aberrant mTOR activity and altered early life USVs firmly be established.

One such model that is not a global knockout model and results in hyperactive mTOR activity in specific areas of the brain is the NS-*Pten* model. These mice have a neuronal subset specific knockout of the *Pten* gene. *PTEN* serves as a negative regulator of the PI3K/Akt/mTOR pathway, therefore deletion of the gene leads to hyperactivity in the signaling cascade (Wu et al., 1998). The primary advantage of this model is that it allows researchers to observe hyperactive mTOR that is localized to the brain. Another advantage is that the NS-*Pten* KO does not diminish the rodent's lifespan to the same extent as the neuronal *Tsc* model, allowing for a more comprehensive behavioral assessment (Meikle et al., 2007; Nguyen et al., 2015). Thus, the NS-*Pten* model is unique in that it allows for a greater understanding of the many cellular and behavioral nuances that arise from aberrant mTOR activity in the brain, something that neither the *Fmr1* nor *Tsc* murine models facilitate to the same degree.

Due to the enlarged window of time that investigators have to examine the behavioral features of NS-*Pten* mice, many aspects of the behavioral and cellular phenotype of this model have been characterized. NS-*Pten* mice have neuronal hypertrophy, increased synaptic density, seizures, and increased levels of phosphorylated Akt, S6, S6K, and FMRP (Ljungberg et al., 2009; Lugo, Smith, Morrison, & White, 2013). NS-*Pten* mice also exhibit social deficits in the social partition and social chamber tests, display increased activity in the open field test, stereotyped and repetitive behavior in the marble burying and board test, less anxiety in the elevated plus maze, and impairments in both contextual learning and trace conditioning (Kwon et al., 2006; Ljungberg et al., 2009; Lugo et al., 2014; Lugo et al., 2013). In summary, NS-*Pten* mice present with an autistic-like phenotype that is in part mediated by hyperactive mTOR. However, one significant area that has not received much attention in the literature is NS-*Pten* KO's early life communicating behavior.

Since NS-*Pten* KO mice also constitute a monogenic hyperactive mTOR model, it is logical to predict aberrant ultrasonic vocalizations similar to those seen in other related models. However, a previous study in our lab found no difference in vocalizations for NS-*Pten* KO pups when using the Ultravox recording system. The microphones used in the Ultravox system detect calls at four parameters generally set to 50, 60, 70, and 80 kHz. Specifically, they are designed to pick up calls within 1 kHz around the frequency that the detector is set to, therefore 8 kHz between each detector could be missed and calls lower than 39 and greater than 81 would not be detected. Recently a full spectrum analysis system, (Avisoft), has become available which has several distinctions that set it apart from Ultravox. Most notably, the full spectrum analysis program records a much

wider range of frequencies, spanning 0-125 kHz. The full spectrum program also allows for the characterization of specific calls, providing a more comprehensive analysis of early life vocalizing behavior.

Another drawback to the previous study is the exclusion of females, as only male vocalizations were recorded and analyzed. Since numerous studies have demonstrated that behaviors, and even some neurological conditions, are directly affected by sex, it is important to also assess females (Clayton & Collins, 2014; Reynolds et al., 2016). Reynolds et al. (2016) reported significant differences in vocalizations between male and female *Fmr1* KO mice. This suggests that investigating USVs in female NS-*Pten* KO mice would not only be extremely fruitful in attaining a comprehensive understanding of the relationship between hyperactive mTOR, aberrant early life vocalizations, and autism, but would also address whether there are sex-specific differences in the mice.

Study Hypotheses

Our hypothesis is based upon a previous publication, Lugo et al., (2014), where we did not find any differences in USVs (Lugo et al., 2014). We therefore hypothesized that there would be no quantitative difference in vocalizations between WT and NS-*Pten* KO mice. However, we hypothesized that there would be qualitative differences present between the groups. We investigated this by eliciting vocalizations from NS-*Pten* KO and WT mice, via a maternal isolation paradigm that included both males and females, on postnatal days (PD) 8 and 11. Analyses of the USVs with SPSS software were conducted to examine both the spectral and temporal variability between WT and KO mouse vocalizations.

CHAPTER THREE

Materials and Methods

This chapter published as: Binder MS, Lugo JN. NS-*Pten* knockout mice show sex- and age-specific differences in ultrasonic vocalizations. *Brain Behav.* 2017;e00857. https://doi.org/10.1002/brb3.857

Animals and Housing

The NS-*Pten* mice used were a FVB-based backcrossed background strain that had been bred for more than 10 generations and have been previously described (RRID:MGI:3714016) (Kwon et al., 2006). NS-*Pten*$^{loxP/+}$ heterozygote parents were bred and used to produce NS-*Pten*$^{+/+}$ wildtype (WT), NS-*Pten*$^{loxP/+}$ heterozygous (HT), and NS-*Pten*$^{loxP/loxP}$KO pups. Pups were clipped on PD 7 and raised with their littermates, as well as both parents, in the home cage. All animals were retained throughout testing. A total of 33 litters, amounting to 54 NS-*Pten* WT and KO pups, were used in this study. They comprised four groups: male WT (*n*= 14), male KO (*n*= 12), female WT (*n*=15), and female KO (*n*=13) mice, all of which were tested in the afternoon during the light cycle. The mice were generated and group housed at Baylor University in a room on a 12-hour light/dark diurnal cycle held at 22^{0}C, with the mice given *ad libitum* access to both food and water. All test procedures were carried out in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals along with Baylor University's Institutional Animal Care and Use Committee.

