Germline Nuclear-predominant Pten Murine Model Exhibits Impaired Social and Perseverative Behavior, Microglial Activation, and Increased Oxytocinergic Activity

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Research

Keywords: PTEN mutation, autism spectrum disorder, mouse model, social impairment, microglia, oxytocin

DOI: https://doi.org/10.21203/rs.3.rs-97746/v1

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Abstract

Background

Autism spectrum disorder (ASD) has a strong genetic etiology. Germline mutation in the tumor suppressor gene \textit{PTEN} is one of the best described monogenic risk cases for ASD. Animal modeling of cell-specific \textit{Pten} loss or mutation has provided insight into how disruptions to the function of PTEN affect neurodevelopment, neurobiology, and social behavior. As such, there is a growing need to understand more about how various aspects of PTEN activity, cell-compartment-specific functions, contribute to certain neurological or behavior phenotypes.

Methods

To understand more about the relationship between Pten localization and downstream effects on neurophenotypes, we generated the nuclear-predominant \textit{Pten}^{Y68H/+} mouse. We subjected the \textit{Pten}^{Y68H/+} mouse to morphological and behavioral phenotyping, including the three-chamber sociability and marble burying tests. We subsequently performed \textit{in vivo} and \textit{in vitro} cellular phenotyping and concluded the work with a transcriptomic survey of the \textit{Pten}^{Y68H/+} cortex, which profiled gene expression.

Results

Despite no significant changes in downstream canonical Pten signaling, we found that the \textit{Pten}^{Y68H/+} mouse presents with macrocephaly, social impairment (i.e., decreased sociability, decreased preference for novel social stimuli, and increased perseverative activity), with significant microglial activation accompanied by enhanced phagocytosis. Because of lack of canonical signaling alterations, we turned to analyzing the neural transcriptomes, which revealed overexpression of many genes involved in neuroinflammation and neuronal function, including oxytocin. Oxytocin transcript was 5-fold overexpressed ($P = 0.0018$) and oxytocin protein was strongly overexpressed in the \textit{Pten}^{Y68H/+} hypothalamus.

Conclusions

The nuclear-predominant \textit{Pten}^{Y68H/+} model has clarified that Pten dysfunction links to microglial pathology and that timed decreased in Pten levels is the provoking insult. Notably, we demonstrate that Pten dysfunction associates with changes in the oxytocin system, an important connection between a prominent ASD risk gene and a potent neuroendocrine regulator of social behavior. Ultimately, the findings from this work may reveal important biomarkers and/or novel therapeutic modalities that could be explored in individuals with germline mutations in \textit{PTEN} with ASD.

Background

It is well established that germline mutations in \textit{PTEN} predispose individuals to autism spectrum disorder (ASD) and ranks among the most common monogenic etiologies [1–13]. We and others have shown that 7–27% of individuals with ASD and concurrent macrocephaly harbor germline mutations in \textit{PTEN}, which
when extrapolated across all cases of ASD may account for 0.5–5% of those cases [5,7,13–16]. In addition to ASD, germline PTEN mutations cause subsets of Cowden syndrome (CS, OMIM #158350), Bannayan-Riley Ruvalcaba Syndrome (BRRS), Proteus syndrome (OMIM #176920), and Proteus-like syndrome [17]. Irrespective of clinical syndrome and pathophysiology, anyone diagnosed with a germline PTEN mutation carries the molecular diagnosis of PTEN Hamartoma Tumor Syndrome (PHTS, OMIM #601728) [17,18].

PTEN has been well characterized as a tumor suppressor gene that removes the 3’ phosphate group from phosphatidylinositol(3,4,5)-triphosphate (PIP3), thereby inhibiting the PI3K/AKT/mTOR signaling pathway, a major growth, survival, and migration pathway [17,19,20]. Beyond this canonical PTEN function, there is a growing body of research exploring the protein phosphatase and non-catalytic activities of PTEN [21,22]. The subcellular localization of PTEN and its importance to neurological phenotypes is of special interest. Recent work suggests that missense mutations in PTEN that disrupt subcellular localization may be more correlated with neurological phenotypes, specifically those associated with ASD-like behaviors [23–25]. Moreover, it has been observed that missense mutation versus other types of mutation are enriched among individuals with ASD [26–29]. However, there are still outstanding questions about the exact impact of PTEN localization on neurological structure and function.

