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Developmental Trajectory and Sex Differences in Auditory Processing in Two Different Mouse Models of Autism Spectrum Disorders

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ABSTRACT OF THE DISSERTATION

Developmental Trajectory and Sex Differences in Auditory Processing in Two Different Mouse Models of Autism Spectrum Disorders

by

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Autism spectrum disorder (ASD) is currently diagnosed in approximately 1 in 44 children in the United States, based on a wide array of symptoms, including sensory dysfunction and abnormal language development. Auditory temporal processing is crucial for speech recognition and language development. Abnormal development of temporal processing may account for ASD language impairments. Sex differences in the development of temporal processing may underlie the differences in language outcomes in male and female children with ASD. To understand mechanisms of potential sex differences in temporal processing requires a preclinical model. However, there are no studies that have addressed sex differences in temporal processing across development in any animal model of ASD. My dissertation research utilizes two different mouse models of ASD that display sensory abnormalities: the *Fmr1* (Fragile X Messenger Ribonucleoprotein 1) knock-out (KO) mouse model of fragile X syndrome (FXS) and a *Pten*-deletion (phosphatase and tensin homolog missing on chromosome 10) model of autism. I utilized a 40 Hz gap-in-noise ASSR (auditory steady state response) paradigm and *in vivo* electrophysiology to measure the cortex's reliability in phase locking to brief

gaps in noise at varying modulation depths to assess temporal processing acuity. The *Fmr1* study results show gap-ASSR deficits in the frontal (FC), but not auditory (AC), cortex in early development (p21, “postnatal day 21”) in male and female KO mice. Unlike male KO mice, female KO mice show wild type-like temporal processing at p30. This research also utilizes a conditional *PTEN* KO model that displays a specific gene KO pattern, occurring exclusively in the hippocampus and layers III-V of the cortex. Results show that abnormal PTEN expression significantly impacts oscillatory activity in male and female mice, including abnormal resting power distributions and decreased phase-locking compared to controls. Overall, deficits become more prominent in adult mice. Notably, in both ASD models, female KO mice showed increased auditory hypersensitivity compared to males, reflected as increased ERP amplitudes. This dissertation research has identified similarities and differences between two different autism models. Further unraveling these circuits may be the key to understanding and treating ASD pathology.

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Chapter 1

Introduction

Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by a lack of social interactions, impaired verbal and nonverbal communication, and repetitive behaviors [Park et al., 2016; Ratajczak, 2011]. The disorders included in this spectrum are autistic disorder, Asperger's disorder, Rett syndrome, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified [Kirkovski et al., 2013; Pickett & London, 2005]. The rate of ASD diagnoses has increased dramatically in the past decade and is currently diagnosed in approximately 1 in 44 children in the United States. [Christensen et al., 2018]. This spectrum of disorders has traditionally been diagnosed within the first three years of life, when differences from age-matched children start to become apparent. ASD is mostly diagnosed behaviorally because no reliable early developmental biomarkers have been identified. Some of the earliest signs recognized in infants include irritability, passivity, difficulties sleeping and eating, and delays in language and social engagement [Park et al., 2016]. By one year of age, abnormalities in visual attention, social responses, motor control, and reactivity become prevalent [Zwaigenbaum et al., 2005]. The core symptoms of ASD are expressed by age 3, making the distinction between ASD and other psychosocial disorders apparent [Park et al., 2016]. Although typically diagnosed in late preschool/early school years, there has been a significant increase in the number of

adults diagnosed, which may be due to the broadened diagnostic criteria in recent years [Park et al., 2016].

The core deficits seen in individuals with ASD include impaired/delayed communication and social interaction as well as sensory hypo- and/or hypersensitivity [Pickett & London, 2005]. Although recognized as a consistent and core abnormality, very little is known about the underlying mechanisms of sensory processing deficits. Moreover, neither developmentally consistent nor robust sensory processing biomarkers have been established for early diagnosis of ASD. A biomarker, or 'biological marker,' is a biological variable associated with the disease of interest that can be measured directly in a patient using sensitive and reliable quantitative measures [Persico et al, 2014]. Identifying biomarkers for ASD has proven to be challenging due to the complexity of the disorder. More specifically, proposed biomarkers may reflect genetic, neurobiological, or epigenetic processes that are not consistent throughout the lifespan, demonstrating a more transient expression pattern. Another potential pitfall in these types of studies is that biomarkers may not actually represent the disorder, but rather the processes that led to it or compensatory responses [Goldani et al., 2014]. Although challenging, the identification of reliable and translatable biomarkers could progress the field by unraveling circuit pathophysiology and underlying mechanisms of ASD, promoting earlier and more reliable diagnoses, predict developmental trajectories, and optimize treatment strategies [Persico et al., 2014].

Language impairments in ASD

Abnormal expressive and receptive language development is consistently reported in humans with ASD. While expressive language refers to the ability to produce

language, receptive language is the ability to comprehend language [Frazier, 2011]. Notably, impairments with language processing, including the phonologic, syntactic, semantic, prosodic, and pragmatic aspects, have been identified [Foss-Feig et al., 2017; Tager-Flusberg & Caronna, 2007; Rapin & Dunn, 2003; Jeste & Nelson, 2009]. Overall, the prosodic and pragmatic aspects of speech are impaired to a greater extent than syntax and semantics in humans with ASD [Sigman & Kim, 1999].

Phonology

Phonology refers to the sound patterns of language. More specifically, the sounds and pronunciation rules that make up a language [Kostyuk et al., 2010]. Phonological speech deficits have been identified in ASD. One study found that that roughly a third of school-age children with ASD demonstrate speech production problems [Shriberg et al., 2001]. Specifically, children with ASD presented with substantially higher rates of speech errors, such as dentalized sibilants (a fricative consonant sound in which the tongue is brought near the roof of the mouth and air is pushed past the tongue to make a hissing sound) and distortions of “r” [Shriberg et al., 2010]. Although these and other abnormalities have been observed, including an MRI study that demonstrated that autistic children process sounds slower than typically developing children, phonology is the area of language least likely impaired in humans with ASD [Redcay & Courchesne, 2008].

Grammar

The research regarding grammar deficits in ASD is inconsistent. Some studies suggest that relatively few problems are present, while others provide evidence that grammar is severely impaired in children with ASD [Eigsti et al., 2007; Eigsti & Bennetto, 2009; Kjelgaard & Tager-Flusberg 2001; Fisher et al., 2005]. One specific deficit

consistently reported across studies is pronoun reversal, which occurs when individuals confuse the first and second pronouns in speech (“you” versus “me”) [Kostyuk et al., 2010; Stefanatos & Baron, 2011]. Other abnormalities seen in humans with ASD include decreased likelihood of producing grammatical morphemes, difficulty with reflexive pronoun interpretation, and using short and long passive verbs [Bartolucci, 1982; Bartolucci et al., 1980; Shulman & Guberman, 2007]. Overall, these abnormalities suggest the inability to process syntactic relationships and interpret syntactic chains. The physiological mechanism behind these deficits are not clear, but it has been reported that the function of processing implicit syntactic relationships is significantly dependent on the hippocampus [Opitz & Friederici, 2007].

Semantic

Children with autism have difficulties with the sematic processing of words. Semantics is the aspect of language that relates to understanding the meaning of words, phrases, and sentences as well as using words appropriately when we speak [Kostyuk et al., 2010]. Specifically, these individuals struggle with abstract words, those that relate to feeling or emotions, and also words that refer to status [Lopez & Leekam, 2003]. These deficits are typically seen as idiosyncratic word use and neologisms in place of generally accepted words for specific objects or events [Stefanatos & Baron, 2011]. Possible explanations have been proposed for these abnormalities. When learning new words, it is likely that children with ASD are not fully attended in the situation and are therefore unable to develop the proper associations between words and objects [Stefanatos & Baron, 2011]. Furthermore, children with ASD also struggle with social referencing, affect sharing, and gaze tracking which would likely impact their ability to

make correct associations between words and objects [Baron-Cohen et al., 1997; Grice et al., 2005].

Pragmatic

Pragmatic language skills are crucial for communicating personal thoughts, ideas, and feelings. These skills include being able to participate in a conversation by taking turns with the other speaker and reacting appropriately to the other person's body language, mood and words. Additionally, pragmatic skills include the ability to maintain a topic or change topics appropriately, maintain appropriate eye-contact during a conversation and the ability to distinguish between informal and formal communication scenarios how to talk and behave formally with some individuals and informally with others [Kostyuk et al., 2010; Kerbel & Grunwell, 1998]. Deficits in this aspect of language are highly correlated with ASD. Specifically, these individuals demonstrate deficiencies in the reciprocity of social communication, unusual or inadequate expression and understanding of ideas, as well as other abnormalities indicative of impaired understanding of social norms and expectations [Stefanatos & Baron, 2011; Rapin & Dunn, 2003; Surian et al. 1996]. These pragmatic impairments have been suggested to be a result of problems with social perception and comprehension [Shields et al., 1996].

Prosodic

Prosody is an important aspect of language and includes the intonation and rhythm of speech [Applebaum et al., 2014]. This key aspect of communication is highly impacted in ASD. Specifically, speech may be inappropriately loud or soft, fast or slow, have no emotional tone and may be generally high pitched. The cadence, rhythm or tempo of verbal productions may also be abnormal, such as misplaced stress on words and increased repetitions [Stefanatos & Baron, 2011]. The neural correlates of prosody

are not fully characterized, but it has been shown that prosody is right hemisphere dominant in children but becomes more bilaterally represented with development [Trauner et al., 1996].

Echolalia

Echolalia is the repetition of words, signs, phrases or sentences spoken by other people. This behavior is highly common in children with ASD, such that a reported 75% of all children with ASD have gone through a phase of echolalic production [Belger et al., 2011]. Although echolalia was once seen as a negative behavior, others have speculated that it may serve specific functions. For example, some individuals may use this repetitive behavior as a way of communication, while others may use the repetition to regulate their own behavior [Kostyuk et al., 2010]. Furthermore, echolalia has also been suggested to serve a variety of pragmatic and linguistic functions as a turn filler, processing aid, self-regulator, or as having expressive communicative intent outside the direct meaning of the echolalic production [Prizant & Duchan, 1981; Stefanatos & Baron, 2011]. The underlying mechanisms of this behavior are beginning to be understood as it is speculated that echolalia may represent compensatory disinhibition of audio-motor processing due to receptive and expressive language deficits [Linetsky et al., 2000; Stefanatos & Baron, 2011]. Although echolalia has been heavily correlated with ASD cases, not all humans with ASD show this behavior nor is it only seen in ASD. Other instances of echolalia include individuals with other language impairments or blindness as well as older individuals with dementia [Yule & Rutter, 1987].

Receptive language

Abnormalities in receptive language are highly reported in individuals with ASD [Chen et al., 2024; Hannant, 2018; Hundry et al., 2010]. However, it is important to note that the severity of these deficits are highly dependent on the type of measurements being used (caregiver reports versus standardized measures versus experimental approaches [Chen et al., 2024]. Furthermore, studies have demonstrated greater impairment in comprehension rather than production skills in toddlers and children with ASD [Luyster et al., 2018; Kjelgaard & Tager-Flusber, 2001]. When looking at the earliest stages of development, the average toddler who understands more than 200 words typically produces only around 45 words [Fenson et al. 1994]. Children with ASD, on the other hand, do not demonstrate this level of comprehension until they can produce an average of 57-126 words, suggesting that children with ASD appear to understand proportionately fewer words than expected based on their expressive vocabularies [Luyster et al., 2008; Charman et al., 2003; Hundry et al., 2010]. Interestingly, this receptive language gap between typically developing children and those with ASD has been shown to widen with age [Chen et al. 2024].

The role of auditory temporal processing in language impairments

It is hypothesized that auditory temporal processing deficits may be the root of these language impairments seen in ASD described above. The ability to make fine temporal discriminations of sound is critical to a wide range of processes including speech recognition, language development and sound localization [Michalewski et al., 2005]. The capacity to follow rapid temporal cues on the order of milliseconds is critical for distinguishing and parsing speech sounds. For example, phoneme discrimination is

critically dependent on the temporal features of speech, including voice-onset time, the duration of frequency transitions, and the silent time between constants and vowels [Mauk & Buonomano, 2004; Lisker & Abramson, 1964; Liberman et al., 1956; Dorman et al., 1979]. Indeed, severe temporal processing impairments have been identified in language disorders, including dyslexia and specific language impairment (SLI) [Tallal & Piercy, 1973; Heath et al., 1999]. Therefore, it is hypothesized that deficits in the ability to process acoustic information presented in rapid succession is disrupting phonological processes and may be responsible for the language impairments seen in individuals with ASD.

Temporal processing of sound can be divided into two categories: temporal integration and temporal acuity/resolution [Eddins & Green, 1995]. Temporal integration describes processing in which the intensity and/or duration of the signal affects the summation of neuronal activity, and temporal acuity/resolution is the minimum time interval within which the auditory system can discriminate between two signals [Eggermont, 2015; Jesteadt et al., 1976; Shinn & Musiek, 2003]. Individuals with ASD show impairments with these temporal aspects of sensory processing, including auditory stimuli duration, onset and offset, and rapid changes within them [Kwakye et al., 2011; Lepistö et al., 2006; Oram Cardy et al., 2005; Orekhova et al., 2009]. One study found that children with ASD had difficulties reproducing the lengths of auditory and visual stimuli while another demonstrated that both children and adults with ASD produced abnormal neural responses to fluctuations in pitch of repeated, sequential auditory stimuli [Szelag et al., 2004; Tecchio et al., 2003; Gomot et al., 2006]. Oram Cardy et al. (2005) demonstrated via magnetoencephalography (MEG) that children with ASD fail to respond to a second stimulus when duos of pure tones are presented in rapid

succession, supporting the idea that rapid temporal processing is impaired in ASD. Additionally, increased gap-detection thresholds have been reported in patients with ASD, a paradigm commonly used to assess auditory temporal processing. Notably, it was shown that impaired gap detection thresholds in children were associated with lower phonological processing scores [Foss-Feig et al., 2017]. These human studies provide support for the hypothesis that auditory temporal processing is critical for many aspects of speech and deficits may be responsible for language impairments seen in ASD. However, very little is known about temporal processing in any animal model of autism. Specifically, it remains unknown if and when the deficits occur across development, what the underlying mechanisms are, and whether the deficits can be targeted with therapeutics.

Sex differences in ASD

A sex bias in ASD diagnosis is well established, with the male:female ratio of diagnosis being ~4:1 [Werling & Geschwind, 2013]. Males with ASD more commonly show externalizing behavior problems, such as aggression, hyperactivity and restricted behaviors, while females show greater internalizing symptoms, including anxiety and depression [Hattier et al., 2011; Mandy et al., 2012; Szatmari et al., 2012; Bolte et al., 2011; Giarelli et al., 2010; Solomon et al., 2012]. Typically developing boys have been shown to have a “weaker” or “slower” capacity for language acquisition, representing more than 70% of late talkers [Adani & Cepanec, 2019]. This sex difference in language development is mirrored in ASD children. For example, Harrop et al. (2021) found significant sex differences in parent-reported early developmental milestones, with females meeting their language milestones earlier than males. These findings align with

what was previously shown by Eriksson et al. (2012), with females developing ahead of males in early communicative gestures, productive vocabulary, and combining words. Additionally, Zubrik et al. (2007) demonstrated that boys with ASD have a greater risk for late language emergence. Although multiple studies have reported a strong link between fetal or early postnatal sex hormone levels and communication and language development, the developmental trajectory and mechanism of this sex difference is not well understood [Lutchmaya et al., 2002; Lombardo et al., 2012; Auyeung et al., 2009; Friederici et al., 2008; Hollier et al., 2013; Schaadt et al., 2015].

ASD-linked genes:

Although most ASD cases are idiopathic, studies have shown that strong genetic components, together with environmental factors present in the early stages of development, can contribute to the pathogenesis of ASD. It is estimated that a genetic cause can be identified in up to 25% of ASD cases, with chromosomal rearrangements and coding-sequence mutations making up ~10–20% and ~5–10% of ASD patients, respectively [Guang et al., 2018; Huguet et al., 2013; Ziats & Rennert, 2016]. A study conducted in 2009 presented a list of 1,237 human genes that have potential links to autism; however, a 2015 study claims that there is strong evidence for a total of 65 ASD candidate genes in addition to syndromic genes that cause syndromes comorbid with ASD [Ziats & Rennert, 2016; Basu et al., 2009; Sanders et al., 2015]. Functions of these genes include cell adhesion molecules, scaffolding proteins, regulators of protein synthesis and degradation, and regulators of chromatin remodeling and transcription [Ziats & Rennert, 2016]. Two genes that exhibit a strong link and comorbidity with ASD include *PTEN* and *Fmr1*.

PTEN

Phosphatase and tensin homolog deleted on chromosome 10 (*Pten*) was first identified as a tumor suppressor gene frequently lost from a region of chromosome 10q23 in multiple cancers, including brain, breast, and prostate, in 1997 [Hopkins et al., 2014; Sansal & Sellers, 2004; Li et al., 1997]. Shortly after its identification, germline *Pten* mutations were linked to a group of inherited clinical syndromes now referred to as PTEN Hamartoma Tumor Syndrome (PHTS) [Endersby & Baker, 2008; Blumenthal & Dennis, 2008]. PTEN is a dual protein and lipid phosphatase that serves as a negative regulator of the PI3K/AKT/mTOR pathway, a pathway known to influence cellular processes including growth, proliferation, survival, apoptosis, metabolism, and cell migration [Endersby & Baker, 2008]. In the brain, components of the PI3K/AKT/mTOR pathway are present at synapses and are essential for synaptogenesis and regulation of dendritic spine morphology [Sawicka & Zukin, 2012]. Growing evidence across fields suggests that dysregulation of this critical pathway may be the underlying cause of numerous diseases, including autism.

Associations between PTEN and autism were discovered as early as 2003, when patients carrying germline *Pten* mutations displayed autistic behavior [Eng, 2003; Reardon et al., 2001; Goffin et al., 2001; Zori et al., 1998]. A small cohort study in 2005 showed an intriguing number of *Pten* mutations in ASD cases with macrocephaly [Butler et al., 2005]. Larger cohorts reflected this initial finding, with *Pten* mutations being found in 17% of macrocephalic ASD cases, further supporting a link between the two [Hobert et al., 2014; Klein et al., 2013; McBride et al., 2010; Varga et al., 2009; Herman et al., 2007; Buxbaum et al., 2007]. PTEN-ASD phenotype characterization was later carried out in humans across an age range of 2-25 years old and was compared to the following

conditions: idiopathic ASD, idiopathic macrocephalic ASD and healthy controls [Tilot et al., 2015]. MRI data collected for the PTEN-ASD group revealed a global increase in white matter volume as well as increased corpus collosum volume [Tilot et al., 2015; Frazier et al., 2015; Frazier, 2019]. This cohort study also found that the PTEN-ASD group had a significantly larger head size compared to the other three as well as lowered IQs, decreased processing speed, working memory, and impaired language development. Specifically, significant delays were identified in their first words and phrases, with some remaining mute or minimally verbal until after 4 years of age [Tilot et al., 2015]. Speech and language delays have been reported across PTEN studies, with prevalence rates between 27 and 57% [Balci et al., 2018; Busa et al., 2015; Lynch et al., 2009]. Given the strong link previously described between temporal processing and speech, these findings suggest that auditory temporal processing deficits may be occurring in *Pten*-deleted individuals. However, further characterization of these deficits and their developmental trajectory has not yet been done in human nor mouse model studies.

A sex-bias in PTEN mutations has been reported in human cohort studies. Notably, females carrying a PTEN mutation had significantly higher cancer risks than males, including thyroid and breast cancers [Nieuwenhuis et al., 2014; Campbell et al., 2001]. Mutations of *Pten* are the most common cause of breast cancers due to their negative regulation of the PI3K/AKT/mTOR pathway, a cancer that is predominately diagnosed in women [Campbell et al., 2001]. This sex-bias is due to the expression of estrogen receptor α (ERα) in 70-80% of breast cancers [Vilgelm et al., 2006]. Hyperactivation of the PI3K/AKT/mTOR pathway in breast cancer cells results in enhanced phosphorylation and the subsequent hyperactivation of ERα, so dual

therapeutics inhibiting both PI3K/AKT/mTOR and ERα are the primary treatments used for breast cancers [Yamnik et al., 2009; Ishida et al., 2018; Ciruelos, 2014; Page et al., 2009]. Furthermore, an interaction between ERα and Group 1 metabotropic receptors (mGluR1 and mGluR5) has been identified specifically in female neurons in multiple brain regions [Martinez et al., 2014; Tabatadze et al., 2015; Tonn Eisinger et al., 2018; Santollo & Daniels, 2019]. In the hippocampus, estradiol acts via ERα to initiate postsynaptic mGluR1-dependent mobilization of the endocannabinoid anandamide to suppress GABA release [Huang & Woolley, 2012]. Additionally, this dual interaction mediates the estradiol effects on hippocampal memory consolidation [Boulware et al., 2013]. ERα-mGluR5 signaling was seen exclusively in female striatal neurons as well [Grove-Strawser et al., 2010]. The hyperactivity of mGluR5 and its subsequent downstream signaling is a mechanism that has been implicated in multiple ASD mouse models, suggesting that an underlying mechanism may be conserved [D'Antoni et al., 2014; Bear et al., 2004]. Whether this mechanism leads to similar phenotypes in these ASD models, such as auditory temporal processing deficits, is yet to be determined. Furthermore, the literature suggests that an ERα-mGluR5 interaction is driving the observed sex-biases seen in various brain regions.

Because germline *Pten* homozygous knockouts are embryonically lethal, numerous mouse models of PTEN mutations have been established to gain a better understanding of how this mutation is related to the autistic phenotypes seen in humans [Di Cristofano et al., 1998]. These models include germline heterozygous loss-of-function, germline homozygous cytoplasm-predominant knock-in, and conditional homozygous loss-of-function [Clipperton-Allen & Page, 2020]. Although all the mouse models developed provide useful information about ASD-relevant cellular and behavioral

deficits, this research will use a conditional *Pten* knock-out (KO) model that utilizes a neuron-specific enolase (*Nse*) promoter-driven cre [Kwon et al., 2006a]. Nse is a glycolytic enolase that is expressed exclusively in neurons. More specifically, the timing of expression for Nse is correlated with the differentiation and maturation of neuronal cells in both the rodent and monkey brain [Marangos et al., 1980]. This model of *Pten* deletion displays a unique KO pattern [Kwon et al., 2006a]. *Cre* activity is first detectable by embryonic day (E)11 in the spinal accessory nerve. The activity is then spread into the spinal cord and peripheral nervous system by E13.5, followed by the ventral spinal cord and dorsal root ganglion at postnatal day (p)0. *Cre* activity becomes detectable in the brain at p2, only in differentiated neurons located within the cortex and hippocampus. At four weeks of age, cre activity remains restricted to the following regions and percentage of neurons in the brain: layers III-V of the cortex (54.5%), dentate gyrus (48.7%), CA3 (37.6%) and polymorphic layer (PML) (58.3%) in the hippocampus. No *cre* activity is detected in glial cells [Kwon et al., 2006a].

The Nse-cre mouse model has been characterized in a variety of autism studies, ranging from cellular to behavioral. A consistent finding across studies is hypertrophy and the subsequent enlargement of the deep cortical layers, dentate gyrus, and PML, causing compression and neuronal loss of the CA1 region [Takeuchi et al., 2013]. Synaptic dysregulation was identified in the hippocampus, with theta burst-induced long-term potentiation (TBS-LTP) being enhanced and mGluR long-term depression (mGluR-LTD) impaired at perforant path-to-dentate granule cell synapses [Takeuchi et al., 2013]. Additionally, Nse-cre PTEN mutants develop abnormalities reminiscent of autism, including macrocephaly, reduced social activity, increased anxiety, and sporadic seizures [Ogawa et al., 2007; Kwon et al., 2006b]. Another study in this model demonstrated

hyperexcitable local neocortical circuits (prolonged, spontaneous persistent activity states known as ‘UP states’) in female, but not male, PTEN KO mice [Molinaro et al., 2024]. Notably, these UP states were successfully rescued by the antagonism of mGluR5 and ERα and an increased interaction was seen between the two receptor types. The Nse-cre KO model has also been used to assess ultrasonic vocalizations (USVs). Binder et al. (2020) demonstrated that KO mice emitted vocalizations of a lower peak frequency, shorter duration, and higher peak amplitude compared to WT mice. KO animals also showed a significantly different distribution of call-types relative to controls, including increased complex and short calls. Overall, this study concluded that Nse-cre KO mice display communication deficits compared to WT controls [Binder et al., 2020].

This research will use this PTEN model due to its unique KO pattern. By eliminating PTEN in this manner, we have the ability to assess the region- and cell-specific contributions that give rise to overall phenotypes. More specifically, this research aims to determine whether deleting PTEN from a portion of cells in layers III-V of the cortex and hippocampal structures impacts auditory temporal processing and sensory responses across development using EEG.

Fmr1

A mutation of the Fragile X Messenger Ribonucleoprotein gene (*Fmr1*) causes fragile X syndrome (FXS), the leading monogenic cause of ASD. *Fmr1* contains a trinucleotide CGG expansion in the 5’ untranslated region (UTR) within the first exon of the gene that ranges from 6-54 repeats in normal individuals [Penagarikano et al., 2007; Wang et al., 2012]. A premutation is caused when the repeat number increases to 60-200 and a full mutation when above 200 [Wang et al., 2012]. This level of expansion causes hypermethylation and silencing of *Fmr1*, thus decreasing expression of fragile X

mental retardation protein (FMRP), the protein product of *Fmr1*. FMRP is an mRNA-binding protein associated with polyribosomes that typically inhibits protein translation [Huber et al., 2002; Leigh & Hagerman, 2020; Darnell et al., 2011]. The targets of FMRP are widespread, placing this protein in an optimal position to cause anomalous neural and circuit function in its absence.

Humans affected by FXS demonstrate intellectual impairment, increased anxiety, cognitive deficits, repetitive behaviors, and abnormal sensory processing [Wisniewski et al., 1991; Cordeiro et al., 2011; Van der Molen et al., 2010; Oakes et al., 2016; Miller et al., 1999; Abbeduto & Hagerman, 1997]. Clinical, behavioral and electrophysiological studies have demonstrated sensory hypersensitivity in humans with FXS across multiple domains, including enhanced electrodermal, tactile, taste, smell, visual, and auditory sensations [Miller et al., 1999; Rogers et al., 2003; Crane et al., 2009; Marco et al., 2011; Takarae et al., 2016; Ethridge et al., 2016]. This phenotype of hypersensitivity is consistent with the *Fmr1* KO mouse model of FXS [Bakker et al., 1994; Bernardet & Crusio, 2006; Mineur et al., 2006; Rotschafer & Razak, 2014]. Notably, *Fmr1* KO mice display abnormal responses to visual, tactile and auditory stimuli similarly to humans, providing a translationally relevant platform to study mechanisms of sensory deficits in humans with FXS [Rotschafer & Razak, 2014; He et al., 2017; Chen & Toth, 2001; Dölen et al., 2007; Arnett et al., 2014; Rais et al., 2018]. EEG recordings from humans with FXS have exhibited altered cortical oscillatory activity that may result in sensory hypersensitivity [Ethridge et al., 2017]. More specifically, increased broadband gamma frequency power was seen in humans with FXS compared to healthy controls [Wang et al., 2017]. When time varying auditory stimuli were used, there was a deficit in narrowband (~40 Hz) evoked gamma synchronization. There is also enhanced

amplitude and reduced habituation of auditory event related potentials (ERPs) in humans with FXS [Ethridge et al., 2019]. These results suggest elevated baseline cortical activity in FXS that disrupts the ability of cortical generators to synchronize their oscillations to time varying stimuli. Enhanced responses to repeated stimuli also indicate elevated ongoing cortical activity. Remarkably similar EEG phenotypes are also seen in the *Fmr1* KO mice: elevated broadband gamma power, reduced narrowband gamma synchronization, increased cortical responses to repeated stimuli, and a longer duration of responses to transient stimuli have been identified in the cortical activity of *Fmr1* KO mice [Rotschafer & Razak, 2013, 2014; Lovelace et al., 2018; Wen et al., 2019].Taken together, the similarities in sensory hypersensitivity behaviors and in sensory EEG phenotypes across humans and mice provide the basis for identification of robust biomarkers and mechanisms that predict impairments in sensory-cognitive functions in FXS.

Humans with FXS show speech deficits, language impairment and delayed language development [Rice et al., 2005; Martin et al., 2013]. Neurophysiology studies have revealed that both children and adults exhibit cortical deficits in auditory temporal processing, a phenotype that is consistent in *Fmr1* KO mice [Castrén et al., 2003; Knoth & Lippé, 2012; Rojas et al., 2001; St Clair et al., 1987; Van der Molen et al., 2012a; Van der Molen et al., 2012b]. More specifically, humans and *Fmr1* KO mice show deficits in both the auditory (AC) and frontal cortex (FC) to produce temporally precise responses to auditory stimuli [Ethridge et al., 2017; Wang et al., 2017; Lovelace et al., 2018]. How and when these auditory temporal processing deficits develop and whether they fluctuate throughout development is incompletely characterized in human and rodent models of FXS. An important finding across developmental studies suggests that many

phenotypes associated with FXS are either seen only transiently or show fluctuations during development [Razak et al., 2020]. These fluctuations throughout development make it difficult to establish consistent biomarkers for diagnoses and determine the optimal time windows for treatment. Preliminary evidence suggests that auditory temporal processing deficits may serve as a consistent biomarker for FXS, as *Fmr1* KO mice demonstrate abnormalities across development. My dissertation research aims to provide insight into critical aspects of FXS that are currently lacking, including identification of robust biomarkers and the optimal time window for treatment. Furthermore, this research will probe the limits of auditory temporal processing in *Fmr1* KO mice across development using EEG. Studying the developmental trajectory will provide insight into the mechanisms of pathophysiology as well as assist in the development of effective therapeutic approaches for optimal treatment.

Converging pathways: mTOR signaling and ASD

Although genetic linked forms of ASD arise as a consequence of mutations in genes with a wide array of molecular functions, the research shows convergence of these varying forms on a common biological pathway (Figure 1.1A). The PI3K/Akt/mTOR pathway is involved in a diverse array of cellular processes, including growth, proliferation, survival, and protein translation that requires a modified guanosine, termed a ‘cap’, at the 5’ end of the RNA [Huber et al., 2015]. Additionally, components of the mTOR pathway are located at synapses where they regulate both dendritic spine morphology and synaptogenesis [Sawicka & Zukin, 2012]. Disruption of this crucial pathway causes a variety of consequences including abnormal synapse formation, neuroinflammation, downregulation of protein synthesis at dendritic spines, and

increased total brain volume [Thomas et al., 2023; Khadem-Reza & Zare, 2022; Winden et al., 2018]. In mammals, mTOR is ubiquitously expressed in all cell types and is recognized as the master regulator of protein synthesis [Thomas et al., 2023].

The schematic in Figure 1.1B (Adapted from Thomas et al., 2023) shows the PI3K/Akt/mTOR pathway. The binding of growth factors to tyrosine kinase receptors activates the phosphoinositide 3-kinase (PI3K). This results in the phosphorylation of phosphatidylinositol-4,5-phosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3) [Weinberg, 2016]. The second messenger PIP3 recruits protein kinase B (Akt) and stimulates its phosphorylation. This phosphorylation causes the inhibition of the tuberous sclerosis complex (TSC) and the subsequent activation of mTORC1. Activation of mTORC1 leads to the phosphorylation of both S6 kinase (p70S6K) and 4EBP1 (eukaryotic initiation factor 4E-binding protein) [Galvin & Hart, 2016; Filomeni et al., 2015]. The S6 kinase activation stimulates protein translation and synthesis. 4EBP1 is bound to eukaryotic translation initiation factor 4E (eIF4E) and inhibits protein translation. Phosphorylation by mTORC1 releases eIF4E from 4EBP1

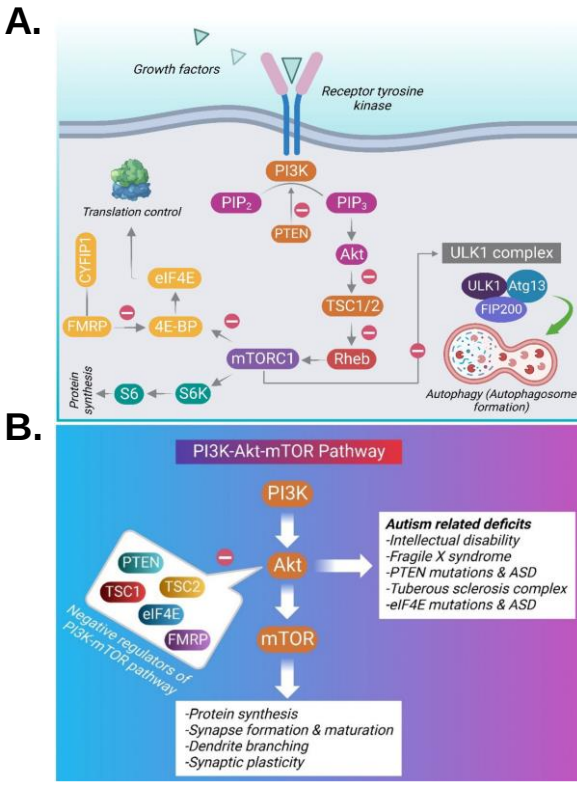

Figure 1.1. Converging pathways: mTOR signaling and ASD. (A) The PI3K/Akt/mTOR pathway. PTEN (upstream) and FMRP (downstream) are negative regulators of mTOR. (B) ASD-linked genes associated with pathway dysfunction. *Figure adapted from Thomas et al., 2023.*

and allows for translation [Chandran et al., 2013]. Activation of this pathway is necessary for the rapid translation of new proteins as well as the induction of translation-dependent long-term synaptic plasticity in response to Gq-coupled receptors, including the mGluR5 receptors [Huber et al., 2015; Hou & Klann, 2004; Volk et al., 2007].

Both PTEN and FMRP serve as negative regulators of this critical pathway. However, PTEN serves as an upstream antagonist while FMRP function is further downstream (Figure 1.1A). PTEN is a lipid phosphatase and de-phosphorylates PIP3 back to PIP2, inhibiting the PI3K/Akt/mTOR pathway from the start. Indeed, autistic behavioral deficits observed in PTEN mutants were reversed following treatment with rapamycin, an mTORC1 inhibitor, suggesting that upregulation of mTOR is impacting these behavioral abnormalities [Zhou et al., 2009]. Furthermore, Zhou et al. (2009) demonstrated that chronic administration of rapamycin prevented both macrocephaly and neuronal hypertrophy in PTEN KO mice. FMRP, on the other hand, works downstream of mTOR and binds to CYFIP1 (cytoplasmic FMRP-interacting protein 1). This will form the FMRP-CYFIP1 complex that inhibits translation by binding to eIF4E. Overactivated mTOR signaling is correlated with elevated cap-dependent translation and impaired synaptic plasticity in *Fmr1* KO mice [Sharma et al, 2010]. Furthermore, PIKE, an upstream activator of mTOR, is an identified target of FMRP [Darnell et al., 2011]. Loss of FMRP releases inhibition of PIKE and causes upregulated levels in KO mice [Gross et al., 2010; Sharma et al., 2010]. PIKE will then stimulate PI3K and subsequently cause the activation of mTOR signaling [Darnell et al, 2011]. Additionally, increased PTEN activity was also identified in *Fmr1* KO mice. Sharma et al. (2010) suggested that the increased PTEN activity may serve as feedback inhibition to compensate for the increased mTOR activity.

Although the current research focuses on PTEN and FMRP, abnormal PI3K/Akt/mTOR signaling has been identified in other ASD mouse models including those of tuberous sclerosis and Rett syndrome (Figure 1.1B) [Auerbach et al., 2011; Ricciardi et al., 2011]. The identification of key signaling pathways is critical for the development of ASD therapeutic targets and the consistency of mTOR dysfunction across ASD models is a promising approach to investigate further.

Characterizing developmental trajectories and sex differences in auditory processing in two different mouse models of ASD

The main goal of this research was to identify robust biomarkers of sensory processing disorders in two different models of ASD. These studies provide novel insights into the developmental trajectory of genotypic- and sex-dependent differences affecting auditory temporal processing in ASD. Additionally, this research utilizes targeted treatments to gain insight into potential therapeutics, critical windows of treatment and underlying mechanism. We recorded EEG responses from freely moving mice as this method is commonly used in humans and therefore makes the research and findings more translatable. Mice were implanted with epidural screw electrodes at various time points throughout development and we recorded brain activity as well as responses to a variety of stimuli designed to assess auditory system function.

Chapter 2: Developmental Delays in Cortical Auditory Temporal Processing in a Mouse Model of Fragile X Syndrome

We quantify auditory temporal processing throughout development in the *Fmr1* knock-out (KO) mouse model of Fragile X Syndrome (FXS), a leading genetic cause of intellectual disability and ASD-associated behaviors. Using epidural electrodes in awake

and freely moving wildtype (WT) and KO mice, we recorded auditory event related potentials (ERP) and auditory temporal processing with a gap-in-noise auditory steady state response (gap-ASSR) paradigm. Mice were recorded at three different ages in a cross-sectional design: postnatal (p)21, p30 and p60. We present evidence that the frontal (FC), but not auditory (AC), cortex shows significant temporal processing deficits at p21 and p30, with poor ability to phase lock to rapid gaps in noise. Temporal processing was similar in both genotypes in adult mice. ERP amplitudes were larger in *Fmr1* KO mice in both AC and FC, consistent with ERP data in humans with FXS.

Chapter 3: Sex Differences During Development in Cortical Temporal Processing and Event Related Potentials in Wild-Type and Fragile X Syndrome Model Mice

We compared the development of auditory temporal processing in male and female WT and *Fmr1* KO mice. Using epidural screw electrodes, we recorded auditory ERP and auditory temporal processing with the gap-in-noise ASSR paradigm at p21 and p30, and p60 ages from both AC and FC of awake, freely moving mice. The results show that ERP amplitudes were enhanced in both sexes of *Fmr1* KO mice across development compared to WT counterparts, with greater enhancement in adult female than adult male KO mice. Gap-ASSR deficits were seen in the FC, but not AC, in early development (p21) in female KO mice. Unlike male KO mice, female KO mice show WT-like temporal processing at p30. There were no temporal processing deficits in the adult mice of both sexes. Male KO mice show slower maturation of temporal processing than females. Female KO mice show stronger hypersensitive responses than males later in development.

Chapter 4: Developmental Trajectory and Sex Differences in Auditory Processing in a PTEN-deletion model of Autism Spectrum Disorders

We recorded epidural EEG signals from the FC and AC cortex in developing (p21 and p30) and adult Nse-cre PTEN mice. We quantified resting EEG spectral power distribution, auditory ERP and temporal processing from awake and freely moving male and female mice. Temporal processing is measured using the gap-in-noise-ASSR paradigm. Male and female KO mice have significantly increased beta power but decreased high frequency oscillations in the AC and FC. Both male and female PTEN KO mice show diminished gap-ASSR responses in the AC and FC compared to control mice. Overall, deficits become more prominent in adult (p60) mice, with KO mice having significantly increased sound evoked power and decreased ASSR responses compared to controls. While both male and female KO mice demonstrated severe temporal processing deficits across development, female KO mice showed increased hypersensitivity compared to males, reflected as increased N1 and P2 amplitudes.

Chapter 5: Impact of acute administration of an mGluR5 antagonist, CTEP, on cortical auditory processing in a PTEN-deletion model of Autism Spectrum Disorders

We aimed to determine whether the group 1 metabotropic receptor mGluR5 was involved in the mechanisms driving these phenotypes, as it has been implicated in several models of ASD, including the Nse-cre *Pten* deletion model. We acutely treated adult PTEN KO mice with CTEP, a specific mGluR5 antagonist, and recorded epidural EEG signals from the FC and AC. We quantified resting EEG spectral power distribution, auditory ERP and temporal processing from awake and freely moving vehicle and CTEP treated male and female mice. The results show that CTEP treatment shifts the resting power distribution in males, such that CTEP treated mice have decreased alpha power

and increased gamma power in the AC compared to vehicle treated males. CTEP treatment showed no significant impact on auditory temporal processing in either sex or cortical region. We also found no treatment effect on ERP amplitudes in males or females. Non-phase locked single trial power is significantly elevated in the AC and FC of CTEP treated females but not males, suggesting a sex specific increase in ‘on-going background activity’ following CTEP treatment.

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Chapter 2

Developmental Delays in Cortical Auditory Temporal Processing in a Mouse Model of Fragile X Syndrome

Abstract

Autism spectrum disorders (ASD) encompass a wide array of debilitating symptoms, including sensory dysfunction and delayed language development. Auditory temporal processing is crucial for speech perception and language development. Abnormal development of temporal processing may account for the language impairments associated with ASD. Very little is known about the development of temporal processing in any animal model of ASD. In the current study, we quantify auditory temporal processing throughout development in the *Fmr1* knock-out (KO) mouse model of Fragile X Syndrome (FXS), a leading genetic cause of intellectual disability and ASD-associated behaviors. Using epidural electrodes in awake and freely moving wildtype (WT) and KO mice, we recorded auditory event related potentials (ERP) and auditory temporal processing with a gap-in-noise auditory steady state response (gap-ASSR) paradigm. Mice were recorded at three different ages in a cross-sectional design: postnatal (p)21, p30 and p60. Recordings were obtained from both auditory and frontal cortices. The gap-ASSR requires underlying neural generators to synchronize responses to gaps of different widths embedded in noise, providing an objective measure of temporal processing across genotypes and age groups. We present evidence that the frontal, but not auditory, cortex shows significant temporal processing deficits at p21 and p30, with poor ability to phase lock to rapid gaps in noise. Temporal processing was similar in both genotypes in adult mice. ERP amplitudes were larger in *Fmr1* KO mice in both auditory

and frontal cortex, consistent with ERP data in humans with FXS. These data indicate cortical region-specific delays in temporal processing development in *Fmr1* KO mice. Developmental delays in the ability of frontal cortex to follow rapid changes in sounds may shape language delays in FXS, and more broadly in ASD.

Introduction

Auditory temporal and spectral modulation cues shape speech recognition [Zeng et al., 2005; Shannon et al., 1995]. Ability to discriminate temporal fine structure is critical for speech processing [Moore, 2008], and the ability to encode subtle differences in temporal modulations is present from a very young age in humans [Cabrera & Gervain et al., 2020]. The inability of the auditory system to process rapidly changing acoustic input during development may disrupt perception of speech, phonological processing and cause language impairments [Tallal et al., 1998]. Temporal processing acuity in infancy predicts language development in ~2 yr old children [Trehub & Henderson, 1996]. Abnormal sensory processing and language development is consistently reported in children with autism spectrum disorders (ASD) [Abbeduto & Hagerman, 1997, Foss-Feig et al., 2017; Tager-Flusberg & Caronna, 2007; Rain & Dunn, 2003; Jeste & Nelson, 2008]. Individuals with ASD show deficits in detection of sound duration, onset and offset, and rapid changes in spectrotemporal properties [Kwakye et al., 2001; Lepistö et al., 2006; Oram Cardy et al., 2005; Orekhova et al., 2009; Meilleur et al., 2020]. Children with ASD show difficulties reproducing the lengths of auditory stimuli, and both children and adults with ASD produce abnormal neural responses to fluctuations in pitch of repeated, sequential auditory stimuli [Szelag et al., 2004; Tecchio et al., 2003; Gomot et al., 2006]. Increased gap-detection thresholds, a paradigm commonly used to assess

auditory temporal processing, are seen in humans with ASD. Notably, impaired gap detection thresholds in children were associated with lower phonological processing scores [Foss-Feig et al., 2017]. These studies in humans provide support for the hypothesis that auditory temporal processing deficits may shape abnormal speech and language function in ASD. A link between abnormal temporal processing and developmental dyslexia has also been proposed, suggesting broader consequences in development [Farmer et al., 1995].

While speech and language function cannot be directly studied in animal models, temporal processing can be quantified. However, the developmental trajectory and underlying neural mechanisms of temporal processing deficits in neurodevelopmental disorders remain unclear and would require a translation-relevant animal model. Identifying when temporal processing deficits arise is critical for determining optimal treatment windows for potential therapeutic tests in pre-clinical models and in clinical studies. Here we present a novel method to assess rapid gap-in-noise temporal processing using EEG recordings in an ASD model mouse, which can be translated relatively easily to humans, and we show robust cortical region-specific developmental delays in auditory temporal processing.

Fragile X syndrome (FXS) is a leading cause of inherited intellectual deficits and ASD-associated behaviors such as repetitive behaviors, sensory, cognitive and social impairments [Wisniewski et al., 1991; Cordeiro et al., 2010; Van der Molen et al., 2010; Oakes et al., 2015; Miller et al., 1999]. Humans with FXS show speech deficits and language impairments [Abbeduto et al., 2007; Abbeduto & Hagerman, 1997; Finestack et al., 2009; Rice et al., 2005]. FXS affects up to 1 in 4000/7000 male/female individuals, respectively, and results from the silencing of the Fragile X Messenger

Ribonucleoprotein (*Fmr1*) gene on the X chromosome [Hall et al., 2009; Wang et al., 2017]. This leads to a partial or complete loss of the Fragile X Messenger Ribonucleoprotein (FMRP) and consequent alterations in synaptic development and plasticity in the brain [Darnell et al., 2011; Huber et al., 2002]. Clinical, behavioral and electrophysiological studies have demonstrated sensory hypersensitivity in humans with FXS across multiple domains [Rogers et al., 2003; Crane et al., 2009; Marco et al., 2011; Takarae et al., 2016; Ethridge et al., 2016; Miller et al., 1999].

Sensory hypersensitivity is also consistently seen in the *Fmr1* KO mouse model of FXS [Bakker et al., 1994; Bernardet & Crusio, 2006; Mineur et al., 2006; Rotschafer & Razak, 2014]. Notably, *Fmr1* KO mice display abnormal responses to auditory stimuli similar to humans, providing a translational platform to study developmental profiles and neural mechanisms of sensory circuit pathophysiology [Razak et al., 2021]. EEG recordings from humans with FXS show altered cortical oscillatory activity that may result in sensory hypersensitivity [Ethridge et al., 2017]. More specifically, increased broadband gamma frequency power was seen in humans with FXS compared to healthy controls [Wang et al., 2017]. When time-varying auditory stimuli were used, there was a deficit in narrowband (~40 Hz) evoked gamma synchronization. There is also enhanced amplitude and reduced habituation of auditory ERPs in humans with FXS [Ethridge et al., 2019]. These results suggest elevated baseline cortical activity in FXS that disrupts the ability of cortical generators to synchronize their oscillations to dynamic stimuli. Enhanced responses to repeated stimuli also indicate elevated ongoing cortical activity. Similar EEG phenotypes are seen in the *Fmr1* KO mice: elevated broadband gamma power, reduced narrowband gamma synchronization and increased cortical responses to repeated stimuli have been identified in *Fmr1* KO mice [Rotschafer & Razak, 2013,

2014; Lovelace et al., 2018; Wen et al., 2019; Jonak et al., 2020]. Taken together, the similarities in sensory hypersensitivity behaviors and in EEG phenotypes across humans and mice indicates that the *Fmr1* KO mouse is a useful model to address sensory dysfunction in FXS.

Developmental abnormalities in cell size and expression of synaptic markers are seen in the auditory brainstem of the *Fmr1* KO mice, a region strongly implicated in high resolution temporal processing [Rotschafer & Cramer, 2017]. This suggests that auditory temporal processing abnormalities may emerge early in development. However, the development of temporal processing has not been studied in the *Fmr1* KO mice, or indeed in any animal model of ASD. The current study tested the hypothesis that both cortical temporal processing and auditory sensitivity deficits are present in the *Fmr1* KO mice from early development. We recorded EEG signals from both the auditory and frontal cortex (AC, FC) in *Fmr1* KO and wildtype (WT) mice at three ages: p21, p30 and p60. To quantify temporal processing fidelity, we utilized a 40 Hz gap-in-noise ASSR (auditory steady state response, henceforth, gap-ASSR) paradigm to assess the cortex's ability to consistently respond to brief gaps in noise at varying modulation depths [Rumschlag & Razak, 2021]. Gap stimuli have been used widely in both humans and mice to study auditory temporal acuity, and EEG recordings can be conducted in humans and mice relatively more easily than single unit recordings [Green, 1971; Anderson & Linden, 2016]. Children with autism show reduced ability to integrate information present in temporal gaps in background sound, providing additional rationale to use gaps-in-noise stimuli to evaluate temporal processing [Groen et al., 2009]. Regional differences in cortical phenotypes are present in *Fmr1* KO mice. In particular, multiple mouse model studies suggest auditory temporal processing abnormalities may

be larger in the FC than AC. For example, Wieczerzak et al. reported reduced recovery of ASSR in FC, compared to AC, following noise induced hearing loss [Wieczerzak et al., 2021]. Lovelace et al. reported a deficit in ASSR in the FC, but not AC, of the adult *Fmr1* KO mouse [Lovelace et al., 2020]. Temporal processing impairments are seen in an auto-immune disorder mouse model with neocortical ectopias in the FC [Clark et al., 2000]. It is not known if these regional differences in any mouse model are present across development, or only at specific ages. Therefore, we compared FC and AC in terms of temporal processing across 3 different ages. Additionally, we assessed the response magnitude of auditory ERP, as they are well characterized in humans with FXS and consistently show enhanced amplitudes. We hypothesized that *Fmr1* KO mice would show a deficit in auditory temporal processing and increased ERP amplitudes compared to WT in both the AC and FC across all 3 developmental time points.