Ultrasonic Vocalizations

In this study USVs were elicited by a maternal isolation paradigm, as this has previously been shown to consistently initiate vocalizing behavior in pups (Shair, 2007). Mice were tested on both postnatal days 8 and 11 because vocalizations typically increase around PD 5, reach their peak around PD 7, and decrease around PD 14 (Branchi, Santucci, Vitale, & Alleva, 1998; Elwood & Keeling, 1982). Prior to testing, the pups were weighed and allowed to habituate in the testing room for 30 minutes. Next, they were removed from their dam in the room temperature home cage and the pups were put into a clean, preheated cage. We then individually tested each mouse by removing the mouse from the warmed cage and placing it in another 22°C cage, as detailed in numerous other studies (Scattoni et al., 2008; Shair, 2007; Tsai et al., 2012). The mouse was then placed within a 40 cm x 40 cm x 30 cm sound-attenuated chamber. Their vocalizations were recorded for 2 minutes using an ultrasonic microphone (CM16/CMPA, Avisoft Bioacoustics, Germany, part #40011) and an ultrasound recording program (UltraSoundGate 116Hb, Avisoft Bioacoustics, part # 41161/41162). Following testing, the pups were placed back in the pre-warmed cage. Once all mice were tested they were returned to their dam.

Ultrasonic Vocalization Analysis

The Avisoft SASLab Pro software (Avisoft SASLabPro, RRID:SCR_014438) was used to perform a Fast Fourier transformation (FFT) on the recorded data, creating a spectrogram with an FTT length of 1024, a time window overlap of 75%, with a 100% Hamming Window, a frequency resolution of 488 Hz, a sampling frequency of 250 kHz, and a time resolution of 1 ms as detailed in Scattoni et al. (2008). The calls were visually

identified using 1 of 10 distinct categories based on internal pitch changes as well as the length and shape of the individual calls. The categories were: complex, harmonic, two syllable, upward, downward, flat, chevron, short, composite, and frequency steps. Complex calls contained one syllable with two or more directional changes in pitch greater than 6.25 kHz. Harmonic calls comprised a single call with additional calls of varying frequencies surrounding the primary call. Two syllable calls contained a flat or downward call with another short, punctuated call near the end. Both upward and downward call types have a continuous increase or decrease in pitch greater than 12.5 kHz, ending with a frequency at least 6.25 kHz more or less than the initial pitch. Flat calls have a consistent beginning and ending of the call, with the pitch frequency deviating less than 3 kHz throughout the call's duration. Chevron calls have an "inverted-U" shape, with a continuous increase in pitch that is greater than 12.5 kHz immediately followed by a decrease in pitch greater than 6.25 kHz. Short calls are less than 5 ms in length. Composite calls contain two harmonically independent components that are emitted at the same time. Frequency steps are calls with an abrupt frequency change that appears as a vertical discontinuous step but with no interruption of time (Scattoni et al., 2008). Additionally, the experimenter was blind to the condition of the animal at the time of scoring.

Behavioral Data Analysis and Statistics

All statistical analyses were conducted using Graphpad Prism 7 software (La Jolla, CA) or SPSS 20.0 (IBM, USA). The differences in the quantity of calls between the groups and across both days were analyzed with a repeated-measures ANOVA with genotype and sex as between-subjects factors. A MANOVA was run to analyze the

differences in the vocalization parameters. This was followed by independent *t*-tests, grouped by genotype, or Mann Whitney *U* tests, when homogeneity of variance assumptions were violated, that detected the specific differences in mean duration, peak frequency, fundamental frequency, and peak amplitude, for each group over each day.

Call type was analyzed with a Pearson Chi-Square, along with individual *z*-tests, to compare significant call type proportions between genotypes, sexes, and postnatal days. A value of $p < .05$, was considered significant for each statistical test, with figures depicting the mean ± standard error of the mean (SEM).

