Female specific dysfunction of sensory neocortical circuits in a mouse model of autism mediated by mGluR5 and Estrogen Receptor α

Gemma Molinaro1, Jacob E. Bowles1, Katilynne Croom3, Darya Gonzalez1, Saba Mirjafary1, Shari Birnbaum2, Khaleel A. Razak4, Jay R. Gibson1, and Kimberly M. Huber1*

1 Department of Neuroscience, O'Donnell Brain Institute, UT Southwestern Medical Center, Dallas, TX, USA
2 Department of Psychiatry, O'Donnell Brain Institute, UT Southwestern Medical Center, Dallas, TX, USA
3Graduate Neuroscience Program, University of California, Riverside, CA.
4Graduate Neuroscience Program, University of California, Riverside, CA; Department of Psychology, University of California, Riverside, CA.

*Correspondence: Kimberly.Huber@UTSouthwestern.edu (K.M.H.)

Summary (203 words)

Autism manifests differently in males and females and the brain mechanisms that mediate these sex-dependent differences are unknown. Here, we demonstrate that deletion of the ASD-risk gene, *Pten,* in neocortical pyramidal neurons (^{NSE}*Pten* KO) results in robust hyperexcitability of local neocortical circuits in female, but not male, mice, observed as prolonged, spontaneous persistent activity states (UP states). Circuit hyperexcitability in females is mediated by enhanced signaling of metabotropic glutamate receptor 5 (mGluR5) and estrogen receptor α (ERα) to ERK and *de novo* protein synthesis. In support of this idea, *Pten* deleted cortical neurons have a female-specific increase in mGluR5 levels and mGluR5-driven protein synthesis rates, mGluR5-ERα complexes are elevated in female cortex and genetic reduction of ERα in *Pten* KO cortical neurons rescues circuit excitability, protein synthesis rates and enhanced neuron size selectively in females. Abnormal timing and hyperexcitability of neocortical circuits in female ^{NSE}*Pten* KO mice are associated with deficits in temporal processing of sensory stimuli and social behaviors as well as mGluR5-dependent seizures. Female-specific cortical hyperexcitability and mGluR5-dependent seizures are also observed in a human disease relevant mouse model, germline *Pten*+/- mice. Our results reveal molecular mechanisms by which sex and a high impact ASD-risk gene interact to affect brain function and behavior.

Introduction:

Autism Spectrum Disorder (ASD) is diagnosed 4 times more often in males than females [1]. However, increasing evidence indicates that ASD is expressed differently in males and females on a behavioral level and affects brain function and connectivity differently. Because of these sex differences, it has been suggested that ASD is not as well detected in females with current ASD diagnostic tests and is more prevalent in females than previously recognized [2-7]. Some symptoms and co-morbidities, such as deficits in sensory processing and epilepsy, are more severe or common in females with autism [8-11]. There is little known of the sex-dependent brain mechanisms that interact with ASD-linked genes and how ASD genes affect female brain physiology and behaviors differently than in males. One prominent ASD gene associated with sex-dependent interactions, at least in cancer, is the *Phosphatase and tensin homolog deleted on chromosome 10* (*PTEN*), a lipid phosphatase that dephosphorylates PIP3 and inactivates PI3K and its downstream signaling to Akt and mTOR [12,13]. PTEN was first discovered as a tumor suppressor and subsequently linked to ASD [14]. Individuals with germline *PTEN* mutations display variable and complex phenotypes including macrocephaly, autism, intellectual disability (ID), and epilepsy that are collectively termed PTEN Hamartoma syndromes (PTHS)[12,15-18]. Importantly, mutations in PTEN or other suppressors of the PI3K/Akt/mTOR pathway are the most common cause of breast cancers; cancers that are ~ 100X more prevalent in women than men [19]. This sex bias is because 70-80% of breast cancers express Estrogen receptor α (ERα) and depend on estrogen signaling for growth [20]. Mutations in *PTEN* or other genes that lead to hyperactivation of Akt/mTOR in breast cancer cells result in enhanced ERα function [21-23]. Thus, combination therapies that inhibit ERα and PI3K/mTOR are most therapeutic for breast cancer [23].

Although there is not a female sex bias in ASD with germline *PTEN* mutations, as there is with breast cancer, the rates of ASD in females with *PTEN* mutations is high (47%) and similar to males (66%)[17]. A mouse model of PTHS, a germline heterozygous deletion of *Pten*, displays female-specific deficits in social behavior [18,24,25]. Similarly, human relevant *Pten* mutation in mice, leading to a cytoplasmic predominant expression of PTEN (*Pten*^{m3m4}), results in a male-selective enhancement in sociability [26]. The sex-dependent effects of PTEN on brain physiology are unknown, nor the molecular mechanisms that lead to sex-dependent behavior deficits with PTEN deletion. ERα is expressed in brain, including cortical neurons, where it has important regulatory functions on different processes such as cognition, anxiety and addiction [27,28]. Many of the effects of estrogen on neurons and behavior are mediated by ERα and rely on activity of metabotropic glutamate receptor 5, a Gq coupled glutamate receptor and candidate therapeutic target in ASD. If or how PTEN deletion in neurons affects ERα or mGluR5 function or if these signaling pathways contribute to brain phenotypes in PTEN loss of function models is also unknown.

To explore the effects of *Pten* deletion on brain function, we studied an established mouse model of PTEN deletion with ASD-relevant behaviors, ^{NSE}*Pten* KO [29]. ^{NSE}*Pten* KO mice have a mosaic deletion of PTEN primarily in Layers 3-5 excitatory neurons. To assess overall cortical circuit function, we measured spontaneous persistent activity states, or UP states, in acute slices of the somatosensory cortex. UP states are driven by the activity of local cortical circuits and are an overall readout of the

balance of excitation and inhibition. UP states also reflect the state of maturation of cortical circuits [30 31]. We have shown that UP state duration is prolonged in another mouse model of ASD, the *Fmr1* knockout (KO) model of Fragile X Syndrome (FXS), reflecting a circuit hyperexcitability. Surprisingly, we observed prolonged UP states in female, but not male, ^{NSE}*Pten* KO mice in contrast to our previous work in male *Fmr1* KO mice. Prolonged UP states in female ^{NSE}*Pten* KO are mediated by enhanced signaling of mGluR5 and ERα and required de novo protein synthesis. We find that *Pten* deletion in L5 neurons results in enhanced mGluR5 levels, mGluR5 and ERα-driven protein synthesis rates and mGluR5-dependent seizures, only in females, but not males. Notably, genetic reduction of ERα in L5 neurons corrects circuit hyperexcitability, enhanced protein synthesis rates and cell size, again, selectively in females. The prolonged cortical circuit activity we observe in slices from female ^{NSE}*Pten* KO is associated with a female selective deficit in temporal processing of sensory stimuli as measured with electroencephalographic (EEG) recordings *in vivo* as well as deficits in social behavior. Female specific cortical circuit hyperexcitability and mGluR5-dependent seizures are also observed in a mouse model of PTHS. Our results suggest a model where deletion of PTEN results in a female specific enhancement of mGluR5-ERα function that drives cortical hyperexcitability through ERK and *de novo* protein synthesis. These results may suggest distinct therapeutic strategies for males and females with PTEN mutations and highlight the importance of studies of ASD risk genes in both males and female brain physiology.

Experimental Procedures

Mice

The mouse lines used are the Congenic NSE-Cre mice [32] crossed with floxed Pten and or floxed Esr1 and the Pten heterozygous mice. Mice were obtained from Dr. Luis Parada (Memorial Sloan Kettering Cancer Center, New York, NY) and from Jackson Laboratory and crossed and maintained as described in the supplemental experimental procedure in the Supplemental Information (SI).

Neocortical Slice preparation and UP state recordings

UP state experiments in somatosensory neocortical slices from male and female mice ages P18-60 were performed and analyzed as described in [33] and SI.

Surface Sensing of Translation (SUnSET) in Brain Slice

To measure protein synthesis in L5 of S1 somatosensory cortex we used the immunohistochemical version of SUnSET [34]. Acute slices were prepared as for UP states and treated as described in supplemental experimental procedure.

Immunohistochemistry

Section obtained from mice perfused with 4% PFA were used for PTEN, Phospho-S6 immunohistochemistry and soma size measurement. Post-fixed slices, re-sectioned into 50 µm sections were used for Puromycin staining after SUnSET. Sections containing the somatosensory cortex were permeabilized and blocked with 0.3% Triton X-100 and 5% goat serum prior to treatment with primary and secondary antibodies (see SI).

Confocal Microscopy and Image quantification

Fluorescence images were taken from L5 of S1 cortex using a Zeiss LSM 710 laser-scanning confocal microscope and analyzed as described in SI. To quantify puromycin and P-S6 fluorescence intensity and soma size, regions of interest (ROIs) were manually drawn around neuronal bodies in the puromycin Channel (red Channel), P-S6 channels (ultrared channel) and Neurotrace channel (blue Channel) of each image with the "Oval" (for puromycin) or "Freehand" tool in FIJI (https://imagej.net/software/fiji/). ROIs in this channel were then used as masks to identify Cre (+) or Cre (-) neurons, depending on PTEN immunofluorescence (Green Channel) and Neun or Neurotrace staining. The puromycin and P-s6 fluorescence intensity of all Cre (-) and Cre (+) neurons was averaged for each section and the mean value of Cre (+) was expressed as % of the Cre (-) neurons for each section. For soma area assessment, raw value (30-50 neurons from 4-6 section/4 mice for each group) were reported as well as average per mouse. In this latter analysis, the area of Cre + neurons were reported as percent of Cre – neurons for each mouse.

mGluR5 Co-Immunoprecipitation in vivo

Neocortices or microdissected L5 S1 from P18-P23 male and female Pten fl/fl Cre (+) or Cre (-) were lysed in coimmunoprecipitation (Co-IP) buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100). Lysate proteins were then tumbled overnight at 4°C with either ERα antibody (Millipore

04-820) or Homer antibody (Santa Cruz Biotechnology, D-3). Dynabeads Protein A or G bead slurry (ThermoFisher) were then added and immunoprecipitated proteins were eluted with 2x SDS Sample Buffer (Bio-Rad) as described in SI.

Immunoblotting

Western blotting was performed using homogenate from S1 cortex, microdissected L5 from S1 and imunoprecipitated proteins as described in SI. Western blot band intensity values were quantified using ImageLab for whole cell lysate bands and for both IP and Co-IP proteins. For analysis of each experiment, the band intensity of each protein was normalized by the housekeeping band intensity (actin or GAPDH) and Co-IP band intensity values were normalized by the intensity of the corresponding IP protein band. (e.g. mGluR5/ERα or mGluR5/Homer).

EEG recordings

EEG recordings were obtained from awake and freely moving mice and performed at P20-23. Recordings were obtained from the AC and FC electrodes, using the occipital screw as reference. The EEG recording set-up has been previously reported [35]. The stimulus used to assess auditory temporal processing is termed the '40 Hz gaps in noise-ASSR' (auditory steady state response, henceforth, 'gap-ASSR') and has been previously reported [36] and described in SI.

Flurothyl induced seizures

Flurothyl (bis(2,2,2-trifluoroethyl) ether) seizure experiments were performed as described [37] (see also SI). Flurothyl exposures were repeated once daily over five consecutive days. Mouse behavior during each flurothyl exposure was video-recorded and latency to the onset of myoclonic and generalized seizure was determined by an investigators (blind to sex and genotype). The mGluR5 specific negative allosteric modulator CTEP was administered 90 min prior fluorothyl exposure.

Behavioral tests

Locomotor Activity, Open Field, Dark-Light Box performance, social approach and fear conditioning were performed as described in SI.

Statistical Analysis

Whenever possible, data was analyzed by an investigator blind to the genotype or sex of mice. Results in figures are graphically represented as mean ± SEM. Statistics were performed in GraphPad Prism. Normal distribution was tested by the D'Agostino & Pearson test. For 2-group comparison, we used a student unpaired 2-tailed t test, or a Mann-Whitney nonparametric test. For multiple group comparison, data were analyzed using ordinary 1-way ANOVA or Kruskal-Wallis (nonparametric) or 2-way ANOVA where appropriate. Sidak posthoc multiple comparison tests were used.