Methods

Mice: All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. Mice were obtained from an in-house breeding colony that originated from Jackson Laboratory (Bar Harbor, ME). The mice used for the study are sighted FVB wild-type (Jax, stock# 004828; WT) and sighted FVB *Fmr1* knock-out (Jax, stock# 004624; *Fmr1* KO). The FVB background strain was chosen (and not C57bl6/J) because our prior developmental work examining cortical parvalbumin and perineuronal nets as well as single unit responses in the auditory cortex and the inferior colliculus have used the FVB strain [Rotschafer & Razak, 2013; Nguyen et al., 2020]. Significant developmental deficits were observed in *Fmr1* KO mice in the FVB strain of mice, predicting temporal processing deficits.

One to five mice were housed in each cage under a 12:12-h light-dark cycle and fed ad libitum. A cross-sectional, as opposed to a longitudinal, design was used in this study as it is impractical to place epidural screw electrodes in brains and skulls that are still developing. The following age ranges and sample sizes were used in this study: WT [p21 (n=10), p30 (n=10), p60 (n=11)] and *Fmr1* KO [p21 (n=10), p30 (n=10), p60 (n=11)]. The ages were selected for this study based on previous findings. Decreased PNN expression surrounding parvalbumin-positive interneurons and cortical hyperexcitability are observed in *Fmr1* KO mice at p21 [Wen et al., 2018]. Additionally, the p14-21 age corresponds to the critical period for responses to simple tones and maturation of tonotopic maps in the auditory cortex [Oswald & Reyes, 2010; Kim et al., 2013]. P30 was chosen because response selectivity to complex sounds has not matured in the auditory cortex until this age [Carrasco et al., 2013]. We chose p60 age group to represent young adulthood. Only male mice were studied.

Surgery: Different groups of mice underwent epidural electrode implant surgery at three developmental timepoints: p18-20, p27-p29, p57-p66. Mice were anesthetized using intraperitoneal (i.p.) injections of either 80/20 mg/kg of ketamine/xylazine (young mice) or 80/10/1 mg/kg ketamine/xylazine/acepromazine (adult mice). The anesthetic state was monitored closely throughout the procedure by toe pinch reflex every 10-15 minutes. Ketamine supplements were given if necessary. ETHIQA-XR (1-shot buprenorphine, 3.25 mg/kg body weight) was administered via subcutaneous injection prior to surgery. Following the removal of fur and skin, and sterilization (alcohol and iodine wipes) of the scalp, an incision was made to expose the scalp. A Foredom dental drill was used to drill ~1mm diameter holes in the skull over the right AC, right FC, and left occipital cortex. The screw positions were determined using skull landmarks and

coordinates previously reported [Wen et al., 2019; Rumschlag & Razak, 2021; Lovelace et al., 2020; Rumschlag et al., 2020] and were based on single unit mapping [Lovelace et al., 2020; Rumschlag et al., 2020; Trujillo et al., 2011; Rumschlag & Razak, 2021; Wen et al., 2019; Rotschafer & Razak, 2014]. The wires extending from three-channel posts were wrapped around 1 mm screws and driven into the pre-drilled holes. Dental cement was applied around the screws, on the base of the post, and exposed skull, to secure the implant. Mice were placed on a heating pad until fully awake and were allowed 48-72 hours for recovery before EEG recordings were made.

EEG recordings: All EEG recordings were obtained from awake and freely moving mice. EEG recordings were performed at three developmental time points: p20-23, p29-31, p59-p70, which we refer to as p21, p30 and p60, respectively. Recordings were obtained from the AC and FC electrodes, using the occipital screw as reference. Mice were placed in an arena where they could move freely during the recording. The arena was inside a Faraday cage placed on a vibration isolation table in a sound-insulated and anechoic booth (Gretch-Ken, OR). Mice were attached to an EEG cable via the implanted post under brief anesthesia with isoflurane. The EEG recording set-up has been previously reported [Rumschlag & Razak, 2021; Rumschlag et al., 2020]. Briefly, the attached cable was connected via a commutator to a TDT (Tucker Davis Technologies, FL) RA4LI/RA4PA headstage/pre-amp, which was connected to a TDT RZ6 multi-I/O processor. OpenEx (TDT) was used to simultaneously record EEG signals and operate the LED light used to synchronize the video and waveform data. TTL pulses were utilized to mark stimulus onsets on a separate channel in the collected EEG data. The EEG signals were recorded at a sampling rate of 24.414 kHz and down-sampled to 1024 Hz for analysis. All raw EEG recordings were visually examined prior to analysis for

artifacts, including loss of signal or signs of clipping, but none were seen. Therefore, no EEG data was rejected. Sound evoked EEGs were recorded as follows.

Auditory ERP: After a 25 minute habituation to the recording arena with no stimuli, narrowband noise pulses (6-12 kHz) were presented at 75 dB SPL (120 repetitions, 100 ms duration, 5ms rise/fall time, 0.25 Hz repetition rate) using a speaker (MF1, Tucker Davis Technologies, FL) situated 20 cm above the floor of the arena. ERP analysis and statistics have been previously described [Rumschlag & Razak, 2021; Rumschlag et al., 2020]. Briefly, the EEG trace was split into trials, using the TTL pulses to mark sound onset. Each trial was baseline corrected, such that the mean of the 250 ms baseline period prior to sound onset was subtracted from the trial trace for each trial. Each trial was then detrended (MATLAB detrend function) and all trials were averaged together. Time-frequency analysis was performed with a dynamic complex Morlet wavelet transform with Gabor normalization. The wavelet parameter was set for each frequency to optimize time-frequency resolution. The non-baseline normalized single trial power (STP) does not correct for mean baseline power levels, allowing for the identification of ongoing 'background activity' during stimulus presentation. To compare the responses across genotype at each developmental time point, a non-parametric permutation test was used, to find clusters of significant values [Maris & Oostenveld, 2007]. First, a t-test was run on each time-frequency point for the two groups being compared, yielding the T-values for all points. T-values corresponding to $p<0.025$ were considered significant. Clusters of significant T-values were found and their area was measured. Next, the group assignments were shuffled randomly, and the t-tests and cluster-measurements were run again on the surrogate groups. This surrogate analysis was performed 2000 times to generate a distribution of cluster sizes that we would expect to find by chance.

Originally identified clusters that were larger than 95% of the surrogate clusters were considered significant. This method allows for the identification of significant differences between groups without performing excessive comparisons.

Gap-ASSR: The stimulus used to assess auditory temporal processing is termed the ’40 Hz gaps-in-noise ASSR’ (auditory steady state response, henceforth, ‘gap-ASSR’) [Rumschlag & Razak, 2021]. The stimulus contains alternating 250 ms segments of noise and gap interrupted noise presented at 75 dB SPL. The gaps are strategically placed 25 ms apart, resulting in a presentation rate of 40 Hz, a rate that produces the strongest ASSR signal when measured from the AC and frontal regions and may reflect the resonance frequency of the underlying neural circuits [Galambos et al., 1981; Pastor et al., 2002; Llinás, 1988; Llinás et al., 1991; Rosanova et al., 2009; Kim et al., 2015; Hwang et al., 2019]. For each gap in noise segment, the gap width and modulation depth are chosen at random. Gaps of 2, 4, 6, 8, 10, or 12 ms widths and modulation depths of 75 and 100% were used. To measure the ability of the cortex to consistently respond to the gaps in noise, inter-trial phase clustering (ITPC) at 40 Hz was measured [Cohen, 2014]. The EEG trace was transformed using a dynamic complex Morlet wavelet transform. The trials corresponding to each parametric pair (gap duration + modulation depth) were grouped together. The ITPC was calculated for each time-frequency point as the average vector for each of the phase unit vectors recorded across trials (trial count >100 trials per parametric pair). The ITPC values at 40 Hz were averaged to extract the mean ITPC for the parametric pairs in the AC and FC.

Statistics: Statistics were performed on GraphPad Prism (ERP) or R (gap-ASSR). To evaluate the effects of genotype (2 levels) and age (3 levels), two-way ANOVA was used for ERP analysis. Post hoc comparisons were carried out with Tukey’s and Bonferroni’s

multiple comparisons test. The ERP data was tested for normality using Shapiro-Wilk test. A three-way repeated measures ANOVA was used for gap-ASSR analysis, with the three factors being genotype (2 levels) X age (3 levels) X gap duration (6 levels). A repeated measures ANOVA was chosen as multiple gap duration data points were collected from a single mouse in a recording session. Mauchly Tests for Sphericity were utilized and corrected for using the Greenhouse-Geisser corrections if necessary. Post hoc contrasts with Sidak corrections for multiple comparisons were used. Cortical regions (AC, FC) and modulation depths (75%, 100%) were analyzed separately. We evaluated the appropriateness of the data for analysis via ANOVA, in particular the assumption of the normality of the residuals. None of the residuals had measures of skewness or kurtosis that exceeded ±2, which is one indication of acceptable normality [West et al., 1995]. Moreover, the residuals were evaluated via quantile-quantile plots. In each of the analyses, the correspondence between the theoretical normal distribution and the obtained residuals was within acceptable bounds.

Results

The main goal of this study was to compare the developmental trajectory of auditory temporal processing and ERPs in WT and *Fmr1* KO mouse auditory and frontal cortex. We predicted that *Fmr1* KO mice would show a deficit in phase locking to rapid gaps in noise and larger ERP amplitudes compared to WT mice across all 3 ages in both AC and FC as markers of temporal processing and hypersensitivity phenotypes, respectively, in FXS.

Abnormal temporal processing is seen in the FC, but not AC, during development

Auditory temporal processing was assessed using a 40 Hz gap-in-noise ASSR stimulus to probe the limits of the auditory and frontal cortices' ability to consistently respond to brief gaps in noise. Decreasing the duration and modulation depth of the gaps reduces the likelihood of consistent response from the cortex, allowing for the detection of deviations between WT and KO mice responses and to track developmental changes. Both AC and FC in mice and humans produce robust 40 Hz ITPC to this type of stimulus, but how the response develops is not known in either species, nor is it known if there is a deficit in FXS [Rumschlag & Razak, 2021; Hwang et al., 2019; Lustenberger et al., 2018].

Figure 2.1 shows gap-ASSR heat maps of ITPC in example WT (Figure 2.1A, C) and *Fmr1* KO (Figure 2.1B, D) mice. In the AC, at p21, or in both AC and FC at p60, there are no clear qualitative differences in the ITPC. However, deficits are clearly seen in the FC at p21, with the KO ITPC barely emerging above background at 40 Hz. Table 2.1 and Figure 2.2 shows the results of full statistical analyses using gap duration, age and genotype as factors.

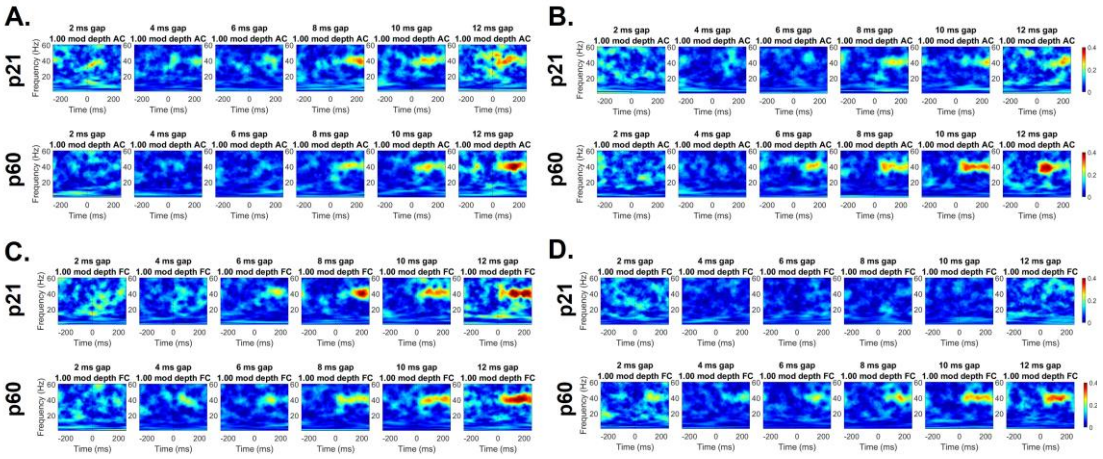

Figure 2.1. Abnormal auditory temporal processing during development in the *Fmr1* KO mice. Individual example heatmaps of ITPC generated at 40Hz at multiple gap durations in p21 and p60 WT (A: AC, C: FC) and *Fmr1* KO (B: AC, D: FC) mice. Qualitative observations of these examples show deficits in cortical temporal processing at p21, but not at p60, in the KO mice. All panels show 100% modulation depth. The onset of the gap-ASSR stimulus is at 0 msec in each panel.

Auditory Cortex

Gap-ASSR ITPC is significantly impacted by gap duration in the AC at both modulation depths. This is expected because it is easier for neural generators to phase lock responses to long gaps compared to short. There was no main effect of age in the AC at either 75% or 100% modulation, but there is an interaction of gap duration x age at 75%, suggesting that ITPC improves with age for longer gaps. Importantly, the genotype comparisons were not significant at any age or modulation depth in the AC (Figure 2.2, 75% modulation – p21: p=0.9223, p30: p=0.9568, p60: p=1.000; 100% modulation – p21: p=0.8664, p30: p=0.6906, p60: p=1.000). Taken together, these data suggest developmental improvement in temporal processing, but no effects of the loss of FMRP, in the auditory cortex at any age.

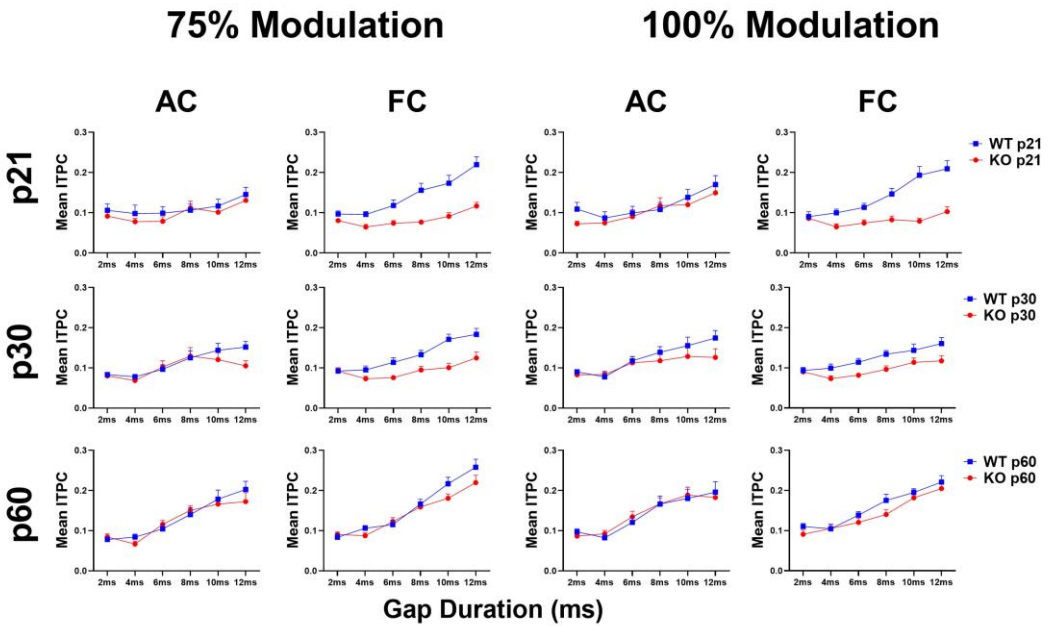

Figure 2.2. Population analysis shows temporal processing deficits in the FC during development in *Fmr1* KO mice. Each plot represents the group average ITPC values. Each row represents a different age group: p21 (top), p30 (middle), and p60 (bottom). The left columns represent AC and FC data at 75% modulation depth and the right columns represent AC and FC data at 100% modulation depth. ITPC increases with gap widths in both genotypes, as expected. *Fmr1* KO mice show significant deficits in the FC, but not the AC, at p21 and p30. Full data analysis is shown in Table 2.1.

Frontal Cortex

Similar to the AC, the frontal cortex showed main effects of gap duration, as expected. However, in contrast to the AC, FC gap-ASSR showed main effects of both age and genotype (Figure 2.2) and a number of relevant interactions (Table 2.1). At both modulation depths, FC responses showed improvement with age indicating a strong developmental regulation of temporal processing in this region. At both modulation depths, *Fmr1* KO neurons showed significant deficits in ITPC compared to WT mice. The genotype X age interactions suggest a delay in ITPC development with adult FC showing no significant deficits. These results indicate a significant delay in the development of temporal processing in the *Fmr1* KO mice.

Evidence of a developmental delay in the FC is shown more directly by collapsing across gaps (Figure 2.3). When collapsed across gap durations, KO mice show a significant ITPC deficit at p21 and p30 in the FC at both modulation depths that is not seen at p60 (75% modulation – p21: $p<0.0001$, p30: $p=0.0022$, p60: $p=0.8372$; 100% modulation – p21: $p<0.0001$, p30: $p=0.0548$, p60: $p=0.6410$). Taken together, these data show improvement in phase locking to gap-ASSR stimuli with development in both AC and FC, and an FC-specific delay in temporal processing in *Fmr1* KO mice.

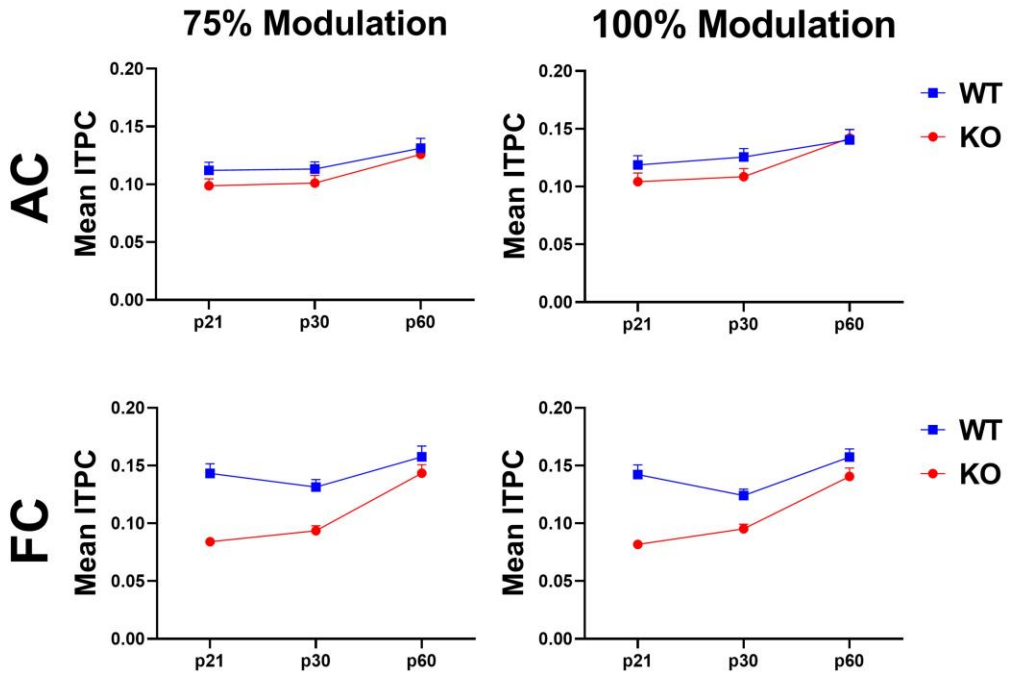

Figure 2.3. Auditory temporal processing improves with age in AC and FC, with developmental delay in the FC. Each plot represents the group average ITPC values collapsed across gap widths. Columns represent different modulation depths and rows represent different cortical regions (Columns – left = 75% modulation, right = 100% modulation; Rows – top = AC, bottom = FC). KO mice show a significant ITPC deficit at p21 and p30 in the FC at both modulation depths, but not at p60. A genotype difference was not seen at any age or modulation depth in the AC.

Table 2.1. Full statistical analysis of gap-ASSR data.

Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
AC	100%	Genotype	F(1,56)=0.8631	0.3568
		Age	F(2,56)=2.5463	0.0874
		Gap Duration	**F(5,280)=24.0444**	**<0.0001**
		Genotype x Gap Duration	F(5,280)=1.5849	0.1986
		Age x Gap Duration	F(10,280)=1.7898	0.1106
		Genotype x Age	F(2,56)=0.3026	0.7401
		Genotype x Age x Gap Duration	F(10,280)=0.6989	0.6392
AC	75%	Genotype	F(1,56)=1.1929	0.2794
		Age	F(2,56)=1.7843	0.1773
		Gap Duration	**F(5,280)=21.2106**	**<0.0001**
		Genotype x Gap Duration	F(5,280)=2.5458	0.0571
		Age x Gap Duration	**F(10,280)=3.2468**	**0.0046**
		Genotype x Age	F(2,56)=0.0719	0.9307
		Genotype x Age x Gap Duration	F(10,280)=0.5839	0.7444
FC	100%	**Genotype**	**F(1,56)=23.7897**	**<0.0001**
		Age	**F(2,56)=12.3904**	**<0.0001**
		Gap Duration	**F(5,280)=20.6491**	**<0.0001**
		Genotype x Gap Duration	**F(5,280)=6.6448**	**<0.0001**
		Age x Gap Duration	**F(10,280)=5.3246**	**<0.0001**
		Genotype x Age	**F(2,56)=3.2364**	**0.0467**
		Genotype x Age x Gap Duration	**F(10,280)=3.5686**	**0.00096**
FC	75%	**Genotype**	**F(1,56)=31.7872**	**<0.0001**
		Age	**F(2,56)=16.0560**	**<0.0001**
		Gap Duration	**F(5,280)=29.1751**	**<0.0001**
		Genotype x Gap Duration	**F(5,280)=10.6887**	**<0.0001**
		Age x Gap Duration	**F(10,280)=5.7920**	**<0.0001**
		Genotype x Age	**F(2,56)=3.9569**	**0.0247**
		Genotype x Age x Gap Duration	F(10,280)=1.0704	0.3839

Three-way repeated measures ANOVA results for gap-ASSR analysis. Mauchly Tests for Sphericity were utilized and p-values were corrected for multiple comparisons using the Greenhouse-Geisser corrections if necessary. See text for post hoc results. Bold text indicates statistical significance (p = or < 0.05).

Development of gap-ASSR single trial power phenotypes in the Fmr1 KO mice

It Is possible that the reduced ITPC in developing *Fmr1* KO mouse FC arises from stimulus induced increases in non-phase locked activity (background noise) as suggested in humans with FXS [Ethridge et al., 2017, 2019]. Therefore, we examined the single trial power (STP) during gap-ASSR stimulation across development and genotypes (Figures 2.4, 2.5 and 2.6). At p21, there was no difference in STP across any of the gaps or cortical regions (Figure 2.4). However, at p30, there was a significant elevation of STP in the KO, compared to the WT mice, and this was seen in both cortical regions (Figure 2.5). The elevation in STP affected gamma band frequencies (25-80 Hz), with no differences in lower frequencies. In the adult group, the direction of STP differences was reversed in the AC, such that the *Fmr1* KO mice showed reduced STP, significantly affecting frequencies <25 Hz (Figure 2.6). However, there were no STP

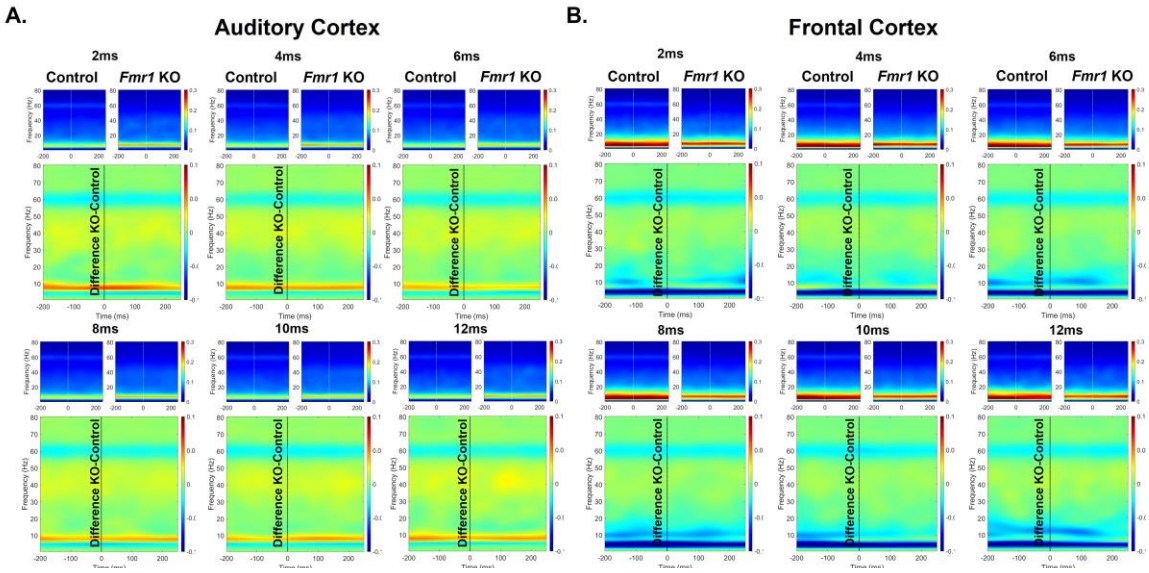

Figure 2.4. No genotype difference in single trial power (STP) of p21 mice during gap-ASSR stimulation. The heatmaps show non-baseline corrected normalized power, where red hues represent increased ongoing background activity, and blue hues represent a decrease. The smaller panels show group average STP at each gap width in WT and *Fmr1* KO mice. The larger panels show the difference between KO and WT. No significant differences were found in STP during the gap-ASSR stimulus in (A) AC or (B) FC at p21.

differences in the FC. These data provide evidence for fluctuating single trial power gap-ASSR phenotypes through development. The lack of concurrence between the STP deficits and the gap-ASSR deficits across both cortical region and age indicates that the temporal processing deficit is not due to sound-induced increases in ongoing background activity.

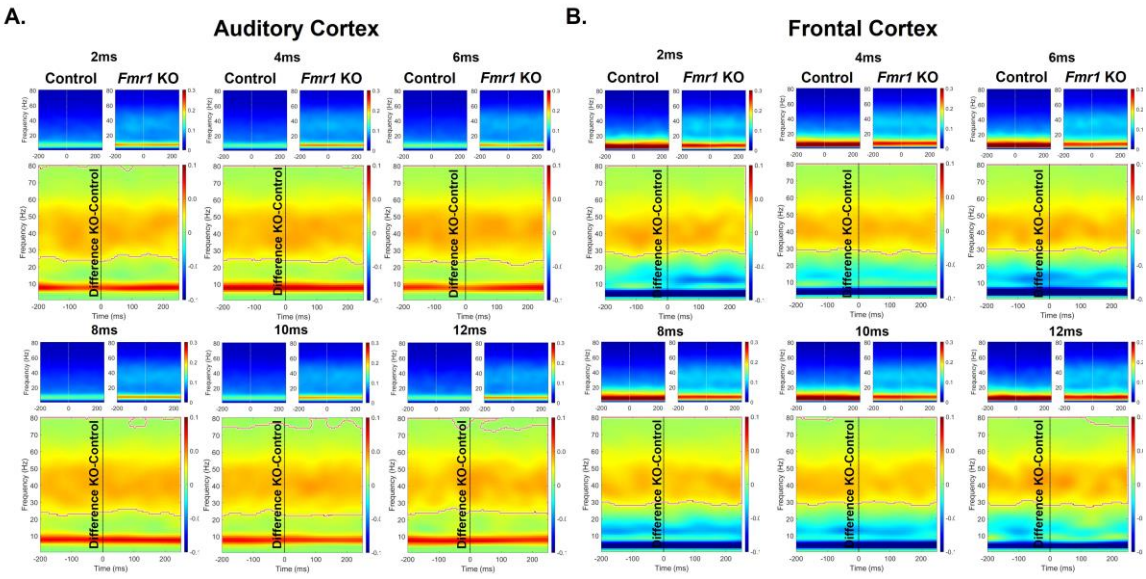

Figure 2.5. Significant elevation of STP in p30 *Fmr1* KO mice during gap-ASSR stimulation. The format of this figure is identical to that of Figure 2.4. Significant differences between genotypes were found using a non-parametric permutation testing approach (see methods). Outlined regions (typically between 25-80 Hz) indicate clusters which are significantly different between WT and KO. *Fmr1* KO mice have increased gamma STP during the gap-ASSR stimulus in (A) AC and (B) FC at p30.

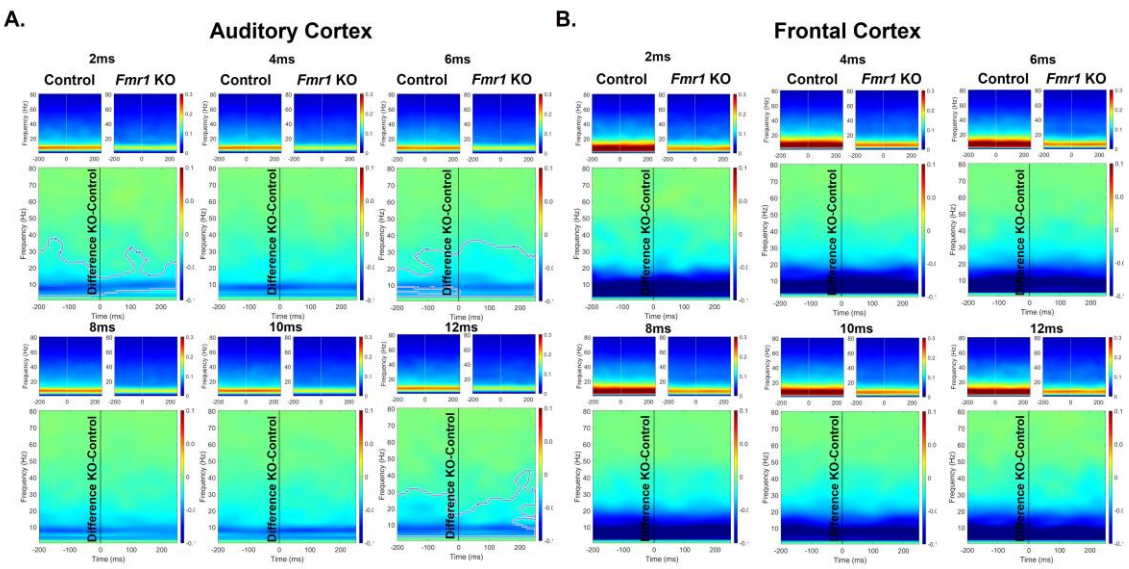

Figure 2.6. No difference or decreased STP in p60 *Fmr1* KO mice during gap-ASSR stimulation. The format of this figure is identical to that of Figure 2.4 and 2.5. Outlined regions (typically <30 Hz) indicate clusters which are significantly different between WT and KO. *Fmr1* KO mice show decreased STP at some gaps during the gap-ASSR stimulus in (A) AC but show no significant clusters in (B) FC at p60. Unlike at p30, there was no increase in STP in KO mice.

Fmr1 KO mice show enhanced ERP amplitude in the AC and FC across development

ERPs consist of a series of voltage fluctuations, referred to as 'waves' (P1, N1, P2), which are evoked at specific latencies after sound onset. Each of the waveforms are associated with the population activity in specific brain regions. Measuring the amplitudes and latencies of these waves allow for the assessment of response synchrony or hypersensitivity to sound presentation. We also characterized non-baseline normalized STP in response to narrowband noise bursts as abnormal power has been identified in humans with FXS during auditory stimulus presentation [Ethridge et al., 2017, 2019]. Table 2.2 and Figures 2.7 and 2.8 show the complete ANOVA analyses of

ERP data across development and genotypes. The major results in the two cortical regions are highlighted below.

Auditory Cortex

ERP P1 amplitude in the AC significantly increases in *Fmr1* KO mice with development (interaction effect: p=0.0002; main effect of age: p=0.0005; KO p21-p60: p<0.0001; KO p30-p60: p=0.0001). These mice also have significantly higher P1 amplitudes compared to WT at p60 (main effect of genotype: p=0.0113; post hoc: p<0.0001). We found a main effect of genotype on N1 amplitudes. Additionally, we report a significant main effect of age on P2 amplitude (p=0.0575). P1 latencies are impacted by age specifically in WT mice, with latencies decreasing with age (main effect of age: p=0.0053; WT p21-p60: p=0.0537). These data show increased ERP amplitudes in the AC of *Fmr1* KO mice as observed consistently in humans with FXS, but indicate early emergence of hypersensitivity.

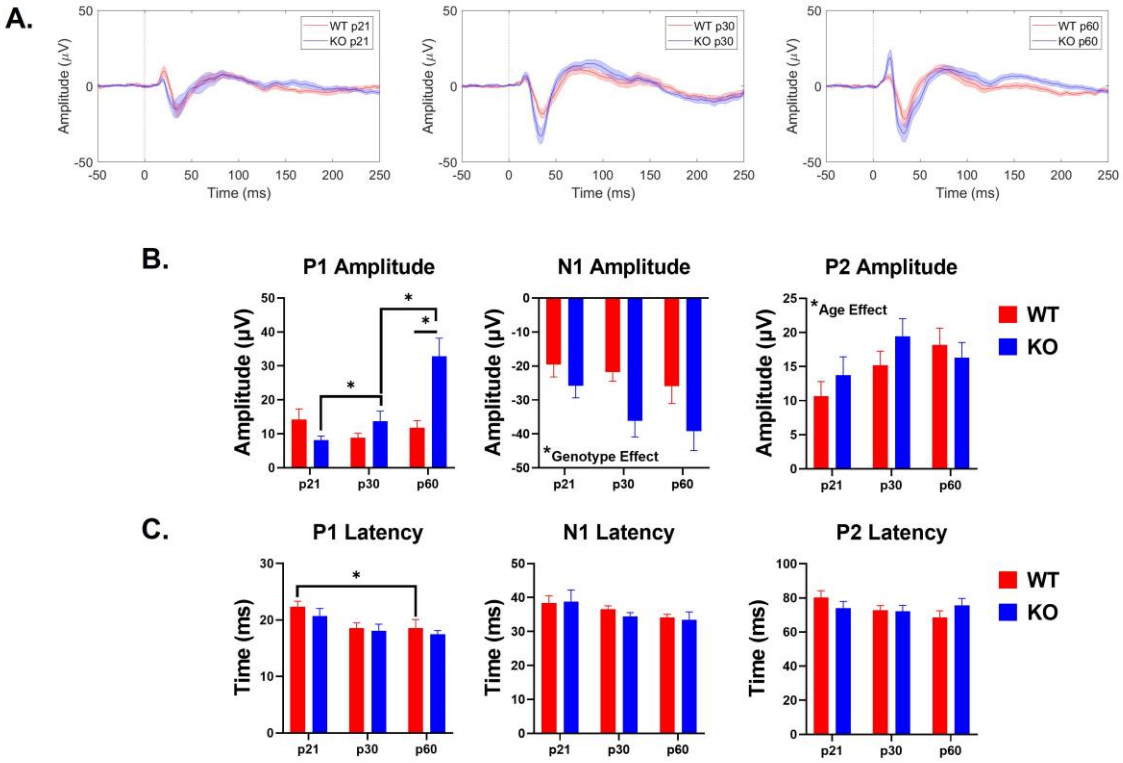

Figure 2.7. Age and genotype impact ERP amplitudes and latencies in the AC. (A) Average ERPs recorded in the AC for WT and KO mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of AC ERP wave amplitudes. P1 amplitude significantly increases in KO mice with development, but not WT mice. Adult KO mice have increased P1 amplitudes compared to WT. Genotype impacts N1 amplitudes. P2 amplitude are affected by age, but not genotype. (C) AC ERP wave latencies. P1 latency decreases with age in WT mice. Full analysis is shown in Table 2.2.

Frontal Cortex

Similar to the AC, *Fmr1* KO mice show a significant increase in P1 amplitude with development in the FC (interaction effect: p=0.034). N1 amplitudes were increased significantly in adult *Fmr1* KO mice (main effect of genotype: p=0.0031; WT-KO p60: p=0.0251). Additionally, P2 amplitudes decrease with age in KO mice (main effect of age: p=0.0159; KO p30-p60: p=0.0510). P2 latency was slower in adult *Fmr1* KO mice

(main effect of age: p=0.0045; WT-KO p60: p=0.0030). These data indicate that *Fmr1* KO mice have abnormally elevated N1/P1 ERP amplitudes in the frontal cortex.

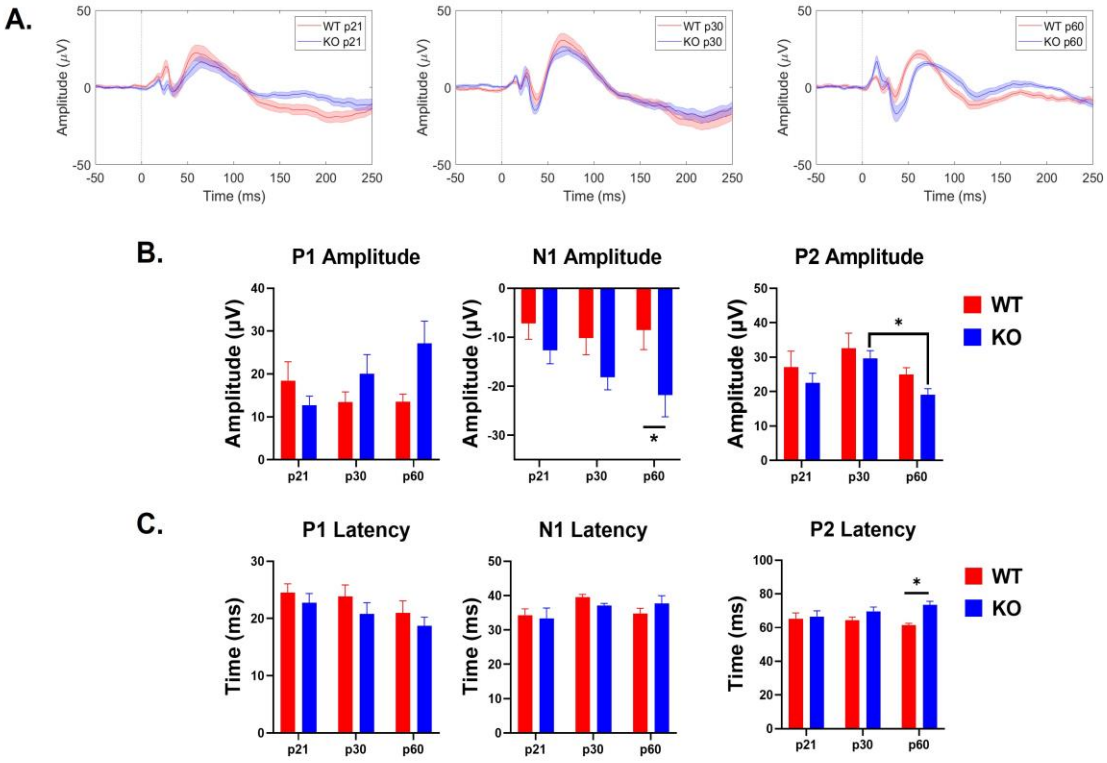

Figure 2.8. Age and genotype impact ERP amplitudes and latencies in the FC. (A) Average ERPs recorded from the FC for WT and KO mice at p21 (left), p30 (middle), and p60 (right). (B) FC ERP wave amplitudes. KO mice show a significant increase in P1 amplitude with development. N1 amplitudes are increased in adult KO mice. P2 amplitudes decrease with age in KO mice. (C) FC ERP wave latencies. P2 latency is increased in adult KO mice. Full analysis is shown in Table 2.2.

Table 2.2. Full statistical analysis of ERP data.

Cortical Region	ERP Component	Factor	ANOVA Results	p-value
AC	P1 Amplitude:	**Age**	**F(2,56)=8.807**	**0.0005**
		Genotype	**F(1,56)=6.863**	**0.0113**
		Age x Genotype	**F(2,56)=9.972**	**0.0002**
	N1 Amplitude:	Age	F(2,56)=2.565	0.0859
		Genotype	**F(1,56)=9.680**	**0.0029**
		Age x Genotype	F(2,56)=0.4776	0.6228
	P2 Amplitude:	**Age**	**F(2,56)=3.007**	**0.0575**
		Genotype	F(1,56)=0.8885	0.3499
		Age x Genotype	F(2,56)=0.9607	0.3888
FC	P1 Amplitude:	Age	F(2,56)=0.9323	0.3997
		Genotype	F(1,56)=2.582	0.1137
		Age x Genotype	**F(2,56)=3.595**	**0.0340**
	N1 Amplitude:	Age	F(2,56)=1.229	0.3002
		Genotype	**F(1,56)=9.559**	**0.0031**
		Age x Genotype	F(2,56)=0.6309	0.5359
	P2 Amplitude:	**Age**	**F(2,56)=4.461**	**0.0159**
		Genotype	F(1,56)=3.105	0.0835
		Age x Genotype	F(2,56)=0.1059	0.8997
AC	P1 Latency:	**Age**	**F(2,56)=5.764**	**0.0053**
		Genotype	F(1,56)=1.320	0.2555
		Age x Genotype	F(2,56)=0.1276	0.8804
	N1 Latency:	Age	F(2,56)=2.884	0.0643
		Genotype	F(1,56)=0.2235	0.6382
		Age x Genotype	F(2,56)=0.1730	0.8416
	P2 Latency:	Age	F(2,56)=1.095	0.3417
		Genotype	F(1,56)=6.018e-005	0.9938
		Age x Genotype	F(2,56)=1.667	0.1981
FC	P1 Latency:	Age	F(2,56)=2.283	0.1114
		Genotype	F(1,56)=2.459	0.1225
		Age x Genotype	F(2,56)=0.0603	0.9415
	N1 Latency:	Age	F(2,56)=2.809	0.0688
		Genotype	F(1,56)=0.0073	0.9323
		Age x Genotype	F(2,56)=1.123	0.3325
	P2 Latency:	Age	F(2,56)=0.1817	0.8343
		Genotype	**F(1,56)=8.761**	**0.0045**
		Age x Genotype	F(2,56)=2.412	0.0989

Two-way ANOVA results for ERP analysis. Post hoc comparisons were done using Tukey's and Bonferroni's multiple comparisons tests and p-values were adjusted accordingly. See text for post hoc results. Bold text indicates statistical significance (p = or < 0.05).

Development of ERP single trial power phenotypes in the Fmr1 KO mice

In addition to ERP peak amplitude and latency, we analyzed STP during the stimulus train used for ERP measurement (Figures 2.9, 2.10). The STP phenotypes were similar to those found with the gap-ASSR paradigm. There was no genotype difference in STP at p21 in either AC (Figure 2.9) or FC (Figure 2.10). At p30, KO mice showed elevated STP in both AC and FC, with effects limited to frequencies between 25-80 Hz. At p60, the KO mouse AC showed reduced STP at frequencies below 60 Hz, but there was no difference in the FC. These results support the idea of developmental fluctuations in background power phenotypes in FXS.

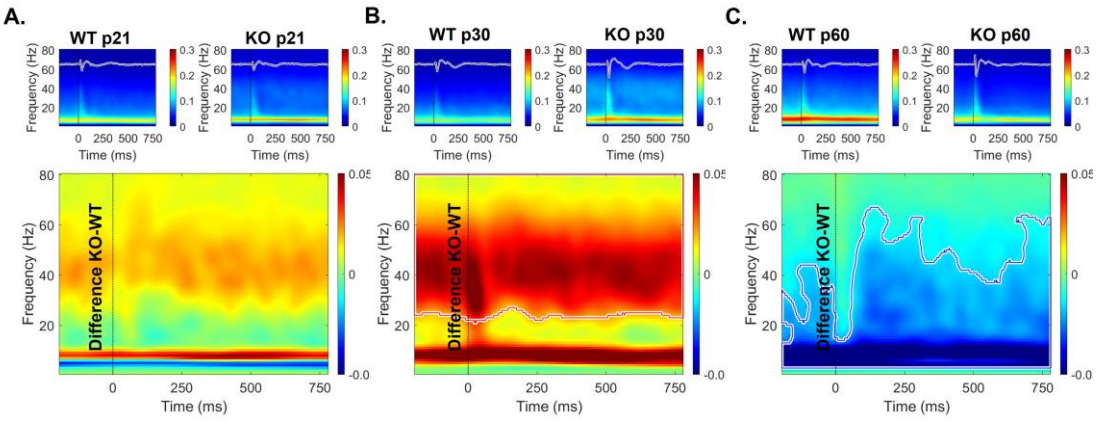

Figure 2.9. Non-baseline normalized STP during ERP stimulation is altered in *Fmr1* KO in the AC during development. The format is similar to Figures 2.4-6, except these are obtained during ERP stimulation. Outlined regions indicate clusters which are significantly different between WT and KO. (A) Young *Fmr1* KO mice show no difference in STP at p21. (B) KO mice have increased background activity in the gamma range at p30. (C) Adult KO mice show decreased STP in the beta and gamma frequency ranges compared to WT.

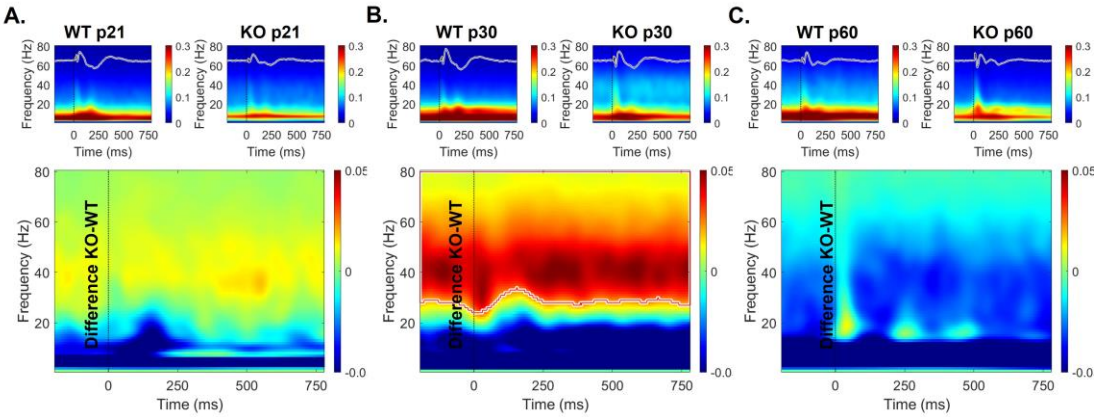

Figure 2.10. Non-baseline normalized STP during ERP stimulation is altered in *Fmr1* KO in the FC during development. Figure format is the same as in Figure 2.4-6. (A) Young *Fmr1* KO mice show no difference in STP at p21. (B) KO mice have increased background activity in the gamma frequencies at p30. (C) Adult *Fmr1* KO mice show no significant difference in STP at p60.