CHAPTER FOUR

Results

This chapter published as: Binder MS, Lugo JN. NS-*Pten* knockout mice show sex- and age-specific differences in ultrasonic vocalizations. *Brain Behav.* 2017;e00857. https://doi.org/10.1002/brb3.857

Quantity of Calls

An analysis of the mean number of calls emitted from each group across both days revealed a significant ANOVA with genotype and sex as the between subjects factors and the number of calls on PD 8 and the number of calls on PD 11 as the within subjects factors. There was a main effect for genotype ($F(1,50) = 7.06$, $p = .01$), sex, ($F(1,50) = 6.96$, $p = .01$), and for the amount of calls emitted on PD 8 when compared to PD 11, ($F(1,50) = 7.33$, $p = .01$). KO mice emitted fewer vocalizations than WT mice and males emitted fewer vocalizations than females (Figure 2a,b). There was a significant reduction in the number of calls that were emitted by WT and KO mice on PD 11 compared with PD 8. Similarly, males and females also emitted fewer calls on PD 11 than on PD 8. There were no interactions found between genotype and sex ($F(1,50) = 2.6$, $p = 0.11$), between genotype over PD 8 and 11 ($F(1,50) = 0.16$, $p = 0.70$), sex over the 2 days ($F(1,50) = 0.1$, $p = 0.76$), or genotype by sex over time ($F(1,50) = 0.7$, $p = 0.4$).

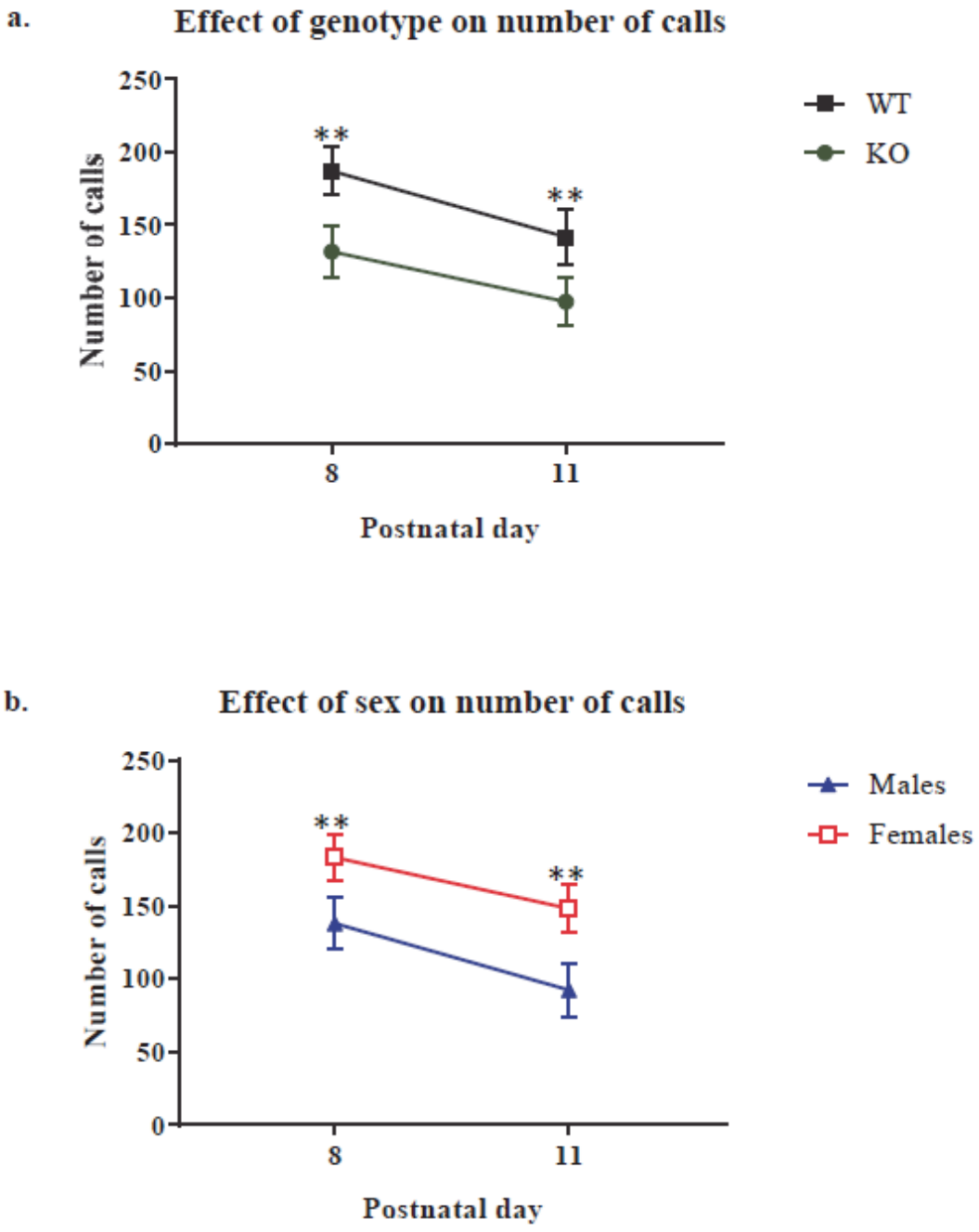

Figure 2. Effect of Genotype and Sex on the Quantity of Calls for PD 8 and PD 11
Mean quantity of calls for genotype and sex on PD 8 and PD 11. (a) WT mice emitted significantly more calls than KO mice, with more USVs being recorded on PD 8 than on PD 11. (b) Females emitted significantly more calls than males, with more USVs being recorded on PD 8 than on PD 11. The data points represent the mean and the error bars represent the standard error of the mean. WT male: $n = 14$, KO male $n = 12$, WT female: $n = 15$, KO female: $n = 13$. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Call Type Compositions