Results

Hyperexcitable sensory neocortical circuits in female mouse model of Pten deletion

To determine the effects of *Pten* deletion on cortical circuit function, we utilized a previously characterized mouse where *Pten* is deleted with *Neuron-specific enolase* (*NSE*) promoter-driven *cre* (^{NSE}*Pten* KO). In brain, NSE-Cre is expressed postnatally (~postnatal day 2) in discrete mature neuronal populations in layers III to V as well as in neurons in the CA3 region and dentate gyrus of the hippocampus [29]. In cortex, NSE-Cre is most abundantly expressed in sensory areas and the majority of neurons are excitatory based on GABA staining >99%, data not shown). Importantly, ^{NSE}*Pten* KO mice show ASD-relevant behaviors such as deficits in social behavior, learning and memory and sensory hypersensitivity and thus may be a good model to study ASD-related circuit alterations with PTEN deletion [29]. To assess sensory cortical circuit function, we performed extracellular multi-unit recordings of spontaneous persistent activity, or UP states, in acute slices of somatosensory barrel neocortex from postnatal day (P) 18-25 male and female mice. Mice were either NSE-Cre(+)/*Ptenfl/fl* or NSE-Cre (-) *Ptenfl/fl* or *Ptenfl/+* littermates, indicated in figures as Cre(+) or Cre(-), respectively (Fig. 1A,B). UP states are a spontaneous, oscillatory (0.5-1 Hz), synchronized firing of neocortical neuron networks driven by recurrent excitatory and inhibitory synaptic circuits and provide a readout of the intact functioning of neocortical circuits [38,39]. The duration of UP states measured in Layers (L) 4, was increased by 56% in female slices compared to Cre (-) controls (Fig. 1C). In contrast, slices from Cre(+) males had normal UP state duration. There was no difference in UP state duration between male and female Cre(-) mice. Similar results were obtained if recordings were performed in L2/3 or L5 (Fig. 1C). UP states were longer in Cre (+) females, but not males, in comparison to Cre (-) controls and there was a significant interaction of sex and genotype on UP state duration in both L2/3 (2 way ANOVA; $F(1, 73) = 5.555$; $p < 0.05$) and in L5 ($F(1, 71) = 7.332$; $p < 0.01$). There was no effect of sex or genotype on the amplitude or frequency of UP states in any layer (Supplementary Table 1). Because similar results were obtained in all layers, subsequent experiments were focused in L4.

During the course of this project, we sometimes observed increases in UP state duration in male Cre(+) mice as compared to male Cre(-). Combining UP state data from all vehicle or un-treated slices across experiments, we observed a small, ~20%, genotypic increase in UP state duration in males (Cre(-); 1145±45 ms; n = 148 slices; Cre(+); 1383±62ms; n= 114; $p < 0.05$) (Fig. 1D). However, the genotypic increase in UP state duration in females was large, ~70%, (Cre (-) 997±31 ms; n = 175; female Cre(+); 1689±58ms; n = 176; $p < 0.001$) and UP states in Cre(+) females were longer than in Cre(+) males ($p < 0.001$), but not different between Cre(-) males and females. This resulted in a significant interaction of sex X genotype on UP state duration ($F(1, 609) = 20.47$; $p < 0.0001$). Averaging UP state duration from all slices per mouse (Fig. 1E) yielded similar results. There was an increase in UP state duration in Cre(+) females, but not males, in comparison to their same sex Cre(-) controls. UP state duration in female Cre(+) was greater than in Cre(+) males and there was a significant interaction of sex X genotype ($F(1, 153) = 11.58$; $p < 0.001$). UP state duration between Cre(-) males and females was not different.

The female specific effects on UP state duration were not attributable to sex-dependent differences in deletion of PTEN in ^{NSE}*Pten* KO mice as measured with fluorescent immunohistochemistry and western blotting. Reflecting the expression pattern of NSE-Cre, we observed a mosaic deletion of PTEN in L5 neurons as indicated by NeuN labeling that was similar in males and females (Female Cre(+); 63±2% of NeuN+ L5 neurons; n=14 slices/3 mice; Male Cre(+); 64±2%; n=10 slices/3 mice; Fig. S1A, B). Total PTEN levels were quantified in L5 enriched cortical lysates which revealed similar decreases of PTEN in male and female Cre(+) mice (Female Cre(+); 76±5%; n=10 mice; Male Cre(+); 72±6% of same sex Cre(-); n= 6 mice; Fig. S1C,D). Taken together these results suggest sex specific circuit hyperexcitability in female *Pten*-deleted cortex.

Pharmacological antagonism of mGluR5 rescues UP states in female Cre(+) mice

Genetic or pharmacological reduction of metabotropic glutamate receptor 5 (mGluR5) activity rescues prolonged UP states in *Fmr1* KO mice [33]. To test whether mGluR5 activity mediates the longer UP states in female Cre(+) mice, we examined the effects of a specific mGluR5 negative allosteric modulator (MTEP). Slices from Cre(+) mice or Cre(-) controls of both sexes were pretreated with vehicle or MTEP (3 µM). We confirmed a robust increase in UP state duration in female Cre(+) mice (218% of Cre(-) controls), that was rescued to normal levels by MTEP. Furthermore, UP state duration in Cre(-) females was unaffected by MTEP and there was a significant interaction of MTEP with genotype ($F(1, 110) = 12.05$; $p < 0.01$; Fig. 2A,B). In males, we observed a small increase in UP state duration in Cre(+) mice (139% of Cre(-) controls; Fig. 2B), and UP state durations in both Cre(+) and Cre(-) males were unaffected by MTEP. These results suggest a sex-specific gain of function of mGluR5 in female Cre(+) mice that drives circuit hyperexcitability.

mGluR5 expression is selectively enhanced in female Cre(+) mice

To determine if mGluR5 levels were altered with *Pten* deletion, we performed western blotting of lysates from somatosensory cortical slices of ^{NSE}*Pten* KO mice where we attempted to enrich for *Pten* KO neurons by microdissection of lower cortical layers, including L5. Western blots of cortical L5-enriched lysates revealed a small, but significant (35%) increase mGluR5 in Cre(+) female mice as compared to Cre(-) controls. mGluR5 protein levels were unaffected in male Cre(+) mice and there was a significant interaction of sex X genotype ($F(1, 32) = 12.80$; $p < 0.01$; Fig. 2C,D). The increase of mGluR5 in Cre(+) female lysates was not associated with changes in protein levels for other glutamate receptors (GluN1 or GluA1) or excitatory synaptic proteins (PSD-95 or Homer) (Fig, 3E). These data suggest a female specific upregulation of mGluR5 protein in PTEN KO neurons. In *Fmr1* KO mice, mGluR5 is less associated with its binding partner Homer, a scaffolding protein that regulates its localization to synapses and signaling to effectors[40 41]. Pharmacological and genetic restoration of mGluR5-Homer scaffolds in *Fmr1* KO mice rescues cortical excitability and seizures in *Fmr1* KO mice [36,41]. Conversely, dissociation of mGluR5 from Homer prolongs cortical UP states [42]. To determine if mGluR5 is dissociated from Homer in ^{NSE}*Pten* KO mice, we performed co-immunoprecipitation of Homer and mGluR5 from lysates of either whole or L5-enriched cortical lysates from male and female mice. In contrast to *Fmr1* KO, we were not able to detect differences in mGluR5 co-IP with Homer in

Cre(+) mice as compared to their same sex Cre(-) controls in either whole or L5 enriched cortical lysates (Fig. S2A,B). This suggests other mechanisms of mGluR5 dysfunction in the female ^{NSE}*Pten* KO females or we are unable to detect changes with a bulk co-IP method because of the mosaic nature of Pten deletion in the ^{NSE}*Pten* KO.

Antagonism or genetic reduction of Estrogen Receptor α (ERα) in L5 neurons rescues prolonged UP states in female ^{NSE}*Pten* KO mice.

A large body of evidence indicates that mGluR5 functionally interacts with estrogen receptor alpha (ERα) specifically in the female brain. mGluR5 mediates the rapid non-genomic effect of ERα on multiple neuronal functions including synaptic transmission, cognition, feeding behavior and others [43 44-46]. Furthermore, mutations in PTEN in breast cancer cells result in enhanced ERα function through phosphorylation of ERα by either by Akt or S6K[21-23]. To determine if ERα activation contributes to circuit dysfunction in female Cre(+) somatosensory cortex we used methyl-piperidino-pyrazole (MPP; 1μM) a selective, high affinity silent antagonist at ERα receptors, which displays > 200-fold selectivity for ERα over ERβ [47]. In females, MPP rescued the increase in UP state duration in Cre(+) mice and had no effect on UP states in Cre(-) mice (Fig. 3A,B). There was also a significant interaction of MPP and genotype on UP state duration ($F(1, 79) = 8.291$; $p < 0.01$). In males, MPP had no effect on UP state duration in either Cre(+) or Cre(-) mice (Fig. 3B). These results suggest, that like mGluR5, there is a selective overactivity of ERα in female Cre(+) mice that promotes UP state duration. To confirm the role of ERα in UP states using a genetic strategy and determine if increased ERα function occurred in the NSE-Cre(+) cortical neurons with *Pten* deletion, we obtained floxed *Esr1* mice and bred them to create mice with reduced ERα levels in NSE-Cre(+) and *Pten* deleted neurons (NSE-Cre/$Pten^{fl/fl}/Esr1^{fl/+}$) and measure UP states in these mice as compared to NSE-Cre/$Pten^{fl/fl}$ and Cre(-) littermates. In females, UP state duration in slices from NSE-Cre/$Pten^{fl/fl}/Esr1^{fl/+}$ mice was reduced as compared to NSE-Cre/$Pten^{fl/fl}$ littermates and equal to durations measured in Cre(-) controls (Fig. 3C, D). In contrast, UP state duration in male Cre(+) mice was unaffected by genetic reduction of ERα (Fig. 3D). Heterozygosity of ERα in cortical neurons without PTEN deletion (NSE-Cre/$Pten^{+/+}/Esr1^{fl/+}$) had no effect on UP state duration in females (Fig. S3A). These results suggest that ERα is overactive and/or dysfunctional in *Pten* deleted NSE-Cre(+) L5 neurons and suggests a cell autonomous regulation of ERα by PTEN that promotes cortical circuit hyperexcitability in females, but not males.

mGluR5-ERα complexes are enhanced in female cortex and in response to active Akt

ERα mediates many non-genomic effects of estradiol by physically or functionally interacting with mGluR1 and mGluR5 in brain regions such as the striatum and hippocampus[48 44]. To determine if mGluR5 physically interacts with ERα in cortex and if this is regulated by sex, we performed co-IP experiments and observed an association of mGluR5 with ERα in cortical lysates that was enhanced, by ~2-fold, in female wildtype mice as compared to males (Fig. 3E, S3B). Total mGluR5 and ERα levels were not different between sexes. In subcellular fractionation experiments, we observed that mGluR5 was present in both nuclear and non-nuclear fractions from cortical lysates and ERα was primarily non-nuclear as previously shown[49,50](Fig. S3C). This suggests that ERα and mGluR5 interact in a non-

nuclear complex in cortex. To determine if mGluR5-ERα interactions were affected by *Pten* we performed a co-IP of ERα and mGluR5 from total cortical lysates of Cre(+) and Cre(-) male and female mice (Fig. S3D,E). We observed a non-significant main effect of genotype ($F(1, 20) = 4.251$; $p = 0.056$) on mGluR5- ERα interactions, with a tendency to increase in Cre(+) mice but did not observe sex-specific effects. The mosaic nature of the NSE-Cre(+) deletion of PTEN in cortical neurons likely prohibits us from detecting molecular changes in whole cortical homogenates. We attempted to co-IP mGluR5- ERα from microdissected cortical slices to enrich for PTEN KO neurons but encountered technical challenges. To overcome challenges of working with endogenous ERα, we expressed ERα together with mGluR5 and Homer2 in HEK293 cells. As we observed in brain, mGluR5 and ERα interact in a complex (Fig. 3F). We observed that coexpression of Homer2 increased mGluR5-ERα interaction (Fig. S3G) so we co-expressed Homer2 in this assay. To mimic the effect of *Pten* knockdown, we overexpressed a constitutively active form of AKT (myrAKT) or a kinase dead (KD-Akt; K179M)[51]. Active AKT increased association of ERα and mGluR5 (Fig. 3F). These results predict that PTEN deletion and enhanced Akt activity increases mGluR5 association with ERα.