Discussion

The major and novel contribution of this study is the identification of developmental trajectories of auditory temporal processing in two cortical regions of WT and *Fmr1* KO mice. We recorded 40 Hz gap-in-noise ASSR from the AC and FC at three different ages as a measure of temporal processing. We also quantified ERP amplitudes/latencies and sound evoked single trial power to determine if abnormally elevated EEG power is developmentally correlated with temporal processing deficits. The results show genotype, cortical region- and age-specific abnormalities in gap-ASSR responses and ERPs. Interestingly, significant developmental delay was seen in gap-ASSR responses in the FC, but not the AC, of *Fmr1* KO mice. ERP N1 amplitudes were larger across development in both AC and FC of the KO mouse. The non-phase locked STP phenotypes showed developmental fluctuations. Between p21 and p30 there was an increase in STP during both gap-ASSR and ERP recordings in the *Fmr1* KO mice,

and at P60 there was a reversal of this phenotype. Taken together, these data provide novel evidence for abnormal development of temporal processing in the frontal cortex, and hypersensitive responses in both auditory and frontal cortex in the *Fmr1* KO mouse model of FXS. The data do not support the notion that hypersensitive cortical responses underlie temporal processing deficits in developing *Fmr1* KO mice as there was no developmental correlation between the two measures. These phenotypes may arise from independent mechanisms. The robust developmental delays in gap-ASSR EEG responses in KO mice provide physiological tools to evaluate underlying mechanisms and identify treatment targets and windows.

The WT mouse shows significant developmental improvement in gap-ASSR EEG responses, providing a reference for other mouse models of other ASD and neurodevelopmental disorders. This is consistent with findings in the rat auditory cortex in which the percentage of neurons with short neural gap detection thresholds increases from juveniles to adults [Zhao et al., 2014]. These neural improvements in gap processing may underlie perceptual improvement in gap detection thresholds in children, a factor that is correlated with improved language in development [Werner et al., 1992; Friedman et al., 2004; Smith et al., 2006; Benasich & Tallal, 2002; Trehub & Henderson,1996].

Expressive and receptive language deficits are consistently reported in FXS, but the underlying mechanisms are unclear [Roberts et al., 2001]. Children with FXS express developmental delays in multiple cognitive categories necessary for language maturation, such as auditory short-term memory and attention [Freund & Reiss, 1991; Ornstein et al., 2008; Bregman et al., 1988; Cornish et al., 2007; Mazzocco et al., 1993; Scerif et al., 2007]. In addition to these cognitive factors, delayed temporal processing

and auditory hypersensitivity may underlie speech and language delays in FXS [4-5]. In developmental disorders and in aging, gap processing has been used to analyze auditory temporal acuity across groups [Bhatara et al., 2013; Gordon-Salant & Fitzgibbons, 1993; Rumschlag & Razak, 2021; Rumschlag et al., 2020]. Increased gap-detection thresholds are seen in children with ASD and impaired gap detection thresholds in children correlate with lower phonological scores [Foss-Feig et al., 2017]. The 40 Hz gap-in-noise-ASSR paradigm used here tests the ability of neural generators of EEGs in the AC and FC to phase lock consistently across trials and can be used in humans with FXS to determine if similar cortical region-specific temporal processing deficits are present in patients. By varying the modulation depth and gap widths, it is possible to compare temporal processing acuity of auditory systems across groups [Green, 1971; Anderson & Linden, 2016]. The cortical mechanisms of gap processing are also beginning to be understood [Weible et al., 2014; Keller et al., 2018]. Future EEG studies in children with FXS should examine if temporal gap processing deficits are present early in development, and if they are related to development of language abilities. This may provide the basis for adaptive training of children with rapidly changing stimuli, including gaps, to improve speech recognition and language [Merzenich et al., 1996].

The current study focused on 40 Hz ASSR for multiple reasons. Gamma band deficits have been consistently observed in humans with FXS and *Fmr1* KO mice across strains and ages [Wang et al., 2017; Ethridge et al., 2017,2019; Lovelace et al., 2018; Wen et al., 2019; Jonak et al., 2020]. There is also a developmental delay in the maturation of parvalbumin-expressing inhibitory neurons and the perineuronal nets that surround them [Wen et al., 2018]. As these structures are involved in generating gamma

band oscillations, we predicted 40 Hz ASSR deficits. Another reason for focusing on the 40 Hz ASSR is that the auditory cortex has a resonance at that frequency, and therefore, produces the largest power in EEG responses at that frequency [Hwang et al., 2019; Baltus & Herrmann, 2015; Hwang et al., 20206]. In addition, the mechanisms of 40 Hz ASSR have been studied, including descriptions of topography across regions and the role of basal forebrain neurons and NMDA receptor function [Sivarao et al., 2016; Kim et al., 2015].

More relevant to speech processing, there is a strong link between gamma band oscillations and phoneme processing, with gamma oscillations parsing speech input in the phoneme range. The slower oscillations (delta-theta) may be more relevant to aspects of intonation and syllabic rates, and other aspects of speech with slower evolution. Based on such observations, the 'asymmetric sampling in time' hypothesis for speech processing has been proposed in which gamma oscillations play a significant role in phoneme processing [Luo & Poeppel, 2012; Giraud & Poeppel, 2012; Peña & Melloni, 2012]. Gamma resolution parsing may provide sufficient cues in separating closely spaced inputs (eg., voice onset time, formant transitions), facilitating speech recognition. It will be very interesting in future studies to examine 10 and 20 Hz ASSRs in the *Fmr1* KO and WT mice, that may allow a prediction of the nature of speech deficits in humans with FXS.

ERP deficits in Fmr1 KO mice

The P1-N1-P2 ERP complex marks the pre-attentive detection of sound and can vary with stimulus features. Consistent with a number of studies in humans with FXS, and our previous studies in adult and developing mice, ERP component amplitudes were

higher in the *Fmr1* KO mice compared to WT mice [Humans: Castrén et al., 2003; Knoth & Lippé, 2012; Rojas et al., 2001; St Clair et al., 1987; Van der Molen et al., 2012a, 2012b; Mice: Lovelace et al., 2018; Wen et al., 2019; Kulinich et al., 2020]. We found N1 amplitude, which are generated from frontal and temporal lobes [Näätänen & Picton, 1987] and marks synchronous activity within the cortex, to be higher in both AC and FC of the KO mice. This is consistent with calcium imaging studies that showed abnormally high synchronous activity in the *Fmr1* KO mouse cortex, and may arise from abnormal activity of parvalbumin positive inhibitory interneurons [Gonçalves et al., 2013; Wen et al., 2018; Assaf & Schiller, 2016]. Reduced habituation of responses in mice [Lovelace et al., 2016] and humans [Ethridge et al., 2016] may also contribute to larger N1 amplitudes because the reported amplitude is the average of responses to multiple trials. We also observed a main effect of genotype and/or genotype X age interactions for P1 amplitude, with KO mice showing larger amplitudes. P1 amplitudes mark thalamocortical input activity, suggesting enhanced input drive of the cortex in the KO mice. This may arise from reduced input layer 4 thalamocortical drive of fast-spiking (putative parvalbumin positive) inhibitory interneurons in the KO cortex as shown by Gibson et al. and Patel et al. [Gibson et al., 2008; Patel et al., 2013]. P2 amplitudes are thought to be related to arousal as auditory input to the mesencephalic reticular activating system contributes to P2 generation [Crowley & Colrain, 2004]. There was no genotype difference in the AC. In the FC, however, there was a trend towards decreased P2 amplitude in the KO mice, suggesting the potential for reduced arousal and attention during development. The enhanced evoked responses and reduced habituation in FXS may lead to reduced ability for auditory change detection as shown by Van der Molen et al. [Van der Molen et al., 2012a]. Such sensory discrimination

deficits may lead to speech and language abnormalities in FXS. A recent study in humans demonstrated a link between habituation and language abilities in children with FXS [An et al., 2022]. Specifically, it was shown that weaker P1 responses to later stimuli in a habituation train as well as larger habituation of P1 was associated with increased receptive and expressive language abilities, suggesting that habituation to repeated tones impacts language abilities in children with FXS.

Enhanced gamma band power in background activity in Fmr1 KO mice

The single trial power (STP) allows for the identification of ongoing 'background activity' during stimulus presentation as it does not correct for mean baseline power levels. It has been suggested that this non-phase locked power reflects relatively slow integrative processes that may impact stimulus or response processing [McKewen et al., 2020]. These processes include top-down and sustained attention, decision-making, and perceptual inference, and are suggested to result from intrinsic network interactions rather than external stimuli [Donner & Siegel, 2011; Seigel & Donner, 2010]. Our results show developmental fluctuations in STP phenotypes in *Fmr1* KO mice, with adolescent KO mice (p30) having increased STP during ERP and gap-ASSR stimuli in the AC and FC compared to WT mice. The increase in STP was seen in the gamma band (30-80 Hz), consistent with data from Ethridge et al. from humans with FXS compared to typically developing control [Ethridge et al., 2019]. Human data, recorded from adolescents and young adults, also shows elevated gamma band STP across multiple stimulus types. Importantly, the elevated gamma power showed correlations with IQ and distractibility. These data suggest increased on-going activity that may be a result of hyperactive network connections across species, and with potential clinical implications

in humans. The reasons why the phenotypes fluctuate over development is unclear. It is also not know if similar age-effects are seen in humans with FXS. With neurodevelopmental disorders, it is sometimes difficult to disambiguate the direct effect of the mutation from the effect of potential compensatory (e.g., homeostatic) adjustments in activity levels.

A previous study (Wen et al., 2019) reported increased resting EEG gamma power in the frontal cortex of adult *Fmr1* KO mice (FVB strain, the same used here)[Wen et al., 2019]. We did not observe increased gamma power in the STP data in the present study. While both resting EEG and sound evoked STP can be considered as background activity, the differences across the two studies can be explained by how these measures are calculated. Resting EEGs are recorded in the absence of any sound stimuli, but the STP calculated is background during sound stimulation. The animal is likely in a different state of arousal in the presence of sounds compared to the resting condition leading to observed differences between the different measures of background gamma activity.

Delayed development of temporal processing in Fmr1 KO mouse frontal cortex

Perhaps the most surprising result of the study is that developmental delays in temporal processing were seen in the FC, but not the AC. These data suggest that FC does not simply inherit auditory responses from the AC, but that additional local processing within the FC and/or auditory pathways that bypass the AC may be involved in producing phase locked responses in the FC. Very little is known regarding mechanisms of auditory processing in the FC. Robust frontal cortex ASSR power is seen in both human [Lustenberg et al., 2018] and mouse EEG recordings [Hwang et al.,

2019]. Indeed, topographical distribution of ASSR power and precision favors more frontal regions in both species. Kim et al. (2015) and Hwang et al. (2019) showed that optogenetic stimulation of GABAergic parvalbumin neurons in the mouse nucleus basalis preferentially increased frontal cortex 40 Hz ASSR oscillations. This suggests independent modulation of ASSR in the FC that may be abnormal in early development in FXS. The idea that FC can robustly mount ASSR, and independently show deficits, is supported by two other lines of evidence. Clark et al. showed in an autoimmune disorder mouse model that gap processing is affected in the FC, while remaining normal in the AC [Clark et al., 2000]. Wieczerzak et al. reported reduced recovery of ASSR in FC, rather than AC, following noise induced hearing loss [Wieczerzak et al., 2021]. The fact that the temporal processing deficits are seen in early development in the FC, and the not the AC in *Fmr1* KO mice, suggests temporal processing deficits may be present across multiple sensory modalities. If a similar developmental regional difference in temporal processing is seen in humans with FXS, this would suggest speech processing and language function may be affected across multiple modalities [Rosenblum, 2008].

An important consequence of abnormal temporal processing in the FC may be related to how FC-AC top-down interactions function during development. FC induces top-down modulation of AC responses in a task- and attention-dependent fashion. Fritz et al. hypothesized that the FC modulates AC neuron receptive fields depending on the task and selective attention [Fritz et al., 2010]. FC-AC connection and its modulation of speech have also been evaluated in humans with FXS. Speech production depends on feedforward control and the synchronization of neural oscillations between the FC and AC. Specifically, the interactions of these two regions allow for comparison of the corollary discharge of intended speech generated from an efference copy of speech to

the actual speech sounds produced, a process essential for making adaptive adjustments to optimize future speech [Schmitt et al., 2019]. A study of humans utilizing a talk-listen paradigm found that in the time window prior to speech production, individuals with FXS have decreased pre-speech activity, including frontotemporal connectivity, as well as increased frontal gamma power compared to controls. These discrepancies brought about less intelligible speech and correlated with increased social communication deficits [Schmitt et al., 2019. Abnormal functional connectivity between FC and AC is also suggested by Zhe et al., (2021) who showed reduced long-range connectivity in *Fmr1* KO mice [Zhang et al., 2021]. Future studies will examine phase connectivity between FC and AC during different sound stimulation paradigms including the gap-ASSR. Taken together, the connections between the FC and AC are essential for shaping sensory responses and disruptions may cause speech and language impairments. A mismatched development pattern between these two regions in humans, as seen in the present study of mice, could possibly give rise to language abnormalities in FXS.

Conclusions

We have identified a developmental delay in auditory temporal processing in the FXS model mouse. The p21-p30 window is a critical period of development in *Fmr1* KO mice that is marked by cortical hyperexcitability and reduced inhibitory interneuron function [Wen et al., 2018,2019; Kulinich et al., 2020]. This delayed development is similar to other studies in *Fmr1* KO mice. For example, in the somatosensory cortex, *Fmr1* KO mice show delayed maturation of GABAergic inhibition and decreased synaptic connectivity that eventually normalize to WT levels in adults [Daw et al., 2007; Bureau et

al., 2008]. Brain development is a precise process that is determined by accurately cued stages of gene expression, molecular guidance cues and intrinsic neuronal activity [Chilton, 2006; Marín et al., 2010]. The timing of these developmental stages, known as critical periods, is imperative for accurate neuronal migration, circuit formation and synaptic refinement [Meredith, 2015]. Disruptions of critical period timelines cause long term impairments in behavioral phenotypes. Even though responses may be normalized in the adult, abnormal critical period development will have long-term consequences for behaviors that build on normal development of responses. For example, developmental delay in FC temporal processing may lead to long term abnormalities in behaviors that depend on accurate temporal processing such as speech, language and binaural processing. In order to effectively treat humans with FXS, it is imperative to understand the developmental trajectory of phenotypes that are likely to be used as clinical outcome measures, as opposed to just adult comparisons. Future studies should evaluate temporal processing across age to determine if similar delays in development are present in humans with FXS, and if the delay relates to language function.

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Chapter 3

Sex Differences During Development in Cortical Temporal Processing and Event Related Potentials in Wild-Type and Fragile X Syndrome Model Mice

Abstract

Autism spectrum disorder (ASD) is currently diagnosed in approximately 1 in 44 children in the United States, based on a wide array of symptoms, including sensory dysfunction and abnormal language development. Boys are diagnosed ~3.8 times more frequently than girls. Auditory temporal processing is crucial for speech recognition and language development. Abnormal development of temporal processing may account for ASD language impairments. Sex differences in the development of temporal processing may underlie the differences in language outcomes in male and female children with ASD. To understand mechanisms of potential sex differences in temporal processing requires a preclinical model. However, there are no studies that have addressed sex differences in temporal processing across development in any animal model of ASD. To fill this major gap, we compared the development of auditory temporal processing in male and female wildtype (WT) and *Fmr1* knock-out (KO) mice, a model of Fragile X Syndrome (FXS), a leading genetic cause of ASD-associated behaviors. Using epidural screw electrodes, we recorded auditory event related potentials (ERP) and auditory temporal processing with a gap-in-noise auditory steady state response (ASSR) paradigm at young (postnatal (p)21 and p30) and adult (p60) ages from both auditory and frontal cortices of awake, freely moving mice. The results show that ERP amplitudes were enhanced in both sexes of *Fmr1* KO mice across development compared to WT counterparts, with greater

enhancement in adult female than adult male KO mice. Gap-ASSR deficits were seen in the frontal, but not auditory, cortex in early development (p21) in female KO mice. Unlike male KO mice, female KO mice show WT-like temporal processing at p30. There were no temporal processing deficits in the adult mice of both sexes. These results show a sex difference in the developmental trajectories of temporal processing and hypersensitive responses in *Fmr1* KO mice. Male KO mice show slower maturation of temporal processing than females. Female KO mice show stronger hypersensitive responses than males later in development. The differences in maturation rates of temporal processing and hypersensitive responses during various critical periods of development may lead to sex differences in language function, arousal and anxiety in FXS.

Introduction

Abnormal sensory processing and delayed language development are core symptoms of ASD [Abbeduto & Hagerman, 1997; Foss-Feig et al., 2017; Tager-Flusberg & Caronna, 2007; Rapin & Dunn, 2003; Jeste & Nelson, 2008]. This spectrum of disorders has traditionally been diagnosed within the first three years of life, when differences from age-matched typically developing children start to become apparent, particularly with sensory issues and language development [Riva et al., 2018]. Deficits in sensory processing have been reported in up to 87% of patients and correlate with autism-related social difficulties [Le Couteur et al., 1989; Lord, 1995; Baum et al., 2015; Hilton et al., 2010; Watson et al., 2011]. A sex bias in ASD diagnosis is well established, with the male:female ratio of diagnosis being ~3.8:1 [Werling & Geschwind, 2013]. Sex differences seen in the maturation rate of language function in typically developing

children [Zubrick et al., 2007; Adani & Cepanec, 2019; Maccoby, 1966; Ramer, 1976; Huttenlocher et al., 1991; Özçalışkan &Goldin-Meadow, 2010] are further enhanced in children with ASD with males showing slower development and/or more impairments [Harrop et al., 2020; Darley & Winitz, 1961; Nelson, 1981]. While multiple studies have suggested a link between fetal or early postnatal sex hormone levels and language development, the developmental trajectory and mechanisms of this sex difference are not well understood [Lutchmaya et al., 2002; Lombardo et al., 2012; Knickmeyer et al., 2008; Auyeung et al., 2009; Friederici et al., 2008; Hollier et al., 2012; Schaadt et al., 2015].

Mutations in the *Fmr1* (Fragile X Messenger Ribonucleoprotein) gene show a strong link and comorbidity with ASD. The silencing of *Fmr1* results in a loss of the Fragile X Messenger Ribonucleoprotein (FMRP) and Fragile X Syndrome (FXS) [Darnell et al., 2011; Huber et al., 2002]. The loss of FMRP causes altered synaptic development and brain plasticity, intellectual deficits, and behaviors related to ASD, including repetitive behaviors, sensory, cognitive, and social impairments [Wisniewski et al., 1991; Cordeiro et al., 2010; Van der Molen et al., 2010; Oakes et al., 2015; Miller et al., 1999; Hall et al., 2009]. Individuals with FXS show abnormal sensory sensitivity and speech and language impairments [Miller et al., 1999; Abbeduto et al., 2007; Finestack et al., 2009; Rice et al., 2005; Martin et al., 2013; Rogers et al., 2003; Crane et al., 2009; Marco et al., 2011; Takarae et al., 2016; Ethridge et al., 2016; Abbeduto & Hagerman, 1997]. As FXS is an X-linked disorder, a strong sex bias is present with ~1 in 4000 males and ~1 in 7000 females affected [Bartholomay et al., 2019]. Males with FXS are on average more impaired in language development than females, but the developmental

mechanisms of sex-differences in language function in FXS are unclear [Kover & Abbeduto, 2010; Abbeduto et al., 2003, 2007; Brady et al., 2004].

Abnormal development of auditory temporal processing may underlie impaired language function. Auditory temporal modulation cues aid speech recognition [Zeng et al., 2005; Shannon et al., 1995], and humans' ability to discriminate temporal cues in sounds is present at a very young age [Moore, 2008; Cabrera & Gervain, 2020]. The inability to process rapidly changing acoustic input during development may interfere with speech perception and phonological processing and may result in language disorders [Tallal et al., 1998]. Individuals with ASD show deficits in detecting sound duration, onset and offset, and rapid spectrotemporal changes [Kwakye et al., 2011; Lepistö et al., 2006; Oram Cardy et al., 2005; Orekhova et al., 2009; Meilleur et al., 2020]. Issues with reproducing auditory stimuli lengths are evident in children with ASD, and both children and adults with ASD display atypical neural responses to pitch fluctuations in sequential, repeated auditory stimuli [Szelag et al., 2004; Tecchio et al., 2003; Gomot et al., 2006]. Increased gap-detection thresholds, commonly used to evaluate auditory temporal processing, are seen in ASD. Importantly, children with poorer gap detection scores were also associated with lower phonological processing scores [Foss-Feig et al., 2017]. These studies provide evidence that deficiencies in auditory temporal processing may influence atypical language function in ASD.

The *Fmr1* KO mouse, an animal model of FXS, exhibits abnormal sensory responses similar to humans, providing a useful platform for studying the developmental patterns and neural mechanisms of sensory circuit dysfunction [Razak et al., 2021]. However, very little is known in terms of sex differences in sensory responses in the *Fmr1* KO mice, or in humans with FXS. Indeed, very little is known about sex

differences in the developmental trajectory of sensory responses in any non-human species, including mice of any genotype. To fill this major gap, we recorded sensory electrophysiological responses in female *Fmr1* KO mice across development in this study and compared the responses to previously published data from male *Fmr1* KO mice using identical methods and ages [Croom et al., 2023]. We tested the hypothesis that sex differences in sensory deficits such as auditory cortical temporal processing and auditory sensitivity emerge in *Fmr1* KO mice from early developmental stages.

We acquired EEG signals from the auditory and frontal cortex (AC, FC) in both *Fmr1* KO and wildtype (WT) mice at three different ages: p21, p30, and p60. A 40 Hz gap-in-noise ASSR (auditory steady state response, hereinafter referred to as gap-ASSR) paradigm was used to measure the cortex's reliability in phase locking to brief gaps in noise at varying modulation depths to assess temporal processing acuity [Rumschlag & Razak, 2021]. Gap stimuli have been used extensively to evaluate auditory temporal precision, and EEG recordings are more readily executed in humans compared to single-unit recordings, and thereby facilitate translational relevance [Green, 1971; Anderson & Linden, 2010]. The gap-ASSR paradigm requires the neural generators to synchronize responses to gaps of different widths in noise, providing an objective measure of temporal processing across genotypes and age groups. Auditory event related potentials (ERPs) are consistently of larger amplitudes in humans with FXS, but potential sex differences in FXS are not known. Therefore, we recorded auditory ERPs in mice to examine possible sex differences in hypersensitive responses during development in the AC and the FC. Our data show earlier maturation of temporal processing in *Fmr1* KO female mice compared to male mice, and a larger enhancement

of ERP amplitudes in KO female than male mice across development, compared to WT mice.

Methods

The following age ranges and sample sizes were used in this study: WT-Females: p21 (n=11), p30 (n=9), p60 (n=8) and *Fmr1* KO-Females: p21 (n=8), p30 (n=9), p60 (n=8)]. The data collected on females were compared to previously published WT and *Fmr1* KO male data [Croom et al., 2023]. None of the female data, or sex comparisons, have been previously published.

All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. Mice were obtained from an in-house breeding colony that originated from Jackson Laboratory (Bar Harbor, ME). The mice used for the study are sighted FVB wild-type (Jax, stock# 004828; WT) and sighted FVB *Fmr1* knock-out (Jax, stock# 004624; *Fmr1* KO). This background strain was chosen because our prior developmental work examining cortical parvalbumin and perineuronal nets as well as single unit responses in the auditory cortex and the inferior colliculus have utilized this same strain [Rotschafer & Razak, 2013; Nguyen et al., 2020]. One to five mice were housed in each cage under a 12:12-h light-dark cycle and fed *ad libitum*. A cross-sectional, as opposed to a longitudinal, design was used in this study as it is impractical to place epidural screw electrodes in brains that are still growing.

The ages selected for the sex difference comparison were based on previous findings. Decreased PNN expression surrounding parvalbumin-positive interneurons and cortical hyperexcitability are observed in *Fmr1* KO mice at p21 [Wen et al., 2018]. Additionally, the p14-21 age corresponds to the critical period for responses to simple

tones and maturation of tonotopic maps in the auditory cortex [Oswald & Reyes, 2010; Kim et al., 2013]. P30 was chosen because response selectivity to complex sounds has not matured in the auditory cortex until this age [Carrasco et al., 2013]. We chose the p60 age group to represent young adulthood. Our previous study in males also showed significant genotype differences in temporal processing at p21 and p30, and normalization at p60. Here we compared developmental trajectories of male and female *Fmr1* KO mice.

Surgery: Different groups of mice underwent epidural electrode implant surgery at three developmental timepoints: p18-20, p27-p29, p57-p66. Surgical procedures have been previously published [Croom et al., 2023; Rumschlag & Razak, 2021; Rumschlag et al., 2020]. Briefly, mice were anesthetized using intraperitoneal (i.p.) injections of either 80/20 mg/kg of ketamine/xylazine (young mice) or 80/10/1 mg/kg ketamine/xylazine/acepromazine (adult mice). The anesthetic state was monitored closely throughout the procedure by toe pinch reflex every 10-15 minutes. ETHIQA-XR (1-shot buprenorphine, 3.25 mg/kg body weight) was administered via subcutaneous injection prior to surgery. An incision was made to expose the scalp following the removal of fur and sterilization (alcohol and iodine wipes) of the scalp. A Foredom dental drill was used to drill ~1mm diameter holes in the skull over the right AC, right FC, and left occipital cortex. The screw positions were determined using skull landmarks and coordinates previously reported [Croom et al., 2023; Rumschlag & Razak, 2021; Rumschlag et al., 2020; Wen et al., 2019; Lovelace et al., 2018]. The wires extending from three-channel posts were wrapped around 1 mm screws and driven into the pre-drilled holes. Dental cement was applied to secure the implant. Mice were placed on a

heating pad until fully awake and were allowed 48-72 hours for recovery before EEG recordings were made.

EEG recordings: All EEG recordings were obtained from awake and freely moving female mice, using methods identical to those published for male mice [Croom et al., 2023]. EEG recordings were performed at three developmental time points: p20-23, p29-31, p59-p70, which we refer to as p21, p30 and p60, respectively. Recordings were obtained from the AC and FC electrodes, using the occipital screw as reference. All recordings were obtained inside a sound-insulated and anechoic booth (Gretch-Ken, OR). Mice were briefly anesthetized with isoflurane and connected to an EEG cable via the implant. Mice were habituated to the recording arena with no stimuli prior to sound evoked recordings. The attached cable was connected via a commutator to a TDT (Tucker Davis Technologies, FL) RA4LI/RA4PA headstage/pre-amp, which was connected to a TDT RZ6 multi-I/O processor. OpenEx (TDT) was used to simultaneously record EEG signals and operate the LED light used to synchronize the video and waveform data. TTL pulses were utilized to mark stimulus onsets on a separate channel in the collected EEG data. The EEG signals were recorded at a sampling rate of 24.414 kHz and down-sampled to 1024 Hz for analysis. All raw EEG recordings were visually examined prior to analysis for artifacts, including loss of signal or signs of clipping. No EEG data were rejected after examination.

Auditory ERP: Narrowband noise stimuli (6-12 kHz bandwidth, 120 repetitions, 100 ms duration, 5ms rise/fall time, 0.25 Hz repetition rate) were presented at 75 dB SPL using a speaker (MF1, Tucker Davis Technologies, FL) situated 20 cm above the floor of the arena. ERP analysis and statistics have been previously described [Croom et al., 2023; Rumschlag & Razak, 2021; Rumschlag et al., 2020]. Briefly, the EEG trace was split into

epochs using the TTL pulses to mark sound onset. Each trial was baseline corrected, such that the mean of the 250 ms baseline period prior to sound onset was subtracted from the trial trace for each trial. Each trial was then detrended (MATLAB detrend function) and all trials were averaged together.

Gap-ASSR: The stimulus used to assess auditory temporal processing is termed the '40 Hz gaps-in-noise ASSR' (auditory steady state response, henceforth, 'gap-ASSR') [Rumschlag & Razak, 2021]. The stimulus contains alternating 250 ms segments of noise and gap interrupted noise presented at 75 dB SPL. The gaps are placed 25 ms apart, resulting in a presentation rate of 40 Hz, a rate that produces the strongest ASSR signal when measured from the AC and frontal regions [Galambos et al., 1981; Pastor et al., 2002; Llinás, 1988; Llinás et al., 1991; Rosanova et al., 2009; Kim et al., 2015; Hwang et al., 2019]. For each gap-in-noise segment, the gap width and modulation depth are chosen at random. Gaps of 2, 4, 6, 8, 10, or 12 ms widths and modulation depths of 75 and 100% were used. To measure the ability of the cortex to consistently respond to the gaps in noise, inter-trial phase clustering (ITPC) at 40 Hz was measured [Cohen, 2014]. The ITPC is based on the distribution of phase angles in the EEG response at 40 Hz (because the stimulus is a 40 Hz train) across all trials and reflects the precise timing of 40 Hz activity in the underlying neural generators. ITPC can be interpreted independently of power. ITPC ranges between 0 and 1, with 0 indicating high variability (uniform distribution) of phase angles across trials, and 1 indicating the same phase angle for every trial. Because ITPC is sensitive to temporal jitter of responses from one trial to the next, this is a useful measure of temporal reliability of responses. The EEG trace was transformed using a dynamic complex Morlet wavelet transform. The trials corresponding to each parametric pair (gap duration + modulation

depth) were grouped together. The ITPC was calculated for each time-frequency point as the average vector for each of the phase unit vectors recorded across trials (trial count >100 trials per parametric pair). The ITPC values at 40 Hz were averaged to extract the mean ITPC for the parametric pairs in the AC and FC.

Statistics: Statistics were performed on GraphPad Prism (ERP) or R (gap-ASSR). To evaluate the effects of genotype (2 levels) and age (3 levels), or sex (2 levels) and age (3 levels), two-way ANOVA was used for ERP analysis. Post hoc comparisons were carried out with Tukey's and Bonferroni's multiple comparisons test. ERP data were tested for normality using Shapiro-Wilk tests. A three-way repeated measures ANOVA was used for the female development gap-ASSR analysis, with the three factors being genotype (2 levels) X age (3 levels) X gap duration (6 levels). Mauchly Tests for Sphericity were utilized and corrected for using the Greenhouse-Geisser corrections if necessary. A two-way repeated measures ANOVA was used for within-genotype sex comparisons at each age, with the two factors being sex (2 levels) X gap duration (6 levels). The Geisser and Greenhouse epsilon hat method was utilized to correct p-values for lack of sphericity using the Greenhouse-Geisser corrections if necessary. A repeated measures ANOVA was chosen as multiple gap duration data points were collected from a single mouse in a recording session. Post hoc contrasts with Sidak corrections for multiple comparisons were used. Cortical regions (AC, FC) and modulation depths (75%, 100%) were analyzed separately. Data were evaluated to ensure ANOVA assumptions were met, in particular the assumption of the normality of the residuals. None of the residuals had measures of skewness or kurtosis that exceeded ±2, which is one indication of acceptable normality [West et al., 1995]. Moreover, the residuals were evaluated via quantile-quantile plots. In each of the

analyses, the correspondence between the theoretical normal distribution and the obtained residuals was within acceptable bounds.

Results

The main goals of this study were to record the developmental trajectory of auditory temporal processing and ERPs in female WT and *Fmr1* KO mouse auditory and frontal cortex and compare these data with previously published data from male counterparts [Croom et al., 2023]. The aim was to determine whether sex differences in evoked responses were present in WT and KO mice throughout development.

Delayed development of temporal processing in the FC of female Fmr1 KO mice

Auditory temporal processing was assessed using a 40 Hz gap-in-noise ASSR stimulus to determine the ability of auditory and frontal cortex (AC, FC) to consistently phase lock to brief gaps in noise. Manipulating the duration and modulation depth of the gaps allows for the identification of differences in temporal acuity between WT and KO mice and to track developmental changes. Figure 3.1 shows inter-trial phase clustering (ITPC) heat maps in the 40 Hz gap-ASSR from example female WT (Fig. 3.1A, C) and *Fmr1* KO (Fig. 3.1B, D) mice (modulation depth of 100%). The y-axis of each panel shows the ITPC at a specific gap generated with the 40 Hz signal, with increasing gaps across columns. Each row marks a different age. In each panel, zero (faint vertical dashed line) on the x-axis marks the onset of the gap ASSR stimulus. The expected ITPC is at 40 Hz because the stimulus is a 40 Hz train. Therefore, the warm colors indicating higher ITPC (see ITPC scale at the right of Figure 3.1) are seen at 40 Hz. Cooler colors indicate relatively low ITPC and are mostly seen for very short gaps, KO mice, and at spectral bands outside 40 Hz.

As expected, both AC and FC are better able to synchronize their responses to longer gaps compared to short gaps (left to right in each row). Across genotypes, there are no qualitative ITPC differences in the AC throughout development (Fig. 3.1A, B). However, in the FC, deficits are clearly seen at p21, with the *Fmr1* KO mice ITPC barely emerging above background at 40 Hz (Fig. 3.1C, D). Genotype differences were not observed at p30 or p60 in female mouse AC and FC.

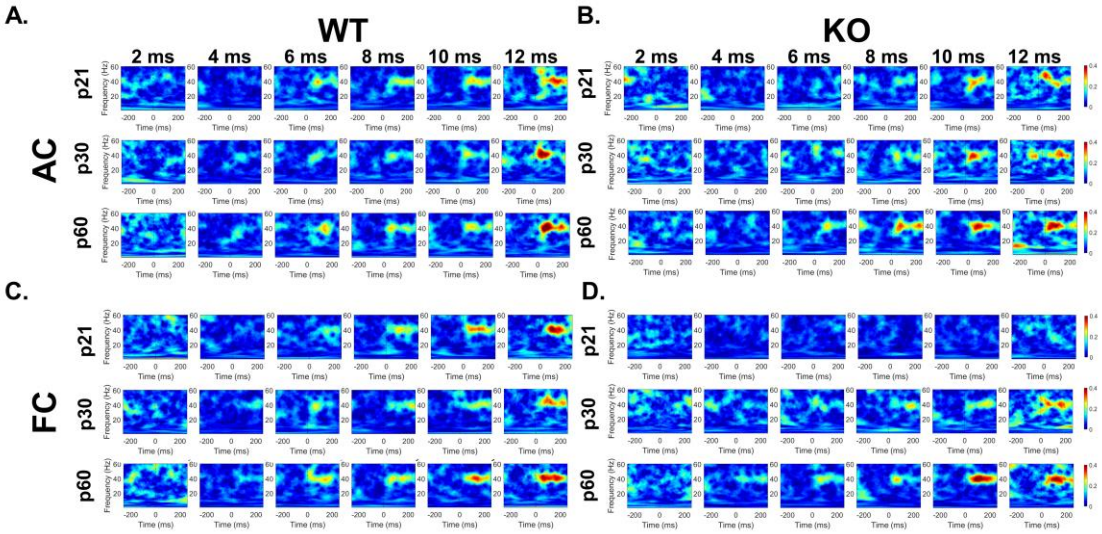

Figure 3.1. Delayed development of temporal processing in the frontal cortex of female *Fmr1* KO mice. Individual example heatmaps of ITPC generated at 40Hz at multiple gap durations in p21, p30, and p60 WT (A: AC, C: FC) and *Fmr1* KO (B: AC, D: FC) female mice. Qualitative observations of these examples show deficits in cortical temporal processing at p21, but not p30 or p60, in the FC KO mice. No deficits are seen in the AC at any age. All panels show 100% modulation depth. The onset of the gap-ASSR stimulus is at 0 msec in each panel.

Quantitative analyses across the population of female mice recorded support these suggestions (Figure 3.2 and Table 3.1). Statistical analyses using gap duration, age and genotype as factors show a main effect of gap duration in the AC and FC. This is not surprising as the cortex responds with more consistent phase angles (less temporal jitter) across trials to the 40 Hz stimulus with longer gap durations. No genotype differences were identified at any age or modulation depth in the AC, similar to

our previously published male data (Figure 3.2, Table 3.1) [Croom et al., 2023]. Figure 3.3 shows average ITPCs collapsed across all the gap durations. In the AC, there are no statistical differences between WT and KO mice at any age. Taken together, these data suggest developmental improvement in temporal processing of female mice, but no effects of the loss of FMRP, in the AC at any age.

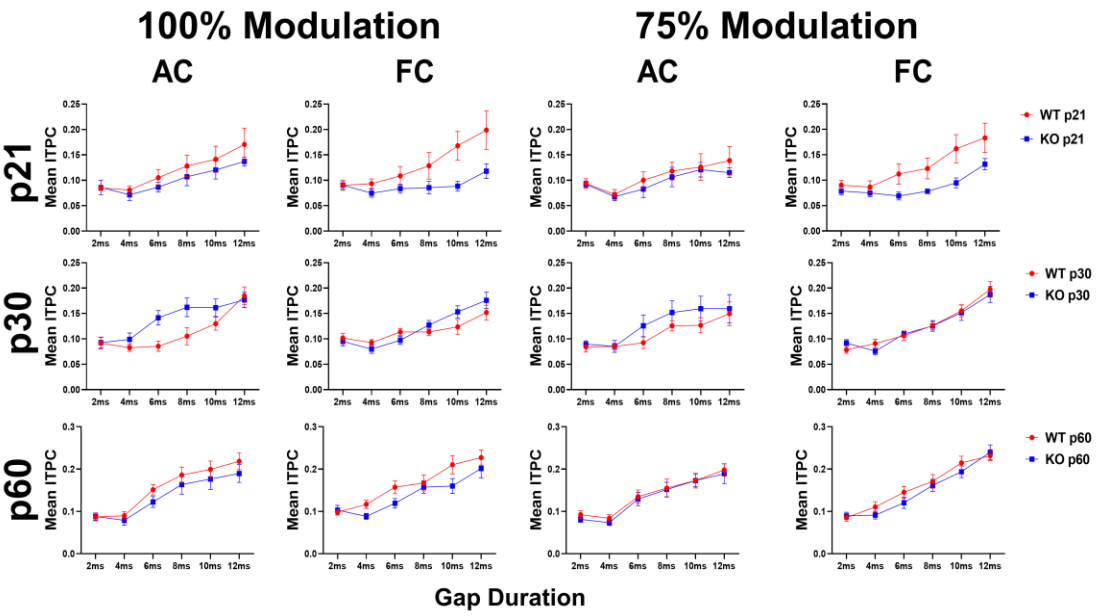

Figure 3.2. Population analysis shows temporal processing deficits in the FC during development in *Fmr1* KO female mice. Each plot represents the group average ITPC values. Each row represents a different age group: p21 (top), p30 (middle), and p60 (bottom). The left columns represent AC and FC data at 100% modulation depth, and the right columns represent AC and FC data at 75% modulation depth. *Fmr1* KO female mice show significant deficits in the FC, but not the AC, at p21. No genotype differences are seen at p30 or p60. Full data results are shown in Table 3.1.

In the frontal cortex, however, significant genotype effects were seen (Figure 3.2, Table 3.1). ITPC increased with age in both WT and *Fmr1* KO females. At both modulation depths, our results show a significant reduction of ITPC in female KO mice at p21 compared to WT female mice (75% MD – p=0.0068; 100% MD – p=0.0026). This deficit is not present at p30 or p60 (p30 – 75% MD p=1.000, 100% MD p=1.000; p60 – 75% MD p=0.9973, 100% MD p=0.3909). Evidence of a developmental delay is shown

more directly by collapsing across gaps, as no significant genotype effect can be seen in the FC at p30 or p60, but a significant reduction is seen in the KO females at P21 (Figure 3.3). Overall, these data show improvement in phase locking to gap-ASSR stimuli during development in both AC and FC in both genotypes, but there is a FC-specific delay in temporal processing in female *Fmr1* KO mice.

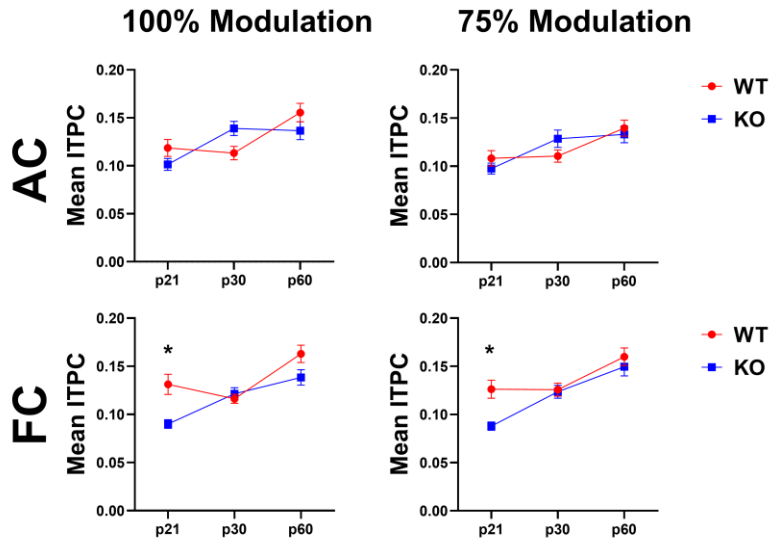

Figure 3.3. Developmental delay in auditory temporal processing in the FC of female *Fmr1* KO mice. Each plot represents the group average ITPC values collapsed across gap widths. Columns represent different modulation depths, and rows represent different cortical regions (Columns – left = 100% modulation, right = 75% modulation; Rows – top = AC, bottom = FC). KO mice show a significant ITPC deficit only at p21 in the FC at both modulation depths, but not at p60. A genotype difference was not seen at any age or modulation depth in the AC.

Table 3.1. Full statistical analysis of female WT and KO gap-ASSR data.

Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
AC	100%	Genotype	F(1,47)=0.9628	0.3315
		Age	F(2,47)=2.5722	0.0870
		Gap Duration	**F(5,235)=3.7481**	**0.0165**
		Genotype x Gap Duration	F(5,235)=0.4926	0.6642
		Age x Gap Duration	F(10,235)=1.2445	0.2913
		Genotype x Age	F(2,47)=1.9964	0.1471
		Genotype x Age x Gap Duration	F(10,235)=1.2189	0.3033
AC	75%	Genotype	F(1,47)=0.3658	0.5482
		Age	F(2,47)=2.1187	0.1315
		Gap Duration	F(5,235)=2.4164	0.0715
		Genotype x Gap Duration	F(5,235)=0.2511	0.8530
		Age x Gap Duration	F(10,235)=1.5942	0.1562
		Genotype x Age	F(2,47)=0.7530	0.4765
		Genotype x Age x Gap Duration	F(10,235)=0.3116	0.9250
FC	100%	**Genotype**	**F(1,47)=6.0378**	**0.01774**
		Age	**F(2,47)=3.6924**	**0.0324**
		Gap Duration	F(5,235)=1.3452	0.2644
		Genotype x Gap Duration	**F(5,235)=3.9951**	**0.0130**
		Age x Gap Duration	F(10,235)=1.9202	0.0935
		Genotype x Age	F(2,47)=1.9326	0.1560
		Genotype x Age x Gap Duration	**F(10,235)=2.5717**	**0.0286**
FC	75%	**Genotype**	**F(1,47)=6.0071**	**0.0180**
		Age	**F(2,47)=6.7788**	**0.0025**
		Gap Duration	**F(5,235)=4.6730**	**0.0037**
		Genotype x Gap Duration	F(5,235)=2.5335	0.0589
		Age x Gap Duration	**F(10,235)=4.0257**	**0.0009**
		Genotype x Age	F(2,47)=1.4508	0.2446
		Genotype x Age x Gap Duration	F(10,235)=1.0932	0.3694

Three-way repeated measures ANOVA results for gap-ASSR analysis. Mauchly Tests for sphericity were utilized and p-values were corrected using the Greenhouse-Geisser method where necessary. See text for post hoc results. Bold text indicates statistical significance (p ≤ 0.05).

Temporal processing matures faster in Fmr1 KO females than males

Figure 3.4 compares *Fmr1* KO female and male data [Croom et al., 2023]. The results show no significant sex difference in the AC at any modulation depth or gap duration. There is a sex difference in the FC at both modulation depths at p30 (p30:

100% MD – p=0.0160; 75% MD – p=0.0034), with female KO mice having significantly higher ITPC compared to males, suggesting that female KO mice have more consistent temporal responses than males across trials at this age. There is no sex difference in *Fmr1* KO mice at p21 or p60. These results show a faster maturation of auditory temporal processing in the FC of KO females compared to males. We also compared male and female WT gap-ASSR responses to test whether this sex difference is unique to *Fmr1* KO mice or is a normative pattern (Figure 3.5). No sex difference was seen in WT mice at any modulation depth in either cortical region. This confirms that female *Fmr1* KO mice show improved auditory temporal processing by p30, while males do not reach WT levels until after p30. Full statistical analysis for WT and KO sex difference gap-ASSR analyses can be found in Tables 3.2 and 3.3.

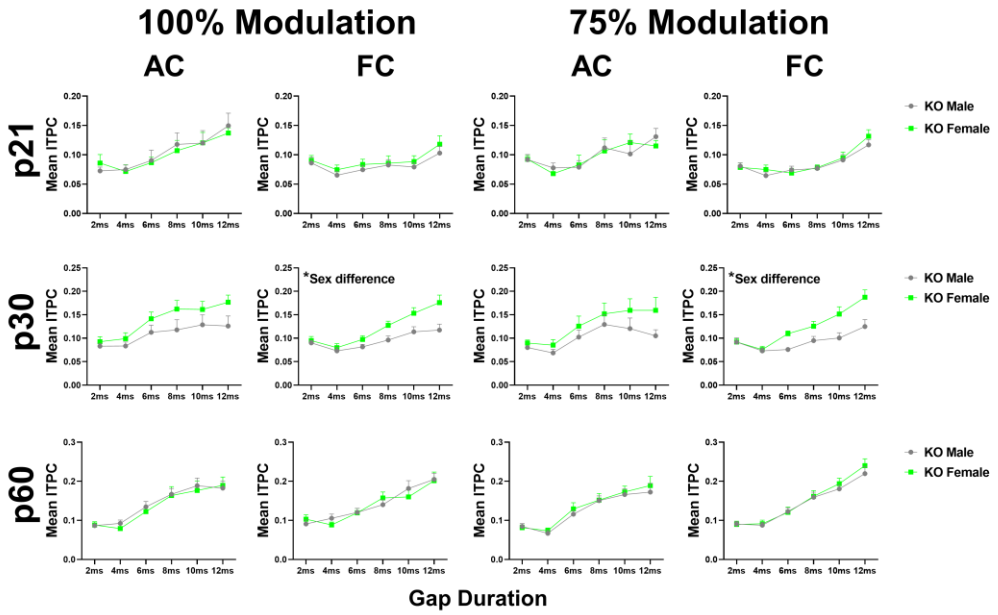

Figure 3.4. Temporal processing matures faster in *Fmr1* KO females than males. Each plot represents the group average ITPC values. Each row represents a different age group: p21 (top), p30 (middle), and p60 (bottom). The left columns represent AC and FC data at 100% modulation depth, and the right columns represent AC and FC data at 75% modulation depth. No significant sex difference in the AC at any modulation depth or gap duration. Female KO mice have significantly higher ITPC in the FC at both modulation depths at p30, but not p21 or p60. Full data results are shown in Table 3.2.

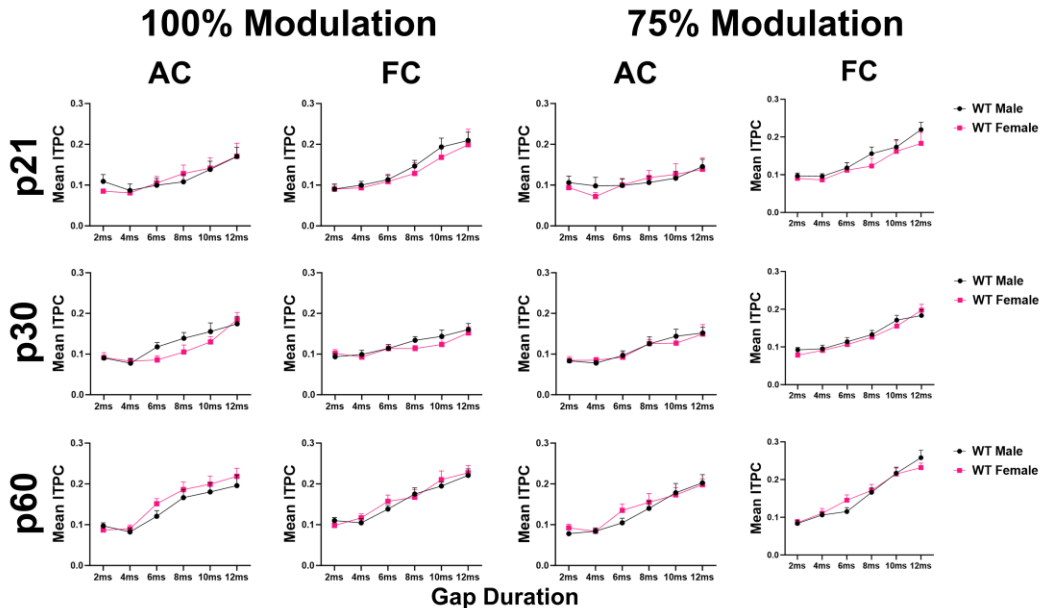

Figure 3.5. No sex difference in auditory temporal processing in WT mice at any in the AC or FC. Each plot represents the group average ITPC values. Each row represents a different age group: p21 (top), p30 (middle), and p60 (bottom). The left columns represent AC and FC data at 100% modulation depth, and the right columns represent AC and FC data at 75% modulation depth. No significant sex difference in the AC or FC at any modulation depth or gap duration. Full data results are shown in Table 3.3.