Even though there were significant genotype and sex differences, we wanted to examine the specific types of calls that were emitted to determine if certain calls are genotype or sex specific. When examining the spectral properties of call types, a Pearson Chi-Square analysis revealed significant population differences between the composition of calls for WT and KO mice emitted on PD 8 ($X^2(7, N = 8718) = 445.83$, $p < .001$) and on PD 11 ($X^2(8, N = 6549) = 101.43$, $p < .001$). Proportional differences detected with z-tests found that on PD 8 KO animals emitted a significantly greater amount of frequency steps and upward calls when compared to WT mice ($p < .05$) (Figure 3a,b). However, KO mice produced significantly less complex, two syllable, chevron, and composite calls ($p < .05$) (Figure 3a,b). On PD 11, KO animals showed a similar pattern, emitting significantly more frequency steps and upward calls ($p < .05$), with WT mice producing more complex and two syllable calls ($p < .05$) (Figure 3c,d).

Sex-specific call type differences were also detected. A Pearson Chi-Square analysis revealed significant population differences between male and female call types for both PD 8 ($X^2(7, N = 8718) = 61.58$, $p < .001$) and PD 11 ($X^2(8, N = 6549) = 53.46$, $p < .001$). Proportional differences detected with *z*-tests found that on PD 8 males emitted significantly more two syllable cries ($p < .05$), whereas females produced more frequency steps calls ($p < .05$) (Figure 3e,f). On PD 11, males emitted more two syllable, short, and downward call types ($p < .05$), while females produced significantly more chevron and frequency steps calls ($p < .05$) (Figure 3g,h).

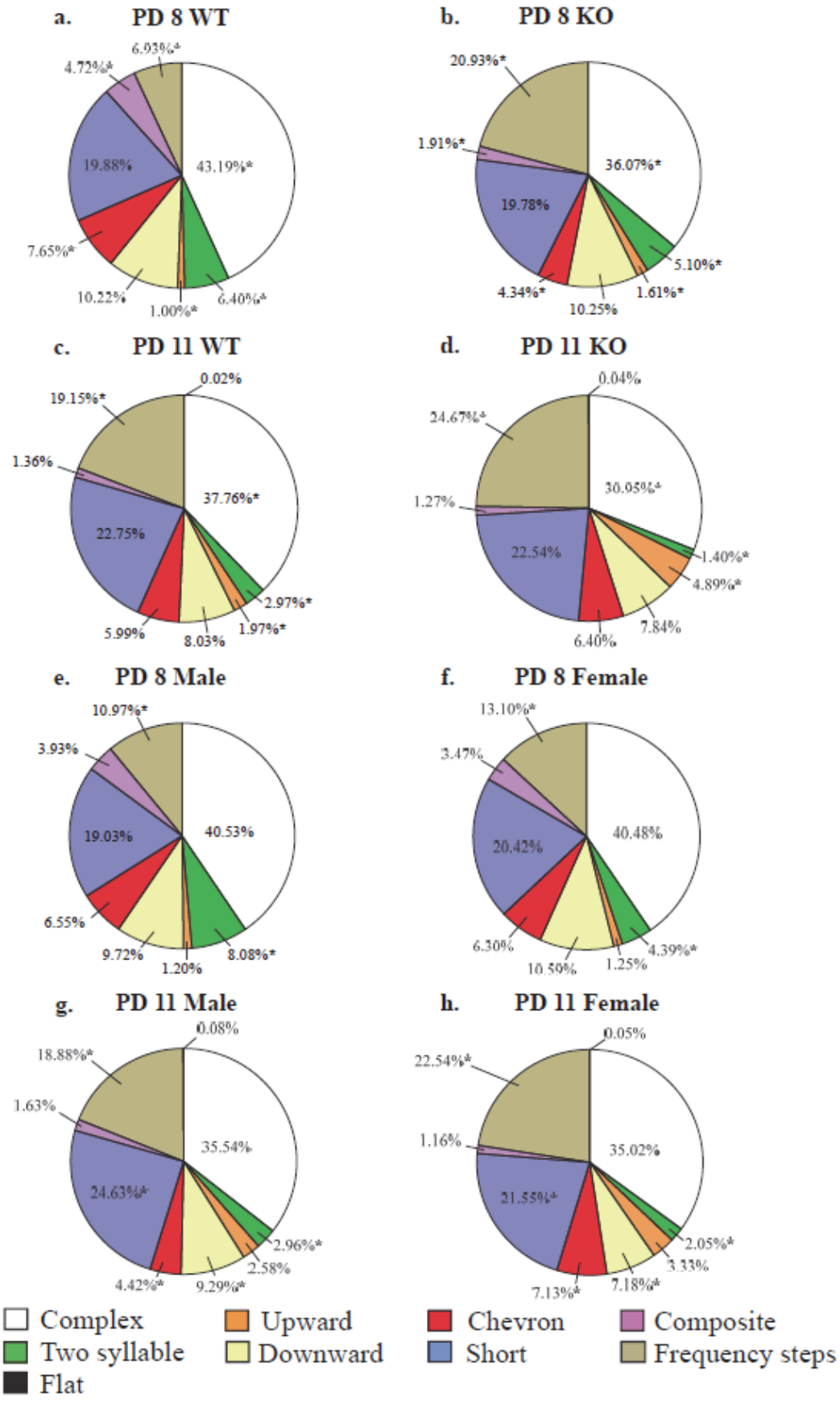
a. PD 8 WT
6.93%*
4.72%*
19.88%
43.19%*
7.65%*
10.22%
1.00%*
6.40%*
b. PD 8 KO
20.93%*
1.91%*
36.07%*
19.78%
5.10%*
4.34%*
1.61%*
10.25%
c. PD 11 WT
0.02%
19.15%*
1.36%
37.76%*
22.75%
2.97%*
5.99%
8.03%
1.97%*
d. PD 11 KO
0.04%
24.67%*
30.95%*
1.27%
22.54%
1.40%*
4.89%*
7.84%
6.40%
e. PD 8 Male
10.97%*
3.93%
19.03%
40.53%
6.55%
9.72%
1.20%
8.08%*
f. PD 8 Female
13.10%*
3.47%
40.48%
20.42%
6.30%
10.50%
1.25%
4.39%*
g. PD 11 Male
0.08%
18.88%*
1.63%
35.54%
24.63%*
2.96%*
2.58%
4.42%*
9.29%*
h. PD 11 Female
0.05%
22.54%*
1.16%
35.02%
21.55%*
2.05%*
3.33%
7.13%*
7.18%*
Complex
Two syllable
Flat
Upward
Downward
Chevron
Short
Composite
Frequency steps