Acute inhibition of ERK activation or protein synthesis corrects UP state duration in female NSE-Cre PTEN KO mice

Candidate signaling pathways downstream of mGluR5 that may regulate circuit excitability include mTORC1, the mitogen-activated kinase ERK, as well as *de novo* protein synthesis [52-59]. To test the role of these candidate pathways in prolonged UP states in female Cre(+) mice, slices were pretreated with either an inhibitor of mTORC1 (rapamycin; 200nM), or the upstream activating kinase of ERK, MEK, U0126 (20 µM) or one of two different protein synthesis inhibitors (anisomycin (20 µM) or cycloheximide (60 µM); Figs. 4,S4). Surprisingly, rapamycin pretreatment (200nM; 2 hr) had no effect on UP state duration in female Cre- or Cre+ mice and failed to correct the prolonged UP states (Fig. S4A,B). U0126 reduced and corrected UP state duration in Cre(+) females to that observed in Cre(-) controls (Fig. 4A,B). There was no effect of U0126 on UP state duration in Cre(-) females. Similarly, anisomycin and cycloheximide both corrected UP state duration in Cre(+) females and had no effect on UP states in Cre(-) controls (Fig. 4A,C,D). Thus, there was a significant interaction of inhibitor with genotype on UP state duration for both anisomycin ($F(1, 57) = 5.236$; $p < 0.05$) and cycloheximide ($F(1, 55) = 8.317$; $p<0.01$). In males, although there was no effect of genotype on UP state duration, there was a main effect of U0126 to reduce UP state duration ($F(1, 51) = 6.076$; $p<0.05$) and this was significant in Cre(+), but not Cre(-) mice (Fig. S4C). Similarly, there were main effects of anisomycin ($F(1, 76) = 15.01$; $p<0.001$) and cycloheximide ($F(1, 41) = 8.443$; $p<0.01$) to reduce UP state duration in males, but this reached statistical significance only in Cre(+), but not Cre(-), males (Fig. S4D, E). Although the UP state duration are not robustly increased in male Cre (+) mice, the UP states are sensitive to ERK and protein synthesis inhibitors. Taken together our results suggest that ERK signaling, and protein synthesis downstream signaling pathways from mGluR5 and ERα promote female selective cortical hyperexcitability in Cre(+) mice.

From these results, we hypothesize that there is enhanced mGluR5 signaling to protein synthesis in female Cre(+) mice that promotes cortical circuit excitability. To test this hypothesis, we measured

protein synthesis rates in male and female cortical neurons with and without *Pten* deletion using surface sensing of translation (SUnSET) in the presence or absence of MTEP. SUnSET measures *de novo* translation by incorporation of the tRNA analog, puromycin[34]. Puromycin incorporation into newly synthetized protein is detected with a puromycin antibody and fluorescence immunohistochemistry (IHC) [60]. Cortical slices from Cre(+) male and female were acutely prepared as for UP state recordings, incubated in puromycin (40 min) with or without MTEP (3µM) and processed for IHC. Neurons were identified using an antibody for NeuN or NeuroTrace and NSE-Cre(+), *Pten* deleted (KO) L5 neurons, were identified with a PTEN antibody. The mosaic nature of NSE-Cre expression allowed us to compare puromycin incorporation in the soma of neighboring Cre(-) or "WT" neurons, identified by positive staining for PTEN and Cre(+) or "KO" neurons that were negative for PTEN in the same section. Preincubation of slices in anisomycin strongly reduced puromycin labelling indicating that it is reflecting *de novo* protein synthesis (Fig. S4F). Analysis of puromycin fluorescence intensity revealed that PTEN KO L5 neurons in females have enhanced puromycin incorporation as compared to neighboring WT neurons (127±3% of WT; n = 30 sections/4 mice) and this increase was reduced by MTEP treatment (113±3%; n = 27/4 mice; Fig. 4E,F). Using a Cre-reporter (TdTomato) to identify Cre(+) neurons, we determined that puromycin intensity is not elevated in female NSE-Cre (+) L5 neurons without *Pten* deletion (Fig. S4G, H). Therefore, the increase in protein synthesis rates in PTEN KO neurons is a result of *Pten* deletion and not an artifact of NSE-Cre expression. In male mice, protein synthesis rates, as measured by puromycin intensity, were elevated in PTEN KO L5 neurons (n=110±2% of neighboring WT, n= 26; $p < 0.0001$; one sample t-test), but this increase was less than observed in females PTEN KO neurons ($p<0.0001$; Sidak's multiple comparison) and unaffected by MTEP (n=112±2%; n=29). As a result, there was a significant interaction of MTEP and sex on puromycin intensity in PTEN KO neurons ($F(1, 108) = 9.293$; $p< 0.01$; Fig. 4F). These results indicate that mGluR5 activity drives protein synthesis rates in PTEN KO neurons but does so only in females. Because inhibition of ERK signaling with U0126 rescued UP state duration in female Cre(+) mice, but rapamycin did not, we tested whether U0126 or rapamycin reduced protein synthesis rates in PTEN KO neurons in female mice (Fig. S4I, J). Surprisingly, we observed that both U0126 and rapamycin pretreatment of slices reduced puromycin incorporation into PTEN KO neurons to a similar degree. These results suggest that inhibition of overall protein synthesis rates does not correlate with rescue of the circuit hyperexcitability and suggests ERK-dependent translation of specific transcripts in PTEN KO neurons drives circuit excitability.

Genetic reduction of ERα levels reduces protein synthesis rates and cell size in PTEN KO L5 neurons from female mice.

Estrogen and ERα promote mRNA translation in neurons and in cancer cells [61-63]. To determine if ERα heterozygosity reduced protein synthesis rates in PTEN KO neurons, we measured puromycin incorporation in neighboring WT (PTEN+) and KO (PTEN(-)) L5 neurons in the cortices of female NSE-Cre/*Pten*$^{fl/fl}$ and NSE-Cre/*Pten*$^{fl/fl}$/*Esr1*$^{fl/+}$ mice (Fig. 5A,B). PTEN KO L5 neurons had a 129±3% increase in puromycin intensity as compared to neighboring WT (Cre-) neurons; n = 17 sections/3 mice. ERα heterozygosity in PTEN KO neurons reduced puromycin intensity to 118±4% of WT (n= 17; p<

0.05) suggesting that ERα contributes to the enhanced protein synthesis rates in female PTEN KO neurons. To determine if ERα contributed to other cellular phenotypes of PTEN KO neurons in a sex-specific manner, we measured cell size[29]. The soma size of PTEN KO L5 neurons were ~40% larger than neighboring WT neurons in both males and females (Fig. 5C-F). ERα heterozygosity reduced the soma size of PTEN KO neurons to ~120% of WT in females, but not had no effect on PTEN KO cell size in males (Fig. 5C-F). PTEN deletion results in activation of the mTORC1-Ribosomal S6 kinase (p70S6K) pathway, observed by phosphorylation (P) of ribosomal protein S6 (P-S6; Ser235/236; [29]). This pathway may contribute to cell size and enhanced protein synthesis rates[64-66]. In female mice, we observe enhanced P-S6 in PTEN KO L5 neurons in comparison to neighboring WT neurons (Fig. S5A, B). ERα heterozygosity reduced P-S6 in PTEN KO neurons. However, the % increase in P-S6 in PTEN KO neurons as normalized to neighboring WT neurons is unchanged by ERα deletion, in contrast to protein synthesis rates. This may be because of the small, trending decrease in P-S6 in Cre- neurons. These results indicate that ERα contributes to the enhanced protein synthesis rates and cell size of PTEN KO neurons in females. Activation of P-S6 may be contributing to ERα driven protein synthesis rates in PTEN KO neurons, but there are likely other signaling pathways that play a role.

Sex specific alterations in cortical temporal processing of sensory stimuli

The prolonged cortical UP states in young female NSE-Cre PTEN KO mice suggests altered timing of cortical circuits which may impact temporal processing of sensory stimuli. High fidelity temporal processing is necessary for speech and language function [67-69] and is affected in individuals with ASD [70-76] . Impaired temporal processing in children is associated with lower phonological processing scores [71]. To determine if there are sex-specific alterations in temporal processing, particularly with rapid gaps in ongoing sensory stimuli, we performed epidural EEG recordings on the auditory (AC) and frontal cortices (FC) in young (P21-23) ^{NSE}*Pten* KO mice. To address temporal acuity, we used a gap-in-noise auditory steady state response (gap-ASSR) paradigm which has been previously used to reveal developmental deficits in temporal processing in an ASD mouse model [77,78]. The gap-ASSR elicits a stead state response by presenting short gaps in continuous noise at 40 Hz. Using short gap widths and shallow amplitude modulation depths (~75%), we compared the limits of temporal processing of cortical circuits across experimental groups. To measure the consistency of cortical responses to the gap-ASSR across trials, we calculated the inter-trial phase clustering (ITPC) at 40Hz. We also measured ITPC during the ASSR without any gaps and termed this “baseline ITPC”. Both male and female Cre+ mice showed reduced ITPC of the gap ASSR measured in the AC and FC with brief (4-6ms) gaps (Fig. 6A, B). However, in females the ITPC during the gap-ASSR was not greater than baseline ITPC, or when no gaps were present. Male Cre+ mice had a greater ITPC during the gap-ASSR as compared to baseline. These results suggest that female Cre+ mice are unable to consistently phase lock their response to brief gaps in sound, and the cortical responses are similar to noise. Male Cre+ mice, however, can consistently register responses to brief gaps in sound, but their responses are not as consistent as Cre- male mice. These results suggest deficits in temporal processing of sensory stimuli in both male and female Cre+ mice and these deficits are more severe in females.

Seizure susceptibility in female NSE-Cre PTEN KO mice

Our slice results suggest an mGluR5-dependent hyperexcitability of cortical circuits in female NSE-Cre PTEN KO mice. To determine if there are sex-specific circuit hyperexcitability *in vivo*, we measured susceptibility to seizures induced with the volatile convulsant flurothyl. The latency to onset to distinct seizure behaviors, myoclonic twitch or tonic-clonic extension, provides a reliable index of seizure threshold in naive mice, and flurothyl seizures are highly penetrant regardless of genetic background [79,80]. At 3 weeks of age, the age of slice for UP state experiments, neither male nor female Cre(+) mice had an increased susceptibility to flurothyl-induced seizures indicating that the cortical excitability we observe in female Cre(+) acute slices is not sufficient to affect seizure latency (data not shown). However, at 6-8 weeks of age both male and female Cre(+) mice had reduced latency to myoclonic twitch and tonic clonic seizure in response to a single flurothyl exposure (Fig. 6C). Because hyperexcitability of cortical circuits in female Cre(+) mice is dependent on mGluR5, we tested whether seizure susceptibility was also dependent on mGluR5 by administering the brain penetrant mGluR5 NAM, CTEP (i.p.; 2mg/kg), prior to flurothyl exposure. CTEP increased the latency to myoclonic twitch in female, but not in male Cre(+) mice (Fig. 6D). CTEP did not affect seizure onset latency in male or female Cre(-) mice. These results indicate that both male and female NSE-Cre PTEN KO mice develop increased susceptibility to seizures as they age, but this is only dependent on mGluR5 in females.

Female-specific behavioral alterations in NSE-Cre PTEN KO mice

Previous work demonstrated ASD-relevant behavioral alterations in NSE-Cre PTEN KO mice such as social interaction deficits, but any sex-dependence of these deficits was not reported[29]. Female mice with a germline heterozygous deletion of *Pten* (*Pten*$^{+/-}$) display deficits in social preference [24,81,82] and we hypothesized that ^{NSE}*Pten* KO mice would exhibit sex-dependent behavioral deficits. To test this hypothesis, we performed a battery of behavioral tests in Cre(+) and Cre(-) male and female mice. In previous work, ^{NSE}*Pten* KO mice exhibited deficits in social interaction, enhanced responses to sensory stimuli, anxiety-like behaviors, seizures, and decreased learning, which are features associated with ASD[29]. Consistent with this previous work, Cre(+) mice displayed a deficit in the three-chamber social interaction test, but this was only observed in females (Fig. 7A). Female Cre(-) mice spent more time sniffing a novel mouse in one chamber as compared to an object in another chamber, whereas female Cre(+) mice sniffed the mouse and object similarly, which resulted in a significant interaction of genotype X chamber ($F(1, 42) = 7.340$; $p < 0.01$). In contrast, male Cre(+) mice performed similarly to male Cre(-) littermates and sniffed a novel mouse more than an object (Genotype X chamber; $F(1, 44) = 0.01$; n.s.). Female Cre(+) mice also displayed an increase in locomotor activity, observed in dim light (females; genotype main effect; $F(1, 31) = 5.874$; $p < 0.05$; Fig. 7B). However, in a brightly lit open field, both male and female Cre(+) mice were hyperlocomotive as assessed by total distance traveled (Fig. S7A). There was no sex or genotype effect on time spent in the center of open field, a measure of anxiety (Fig. S7A). In another measure of anxiety, the dark-light box, male Cre(+) mice had decreased time spent in the light side of the box, but females also had a strong trend towards decreased time in light (Fig. S7B). These results suggest a tendency for increased anxiety in both male and female Cre(+) mice. To assess potential sex-dependence in a learning task, we assayed delay cue and context-

dependent fear, or threat, conditioning. Female Cre(+) mice displayed enhanced cue-induced fear, as measured by % time freezing (Fig. 7C). Baseline freezing and context-induced freezing were normal. Male Cre(+) had normal freezing at baseline and in response to cue or context. These results suggest female specific effects of NSE-Cre mediated *Pten* deletion in mice on social preference, locomotion and fear similar to other *Pten* deletion models [25].