Table 3.2. Full statistical analysis of male and female KO gap-ASSR data.

Age	Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
P21	AC	100%	Interaction	F(5,80)=0.3211	0.8989
			Gap Duration	**F(2.708,43.34)=11.06**	**<0.0001**
			Sex	F(1,16)=0.02711	0.8713
P21	AC	75%	Interaction	F(5,80)=0.8956	0.4882
			Gap Duration	**F(3.233,51.73)=8.822**	**<0.0001**
			Sex	F(1,16)=0.0053	0.9424
P21	FC	100%	Interaction	F(5,80)=0.1754	0.9711
			Gap Duration	**F(3.740,59.84)=7.125**	**0.0001**
			Sex	F(1,16)=0.6813	0.4213
P21	FC	75%	Interaction	F(5,80)=0.5624	0.7285
			Gap Duration	**F(3.847,61.55)=16.67**	**<0.0001**
			Sex	F(1,16)=0.4218	0.5252
P30	AC	100%	Interaction	F(5,85)=1.187	0.3225
			Gap Duration	**F(2.628,44.67)=14.42**	**<0.0001**
			Sex	F(1,17)=2.700	0.1187
P30	AC	75%	Interaction	F(5,85)=0.8130	0.5436
			Gap Duration	**F(3.024,51.41)=9.528**	**<0.0001**
			Sex	F(1,17)=2.037	0.1716
P30	FC	100%	**Interaction**	**F(5,85)=3.911**	**0.0031**
			Gap Duration	**F(3.160,53.73)=26.73**	**<0.0001**
			Sex	**F(1,17)=7.150**	**0.0160**
P30	FC	75%	**Interaction**	**F(5,85)=3.967**	**0.0028**
			Gap Duration	**F(2.962,50.36)=21.45**	**<0.0001**
			Sex	**F(1,17)=11.53**	**0.0034**
P60	AC	100%	Interaction	**F(5,85)=0.2513**	0.9381
			Gap Duration	**F(2.405,40.88)=29.66**	**<0.0001**
			Sex	F(1,17)=0.1049	0.7500
P60	AC	75%	Interaction	**F(5,85)=0.1959**	0.9633
			Gap Duration	**F(2.455,41.73)=30.59**	**<0.0001**
			Sex	F(1,17)=0.2712	0.6092
P60	FC	100%	Interaction	F(5,85)=0.9318	0.4647
			Gap Duration	**F(2.910,49.46)=28.83**	**<0.0001**
			Sex	F(1,17)=0.0223	0.8831
P60	FC	75%	Interaction	F(5,85)=0.4046	0.8444
			Gap Duration	**F(3.577,60.81)=64.18**	**<0.0001**
			Sex	F(1,17)=0.2619	0.6154

Two-way repeated measures ANOVA results for gap-ASSR analysis comparing male and female Fmr1 KO mice. Sex differences are only seen at p30 in the FC. Degrees of freedom and p-values were corrected for lack of sphericity using the Greenhouse-Geisser method. Bold text indicates statistical significance ($p < 0.05$).

Table 3.3. Full statistical analysis of male and female WT gap-ASSR data.

Age	Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
P21	AC	100%	Interaction	F(5,95)=0.7051	0.6210
			Gap Duration	**F(1.855,32.25)=13.45**	**<0.0001**
			Sex	F(1,19)=3.482e-005	0.9954
P21	AC	75%	Interaction	F(5,95)=0.8763	0.5001
			Gap Duration	**F(2.294,43.58)=6.872**	**0.0017**
			Sex	F(1,19)=0.0296	0.8650
P21	FC	100%	Interaction	F(5,95)=0.2110	0.9571
			Gap Duration	**F(2.161,41.07)=21.59**	**<0.0001**
			Sex	F(1,19)=0.2328	0.6350
P21	FC	75%	Interaction	F(5,95)=0.7227	0.6080
			Gap Duration	**F(1.762,33.49)=29.02**	**<0.0001**
			Sex	F(1,19)=0.6102	0.4444
P30	AC	100%	Interaction	F(5,85)=1.923	0.0989
			Gap Duration	**F(4.066,69.12)=26.12**	**<0.0001**
			Sex	F(1,17)=0.6887	0.4181
P30	AC	75%	Interaction	F(5,85)=0.2505	0.9385
			Gap Duration	**F(2.749,46.74)=13.02**	**<0.0001**
			Sex	F(1,17)=0.0496	0.8264
P30	FC	100%	Interaction	F(5,85)=0.7688	0.5748
			Gap Duration	**F(3.748,63.72)=12.81**	**<0.0001**
			Sex	F(1,17)=0.5620	0.4637
P30	FC	75%	Interaction	F(5,85)=0.6334	0.6748
			Gap Duration	**F(3.708,63.04)=38.15**	**<0.0001**
			Sex	F(1,17)=0.3719	0.5500
P60	AC	100%	Interaction	F(5,85)=0.6398	0.6699
			Gap Duration	**F(2.576,43.80)=32.67**	**<0.0001**
			Sex	F(1,17)=0.6390	0.4351
P60	AC	75%	Interaction	F(5,85)=0.7737	0.5714
			Gap Duration	**F(2.294,39.00)=35.44**	**<0.0001**
			Sex	F(1,17)=0.2400	0.6304
P60	FC	100%	Interaction	F(5,85)=0.7762	0.5696
			Gap Duration	**F(3.919,66.63)=44.83**	**<0.0001**
			Sex	F(1,17)=0.1551	0.6986
P60	FC	75%	Interaction	F(5,85)=1.598	0.1694
			Gap Duration	**F(3.677,62.51)=76.88**	**<0.0001**
			Sex	F(1,17)=0.0253	0.8754

Two-way repeated measures ANOVA results for gap-ASSR analysis comparing male and female WT mice. No sex differences were present at any age. Degrees of freedom and p-values were corrected for lack of sphericity using the Greenhouse-Geisser epsilon-hat method. Bold text indicates statistical significance ($p < 0.05$).

Female Fmr1 KO mice show enhanced cortical ERP amplitudes across development

ERPs consist of a series of voltage fluctuations, referred to as 'waves' (P1, N1, P2). These waves are evoked at specific latencies after sound onset and are associated with the population activity in specific brain regions. Measuring the amplitudes and latencies of these waves allow for the assessment of neuronal response synchrony or hypersensitivity to sound presentation. Table 3.4 and Figures 3.6-3.7 show the complete ANOVA results of female WT and *Fmr1* KO ERP data across development and genotypes.

Auditory Cortex ERP

All three peaks (P1, N1 and P2) show a larger increase with age in the *Fmr1* KO female mice compared to WT females, resulting in significant age-dependent ERP amplitude differences (Figure 3.6A). ERP P1 amplitude increases with age in female *Fmr1* KO mice (interaction effect: $p=0.0507$; main effect of age: $p<0.0001$; KO p21-30: $p=0.0202$; KO p21-60: $p<0.0001$). P1 amplitude is also significantly increased in KO females compared to WT at p30 and p60 (main effect of genotype: $p=0.0010$; p30: $p=0.0341$; p60: $p=0.0054$) (Figure 3.6B). N1 amplitude increases with age in KO mice, but only shows a significant genotype effect at p60 (main effect of age: $p<0.001$; KO p21-60: $p=0.0006$; KO p30-60: $p=0.0006$; main effect of genotype: $p=0.0005$; p60: $p=0.0048$) (Figure 3.6B). Similarly, P2 amplitude increased with age in KO mice and was significantly elevated compared to WT at p60 (main effect of age: $p=0.0257$; KO p30-60: $p=0.0058$; main effect of genotype: $p=0.0059$; p60: $p=0.0226$) (Figure 3.6B). No genotype or age differences were seen in P1 or P2 latencies, but N1 latency decreased with age in WT females (main effect of age: 0.0059; WT p21-30: $p=0.0504$; WT p21-60:

p=0.0010) (Figure 3.6C). These data show increased ERP amplitudes in the AC of female *Fmr1* KO mice as observed consistently in humans with FXS. Furthermore, this hypersensitivity increases with age in female KO mice.

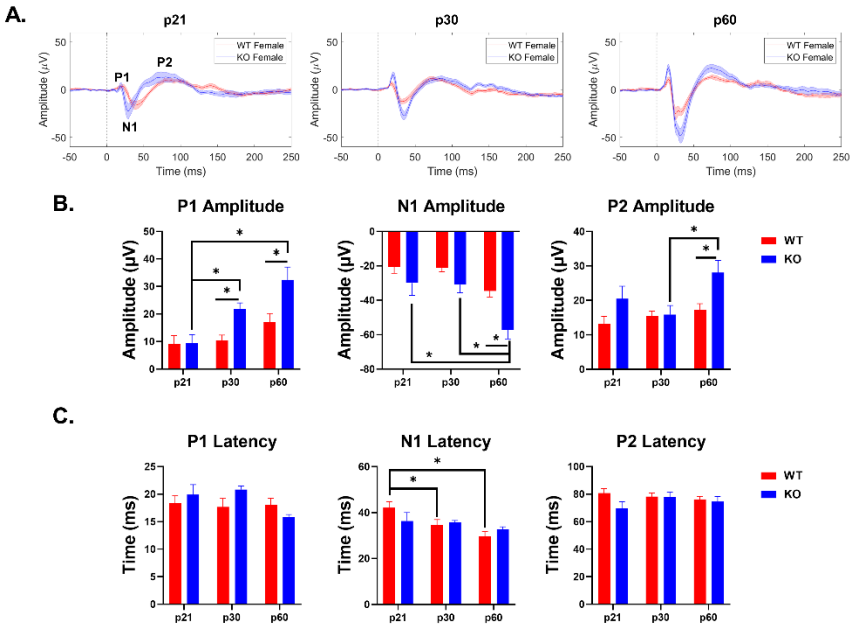

Figure 3.6. Age and genotype impact ERP amplitudes and latencies in the AC of female mice. (A) Average ERPs recorded in the AC for WT and KO female mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of AC ERP wave amplitudes. P1 amplitude significantly increases in KO mice with development, but not WT mice. KO mice have increased P1 amplitudes compared to WT at p30 and p60. N1 and P2 amplitudes are enhanced in adult KO females compared to WT and increase with age. (C) AC ERP wave latencies. N1 latency decreases with age in WT mice. Full data results are shown in Table 3.4.

Frontal Cortex ERP

As in the AC, frontal cortex ERP amplitudes show a more pronounced developmental increase in female KO mice, compared to WT females (Figure 3.7A). ERP P1 and N1 amplitudes increase with age in KO female mice (P1 – interaction effect: p=0.0318; main effect of age: p=0.0021; KO p21-p60: p=0.0002; KO p30-p60: p=0.0277; N1 – interaction effect: 0.0109; main effect of age: p<0.0001; KO p21-60: p<0.0001; KO p30-p60: p=0.0011) (Figure 3.7B). N1 and P2 amplitudes increase with age in WT female mice (N1 – main effect of age: p<0.0001; WT p30-p60: p=0.0314; P2 – main effect of age: p=0.0283; WT p21-60: p=0.0249) (Figure 3.7B). Female KO mice have increased N1 amplitudes at p30 and p60 compared to WT (main effect of genotype: 0.0014; p30: p=0.0354; p60: p=0.0019) (Figure 3.7B). N1 and P2 latencies showed developmental fluctuations in WT and KO mice, respectively (N1 – main effect of age: 0.0020; WT p30-p60: 0.0440; P2 – main effect of age: 0.0365; KO p21-30: p<0.0001;

KO p21-60: p=0.0034) (Figure 3.7C). These data show increased ERP amplitudes, specifically N1, in the FC of female *Fmr1* KO mice.

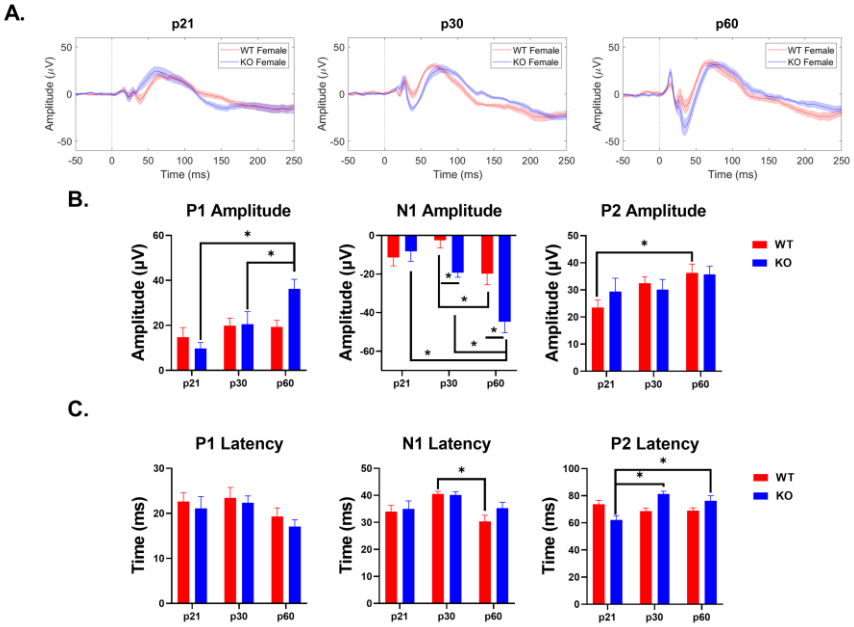

Figure 3.7. Age and genotype impact ERP amplitudes and latencies in the FC of female mice. (A) Average ERPs recorded in the FC for WT and KO female mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of FC ERP wave amplitudes. P1 amplitude significantly increases in KO mice with development, but not WT mice. N1 amplitudes are enhanced in KO females at p30 and p60. N1 amplitudes increase with age in WT and KO females. P2 amplitudes increase with age in WT mice. (C) FC ERP wave latencies. N1 latency decreases with age in WT mice. P2 latency fluctuates with age in KO mice. Full data results are shown in Table 3.4.

Table 3.4. Full statistical analysis of female development ERP data.

Cortical Region	ERP Component	Factor	ANOVA Results	p-value
AC	P1 Amplitude:	**Interaction**	**F(2,47)=3.179**	**0.0507**
		Age	**F(2,47)=12.03**	**<0.0001**
		Genotype	**F(1,47)=12.39**	**0.0010**
	N1 Amplitude:	Interaction	F(2,47)=1.292	0.2843
		Age	**F(2,47)=12.50**	**<0.0001**
		Genotype	**F(1,47)=13.94**	**0.0005**
	P2 Amplitude:	Interaction	F(2,47)=2.022	0.1438
		Age	**F(2,47)=3.960**	**0.0257**
		Genotype	**F(1,47)=8.327**	**0.0059**
FC	P1 Amplitude:	**Interaction**	**F(2,47)=3.713**	**0.0318**
		Age	**F(2,47)=7.045**	**0.0021**
		Genotype	F(1,47)=1.562	0.2175
	N1 Amplitude:	**Interaction**	**F(2,47)=4.987**	**0.0109**
		Age	**F(2,47)=14.40**	**<0.0001**
		Genotype	**F(1,47)=11.60**	**0.0014**
	P2 Amplitude:	Interaction	F(2,47)=0.8274	0.4434
		Age	**F(2,47)=3.851**	**0.0283**
		Genotype	F(1,47)=0.1218	0.7287
AC	P1 Latency:	Interaction	F(2,47)=2.187	0.1235
		Age	F(2,47)=1.931	0.1564
		Genotype	F(1,47)=0.6171	0.4361
	N1 Latency:	Interaction	F(2,47)=2.009	0.1455
		Age	**F(2,47)=5.729**	**0.0059**
		Genotype	F(1,47)=0.0706	0.7916
	P2 Latency:	Interaction	F(2,47)=1.445	0.2461
		Age	F(2,47)=0.4215	0.6585
		Genotype	F(1,47)=2.342	0.1327
FC	P1 Latency:	Interaction	F(2,47)=0.0354	0.9643
		Age	F(2,47)=2.823	0.0695
		Genotype	F(1,47)=0.9239	0.3414
	N1 Latency:	Interaction	F(2,47)=0.8245	0.4447
		Age	**F(2,47)=7.105**	**0.0020**
		Genotype	F(1,47)=1.141	0.2909
	P2 Latency:	**Interaction**	**F(2,47)=11.18**	**0.0001**
		Age	**F(2,47)=3.555**	**0.0365**
		Genotype	F(1,47)=1.494	0.2277

Two-way ANOVA results for ERP analysis. Post hoc comparisons were done using Tukey's and Bonferroni's multiple comparisons tests. See text for post hoc results. Bold text indicates statistical significance (p = or < 0.05).

Development of WT and Fmr1 KO male and female ERP phenotypes

Auditory cortex – WT mice

ERP P1 amplitudes were not impacted by age or sex in the AC. However, both N1 and P2 amplitudes were affected by age (N1 – main effect of age: p=0.0188; P2 –

main effect of age: p=0.0264) (Figure 3.8B). Specifically, female and male N1 and P2 amplitudes increased with age, respectively (N1 – female p21-p60: p=0.0330; P2 – male p21-p60: p=0.0309) (Figure 3.8B). N1 and P2 latencies were also impacted by age (N1 – main effect of age: p=0.0004; P2 – main effect of age: p=0.0519) (Figure 3.8C). N1 latency decreased with age in female mice (p21-p30: p=0.0233; p21-p60: p=0.0002) (Figure 3.8C). P2 latency decreased with age in males (p21-p60: p=0.0316) (Figure 3.8C). No sex differences were seen in any wave amplitude or latency. Overall, these results suggest that responses are similar in male and female WT mice throughout development in the AC. Table 3.5 shows the complete ANOVA analyses of male and female WT AC ERP data across development.

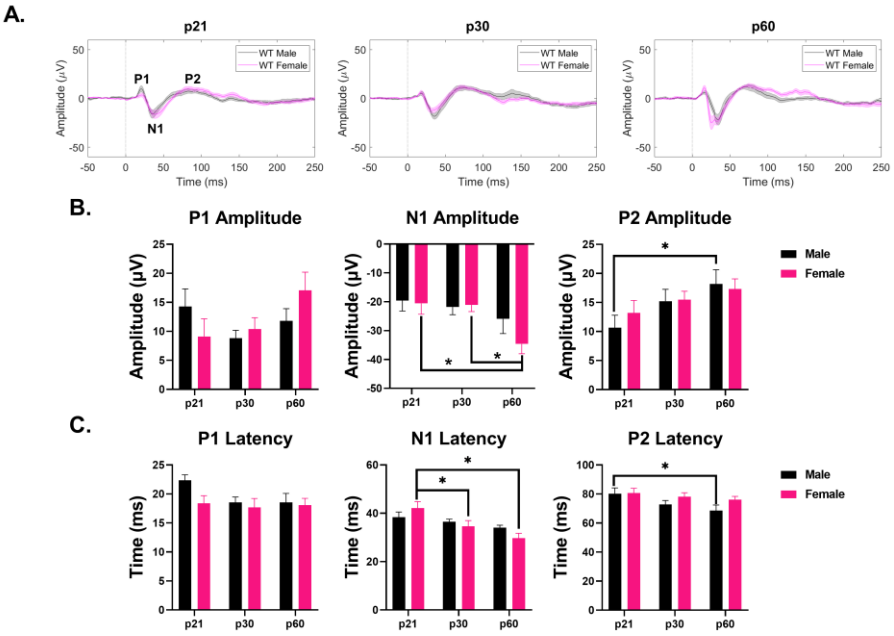

Figure 3.8. No sex difference in ERP amplitudes or latencies in the AC of WT mice. (A) Average ERPs recorded in the AC for WT male and female mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of AC ERP wave amplitudes. N1 amplitudes increase with age in females. P2 amplitudes increase with age in males. (C) AC ERP wave latencies. N1 latency decreases with age in females. P2 latency decreases with age in males. Full data results are shown in Table 3.5.

Frontal cortex – WT mice

ERP wave amplitudes were not affected by age or sex in the FC (Figure 3.9B). N1 latency showed developmental fluctuations in female mice (main effect of age: p=0.0002; p21-p30: p=0.0253; p30-p60: p=0.0009) (Figure 3.9C). A significant sex difference was identified in P2 latencies at p21 (main effect of sex: p=0.0011; p21: p=0.0379) (Figure 3.9C). These results suggest no significant sex difference in ERP peak amplitudes in the FC of WT mice during development. Table 3.5 shows the complete ANOVA analyses of male and female WT FC ERP data across development.

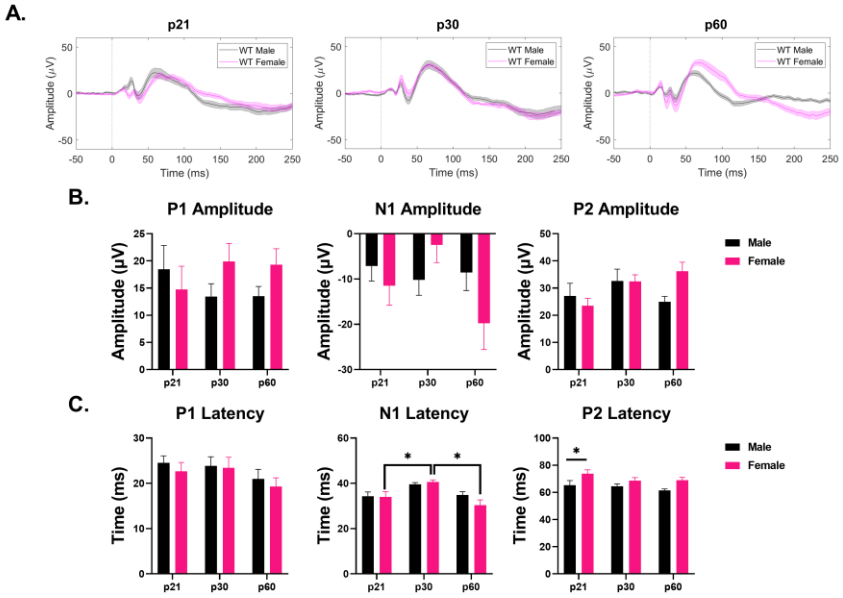

Figure 3.9. Sex difference in ERP latencies in the FC of WT mice. (A) Average ERPs recorded in the FC for WT male and female mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of FC ERP wave amplitudes. No impact of age or sex on any ERP wave amplitude. (C) FC ERP wave latencies. N1 latency fluctuates with age in females. P2 latency is increased in female WT mice compared to males. Full data results are shown in Table 3.5.

Table 3.5. Full statistical analysis of WT development ERP data.

Cortical Region	ERP Component	Factor	ANOVA Results	p-value
AC	P1 Amplitude:	Interaction	F(2,53)=2.202	0.1206
		Age	F(2,53)=1.729	0.1873
		Sex	F(1,53)=0.0689	0.7939
	N1 Amplitude:	Interaction	F(2,53)=0.8576	0.4300
		Age	**F(2,53)=4.289**	**0.0188**
		Sex	F(1,53)=0.9495	0.3343
	P2 Amplitude:	Interaction	F(2,53)=0.3466	0.7087
		Age	**F(2,53)=3.895**	**0.0264**
		Sex	F(1,53)=0.1412	0.7086
FC	P1 Amplitude:	Interaction	F(2,53)=1.460	0.2415
		Age	F(2,53)=0.0029	0.9971
		Sex	F(1,53)=1.067	0.3062
	N1 Amplitude:	Interaction	F(2,53)=2.609	0.0830
		Age	F(2,53)=1.770	0.1803
		Sex	F(1,53)=0.6039	0.4405
	P2 Amplitude:	Interaction	F(2,53)=2.569	0.0862
		Age	F(2,53)=2.502	0.0916
		Sex	F(1,53)=0.8089	0.3725
AC	P1 Latency:	Interaction	F(2,53)=1.142	0.3268
		Age	F(2,53)=1.935	0.1544
		Sex	F(1,53)=2.848	0.0974
	N1 Latency:	Interaction	F(2,53)=2.322	0.1080
		Age	**F(2,53)=9.198**	**0.0004**
		Sex	F(1,53)=0.2755	0.6018
	P2 Latency:	Interaction	F(2,53)=0.6371	0.5328
		Age	F(2,53)=3.130	0.0519
		Sex	F(1,53)=2.747	0.1034
FC	P1 Latency:	Interaction	F(2,53)=0.078	0.9250
		Age	F(2,53)=1.919	0.1568
		Sex	F(1,53)=0.6243	0.4330
	N1 Latency:	Interaction	F(2,53)=1.279	0.2867
		Age	**F(2,53)=9.972**	**0.0002**
		Sex	F(1,53)=0.7294	0.3969
	P2 Latency:	Interaction	F(2,53)=0.4331	0.6508
		Age	F(2,53)=1.793	0.1764
		Sex	**F(1,53)=11.90**	**0.0011**

Two-way ANOVA results for ERP analysis. Post hoc comparisons were done using Tukey's and Bonferroni's multiple comparisons tests. See text for post hoc results. Bold text indicates statistical significance ($p < 0.05$).

Auditory cortex – Fmr1 KO mice

ERP P1 amplitudes increased with age in male and female KO mice (main effect of age: $p<0.0001$; male – p21-p60: $p<0.0001$, p30-p60: $p=0.0008$; female – p21-p60:

p=0.0004) (Figure 3.10B). N1 amplitude significantly increased with age only in females (main effect of age: p=0.0010; p21-p60: p=0.0036; p30-60: p=0.0039) (Figure 3.10B). P2 amplitude was significantly elevated in adult female KO and increased with age (interaction effect: p=0.0285 main effect of sex: p=0.0378; p60: p=0.0156) (Figure 3.10B). P1 latency significantly decreased with age in females (main effect of age: p=0.0055; p21-p60: p=0.0532; p30-60: p=0.0106), but no sex or age difference was seen in N1 or P2 latencies (Figure 3.10C). The results suggest that adult female *Fmr1* KO mice have increased hypersensitivity, based on ERP amplitudes, compared to adult males in the AC. Table 3.6 shows the complete ANOVA analyses of male and female *Fmr1* KO AC ERP data across development.

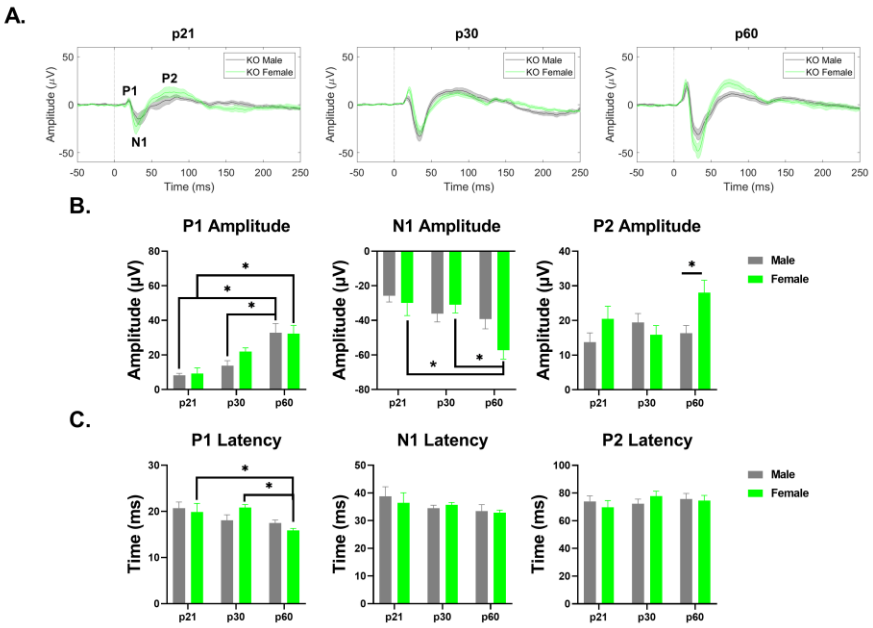

Figure 3.10. Sex difference in ERP amplitudes in the AC of *Fmr1* KO mice. (A) Average ERPs recorded in the AC for *Fmr1* KO male and female mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of AC ERP wave amplitudes. P1 amplitudes increase with age in male and female KO mice. N1 amplitudes increase with age in females. P2 amplitudes are significantly higher in adult female KO mice. (C) AC ERP wave latencies. P1 latency decreases with age in KO females. Full data results are shown in Table 3.6.

Frontal cortex – Fmr1 KO mice

Both P1 and N1 amplitudes increased with age in female KO mice (P1 – main effect of age: p=0.0001; p21-60: p=0.0006; p30-p60: p=0.0475; N1 – main effect of age: p<0.0001; p21-p60: p<0.0001; p30-p60: p=0.0002) (Figure 3.11B). However, only P1 amplitudes significantly increased with age in male KO (p21-p60: p=0.0424) (Figure 3.11B). Female KO have significantly increased N1 and P2 amplitudes compared to males, which increases age (N1 – interaction effect: p=0.0026; main effect of sex: p=0.0478; p60: p=0.0004; P2 – interaction effect: p=0.0376; main effect of sex: p=0.0027; p60: p=0.0011) (Figure 3.11B). Main effects of age were identified in P1 and P2 latencies, with P2 latencies increasing during development in females (P1 – main effect of age: 0.0570; P2 – interaction effect: p=0.0273; main effect of age: p=0.0004; p21-p30: p=0.0001; p21-60: p=0.0068) (Figure 3.11C). Overall, these results suggest that ERP amplitudes increase with age in both males and females, but female *Fmr1* KO mice develop increased hypersensitivity with age compared to males in the FC. Table 3.6 shows the complete ANOVA analyses of male and female *Fmr1* KO FC ERP data across development.

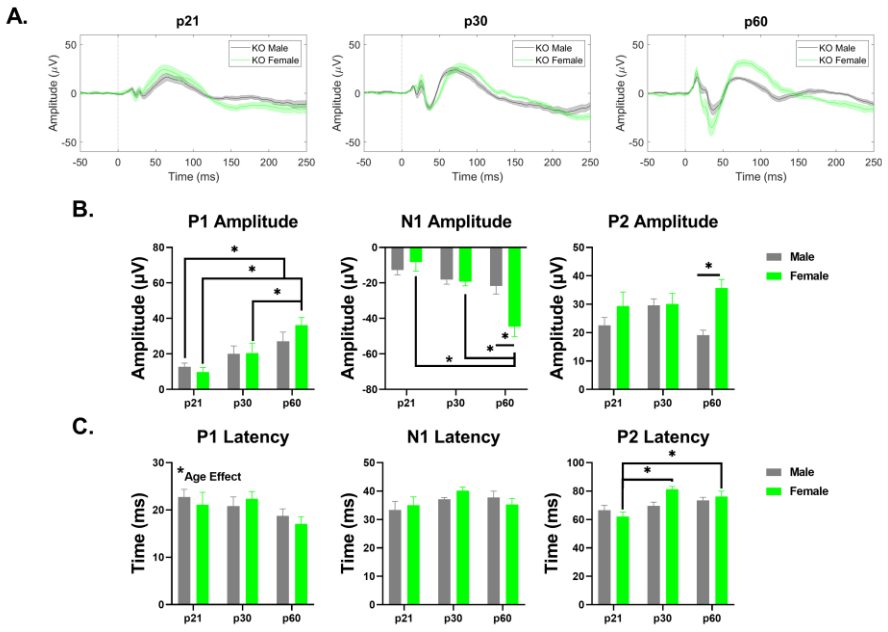

Figure 3.11. Sex difference in ERP amplitudes in the FC of *Fmr1* KO mice. (A) Average ERPs recorded in the FC for *Fmr1* KO male and female mice at p21 (left), p30 (middle), and p60 (right). (B) Population averages of FC ERP wave amplitudes. P1 amplitudes increase with age in male and female KO mice. N1 amplitudes increase with age in females. N1 and P2 amplitudes are significantly higher in adult female KO mice. (C) FC ERP wave latencies. P1 latency is impacted by age. P2 latency increases with age in KO females. Full data results are shown in Table 3.6.

Table 3.6. Full statistical analysis of KO development ERP data.

Cortical Region	ERP Component	Factor	ANOVA Results	p-value
AC	P1 Amplitude:	Interaction	F(2,50)=0.7898	0.4595
		Age	**F(2,50)=21.28**	**<0.0001**
		Sex	F(1,50)=0.9544	0.3333
	N1 Amplitude:	Interaction	F(2,50)=2.486	0.0935
		Age	**F(2,50)=7.916**	**0.0010**
		Sex	F(1,50)=1.747	0.1922
	P2 Amplitude:	**Interaction**	**F(2,50)=3.825**	**0.0285**
		Age	F(2,50)1.903	0.1598
		Sex	**F(1,50)=4.555**	**0.0378**
FC	P1 Amplitude:	Interaction	F(2,50)=1.002	0.3743
		Age	**F(2,50)=10.83**	**0.0001**
		Sex	F(1,50)=0.3887	0.5358
	N1 Amplitude:	**Interaction**	**F(2,50)=6.737**	**0.0026**
		Age	**F(2,50)=16.96**	**<0.0001**
		Sex	**F(1,50)=4.119**	**0.0478**
	P2 Amplitude:	**Interaction**	**F(2,50)=3.507**	**0.0376**
		Age	F(2,50)=0.8073	0.4518
		Sex	**F(1,50)=9.931**	**0.0027**
AC	P1 Latency:	Interaction	F(2,50)=2.242	0.1168
		Age	**F(2,50)=5.773**	**0.0055**
		Sex	F(1,50)=0.0156	0.9008
	N1 Latency:	Interaction	F(2,50)=0.2806	0.7565
		Age	F(2,50)=1.695	0.1940
		Sex	F(1,50)=0.0838	0.7734
	P2 Latency:	Interaction	F(2,50)=0.7940	0.4576
		Age	F(2,50)=0.4282	0.6540
		Sex	F(1,50)=0.0013	0.9714
FC	P1 Latency:	Interaction	F(2,50)=0.5262	0.5941
		Age	F(2,50)=3.034	0.0570
		Sex	F(1,50)=0.1518	0.6984
	N1 Latency:	Interaction	F(2,50)=0.8430	0.4364
		Age	F(2,50)=1.986	0.1479
		Sex	F(1,50)=0.1684	0.6833
	P2 Latency:	**Interaction**	**F(2,50)=3.872**	**0.0273**
		Age	**F(2,50)=9.160**	**0.0004**
		Sex	F(1,50)=2.031	0.1603

Two-way ANOVA results for ERP analysis. Post hoc comparisons were done using Tukey's and Bonferroni's multiple comparisons tests. See text for post hoc results. Bold text indicates statistical significance ($p < 0.05$).

Discussion

The major novel contribution of this study is the identification of sex differences in the developmental trajectories of auditory temporal processing and auditory ERP amplitudes in *Fmr1* KO mice (summary in Table 3.7). The results show genotype, cortical region and age-specific abnormalities in gap-ASSR responses and ERPs in female

mice. A significant developmental delay was seen in gap-ASSR responses in the FC, but not the AC, of female *Fmr1* KO mice compared to WT female mice. However, when compared to male *Fmr1* KO mice, female *Fmr1* KO mice show faster maturation of temporal processing. ERP amplitudes were significantly higher in *Fmr1* KO females than WT females throughout development in the AC and FC, compared to female WT mice. However, adult *Fmr1* KO mice displayed sex differences, with females showing increased N1 and P2 amplitudes compared to males. There were no sex differences in temporal processing or ERP amplitudes in WT mouse cortex indicating that the KO sex differences are not normative, and are related to the loss of FMRP. Taken together, these data show diverging trajectories of ERP and temporal processing phenotypes in female *Fmr1* KO mice, with earlier normalization of temporal processing, but more hypersensitive responses with development, compared to KO males.

Stimuli	Cortical Region	p21		p30		p60	
		Male	Female	Male	Female	Male	Female
Gap-ASSR	AC	*NO*	*NO*	*NO*	*NO*	*NO*	*NO*
	FC	*YES*	*YES*	*YES*	*NO*	*NO*	*NO*
ERP	AC	*NO*	*NO*	*NO*	*YES*	*YES*	*YES*
	FC	*NO*	*NO*	*NO*	*YES*	*YES*	*YES*

Table 3.7. Summary of genotype differences of both sexes in gap-ASSR/ERP measures across development. 'Yes' and 'No' indicates whether a genotype effect was present between WT and Fmr1-KO mice.

EEG recordings from humans with FXS demonstrate altered cortical oscillatory activity, including elevated broadband gamma power and reduced phase locking to auditory spectrotemporal modulations, particularly ~40 Hz. Increased ERP amplitudes are also commonly seen across studies of humans with FXS [Ethridge et al., 2019; Castrén et al., 2003; Knoth & Lippé, 2012; Rojas et al., 2001; St Clair et al., 1987; Van der Molen et al., 2012a, 2012b]. Sex differences in EEG responses in FXS have not received much attention, however, compared to behavioral studies [Petroni et al., 2022;

Nolan et al., 2017; Quin et al., 2005; Schmitt et al., 2023]. Ethridge et al. (2019) showed that in the resting state, females with FXS showed increased alpha power relative to typically-developing females, whereas a reduction in alpha power is seen in male FXS patients [Ethridge et al., 2019; Van der Molen & Van der Molen, 2013; Wang et al., 2017]. In a follow-up study, Smith et al. (2021) show that males with FXS have a lower peak alpha frequency, but not females [Smith et al., 2021]. Additionally, females with FXS show stronger phase locking to spectrotemporally modulated sounds than FXS males [Ethridge et al., 2019]. These findings suggest sex differences in EEG responses in humans with FXS. Our findings of a female advantage in temporal processing in *Fmr1* KO mice is consistent with the human studies.

One major finding of this study is the elevated ERP amplitudes in female *Fmr1* KO mice compared to WT females, and compared to male *Fmr1* KO mice, in both cortical regions. The P1-N1-P2 ERP complex marks the pre-attentive detection of sound and can vary with stimulus features. P1 and N1 amplitudes mark initial sound detection, including thalamocortical input and primary auditory cortex activity, respectively. P2 amplitudes are thought to be related to arousal as auditory input to the mesencephalic reticular activating system contributes to P2 generation [Crowley & Colrain, 2004]. Because N1 and P2 are generated by structures involved in early auditory processing, their enhancement, which is commonly seen in FXS, may reflect altered perception of auditory stimulus [Näätänen et al., 2007]. Our data shows that female KO mice have significantly larger P2 amplitudes in both AC and FC compared to male KO mice. This suggests enhanced activation of the arousal component in female *Fmr1* KO mice. Arousal, along with anxiety and avoidance, represent three key behaviors exhibited in response to acute, potential, and sustained threats. Furthermore, these are the typical

responses to aversive or dangerous stimuli. Previous studies suggest that the dysregulation of these responses can result in clinical manifestation of emotional disorders, including anxiety and depression [Bartholomay et al., 2019]. It is possible in humans with FXS that evoked sensory responses are larger in female patients compared to males. Gesi et al., (2021) found that adult females with ASD reported significantly higher scores than men in the hyper/hyporeactivity to sensory input domain, but clearly additional studies are needed to determine if robust sex differences are seen in abnormal sensory sensitivity in humans with ASD, as suggested by our preclinical data [Gesi et al., 2021]. While males with ASD more commonly show externalizing behavior problems, such as aggression, hyperactivity and restricted behaviors, females with FXS show greater internalizing symptoms, including anxiety and depression, as well as social difficulties [Hattier et al., 2011; Mandy et al., 2011; Szatmari et al., 2011; Bölte et al., 2011; Giarelli et al., 2010; Solomon et al., 2011]. These opposing symptomologies may be due in part to increased activation of the arousal system in females with FXS. Increased N1 amplitudes were seen in female FC (and a trending increase of N1 in AC), compared to male KO mice suggesting that hypersensitive cortical responses are further enhanced in female mice. No sex differences were seen in WT ERP amplitudes suggesting a deviation in KO females from a normative trajectory. The difference between male and female KO mice in ERP amplitude is largest in the P60 group, suggesting a late developing sex difference in hypersensitivity.

A second major finding of this study is that temporal processing matures faster in the female *Fmr1* KO mice, compared to males. In male KO mice, reduced ITPC is seen in the FC at both p21 and p30, but in the female KO mice, reduced ITPC is only present at p21. While tonotopic maps and the balance between excitatory and inhibitory inputs

are established earlier in development (<p21), the p30-40 window is a critical period for development of selectivity for spectrotemporally complex sounds in the mouse auditory cortex [Oswald & Reyes, 2008, 2010; Kim et al., 2013; Carrasco et al., 2013; 71-73, Meng et al., 2019; Bhumika et al., 2019; Nakamura et al., 2020]. Impairments in temporal processing during this time window in male *Fmr1* KO mice will lead to abnormal development of cortical selectivity for complex sounds, and consequently to long-term abnormalities in auditory processing. In female *Fmr1* KO mice, temporal processing is WT-like before p30 and this earlier maturation may result in less severe long term consequences in processing of complex sounds. Disruptions of critical period timelines cause long term impairments in behavioral phenotypes. Although these behaviors might appear normalized by adulthood, any irregularities during key developmental phases will have long-term consequences for behaviors that build on normal development of responses. For example, developmental delay in FC temporal processing may lead to long term abnormalities in behaviors that depend on accurate temporal processing such as speech, language and binaural processing. Given the importance of selectivity to spectrotemporal cues in the development of human speech and language function, a similar delay in development of normal temporal processing in males with FXS, compared to females, will result in sex differences in long term deficits in language function. Studies of development of temporal processing and associations with language function are needed in male and female children with FXS.

The development of temporal response properties in the primary auditory cortex of both mice and rats has been shown to be cell-type specific [Froemke et al., 2011; Cai et al., 2017]. Although inhibitory responses mature later than excitatory responses, regular-spiking neurons (putative excitatory cells) demonstrate weaker stimulus-following

ability compared with fast-spiking (putative inhibitory) neurons [Cai et al., 2017; Knipper et al., 2022]. Postsynaptic current duration also differs in the developing auditory cortex, such that inhibitory currents are prolonged compared to excitatory and cause a slower following capacity of two closely timed stimuli [Cai et al., 2017; Kotak et al., 2008; Takesian et al., 2012; Oswald & Reyes, 2010]. Although the inhibitory duration gradually shortens with development, the longer durations could cause overlap and summation of inhibitory inputs evoked by closely following stimuli, such as in the beginning and end of a gap. Inhibitory dysfunction in FXS is well-established. Nomura et al. (2017) demonstrated a delay in the maturation of the intrinsic properties of fast-spiking interneurons in the sensory cortex as well as a deficit in the formation of excitatory synaptic inputs on to these neurons in *Fmr1* KO mice at p9 [Nomura et al., 2017]. Inhibitory circuits have been implicated in gap detection, however it has been suggested that they provide dynamic gain control over local activity rather than play a specialized role in gap detection. Specifically, Keller et al. (2018) showed that parvalbumin-positive interneurons have stronger on- and off-responses as well as post-response suppression compared to pyramidal neurons. Similar properties were seen for white noise bursts, suggesting that these are generalized response properties of parvalbumin-positive cells [Keller et al., 2018]. Given the impairment of inhibition in FXS, future studies should investigate the role of inhibitory cell types using gap detection paradigms. While these mechanisms may underlie improved temporal processing with age, there appears to be no sex differences in the WT mice. How sex differences emerge in the *Fmr1* KO mice has not been explored in terms of cell-type specific responses. However, it should be noted that temporal processing deficits may arise purely from local cortical circuit deficits and/or from subcortical deficits (including brainstem deficits) [Nguyen et al., 2020;

McCullagh et al., 2020]. FMRP is normally expressed along most of the auditory pathway, and future studies should examine the effects of regional FMRP loss along the auditory pathway on temporal processing development in male and female mice.

A consistent phenotype seen in both male and female *Fmr1* KO mice is that developmental delays in temporal processing are seen in the FC, but not the AC. These findings suggest two key points. Firstly, a lack of deficit in the AC suggests that the FC does not simply inherit auditory responses from the AC, but rather additional local processing within the FC and/or auditory pathways that bypass the AC may be involved in producing phase locked responses in the FC. Secondly, the dichotomy of maturation in males and females could bring about long-term consequences in the FC related to top-down interactions and could possibly give rise to the opposing timelines of language development seen in humans with FXS. The FC induces top-down modulation of AC responses in a task- and attention-dependent manner [Fritz et al., 2010]. FC-AC connection and its modulation of speech have also been evaluated in humans with FXS. Speech production depends on feedforward control and the synchronization of neural oscillations between the FC and AC. Specifically, the interactions of these two regions allow for comparison of the corollary discharge of intended speech generated from an efference copy of speech to the actual speech sounds produced, a process essential for making adaptive adjustments to optimize future speech [Schmitt et al., 2019]. Furthermore, top-down corticothalamic projections to the medial geniculate body have been shown to influence temporal processing and stimulus encoding [Kommajosyula et al., 2021]. Atypical regional connectivity patterns, with both hyper- and hypo-connectivity are observed in ASD [Müller et al., 2011; O'Reilley et al., 2017]. Long-range connectivity appears to be reduced, while local connectivity may be increased. The few studies in

FXS that have examined cross-regional or cross-frequency coupling show abnormal connectivity. However, even fewer studies have examined sex differences in connectivity. Wang et al. (2017) used both males and females with FXS and found increased theta-to-gamma but decreased alpha-to-gamma band amplitude coupling in resting EEG signals in both sexes [Wang et al., 2017]. Schmitt et al. (2022) also reported gamma band hyper-connectivity and alpha band hypo-connectivity within frontal cortex in individuals with FXS, but once again found there to be no sex difference [Schmitt et al., 2022]. Future studies should examine sex differences in FC-AC functional connectivity during development in humans with FXS to identify potential correlations with abnormal language development.

The mechanisms responsible for the earlier maturation of temporal processing in the female *Fmr1* KO mice are unclear. A recent human study discovered a prolonged alpha state during the pre-stimulus period of an auditory evoked task in females with FXS. Norris et al. (2022) hypothesized that the length of time spent in alpha may reflect a compensatory mechanism that could potentially 'rescue' sensory processing abilities [Norris et al., 2022]. Therefore, the sustained alpha state identified in females could account for improvements seen in females versus males with FXS. Another potential mechanism involves a sex-specific interaction between Group 1 metabotropic receptors (mGluR1 and mGluR5) and estrogen receptor α (ERα). This is an intriguing hypothesis as elevated mGluR5 signaling is heavily reported in FXS [Huber et al., 2002; Bear et al., 2004; Darnell & Klann, 2013]. This sex-specific interaction between Group 1 metabotropic receptors and ERα has been identified specifically in female neurons in multiple brain regions [Martinez et al., 2014; Tabatadze et al., 2015; Tonn Eisinger et al., 2018; Santollo & Daniels, 2019]. In the hippocampus, estradiol acts via ERα to initiate

postsynaptic mGluR1-dependent mobilization of the endocannabinoid anandamide to suppress GABA release [Huang & Woolley, 2012]. Additionally, this dual interaction has been shown to mediate the estradiol effects on hippocampal memory consolidation [Boulware et al., 2013]. ERα-mGluR5 signaling was seen exclusively in female striatal neurons as well [Grove-Strawser et al., 2010]. This interaction of receptors has not been investigated in rodent models of FXS. Future studies should evaluate this sex-specific mechanism in female neurons as it could provide an explanation for the sex differences seen in *Fmr1* KO mice.

Conclusions

This is the first study to test and report sex differences during development in sensory processing in any ASD animal model. In terms of temporal processing, we used the 40 Hz ASSR paradigm, which models phonemic rates in speech [Luo & Poeppel, 2012]. Slower oscillations (delta to theta) may be more relevant to aspects of intonation and syllabic rates, and other aspects of speech with slower rates. Future studies will examine 10 and 20 Hz ASSRs in the *Fmr1* KO and WT mice, that may allow a prediction of the nature of speech deficits in humans with FXS. Given the robust sex differences and different trajectories of temporal processing versus hypersensitivity phenotypes in male and female mice with an identical gene knockout, future studies should examine possible role of gonadal hormones in the emergence of sex differences, either with gonadectomy at specific ages, or implants to release hormones over a specific time window. The peri-pubertal window is a critical period of development in *Fmr1* KO mice that is marked by cortical hyperexcitability and reduced inhibitory interneuron function [Wen et al., 2018, 2019; Kulinich et al., 2020]. However, these studies were carried out

only in male mice. Future studies will characterize these developmental milestones in female *Fmr1* KO mice. In order to effectively treat humans with FXS, it is imperative to understand the sex differences and the developmental trajectory of phenotypes that are likely to be used as clinical outcome measures, as opposed to just adult male comparisons. The differing trajectories of temporal processing and hypersensitivity in female compared to male KO mice suggests that more developmental studies of human females with FXS are needed. Future studies in humans with FXS should evaluate temporal processing across age in both males and females to determine if similar delays in development are present, and if the delay relates to language function.