Figure 3. Call Type Composition per Genotype, Sex, and Day. (a) Call types for PD 8 WT mice. (b) Call types for PD 8 KO mice. (a,b) PD 8 KO animals emitted more frequency steps and upward calls, but fewer complex, two syllable, chevron, and composite calls when compared to WT mice. (c) Call types for PD 11 WT mice. (d) Call types for PD 11 KO mice. (c,d) PD 11 KO animals emitted more frequency steps and upward calls, but less complex and two syllable calls when compared to WT mice. (e) Call types for PD 8 males. (f) Call types for PD 8 females. (e,f) PD 8 males emitted more two syllable cries, but less frequency steps calls when compared to females. (g) Call types for PD 11 males. (h) Call types for PD 11 females. (g,h) PD 11 males emitted more two syllable, short, and downward call types, but fewer chevron and frequency steps calls when compared to females. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Spectral and Temporal Characteristics

We then analyzed the spectral and temporal characteristics of ultrasonic vocalizations for PD 8. Our analyses revealed a significant MANOVA for PD 8, with genotype and sex as the fixed factors and mean duration, peak frequency, fundamental frequency, and peak amplitude as dependent factors. There was a main effect for genotype ($F(4,8711) = 73.09$, $p < .001$), sex ($F(4,8711) = 35.98$, $p < .001$), and an interaction between genotype and sex ($F(4,8711) = 8.03$, $p < .001$). We then examined the specific between-subjects effects. There was a main effect of genotype for mean duration ($F(1,8714) = 24.1$, $p < 0.001$), peak amplitude ($F(1,8714) = 249.0$, $p < 0.001$), and peak frequency ($F(1,8714) = 49.4$, $p < 0.001$), but not for the mean fundamental frequency ($F(1,8714) = 1.13$, $p = 0.29$). There were main effects of sex for the mean duration ($F(1,8714) = 12.9$, $p < 0.001$), fundamental frequency ($F(1,8714) = 5.4$, $p < 0.05$), peak amplitude ($F(1,8714) = 110.0$, $p < 0.001$), and peak frequency ($F(1,8714) = 43.9$ $p < 0.001$). There was an interaction between genotype and sex for mean duration ($F(1,8714) = 9.5$, $p < 0.01$), fundamental frequency ($F(1,8714) = 6.7$, $p < 0.05$), and peak amplitude ($F(1,8714) = 10.8$, $p < 0.001$), but not for the mean peak frequency ($F(1,8714) = 0.001$, $p = 0.97$).