Female specific circuit excitability in a mouse model of PTEN Hamartoma Syndrome (PTHS)

To determine if there were sex-dependent alterations in cortical excitability in a mouse model with construct validity for individuals with *PTEN* mutations, we examine UP states in acute slices of somatosensory cortex from mice with germline haploinsufficiency of *Pten* (*Pten*$^{+/-}$). In contrast to ^{NSE}*Pten* KO mice, we did not observe altered UP state duration in slices from juvenile female, or male, *Pten*$^{+/-}$ mice (P18-25; Fig. S8A). However, in young adult mice (6-8 weeks), we observed a female-specific cortical circuit hyperexcitability in *Pten*$^{+/-}$ mice (Fig. 8A,B) measured as an increase in the number of UP states and total time spent in an UP state (Fig. 8A,B). In *Pten*$^{+/-}$ males, UP state duration was reduced, and the number of UP states and total time spent in an UP state was unchanged. There was a significant interaction of genotype X sex for both UP state number ($F(1, 193) = 5.041$; $p < 0.05$) and time in UP state ($F(1, 193) = 7.671$; $p < 0.01$). Surprisingly, wild-type females had fewer UP states and less time in an UP state when compared to wild-type males. These results reveal female selective increase in excitability of cortical circuits with germline haploinsufficiency of *Pten*, a human-disease relevant model of loss of function mutations in *PTEN*.

We next tested if there were a sex-dependent seizure susceptibility in *Pten*$^{+/-}$ mice in response to flurothyl treatment. We observed a main effect of *Pten*$^{+/-}$ to reduce the latency for onset of myoclonic twitch and tonic clonic seizures but did not observe a female specific reduction in seizure latency (Fig. S8B). However, in a repeated flurothyl treatment paradigm (daily for 5 days), female *Pten*$^{+/-}$ mice had an increased probability of death during flurothyl treatment as compared to wild-type females (Fig. 8C). Male *Pten*$^{+/-}$ mice died at similar rate to wild-type males. To determine if the flurothyl-induced death was mediated by mGluR5, we pretreated mice daily with CTEP (i.p.) 90 min prior to flurothyl. CTEP treatment resulted in greater survival of *Pten*$^{+/-}$ females on the first day of flurothyl exposure and over the course of a 3 day repeated exposure (Fig. 8D; S8C). In contrast, CTEP did not affect survival of male *Pten*$^{+/-}$ mice. CTEP did not affect the latency to myoclonic twitch or tonic-clonic seizure in male or female *Pten*$^{+/-}$ mice and had no effect on survival of wild-type mice with repeated flurothyl (Fig. S8C, D). These results reveal a female-specific, mGluR5-dependent seizure severity in a mouse model of human *PTEN* haploinsufficiency.

Discussion

Understanding how ASD genes affect and interact with sex-specific brain functions and signaling pathways will be key to developing more personalized therapeutics for individuals. *Pten* mutations have a strong female bias for causing breast cancer, but the sex-specific roles of PTEN in the

brain are relatively unknown. Here we identify a female-specific hyperexcitability of cortical circuits and an mGluR5-dependent seizure severity in mice with postnatal deletion of *Pten* in L5 neurons or with germline heterozygous deletion of *Pten*. Using the ^{NSE}*Pten* KO mouse model, we identified a female specific, cell autonomous effect of *Pten* deletion in L5 neurons that results in enhanced signaling of mGluR5-ERα to protein synthesis that drives hyperexcitability and abnormal timing of developing cortical circuits. This abnormal timing of cortical circuit correlates with reduced temporal processing of sensory stimuli in young female ^{NSE}*Pten* KO mice, as measured by the gap-ASSR. Young adult ^{NSE}*Pten* KO displayed female specific ASD-relevant behaviors, such as reduced social interaction, hyperactivity and enhanced fear learning. Our results reveal a molecular mechanism for the sex-specific effects of loss of function of an ASD-risk gene on neuronal and circuit function and suggest distinct pharmacological strategies, based on sex, for individuals with *PTEN* mutations and perhaps others genetic causes of ASD.

Female selective hyperactivity of mGluR5 signaling drives excitability in PTEN KO cortical neurons

Our results suggest that the enhanced signaling mGluR5-ERα underlies the female-specific effects of *Pten* deletion. Prolonged UP states, enhanced protein synthesis rates and seizure susceptibility in ^{NSE}*Pten* KO mice was dependent on mGluR5 in females, but not males. Antagonism or genetic reduction of ERα corrected UP states and protein synthesis rates in female, but not male, ^{NSE}*Pten* KO mice. Previous work in rodents has shown that estrogen signals through mGluR5 selectively in female brains and this contributes to sex-dependent effects of estrogen in drug addiction, learning and memory and other behaviors [45,83]. Consistent with these studies, we observe enhanced mGluR5- ERα complexes in female cortex. In addition, *Pten* deletion results in a female specific enhancement of mGluR5 levels in L5-enriched cortical lysates. Together, these results point to enhanced mGluR5-ERα function or signaling in female PTEN KO neurons which drives ERK activation and protein synthesis. In further support of this idea, genetic reduction in ERα reduced protein synthesis rates and cell size in L5 PTEN KO neurons, in a cell autonomous fashion. Thus, our results suggest direct effects of PTEN on mGluR5-ERα within L5 neurons. Hyperactivation of Akt in breast cancer cells results in phosphorylation and constitutive activity of ERα that further drives activation of PI3K/mTOR and tumor growth [21-23,84]. Pten deletion in neurons may results in similar alterations in ERα phosphorylation and activity.

Dysfunction of mGluR5 signaling to ERK and protein synthesis: Convergence of ASD genes

Like female ^{NSE}*Pten* KO, prolonged UP states and other measures of hyperexcitability in male *Fmr1* KO mice are dependent on mGluR5 [33], ERK (Fig. S4E) [58] and *de novo* protein synthesis [59] (Molinaro and Huber, unpublished). Acute antagonism of ERK activation and *de novo* protein synthesis, but, surprisingly, not mTORC1, corrected long UP states in female ^{NSE}*Pten* KO slices. Although rapamycin reduced protein synthesis rates in PTEN KO neurons, it did not correct circuit excitability, in contrast to the inhibitor of ERK activation. Similarly, in the *Fmr1* KO, inhibition of ERK, but not mTORC1 has been implicated in circuit hyperexcitability and seizures[57,58,85]. This suggests that an ERK-dependent signaling pathway(s) promotes translation of specific mRNA transcripts that lead to circuit hyperexcitability with loss of function of either *Fmr1* or *Pten*. Thus, our results reveals a

point of convergence between these two ASD genes, with the exception that in the PTEN KO, these effects are only observed in females. In *Fmr1* KO mice, mGluR5 is less associated with its scaffolding protein Homer[40,86]. Restoration of mGluR5-Homer interactions in *Fmr1* KO mice corrects prolonged UP states, seizures, and some behaviors [36,41]. In ^{NSE}*Pten* KO, we were unable to detect changes in mGluR5-Homer in either sex, which may have been because of the mosaic nature of *Pten* deletion. Alternatively, mGluR5 may be activated in PTEN KO neurons through its interaction with a hyperactive ERα. However, in both models, dysfunctional mGluR5 drives elevated mRNA translation rates and cortical circuit hyperexcitability. Deletion of *Tsc2*, another ASD-risk gene and suppressor of mTORC1 activation, results in mGluR5-dependent hyperexcitability or seizures in mice. The sex-dependence of this effect was either not tested[53] or evident [87]. As in breast cancer, one may expect dysregulation of ERα in neurons with loss of function mutations in suppressors of PI3K and sex-dependent effects on brain function and behavior.

Developmental regulation of PTEN phenotypes and role of estrogen

The sex-specific, ERα -dependent cortical circuit dysfunction in ^{NSE}*Pten* KO was observed in young, pre-pubertal mice (P18-25), when circulating testosterone and estrogen levels are low[88]. Our results with MPP suggest that estrogen is necessary to drive ERα activity and long UP states. If so, brain synthesized estrogen may mediate ERα activation in this context. Observing sex-specific effects of *Pten* deletion young mice, before puberty, also suggests that the PTEN interacts with mechanisms of sexual differentiation of the brain; a process that occurs during the first few postnatal days in rodents [88]. Hyperexcitability of cortical circuits was also observed in female *Pten*$^{+/-}$ but not until the young adult stage (6-8 week). This may be a consequence of the heterozygous expression of *Pten* that maintains circuit function in developing cortex. Although reductions in seizure latency were not sex-dependent in ^{NSE}*Pten* KO mice, seizures were sensitive to CTEP only in females. We did observe enhanced seizure severity in female *Pten*$^{+/-}$ mice, as compared to males, which we measured as an increased probability of death. This was also selectively prevented in females by CTEP. These results are consistent with a female specific, enhanced function of mGluR5 that leads to circuit hyperexcitability with *Pten* deletion. Epilepsy is more common in females with autism, as compared to males[89,90] and brain synthesized estrogen promotes seizures [91]. This suggests there may be roles for estrogen and/or mGluR5- ERα signaling in epilepsy in females with autism. In this study, we did not control for estrus cycle in phenotypes in adult female *Pten*$^{+/-}$ mice and would predict that estrus cycle and circulating estrogen would modulate these phenotypes.

Sex-specific deficits in temporal sensory processing in young ^{NSE}*Pten* KO

UP states reflect the balance of excitation and inhibition in cortical circuits and provide a measure of dynamic circuit function [30,38]. By measuring UP states we revealed altered function of developing cortical circuits in the form of prolonged circuit activity as well as an overall increase in circuit activity in females with *Pten* deletion. The contribution of this prolonged circuit activity and hyperexcitability to *in vivo* cortical function is unknown but may contribute to the reduced ability of cortical circuits to rapidly respond and synchronize to changing sensory stimuli, as revealed here by the reduced ability of female ^{NSE}*Pten* KO mice to consistently respond to brief gaps in a steady state sound.

Our previous work in the Fragile X Syndrome mouse model (*Fmr1* KO) identified prolonged duration of cortical UP states and reduced phase loking to rapid gaps in sensory stimuli, particularly in developing mice[78]. Because *Fmr1* is X-linked, we only tested males in these studies. Similar to ^{NSE}*Pten* KO, cortical circuits of juvenile *Fmr1* KO mice have a reduced ability to synchronize to spectrotemporally dynamic sounds at 40-80 Hz [92]. These results suggest similar mechanisms of cortical circuit dysfunction in developing ^{NSE}*Pten* KO and *Fmr1* KO that results in prolonged circuit activity and deficits in the temporal processing of sensory stimuli. In male ^{NSE}*Pten* KO, we observed smaller and less reliable increases in cortical UP state duration and smaller deficits in the gap-ASSR. These results indicate that effects of PTEN deletion are not exclusive to females but are more robust in females as compared to males. Responses to narrow gaps in sounds are commonly used measures of auditory acuity that shows prolonged maturation in children and predicts phonological aspects of speech [71]. These data suggest that observed sex-dependent differences in gap processing may underlie abnormal speech recognition in females to a greater extent than males with *PTEN* mutations. Girls with ASD have more severe deficits in sensory processing as compared to boys, particularly in the auditory and somatosensory domains [8]. Studies of sensory circuits and their development across sexes in ASD mouse models may contribute to understanding these deficits and developing therapies.