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Chapter 4

Developmental Trajectory and Sex Differences in Auditory Processing in a PTEN-deletion model of Autism Spectrum Disorders

Abstract

Autism Spectrum Disorders (ASD) encompass a wide array of debilitating symptoms, including severe sensory deficits and abnormal language development. Sensory deficits early in development may lead to broader symptomatology in adolescents and adults. The mechanistic links between ASD risk genes, sensory processing and language impairment are unclear. There is also a sex bias in ASD diagnosis and symptomatology. The current study aims to identify the developmental trajectory and genotype- and sex-dependent differences in auditory sensitivity and temporal processing in a *Pten*-deletion (phosphatase and tensin homolog missing on chromosome 10) mouse model of ASD. Auditory temporal processing is crucial for speech recognition and language development and deficits will cause language impairments. However, very little is known about the development of temporal processing in ASD animal models, and if there are sex differences. To address this major gap, we recorded epidural electroencephalography (EEG) signals from the frontal (FC) and auditory (AC) cortex in developing and adult Nse-cre PTEN mice, in which *Pten* is deleted in specific cortical layers (layers III-V) and the dentate gyrus (PTEN KO). We quantified resting EEG spectral power distribution, auditory event related potentials (ERP) and temporal processing from awake and freely moving male and female mice. Temporal processing is measured using a gap-in-noise-ASSR (auditory steady state response) stimulus

paradigm. The experimental manipulation of gap duration and modulation depth allows us to measure cortical entrainment to rapid gaps in sounds. Temporal processing was quantified using inter-trial phase clustering (ITPC) values that account for phase consistency across trials. The results show genotype differences in resting power distribution in PTEN KO mice throughout development. Male and female KO mice have significantly increased beta power but decreased high frequency oscillations in the AC and FC. Both male and female PTEN KO mice show diminished ITPC in their gap-ASSR responses in the AC and FC compared to control mice. Overall, deficits become more prominent in adult (p60) mice, with KO mice having significantly increased sound evoked power and decreased ITPC compared to controls. While both male and female KO mice demonstrated severe temporal processing deficits across development, female KO mice showed increased hypersensitivity compared to males, reflected as increased N1 and P2 amplitudes. These data identify a number of novel sensory processing deficits in a PTEN-ASD mouse model that are present from an early age. Abnormal temporal processing and hypersensitive responses may contribute to abnormal development of language function in ASD.

Introduction

Autism spectrum disorders (ASD) are characterized by reduced social interactions, impaired verbal and nonverbal communication, and repetitive behaviors [Park et al., 2016; Ratajczak, 2011; Kirkovski et al., 2013; Pickett & London, 2005]. The rate of ASD diagnoses is ~ 1 in 44 children in the United States [Christensen et al., 2018]. ASD has traditionally been diagnosed within the first three years of life, with some of the earliest signs recognized in infants being delays in language and social

engagement [Park et al., 2016]. The core symptoms of ASD such as abnormal sensory sensitivity and processing [Picket & London, 2005], may lead to speech and language deficits, but very little is known about the underlying mechanisms of language deficits in ASD.

Language impairments in ASD include syntactic, semantic, and pragmatic aspects [Foss-Feig et al., 2017; Tager-Flusberg & Caronna, 2007; Rapin & Dunn, 2003; Jeste & Nelson, 2009]. It is hypothesized that auditory spectrotemporal processing deficits may be at the root of speech and language impairments. The ability to make fine temporal discriminations of sound is critical to speech recognition, language development and sound localization [Michalewski et al., 2005]. Temporal processing of sound can be broadly divided into two categories: temporal integration and temporal acuity/resolution [Eddins & Green, 1995]. Temporal integration describes processing in which the sound level and/or duration of the signal affects the summation of neuronal activity, and temporal acuity/resolution is the minimum time interval within which the auditory system can resolve two signals [Eggermont, 2015; Jesteadt et al., 1976; Shinn & Musiek, 2003]. Individuals with ASD show impairments with both these temporal aspects, including discrimination of sound duration, onset and offset, and rapid changes within them [Kwakye et al., 2011; Lepistö et al., 2006; Oram Cardy et al., 2005; Orekhova et al., 2009]. Children with ASD show difficulties reproducing the lengths of auditory and visual stimuli. Children and adults with ASD produce abnormal neural responses to fluctuations in pitch of repeated, sequential auditory stimuli [Szelag et al., 2004; Tecchio et al., 2003; Gomot et al., 2006]. Additionally, increased gap-detection threshold has been reported in ASD, a paradigm commonly used to assess auditory temporal acuity. Impaired gap detection thresholds in children were associated with

lower phonological processing scores [Foss-Feig et al., 2017]. These studies suggest that temporal processing is critical for many aspects of speech recognition, and deficits may lead to impaired language development in ASD.

A genetic cause may be identified in up to 25% of ASD, with chromosomal rearrangements and coding-sequence mutations responsible for ~10–20% and ~5–10% of ASD cases, respectively [Guang et al., 2018; Huguet et al., 2013; Ziats & Rennert, 2016]. One gene that exhibits a strong link and comorbidity with ASD is phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN is a negative regulator of the PI3K/AKT/mTOR pathway that influences cellular processes including growth, proliferation, survival, apoptosis, metabolism, and cell migration [Endersby & Baker, 2008]. In the brain, components of the PI3K/AKT/mTOR pathway are present at synapses and are essential for synaptogenesis and regulation of dendritic spine morphology [Sawicka & Zukin, 2012]. Growing evidence suggests that dysregulation of this critical pathway may be the underlying cause of numerous neurodevelopmental disorders, including ASD.

Humans carrying germline PTEN mutations display ASD-related behaviors [Eng, 2003; Reardon et al., 2001; Goffin et al., 2001; Zori et al., 1998]. A small cohort study showed a number of PTEN mutations in ASD cases with macrocephaly [Butler et al., 2005]. Larger cohort studies reflected this initial finding, with PTEN mutations being found in 17% of macrocephalic ASD cases [Hobert et al., 2014; Klein et al., 2013; McBride et al., 2010; Varga et al., 2009; Herman et al., 2007; Buxbaum et al., 2007]. Humans with PTEN-ASD have significant intellectual impairment, decreased working memory and impaired language development [Tilot et al., 2015; Frazier et al., 2015; Frazier, 2019]. The underlying mechanisms of abnormal or delayed language function in

PTEN-ASD, or indeed in other forms of ASD, remain unclear. The link between auditory temporal processing and speech and language indicates that studying temporal processing in animal models is a feasible and important bridge to understand mechanisms of language dysfunction in ASD. Therefore, the first major goal of this study was to quantify the development of temporal processing in a PTEN mouse model of ASD.

Mutations of PTEN are the most common cause of breast cancers due to their negative regulation of the PI3K/AKT/mTOR pathway, a cancer that is predominantly diagnosed in women [Campbell et al., 2001]. This sex bias is due to the expression of estrogen receptor α (ERα) in 70-80% of breast cancers [Vilgelm et al., 2006]. Hyperactivation of the PI3K/AKT/mTOR pathway in breast cancer cells results in enhanced phosphorylation and the subsequent hyperactivation of ERα [Yamnik et al., 2009; Ishida et al., 2018; Ciruelos, 2014; Page et al., 2009]. Furthermore, an interaction between ERα and Group 1 metabotropic receptors (mGluR1 and mGluR5) has been identified specifically in female neurons in multiple brain regions, including the hippocampus and striatum [Martinez et al., 2014; Tabatadze et al., 2015; Tonn Eisinger et al., 2018; Santollo & Daniels, 2019]. A recent study demonstrated hyperexcitable local neocortical circuits in female, but not male, PTEN KO mice, observed as prolonged, spontaneous persistent activity states (UP states) [Molinaro et al., 2024]. However, it is currently unclear if this sex bias in PTEN deletions promote abnormal phenotypes in the sensory responses of females *in vivo.* Therefore, the second major goal of this study was to determine if sex differences are present in the development of temporal processing and sensory sensitivity in the PTEN mouse model of ASD.

We studied the Nse-cre PTEN mouse model (hereafter referred to as 'PTEN KO') in which PTEN is reduced in layers III-V of the neocortex and the dentate gyrus of the hippocampus. Nse-cre PTEN mutants develop ASD-related abnormalities, including reduced social activity, increased anxiety, and seizures [Ogawa et al., 2007; Kwon et al., 2006b]. Furthermore, PTEN KO mice demonstrate increased activity in response to sensory stimuli in a pre-pulse inhibition paradigm [Kwon et al., 2006b]. Reminiscent of human macrocephaly, a consistent finding across studies of this mouse model is hypertrophy and the subsequent enlargement of the deep cortical layers and dentate gyrus causing compression and neuronal loss [Takeuchi et al., 2013]. More specifically, this model provides us with the opportunity to test whether and how sex-differences in sensory processing abnormalities may arise during development in an ASD model with cortical abnormalities. Here we test the hypothesis that PTEN KO mice show abnormal oscillatory activity and greater auditory sensitivity and temporal processing abnormalities. We also quantified sex differences by recording from male and female mice. Our data show that abnormal PTEN expression in the cortex significantly impacts oscillatory activity in male and female mice at rest and during sound-evoked responses. Overall, we report considerable genotype differences across development in both male and female mice, with increasing impairments in the adult mice, compared to early development.

Methods

Mice: All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside (Reference number: 20220020; Approval date: 07/19/2022). Because germline PTEN homozygous knockouts are embryonically lethal,

mice with non-lethal PTEN mutations have been established to study cellular/circuit dysfunctions and ASD phenotypes [Di Cristofano et al., 1998]. These models include germline heterozygous loss-of-function, germline homozygous cytoplasm-predominant knock-in, and conditional homozygous loss-of-function [Clipperton-Allen & Page, 2020]. The present study used a conditional PTEN knock-out (KO) mouse model that utilizes a neuron-specific enolase (Nse) promoter-driven cre [Kwon et al., 2006a]. This PTEN model provides the ability to assess the region- and cell-type specific contributions that give rise to overall phenotypes. Nse is a glycolytic enolase that is expressed exclusively in neurons. Cre activity becomes detectable in the brain at p2 in differentiated neurons located within the cortex and hippocampus. At four weeks of age, cre activity remains restricted to the following regions and percentage of neurons in the brain: layers III-V of the cortex (54.5%), dentate gyrus (48.7%), CA3 (37.6%) and polymorphic layer (PML) (58.3%) in the hippocampus. No cre activity is detected in glial cells [Kwon et al., 2006a].

PTEN-mutant mice (*Nse-cre; Pten*$^{flox/flox}$) were generated by breeding male *PTEN*$^{flox/flox}$ mice (JAX, stock #006440) and female *Nse-cre; PTEN*$^{flox/WT}$ mice on the C57bl6/J background (Provided by Dr. Kimberly Huber, UTSW). This breeding arrangement produces litters containing three different genotypes: control (*cre* negative), PTEN-heterozygous (*Nse-cre; Pten*$^{flox/WT}$), and PTEN-KO (*Nse-cre; Pten*$^{flox/flox}$). Male and female control and PTEN-KO mice were used for the current study. All mice were genotyped via toe clipping (Transnetyx) and studies were done before the onset (<p80) of functional age-related hearing loss in the C57bl6/J strain of mice [Johnson et al., 1997]. One to five mice were housed in each cage under a 12:12-h light-dark cycle and fed *ad libitum*. A cross-sectional, as opposed to a longitudinal, design was used in this study as it is impractical to place epidural screw electrodes in brains and skulls that are

still developing. The following age ranges and sample sizes were used in this study: Control [Males: p21 (n=15), p30 (n=15), p60 (n=12); Females: p21 (n=11), p30 (n=11), p60 (n=15)] and *PTEN* KO [Males: p21 (n=12), p30 (n=10), p60 (n=10); Females: p21 (n=12), p30 (n=10), p60 (n=12)].

Surgery: Different groups of mice underwent epidural electrode implantation surgery at three age ranges: p18-20, p27-p29, p57-p66. Surgical procedures have been previously published [Croom et al. 2023, Rumschlag et al., 2021; Rumschlag & Razak, 2021]. Briefly, mice were anesthetized using intraperitoneal (i.p.) injections of either 80/20 mg/kg of ketamine/xylazine (young mice) or 80/10/1 mg/kg ketamine/xylazine/acepromazine (adult mice). The anesthetic state was monitored throughout the procedure by toe pinch reflex every 10-15 minutes. ETHIQA-XR (1-shot buprenorphine, 3.25 mg/kg body weight) was administered via subcutaneous injection prior to surgery. An incision was made to expose the scalp following the removal of fur, and sterilization (alcohol and iodine wipes) of the scalp. A Foredom dental drill was used to drill ~1mm diameter holes in the skull over the right AC, right FC, and left occipital cortex. The screw positions were determined using skull landmarks and coordinates previously reported [Wen et al., 2019; Rumschlag & Razak, 2021; Lovelace et al., 2018; Lovelace et al., 2020; Rumschlag et al., 2021] and were based on single unit recordings [Rotschafer & Razak 2014; Wen et al., 2019; Trujillo et al., 2011]. The wires extending from three-channel posts were wrapped around 1 mm screws and driven into the pre-drilled holes. Dental cement was applied to secure the implant. Mice were placed on a heating pad until fully awake and were allowed 48-72 hours for recovery before EEG recordings were made.

EEG recordings: All EEG recordings were obtained from awake and freely moving mice. EEG recordings were performed at three developmental time points: p20-23, p29-31, p59-p70, which we refer to as p21, p30 and p60, respectively. Recordings were obtained from the AC and FC electrodes, using the occipital screw as reference. Mice were placed in an arena inside a Faraday cage. The cage was located on a vibration isolation table in a sound-insulated and anechoic booth (Gretch-Ken, OR). Mice were briefly anesthetized with isoflurane and attached to an EEG cable via the implanted screws and then habituated to the recording arena with no stimuli for 15 minutes prior to sound evoked recordings. The EEG recording set-up has been previously reported [Rumschlag et al., 2021; Rumschlag & Razak, 2021; Croom et al., 2023]. Briefly, the attached cable was connected via a commutator to a TDT (Tucker Davis Technologies, FL) RA4LI/RA4PA headstage/pre-amp, which was connected to a TDT RZ6 multi-I/O processor. OpenEx (TDT) was used to simultaneously record EEG signals and operate the LED light used to synchronize the video and waveform data. TTL pulses were utilized to mark stimulus onsets on a separate channel in the collected EEG data. The EEG signals were recorded at a sampling rate of 24.414 kHz and down-sampled to 1024 Hz for analysis. All raw EEG recordings were visually examined prior to analysis for artifacts, including loss of signal or signs of clipping. No EEG data collected in this study was rejected after examination.

Resting: For resting EEGs (no sound stimulus presented), mice were habituated in the arena for 15 minutes followed by 5 minutes of recording. Power spectral densities were analyzed in both AC and FC. The traces were split into Hanning-windowed 1-second segments with 50% overlap to avoid edge artifacts and spectral splatter and then transformed to the frequency domain via Fourier transform [Rumschlag et al., 2021]. The

average power was calculated by averaging the spectra from each of the 1-second segments and were then split into frequency bands (theta: 3-7 Hz, alpha: 8-13 Hz, beta: 14-29 Hz, low gamma: 30-59 Hz, high gamma: 61-100 Hz, and high-frequency oscillations (HFO): 101-250 Hz). Gamma power was split to low and high ranges as studies have suggested that rhythms in the 30–60 Hz band and higher frequency broadband gamma (>50 Hz) are distinct and generated by different mechanisms [Dvorak & Fenton, 2014; Balakrishnan & Pearce 2015]. The lower gamma frequency rhythm may reflect parvalbumin cell activity while the higher gamma frequency and HFO may be related to spiking activity near the electrodes [Ray & Maunsell 2011; Buzsáki & Wang 2012]. The relative power of each frequency band was used in the analysis, representing the power within each frequency band relative to the total power.

Auditory ERP: Narrowband noise stimuli (6-12 kHz, 120 repetitions, 100 ms duration, 5ms rise/fall time, 0.25 Hz repetition rate) were presented at 75 dB SPL using a speaker (MF1, Tucker Davis Technologies, FL) situated 20 cm above the floor of the arena. The EEG trace was split into trials, using the TTL pulses to mark sound onset [Croom et al., 2023; Rumschlag & Razak 2021; Rumschlag et al., 2021]. Each trial was baseline corrected, such that the mean of the 250 ms baseline period prior to sound onset was subtracted from the trial trace for each trial. Each trial was then detrended (MATLAB detrend function) and all trials were averaged together. Time-frequency analysis was performed with a dynamic complex Morlet wavelet transform with Gabor normalization. The wavelet parameter was set for each frequency to optimize time-frequency resolution. The non-baseline normalized single trial power (STP) does not correct for mean baseline power levels, allowing for the identification of ongoing ‘background activity’ during stimulus presentation. To compare the responses across genotype at

each developmental time point, a non-parametric permutation test was used, to find clusters of significant values [Maris & Oostenveld, 2007]. First, a t-test was run on each time-frequency point for the two groups being compared, yielding the values of the *t* statistic for all points. The values of the *t* statistic corresponding to $p<0.025$ were considered significant. Clusters of significant *t* values were found and their area was measured. Next, the group assignments were shuffled randomly, and the t-tests and cluster-measurements were run again on the surrogate groups. This surrogate analysis was performed 2000 times to generate a distribution of cluster sizes that we would expect to find by chance. Originally identified clusters that were larger than 95% of the surrogate clusters were considered significant. This method allows for the identification of significant differences between groups without performing excessive comparisons.

Gap-ASSR: The stimulus used to assess auditory temporal processing is termed the '40 Hz gaps-in-noise ASSR' (auditory steady state response, henceforth, 'gap-ASSR') [Rumschlag & Razak, 2021; Croom et al., 2023]. The stimulus contains alternating 250 ms segments of noise and gap interrupted noise presented at 75 dB SPL. The gaps are placed 25 ms apart, resulting in a presentation rate of 40 Hz, a rate that produces the strongest ASSR signal when measured from the AC and frontal regions and may reflect the resonance frequency of the underlying neural circuits [Galambos et al., 1981; Pastor et al., 2002; Llinas, 1988; Llinas et al., 1991; Rosanova et al., 2009; Kim et al., 2015; Hwang et al., 2019]. For each gap in noise segment, the gap duration and modulation depth are chosen at random. Gaps of 2, 4, 6, 8, 10, or 12 ms durations and modulation depths of 75 and 100% were used. To measure the ability of the cortex to consistently respond to the gaps in noise, inter-trial phase clustering (ITPC) at 40 Hz was measured [Cohen, 2014]. ITPC measures the phase consistency of the recorded signal across

multiple trials. The ITPC is based on the distribution of phase angles in the EEG response at 40 Hz across all trials and reflects the precise timing of 40 Hz activity in the underlying neural generators. If the cortical regions are consistently responding to a specific gap width across multiple trials, the ITPC values will be high (maximum of 1). If the response phase angle is not consistent across trials, the expected ITPC value will be significantly lower (minimum of zero). The EEG trace was transformed using a dynamic complex Morlet wavelet transform. The trials corresponding to each parametric pair (gap duration + modulation depth) were grouped together. The ITPC was calculated for each time-frequency point as the average vector for each of the phase unit vectors recorded across trials (trial count >100 trials per parametric pair). The ITPC values at 40 Hz were averaged to extract the mean ITPC for the parametric pairs in the AC and FC.

Statistics: Statistics were performed on GraphPad Prism (ERP) or R (gap-ASSR). To evaluate the effects of genotype (2 levels), age (3 levels) and sex (2 levels), three-way ANOVA was used for resting and ERP analysis. Post hoc comparisons were carried out using the estimated marginal means and p-values with adjusted with Bonferroni correction. The resting and ERP data was tested for normality using Shapiro-Wilk tests. A four-way repeated measures ANOVA was used for gap-ASSR analysis, with the four factors being genotype (2 levels) X age (3 levels) X sex (2 levels) X gap duration (6 levels). A repeated measures ANOVA was chosen as multiple gap duration data points were collected from a single mouse in a recording session. Mauchly Tests for Sphericity were utilized and corrected for using the Greenhouse-Geisser corrections if necessary. Post hoc contrasts with Sidak corrections for multiple comparisons were used. Cortical regions (AC, FC) and modulation depths (75%, 100%) were analyzed separately. We evaluated the appropriateness of the data for analysis via ANOVA, in particular the

assumption of the normality of the residuals. None of the residuals had measures of skewness or kurtosis that exceeded ±2, which is one indication of acceptable normality [West et al., 1995]. Moreover, the residuals were evaluated via quantile-quantile plots. In each of the analyses, the correspondence between the theoretical normal distribution and the obtained residuals was within acceptable bounds.

Results

ASD-linked genes, such as PTEN, may interact with sex-specific pathways, leading to the different behavioral and neuroanatomical phenotypes in males and females [Ferri et al., 2018; Werling et al., 2016; Evans et al., 2019]. However, very little is known about development or sex differences in sensory processing in PTEN mutations, or indeed in other ASD animal models. The main goal of this study was to fill this major gap by characterizing the developmental trajectory of cortical resting EEG and sound-evoked responses in the Nse-cre PTEN-deletion model. We tested the hypothesis that sex-specific cortical hyperexcitability, abnormal resting power distributions, and temporal processing deficits would be present in PTEN model mice compared to control mice throughout development.

Male and female PTEN KO mice have increased beta power and decreased high gamma power and high frequency oscillations

We quantified resting (no stimulus) spectral power distribution in male and female control and PTEN KO mice in the auditory cortex (AC) and frontal cortex (FC) at three developmental time points (p21, p30 and p60). Figure 4.1 and Tables 4.1-4.2 show the results of the full statistical analyses of resting spectral power. Post hoc comparisons

can be found in Supplementary Tables 4.1-22. The major genotype difference in both cortical regions is increased beta power and a reduction in high gamma power and high frequency oscillations.

Auditory Cortex

There is a significant impact of age across all frequency bands in the AC (Figure 4.1, top row; Supplementary Tables 4.1-6). Beta power was significantly enhanced in both male and female KO mice compared to their respective controls across development (Age x Genotype interaction: $p<0.0001$) (Figure 4.1C). This genotype effect was present in females as early as p21, but only became significant in males at p30 and p60 (Supplementary Table 4.7). Similar phenotype differences between KO males and females were seen in low gamma power (Figure 4.1D). Female KO mice showed significantly increased low gamma power compared to female controls at p21 and p30, while male KO mice showed increased power at p30 and p60 (Supplementary Table 4.8). A sex difference between KO males and females was also identified in low gamma power, which differed across ages (Age x Sex interaction: $p=0.0082$). Notably, adult KO males have significantly increased low gamma power compared to KO females (Supplementary Table 4.9). An age x sex x genotype interaction was also identified, suggesting that genotype differences in low gamma power varied between the sexes across development (Age x Sex x Genotype interaction: $p=0.0149$). Age and sex significantly impacted high gamma power (Age main effect: $p=0.0136$; Sex main effect: $p=0.0424$) (Figure 4.1E, Supplementary Table 4.10). Power in the high gamma frequency band was lower in adult KO females compared to control females (Supplementary Table 4.11). No genotype differences in high gamma power were seen

in males at any age. HFO were also impacted by age (Age main effect: p=0.0031) (Figure 4.1F). Both male and female KO mice showed a significant decrease in HFO compared to their respective controls at p30 and p60, but only males showed decreased levels at p21 (Figure 4.1F, Supplementary Table 4.12). Taken together, these results suggest a shift in the relative spectral power distribution in KO mice in the AC, such that beta power increases with age while higher frequencies (high gamma and HFO) decrease. Furthermore, phenotype differences between KO males and females are seen in beta, low and high gamma power, as well as HFO.

Frontal cortex

Similarly to the AC, age significantly impacted all the frequency bands (Figure 4.1, bottom row; Supplementary Tables 4.13-18). Theta power was affected by genotype in the FC (Genotype main effect: p=0.0470) (Figure 4.1G, Supplementary Table 4.19). An age x genotype interaction was identified for alpha power, suggesting that the effect of genotype differed across ages (Age x Sex interaction: p=0.0108) (Figure 4.1H). Similarly to the AC, beta power was significantly enhanced in both male and female KO mice compared to their respective controls across development (Age x Genotype interaction: p<0.0001) (Figure 4.1I). This genotype effect was present in females as early as p21, but only became significant in males at p30 and p60 (Supplementary Table 4.20). Power in the high gamma frequency band was lower in both male and female KO mice throughout development (Age x Genotype interaction: p=0.0109) (Figure 4.1K). Notably, high gamma power was significantly lower in adult male and female PTEN KO mice (Supplementary Table 4.21). A similar trend was identified in HFO, such that HFO

decreased with age in male and female KO mice, dropping below control levels by p30 (Age x Genotype interaction: p=0.0290)(Figure 4.1L, Supplementary Table 4.22).

Taken together, with reduced PTEN expression in the cortex, a major outcome in both AC and FC, is the increase in resting EEG beta power and a decrease in high gamma power and high frequency oscillations. In both male and female control mice, beta power remained relatively stable across development, but PTEN mutation leads to an increased beta power with age and this effect was observed earlier in females, as compared to males. High frequency oscillations remain stable throughout development in control mice. However, the power in high frequency oscillations steadily decline with age in PTEN mutant mice.

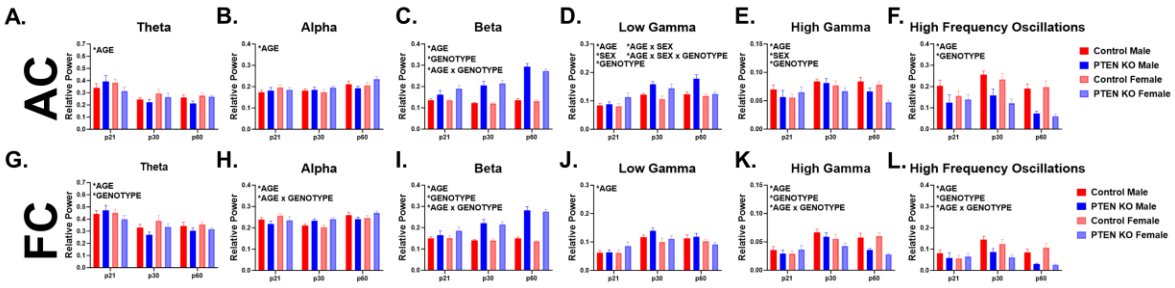

Figure 4.1. Abnormal resting spectral power distributions in the auditory and frontal cortices in Male and female PTEN KO mice. Relative resting spectral power distribution in male and female control and PTEN KO mice in the AC (A-F) and FC (G-L) across development. Panels indicate main and interaction effects. Male and female PTEN KO mice have increased beta power (AC: C, FC: I) and decreased high gamma power (AC: E, FC: K) and high frequency oscillations (AC: E, FC: L) compared to control mice. Error bars show SEM. Full ANOVA analysis can be found in Tables 4.1 and 4.2. Post hoc comparisons can be found in Supplementary Tables 4.1-22.

Table 4.1. Full statistical analysis of resting data from the AC.

Cortical Region	Frequency	Factor	ANOVA Results	p-value
AC	Theta:	**Age**	**F(2,133)=16.37**	**<0.0001**
		Sex	F(1,133)=1.519	0.2199
		Genotype	F(1,133)=1.458	0.2293
		Age x Sex	F(2,133)=1.467	0.2343
		Age x Genotype	F(2,133)=0.1502	0.8606
		Sex x Genotype	F(1,133)=0.6565	0.4193
		Age x Sex x Genotype	F(2,133)=2.012	0.1378
	Alpha:	**Age**	**F(2,133)=6.468**	**0.0021**
		Sex	F(1,133)=2.544	0.1131
		Genotype	F(1,133)=0.5771	0.4488
		Age x Sex	F(2,133)=0.4667	0.6281
		Age x Genotype	F(2,133)=0.3303	0.7193
		Sex x Genotype	F(1,133)=1.189	0.2776
		Age x Sex x Genotype	F(2,133)=2.030	0.1354
	Beta:	**Age**	**F(2,133)=25.34**	**<0.0001**
		Sex	F(1,133)=0.1268	0.7224
		Genotype	**F(1,133)=211.1**	**<0.0001**
		Age x Sex	F(2,133)=1.510	0.2246
		Age x Genotype	**F(2,133)=24.94**	**<0.0001**
		Sex x Genotype	F(1,133)=0.4319	0.5122
		Age x Sex x Genotype	F(2,133)=0.9978	0.3714
	Low Gamma:	**Age**	**F(2,133)=27.68**	**<0.0001**
		Sex	**F(1,133)=3.832**	**0.0524**
		Genotype	**F(1,133)=28.02**	**<0.0001**
		Age x Sex	**F(2,133)=4.986**	**0.0082**
		Age x Genotype	F(2,133)=0.9753	0.3798
		Sex x Genotype	F(1,133)=0.3737	0.5420
		Age x Sex x Genotype	**F(2,133)=4.340**	**0.0149**
	High Gamma:	**Age**	**F(2,133)=4.443**	**0.0136**
		Sex	**F(1,133)=4.198**	**0.0424**
		Genotype	**F(1,133)=6.728**	**0.0106**
		Age x Sex	F(2,133)=0.5232	0.5938
		Age x Genotype	F(2,133)=2.690	0.0716
		Sex x Genotype	F(1,133)=0.0107	0.91770
		Age x Sex x Genotype	F(2,133)=1.797	0.1699
	HFO:	**Age**	**F(2,133)=6.037**	**0.0031**
		Sex	F(1,133)=1.231	0.2692
		Genotype	**F(1,133)=41.59**	**<0.0001**
		Age x Sex	F(2,133)=0.2642	0.7682
		Age x Genotype	F(2,133)=2.816	0.0634
		Sex x Genotype	F(1,133)=0.0899	0.7648
		Age x Sex x Genotype	F(2,133)=0.8485	0.4304

Three-way ANOVA results for resting EEG analysis. Bold text indicates statistical significance (p = or < 0.05).

Table 4.2. Full statistical analysis of resting data from the FC.

Cortical Region	Frequency	Factor	ANOVA Results	p-value
FC	Theta:	**Age**	**F(2,133)=18.13**	**<0.0001**
		Sex	F(1,133)=0.6159	0.4340
		Genotype	**F(1,133)=4.021**	**0.0470**
		Age x Sex	F(2,133)=2.310	0.1033
		Age x Genotype	F(2,133)=0.5568	0.5744
		Sex x Genotype	F(1,133)=0.4694	0.4945
		Age x Sex x Genotype	F(2,133)=0.7431	0.4776
	Alpha:	**Age**	**F(2,133)=7.336**	**0.0010**
		Sex	F(1,133)=1.597	0.2085
		Genotype	F(1,133)=0.3974	0.5295
		Age x Sex	F(2,133)=0.5227	0.5941
		Age x Genotype	**F(2,133)=4.684**	**0.0108**
		Sex x Genotype	F(1,133)=1.671	0.1984
		Age x Sex x Genotype	F(2,133)=0.8859	0.4148
	Beta:	**Age**	**F(2,133)=16.04**	**<0.0001**
		Sex	F(1,133)=0.0059	0.9387
		Genotype	**F(1,133)=126.9**	**<0.0001**
		Age x Sex	F(2,133)=0.7933	0.4545
		Age x Genotype	**F(2,133)=21.05**	**<0.0001**
		Sex x Genotype	F(1,133)=0.3149	0.5756
		Age x Sex x Genotype	F(2,133)=0.2766	0.7588
	Low Gamma:	**Age**	**F(2,133)=23.35**	**<0.0001**
		Sex	F(1,133)=2.653	0.1057
		Genotype	F(1,133)=2.323	0.1298
		Age x Sex	F(2,133)=2.961	0.0552
		Age x Genotype	F(2,133)=0.8676	0.4223
		Sex x Genotype	F(1,133)=0.0242	0.8765
		Age x Sex x Genotype	F(2,133)=1.010	0.3668
	High Gamma:	**Age**	**F(2,133)=13.04**	**<0.0001**
		Sex	F(1,133)=1.957	0.1642
		Genotype	**F(1,133)=10.55**	**0.0015**
		Age x Sex	F(2,133)=1.284	0.2804
		Age x Genotype	**F(2,133)=4.676**	**0.0109**
		Sex x Genotype	F(1,133)=0.0041	0.9487
		Age x Sex x Genotype	F(2,133)=1.049	0.3532
	HFO:	**Age**	**F(2,133)=6.889**	**0.0014**
		Sex	F(1,133)=0.4948	0.4830
		Genotype	**F(1,133)=19.58**	**<0.0001**
		Age x Sex	F(2,133)=0.7790	0.4609
		Age x Genotype	**F(2,133)=3.637**	**0.0290**
		Sex x Genotype	F(1,133)=0.0004	0.09828
		Age x Sex x Genotype	F(2,133)=0.6476	0.5250

Three-way ANOVA results for resting EEG analysis. Bold text indicates statistical significance (p = or < 0.05).

Auditory cortical temporal processing deficits are present in PTEN KO males and females across development

Auditory temporal processing was quantified using a 40 Hz gap-in-noise ASSR stimulus to assess the ability of the cortex to consistently phase lock to brief gaps in noise. Manipulating gap duration and modulation depth of the ASSR stimulus allows for a comparison of temporal processing across experimental groups and development. Figure 4.2 shows example gap-ASSR heat maps of AC and FC ITPC for individual control (Figure 4.2A and 4.2C) and PTEN KO (Figure 4.2B and 4.2D) mice. Each panel shows the ITPC at a specific gap generated with the 40Hz stimulus, with increasing gaps across columns and each row showing a different age. In each panel, zero on the x-axis (faint dashed line) marks the onset of the gap ASSR stimulus. The expected ITPC is at 40 Hz because the stimulus is a 40 Hz train. Therefore, the warm colors indicating higher ITPC are seen at 40 Hz. Cooler colors indicate relatively low ITPC and are mostly seen for very short gaps, the KO mouse data, and at spectral bands outside 40 Hz. As expected, both AC and FC in control mice are better able to synchronize their responses to longer gaps compared to short gaps (Figure 4.2A and 4.2C). However, major deficits are seen in both cortical regions of the PTEN KO mouse, with the ITPC not emerging above background at 40 Hz at any age or gap duration (Figure 4.2B and 4.2D). The complete four-way ANOVA analysis of this data (age x sex x genotype x gap duration) are shown in Tables 4.3-4.4. Post hoc comparisons are shown in Supplementary Table 4.23.

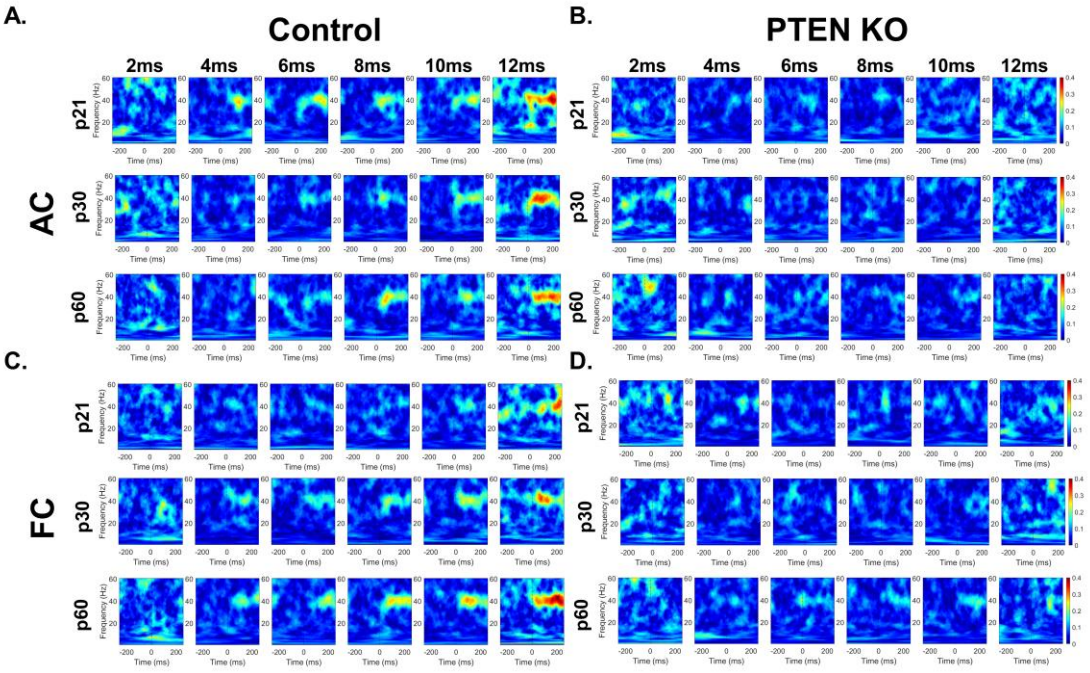

Figure 4.2. Impaired temporal processing in PTEN KO mice. Individual example heatmaps of ITPC generated at 40Hz for different gap durations in p21, p30, and p60 control (A: AC, C: FC) and PTEN KO (B: AC, D: FC) female mice. The same example mouse is used for the AC and FC. Each panel shows the ITPC (scale is seen at the right edge of the figure, warmer colors mean greater ITPC) obtained at a specific gap width. Sound onset in each panel is at 0msec. Each column shows ITPC for the same gap width, with the gap width increasing from left to right. As expected, ITPC increases with increasing gap width. The y-axis of each panel is the range of frequencies analyzed for ITPC. ITPC is maximum around 40 Hz, which was the repetition rate of ASSR stimulus train. Qualitative observations of these examples show clear deficits in cortical temporal processing across development in both cortical regions. All panels show 100% modulation depth. The onset of the gap-ASSR stimulus is at 0 msec in each panel.

Auditory Cortex

As expected, there is a significant impact of gap duration on ITPC in the AC. Three interaction effects were identified at both modulation depths: genotype x gap duration, genotype x age x gap duration, and genotype x sex x gap duration. These interactions suggest that the genotype differences between control and PTEN KO mice vary across the ages and sexes at different gap durations. At 100% modulation, male PTEN KO have impaired temporal processing across development compared to control

males (Figure 4.3, right AC column). A significant impairment was only seen at p21 and p60 in KO males at 75% modulation (Figure 4.3, left AC column). Deficits in KO females show a different developmental trajectory in the AC. Young KO females show a significant decrease in ITPC compared to control females at p21 with 75% modulation (Figure 4.4, left AC column). No difference between female control and KO were seen at p30 at either modulation depth, demonstrating a phenotype difference between male and female KO mice. Notably, both male and female adult PTEN KO mice have severely impaired temporal processing in the AC compared to their respective controls (Figures 4.3 and 4.4). This genotype effect can be seen in Figure 4.5A, which shows average ITPCs collapsed across all the gap durations. Overall, no significant sex differences were seen in control or PTEN KO mice (Figure 4.6). Taken together, these results suggest that temporal processing is impaired in the AC of young male and female PTEN KO mice, and the impairment worsens with age.

Frontal Cortex

Similar to the AC, ITPC improves with increasing gap duration in the FC. An age x gap duration and genotype x gap duration interaction were seen at 100% modulation, but not 75%. Notably, at 100% modulation, both male and female KO mice showed significantly decreased ITPC compared to their respective controls across development in the FC. This genotype effect can be seen in Figure 4.5B, which shows average ITPCs collapsed across all the gap durations. KO males demonstrated the same severity in deficits at 75% modulation (Figure 4.3, left FC column). However, ITPC was significantly decreased at p21 and p60 in Female KO mice but not p30 at 75% modulation (Figure 4.4, left FC column). These results suggest developmental fluctuations in female PTEN

KO mice temporal processing. Much like the AC, no sex differences were identified in control or PTEN KO mice (Figure 4.6). Taken together, these results suggest that temporal processing is significantly impaired in the FC of male KO mice across development. Although these deficits are present in young and adult female KO, the temporal processing capacity of KO females fluctuates with age. Overall, ITPC deficits worsen with age in male and female KO mice in the AC and FC.

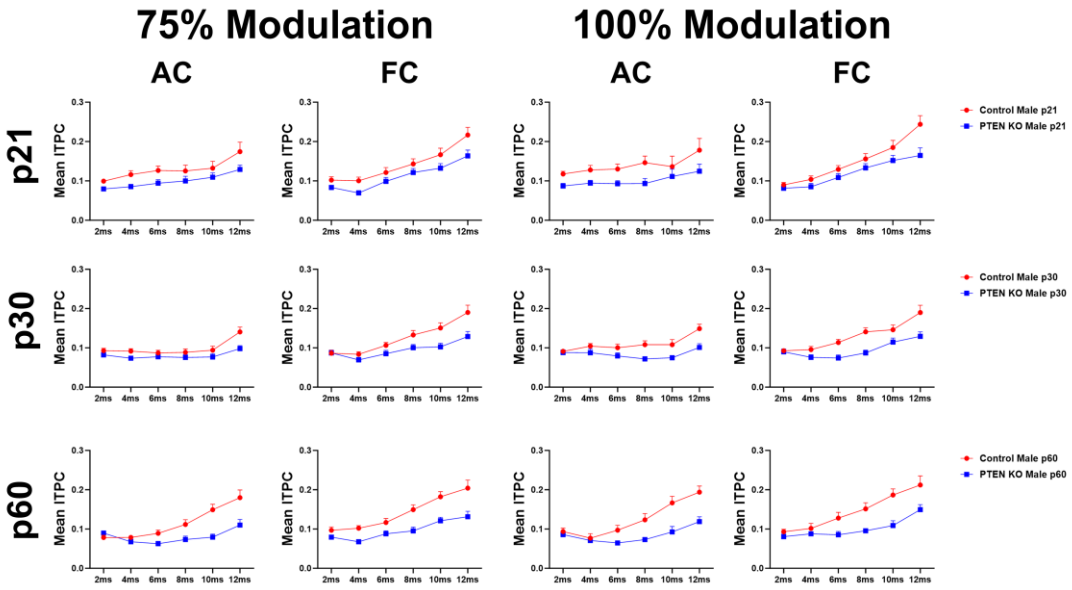

Figure 4.3. Population analysis shows temporal processing deficits in the AC and FC during development in PTEN KO male mice. Each plot represents the group average ITPC values. Each row represents a different age group: p21 (top), p30 (middle), and p60 (bottom). The left columns represent AC and FC data at 75% modulation depth, and the right columns represent AC and FC data at 100% modulation depth. PTEN KO male mice show significant deficits in both cortical regions across development. Error bars show SEM. The complete four-way ANOVA analysis of this data is shown in Tables 4.3 and 4.4. Post hoc comparisons are shown in Supplementary Table 4.23.

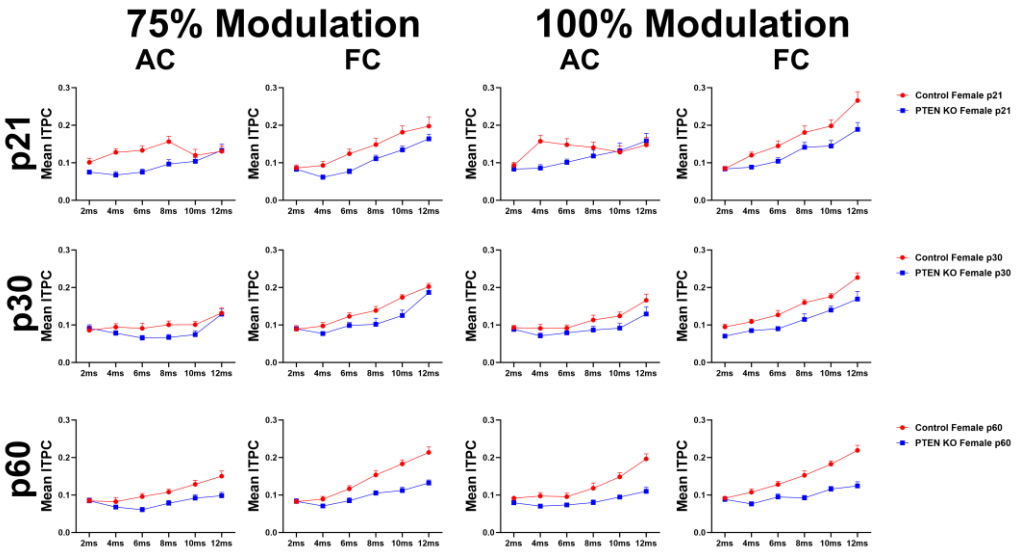

Figure 4.4. Population analysis shows temporal processing deficits in the AC and FC during development in PTEN KO female mice. Each plot represents the group average ITPC values. Each row represents a different age group: p21 (top), p30 (middle), and p60 (bottom). The left columns represent AC and FC data at 75% modulation depth, and the right columns represent AC and FC data at 100% modulation depth. PTEN KO female mice show significant deficits in both cortical regions across development. No significant difference between PTEN KO and control females were seen in the AC at p30. Error bars show SEM. The complete four-way ANOVA analysis of this data is shown in Tables 4.3 and 4.4. Post hoc comparisons are shown in Supplementary Table 4.23.

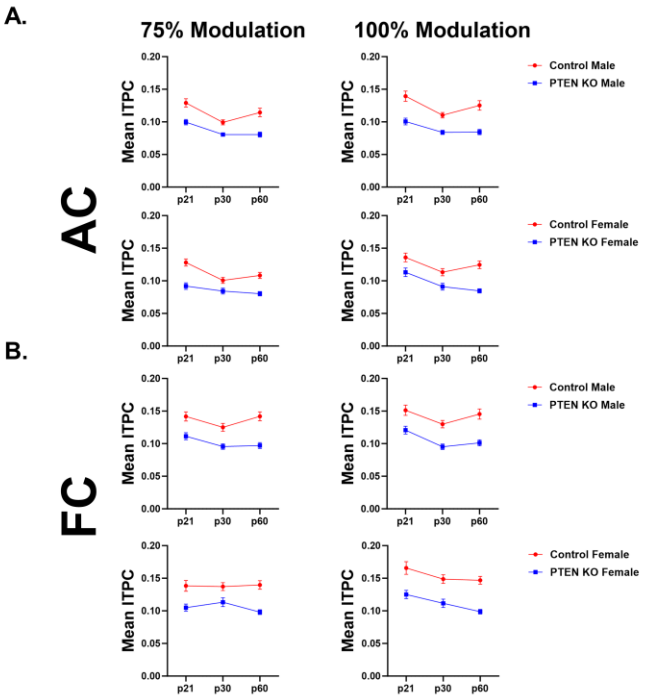

Figure 4.5. Impaired auditory temporal processing in the AC and FC of male and female PTEN KO mice. Each plot represents the group average ITPC values from the AC (A) and FC (B) collapsed across all the gap widths. Columns represent different modulation depths, and rows represent the different sexes (Columns – left = 75% modulation, right = 100% modulation; Rows – top = males, bottom = females). KO mice show a significant ITPC deficit across development in the AC and FC. Significance was not reached in females in the AC at p30. Error bars show SEM.

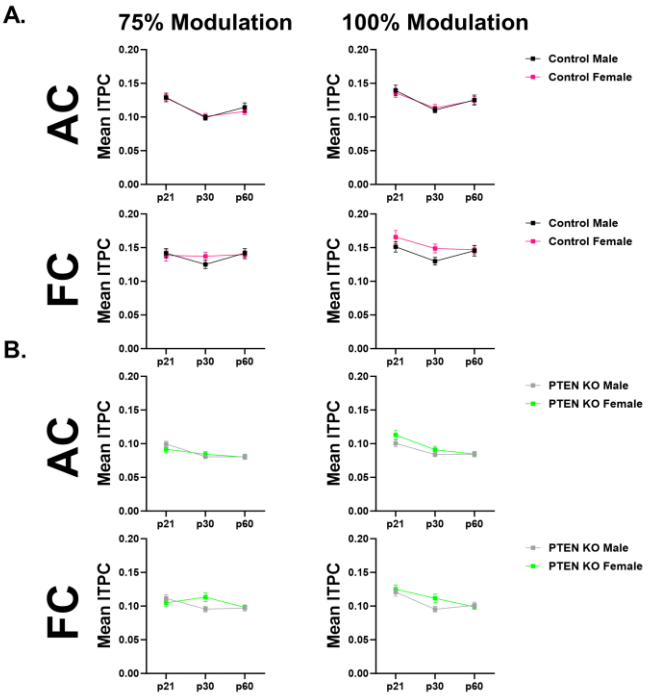

Figure 4.6. No sex difference in auditory temporal processing in control or PTEN KO mice at any age in the AC or FC. Each plot represents the group average (A: control, B: PTEN KO) ITPC values collapsed across all the gap widths. Columns represent different modulation depths, and rows represent different cortical regions (Columns – left = 75% modulation, right = 100% modulation; Rows – top = AC, bottom = FC). No significant sex difference in either genotype in the AC or FC at any age. Error bars show SEM.