In order to interrogate the effects of PTEN mislocalization, we developed two complementary mouse models of germline Pten mutation. One model exhibits cytoplasmic-predominant expression of Pten, the Pten<sup>m3m4</sup> model, while the other exhibits nuclear-predominant expression of Pten, the Pten<sup>Y68H</sup> model. In the cytoplasmic-predominant model, we found no significant morphological or behavioral changes in Pten<sup>m3m4/+</sup> mice, but in the homozygous Pten<sup>m3m4/m3m4</sup> mice we observed dramatic macrocephaly and a sex-dependent increase in social motivation with severe deficits in motor coordination [23]. Additionally, we performed extensive cellular phenotyping on the Pten<sup>m3m4</sup> model, finding hypertrophy of neuronal somas, astrogliosis, dysmyelination, stunted maturation of neural stem cells (NSCs), precocious differentiation of oligodendrocyte progenitor cells (OPCs), and microgliosis, specifically cell-autonomous microglial activation and increased phagocytic response [23,26,30–32]. Our molecular characterization of the Pten<sup>m3m4</sup> model included an RNA sequencing experiment that found that the neural transcriptome included many genes relevant to human idiopathic ASD [24]. In contrast to the cytoplasmic-predominant model, the nuclear-predominant Pten mutant has never been characterized or subject to study. The Y68H mutation, which induces nuclear-predominant Pten expression, especially in metabolically stressed cells [33,34], is likely to affect neurological function as it is a mutation that has been identified in PHTS patients diagnosed with ASD. Therefore, we hypothesize that the nuclear-predominant Y68H mutation in Pten contributes to deficiencies in social behavior as well as pathological changes in neuronal and glial function, but perhaps via a different mechanism.

**Methods**

**Animals**

We generated Pten<sup>Y68H/+</sup> mice on a C57BL/6J (Jackson Laboratory, Bar Harbor, Milwaukee) background by introducing one missense mutation into exon three of the mouse Pten gene, specifically Pten c.202 T>C, via
standard cre-lox methodology (Fig. 1A). This mutation targets the sequence analogous to the ATP-binding motif B found in human PTEN[33,34]. Mice were backcrossed onto C57BL/6J (Jackson Laboratory) inbred strain for five generations to reduce the impact of genetic heterogeneity on the results. Genotyping was performed on genomic DNA from clipped toes per the Jackson Laboratory protocol using modified PCR primers: Y68H F1, 5’-GTTCACAGCTGTTAGG -3’, and Y68H R1, 5’-TGTACCCAGTCACAGACTTCC -3’. Mice were maintained on a 14:10 light: dark cycle with access to food and water \textit{ad libitum}. The room temperature was maintained between 18 and 26°C. Animals were euthanized via CO$_2$ asphyxiation followed by cervical dislocation. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Cleveland Clinic. Additionally, for all experiments described, we utilized only male mice for our experiments.

**Western blot analysis**

Cortical regions of the brain were isolated, snap-frozen, and stored at -80°C. Tissue was thawed on ice and lysed in RIPA buffer (10 mM Tris-Cl [pH 8], 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl), containing phosphatase inhibitor #2 (Sigma, St. Louis, Missouri, #P5726-5ML), phosphatase inhibitor #3 (Sigma, #P0044-5ML), and protease inhibitor (Sigma, #P8345-5ML). Lysates were quantified for protein concentration using bicinchoninic acid assay (BCA) assay, equalized to a concentration of 1 µg/µl of protein per sample, and finally 20 µg of protein was loaded to a 4-15% gradient polyacrylamide gel for SDS-PAGE separation. The separated proteins were transferred to a nitrocellulose membrane and blocked overnight at 4°C in 3% bovine serum albumin (BSA) in 1X Tris-buffered saline, containing 0.2% Tween-20 (TBST). Membranes were then washed with TBST and incubated with experiment-specific primary antibodies diluted in bovine serum albumin (BSA) overnight at 4°C. The following antibodies were used: PTEN (1:5000, #ABM-2025, Cascade Bioscience, Winchester, Massachusetts), IBA1 (1:500, #019-19741, Wako, Bellwood, Virginia), MBP (1:1000, MAB386, EMD Millipore, Burlington, Massachusetts), PLP (1:1000, ab28486, Abcam, Cambridge, Massachusetts), GAPDH (1:5000, 2118L, Cell Signaling, Danvers, Massachusetts), HSP90 (1:1000, 4874, Cell Signaling), Lamin A/C (1:1000, 2032, Cell Signaling), Beta-actin (1:500, AM4302, Thermo-Fisher, Waltham, Massachusetts), phospho-AKT Ser473 (1:1000, 9271, Cell Signaling), AKT (1:1000, 4691, Cell Signaling), phospho-ERK1/2 (1:1000, 9101, Cell Signaling), ERK1/2 (1:1000, 9102, Cell Signaling), phospho-S6 (1:1000, 4858S, Cell Signaling), and C1q (1:500, ab71940, Abcam). We removed the primary antibody solution and performed three washes, 10 minutes per wash, with TBST. Blots were probed with goat anti-mouse secondary antibody IRDye800CW (1:20,000, #213965, LI-COR, Lincoln, Nebraska) or goat anti-rabbit IRDye680 (1:20,000, #213971, LI-COR) diluted in BSA, for two hours at room temperature. The membranes were washed three times, 10 minutes each in TBST, and imaged using the Odyssey CLx imaging system (LI-COR). Using ImageJ (National Institute of Health, Bethesda, Maryland, 1995), we performed densitometry analysis on these images to quantify protein expression.