Deficits in female specific behaviors in mouse models of PTEN deletion

Increasing evidence indicates that there are sex-specific effects on social behavior in multiple mouse models of PTEN loss of function [25]. Most notably, female, but not male, *Pten*$^{+/-}$ mice have deficits in social preference[18,24,25]. Interestingly, male *Pten*^{m3m4} mutant mice, expressing a cytoplasmic predominant form of PTEN, exhibit enhanced sociability[26]. Consistent with these studies, we demonstrate a female-specific deficit in social interaction in ^{NSE}*Pten* KO mice. *Pten*$^{+/-}$ mice have female specific alterations in fear conditioning, as we observe in the ^{NSE}*Pten* KO mice [25]. We observed a female specific hyperlocomotion under dim light, but not in the open field, where both males and females were hyperlocomotive. Modulators of mGluR5, either positive or negative, affect and correct behaviors in adult mouse ASD models caused by loss of function mutation in suppressors of mTOR or mRNA translation[93-95] and inbred strains [96] but sex-specific effects were not reported. Similarly, the role of brain ERα in sex-specific ASD-relevant behaviors has not been reported in any mouse models. Modulation of mGluR5 and/or ERα may have sex-specific on behavioral phenotypes in mouse models of *Pten* deletion and other ASD models.

It is increasingly recognized that ASD manifests differently in females and males. For example, as discussed, females with autism are more likely to have epilepsy and more severe problems in sensory processing in some modalities. Here we demonstrate female selective hyperexcitability of sensory circuits, enhanced severity of seizures, and a female specific pharmacology of circuit function with loss of function of a high-impact ASD-risk gene. Importantly, we reveal roles for brain estrogen receptors in ASD-relevant phenotypes and suggests that ASD- risk genes that regulate the PI3K pathway, as in cancer, also regulate ERα in the brain and may give rise to sex-specific alterations in brain function and behavior. Our results also predict sex-specific efficacy in therapeutic strategies for individuals with *PTEN* mutations and perhaps more generally in ASD.

Credit authorship contribution statement

Gemma Molinaro: Conceptualization, Investigation, Formal analysis, Writing – original draft. Jacob E. Bowles: Investigation. Katilynne Croom: Investigation, Formal analysis. Darya Gonzalez: Investigation. Saba Mirjafary Investigation. Shari Birnbaum: Conceptualization, Supervision. Khaleel A. Razak: Conceptualization, Supervision, Formal analysis. Jay R. Gibson: Conceptualization, Supervision, Validation. Kimberly M. Huber: Conceptualization, Formal analysis, Writing – original draft, Supervision, Funding acquisition.

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Figure legends

Figure 1. Female specific cortical circuit hyperexcitability in acute slices of ^{NSE}*Pten* KO mice. A. Schematic of thalamocortical slice of somatosensory cortex and extracellular multiunit recording in Layer (L) 4. **B.** Representative spontaneous, persistent activity states, or UP states from extracellular recordings in L4 of male and female *Pten*$^{fl/fl}$ Cre (-) and *Pten*$^{fl/fl\text{-}NSE\text{-}Cre}$ Cre (+) somatosensory cortex. Dashed lines indicate shorter epochs of the longer traces which are expanded to show more detail. Scale bars = 0.2mV/8 or 0.8 sec. **C.** Group average UP state duration in male and female Cre (-) and Cre (+) mice in L4, L2/3 and L5. (N= 14-22 slices from 4-6 mice/sex/genotype). **D,E.** UP state durations for vehicle or un-treated slices across experiments recorded in L4 of males and females Cre (-) and Cre (+) mice. Data are shown averaging UP state duration per slice (D) or per mouse (E) (Females; Cre (-); n=175 slices from 45 mice; Cre (+); n=176 slices from 43 mice; Males; Cre (-); n=148 slices from 34 mice; Cre (+); n=131 slices from 35 mice). For all figures, data are represented as mean ± SEM. Two-way ANOVA with Sidak for multiple comparisons. For all figures, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0005$, $^{****}p < 0.0001$.

Figure 2. Prolonged UP states in Female ^{NSE}*Pten* KO mice are rescued by an mGluR5 negative allosteric modulator (NAM) and associated with increased mGluR5 in cortical Layer 5. A. Representative UP states from female Cre (-) and Cre (+) slices pretreated with vehicle or the mGluR5 selective NAM MTEP (3 μM; 2 hours). Scale bars= 0.1 mV/0.8 sec. **B.** Left: Group data show that MTEP corrected UP state duration in female Cre (+) mice without affecting UP states in Cre (-) mice. Right: MTEP has no effect on UP state duration in slices from male Cre(+) or Cre (-) mice. N= 25-45 slices from 4-8 mice/genotype/sex. **C**. Left: Layer (L)5 was microdissected from slices of ^{NSE}*Pten* KO mice to enrich for PTEN KO neurons. Right: Representative western blots from L5 lysates prepared from male and female Cre (-) and Cre (+) mice. **D.** Quantified group data show that mGluR5 levels are elevated in L5 of female, but not male, Cre (+) mice as compared to Cre(-) same sex littermates. **E.** Quantification of mGluR5 interacting proteins, Homer and ERα, AMPA or NMDA receptors, or PSD-95 revealed no changes in L5 of Cre(+) mice of either sex.

Figure 3. Estrogen Receptor α (ERα) is required in L5 neurons for prolonged UP states and is more associated with mGluR5 in females. A. Representative UP states from female Cre (-) and Cre (+) slices pretreated with vehicle or the ERα selective antagonist (MPP, 1 μM; 1.5-2 hours). Scale bars= 0.1 mV/0.8 sec. **B.** Left: Group data show that MPP reduced UP state duration in female Cre (+) mice without affecting UP states in Cre (-) mice. Right: UP state duration was unaffected by MPP in male Cre (+) and Cre (-) mice. N=16-32 slices from 4-8 mice/sex/genotype. **C.** Representative UP states from female *NSE-Cre/Pten*$^{fl/fl}$ (Cre (+)) and Cre(-) mice and *NSE-Cre/Pten*$^{fl/fl}$*/Esr1*$^{fl/+}$, with a genetic reduction of ERα in NSE-Cre(+) neurons. Scale bars= 0.1 mV/0.8 sec **D.** Genetic reduction of ERα expression reduces UP state duration in female (left), but not male (right), Cre (+) mice. N= 20-40 slices from 3-8 mice/sex/genotype. **E.** Left: Representative western blots from ERα-mGluR5 co-IP and inputs from cortical lysates prepared from male (M) and female (F) wildtype littermates. Right: Quantified group data shows that cortical ERα-mGluR5 interactions are enhanced females as compared to males. (n = 5 female and 6 male mice). **F.** Active AKT increased association of ERα and mGluR5 in HEK cells.

Representative western blots (left) and quantification (right) show co-IP of Flag-mGluR5 and HA-ERα from lysates of HEK-293T cells co-expressing either constitutively active AKT (myrAKT; M) or a kinase dead (KD) AKT. N= 6.

Figure 4. Inhibitors of ERK activation and *de novo* protein synthesis correct prolonged UP states in female ^{NSE}*Pten* KO mice. A. Representative UP states from female Cre (-) and Cre(+) mice pre-incubated in vehicle, U0126 (20 µM, 2 hrs), anisomycin (20 µM, 1hr) or cycloheximide (60 µM, 1 hr). Scale bars= 0.05 mV/1 sec. **B, C, D**. Group data of average UP state duration show that inhibitors of ERK activation or protein synthesis reduce UP state duration in slices from female Cre (+) mice and have no effect on UP state duration in Cre (-) mice. N= 12-30 slices from 3-6 mice/genotype/condition. Two-way ANOVA, Dunnet's (B) or Sidak's multiple comparison tests (C,D). **E.** Representative images of puromycin immunolabeling (red) of L5 neurons in cortical sections from male and female Cre (+) mice pre-treated with vehicle or MTEP (3 µM; 2 hr). NeuN (blue) and PTEN (green) immunolabeling was used to identify PTEN KO (filled arrows) and neighboring PTEN+ neurons (open arrows). Scale bar: 30 µm. **F.** Quantified group data of puromycin fluorescent intensity of PTEN KO neurons normalized to PTEN+ neurons within the same section. Puromycin intensity of PTEN KO neurons was enhanced in sections from females and greater than in PTEN KO neurons from male mice. MTEP pretreatment reduced puromycin labeling in female, but not male, mice. N= 26-30 sections from 4 mice/sex.

Figure 5. ERα promotes protein synthesis rates and soma size in *Pten* KO L5 neurons from female mice. A. Representative images of puromycin immunolabeling (red) of L5 neurons in cortical sections from female NSE-Cre/*Pten*$^{fl/fl}$ or NSE-Cre/*Pten*$^{fl/fl}$/*Esr1*$^{fl/+}$ mice. NeuN (blue) and PTEN (green) immunolabeling identify PTEN KO (filled arrows) and neighboring PTEN+ neurons (open arrows). Scale bar: 30 µm. **B.** Quantified group data demonstrates that heterozygous deletion of *Esr1* in PTEN KO L5 neurons reduces puromycin intensity as normalized to neighboring PTEN+ neurons. N= 17 sections from 3mice/genotype; t-test. **C,E.** Representative images of Nissl staining (blue) of L5 neurons in cortical sections from female (C) and male (E) NSE-Cre/*Pten*$^{fl/fl}$ or NSE-Cre/*Pten*$^{fl/fl}$/*Esr1*$^{fl/+}$ mice. PTEN (green) immunolabeling identify PTEN KO (filled arrows) and neighboring PTEN+ neurons (open arrows). Scale bar = 40 µm. **D, F.** Raw (left) and normalized (right) mouse averages show an increase in soma size of PTEN KO L5 neurons that is reduced by genetic reduction of *Esr1* in females (D), but not in males (F). N = 20-21 sections from 3-4 mice/sex. Mann Whitney test.

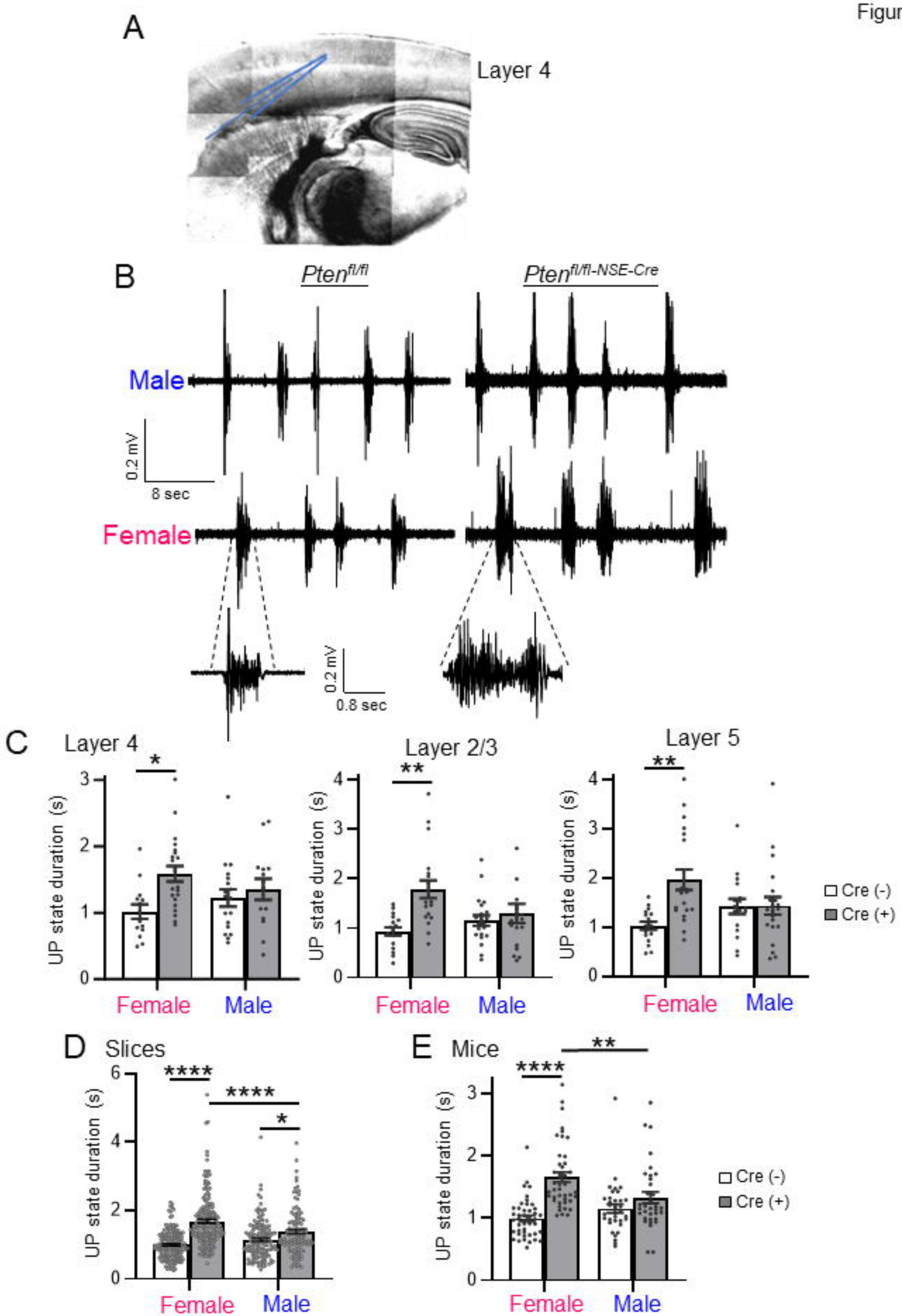
Figure 6. **Deficits in temporal processing of sensory stimuli and mGluR5-dependent seizures female ^{NSE}*Pten* KO mice. A, B** (Left) Average ITPC heatmaps of P21 female and male Cre(-) and Cre(+) mice from the auditory cortex (A) or frontal cortex (B). Heatmaps show the average ITPC