Table 4.3. Full statistical analysis of gap-ASSR data from the AC.

Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
AC	100%	**Age**	**F(2,132)=3.0068**	**0.0528**
		Sex	F(1,132)=1.0611	0.3048
		Genotype	F(1,132)=3.2258	0.0747
		Gap Duration	**F(5,550)=7.6570**	**0.0001**
		Age x Sex	F(2,132)=0.2437	0.7840
		Age x Genotype	F(2,132)=0.7152	0.4909
		Age x Gap duration	F(10,660)=0.8579	0.5115
		Genotype x Sex	F(1,132)=0.8735	0.3517
		Genotype x Gap duration	**F(5,660)=4.0893**	**0.0106**
		Sex x Gap Duration	F(5,660)=1.2697	0.2846
		Genotype x Age x Sex	F(2,132)=0.2129	0.8085
		Genotype x Age x Gap Duration	**F(10,660)=3.3271**	**0.0056**
		Genotype x Sex x Gap Duration	**F(5,660)=2.8518**	**0.0458**
		Age x Sex x Gap Duration	F(10,660)=0.4911	0.7866
		Age x Sex x Genotype x Gap Duration	F(10,660)=0.9340	0.4603
AC	75%	Age	F(2,132)=0.7366	0.4806
		Sex	F(1,132)=0.6214	0.4319
		Genotype	**F(1,132)=12.586**	**0.0005**
		Gap Duration	**F(5,550)=7.4050**	**<0.0001**
		Age x Sex	F(2,132)=0.3193	0.7272
		Age x Genotype	F(2,132)=0.9153	0.4029
		Age x Gap duration	F(10,660)=1.7239	0.1114
		Genotype x Sex	F(1,132)=0.2398	0.6251
		Genotype x Gap duration	**F(5,660)=4.0176**	**0.0071**
		Sex x Gap Duration	F(5,660)=0.4914	0.6944
		Genotype x Age x Sex	F(2,132)=0.2209	0.8021
		Genotype x Age x Gap Duration	**F(10,660)=2.3487**	**0.0289**
		Genotype x Sex x Gap Duration	**F(5,660)=2.9083**	**0.0328**
		Age x Sex x Gap Duration	F(10,660)=0.7466	0.6165
		Age x Sex x Genotype x Gap Duration	F(10,660)=0.8829	0.5098

Four-way repeated measures ANOVA results for gap-ASSR analysis. Mauchly Tests for Sphericity were utilized and p-values were corrected for multiple comparisons using the Greenhouse-Geisser corrections if necessary. Bold text indicates statistical significance (p = or < 0.05).

Table 4.4. Full statistical analysis of gap-ASSR data from the FC.

Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
FC	100%	Age	F(2,132)=2.5674	0.0805
		Sex	F(1,132)=0.1366	0.7122
		Genotype	**F(1,132)=10.9466**	**0.0012**
		Gap Duration	**F(5,660)=19.1439**	**<0.0001**
		Age x Sex	F(2,132)=0.5827	0.5598
		Age x Genotype	F(2,132)=0.2345	0.7913
		Age x Gap duration	**F(10,660)=2.4168**	**0.0210**
		Genotype x Sex	F(1,132)=0.3709	0.5435
		Genotype x Gap duration	**F(5,660)=3.3317**	**0.0156**
		Sex x Gap Duration	F(5,660)=0.7452	0.5395
		Genotype x Age x Sex	F(2,132)=0.0566	0.9450
		Genotype x Age x Gap Duration	F(10,660)=0.7967	0.5855
		Genotype x Sex x Gap Duration	F(10,660)=0.4271	0.7555
		Age x Sex x Gap Duration	F(10,660)=1.5914	0.1392
		Age x Sex x Genotype x Gap Duration	F(10,660)=0.7403	0.6322
FC	75%	Age	F(2,132)=0.8067	0.4485
		Sex	F(1,132)=0.3269	0.5684
		Genotype	**F(1,132)=7.7696**	**0.0061**
		Gap Duration	**F(5,660)=20.3753**	**<0.0001**
		Age x Sex	F(2,132)=1.0442	0.3548
		Age x Genotype	F(2,132)=0.5831	0.5595
		Age x Gap duration	F(10,660)=1.9025	0.0682
		Genotype x Sex	F(1,132)=0.0395	0.8428
		Genotype x Gap duration	F(5,660)=1.5913	0.1836
		Sex x Gap Duration	F(5,660)=0.5591	0.6672
		Genotype x Age x Sex	F(2,132)=0.0735	0.9291
		Genotype x Age x Gap Duration	F(10,660)=1.5141	0.1611
		Genotype x Sex x Gap Duration	F(5,550)=1.0601	0.3711
		Age x Sex x Gap Duration	F(10,660)=1.1000	0.3619
		Age x Sex x Genotype x Gap Duration	F(10,660)=0.7646	0.6159

Four-way repeated measures ANOVA results for gap-ASSR analysis. Mauchly Tests for Sphericity were utilized and p-values were corrected for multiple comparisons using the Greenhouse-Geisser corrections if necessary. Bold text indicates statistical significance (p = or < 0.05).

Male and female PTEN KO mice exhibit abnormal ERP amplitudes in the AC and FC

ERPs consist of a series of voltage fluctuations, referred to as 'waves' (P1, N1, P2) (Figures 4.7A and 4.7C). These waves are evoked at specific latencies after sound onset and are associated with the population activity in specific brain regions. Measuring the amplitudes of these waves allows for the assessment of hypersensitivity to sound presentation. We also characterized non-baseline normalized STP in response to narrowband noise bursts, as abnormal power ('background activity') during sound-evoked responses show clinical correlations in humans with autism [Ethridge et al., 2017; Ethridge et al., 2019]. Figures 4.7-9 and Table 4.5 show the complete ANOVA analyses of control and PTEN KO male and female ERP data across development and genotypes. Post hoc comparisons for the AC and FC are shown in Supplementary Tables 4.24-29 and Supplementary Tables 4.30-33, respectively.

Auditory Cortex

P1 amplitude was significantly impacted by both genotype and sex in the AC. Specifically female and male PTEN KO mice had significantly decreased P1 amplitudes at p21 and p30, respectively, compared to their controls (Figure 4.7B, Supplementary Table 4.24). Sex differences were only seen in control mice, such that females had increased P1 amplitudes at p21 and p60 (Figure 4.7B, Supplementary Table 4.25). N1 amplitudes were increased in young male KO mice, but no significant genotype effect was seen in females at any age (Supplementary Table 4.26). Additionally, KO males showed a decrease in N1 amplitude with age (Figure 4.7B, Supplementary Table 4.27). Sex differences were identified in control and KO mice. Specifically, control females had

increased N1 amplitudes compared to control males at p60 (Figure 4.7B, Supplementary Table 4.28). KO females showed increased N1 and P2 amplitudes compared to males at p30 and p60, respectively (Figure 4.7B, Supplementary Tables 4.28-29). In addition to ERP peak amplitudes, we analyzed non-baseline normalized single trial power (STP) during the stimulus train used for ERP measurement. No changes in power were seen at p21 in either sex (Figures 4.8A and 4.9A). However, STP was significantly increased in PTEN KO males and females at p30 and p60 in the AC, suggesting a developmental increase in ongoing 'background activity' in PTEN KO mice (Figures 4.8A and 4.9A).

Frontal Cortex

Similar to the AC, P1 amplitude was significantly impacted by both genotype and sex in the FC (Figure 4.7D, Supplementary Tables 4.30-31). Additionally, P1 amplitude was also significantly decreased in young female PTEN KO mice (Figure 4.7D, Supplementary Table 4.30). Phenotype differences between the sexes were also identified for N1 amplitudes, such that N1 amplitude was increased in KO males at p21 and in females at p60 (Figure 4.7D, Supplementary Table 4.32). KO males also showed developmental fluctuations in N1 amplitude (Figure 4.7D, Supplementary Table 4.33). An age x genotype interaction was identified for P2 amplitudes, suggesting that the effect of genotype changes across ages (interaction effect: $p=0.0032$). FC STP results were similar to the AC with PTEN KO males and females showing significantly increased STP at p30 and p60, but no genotype difference at p21 (Figures 4.8B and 4.9B).

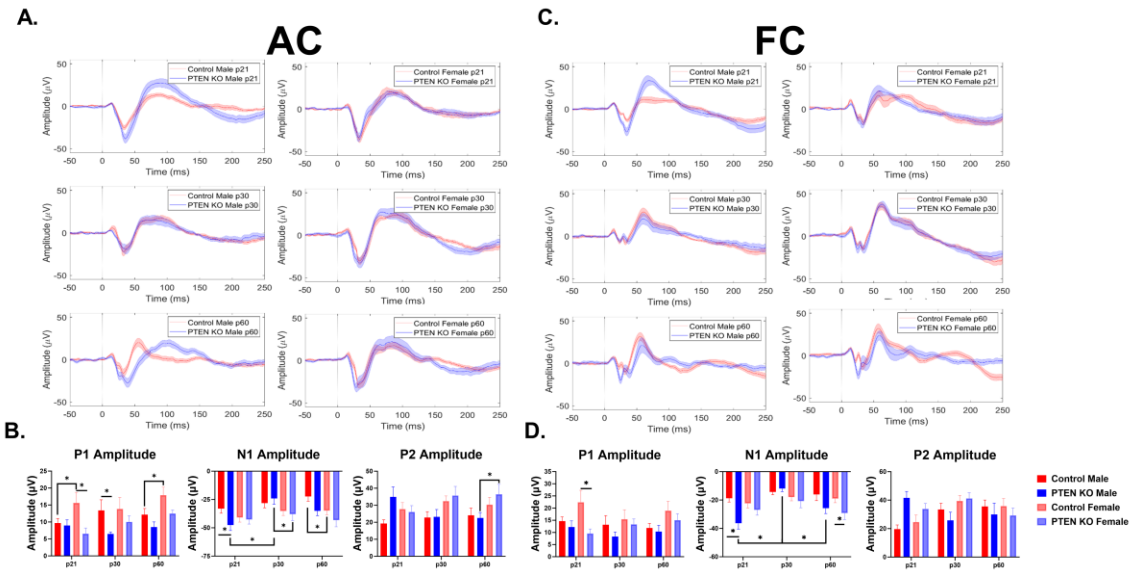

Figure 4.7. Age, genotype, and sex impact ERP amplitudes in the AC and FC. (A) Average ERPs recorded in the AC for control and KO male (left) and female (right) mice at p21 (top), p30 (middle), and p60 (bottom). (B) Population averages of AC ERP wave amplitudes. Female and male PTEN KO mice have decreased P1 amplitudes at p21 and p30, respectively, compared to their controls. Control females have increased P1 amplitudes at p21 and p60 compared to control males. N1 amplitudes are increased in young male KO mice but decrease with age. Control females have increased N1 amplitudes compared to control males at p60. KO females show increased N1 and P2 amplitudes compared to KO males at p30 and p60, respectively. (C) Average ERPs recorded in the FC for control and KO male (left) and female (right) mice at p21 (top), p30 (middle), and p60 (bottom). (D) Population averages of FC ERP wave amplitudes. P1 amplitude was significantly decreased in young female PTEN KO mice. N1 amplitude was increased in KO males at p21 and in KO females at p60. KO males show developmental fluctuations in N1 amplitude. An age x genotype interaction was identified for P2 amplitudes. Error bars show SEM. The complete ANOVA analysis can be found in Table 4.5. Post hoc comparisons for the AC and FC are shown in Supplementary Tables 4.24-29 and Supplementary Tables 4.30-33, respectively.

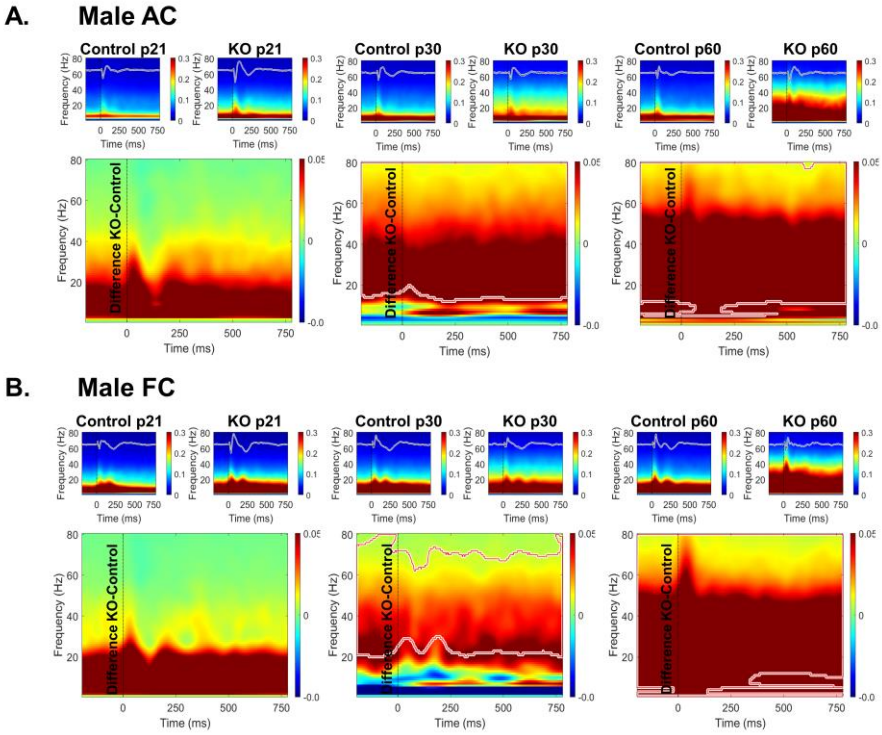

Figure 4.8. Elevated background noise power in PTEN KO male mice. Non-baseline normalized STP during ERP stimulation is altered in PTEN KO males in the AC and FC during development. The heatmaps show non-baseline corrected normalized power from the AC (A) and FC (B), where warm hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP for control and PTEN KO mice. The larger panels show the difference between KO and control. Outlined regions indicate clusters which are significantly different between control and KO. (A) STP was significantly increased in PTEN KO males p30 and p60 in the AC. (B) STP was significantly increased in PTEN KO males at p30 and p60 in the FC.

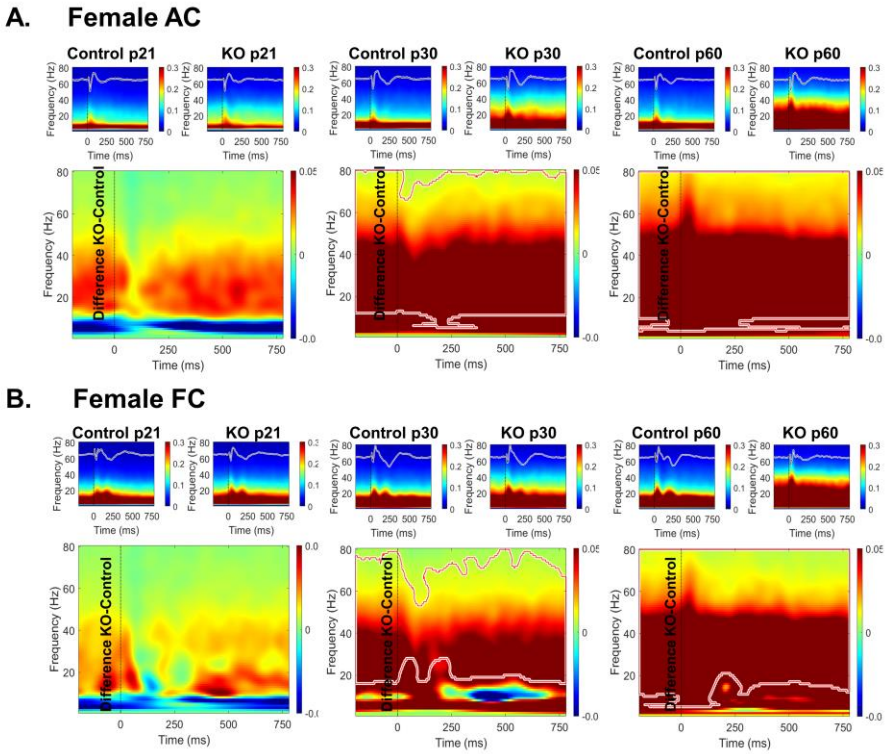

Figure 4.9. Elevated background noise power in PTEN KO female mice. Non-baseline normalized STP during ERP stimulation is altered in female PTEN KO mice in the AC and FC during development. The heatmaps show non-baseline corrected normalized power from the AC (A) and FC (B). Color scheme, scale and implication of contour lines are the same as in Figure 8. (A) STP was significantly increased in PTEN KO females p30 and p60 in the AC. (B) STP was significantly increased in PTEN KO females at p30 and p60 in the FC.

Table 4.5. Full statistical analysis of ERP data.

Cortical Region	ERP Component	Factor	ANOVA Results	p-value
AC	P1 Amplitude:	Age	F(2,133)=1.448	0.2386
		Sex	**F(1,133)=5.105**	**0.0255**
		Genotype	**F(1,133)=15.02**	**0.0002**
		Age x Sex	F(2,133)=0.6202	0.5394
		Age x Genotype	F(2,133)=0.0371	0.9636
		Sex x Genotype	F(1,133)=0.8076	0.3704
		Age x Sex x Genotype	F(2,133)=1.693	0.1879
	N1 Amplitude:	**Age**	**F(2,133)=5.001**	**0.0081**
		Sex	**F(1,133)=8.122**	**0.0051**
		Genotype	**F(1,133)=5.309**	**0.0228**
		Age x Sex	F(2,133)=1.357	0.2609
		Age x Genotype	F(2,133)=1.648	0.1964
		Sex x Genotype	F(1,133)=0.4325	0.5119
		Age x Sex x Genotype	F(2,133)=1.192	0.3067
	P2 Amplitude:	Age	F(2,133)=0.1637	0.8492
		Sex	**F(1,133)=8.176**	**0.0049**
		Genotype	F(1,133)=2.250	0.1360
		Age x Sex	F(2,133)=2.218	0.1128
		Age x Genotype	F(2,133)=0.4778	0.6212
		Sex x Genotype	F(1,133)=0.2052	0.6513
		Age x Sex x Genotype	F(2,133)=2.500	0.0859
FC	P1 Amplitude:	Age	F(2,133)=0.4910	0.6131
		Sex	**F(1,133)=5.073**	**0.0259**
		Genotype	**F(1,133)=6.657**	**0.0110**
		Age x Sex	F(2,133)=0.3230	0.7246
		Age x Genotype	F(2,133)=0.7765	0.4621
		Sex x Genotype	F(1,133)=0.8935	0.3462
		Age x Sex x Genotype	F(2,133)=1.157	0.3177
	N1 Amplitude:	**Age**	**F(2,133)=8.117**	**0.0005**
		Sex	F(1,133)=1.159	0.2836
		Genotype	**F(1,133)=12.32**	**0.0006**
		Age x Sex	F(2,133)=1.813	0.1672
		Age x Genotype	F(2,133)=2.843	0.0618
		Sex x Genotype	F(1,133)=0.3717	0.5431
		Age x Sex x Genotype	F(2,133)=1.825	0.1652
	P2 Amplitude:	Age	F(2,133)=1.065	0.3477
		Sex	F(1,133)=1.163	0.2828
		Genotype	F(1,133)=0.6117	0.4356
		Age x Sex	F(2,133)=1.838	0.1631
		Age x Genotype	**F(2,133)=5.986**	**0.0032**
		Sex x Genotype	F(1,133)=0.0657	0.7980
		Age x Sex x Genotype	F(2,133)=1.288	0.2792

Three-way ANOVA results for ERP EEG analysis. Bold text indicates statistical significance (p = or < 0.05).

Discussion

Examination of sensory processing in the Nse-cre based PTEN KO mice reveal a number of major and novel genotype-, developmental- and sex-dependent differences. The first major result of this study is the significant impact of cortical PTEN loss on oscillatory activity in the AC and FC. In resting conditions, the overall relative power spectrum is shifted in male and female KO mice, such that beta power is increased, and high frequency oscillations are decreased (Figure 4.1). Second, there is a significant impairment of auditory temporal processing in male and female KO mice across development (Figures 4.2-5). Third, PTEN KO mice displayed abnormal cortical ERP amplitudes throughout development (Figure 4.7) ERP amplitude results also showed sex differences in the KO, but not WT, mice (Figure 4.7). Lastly, the non-phase locked single trial power is elevated in male and female KO mice compared to their respective controls at p30 and p60, suggesting a developmental increase in on-going 'background activity' in the AC and FC of KO mice (Figures 4.8-9). Overall, these data suggest that irregular oscillatory activity and auditory processing deficits in PTEN ASD model mice are present from an early age and continue to worsen with age. These data identify a number of sensory processing biomarkers that can be tested in humans with PTEN mutations in future studies. Objective electrophysiological biomarkers may lead to more robust translational approaches for treatments.

A potential explanation for the dramatic decline with age of EEG phenotypes in PTEN KO mice is macrocephaly. The Nse-cre PTEN model creates a mosaic knock-out pattern in excitatory neurons of layers III-V of the cortex and hippocampal regions (dentate gyrus, CA3, and polymorphic layer) [Kwon et al., 2006a]. The cre activity, driven by a neuron-specific enolase promoter, is first detectable by embryonic day (E) 11.5 in

the cranial/spinal accessory nerve and evident by p2 in the cerebral cortex and hippocampus, specifically in postmitotic neurons [Kwon et al., 2006a]. By four weeks of age, the Nse-cre activity is most abundant in the sensory cortex [Kwon et al., 2006a]. The loss of PTEN causes the upregulation of the PI3K/AKT/mTOR pathway, a pathway shown to promote processes linked to cell growth [Kim & Guan, 2019]. Without the negative regulation of PTEN, progressive macrocephaly occurs by p60 in Nse-cre mice, making the brains significantly larger than those of littermate controls [Kwon et al., 2006b]. At the cellular level, soma enlargement, and eventual hypertrophy, as well as neuronal outgrowth are observed in areas with cre activity. This causes the compression of surrounding areas, including the CA1 region of the hippocampus. Since these structural changes develop over time, it is likely that the anatomical changes bring about significant functional abnormalities in Nse-cre PTEN KO brains, causing the most severe deficits to be seen in adult KO. However, it is important to note that synaptic plasticity deficits were still identified in young Nse-cre PTEN KO mice prior to the onset of visible morphological abnormalities [Takeuchi et al., 2013]. Specifically, Takeuchi et al. (2013) reported that theta burst-induced long-term potentiation and mGluR-dependent long-term depression are dysregulated at medial perforant path-to-dentate gyrus synapses of Nse-Cre PTEN KO mice before the onset of visible morphological abnormalities. This suggests that other mechanisms, such as abnormal synaptic plasticity, may be responsible for the deficits seen in younger mice at p21 and p30. Indeed, the PI3K/AKT/mTOR pathway has been implicated in synaptic plasticity, such that PI3K is associated with both long-term depression (LTD) and long-term potentiation (LTP) [Sánchez-Castillo et al., 2022]. Removing PTEN regulation from this pathway is likely

causing significant dysfunction in both young and adult Nse-cre KO mice and may contribute to abnormal phenotypes.

A homozygous PTEN deletion is embryonically lethal, causing death before E8 in developing mice [Di Cristofano et al., 1998]. This has led to the development of numerous PTEN deletion mouse models, including, but not limited to, a PTEN heterozygous, Nkx2.1-cre, CamKIIα-cre, and GFAP-cre [Clipperton-Allen & Page, 2015; Fraser et al., 2008; Sperow et al., 2012; Vogt et al., 2015]. This is the first time in vivo sensory EEG responses have been recorded in PTEN mice across development, so a direct comparison across models is not possible. It appears that the impact of PTEN loss in each of these models depends on the timeline in which PTEN is deleted. For example, PTEN deletion in the CamKIIα-cre model occurs between the age of 2-8 weeks in excitatory forebrain neurons. Loss of PTEN at this developmental stage does not result in the aberrant neuronal growth and macrocephaly consistently seen in other models, but instead causes synaptic dysfunction including decreased LTP and LTD [Sperow et al., 2012]. Another commonly used approach to study the loss of PTEN utilizes viral injections, providing a more localized knock-out pattern. Xiong et al. (2012) conditionally knocked-out PTEN in the auditory cortex and found that PTEN deletion caused both local and long range hyperconnectivity.

A key finding of this study is the overall shift in the relative power spectrum during rest in PTEN KO mice. Our data show that beta power significantly increases with age in the AC and FC while high gamma power and high frequency oscillations decline. These shifts differ from the typical ASD resting power distribution. Interestingly, the power distributions in humans and mouse models of ASD demonstrate an opposing pattern, such that beta power does not change, and gamma power is significantly

increased [Neo et al., 2023; Wang et al., 2013; Lovelace et al., 2018]. The cognitive implications of these resting power distribution abnormalities are not well understood. Beta oscillations are associated with task engagement, motor control, and general alertness [Neuhaus et al., 2021]. It has been shown that decreased beta power in humans causes less engagement in information processing as well as decreased attention [Pitchford & Arnell, 2019]. Abnormally elevated beta power at rest may reduce the dynamic range of task-dependent changes in this spectral band. Busch et al. (2019) found that humans with PTEN-ASD demonstrated reduced performance on measure of attention as well as motor control, however EEG phenotypes were not characterized [Busch et al., 2019]. Because attention influences sensory processing, the increased beta power seen here in PTEN KO mice could impact overall alertness and attention during the sound presentations, and ultimately cause abnormalities in responses [Fritz et al., 2010; Price & Moncrieff, 2021]. High frequency oscillations represent local spiking activity and the decrease seen in KO males and females may be a result of the morphological changes and compression occurring with age in the cortex, making neurons in those regions less functional. High frequency oscillations have also been implicated in other physiological processes. For example, it was shown in humans that high frequency oscillations are associated with memory encoding and recall [Kucewicz et al., 2014]. Another study hypothesized that these oscillations are reflective of information processing, specifically local GABAergic networks responding to thalamocortical input [Hashimoto et al., 1996]. Taken together, the dramatic decrease of high frequency oscillations seen in PTEN KO mice may reflect processing deficits at the cortical circuit level.

Cortical beta and gamma band oscillations have been linked to the activity of subtypes of inhibitory interneurons. Specifically, the beta frequency band has been linked to somatostatin (SOM) interneurons and the gamma frequency band to parvalbumin (PV) interneurons in the visual cortex [Chen et al., 2017]. Furthermore, PV interneurons play a role in de-synchronization of neural activity, whereas SOM interneurons promote the synchronization of neural activity. There are currently very few studies that have investigated the relationship between PTEN and PV/SOM interneurons, none of which were carried out in the Nse-cre PTEN model. One behavioral study found that deleting PTEN in PV and SOM expressing interneurons gave rise to ASD behaviors, including hyperactivity and anxiety [Shin et al., 2021]. Vogt et al. (2015) utilized a Nkx-2.1-cre PTEN model which causes embryonic deletion of PTEN in the medial ganglionic eminence (MGE). The majority of interneurons arise from the MGE and the loss of PTEN led to altered distribution of MGE-derived cells. Specifically, the study showed a preferential loss of SOM interneurons and increased PV interneurons, shifting the PV/SOM ratio in the cortex [Vogt et al., 2015]. These studies suggest a link between PTEN and PV/SOM interneurons that may underlie the EEG impairments we discovered here. Furthermore, if there are changes in PV or SOM neurons in the NSE-Cre model, they are non-cell autonomous. It is possible that the output from *Pten* deleted L5 neurons onto PV or Som neurons are irregular and impacting beta power. Future studies should test PV/SOM interneuron development and function in PTEN KO mice.

Auditory temporal processing is critical for development of speech processing and language function in humans. Testing temporal processing in mice may provide a bridge to understand dysfunctions in complex auditory processing in ASD. A major

finding of our study is that auditory temporal processing is impaired in PTEN KO mice throughout development. These results align with symptoms displayed in humans with PTEN-ASD. Specifically, a cohort study found that individuals with PTEN-ASD had decreased processing speed and impaired language development. Significant delays were identified in their first words and phrases, with some remaining mute or minimally verbal until after 4 years of age [Tilot et al., 2015; Frazier et al., 2015; Fraiser, 2019]. In developmental disorders and in aging, gap processing has been used to analyze auditory temporal acuity across groups [Rumschlag & Razak, 2021; Rumschlag et al., 2020; Bhatara et al., 2013; Gordon-Salant & Fitzgibbons, 1993]. Increased gap-detection thresholds are seen in children with ASD and impaired gap detection thresholds in children correlate with lower phonological scores [Foss-Feig et al., 2017]. The mechanism behind gap induced ASSR is not clear, however studies have suggested that subcortical regions may be involved in this response. For example, optogenetic stimulation of GABAergic parvalbumin neurons in the mouse basal forebrain preferentially increased frontal cortex 40 Hz ASSR oscillations [Kim et al., 2015; Hwang et al., 2019]. Additionally, top-down corticothalamic projections to the medial geniculate body influence temporal processing and stimulus encoding [Kommajoysula et al., 2021]. Another study identified a cortico-collicular gap detection circuit that amplified cortical gap responses, specifically for brief gaps [Weible et al., 2020]. In the Nse-cre PTEN deletion model, the loss of PTEN and the subsequent anatomical and functional deficits that occur in layer V could be impacting the circuitry necessary to consistently respond to the brief gaps in noise, as corticofugal projections from layer V of the auditory cortex extend to the midbrain [Games & Winer, 1988].

The gap-ASSR stimulus at 40 Hz was chosen for this study due to its relevance to aspects of speech processing. Gamma oscillations may parse speech input in the phoneme range. Oscillations in the delta and theta frequency ranges may relate to slower rhythms in speech such as intonation and syllabic rates. Based on such observations, the ‘asymmetric sampling in time’ hypothesis for speech processing has been proposed in which gamma oscillations play a significant role in phoneme processing [Luo & Poeppel, 2012; Giraud & Poeppel, 2012; Pena & Melloni, 2012] by separating inputs that require high temporal resolution such as voice onset time and formant transition. Speech abnormalities have not been fully characterized in individuals with PTEN-ASD, however the findings of our study suggest future studies should characterize auditory acuity in humans with PTEN related ASD.

The P1-N1-P2 ERP complex marks the pre-attentive detection of sound and can vary with stimulus features. P1 and N1 amplitudes correlate with initial sound detection, including thalamocortical input and primary auditory cortex activity, respectively. PTEN KO mice displayed abnormal cortical ERP amplitudes throughout development. Specifically, P1 amplitudes were decreased in KO mice compared to their respective controls. These results suggest a decrease in thalamocortical input to cortical regions. The development of corticothalamic projections begins as early as E12 in mice, as thalamocortical axons begin extending towards the cortex [Huerga-Gomez et al., 2023]. These projections invade the cortical plate at E17, and reach their final destination in the cortex, primarily layers IV and Vb, during the first postnatal week of life. Because cre becomes active in the Nse-cre model by p2 in the brain, it is possible that neurons in layer IV are not receptive to the incoming thalamic projections and are unable to make the appropriate connections, therefore causing decreased P1 amplitudes in KO mice.

We also found that N1 amplitudes, which mark synchronous activity within the cortex, increased in KO mice. Specifically, N1 amplitudes were increased in young male KO mice in the AC and FC, but only in adult KO females in the FC. This opposing relationship between P1 and N1 amplitudes in KO mice could possibly reflect cortical gain at the cortical level in response to abnormally reduced thalamocortical input [Resnik & Polley, 2021]. The enhancement of N1 amplitudes also suggests increased hypersensitivity to the stimuli and is a consistent phenotype seen in numerous ASD studies including in Fragile X Syndrome (FXS), in both humans and mice [Castren et al., 2003; Knoth & Lippe, 2012; Rojas et al., 2001; St. Clair et al., 1987; Van de Molen et al., 2012a; Van de Molen et al., 2012b; Kulinich et al., 2020; Lovelace et al., 2018; Wen et al., 2019; Jonak et al., 2020]. Although PTEN and FXS forms of ASD are caused by two different genes, a common mechanism may underlie these similar phenotypes. Abnormal sensitivity has been reported in both human and animals with PTEN deletion. Notably, humans with PTEN-ASD showed severely impaired sensory functioning compared to age-matched controls, particularly these individuals tested as under-responsive and show decreased taste and smell sensitivity [Busch et al., 2019]. The Nse-cre PTEN model mice also demonstrate exaggerated responses to sensory stimuli in a pre-pulse inhibition paradigm. Specifically, mutants exhibited increased startle responses to a white noise stimulus [Kwon et al., 2006b]. PTEN loss in the Nse-cre model has been shown to increase axonal growth in the dentate gyrus, such that axonal processes were more abundant and projected to a broader area [Kwon et al., 2006b]. Furthermore, this excessive growth led to abnormal synapses, including an increase in presynaptic vesicle numbers compared to controls. Although these phenotypes have not been studied in the cortex, it is likely that similar abnormalities occur, and increased

synapses or aberrant connections could cause increased N1 amplitudes in KO mice. Taken together, P1 and N1 are generated by structures involved in early auditory processing and differences in their amplitudes compared to controls may ultimately reflect altered perception of auditory stimulus.

The single trial power (STP) allows for the identification of ongoing 'background activity' during stimulus presentation as it does not correct for mean baseline power levels. It has been suggested that this non-phase locked power reflects relatively slow integrative processes that may impact stimulus or response processing [McKewen et al., 2020]. These processes include top-down and sustained attention, decision-making, and perceptual inference, and are suggested to result from intrinsic network interactions rather than external stimuli [Donner & Siegel, 2011; Siegel & Donner, 2010]. Our results show a developmental increase in STP in PTEN KO mice compared to controls. Specifically, no STP phenotype differences were seen at p21 in either sex but STP was significantly increased in PTEN KO mice at p30 and p60 in the AC and FC. Increased STP has been seen in humans with FXS and the *Fmr1* KO mouse model [Ethridge et al., 2019; Wen et al., 2019; Croom et al., 2023]. In humans with FXS, elevated STP showed correlations with IQ and distractibility. These data suggest elevated on-going background activity from hyperactive networks in ASD may have clinical implications in humans. Future studies should be carried out in humans with PTEN deletion to determine whether similar correlations exist.

A key hypothesis of this study was female PTEN KO mice would show greater auditory sensitivity and processing abnormalities than PTEN KO males. This hypothesis was supported by previous work done in the Nse-cre model. Molinaro et al., (2024) showed prolonged and spontaneous persistent activity states (UP states) in female but

not male KO mice at P21, suggesting sex-specific circuit hyperexcitability. Furthermore, sex differences were also seen in social behavior and seizure-induced mortality in adults. In the current study, we show significant sex differences in PTEN KO mice auditory cortex in resting and sound-evoked EEG phenotypes. Specifically, young, P21, female KO mice show increases in resting beta power and adult male KO mice have increased low gamma power compared to adult females. Abnormalities in resting EEG power have also been identified in other genetically linked forms of ASD in humans, including FXS and Rett syndrome. Specifically, males with FXS and Rett syndrome show increased and decreased gamma power compared to females, respectively [Smith et al., 2021; Sysoeva et al., 2023]. The current study also identified sex differences in evoked responses, such that female KO mice show increased N1 and P2 amplitudes compared to males at p30 and p60, respectively. Although N1 amplitudes were increased in both KO males and females compared to their respective controls, no sex difference was seen in control mice at that age. This suggests that hypersensitive cortical responses are further enhanced in female KO mice compared to males. P2 amplitudes are suggested to be related to arousal as auditory input to the mesencephalic reticular activating system contributes to P2 generation [Crowley & Colrain, 2004]. Furthermore, KO females show increased locomotor activity compared to males, which may be a result of increased activation of this arousal circuit [Molinaro et al., 2024]. These results are similar to sex differences seen in FXS model mice, such that female *Fmr1* KO mice have increased N1 and P2 amplitudes compared to KO males [Croom et al., 2024]. The similarities in sex difference between these different ASD models provide evidence that there may be a fundamental issue at the circuit level that is shared among autisms. Several lines of evidence, both in human and animal models, suggest inhibitory circuit

dysfunction may be a fundamental issue [Maloney et al., 2013; Voineagu et al., 2011]. For example, one of the most commonly observed copy number variations in ASD is located on the chromosome 15q11 to q13 region, which contains several genes coding for subunit variants for GABA receptors [Coghlan et al., 2012]. Deletions involving these regions have been associated with Angelman or Prader–Willi syndromes which show a high comorbidity with ASD. Additionally, disruption of the GABAergic system is sufficient to generate the Rett syndrome phenotype in $Mecp2^{-/-}$ mice [Chao et al., 2010]. Taken together, the similarities between different autism models suggest shared mechanisms and unraveling these circuits may be the key to understanding and treating ASD pathology.

Conclusion

This is the first study to comprehensively characterize resting and sound-evoked EEG phenotypes as well as sex differences during development in a mouse model of ASD with PTEN loss of function. ASD manifests differently in females and males, however little progress has been made in understanding underlying mechanisms due to a lack of robust and objective outcome measures in animal models. Here we demonstrate that loss of PTEN in cortical layers III-V and the hippocampus cause abnormal relative resting power distribution as well as sensory responses in a sex-specific manner. Female KO mice showed increased hypersensitivity compared to males. These studies now provide specific physiological probes to examine sex differences from a mechanistic perspective in an ASD model.

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Supplementary Table 4.1. Post hoc testing for the effect of age on relative theta power in AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	**p21**	p30	.087	.043	.136
			p60	**.105**	**.040**	**.030**
		p30	p21	-.087	.043	.136
			p60	.018	.040	1.000
		p60	**p21**	**-.105**	**.040**	**.030**
			p30	-.018	.040	1.000
	Male	**p21**	**p30**	**.098**	**.037**	**.028**
			p60	.080	.039	.131
		p30	**p21**	**-.098**	**.037**	**.028**
			p60	-.018	.039	1.000
		p60	p21	-.080	.039	.131
			p30	.018	.039	1.000
PTEN KO	Female	p21	p30	.049	.043	.791
			p60	.046	.041	.806
		p30	p21	-.049	.043	.791
			p60	-.003	.043	1.000
		p60	p21	-.046	.041	.806
			p30	.003	.043	1.000
	Male	**p21**	**p30**	**.171**	**.043**	**<.001**
			p60	**.182**	**.043**	**<.001**
		p30	**p21**	**-.171**	**.043**	**<.001**
			p60	.011	.045	1.000
		p60	**p21**	**-.182**	**.043**	**<.001**
			p30	-.011	.045	1.000

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.2. Post hoc testing for the effect of age on relative alpha power in AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	.022	.018	.722
			p60	-.010	.017	1.000
		p30	p21	-.022	.018	.722
			p60	-.032	.017	.194
		p60	p21	.010	.017	1.000
			p30	.032	.017	.194
	Male	p21	p30	-.008	.016	1.000
			p60	-.039	.017	.066
		p30	p21	.008	.016	1.000
			p60	-.031	.017	.194
		p60	p21	.039	.017	.066
			p30	.031	.017	.194
PTEN KO	Female	**p21**	p30	-.012	.018	1.000
			p60	**-.052**	**.018**	**.012**
		p30	p21	.012	.018	1.000
			p60	-.040	.018	.094
		p60	**p21**	**.052**	**.018**	**.012**
			p30	.040	.018	.094
	Male	p21	p30	-.003	.018	1.000
			p60	-.010	.018	1.000
		p30	p21	.003	.018	1.000
			p60	-.007	.019	1.000
		p60	p21	.010	.018	1.000
			p30	.007	.019	1.000

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.3. Post hoc testing for the effect of age on relative beta power in AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	.015	.016	1.000
			p60	.005	.015	1.000
		p30	p21	-.015	.016	1.000
			p60	-.010	.015	1.000
		p60	p21	-.005	.015	1.000
			p30	.010	.015	1.000
	Male	p21	p30	.014	.014	.936
			p60	.000	.015	1.000
		p30	p21	-.014	.014	.936
			p60	-.014	.015	1.000
		p60	p21	.000	.015	1.000
			p30	.014	.015	1.000
PTEN KO	Female	**p21**	p30	-.024	.016	.424
			p60	**-.082**	**.016**	**<.001**
		p30	p21	.024	.016	.424
			p60	**-.058**	**.016**	**.002**
		p60	**p21**	**.082**	**.016**	**<.001**
			p30	**.058**	**.016**	**.002**
	Male	**p21**	**p30**	**-.042**	**.016**	**.034**
			p60	**-.130**	**.016**	**<.001**
		p30	**p21**	**.042**	**.016**	**.034**
			p60	**-.088**	**.017**	**<.001**
		p60	**p21**	**.130**	**.016**	**<.001**
			p30	**.088**	**.017**	**<.001**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.4. Post hoc testing for the effect of age on relative low gamma power in AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	**p21**	p30	-.026	.014	.206
			p60	**-.037**	**.013**	**.016**
		p30	p21	.026	.014	.206
			p60	-.011	.013	1.000
		p60	**p21**	**.037**	**.013**	**.016**
			p30	.011	.013	1.000
	Male	**p21**	**p30**	**-.038**	**.012**	**.005**
			p60	**-.040**	**.013**	**.006**
		p30	**p21**	**.038**	**.012**	**.005**
			p60	-.001	.013	1.000
		p60	**p21**	**.040**	**.013**	**.006**
			p30	.001	.013	1.000
PTEN KO	Female	p21	p30	-.031	.014	.090
			p60	-.010	.013	1.000
		p30	p21	.031	.014	.090
			p60	.020	.014	.437
		p60	p21	.010	.013	1.000
			p30	-.020	.014	.437
	Male	**p21**	**p30**	**-.070**	**.014**	**<.001**
			p60	**-.090**	**.014**	**<.001**
		p30	**p21**	**.070**	**.014**	**<.001**
			p60	-.020	.015	.532
		p60	**p21**	**.090**	**.014**	**<.001**
			p30	.020	.015	.532

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.5. Post hoc testing for the effect of age on relative high gamma power in AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	-.021	.011	.144
			p60	-.022	.010	.081
		p30	p21	.021	.011	.144
			p60	-.001	.010	1.000
		p60	p21	.022	.010	.081
			p30	.001	.010	1.000
	Male	p21	p30	-.014	.009	.380
			p60	-.014	.010	.440
		p30	p21	.014	.009	.380
			p60	.000	.010	1.000
		p60	p21	.014	.010	.440
			p30	.000	.010	1.000
PTEN KO	Female	p21	p30	-.001	.011	1.000
			p60	.018	.010	.244
		p30	p21	.001	.011	1.000
			p60	.020	.011	.219
		p60	p21	-.018	.010	.244
			p30	-.020	.011	.219
	Male	p21	p30	-.025	.011	.066
			p60	-.010	.011	1.000
		p30	p21	.025	.011	.066
			p60	.015	.011	.525
		p60	p21	.010	.011	1.000
			p30	-.015	.011	.525

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.6. Post hoc testing for the effect of age on relative HFO in AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	-.078	.037	.109
			p60	-.041	.034	.687
		p30	p21	.078	.037	.109
			p60	.036	.034	.870
		p60	p21	.041	.034	.687
			p30	-.036	.034	.870
	Male	p21	p30	-.053	.031	.289
			p60	.012	.033	1.000
		p30	p21	.053	.031	.289
			p60	.064	.033	.167
		p60	p21	-.012	.033	1.000
			p30	-.064	.033	.167
PTEN KO	Female	p21	p30	.018	.037	1.000
			p60	.080	.035	.075
		p30	p21	-.018	.037	1.000
			p60	.062	.037	.292
		p60	p21	-.080	.035	.075
			p30	-.062	.037	.292
	Male	p21	p30	-.034	.037	1.000
			p60	.052	.037	.489
		p30	p21	.034	.037	1.000
			p60	.086	.039	.084
		p60	p21	-.052	.037	.489
			p30	-.086	.039	.084

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.7. Post hoc testing for the effect of genotype on relative beta power in AC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	**p21**	**-.055**	**.016**	**<.001**
	p30	**-.094**	**.017**	**<.001**
	p60	**-.142**	**.015**	**<.001**
Male	p21	-.027	.015	.074
	p30	**-.082**	**.016**	**<.001**
	p60	**-.157**	**.016**	**<.001**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.8. Post hoc testing for the effect of genotype on relative low gamma power in AC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	**p21**	**-.033**	**.014**	**.017**
	p30	**-.038**	**.014**	**.009**
	p60	-.006	.013	.622
Male	p21	-.005	.013	.695
	p30	**-.037**	**.013**	**.007**
	p60	**-.055**	**.014**	**<.001**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.9. Post hoc testing for the effect of sex on relative low gamma power in AC.

Genotype	Age	Mean Difference	Standard Error	Adjusted p-value
Control	p21	-.002	.013	.867
	p30	-.015	.013	.249
	p60	-.005	.013	.694
PTEN KO	p21	.026	.013	.056
	p30	-.014	.015	.345
	p60	**-.054**	**.014**	**<.001**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.10. Post hoc testing for the effect of sex on relative high gamma power in AC.