**Behavior Testing**

To assess changes in social behavior, we employed the three-chamber sociability test according to a previously reported protocol [23,35]. Mice were placed in a center chamber for five minutes and then
returned to their original cage. Next, the assessment consisted of a 10-minute trial, where the test mouse was returned to the central chamber and given a choice between two identical containers, one chamber containing a mouse, and the other an empty chamber. In order to measure preference for social novelty, the 10-minute trial was repeated two days later with a familiar mouse in one chamber and a novel mouse in the other. Time spent in each chamber and time spent in close contact with the containers were recorded and quantified using Noldus EthoVision software (Wageningen, Netherlands).

To assess repetitive behavior, we administered the marble burying test to our mice per a previously published protocol [36]. This trial is performed by placing 20 marbles atop clean bedding material and placing the trial mouse into the case for a 30-minute session. Upon completion of the trial the number of buried and non-buried marbles was scored.

**Primary microglia cell culture**

Mixed glia were obtained by trypsinization of postnatal day 2 (P2) cortices followed by plating on poly-D-lysine coated T-75 culture flasks. Mixed glia cultures, were maintained in DMEM (Lerner Research Institute Media Core, Cleveland, OH) with 10% FBS and 1% Penicillin and Streptomycin (Pen/Strep). Once the mixed glia cultures reached confluency at approximately DIV 10, they were agitated for one hour at 170 RPM. At this point, the supernatant was removed and spun down at 1200 RMP in order to isolate primary microglia. Isolated microglia were resuspended in DMEM with 10% FBS and 1% Pen/Strep and seeded on poly-D-lysine coated glass cover slips subsequently used for immunofluorescence staining and phagocytic assays at DIV 3 post-shaking.

**Phagocytosis assay**

We plated primary microglia at a density of $1 \times 10^5$ in a 12-well dish with PDL-coated coverslips for 48 hours in a 37°C cell incubator with 5% CO$_2$ and 100% humidity. Next, we blocked 1 μm fluorescent beads (Sigma-Aldrich, #L1030) in FBS for one hour at 37°C at a ratio of 1:5 v/v. Fluorescent beads were diluted with DMEM to reach a final concentration of 0.01% (v/v). Microglial culture media was replaced with 250 μl DMEM containing beads, and incubated for one hour at 37°C in a cell incubator. Cultures were washed thoroughly five times with ice-cold PBS (Lerner Research Institute Media Core) and fixed in ice-cold methanol prior to immunofluorescent staining for Iba1 (1:500, #019-19741, Wako).

**Immunofluorescence staining of brain tissue**

Mice were euthanized and perfused with approximately 50 ml of 1X PBS. Brain tissue was then extracted and fixed in 4% PFA (pH = 7) for 24 hours at 4°C. Brains were then washed three times with PBS and cryoprotected in 30% sucrose dissolved in PBS for 94 hours at 4°C. Frozen brain sections were cut coronally to a width 10 μm on a cryostat and mounted on polarized glass slides (Fisherbrand Superfrost Plus microscope slides, #12-550-15, Fisher Scientific, Waltham, MA). OCT was removed by washing slides in PBS for 10 minutes and tissue was permeabilized with 3% Triton-X dissolved in PBS for 10 minutes. Slides were next washed three times for five minutes each in PBS and probed with experiment specific primary antibodies: Iba1 (1:500, #019-19741, Wako), Plp (1:1000, ab28486, Abcam), NeuN (1:250, MAB377, EMD
Millipore), Olig2 (1:250, ab9610, Abcam), S100b (1:200, ab52642, Abcam), Gfap (1:250, sc-33673, Santa
cruz), Oxt (1:250, ab212193, Abcam), Pten (1:5000, #ABM-2025, Cascade Bioscience) and incubated
overnight at 4°C. The following day, slides were washed with PBS for three times five minutes each. This
was followed by incubation with secondary antibody for two hours: goat anti-mouse Alexa Fluor 568
(1:2000, #A11031, Thermo-Fisher) and goat anti-rabbit Alexa Fluor 488 (1:2000, #A11008, Thermo-Fisher).
Post incubation, slides were washed and mounted in Vectashield medium with DAPI (Vector Laboratories,
Burlingame, CA), coverslipped, and sealed with nail polish.