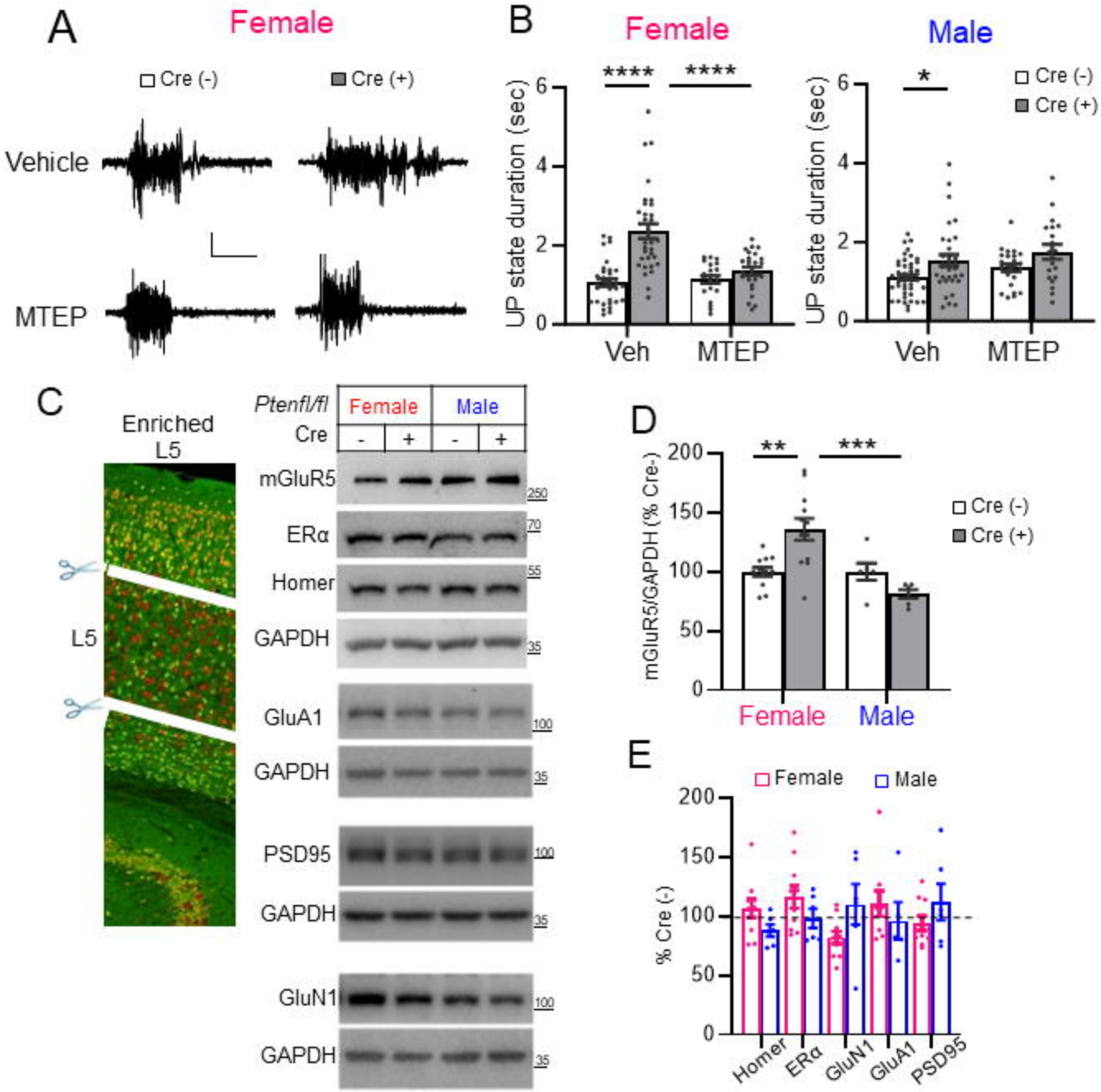
produced across presentations of the 6ms/75% modulated gap. **A, B** (Right) Both male and female Cre(+) mice show ITPC deficits when presented with gap interrupted noise (+ Gap). Cre(+) females' ITPC during the gap-ASSR was not greater than baseline levels (unmodulated noise). Male Cre(+) mice significantly increased ITPC during the gap-ASSR as compared to baseline. **C.** Flurothyl-induced seizure in 6-8 weeks male and female Cre (+) and Cre (-) mice. Latency to myoclonic twitch is similarly decreased in male and female Cre (+) mice. (n=6-9 mice/sex/genotype). **D.** Acute administration of the mGluR5 NAM CTEP increases latency to myoclonic twitch in female Cre (+) mice (left) but has no effect in male Cre (+) mice (right). N=6-9 mice/sex/genotype/treatment.

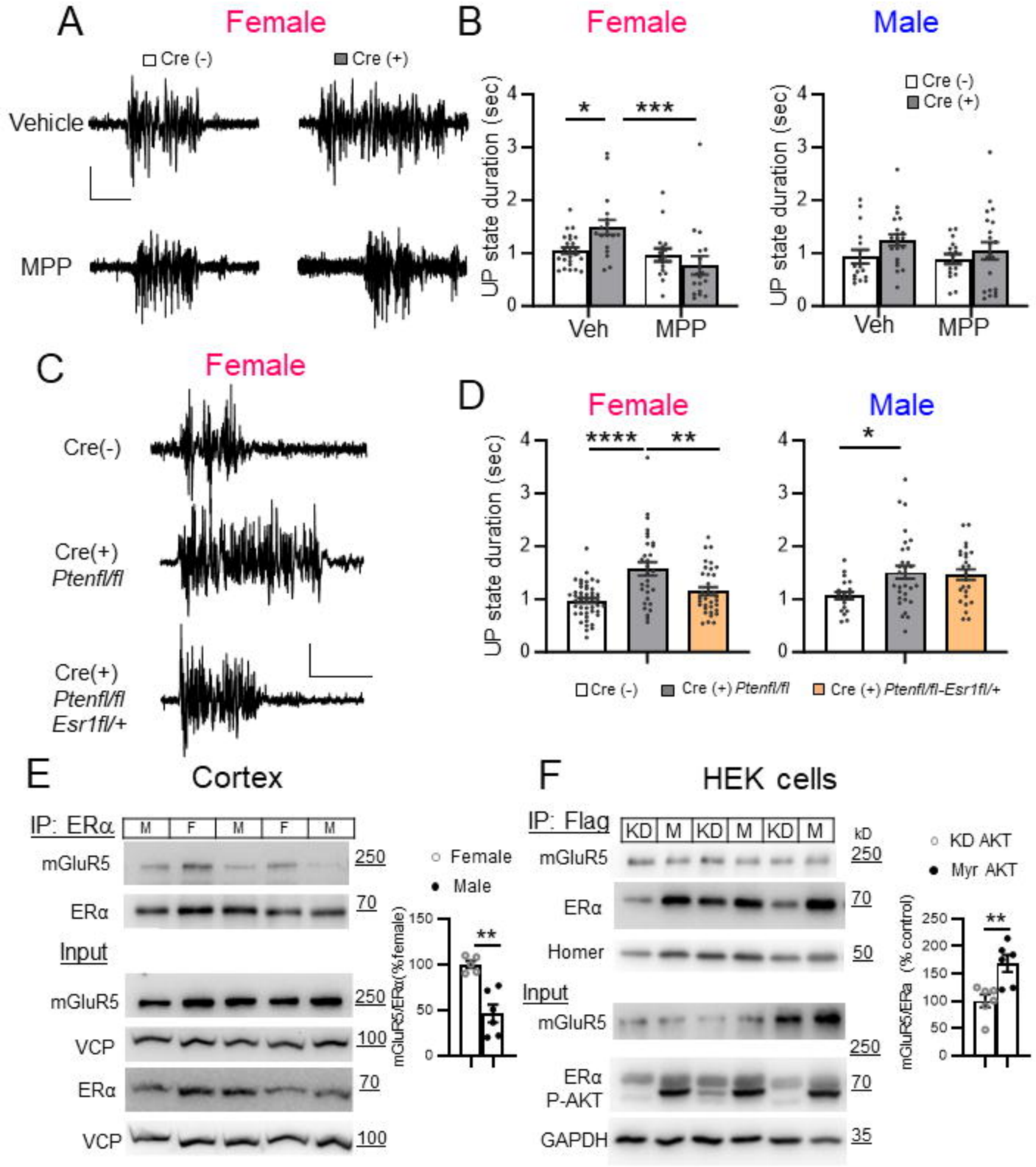
Figure 7. Female specific behavioral alterations in ^{NSE}*Pten* KO mice. **A.** Reduced sociability, measured as time sniffing, in the three-chamber social interaction test is displayed by female, but not male Cre (+) mice. N= 20-26 mice/sex/genotype. **B.** Female specific increase in locomotor activity in Cre (+) mice measured as infrared beam breaks. (Females; n= 18 Cre (-) and 15 Cre (+) mice; Males; n= 18 Cre (-) and 15 Cre (+) mice). **C.** Freezing to the cue in delay fear conditioning is enhanced in female, but not male Cre (+) mice. Baseline and context-dependent freezing are not different between sex or genotype. N= 17-25 mice/sex/genotype.