Genotype	Age	Mean Difference	Standard Error	Adjusted p-value
Control	p21	-.014	.010	.157
	p30	-.007	.010	.489
	p60	-.006	.010	.531
PTEN KO	p21	.009	.010	.392
	p30	-.015	.011	.194
	p60	-.019	.011	.083

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.11. Post hoc testing for the effect of genotype on relative high gamma power in AC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	-.010	.011	.362
	p30	.010	.011	.346
	p60	**.031**	**.010**	**.002**
Male	p21	.013	.010	.170
	p30	.003	.010	.797
	p60	.018	.011	.095

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.12. Post hoc testing for the effect of genotype on relative HFO in AC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	.017	.036	.643
	p30	**.113**	**.038**	**.003**
	p60	**.138**	**.033**	**<.001**
Male	**p21**	**.078**	**.033**	**.021**
	p30	**.097**	**.035**	**.007**
	p60	**.118**	**.037**	**.002**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.13. Post hoc testing for the effect of age on relative theta power in FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	.065	.044	.436
			p60	.095	.041	.067
		p30	p21	-.065	.044	.436
			p60	.030	.041	1.000
		p60	p21	-.095	.041	.067
			p30	-.030	.041	1.000
	Male	**p21**	**p30**	**.111***	**.038**	**.012**
			p60	**.096**	**.040**	**.054**
		p30	**p21**	**-.111**	**.038**	**.012**
			p60	-.014	.040	1.000
		p60	**p21**	**-.096**	**.040**	**.054**
			p30	.014	.040	1.000
PTEN KO	Female	p21	p30	.063	.045	.484
			p60	.082	.042	.166
		p30	p21	-.063	.045	.484
			p60	.019	.045	1.000
		p60	p21	-.082	.042	.166
			p30	-.019	.045	1.000
	Male	**p21**	**p30**	**.201**	**.045**	**<.001**
			p60	**.169**	**.045**	**<.001**
		p30	**p21**	**-.201**	**.045**	**<.001**
			p60	-.033	.046	1.000
		p60	**p21**	**-.169**	**.045**	**<.001**
			p30	.033	.046	1.000

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.14. Post hoc testing for the effect of age on relative alpha power in FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	**p21**	**p30**	**.054**	**.017**	**.007**
			p60	.010	.016	1.000
		p30	**p21**	**-.054**	**.017**	**.007**
			p60	**-.044**	**.016**	**.024**
		p60	p21	-.010	.016	1.000
			p30	**.044**	**.016**	**.024**
	Male	p21	p30	.028	.015	.177
			p60	-.021	.016	.569
		p30	p21	-.028	.015	.177
			p60	**-.049**	**.016**	**.007**
		p60	p21	.021	.016	.569
			p30	**.049**	**.016**	**.007**
PTEN KO	Female	p21	p30	-.006	.017	1.000
			p60	-.035	.017	.112
		p30	p21	.006	.017	1.000
			p60	-.029	.017	.296
		p60	p21	.035	.017	.112
			p30	.029	.017	.296
	Male	p21	p30	-.015	.017	1.000
			p60	-.023	.017	.597
		p30	p21	.015	.017	1.000
			p60	-.008	.018	1.000
		p60	p21	.023	.017	.597
			p30	.008	.018	1.000

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.15. Post hoc testing for the effect of age on relative beta power in FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	.011	.018	1.000
			p60	.015	.017	1.000
		p30	p21	-.011	.018	1.000
			p60	.004	.017	1.000
		p60	p21	-.015	.017	1.000
			p30	-.004	.017	1.000
	Male	p21	p30	.009	.015	1.000
			p60	.001	.016	1.000
		p30	p21	-.009	.015	1.000
			p60	-.008	.016	1.000
		p60	p21	-.001	.016	1.000
			p30	.008	.016	1.000
PTEN KO	Female	**p21**	p30	-.029	.018	.332
			p60	**-.089**	**.017**	**<.001**
		p30	p21	.029	.018	.332
			p60	**-.060**	**.018**	**.003**
		p60	**p21**	**.089**	**.017**	**<.001**
			p30	**.060**	**.018**	**.003**
	Male	**p21**	**p30**	**-.057**	**.018**	**.006**
			p60	**-.117**	**.018**	**<.001**
		p30	**p21**	**.057**	**.018**	**.006**
			p60	**-.060**	**.019**	**.006**
		p60	**p21**	**.117**	**.018**	**<.001**
			p30	**.060**	**.019**	**.006**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.16. Post hoc testing for the effect of age on relative low gamma power in FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	**p21**	**p30**	**-.039**	**.016**	**.044**
			p60	**-.041**	**.015**	**.017**
		p30	**p21**	**.039**	**.016**	**.044**
			p60	-.002	.015	1.000
		p60	**p21**	**.041**	**.015**	**.017**
			p30	.002	.015	1.000
	Male	**p21**	**p30**	**-.055**	**.013**	**<.001**
			p60	**-.051**	**.014**	**.001**
		p30	**p21**	**.055**	**.013**	**<.001**
			p60	.005	.014	1.000
		p60	**p21**	**.051**	**.014**	**.001**
			p30	-.005	.014	1.000
PTEN KO	Female	p21	p30	-.026	.016	.320
			p60	-.006	.015	1.000
		p30	p21	.026	.016	.320
			p60	.020	.016	.643
		p60	p21	.006	.015	1.000
			p30	-.020	.016	.643
	Male	**p21**	**p30**	**-.075**	**.016**	**<.001**
			p60	**-.055**	**.016**	**.002**
		p30	**p21**	**.075**	**.016**	**<.001**
			p60	.020	.016	.665
		p60	**p21**	**.055**	**.016**	**.002**
			p30	-.020	.016	.665

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.17. Post hoc testing for the effect of age on relative high gamma power in FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	**p21**	**p30**	**-.026**	**.009**	**.019**
			p60	**-.031**	**.009**	**.002**
		p30	**p21**	**.026**	**.009**	**.019**
			p60	-.005	.009	1.000
		p60	**p21**	**.031**	**.009**	**.002**
			p30	.005	.009	1.000
	Male	**p21**	**p30**	**-.031**	**.008**	**<.001**
			p60	**-.022**	**.009**	**.038**
		p30	**p21**	**.031**	**.008**	**<.001**
			p60	.009	.009	.864
		p60	**p21**	**.022**	**.009**	**.038**
			p30	-.009	.009	.864
PTEN KO	Female	p21	p30	-.006	.009	1.000
			p60	.009	.009	.988
		p30	p21	.006	.009	1.000
			p60	.015	.009	.335
		p60	p21	-.009	.009	.988
			p30	-.015	.009	.335
	Male	**p21**	**p30**	**-.030**	**.009**	**.006**
			p60	-.007	.009	1.000
		p30	**p21**	**.030**	**.009**	**.006**
			p60	.023	.010	.066
		p60	p21	.007	.009	1.000
			p30	-.023	.010	.066

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.18. Post hoc testing for the effect of age on relative HFO in FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	**p21**	**p30**	**-.067**	**.026**	**.032**
			p60	-.049	.024	.124
		p30	**p21**	**.067**	**.026**	**.032**
			p60	.017	.024	1.000
		p60	p21	.049	.024	.124
			p30	-.017	.024	1.000
	Male	**p21**	**p30**	**-.064**	**.022**	**.013**
			p60	-.005	.023	1.000
		p30	**p21**	**.064**	**.022**	**.013**
			p60	**.059**	**.023**	**.038**
		p60	p21	.005	.023	1.000
			p30	**-.059**	**.023**	**.038**
PTEN KO	Female	p21	p30	.003	.026	1.000
			p60	.039	.025	.348
		p30	p21	-.003	.026	1.000
			p60	.036	.026	.511
		p60	p21	-.039	.025	.348
			p30	-.036	.026	.511
	Male	p21	p30	-.028	.026	.842
			p60	.028	.026	.828
		p30	p21	.028	.026	.842
			p60	.056	.027	.117
		p60	p21	-.028	.026	.828
			p30	-.056	.027	.117

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.19. Post hoc testing for the effect of genotype on relative theta power in FC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	.052	.043	.232
	p30	.050	.045	.274
	p60	.039	.040	.337
Male	p21	-.031	.040	.439
	p30	.059	.042	.164
	p60	.041	.045	.358

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.20. Post hoc testing for the effect of genotype on relative beta power in FC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	**p21**	**-.036**	**.018**	**.045**
	p30	**-.076**	**.018**	**<.001**
	p60	**-.140**	**.016**	**<.001**
Male	p21	-.015	.016	.371
	p30	**-.081**	**.017**	**<.001**
	p60	**-.132**	**.018**	**<.001**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.21. Post hoc testing for the effect of genotype on relative high gamma power in FC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	-.008	.009	.417
	p30	.012	.010	.207
	p60	**.032**	**.009**	**<.001**
Male	p21	.007	.009	.444
	p30	.008	.009	.404
	p60	**.021**	**.009**	**.026**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.22. Post hoc testing for the effect of genotype on relative HFO in FC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	-.008	.025	.748
	p30	**.062**	**.026**	**.021**
	p60	**.080**	**.023**	**<.001**
Male	p21	.022	.023	.348
	p30	**.058**	**.025**	**.020**
	p60	**.055**	**.026**	**.034**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.23. Post hoc testing for the effect of genotype on gap-ASSR data.

Cortical Region	Modulation Depth	Sex	Age	Mean Difference	Standard error	Adjusted p-value
AC	100%	Female	p21	-0.0228	0.00876	0.1988
			p30	-0.0221	0.00893	0.2771
			p60	**-0.0400**	**0.00792**	**<0.0001**
		Male	**p21**	**-0.0388**	**0.00792**	**<0.0001**
			p30	**-0.0263**	**0.00835**	**0.0388**
			p60	**-0.0409**	**0.00876**	**0.0001**
	75%	Female	**p21**	**-0.0363**	**0.00876**	**0.0008**
			p30	-0.0166	0.00893	0.7937
			p60	**-0.0280**	**0.00792**	**0.0099**
		Male	**p21**	**-0.0295**	**0.00792**	**0.0047**
			p30	-0.0185	0.00835	0.4754
			p60	**-0.0339**	**0.00876**	**0.0026**
FC	100%	Female	**p21**	**-0.0405**	**0.00876**	**0.0001**
			p30	**-0.0373**	**0.00893**	**0.0007**
			p60	**-0.0482**	**0.00792**	**<0.0001**
		Male	**p21**	**-0.0305**	**0.00792**	**0.0029**
			p30	**-0.0347**	**0.00835**	**0.0008**
			p60	**-0.0442**	**0.00876**	**<0.0001**
	75%	Female	**p21**	**-0.0335**	**0.00876**	**0.0032**
			p30	-0.0240	0.00893	0.1587
			p60	**-0.0417**	**0.00792**	**<0.0001**
		Male	**p21**	**-0.0303**	**0.00792**	**0.0032**
			p30	**-0.0294**	**0.00835**	**0.0103**
			p60	**-0.0448**	**0.00876**	**<0.0001**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted using Sidak method. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.24. Post hoc testing for the effect of genotype on P1 amplitude in the AC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	**p21**	**9.094**	**3.178**	**.005**
	p30	3.808	3.327	.254
	p60	5.411	2.949	.069
Male	p21	.773	2.949	.794
	p30	**6.985**	**3.109**	**.026**
	p60	3.660	3.260	.264

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.25. Post hoc testing for the effect of sex on P1 amplitude in AC.

Genotype	Age	Mean Difference	Standard Error	Adjusted p-value
Control	**p21**	**5.936**	**3.023**	**.052**
	p30	.416	3.023	.891
	p60	**5.762**	**2.949**	**.053**
PTEN KO	p21	-2.385	3.109	.444
	p30	3.594	3.405	.293
	p60	4.011	3.260	.221

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.26. Post hoc testing for the effect of genotype on N1 amplitude in the AC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	1.651	6.481	.799
	p30	2.796	6.784	.681
	p60	8.430	6.013	.163
Male	**p21**	**14.568**	**6.013**	**.017**
	p30	-4.067	6.338	.522
	p60	12.665	6.648	.059

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.27. Post hoc testing for the effect of age on N1 amplitude in the AC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	-5.679	6.620	1.000
			p60	-6.155	6.163	.959
		p30	p21	5.679	6.620	1.000
			p60	-.476	6.163	1.000
		p60	p21	6.155	6.163	.959
			p30	.476	6.163	1.000
	Male	p21	p30	-4.902	5.669	1.000
			p60	-10.735	6.013	.229
		p30	p21	4.902	5.669	1.000
			p60	-5.834	6.013	1.000
		p60	p21	10.735	6.013	.229
			p30	5.834	6.013	1.000
PTEN KO	Female	p21	p30	-4.534	6.648	1.000
			p60	.624	6.338	1.000
		p30	p21	4.534	6.648	1.000
			p60	5.157	6.648	1.000
		p60	p21	-.624	6.338	1.000
			p30	-5.157	6.648	1.000
	Male	**p21**	**p30**	**-23.537**	**6.648**	**.002**
			p60	-12.638	6.648	.178
		p30	**p21**	**23.537**	**6.648**	**.002**
			p60	10.898	6.943	.357
		p60	p21	12.638	6.648	.178
			p30	-10.898	6.943	.357

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.28. Post hoc testing for the effect of sex on N1 amplitude in AC.

Genotype	Age	Mean Difference	Standard Error	Adjusted p-value
Control	p21	-7.877	6.163	.203
	p30	-7.100	6.163	.251
	p60	**-12.458**	**6.013**	**.040**
PTEN KO	p21	5.039	6.338	.428
	p30	**-13.964**	**6.943**	**.046**
	p60	-8.223	6.648	.218

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.29. Post hoc testing for the effect of sex on P2 amplitude in AC.

Genotype	Age	Mean Difference	Standard Error	Adjusted p-value
Control	p21	8.427	5.793	.148
	p30	9.679	5.793	.097
	p60	6.247	5.652	.271
PTEN KO	p21	-8.831	5.958	.141
	p30	12.588	6.527	.056
	p60	**13.935**	**6.249**	**.027**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.30. Post hoc testing for the effect of genotype on P1 amplitude in the FC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	**p21**	**12.892**	**4.437**	**.004**
	p30	2.101	4.644	.652
	p60	3.884	4.117	.347
Male	p21	2.453	4.117	.552
	p30	4.861	4.339	.265
	p60	1.440	4.551	.752

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.31. Post hoc testing for the effect of sex on P1 amplitude in FC.

Genotype	Age	Mean Difference	Standard Error	Adjusted p-value
Control	p21	7.736	4.219	.069
	p30	2.243	4.219	.596
	p60	7.143	4.117	.085
PTEN KO	p21	-2.702	4.339	.534
	p30	5.003	4.753	.294
	p60	4.698	4.551	.304

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.32. Post hoc testing for the effect of genotype on N1 amplitude in the FC.

Sex	Age	Mean Difference	Standard Error	Adjusted p-value
Female	p21	4.588	4.995	.360
	p30	2.731	5.229	.602
	p60	**10.163**	**4.635**	**.030**
Male	**p21**	**17.536**	**4.635**	**<.001**
	p30	-2.287	4.886	.640
	p60	9.583	5.124	.064

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Supplementary Table 4.33. Post hoc testing for the effect of age on N1 amplitude in the FC.

Genotype	Sex	Age	Age	Mean Difference	Standard Error	Adjusted p-value
Control	Female	p21	p30	-4.357	5.103	1.000
			p60	-3.353	4.750	1.000
		p30	p21	4.357	5.103	1.000
			p60	1.004	4.750	1.000
		p60	p21	3.353	4.750	1.000
			p30	-1.004	4.750	1.000
	Male	p21	p30	-4.554	4.370	.898
			p60	-2.719	4.635	1.000
		p30	p21	4.554	4.370	.898
			p60	1.836	4.635	1.000
		p60	p21	2.719	4.635	1.000
			p30	-1.836	4.635	1.000
PTEN KO	Female	p21	p30	-6.214	5.124	.682
			p60	2.223	4.886	1.000
		p30	p21	6.214	5.124	.682
			p60	8.437	5.124	.306
		p60	p21	-2.223	4.886	1.000
			p30	-8.437	5.124	.306
	Male	**p21**	**p30**	**-24.378**	**5.124**	**<.001**
			p60	-10.672	5.124	.118
		p30	**p21**	**24.378**	**5.124**	**<.001**
			p60	**13.706**	**5.352**	**.035**
		p60	p21	10.672	5.124	.118
			p30	**-13.706**	**5.352**	**.035**

Post hoc comparisons were carried out using the estimated marginal means and p-values were adjusted with Bonferroni correction. Bold text indicates statistical significance (p = or < 0.05).

Chapter 5

Impact of acute administration of an mGluR5 antagonist, CTEP, on cortical auditory processing in a PTEN-deletion model of Autism Spectrum Disorders

Abstract

Symptoms of Autism Spectrum Disorders (ASD) include severe sensory sensitivity and processing deficits which may lead to broader symptomology including delayed language. The mechanistic links between ASD risk-genes and sensory processing remain unclear. Abnormal temporal processing may lead to speech and language deficits in ASD. The developmental trajectories and genotype- and sex-dependent differences in auditory temporal processing are beginning to be elucidated in transgenic animal models of ASD. We recently showed in the Nse-cre *Pten*-deletion model (phosphatase and tensin homolog missing on chromosome 10) of ASD that deleting PTEN in the cortex significantly impacts oscillatory activity and auditory temporal processing. Specifically, loss of PTEN in cortical layers III-V and the dentate gyrus results in genotype differences in resting electroencephalograph (EEG) recordings and sound evoked responses in both males and females. The underlying mechanisms behind these abnormalities are not well understood. In the current study, we tested whether abnormal activity of group 1 metabotropic receptor mGluR5 was involved in these phenotypes. mGluR5 has been implicated in several models of ASD, including the Nse-cre *Pten* deletion model (PTEN KO). We acutely treated adult PTEN KO mice with CTEP, a specific mGluR5 antagonist, and recorded epidural EEG signals from the frontal (FC) and auditory (AC) cortex. Resting EEG spectral power distribution, auditory event

related potentials (ERP) and temporal processing were quantified from awake and freely moving vehicle- and CTEP-treated male and female mice. Temporal processing was measured using a gap-in-noise-ASSR (auditory steady state response) stimulus paradigm. The experimental manipulation of gap duration and modulation depth allows us to measure cortical entrainment to rapid gaps in sounds. Temporal processing was quantified using inter-trial phase clustering (ITPC) values that account for phase consistency across trials. The results show that CTEP treatment shifts the resting power distribution in males, such that CTEP treated mice have decreased alpha power and increased gamma power in the AC compared to vehicle treated males. CTEP treatment showed no significant impact on auditory temporal processing in either sex or cortical region. The results also show no treatment effect on ERP amplitudes in males or females. Lastly, non-phase locked single trial power is significantly elevated in the AC and FC of CTEP treated females but not males, suggesting a sex specific increase in ‘on-going background activity’ following CTEP treatment. Overall, these data demonstrate that the irregular oscillatory activity and auditory processing deficits previously identified in the Nse-PTEN ASD model are not significantly improved by CTEP treatment, indicating that abnormal mGluR5 activity alone may not drive the EEG phenotypes. However, the differential impact of treatment across the sexes suggests that mGluR5 may be involved in sex-specific mechanisms that underlie the abnormal oscillatory activity.

Introduction

Autism spectrum disorders (ASD) are characterized by reduced social interactions, impaired verbal and nonverbal communication, and repetitive behaviors

[Park et al., 2016; Ratajczak, 2011; Kirkovski et al., 2013; Pickett & London, 2005]. The rate of ASD diagnoses is ~ 1 in 44 children in the United States [Christensen et al., 2018]. ASD has traditionally been diagnosed within the first three years of life, with some of the earliest signs recognized in infants being delays in language and social engagement [Park et al., 2016]. Sensory abnormalities are consistently associated with ASD and are considered a core diagnostic criterion [Picket & London, 2005]. Early developmental sensory deficits may lead to speech and language deficits, but very little is known about the underlying mechanisms of language deficits in ASD.

Auditory temporal processing is a critical aspect of speech processing and normal development of language function. We recently showed significant developmental deficits in auditory temporal processing along with hypersensitive responses and increased cortical background noise in a mouse model of Phosphatase and Tensin homolog deleted on chromosome 10 (*PTEN*) associated ASD. PTEN is a negative regulator of the PI3K/AKT/mTOR pathway that influences cellular processes including growth, proliferation, survival, apoptosis, metabolism, and cell migration [Endersby & Baker, 2008]. In the brain, components of the PI3K/AKT/mTOR pathway are present at synapses and are essential for synaptogenesis and regulation of dendritic spine morphology [Sawicka & Zukin, 2012]. Humans carrying germline PTEN mutations and abnormal PI3K/AKT/mTOR signaling display ASD-related behaviors [Eng, 2003; Reardon et al., 2001; Goffin et al., 2001; Zori et al., 1998]. Butler et al., (2005) showed a number of PTEN mutations in ASD cases with macrocephaly. PTEN mutations were found in 17% of macrocephalic ASD cases [Hobert et al., 2014; Klein et al., 2013; McBride et al., 2010; Varga et al., 2009; Herman et al., 2007; Buxbaum et al., 2007]. Humans with PTEN-ASD have significant intellectual impairment, decreased working

memory and impaired language development [Tilot et al., 2015; Frazier et al., 2015; Frazier, 2019].

A sex-bias in PTEN mutations has been reported in human cohort studies. Notably, females carrying a PTEN mutation had significantly higher cancer risks than males, including thyroid and breast cancers [Nieuwenhuis et al., 2014; Riegert-Johnson et al., 2010]. Mutations of PTEN are a common cause of breast cancers due to their negative regulation of the PI3K/AKT/mTOR pathway, a cancer that is predominately diagnosed in women [Campbell et al., 2001]. This sex-bias is due to the expression of estrogen receptor α (ERα) in 70-80% of breast cancers [Vilgelm et al., 2006]. Hyperactivation of the PI3K/AKT/mTOR pathway in breast cancer cells results in enhanced phosphorylation and the subsequent hyperactivation of ERα, so dual therapeutics inhibiting both PI3K/AKT/mTOR and ERα are the primary treatments used for breast cancers [Yamnik et al., 2009; Ishida et al., 2018; Ciruelos, 2014; Page et al., 2009]. Furthermore, an interaction between ERα and Group 1 metabotropic receptors (mGluR1 and mGluR5) has been identified specifically in female neurons in multiple brain regions [Martinez et al., 2014; Tabatadze et al., 2015; Tonn Eisinger et al., 2018; Santollo & Daniels, 2019]. In the hippocampus, estradiol acts via ERα to initiate postsynaptic mGluR1-dependent mobilization of the endocannabinoid anandamide to suppress GABA release [Huang & Woolley, 2012]. Additionally, this dual interaction has been shown to mediate the estradiol effects on hippocampal memory consolidation [Boulware et al., 2013]. ERα-mGluR5 signaling was seen exclusively in female striatal neurons as well [Grove-Strawser et al., 2010]. This interaction has recently been reported in the cortex of the Nse-cre PTEN mouse model (hereafter referred to as ‘PTEN KO’) in which PTEN is reduced in layers III-V of the neocortex and the dentate gyrus of

the hippocampus. Specifically, hyperexcitable local neocortical circuits (prolonged, spontaneous persistent activity states known as ‘UP states’) were found in female, but not male, PTEN KO mice [Molinaro et al., 2024]. Notably, these UP states were successfully rescued by the antagonism of mGluR5 and ERα and an increased interaction was seen between the two receptor types. These results suggest that a PTEN-linked sex-differences occur in the cortical responses, promoting abnormal phenotypes in females *in vitro*, and the underlying mechanism involves mGluR5 and ERα.

In a recent study, we recorded sensory electrophysiological responses (EEG) in control and PTEN KO mice across development and found that decreased PTEN expression in the cortex significantly impacted resting EEG oscillatory activity in male and female mice and during sound evoked responses [Croom et al., 2024b]. Sex differences were found in relative resting power distributions and sound evoked responses. In particular, adult female PTEN KO mice showed larger evoked responses compared to male KO mice and WT mice. Elevated mGluR5 activity may underlie abnormal auditory responses [Molinaro et al., 2024]. We tested this hypothesis in the current study using acute injections of a potent mGluR5 antagonist, CTEP (chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine) in adult PTEN KO mice prior to EEG recordings. Our data show that the effects of acute CTEP treatment on oscillatory activity are minimal in both sexes, suggesting that mGluR5 activity alone is not likely not responsible for the deficits seen in KO mice.

Methods

Mice: All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. Because germline PTEN homozygous knockouts are embryonically lethal, mice with non-lethal PTEN mutations have been established to study cellular/circuit dysfunctions and ASD phenotypes [Di Cristofano et al., 1998]. The present study used a conditional PTEN knock-out (KO) mouse model that utilizes a neuron-specific enolase (Nse) promoter-driven cre [Kwon et al., 2006a; Molinaro et al., 2024; Croom et al., 2024b]. Nse is a glycolytic enolase that is expressed exclusively in neurons. Cre activity becomes detectable in the brain at p2, in differentiated neurons located within the cortex and hippocampus. At four weeks of age, cre activity remains restricted to the following regions and percentage of neurons in the brain: layers III-V of the cortex (54.5%), dentate gyrus (48.7%), CA3 (37.6%) and polymorphic layer (PML) (58.3%) in the hippocampus. No cre activity is detected in glial cells [Kwon et al., 2006a].

PTEN-mutant mice (*Nse-cre; Pten*$^{flox/flox}$) were generated by breeding male *PTEN*$^{flox/flox}$ mice (JAX, stock #006440) and female *Nse-cre; PTEN*$^{flox/WT}$ mice on the C57bl6/J background (Provided by Dr. Kimberly Huber, UTSW). This breeding arrangement produces litters containing three different genotypes: control (*cre* negative), PTEN-heterozygous (*Nse-cre; Pten*$^{flox/WT}$), and PTEN-KO (*Nse-cre; Pten*$^{flox/flox}$). Male and female PTEN-KO mice were used for the current study. Data collected on vehicle and CTEP treated KO mice were compared to previously published naïve male and female KO data [Croom et al., 2024b]. All mice were genotyped via toe clipping (Transnetyx) and studies were done before the onset (<p80) of functional age-related hearing loss in the C57bl6/J strain of mice [Johnson et al., 1997]. The following sample sizes were used

in this study: Vehicle treated [Males: n=10, Females: n=11], CTEP treated [Males: n=10, Females: n=11]. To ensure that vehicle treated PTEN KO mice exhibited abnormal auditory responses as seen in naïve PTEN KO mice, we compared data from these two groups of mice. The data for naïve PTEN KO mice used for this comparison has been published previously [n=10 males and 12 females, Croom et al., 2024b].

Surgery: Adult mice underwent epidural electrode implantation surgery at ~p58-p65. This specific age range was chosen as the largest deficits were seen in adult PTEN KO mice, and female KO mice showed larger amplitudes of event related potentials (ERP) than male KO mice at this age. Surgical procedures have been previously published [Croom et al. 2023, 2024a, 2024b; Rumschlag et al., 2021; Rumschlag & Razak, 2021]. Briefly, mice were anesthetized using intraperitoneal (IP) injections of 80/10/1 mg/kg ketamine/xylazine/acepromazine adult mice. The anesthetic state was monitored throughout the procedure by toe pinch reflex every 10-15 minutes. ETHIQA-XR (1-shot buprenorphine, 3.25 mg/kg body weight) was administered via subcutaneous injection prior to surgery. An incision was made to expose the scalp following the removal of fur, and sterilization (alcohol and iodine wipes) of the scalp. A Foredom dental drill was used to drill ~1mm diameter holes in the skull over the right AC, right FC, and left occipital cortex. The screw positions were determined using skull landmarks and coordinates previously reported [Wen et al., 2019; Rumschlag & Razak, 2021; Lovelace et al., 2018; Lovelace et al., 2020; Rumschlag et al., 2021] and were based on single unit recordings [Rotschafer & Razak 2014; Wen et al., 2019; Trujillo et al., 2011]. The wires extending from three-channel posts were wrapped around 1 mm screws and driven into the pre-drilled holes. Dental cement was applied to secure the implant. Mice were placed on a

heating pad until fully awake and were allowed 48-72 hours for recovery before EEG recordings were made.

Drug procedures: The mGluR5 specific negative allosteric modulator CTEP was chosen for these experiments due to its crossing of blood-brain barrier, selective binding and long half-life of 18 hours [Lindemann et al., 2011]. Adult mice were acutely administered either 2 mg/kg CTEP (prepared fresh daily) or vehicle (0.9% NaCl, 0.3% Tween-80) via IP injection 90 minutes prior to EEG recording, as previously published [Molinaro et al., 2024].

EEG recordings: All EEG recordings were obtained from awake and freely moving mice. EEG recordings were performed at ~p59-p70. Recordings were obtained from the AC and FC electrodes, using the occipital screw as reference. Mice were placed in an arena inside a Faraday cage. The cage was located on a vibration isolation table in a sound-insulated and anechoic booth (Gretch-Ken, OR). Mice were briefly anesthetized with isoflurane and attached to an EEG cable via the implanted screws and then habituated to the recording arena with no stimuli for 15 minutes prior to sound evoked recordings. The EEG recording set-up has been previously reported [Rumschlag et al., 2021; Rumschlag & Razak, 2021; Croom et al., 2023, 2024a, 2024b]. Briefly, the attached cable was connected via a commutator to a TDT (Tucker Davis Technologies, FL) RA4LI/RA4PA headstage/pre-amp, which was connected to a TDT RZ6 multi-I/O processor. OpenEx (TDT) was used to simultaneously record EEG signals and operate the LED light used to synchronize the video and waveform data. TTL pulses were utilized to mark stimulus onsets on a separate channel in the collected EEG data. The EEG signals were recorded at a sampling rate of 24.414 kHz and down-sampled to 1024 Hz for analysis. All raw EEG recordings were visually examined prior to analysis for

artifacts, including loss of signal or signs of clipping. No EEG data collected in this study was rejected after examination.

Resting EEG: For resting EEGs (no sound stimulus presented), mice were habituated in the arena for 15 minutes followed by 5 minutes of recording. Power spectral densities were analyzed in both AC and FC. The traces were split into Hanning-windowed 1-second segments with 50% overlap to avoid edge artifacts and spectral splatter and then transformed to the frequency domain via Fourier transform [Rumschlag et al., 2021]. The average power was calculated by averaging the spectra from each of the 1-second segments and were then split into frequency bands (theta: 3-7 Hz, alpha: 8-13 Hz, beta: 14-29 Hz, low gamma: 30-59 Hz, high gamma: 61-100 Hz, and high-frequency oscillations (HFO): 101-250 Hz). Gamma power was split to low and high ranges as studies have suggested that rhythms in the 30–60 Hz band and higher frequency broadband gamma (>50 Hz) are distinct and generated by different mechanisms [Dvorak & Fenton, 2014; Balakrishnan & Pearce 2015].

Auditory ERP: Narrowband noise stimuli (1-12 kHz, 120 repetitions, 100 ms duration, 5ms rise/fall time, 0.25 Hz repetition rate) were presented at 75 dB SPL using a speaker (MF1, Tucker Davis Technologies, FL) situated 20 cm above the floor of the arena. The EEG trace was split into trials, using the TTL pulses to mark sound onset [Croom et al., 2023, 2024a, 2024b; Rumschlag & Razak 2021; Rumschlag et al., 2021]. Each trial was baseline corrected, such that the mean of the 250 ms baseline period prior to sound onset was subtracted from the trial trace for each trial. Each trial was then detrended (MATLAB detrend function) and all trials were averaged together. Time-frequency analysis was performed with a dynamic complex Morlet wavelet transform with Gabor normalization. The wavelet parameter was set for each frequency to optimize time-

frequency resolution. The single trial power (STP) measurement does not correct for mean baseline power levels, allowing for the identification of ongoing 'background activity' during stimulus presentation. To compare the responses across treatment conditions and sexes, a non-parametric permutation test was used, to find clusters of significant values [Maris & Oostenveld, 2007]. First, a t-test was run on each time-frequency point for the two groups being compared, yielding the T-values for all points. T-values corresponding to $p<0.025$ were considered significant. Clusters of significant T-values were found and their area was measured. Next, the group assignments were shuffled randomly, and the t-tests and cluster-measurements were run again on the surrogate groups. This surrogate analysis was performed 2000 times to generate a distribution of cluster sizes that we would expect to find by chance. Originally identified clusters that were larger than 95% of the surrogate clusters were considered significant. This method allows for the identification of significant differences between groups without performing excessive comparisons.

Gap-ASSR: The stimulus used to assess auditory temporal processing is termed the '40 Hz gaps-in-noise ASSR' (auditory steady state response, henceforth, 'gap-ASSR') [Rumschlag & Razak, 2021; Croom et al., 2023, 2024a, 2024b]. The stimulus contains alternating 250 ms segments of noise and gap interrupted noise presented at 75 dB SPL. The gaps are placed 25 ms apart, resulting in a presentation rate of 40 Hz, a rate that produces the strongest ASSR signal when measured from the AC and frontal regions and may reflect the resonance frequency of the underlying neural circuits [Galambos et al., 1981; Pastor et al., 2002; Llinas, 1988; Llinas et al., 1991; Rosanova et al., 2009; Kim et al., 2015; Hwang et al., 2019]. For each gap in noise segment, the gap duration and modulation depth are chosen at random. Gaps of 2, 4, 6, 8, 10, or 12 ms

durations and modulation depths of 75 and 100% were used. To measure the ability of the cortex to consistently respond to the gaps in noise, inter-trial phase clustering (ITPC) at 40 Hz was measured [Cohen, 2014]. ITPC measures the phase consistency of the recorded signal across multiple trials. The ITPC is determined by the distribution of phase angles in the EEG response across trials at each gap duration and reflects the precise timing of 40 Hz activity in the underlying neural generators. If the cortical regions are consistently responding to a specific gap duration across trials, the ITPC values will be high, reaching a maximum of 1. If the response is not consistent across trials, the expected ITPC value will be significantly lower (minimum of zero). The EEG trace was transformed using a dynamic complex Morlet wavelet transform. The trials corresponding to each parametric pair (gap duration + modulation depth) were grouped together. The ITPC was calculated for each time-frequency point as the average vector for each of the phase unit vectors recorded across trials (trial count >100 trials per parametric pair). The ITPC values at 40 Hz were averaged to extract the mean ITPC for the parametric pairs in the AC and FC.

Statistics: Statistics were performed on GraphPad Prism. To evaluate the effects of treatment (2 levels) and sex (2 levels), two-way ANOVAs were used for resting and ERP analysis. Post hoc comparisons were carried out with Bonferroni's multiple comparisons test and p-values were adjusted accordingly. The resting and ERP data was tested for normality using Shapiro-Wilk tests. Unpaired t-tests were used for resting and ERP data to compare naïve male and female KO to vehicle treated male and female KO. A three-way repeated measures ANOVA was used for vehicle vs CTEP treated gap-ASSR analysis, with the three factors being treatment (2 levels) X sex (2 levels) X gap duration (6 levels). A two-way repeated measures ANOVA was used for naïve vs vehicle treated

gap-ASSR analysis, with the two factors being gap duration (6 levels) X condition (2 levels). A repeated measures ANOVA was chosen as multiple gap duration data points were collected from a single mouse in a recording session. Mauchly Tests for Sphericity were utilized and corrected for using the Greenhouse-Geisser corrections if necessary. Cortical regions (AC, FC) and modulation depths (75%, 100%) were analyzed separately.

Results

Our previous study characterized the developmental trajectory of cortical resting EEG and sound-evoked responses in the Nse-cre PTEN-deletion model (Croom et al., 2024b). Results of that study showed abnormal relative resting power distributions, auditory temporal processing deficits, and hypersensitivity. While both male and female KO mice demonstrated temporal processing deficits across development, adult female KO mice showed increased ERP amplitudes compared to males. The main goal of this study was to determine whether an antagonist of mGluR5 receptor improves these phenotypes in adult PTEN KO mice. We hypothesized that mGluR5 antagonism with CTEP would improve EEG phenotypes to a greater extent in KO females, compared to males, due to the sex-bias seen in mGluR5-ERα interactions.

Male KO mice have decreased alpha and increased gamma power in the AC following CTEP treatment

We quantified resting (no stimulus) spectral power distribution in naïve (no injection) adult male and female KO mice to vehicle-treated male and female KO mice to ensure vehicle treatment alone was not impacting phenotypes in the auditory cortex (AC) and frontal cortex (FC). Figure 5.1 and Table 5.1 show the results of the full

statistical analyses of resting spectral power. We then compared adult vehicle treated male and female PTEN KO mice to age- and sex-matched KO mice who were acutely treated with CTEP. Figure 5.2 and Table 5.2 show this analysis.

When comparing vehicle treated and naïve mice, no differences were seen in the AC of males or females (Figure 5.1A-F). The subsequent analysis showed that CTEP treatment did alter resting power distributions in the AC (Figure 5.2). The major treatment effect was decreased alpha and increased gamma power in the AC of male, but not female, mice. Alpha power was significantly impacted by both drug treatment and sex. Specifically, CTEP treated PTEN KO males had decreased alpha power compared to CTEP treated KO females ($p=0.0068$) (Figure 5.2B). CTEP treatment also caused a significant reduction of alpha power in KO males (Figure 5.2B). Low gamma power was impacted by sex (main effect: $p=0.0435$) (Figure 5.2D). CTEP treated males had significantly more power in the low and high gamma frequency bands compared to vehicle treated males (low gamma: $p=0.0162$; high gamma: $p=0.0270$) (Figure 5.2D-E). Additionally, CTEP treated males had increased high gamma power compared to CTEP treated females ($p=0.0260$) (Figure 5.2E). A main effect of sex was also seen for high frequency oscillations (main effect: $p=0.0310$) (Figure 5.2F). These data show that CTEP treatment alters male KO mice relative resting power distributions in the AC, compared to vehicle.

Similarly to the AC, no differences were seen between naïve and vehicle treated male and female KO mice in the FC (Figure 5.1G-L). When comparing vehicle and CTEP treated mice, a significant main effect of drug treatment was seen in the alpha and low gamma frequency bands (alpha: $p=0.0504$; low gamma: $p=0.0143$) (Figure 5.1H, 5.1J). Additionally, high gamma power was significantly impacted by sex ($p=0.0466$)

(Figure 5.1K). Taken together, these results show that CTEP caused sex and region-specific actions on relative resting power distributions. Specifically, CTEP treatment lowered alpha power and increased high gamma power in the AC, but not FC, of male KO mice.

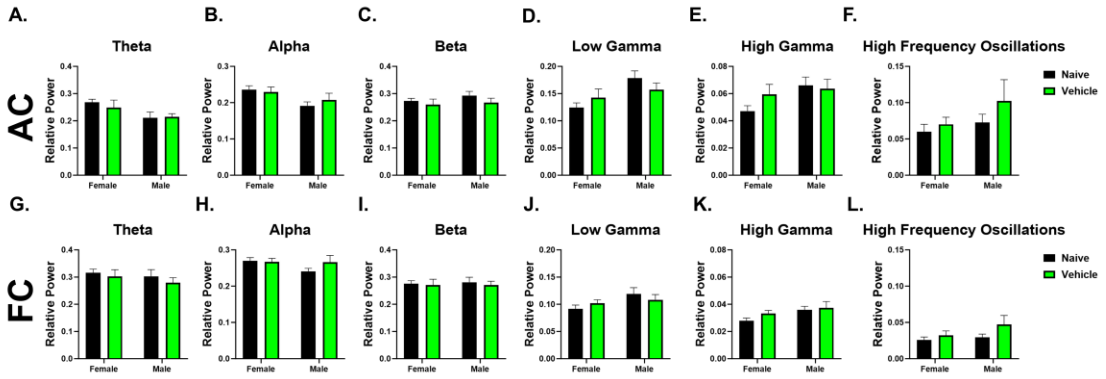

Figure 5.1. No impact of vehicle treatment on resting spectral power distribution in PTEN KO mice. Relative resting spectral power distribution in male and female naïve and vehicle treated PTEN KO mice in the AC (A-F) and FC (G-L). No effect of vehicle treatment can be seen in either sex or cortical region.

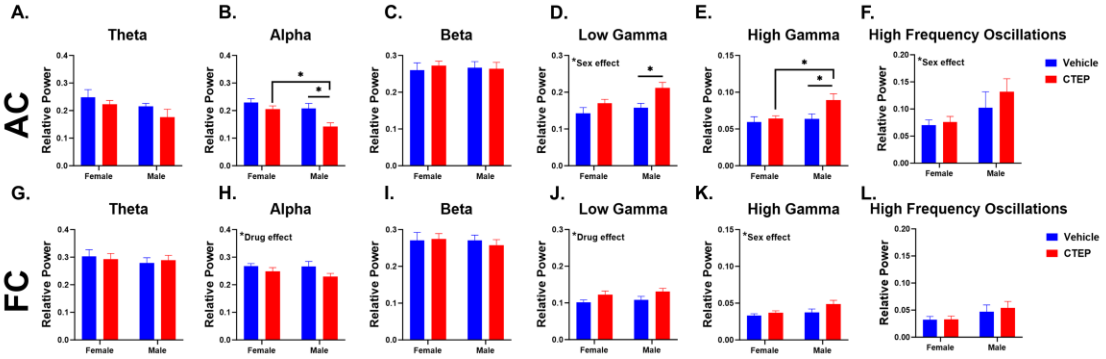

Figure 5.2. Male KO mice have decreased alpha and increased gamma power in the AC following CTEP treatment. Relative resting spectral power distribution in male and female vehicle and CTEP treated PTEN KO mice in the AC (A-F) and FC (G-L). Panels indicate main effects and post hoc comparisons. CTEP treatment decreased alpha (B) and increased gamma power (D,E) in the AC of male mice. CTEP treated PTEN KO males have decreased alpha power compared to CTEP treated KO females (B). Low gamma power and high frequency oscillations were impacted by sex in the AC (D,F). CTEP treated males have increased high gamma power compared to CTEP treated females (D). A significant main effect of drug treatment is seen in the alpha and low gamma frequency bands in the FC (H,J). High gamma power significantly impacted by sex in the FC (K).

Table 5.1. Full statistical analysis of resting EEG data for naïve and vehicle treated mice.

Sex	Cortical Region	Frequency	(t,df)	p-value
Female	AC	Theta:	t(21)=0.6917	0.4967
		Alpha:	t(21)=0.3932	0.6981
		Beta:	t(21)=0.6182	0.5431
		Low Gamma:	t(21)=1.053	0.3045
		High Gamma:	t(21)=1.551	0.1358
		HFO:	t(21)=0.7255	0.4761
	FC	Theta:	t(21)=0.4873	0.6311
		Alpha:	t(21)=0.2091	0.8364
		Beta:	t(21)=0.2082	0.8371
		Low Gamma:	t(21)=1.081	0.2921
		High Gamma:	t(21)=1.682	0.1074
		HFO:	t(21)=0.8522	0.4037
Male	AC	Theta:	t(18)=0.1488	0.8834
		Alpha:	t(18)=0.7412	0.4681
		Beta:	t(18)=1.154	0.2636
		Low Gamma:	t(18)=1.168	0.2579
		High Gamma:	t(18)=0.2542	0.8022
		HFO:	t(18)=0.9360	0.3617
	FC	Theta:	t(18)=0.7602	0.4570
		Alpha:	t(18)=1.239	0.2314
		Beta:	t(18)=0.4404	0.6649
		Low Gamma:	t(18)=0.7119	0.4856
		High Gamma:	t(18)=0.2690	0.7910
		HFO:	t(18)=1.308	0.2073

Unpaired t-tests were used to compare naïve male and female KO to vehicle treated male and female KO. Bold text indicates statistical significance (p = or < 0.05).

Table 5.2. Full statistical analysis of resting EEG data for vehicle and CTEP treated mice.

Cortical Region	Frequency	Factor	ANOVA Results	p-value
AC	Theta:	Treatment	F(1,38)=2.096	0.1559
		Sex	F(1,38)=3.284	0.0779
		Treatment x Sex	F(1,38)=0.0965	0.7577
	Alpha:	**Treatment**	**F(1,38)=9.499**	**0.0038**
		Sex	**F(1,38)=8.865**	**0.0050**
		Treatment x Sex	F(1,38)=2.068	0.1586
	Beta:	Treatment	F(1,38)=0.0746	0.7861
		Sex	F(1,38)=0.0029	0.9568
		Treatment x Sex	F(1,38)=0.2059	0.6526
	Low Gamma:	**Treatment**	**F(1,38)=9.243**	**0.0043**
		Sex	**F(1,38)=4.361**	**0.0435**
		Treatment x Sex	F(1,38)=1.009	0.3215
	High Gamma:	**Treatment**	**F(1,38)=4.878**	**0.0333**
		Sex	**F(1,38)=4.593**	**0.0386**
		Treatment x Sex	F(1,38)=2.380	0.1312
	HFO:	Treatment	F(1,38)=0.7940	0.3785
		Sex	**F(1,38)=5.017**	**0.0310**
		Treatment x Sex	F(1,38)=0.3631	0.5504
FC	Theta:	Treatment	F(1,38)=1.26e-006	0.9991
		Sex	F(1,38)=0.4670	0.4985
		Treatment x Sex	F(1,38)=0.2001	0.6572
	Alpha:	**Treatment**	**F(1,38)=4.084**	**0.0504**
		Sex	F(1,38)=0.5227	0.4741
		Treatment x Sex	F(1,38)=0.4257	0.5180
	Beta:	Treatment	F(1,38)=0.0877	0.7686
		Sex	F(1,38)=0.2227	0.6397
		Treatment x Sex	F(1,38)=0.2409	0.6264
	Low Gamma:	**Treatment**	**F(1,38)=6.593**	**0.0143**
		Sex	F(1,38)=0.7084	0.4052
		Treatment x Sex	F(1,38)=0.0096	0.9224
	High Gamma:	Treatment	F(1,38)=3.883	0.0561
		Sex	**F(1,38)=4.232**	**0.0466**
		Treatment x Sex	F(1,38)=0.9191	0.3438
	HFO:	Treatment	F(1,38)=0.1936	0.6624
		Sex	F(1,38)=3.762	0.0599
		Treatment x Sex	F(1,38)=0.1203	0.7307

Two-way ANOVA results for resting EEG analysis. Bold text indicates statistical significance (p = or < 0.05).

Auditory temporal processing deficits are not corrected by CTEP treatment

Auditory temporal processing was quantified using a 40 Hz gap-in-noise ASSR stimulus to assess the ability of the cortex to consistently phase lock to brief gaps in noise. Manipulating gap duration and modulation depth of the stimulus allows for a

comparison of temporal processing across experimental groups. Figure 5.3 shows example gap-ASSR heat maps of AC and FC ITPC for individual vehicle (Figure 5.3A and 5.3C) and CTEP treated (Figure 5.3B and 5.3D) mice. Each panel shows the ITPC at a specific gap generated with the 40 Hz stimulus, with increasing gaps across columns and each row showing a different age. In each panel, zero on the x-axis (faint dashed line) marks the onset of the gap ASSR stimulus. The ITPC is strongest at 40 Hz because the stimulus is a 40 Hz train. The warm colors indicating higher ITPC are at 40 Hz. Cooler colors indicate relatively low ITPC and are expected for very short gaps and at spectral bands outside 40 Hz. Matching our previously published naïve KO results, major deficits are seen in both cortical regions of the vehicle and CTEP treated mice. Specifically, the ITPC does not emerge above background at 40 Hz for any gap duration in the vehicle or CTEP treated example (Figure 5.3). The complete three-way ANOVA analysis of this data (gap duration x drug x sex) is shown in Table 5.3. Table 5.4 shows the two-way ANOVA analysis for naïve and vehicle treated PTEN KO males and females.

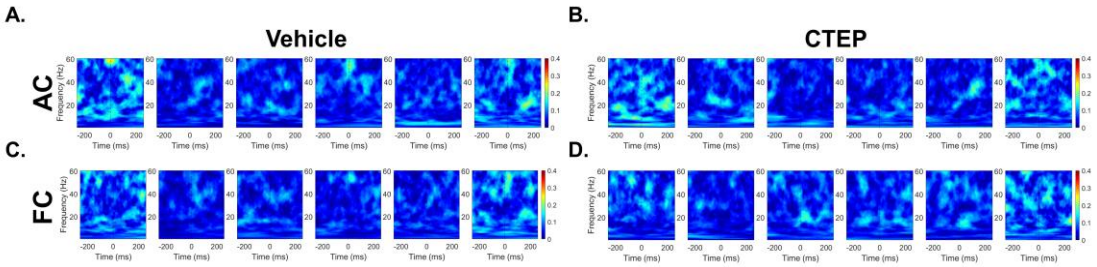

Figure 5.3. Auditory temporal processing deficits are not corrected by CTEP treatment. Individual example heatmaps of ITPC generated at 40Hz for different gap durations in vehicle (A: AC, C: FC) and CTEP treated (B: AC, D: FC) PTEN KO mice. The same example mouse is used for the AC and FC. Each panel shows the ITPC (scale is seen at the right edge of the figure, warmer colors mean greater ITPC) obtained at a specific gap width. Sound onset in each panel is at 0msec. Each column shows ITPC for the same gap width, with the gap width increasing from left to right. The y-axis of each panel is the range of frequencies analyzed for ITPC. ITPC is maximum around 40 Hz, which was the repetition rate of ASSR stimulus train. Qualitative observations of these examples show clear deficits in cortical temporal processing in both vehicle and CTEP treated mice in the AC and FC. All panels show 100% modulation depth.

Auditory Cortex

As expected, there was a significant impact of gap duration on ITPC in the AC at both modulation depths ($p<0.0001$). Although no interactions or main effects of treatment and sex were identified at 75% modulation, a gap duration x sex x drug interaction and a main effect of drug treatment were seen at 100%, suggesting that the impact of drug treatment may vary across the sexes at different gap durations (interaction effect: $p=0.0006$; main effect of drug treatment: $p=0.0170$) (Figure 5.4). This result is due to a significant difference between vehicle and CTEP treated females, specifically at longer gap durations (12ms), with ITPC decreasing in CTEP treated females (Figure 5.4, top row). No significant differences between vehicle and CTEP treated males were seen for either modulation depth or cortical region (Figure 5.4, bottom row). Similarly, no differences between naïve and vehicle treated males and females were identified in the AC (Figure 5.5).

Frontal Cortex

Similar to the AC, there was a significant effect of gap duration on ITPC in the FC at both modulation depths ($p<0.0001$). However, no interactions or main effects of treatment and sex were identified in the FC (Figure 5.4). No differences were seen between naïve and vehicle treated males and females in the FC (Figure 5.5). Taken together, these results suggest that temporal processing deficits are not improved by CTEP treatment in adult male or female KO mice. Overall, ITPC values remained low after treatment, resembling naïve KO phenotypes.