Due to the interaction of group and sex we performed independent *t*-tests or non-parametric Mann-Whitney *U* tests between WT and KO mice per sex. Significant differences between WT and KO mice on PD 8 were detected, with KO males emitting calls of a shorter average duration ($t(3588) = 5.08$, $p < .001$) (Figure 4a) and lower mean peak amplitude ($U = 1217712$, $p < .001$), than WT males (Figure 4d). Female KO mice on PD 8 displayed calls with a higher mean peak frequency ($U = 2853297$, $p < .001$)

(Figure 4b), a lower mean fundamental frequency ($U = 2948392$, $p = .04$) (Figure 4c), and a lower mean peak amplitude ($U = 2386907$, $p < .001$) than WT females (Figure 4d).

Analyses of the spectral and temporal characteristics of ultrasonic vocalizations revealed a significant MANOVA for PD 11 with genotype and sex as the fixed factors and mean duration, peak frequency, fundamental frequency, and peak amplitude as dependent factors. There was a main effect for genotype ($F(4,6542) = 151.55$, $p < .001$), sex ($F(4,6542) = 37.18$, $p < .001$), and an interaction between genotype and sex ($F(4,6542) = 22.88$, $p <.001$). The between subjects analyses revealed a main effect of genotype for mean duration ($F(1,6545) = 123.8$, $p < 0.001$), fundamental frequency ($F(1,6545) = 157.7$, $p < 0.001$), and peak frequency ($F(1,6545) = 408.7$, $p < 0.001$), but not for mean peak amplitude ($F(1,6545) = 0.007$, $p = 0.93$). There was a main effect of sex for mean duration ($F(1,6545) = 27.8$, $p < 0.001$), peak amplitude ($F(1,6545) = 32.1$, $p < 0.001$), and peak frequency ($F(1,6545) = 4.3$, $p < 0.05$), but not for the mean fundamental frequency ($F(1,6545) = 0.1$, $p = 0.76$). There were several statistically significant interactions found between genotype and sex. The differences were found in the mean fundamental frequency ($F(1,6545) = 52.9$, $p < 0.001$), peak amplitude ($F(1,6545) = 28.9$, $p < 0.001$), and peak frequency ($F(1,6545) = 24.3$, $p < 0.001$), but not for the mean duration ($F(1,6545) = 1.2$, $p = 0.27$).

When examining males on PD 11, KO mice emitted calls of an average shorter duration ($U = 625954$, $p < .001$) (Figure 4e), higher mean peak frequency ($t(2397) = 9.33$, $p < .001$) (Figure 4f), higher mean fundamental frequency ($U = 658154$, $p < .001$) (Figure 4g), and lower mean peak amplitude ($U = 676598$, $p < .001$) than WT males (Figure 4h). Similarly, when analyzing females on PD 11, KO mice emitted cries of a

shorter mean duration ($U = 1487283$, $p < .001$) (Figure 4e), higher mean peak frequency ($U = 1161870$, $p < .001$) (Figure 4f) and a higher mean fundamental frequency ($U = 1305243$, $p < .001$) than WT females (Figure 4g).