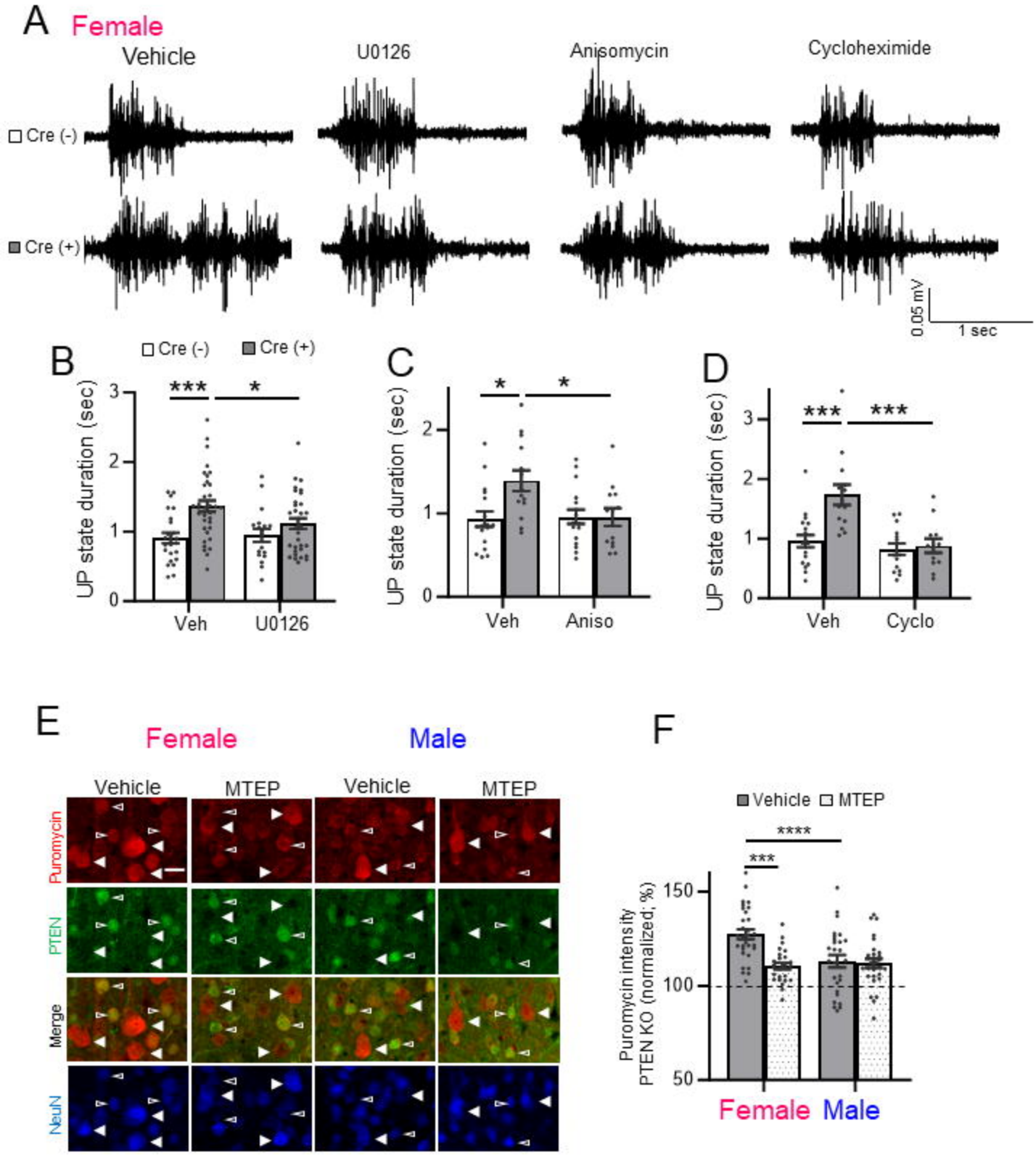
Figure 8. Female selective circuit excitability and mGluR5-dependent seizure severity in a mouse model of PTEN Hamartoma syndrome. **A.** Representative UP states from extracellular recordings in L4 of somatosensory cortex from 6-8 week old male and female *Pten*$^{+/-}$ mice. Scale bars = 100µV/7.5sec. **B.** Group averages of UP state duration, frequency and total time in an UP state in female and male *Pten*$^{+/-}$ slices. N= 43-58 slices from 8-12 mice/sex/genotype. **C.** Survival curve of female and male *Pten*$^{+/-}$ mice (P90-100) with repeated, daily flurothyl exposure. Female, but not male, *Pten*$^{+/-}$ mice have a reduced rate of survival as compared to same sex littermates. Mantel-Cox test. N=10-14 mice/sex/genotype. **D.** Pre-treatment with CTEP increased survival of female, but not male, *Pten*$^{+/-}$ mice on the first day of flurothyl exposure. Cumulative number of Pten+/- mice that died or survived on day 1 of flurothyl exposure. N=13-17 mice/sex/treatment condition. Chi-square test.

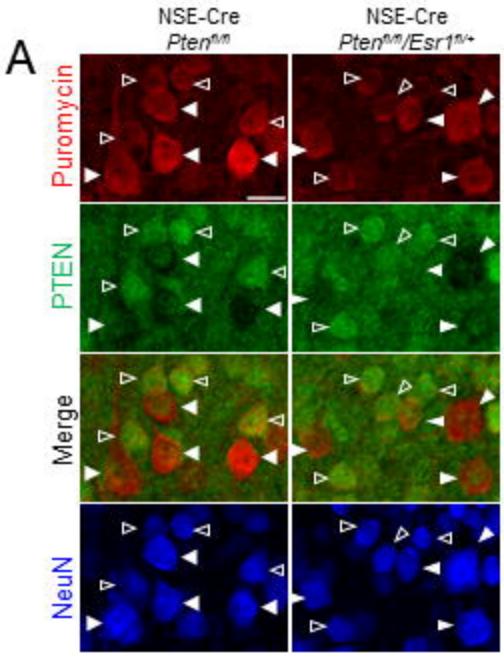
Figure 1

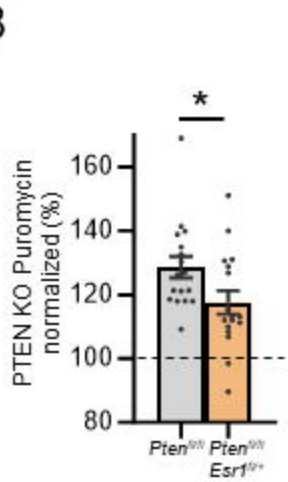

A
Layer 4
B
Pten fl/fl
Pten fl/fl-NSE-Cre
Male
Female
0.2 mV
8 sec
0.2 mV
0.8 sec
C
Layer 4
Layer 2/3
Layer 5
UP state duration (s)
Female
Male
Cre (-)
Cre (+)
D Slices
E Mice

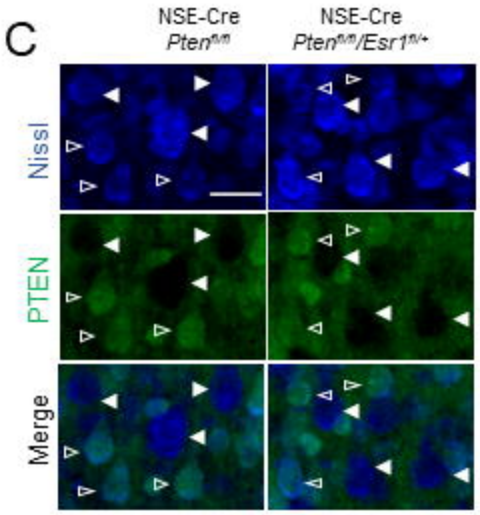

A
Female
Cre (-)
Cre (+)
Vehicle
MTEP
B
Female
Male
UP state duration (sec)
****
****
*
Cre (-)
Cre (+)
Veh
MTEP
C
Enriched L5
L5
Ptenfl/fl
Female
Male
Cre
mGluR5
ERα
Homer
GAPDH
GluA1
PSD95
GluN1
250
70
55
35
100
D
mGluR5/GAPDH (% Cre-)
**
***
E
% Cre (-)
Homer
ERα
GluN1
GluA1
PSD95

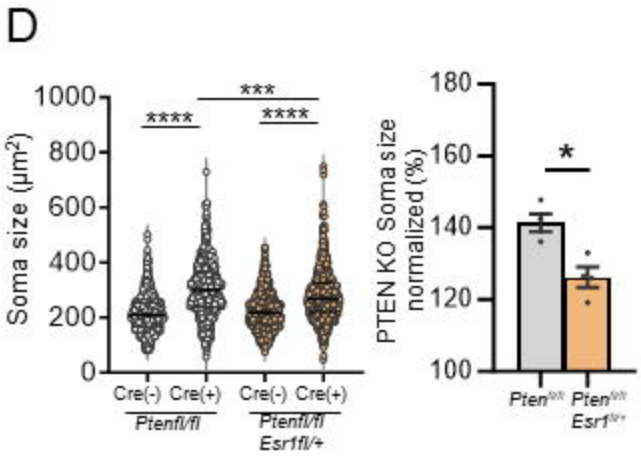

A
Female
Cre (-)
Cre (+)
Vehicle
MPP
B
Female
Male
UP state duration (sec)
Veh
MPP
C
Female
Cre(-)
Cre(+)
Ptenfl/fl
Cre(+)
Ptenfl/fl
Esr1fl/+
D
Female
Male
Cre (-)
Cre (+) Ptenfl/fl
Cre (+) Ptenfl/fl-Esr1fl/+
E
Cortex
IP: ERα
M F M F M
mGluR5
ERα
Input
VCP
250
70
100
Female
Male
mGluR5/ERα(%female)
F
HEK cells
IP: Flag
KD M KD M KD M
kD
Homer
50
P-AKT
GAPDH
35
KD AKT
Myr AKT
mGluR5/ERa (% control)

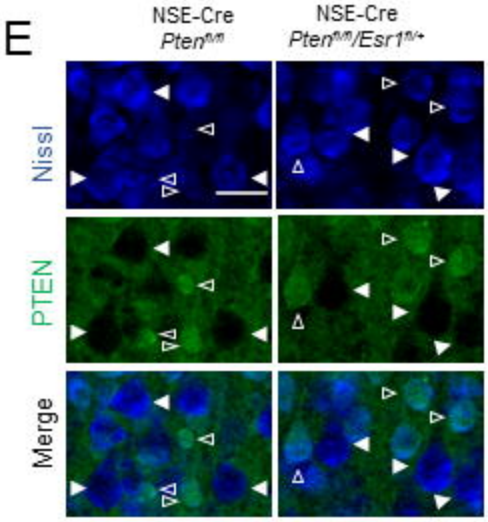

A
Female
Vehicle
U0126
Anisomycin
Cycloheximide
Cre (-)
Cre (+)
0.05 mV
1 sec
B
Cre (-)
Cre (+)
UP state duration (sec)
***
*
Veh
U0126
C
*
*
Veh
Aniso
D
***
***
Veh
Cyclo
E
Female
Male
Vehicle
MTEP
Vehicle
MTEP
Puromycin
PTEN
Merge
NeuN
F
Vehicle
MTEP
****
***
Puromycin intensity
PTEN KO (normalized; %)
150
100
50
Female
Male


A
NSE-Cre
Pten fl/fl
NSE-Cre
Pten fl/fl/Esr1 fl/+
Puromycin
PTEN
Merge
NeuN


B
*
PTEN KO Puromycin normalized (%)
160
140
120
100
80
Pten fl/fl
Pten fl/fl Esr1 fl/+


C
NSE-Cre
Pten fl/fl
NSE-Cre
Pten fl/fl/Esr1 fl/+
Nissl
PTEN
Merge


D
Soma size (μm²)
1000
800
600
400
200
0
****
***
****
Cre(-)
Cre(+)
Cre(-)
Cre(+)
Ptenfl/fl
Ptenfl/fl Esr1fl/+
PTEN KO Soma size normalized (%)
180
160
140
120
100
*
Pten fl/fl
Pten fl/fl Esr1 fl/+


E
NSE-Cre
Pten fl/fl
NSE-Cre
Pten fl/fl/Esr1 fl/+
Nissl
PTEN
Merge

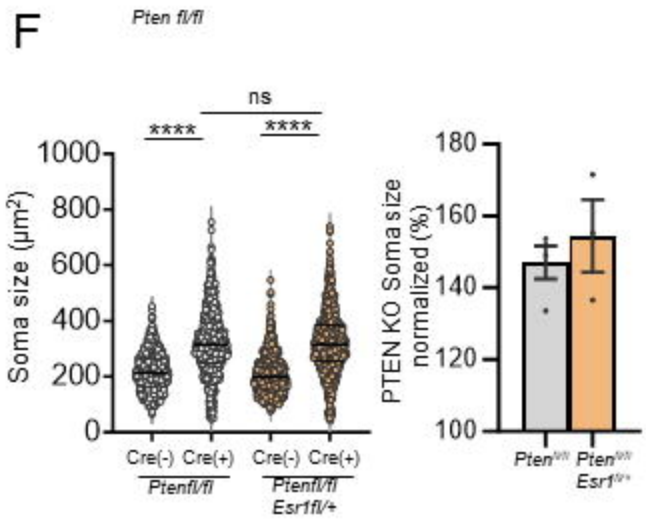

F
Pten fl/fl
Soma size (μm²)
1000
800
600
400
200
0
ns
****
****
Cre(-)
Cre(+)
Cre(-)
Cre(+)
Ptenfl/fl
Ptenfl/fl Esr1fl/+
PTEN KO Soma size normalized (%)
180
160
140
120
100
Pten fl/fl
Pten fl/fl Esr1 fl/+