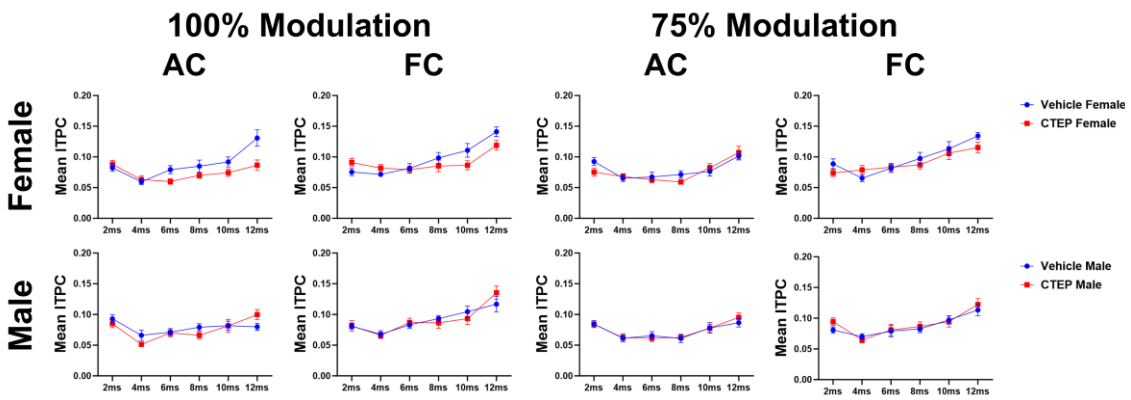

Figure 5.4. Population analysis shows no impact of CTEP treatment on temporal processing deficits in male or female PTEN KO mice. Each plot represents the group average ITPC values for vehicle and CTEP treated females (top row) and males (bottom row). The left columns represent AC and FC data at 100% modulation depth, and the right columns represent AC and FC data at 75% modulation depth. A gap duration x sex x drug interaction and a main effect of drug treatment are seen at 100% modulation depth in the AC. CTEP treatment significantly decreased ITPC in the AC of females at longer gap durations (100% modulation). No interactions or main effects of treatment and sex were identified in the FC. The complete three-way ANOVA analysis of this data is shown in Table 5.3.

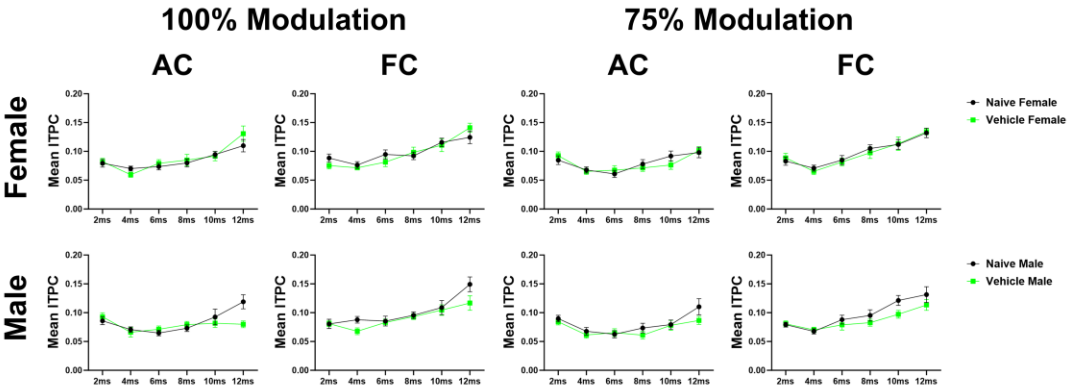

Figure 5.5. No impact of vehicle treatment on auditory temporal processing in PTEN KO mice. Each plot represents the group average ITPC values for naïve and vehicle treated females (top row) and males (bottom row). The left columns represent AC and FC data at 100% modulation depth, and the right columns represent AC and FC data at 75% modulation depth. No differences are seen between naïve and vehicle treated males and females in either cortical region. The complete two-way ANOVA analysis of this data is shown in Table 5.4.

Table 5.3. Full statistical analysis of gap-ASSR data for vehicle and CTEP treated mice.

Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
AC	100%	**Gap Duration**	**F(4.38,166.5)=15.41**	**<0.0001**
		Sex	F(1,38)=1.164	0.2874
		Treatment	**F(1,38)=6.231**	**0.0170**
		Gap duration x Sex	F(5,190)=1.295	0.2677
		Gap duration x Treatment	F(5,190)=0.4546	0.8096
		Sex x Treatment	F(1,38)=2.749	0.1056
		Gap duration x Sex x Treatment	**F(5,190)=4.566**	**0.0006**
AC	75%	**Gap Duration**	**F(4.033,153.2)=20.10**	**<0.0001**
		Sex	F(1,38)=1.552	0.2204
		Treatment	F(1,38)=0.1109	0.7410
		Gap duration x Sex	F(5,190)=0.6264	0.6798
		Gap duration x Treatment	F(5,190)=0.9194	0.4695
		Sex x Treatment	F(1,38)=0.4452	0.5086
		Gap duration x Sex x Treatment	F(5,190)=0.5673	0.7250
FC	100%	**Gap Duration**	**F(3.086,144.6)=29.21**	**<0.0001**
		Sex	F(1,38)=0.2537	0.6174
		Treatment	F(1,38)=0.4092	0.5262
		Gap duration x Sex	F(5,190)=0.4202	0.8343
		Gap duration x Treatment	F(5,190)=1.720	0.1319
		Sex x Treatment	F(1,38)=0.5536	0.4614
		Gap duration x Sex x Treatment	F(5,190)=1.918	0.0932
FC	75%	**Gap Duration**	**F(4.171,158.5)=26.28**	**<0.0001**
		Sex	F(1,38)=1.256	0.2694
		Treatment	F(1,38)=0.0939	0.7609
		Gap duration x Sex	F(5,190)=0.8828	0.4937
		Gap duration x Treatment	F(5,190)=0.2803	0.9235
		Sex x Treatment	F(1,38)=1.183	0.2835
		Gap duration x Sex x Treatment	F(5,190)=1.626	0.1549

Three-way repeated measures ANOVA results for gap-ASSR analysis. P-values were corrected for multiple comparisons using the Greenhouse-Geisser corrections if necessary. Bold text indicates statistical significance (p = or < 0.05). 'Treatment' indicates vehicle vs CTEP comparisons.

Table 5.4. Full statistical analysis of gap-ASSR data for naïve and vehicle treated mice.

Sex	Cortical Region	Modulation Depth	Factor/Interaction	ANOVA Results	Adjusted p-value
Female	AC	100%	**Gap Duration**	**F(3.452,72.49)=13.97**	**<0.0001**
			Condition	F(1,21)=0.3741	0.5473
			Gap x Condition	F(5,105)=1.062	0.3858
		75%	**Gap Duration**	**F(3.558,74.73)=7.409**	**<0.0001**
			Condition	F(1,21)=0.0697	0.7943
			Gap x Condition	F(5,105)=0.7817	0.5651
	FC	100%	**Gap Duration**	**F(4.474,93.94)=20.27**	**<0.0001**
			Condition	F(1,21)=0.1082	0.7454
			Gap x Condition	F(5,105)=1.460	0.2093
		75%	**Gap Duration**	**F(3.669,77.05)=25.33**	**<0.0001**
			Condition	F(1,21)=0.0297	0.8647
			Gap x Condition	F(5,105)=0.2993	0.9123
Male	AC	100%	**Gap Duration**	**F(4.040,72.73)=5.833**	**0.0004**
			Condition	F(1,18)=1.055	0.3180
			Gap x Condition	**F(5,90)=2.911**	**0.0175**
		75%	**Gap Duration**	**F(3.411,61.39)=6.975**	**0.0002**
			Condition	F(1,18)=2.174	0.1576
			Gap x Condition	F(5,90)=0.7791	0.5674
	FC	100%	**Gap Duration**	**F(3.595,64.72)=14.60**	**<0.0001**
			Condition	F(1,18)=2.174	0.1576
			Gap x Condition	F(5,90)=1.432	0.2204
		75%	**Gap Duration**	**F(3.559,64.07)=13.36**	**<0.0001**
			Condition	F(1,18)=3.404	0.0815
			Gap x Condition	F(5,90)=0.9235	0.4697

Two-way repeated measures ANOVA results for comparisons. P-values were corrected for multiple comparisons using the Greenhouse-Geisser corrections if necessary. Bold text indicates statistical significance (p = or < 0.05). 'Condition' indicates vehicle vs naïve comparisons.

CTEP treatment does not affect ERP amplitudes but differentially impacts single trial power in females

ERPs consist of a series of voltage fluctuations, referred to as 'waves' (P1, N1, P2). These waves are evoked at specific latencies after sound onset and are associated with the population activity in specific brain regions. Measuring the amplitudes of these waves allows for the assessment of auditory response magnitudes and synchrony. We previously showed that P1 amplitudes were decreased and N1 amplitudes were

increased in naïve adult PTEN KO mice. We found there to be no significant difference between naïve and vehicle treated KO mice in the AC or FC (Figure 5.6, Table 5.5). CTEP injection had no impact on any ERP component in the AC or FC, suggesting that abnormalities may not involve greater mGluR5 activation (Figure 5.7, Table 5.6). Additionally, no sex differences were seen in either cortical region (Figure 5.7, Table 5.6).

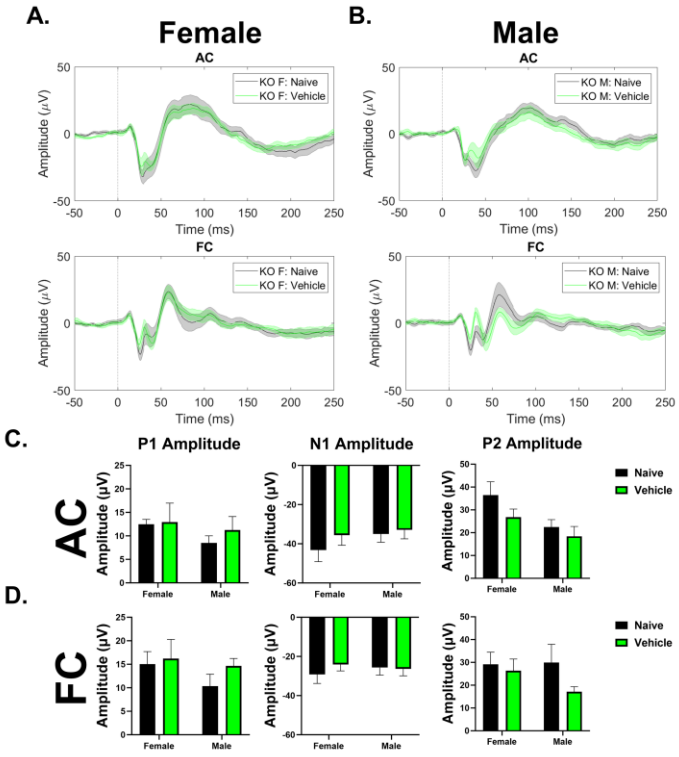

Figure 5.6. No impact of vehicle treatment on ERP amplitudes in PTEN KO mice. (A) Average ERPs recorded for naïve and vehicle treated PTEN KO females in the AC (top) and FC (bottom). (B) Average ERPs recorded for naïve and vehicle treated PTEN KO males in the AC (top) and FC (bottom). (C) Population averages of AC ERP wave amplitudes. No significant difference between naïve and vehicle treated KO mice in the AC. (D) Population averages of FC ERP wave amplitudes. No significant difference between naïve and vehicle treated KO mice in the FC.

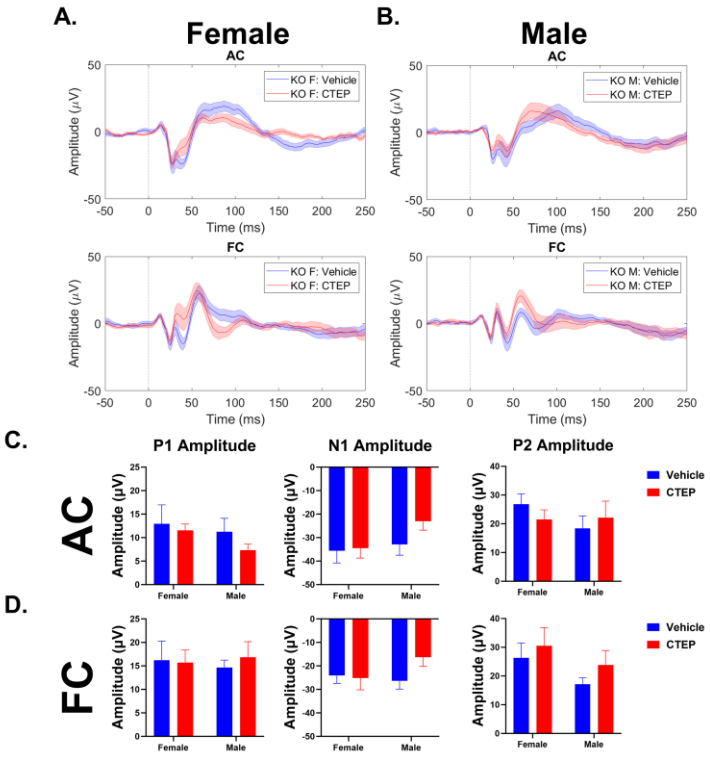

Figure 5.7. CTEP treatment does not affect ERP amplitudes in PTEN KO mice. (A) Average ERPs recorded for vehicle and CTEP treated PTEN KO females in the AC (top) and FC (bottom). (B) Average ERPs recorded for vehicle and CTEP treated PTEN KO males in the AC (top) and FC (bottom). (C) Population averages of AC ERP wave amplitudes. No significant difference between vehicle and CTEP treated KO mice in the AC. (D) Population averages of FC ERP wave amplitudes. No significant difference between vehicle and CTEP treated KO mice in the FC.

We characterized non-baseline normalized STP in response to narrowband noise bursts, as abnormal power ('background activity') during sound-evoked responses show clinical correlations in humans with autism [Ethridge et al., 2017; Ethridge et al., 2019]. We previously showed that STP was significantly increased in naïve adult PTEN KO males and females compared to controls [Croom et al., 2024b]. Additionally, we found there to be no significant difference between naïve and vehicle treated KO mice in either cortical region (Figures 5.8 and 5.9). CTEP treatment does not affect STP in male and female PTEN KO mice compared to vehicle in the AC (Figure 5.10) or FC (Figure 5.11). We also compared the STP of vehicle treated males and females as well CTEP treated males and females to determine whether CTEP treatment was affecting the sexes differentially. In both the AC and FC, there was no difference between vehicle treated

males and females (Figure 5.12A, Figure 5.13A). However, CTEP treated females showed significantly increased STP compared CTEP treated males in both cortical regions (Figure 5.12B, Figure 5.13B). Taken together, the ERP results suggest that overall synchrony of responses is not impacted by CTEP treatment in PTEN KO mice, but treatment does differentially impact STP in the sexes. Specifically, females show increased ongoing background activity following CTEP treatment.

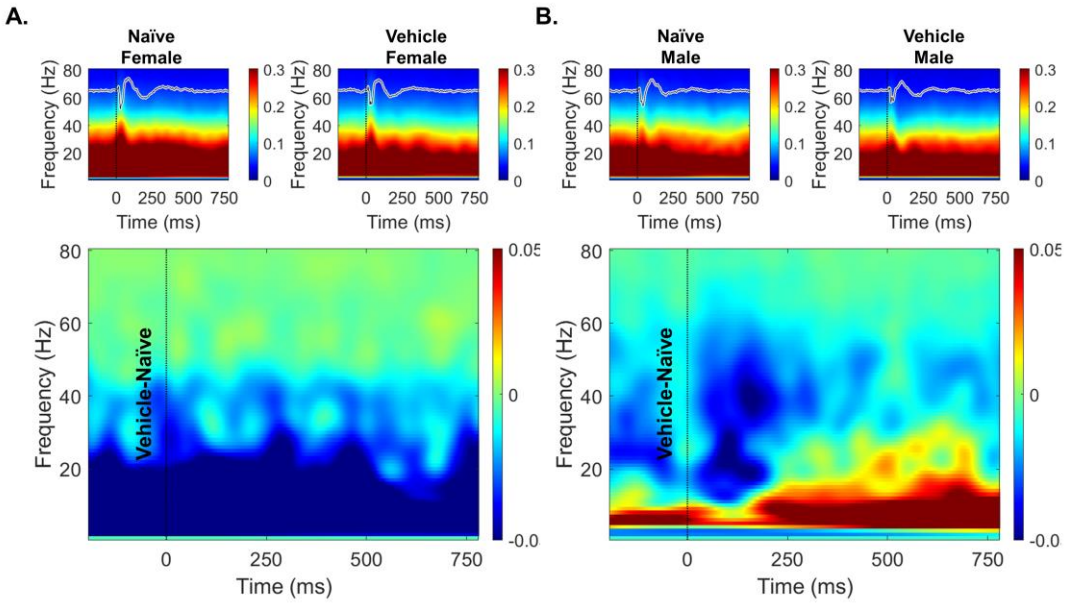

Figure 5.8. No impact of vehicle treatment on STP in the AC PTEN KO mice. The heatmaps show non-baseline corrected normalized power, where warmer hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP in naïve (left) and vehicle treated (right) female (A) and male (B) PTEN KO mice. The larger panels show the difference between vehicle treated and naïve mice. No significant differences were found in STP in females (A) or males (B) in the AC.

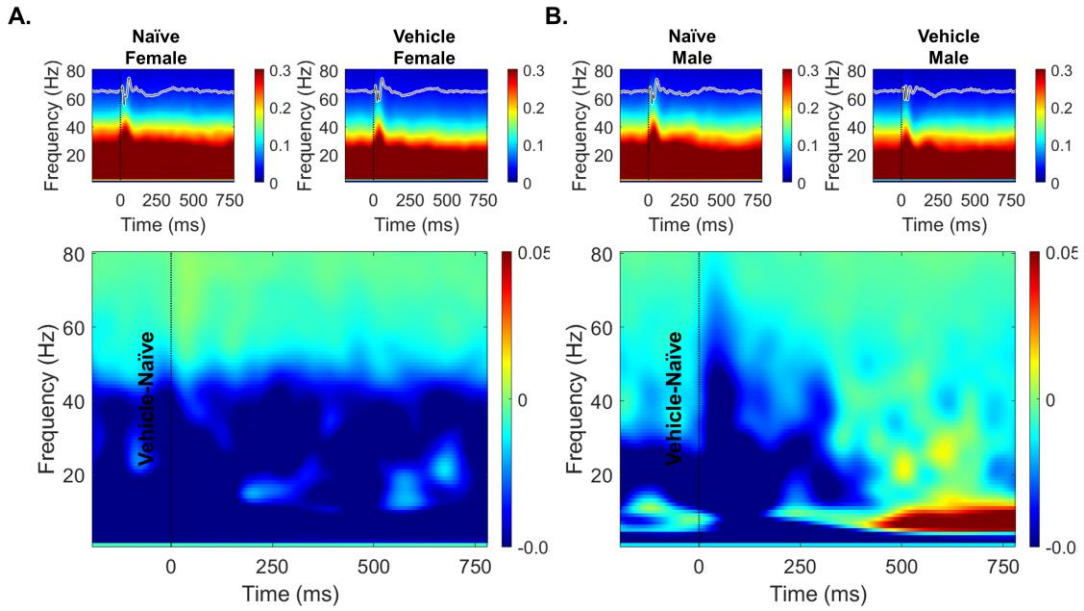

Figure 5.9. No impact of vehicle treatment on STP in the FC PTEN KO mice. The heatmaps show non-baseline corrected normalized power, where warmer hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP in naïve (left) and vehicle treated (right) female (A) and male (B) PTEN KO mice. The larger panels show the difference between vehicle treated and naïve mice. No significant differences were found in STP in females (A) or males (B) in the FC.

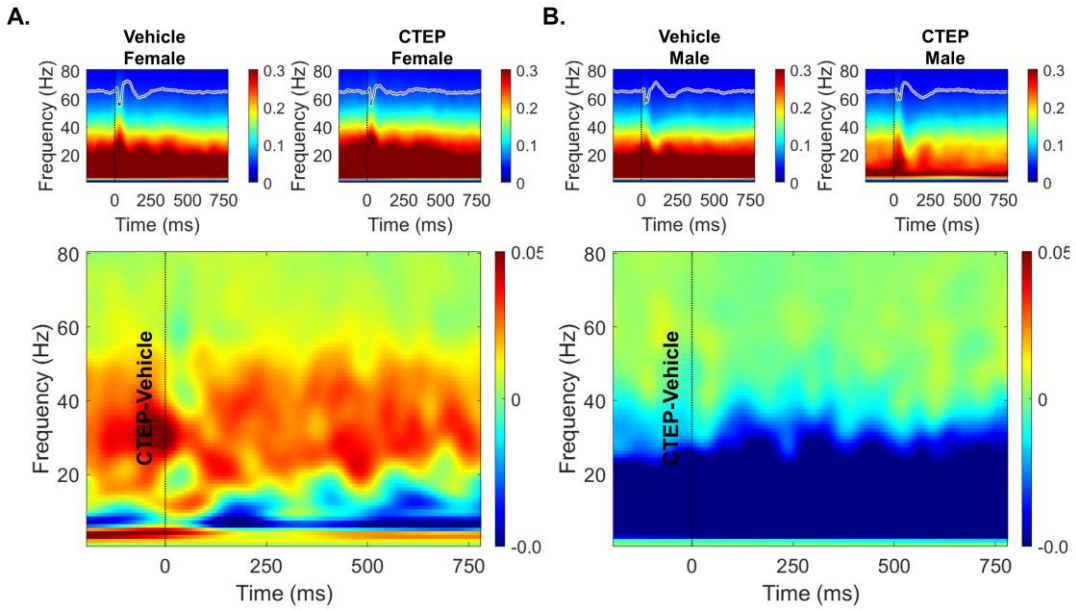

Figure 5.10. CTEP treatment does not affect STP in the AC of PTEN KO mice. The heatmaps show non-baseline corrected normalized power, where warmer hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP in vehicle (left) and CTEP treated (right) female (A) and male (B) PTEN KO mice. The larger panels show the difference between CTEP and vehicle treated mice. No significant differences were found in STP in vehicle and CTEP treated females (A) or males (B) in the AC.

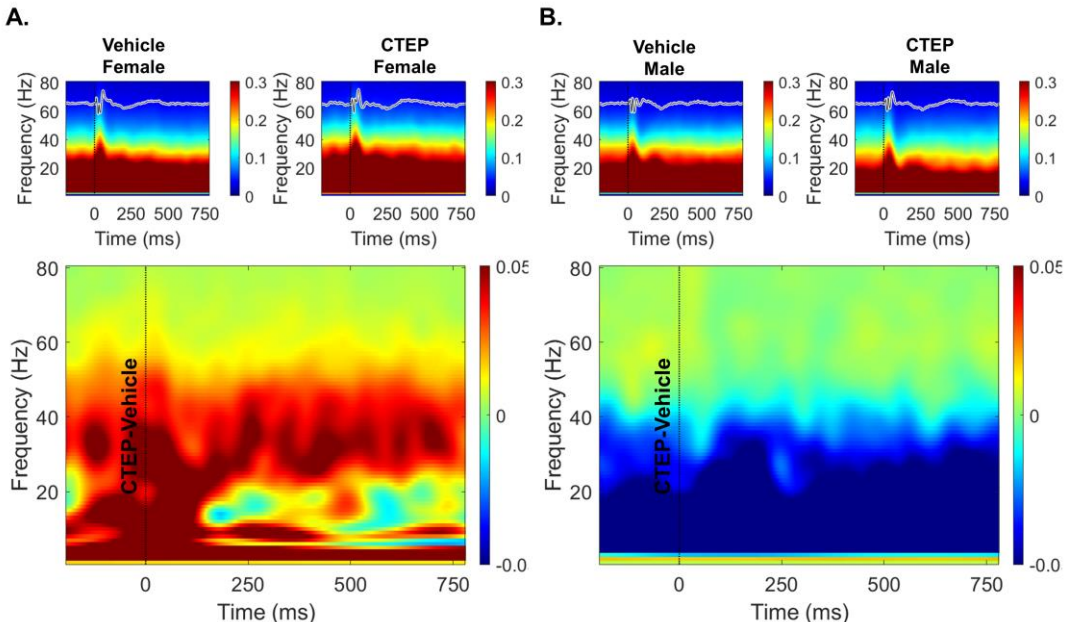

Figure 5.11. CTEP treatment does not affect STP in the FC of PTEN KO mice. The heatmaps show non-baseline corrected normalized power, where warmer hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP in vehicle (left) and CTEP treated (right) female (A) and male (B) PTEN KO mice. The larger panels show the difference between CTEP and vehicle treated mice. No significant differences were found in STP in vehicle and CTEP treated females (A) or males (B) in the FC.

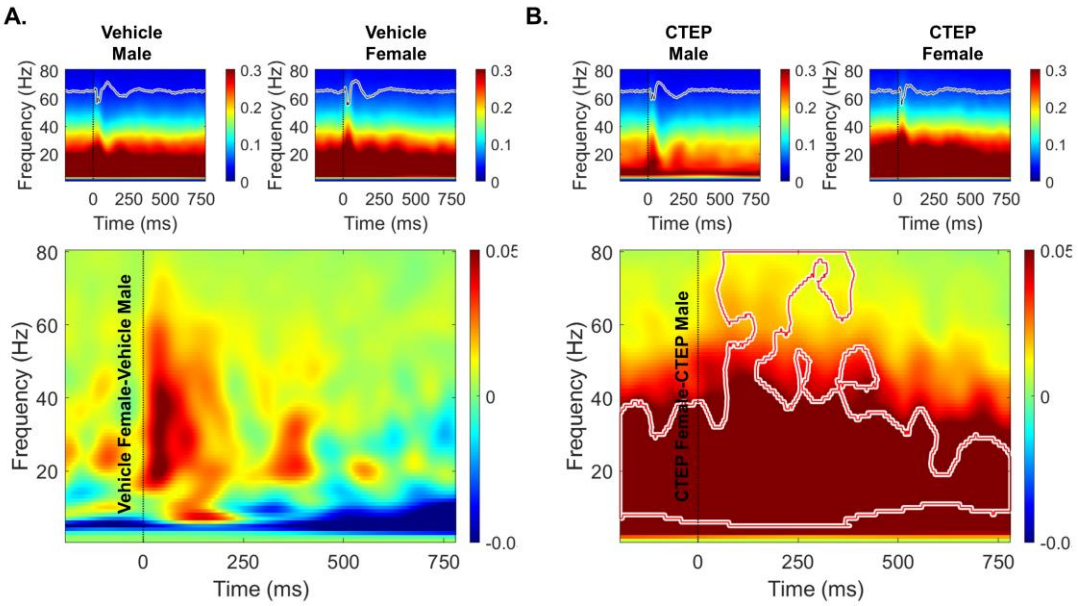

Figure 5.12. CTEP treatment significant increases STP in the AC of female PTEN KO mice. The heatmaps show non-baseline corrected normalized power, where warmer hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP in the male (left) and female (right) vehicle (A) and CTEP treated (B) PTEN KO mice. The larger panels show the difference between the sexes. Outlined regions indicate clusters which are significantly different between males and females. No sex difference were found in STP following vehicle treatment (A). CTEP treatment significantly increased STP in PTEN KO females (B).

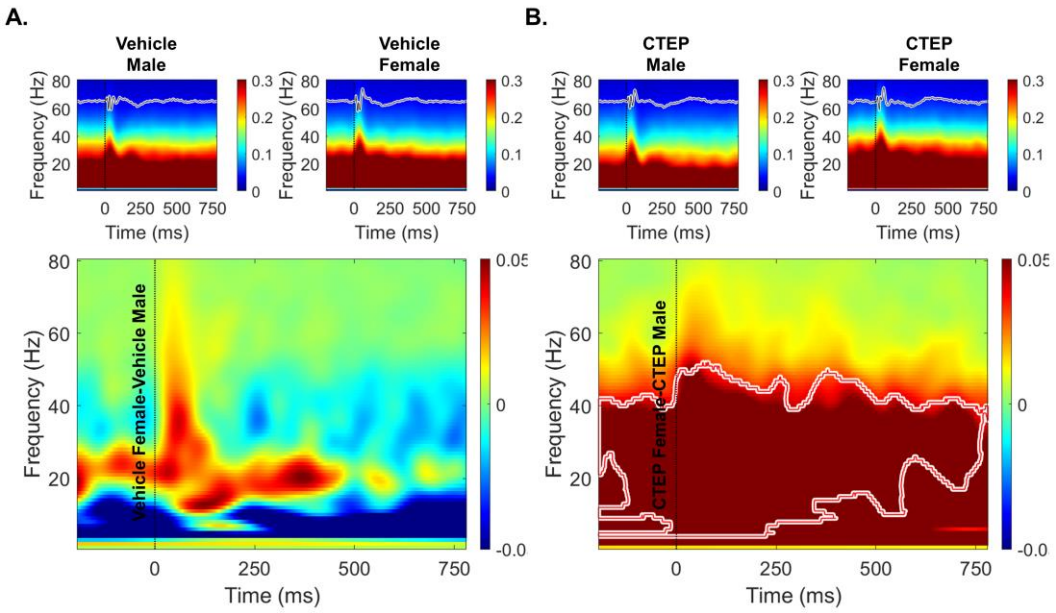

Figure 5.13. CTEP treatment significant increases STP in the FC of female PTEN KO mice. The heatmaps show non-baseline corrected normalized power, where warmer hues represent increased ongoing background activity, and cooler hues represent a decrease. The smaller panels show group average STP in the male (left) and female (right) vehicle (A) and CTEP treated (B) PTEN KO mice. The larger panels show the difference between the sexes. Outlined regions indicate clusters which are significantly different between males and females. No sex difference were found in STP following vehicle treatment (A). CTEP treatment significantly increased STP in PTEN KO females (B).

Table 5.5. Full statistical analysis of ERP EEG data for naïve and vehicle treated mice.

Sex	Cortical Region	Frequency	(t,df)	p-value
Female	AC	P1 Amplitude	t(21)=0.1080	0.9151
		N1 Amplitude	t(21)=0.9625	0.3468
		P2 Amplitude	t(21)=1.366	0.1864
	FC	P1 Amplitude	t(21)=0.2436	0.8099
		N1 Amplitude	t(21)=0.8779	0.3899
		P2 Amplitude	t(21)=0.3843	0.7046
Male	AC	P1 Amplitude	t(18)=0.8461	0.4086
		N1 Amplitude	t(18)=0.3323	0.7435
		P2 Amplitude	t(18)=0.7582	0.4581
	FC	P1 Amplitude	t(18)=1.437	0.1678
		N1 Amplitude	t(18)=0.1370	0.8926
		P2 Amplitude	t(18)=1.547	0.1393

Unpaired t-tests were used to compare naïve male and female KO to vehicle treated male and female KO. Bold text indicates statistical significance (p = or < 0.05).

Table 5.6. Full statistical analysis of ERP EEG data for vehicle and CTEP treated mice.

Cortical Region	Frequency	Factor	ANOVA Results	p-value
AC	P1 Amplitude	Treatment	F(1,38)=0.9635	0.3325
		Sex	F(1,38)=1.183	0.2837
		Treatment x Sex	F(1,38)=0.2126	0.6474
	N1 Amplitude	Treatment	F(1,38)=1.464	0.2337
		Sex	F(1,38)=2.438	0.1267
		Treatment x Sex	F(1,38)=0.9485	0.3363
	P2 Amplitude	Treatment	F(1,38)=0.0348	0.8530
		Sex	F(1,38)=0.8176	0.3716
		Treatment x Sex	F(1,38)=1.096	0.3017
FC	P1 Amplitude	Treatment	F(1,38)=0.0714	0.7907
		Sex	F(1,38)=0.0044	0.9474
		Treatment x Sex	F(1,38)=0.1878	0.6672
	N1 Amplitude	Treatment	F(1,38)=1.272	0.2664
		Sex	F(1,38)=0.6661	0.4194
		Treatment x Sex	F(1,38)=1.912	0.1749
	P2 Amplitude	Treatment	F(1,38)=1.190	0.2822
		Sex	F(1,38)=2.534	0.1197
		Treatment x Sex	F(1,38)=0.0629	0.8032

Two-way ANOVA results for ERP EEG analysis. Bold text indicates statistical significance (p = or < 0.05).

Discussion

The present study tested the hypothesis that abnormal mGluR5 receptor activity in Nse-cre PTEN KO mice causes abnormal auditory responses. We tested the prediction that an mGluR5 receptor antagonist, CTEP, will increase ITPC to gap-ASSR stimuli, reduce ERP amplitudes and alter power spectral distributions. However, we found that CTEP treatment shows minimal effects on auditory processing and resting cortical EEG measures in Nse-cre PTEN KO mice. The impact of treatment does differ across the sexes. In resting conditions, the overall relative power spectrum is shifted in males, such that CTEP treated mice have decreased alpha power and increased gamma power in the AC. Adult PTEN KO mice have severe auditory temporal processing deficits, shown as decreased ITPC during the gap-ASSR stimulus, and

CTEP treatment showed no improvement of these abnormalities in either cortical region. The results also show no treatment effect on ERP amplitudes in males or females. Lastly, non-phase locked single trial power is significantly elevated in the AC and FC of CTEP treated females but not males, suggesting a sex specific increase in 'on-going background activity' following CTEP treatment. Additionally, we found there to be no difference between naïve and vehicle treated mice in resting or sound evoked responses, showing that CTEP treatment is causing these differences. Overall, the data suggests that irregular oscillatory activity and auditory processing deficits identified in the Nse-PTEN ASD model are not occurring in vivo via abnormal mGluR5 activity alone. However, the differential impact of treatment across the sexes suggests that mGluR5 may be involved in sex-specific mechanisms that underlie the abnormal oscillatory activity.

We previously showed that irregular oscillatory activity and auditory processing deficits in PTEN ASD model mice are present from an early age and continue to worsen with age. We hypothesized that this significant decline with age may be due to severe macrocephaly occurring in these mice. The cre activity in this conditional model is driven by a neuron specific enolase promoter and is first detectable by embryonic day (E) 11.5 in the cranial/spinal accessory nerve. It becomes evident by p2 in the cerebral cortex and hippocampus, specifically in postmitotic neurons, and becomes most abundant in the sensory cortex by four weeks of age [Kwon et al., 2006a]. The loss of PTEN causes the upregulation of the PI3K/AKT/mTOR pathway, a pathway shown to promote processes linked to cell growth [Kim & Guan, 2019]. Without the negative regulation of PTEN, progressive macrocephaly occurs by p60 in Nse-cre mice, making the brains significantly larger than those of littermate control [Kwon et al., 2006b]. At the cellular

level, soma enlargement, and eventual hypertrophy, as well as neuronal outgrowth is observed in areas with cre activity. This causes the compression of surrounding areas, including the CA1 region of the hippocampus. Since these structural changes develop over time, it is likely that the anatomical changes bring about significant functional abnormalities in Nse-cre PTEN KO brains, causing the most severe deficits to be seen in adult KO. Although acute CTEP treatment did increase the latency of seizure behavior in adult KO females, it is possible that the oscillatory activity in the brain cannot be rescued at this late state [Molinaro et al., 2024]. Future studies should analyze the impact of acute CTEP treatment earlier in development, prior to the onset of these gross morphological changes.

A key finding from this study was the impact of CTEP treatment on the relative resting power distribution in males. Specifically, the results show decreased alpha power and increased gamma power. Alpha waves are seen during a normal wakeful state and have been linked to attention, perceptual processing, and semantic memory [Knyazev et al., 2006]. Gamma band activity has been linked to a variety of cortical functions, including perception, attention, memory, synaptic plasticity, and motor control [Uhlhaas et al., 2008]. Interestingly, both the alpha and gamma frequencies have sparked great interest in autism research and have been proposed as viable biomarkers for diagnosis. For example, a recent literature review, including 1246 autistic and 1455 neurotypical individuals across 41 studies, found that autistic individuals exhibited reduced relative alpha and increased gamma power [Neo et al., 2023]. These two frequency bands are also implicated in fragile X syndrome, another genetically linked form of ASD. Specifically, resting state studies of humans and mouse models of FXS have demonstrated reduced alpha and increased gamma power compared to controls [Van

der Molen & Van der Molen, 2013; Lovelace et al., 2018; Jonak et al., 2020; Wang et al., 2017]. We previously showed that adult PTEN KO mice had significantly decreased gamma power compared to controls [Croom et al., 2024b]. Because we found there to be no difference between the gamma power in naïve and vehicle treated KO mice, CTEP treatment may be rescuing the deficit. No effect of genotype on alpha power was seen in our previous study, however a recent study did demonstrate that alpha power was decreased in mGluR5 KO mice, suggesting that this result may be due to the blocking mGluR5 activity by CTEP [Aguilar et al., 2020].

Both male and female PTEN KO mice show severe auditory temporal processing deficits [Croom et al., 2024b]. Specifically, we showed that developing (p21 and p30) and adult (p60) KO mice have significantly decreased ITPC compared to age-matched controls in both the AC and FC. Our current study found no significant impact of CTEP treatment in adult mice, suggesting that mGluR5 function is not necessary for the temporal fidelity of cortical responses. Several studies have inquired about mGluRs' role in auditory processing as they are widely expressed along the auditory pathway [Tang & Lu, 2018; Lu, 2014; Ferraguti & Shigemoto, 2006; Shigemoto et al., 1993]. For example, Voytenko & Galazyuk (2011) demonstrated that group I mGluRs modulate neuronal firing in a facilitating manner in the inferior colliculus. Additionally, mGluR-triggered temporally patterned activity was seen in the medial nucleus of the trapezoid body (MNTB), a phenomenon that was eliminated in mGluR5 KO mice [Wang et al., 2023]. These findings propose a potential role for mGluRs in temporal processing modulation, boosting the rationale for the current study. The underlying mechanism of the gap-ASSR is currently not completely characterized. Several studies have shown evidence for the contribution of frontal generators towards 40 Hz ASSRs. For example, Kim et al. (2015)

and Hwang et al. (2019) showed that optogenetic stimulation of GABAergic parvalbumin neurons in the basal forebrain preferentially increased frontal cortex 40 Hz ASSR oscillations. Another proposed mechanism relies on the glutamatergic N-methyl-D-aspartate (NMDA) receptors. Specifically, the NMDA receptor activation on parvalbumin interneurons appears to be critical for the 40 Hz ASSR. Evidence for this hypothesis was found in pharmacological studies. Sivarao et al. (2016) utilized ketamine, an NMDA receptor antagonist, and found that receptor channel occupancy is related to the modulation of both power and phase-locking of 40-Hz ASSRs, such that lower ketamine dosages cause an increase in spectral power and ITPC, while higher doses caused decreased 40-Hz ASSRs. Indeed, structural and functional interactions between mGluRs and NMDA receptors have been identified. Specifically, activation of NMDA receptors provides faciliatory regulation of mGluR5 responses [Chen et al., 2011; Alagarsamy et al., 2002; Mao & Wang, 2002]. Additionally, activation of mGluR5 induce a selective potentiation of NMDA-evoked currents [Awad et al., 2000; Pisani et al., 2001]. It is possible that blocking mGluR5 activity with CTEP depresses NMDA receptor activity in PTEN KO mice. Taken together, future studies should investigate these mechanisms to determine not only their role in gap-ASSR, but also whether mechanistic abnormalities are present in PTEN KO mice.

The single trial power (STP) allows for the identification of ongoing ‘background activity’ during stimulus presentation as it does not correct for mean baseline power levels. It has been suggested that this non-phase locked power reflects relatively slow integrative processes that may impact stimulus or response processing [McKewen et al., 2020]. These processes include top-down and sustained attention, decision-making, and perceptual inference, and are suggested to result from intrinsic network interactions

rather than external stimuli [Donner & Siegel, 2011; Siegel & Donner, 2010]. We previously showed a developmental increase in STP in PTEN KO mice compared to controls. Specifically, no STP phenotype differences were seen at p21 in either sex but STP was significantly increased in PTEN KO mice at p30 and p60 in the AC and FC [Croom et al., 2024b]. Our current study identified a treatment x sex interaction, such that CTEP treatment differentially impacted males and females. Specifically, CTEP treated females had increased STP compared to CTEP treated males. No significant differences were seen between vehicle treated males and females or naïve and vehicle treated animals, suggesting that this increase in STP in females is due to CTEP. Increased levels of background activity are not seen as a positive phenotype in other autism models. For example, increased STP has been seen in humans with FXS and the *Fmr1* KO mouse model of FXS [Ethridge et al., 2019; Wen et al., 2019; Croom et al., 2023]. In humans with FXS, elevated STP showed correlations with IQ and distractibility. Overall, these data suggest that CTEP treatment is increasing STP, specifically in females, and enhancing ASD phenotypes.

The current study aimed to determine whether mGluR5 dysfunction was involved in the abnormal oscillatory activity of PTEN KO mice. This hypothesis was based on recent literature suggesting a sex bias in PTEN mutations as a result of mGluR5 and ERα interactions. Molinaro et al., (2024) showed prolonged and spontaneous persistent activity states (UP states) in female but not male Nse-cre PTEN KO mice at p21, suggesting sex-specific circuit hyperexcitability. In adults, sex differences were seen in social behavior and seizure-induced mortality. Furthermore, female PTEN deleted cortical neurons demonstrated an increase in mGluR5 levels and mGluR5-driven protein synthesis rates as well as mGluR5-ERα complexes [Molinaro et al., 2024]. Abnormalities

were able to be rescued with CTEP and MTEP treatment, another mGluR5 negative allosteric modulator. We previously showed that male and female Nse-cre PTEN KO mice also have significant irregularities in resting power distributions and sound evoked responses. However, neither males or females in the cohort of mice used for the current study were able to be rescued with acute CTEP treatment. Indeed, CTEP treatment has been shown to be beneficial for other ASD models, including FXS, Rett's syndrome, and tuberous sclerosis complex (TSC) [Tao et al., 2016; Stoppel et al., 2021; Kelly et al., 2018]. It is possible that a more chronic dosage plan is necessary in order to see improvements in in vivo EEG phenotypes in PTEN KO mice. For example, Kelly et al. (2018) demonstrated that chronic, but not acute, CTEP treatment significantly reduced seizure frequency and total seizure time in the *Tsc2* mutant mouse model of ASD. Furthermore, sex differences in dosage amounts have also been identified with CTEP. Specifically, Li et al. (2022) demonstrated in a mouse model of Huntington's disease that female mice required a longer treatment duration with CTEP than male mice to show improvement in certain phenotypes, suggesting that sex has an impact on the efficacy of CTEP treatment. Overall, these findings suggest that a more chronic approach in CTEP treatment may be beneficial in PTEN KO mice. Additionally, the current study did not investigate the role of ERα and its interactions with mGluR5. Molinaro et al. (2024) demonstrated that genetic reduction of ERα in PTEN KO cortical neurons also rescued circuit excitability. Future studies should examine the impact of ERα on cortical EEG phenotypes as well as its association with mGluR5.

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Chapter 6

Conclusion

Autism Spectrum Disorders (ASD) encompass a wide array of debilitating symptoms, including severe sensory deficits and abnormal language development. Sensory deficits early in development may lead to broader symptomatology in adolescents and adults. The mechanistic links between ASD risk genes, sensory processing and language impairment are unclear. There is also a sex bias in ASD diagnosis and symptomatology that is not fully understood. Additionally, very little is known about the development of temporal processing in any animal model of ASD. To fill these gaps in the field, my dissertation research is centered on sensory processing in two different mouse models of ASD. However, these approaches and stimuli could have an impact in furthering our understanding of other neurodevelopmental disorders. The need for physiologically relevant and easily measurable biomarkers is critical for progressing the field, including diagnosis and treatment development. Furthermore, the methodology (EEG) used to obtain them is easily replicable in human work.

Chapters 2 and 3 characterized the auditory temporal processing capabilities of male (Chapter 2) and female (Chapter 3) *Fmr1* KO mice across development. Chapter 2 presented evidence that the frontal (FC), but not auditory (AC), cortex of male KO mice shows significant temporal processing deficits at p21 and p30, with poor ability to phase lock to rapid gaps in noise. However, temporal processing was similar in both genotypes in adult mice, suggesting a cortical region-specific delay. ERP amplitudes were also significantly larger in male *Fmr1* KO mice compared to controls in both auditory and

frontal cortex, consistent with ERP data in humans with FXS. Chapter 3 focused on female *Fmr1* KO phenotypes across development as well as sex differences. Gap-ASSR deficits were seen in the FC, but not AC, in early development (p21) in female KO mice. Unlike male KO mice, female KO mice show WT-like temporal processing at p30, providing evidence that temporal processing matures slower in male KO mice. ERP amplitudes were also enhanced in female KO across development compared to WT counterparts. Additionally, female KO mice showed stronger hypersensitive responses than males later in development. The differences in maturation rates of temporal processing and hypersensitive responses during various critical periods of development may lead to sex differences in language function, arousal and anxiety in FXS.

Chapter 4 identified the developmental trajectory and genotype- and sex-dependent differences in auditory sensitivity and temporal processing in a *Pten*-deletion mouse model of ASD. The results showed genotype differences in resting power distribution in PTEN KO mice throughout development, such that male and female KO mice have significantly increased beta power but decreased high frequency oscillations in the AC and FC. Both male and female PTEN KO mice showed diminished ITPC in their gap-ASSR responses in the AC and FC compared to control mice. Notably, deficits became more prominent in adult mice, with KO mice having significantly increased sound evoked power and decreased ITPC compared to controls. Female KO mice showed increased hypersensitivity compared to males, reflected as increased N1 and P2 amplitudes. Taken together, these data identified novel sensory processing deficits in a PTEN-ASD mouse model that are present from an early age and provide a strong foundation for future studies.

Chapter 5 tested whether abnormal activity of group 1 metabotropic receptor mGluR5 is involved in the phenotypes identified in PTEN KO mice in Chapter 4. The results showed that acute CTEP treatment shifted the resting power distribution in males, such that CTEP treated mice had decreased alpha power and increased gamma power in the AC compared to vehicle treated males. CTEP treatment showed no significant impact on auditory temporal processing in either sex or cortical region. The results also showed no treatment effect on ERP amplitudes in males or females. Non-phase locked single trial power was significantly elevated in the AC and FC of CTEP treated females but not males, suggesting a sex specific increase in 'on-going background activity' following CTEP treatment. Overall, these data demonstrated that the irregular oscillatory activity and auditory processing deficits previously identified in the PTEN ASD model were not significantly improved by CTEP treatment, indicating that abnormal mGluR5 activity alone may not be driving the EEG phenotypes. However, the differential impact of treatment across the sexes suggests that mGluR5 may be involved in sex-specific mechanisms that underlie the abnormal oscillatory activity.

Taken together, this dissertation characterized the developmental trajectories of EEG phenotypes in two different ASD mouse models and identified both genotype-, sex- and cortical region-specific differences. Although there are significant underlying model differences between the two (Global KO – FXS/Specific, conditional KO – PTEN), similarities were identified, including auditory temporal processing deficits early in development and increased hypersensitivity in adult females. These key findings provide evidence that there may be a fundamental issue at the circuit level that is shared among autisms. Overall, this dissertation research establishes a strong foundation for identifying

this core circuit deficit which would allow for earlier, more accurate diagnoses in patients as well as more effective therapeutic approaches.

Future Studies

An important motivation for this dissertation research was translatability. The gap-ASSR stimulus paradigm requires no behavioral training and can easily be replicated in humans at any age. The next step currently underway for the FXS mouse model is to use a 30-channel multielectrode array (MEA) system with the gap-ASSR stimulus. The use of this system versus the 2-channel system used for the current study will allow for more region- and hemispheric- specific analysis. Following the completion of MEA characterization, the same gap-ASSR stimulus can be used in humans as well, allowing for consistency across study methodologies. It is critical that temporal processing is evaluated across ages in both males and females with FXS to determine if similar delays in development are present, and if the delay relates to language function. Additionally, connectivity studies are currently being carried out for the *Fmr1* KO male and female mice throughout development. The few studies in FXS that have examined cross-regional or cross-frequency coupling show abnormal connectivity. However, even fewer studies have examined sex differences in connectivity. Given the cortical region-specific deficits seen in this dissertation research, understanding the connectivity between regions may elucidate potential mechanisms underlying abnormal phenotypes.

This dissertation research is the first to characterize EEG phenotypes in the Nse-PTEN KO mouse model and therefore provides numerous questions to explore further. First, we hypothesized that an interaction between mGluR5 and ERα was driving abnormal oscillatory and sound-evoked activity in female PTEN KO. Although this

research suggests that mGluR5 alone is not causing these phenotypes, future studies should examine the effects of CTEP treatment at an earlier stage of development, such as p21. Earlier intervention may bring about more beneficial results. Furthermore, the role of ERα should be investigated via pharmacological or genetic approaches. For example, MPP (methyl piperidino pyrazole) is a highly selective ERα antagonist and has been shown to have effects on PTEN KO mice *in vitro*. Additionally, utilizing a dual deletion mouse model in which both PTEN and ERα genes have been removed from the same neurons may provide insight into whether ERα activity is responsible for the abnormal female PTEN-mutated cortical oscillations and auditory temporal processing. Finally, the loss of PTEN in the Nse-cre model is limited to layers III-V of the cortex and likely disrupting both input and output connection to/from the auditory cortex. Future studies exploring these connections, such as tract tracings, would allow for a better understanding of the underlying mechanisms of the characterized EEG phenotypes.