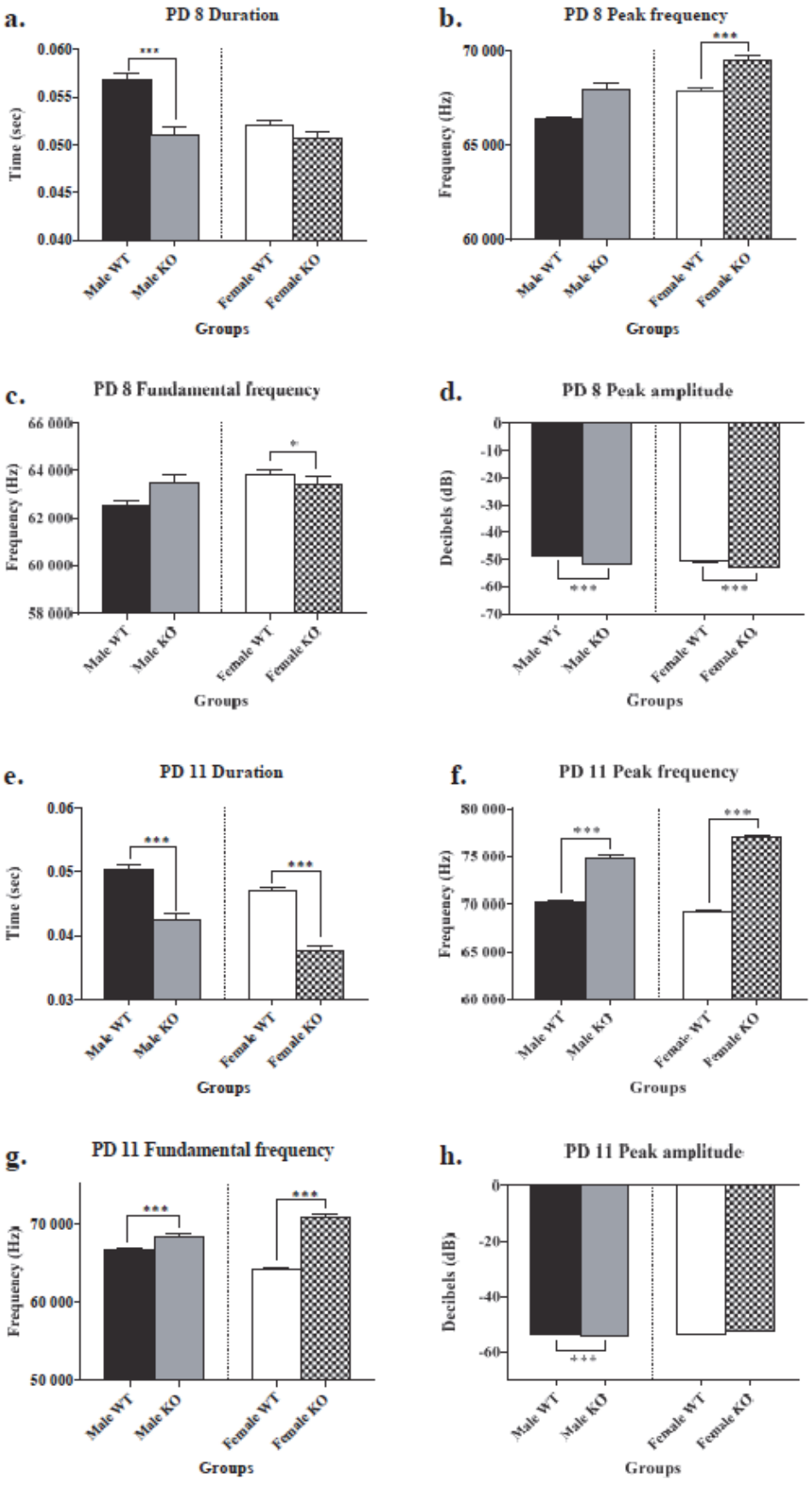
a.
PD 8 Duration
Time (sec)
0.060
0.055
0.050
0.045
0.040
***
Male WT
Male KO
Female WT
Female KO
Groups
b.
PD 8 Peak frequency
Frequency (Hz)
70 000
65 000
60 000
***
Male WT
Male KO
Female WT
Female KO
Groups
c.
PD 8 Fundamental frequency
Frequency (Hz)
66 000
64 000
62 000
60 000
58 000
*
Male WT
Male KO
Female WT
Female KO
Groups
d.
PD 8 Peak amplitude
Decibels (dB)
0
-10
-20
-30
-40
-50
-60
-70
***
***
Male WT
Male KO
Female WT
Female KO
Groups
e.
PD 11 Duration
Time (sec)
0.06
0.05
0.04
0.03
***
***
Male WT
Male KO
Female WT
Female KO
Groups
f.
PD 11 Peak frequency
Frequency (Hz)
80 000
75 000
70 000
65 000
60 000
***
***
Male WT
Male KO
Female WT
Female KO
Groups
g.
PD 11 Fundamental frequency
Frequency (Hz)
70 000
60 000
50 000
***
***
Male WT
Male KO
Female WT
Female KO
Groups
h.
PD 11 Peak amplitude
Decibels (dB)
0
-20
-40
-60
***
Male WT
Male KO
Female WT
Female KO
Groups

Figure 4. Spectral Characteristics of the Vocalizations. Mean duration, peak frequency, fundamental frequency, and peak amplitude for WT and KO mice on PD 8 and PD 11. Male KO mice on PD 8 emitted calls of a shorter mean duration (a), and a lower mean peak amplitude (d) than male WT mice. Female KO mice on PD 8 emitted calls at a higher mean peak frequency (b), a lower fundamental frequency (c), and a lower mean peak amplitude (d) than WT females. Male KO mice on PD 11 emitted USVs of a shorter mean duration (e), higher mean peak frequency (f), higher mean fundamental frequency (g), and a lower mean peak amplitude (h) than WT males. Female KO mice on PD 11 emitted calls of a shorter mean duration (e), higher mean peak frequency (f), and a higher mean fundamental frequency (g) than WT females. The bars represent the mean and the error bars represent the standard error of the mean. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

CHAPTER FIVE

Discussion

This chapter published as: Binder MS, Lugo JN. NS-*Pten* knockout mice show sex- and age-specific differences in ultrasonic vocalizations. *Brain Behav.* 2017;e00857. https://doi.org/10.1002/brb3.857

In this study, we compared the quantity, call types, and spectral characteristics of neonatal ultrasonic vocalizations in WT and NS-*Pten* KO male and female pups. We found that NS-*Pten* KO mice emitted significantly fewer vocalizations than WT mice. Additionally, male pups emitted fewer vocalizations than females on PD 8 and 11. Overall, there was a general reduction in the number of calls from PD 8 to PD 11. The types of calls emitted were also different between WT and KO mice. Lastly, significant differences in the acoustic and temporal structures of the calls were revealed. Specifically, NS-*Pten* KO males on PD 8 emitted calls with a shorter mean duration and lower mean peak amplitude. NS-*Pten* KO females had calls of a higher mean peak frequency, lower fundamental frequency, and a lower peak amplitude. On PD 11, NS-*Pten* KO males emitted calls with a shorter mean duration, higher peak frequency, higher fundamental frequency, and a lower mean peak amplitude, whereas NS-*Pten* KO females emitted calls of a shorter mean duration, higher peak frequency, and a higher mean fundamental frequency.