Figure 6

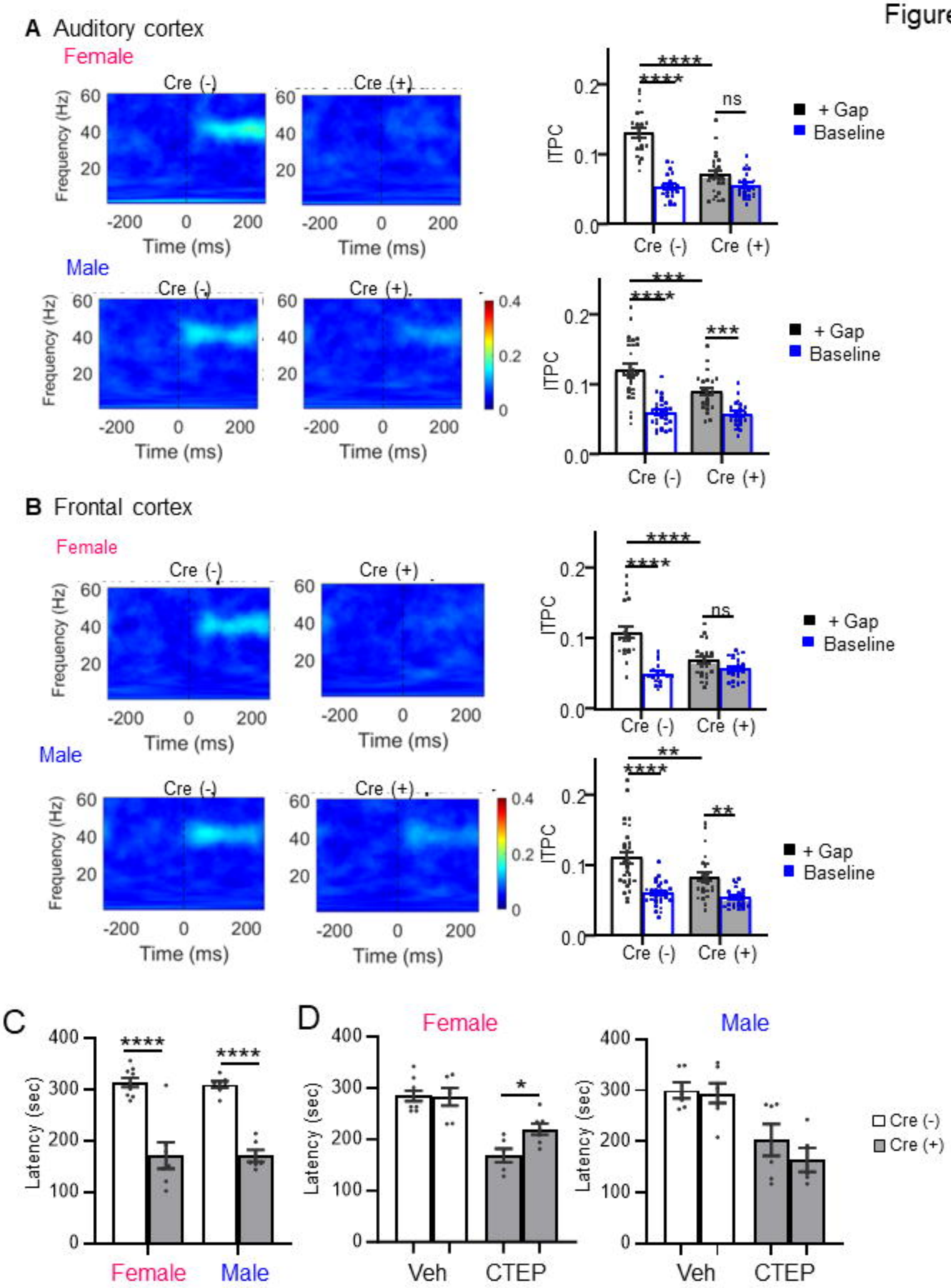
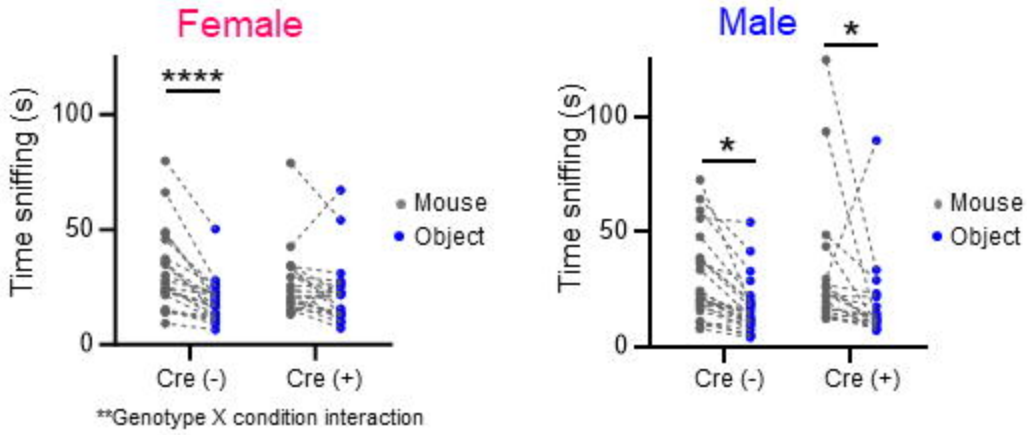
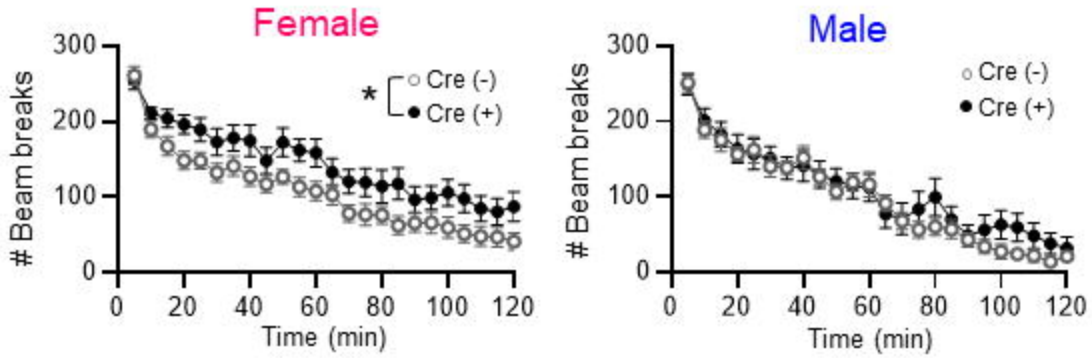

A Auditory cortex
Female
Cre (-)
Cre (+)
Frequency (Hz)
Time (ms)
ITPC
+ Gap
Baseline
ns
Male
B Frontal cortex
C
Latency (sec)
Female
Male
D
Veh
CTEP
Cre (-)
Cre (+)

Figure 7

A Social Interaction



B Locomotor activity



C Fear conditioning

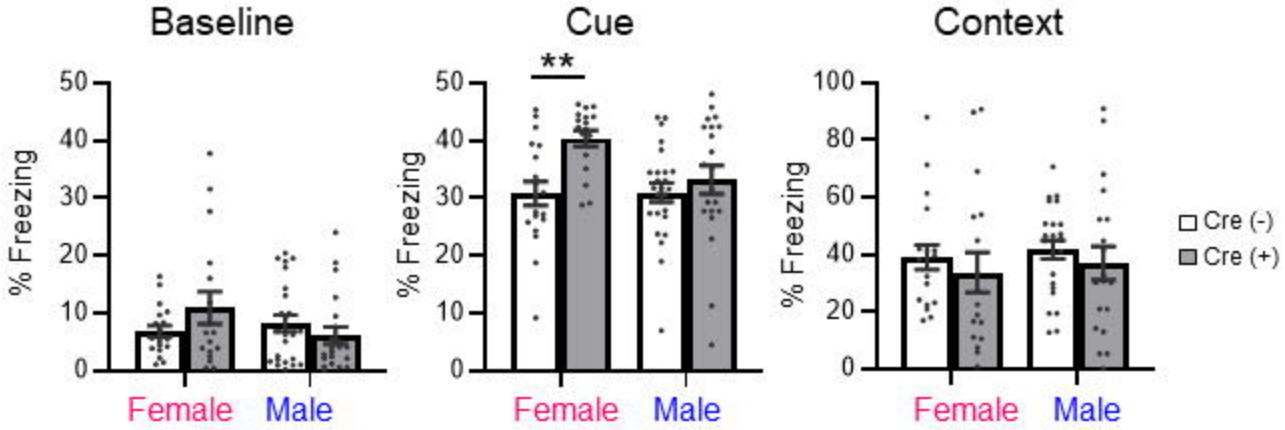
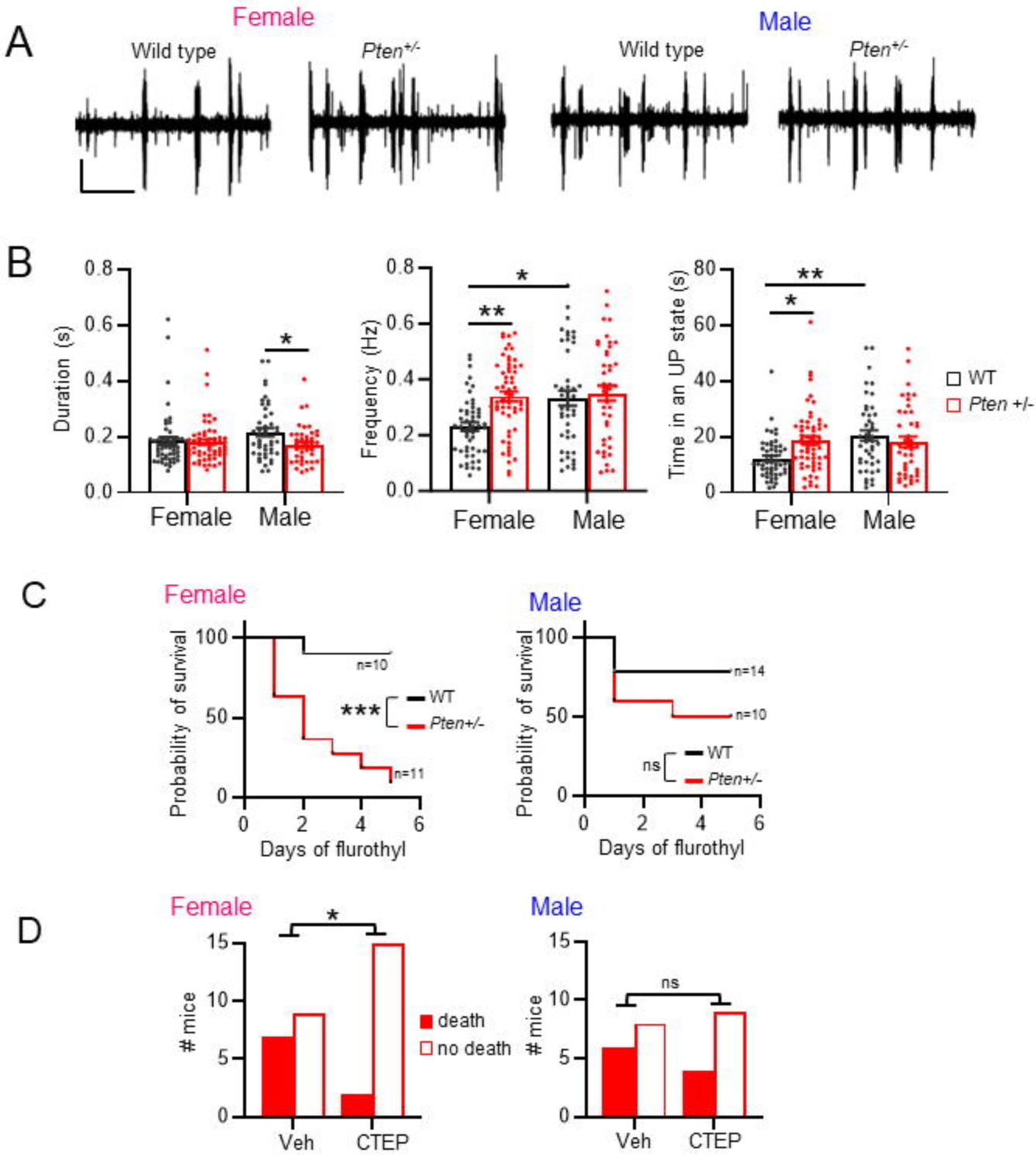


Figure 8


A
Female
Wild type
Pten+/-
Male
Wild type
Pten+/-
B
Duration (s)
0.8
0.6
0.4
0.2
0.0
*
Female
Male
Frequency (Hz)
0.8
0.6
0.4
0.2
0.0
*
**
Female
Male
Time in an UP state (s)
80
60
40
20
0
**
*
Female
Male
WT
Pten +/-
C
Female
Probability of survival
100
50
0
n=10
***
WT
Pten+/-
n=11
0
2
4
6
Days of flurothyl
Male
Probability of survival
100
50
0
n=14
n=10
ns
WT
Pten+/-
0
2
4
6
Days of flurothyl
D
Female
*
mice
15
10
5
0
death
no death
Veh
CTEP
Male
ns
mice
15
10
5
0
Veh
CTEP