Perhaps the most important finding in this study was the aberrant vocalizations seen in NS-*Pten* KO mice. Similarly deviant vocalizations have also been observed in *Fmr1* and *Tsc1* deficient pups, therefore our results contribute to a consistency of communication deficits found across several mTOR models (Reynolds et al., 2016; Tsai

et al., 2012; Young, Schenk, Yang, Jan, & Jan, 2010). Specifically, *Fmr1* KO pups were shown to emit significantly fewer vocalizations than their WT counterparts when separated from their mother (Reynolds et al., 2016). In contrast, *Tsc1* HT pups emitted significantly more vocalizations than their controls (Tsai et al., 2012). Thus, while the particular quantity of vocalizations emitted is not always constant across different mTOR models, the underlying aberrant vocalizing patterns are still observed, demonstrating a consistency between our findings and those in related studies.

Another congruous finding between our results and those seen in another mTOR model is the average length of the vocalization emitted. Both male and female *Fmr1* KO pups have been shown to exhibit a decreased call duration when compared to wild types, resembling our findings in the current study (Reynolds et al., 2016). Call duration is significant as dams have been shown to prefer a longer call over a shorter one (Smith, 1976). Dams have even been shown to not respond to vocalizations lasting under 30 ms (Ehret, 1992). Taken together, this indicates that mTOR dysfunction may put the corresponding KO pups at a disadvantage in eliciting their dams' response due to their decreased call length.

While the results garnered from our study do appear to fit in well with the literature as a whole, there is one notable exception, as the current findings are contrary to a similar study conducted previously in our lab. Specifically, a prior study from our lab reported no difference in the quantity of vocalizations for NS-*Pten* KO mice on PD 10 and 12 (Lugo et al., 2014). This discrepancy is perhaps best explained by the different software programs used to record the USVs. Whereas the current study utilized a full spectrum analysis program, Lugo *et al.,* (2014) employed the Ultravox system. The full

spectrum analysis equipment uses a broad-spectrum microphone that is able to identify frequencies ranging from 0 to 125 kHz, allowing for the detection of all possible neonatal cries (Avisoft Bioacoustics). Conversely, the Ultravox system utilizes microphones set to a specific frequency; for the study in question they were set to 40, 50, 60 and 70 kHz (Lugo et al., 2014). The detectors are designed to pick up calls within 1 kHz around the frequency that the detector is set to. Therefore, approximately 8 kHz between each detector could be missed and calls lower than 39 and greater than 81 would not be detected. As mice are able to vocalize anywhere between 30 to 90 kHz, it is quite possible that some of the calls were simply not detected using the Ultravox system. Additionally, the full spectrum analysis program allows the experimenter to delete background noise that may have been incorrectly detected as a call; no such option exists for the Ultravox system. While no study has directly compared the vocalizations recorded in the Ultravox program to the Avisoft program, the numerous differences underscoring these two systems form the most likely explanation for any variation present in the results.

While the current vocalization literature is promising, future studies could look more closely at the USV patterns of females in various models, as this presents an area that is currently underrepresented. Future studies could also examine other characteristics of calls in mTOR models such as the average peak amplitude, peak frequency, or fundamental frequency, to provide the current study's findings more context. Additionally, measures of maternal behavior could be investigated. Specifically, maternal retrieval tests, wherein the dam would choose between the recordings of WT or KO USVs, could provide a valuable insight into the relationship between altered neonatal

cries and the corresponding maternal response. Studies could also add in more time points to see if the developmental trajectory of call frequency is altered in NS-*Pten* KO mice. Lastly, studies could investigate drug-based interventions to see if the atypical vocalization behavior seen in mTOR models normalizes in the presence of a treatment.

Overall, our results demonstrate a striking consistency in aberrant ultrasonic vocalizations that is seen across numerous mTOR models. Furthermore, there is evidence to suggest that the temporal characteristic of the calls, their average duration, is also uniformly impacted. In conclusion, through examining an underrepresented model, this study addressed a significant deficit in the mTOR vocalization literature. Additionally, it not only provides context for findings in the *Fmr1* and *Tsc1* models, but also further implicates the mTOR pathway as playing a potential causative role in aberrant vocalizations in neonates. Perhaps most importantly, the current study significantly contributes to the characterization of a molecular mechanism responsible for an autistic like phenotype, thereby partially elucidating the underlying disorder. Thus, as our study helps to facilitate a better understanding of the NS-*Pten* phenotype it is, by proxy, also integral to garnering a better understanding of autism.

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