**In vitro immunofluorescence staining**

We cultured primary microglia on poly-D-lysine (PDL)-coated cover slips until DIV 14. Microglia were
washed with ice-cold PBS and fixed in ice-cold methanol for two minutes. This was followed by three
washes for five minutes each with ice-cold PBS. We then permeabilized the microglia with 0.03% Triton X-
100 dissolved in PBS for four minutes. Next, cells were blocked with 10% normal goat serum for one hour at
room temperature, followed by incubation with primary antibody Iba1 (1:500, #019-19741, Wako) diluted in
10% normal goat serum in PBS. Cells were then incubated in primary antibody overnight at 4°C. The
following day cells were washed with PBS three times for five minutes and secondary was added, goat anti-
mouse Alexa Fluor 568 secondary antibody (1:2000, #A11031, Thermo-Fisher) diluted in 10% normal goat
serum in PBS. The cells were incubated in secondary antibody for two hours at room temperature, washed
with PBS three times for five minutes, and coverslipped with Vectashield medium with DAPI (Vector
Laboratories).

**Immunofluorescence quantification**

We captured images of brain sections and primary microglia as confocal images using a Leica TCS-SP8-
AOBS inverted confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany). Brain sections and
microglia cultures were imaged with a minimum of N = 3 biological replicates. ImageJ software was used
to measure area and intensity of the stain and calculated integrated density of brain images. Additionally,
ImageJ was used to measure area of stain per microglia *in vivo* to assess morphological changes.

**Transcriptomic data analysis**

We isolated total RNA from the cortex of eight *Pten* Y68H/+ mice and seven *Pten* */+* mice. Aliquots of roughly
60 ng/μL total RNA (average RIN score = 9.1; Additional file 1: Table S1) were prepared (TruSeq Stranded
Total RNA – RiboZero Gold, Illumina, San Diego, CA) and then sequenced using an Illumina NOVA-Seq. The
resulting Fastq sequences were subject to standard processing and quality control (QC) evaluation, using
MultiQC v1.9 ([https://multiqc.info/](https://multiqc.info/)). Then, we performed an alignment to a the mouse reference genome
(mm10) using Spliced Transcripts Alignment to a Reference (STAR) 2.7.5
([https://github.com/alexdobin/STAR](https://github.com/alexdobin/STAR)) [37–39] and repeated a quality control evaluation using MultiQC
v1.9. One *Pten* */+* sample and three *Pten* Y68H/+ samples were discarded due to a high proportion of
repetitive sequences and generally poor alignment statistics (Additional file 1: Fig S1). Additionally, we used
Salmon 1.8.0 ([https://bioconductor.org/packages/release/workflows/html/rnaseqDTU.html](https://bioconductor.org/packages/release/workflows/html/rnaseqDTU.html)) as an
alternative method to count reads mapping to a present index of known cDNA transcripts. Subsequently, we
performed DeSeq2 1.28.1 on STAR-aligned counts and Salmon-produced counts to assess differential expression (DE). These two methods were used to ensure concordance between both approaches. Genes experiencing DE were analyzed in RStudio 1.2.5001 using R 4.0.0 to construct volcano plots and heatmaps. Generally, a p-value ($P < 0.05$), fold change ($\log_2(\text{Fold Change}) \geq 1.0$ or $\log_2(\text{Fold Change}) \leq -1.0$), and count (RPKM > 10) thresholds were used for these analyses. In order to assess the biological impact of the DE results, we used STRING (https://string-db.org/) and Ingenuity Pathway Analysis (Qiagen, Redwood City, California) software.

**Statistical analysis**

We analyzed normally distributed data using a one-way analysis of variance (ANOVA) or Student’s t-test, where appropriate (GraphPad Prism 8). After performing a one-way ANOVA (F), we performed a post-hoc Tukey-Kramer analysis. When data were not normally distributed, we performed non-parametric analyses including Mann-Whitney U and Kruskall-Wallis tests (H), where appropriate (Graph Pad Prism 8). P-values that are less than 0.05 were considered statistically significant.

**Results**

$Pten^{Y68H/+}$ mice exhibit increased nuclear Pten localization and increased brain mass

We originally observed the $PTEN^{Y68H}$ mutation in PHTS individuals diagnosed with ASD and found that this particular mutation was sufficient to disrupt the subcellular partitioning of PTEN protein, resulting in relatively predominant nuclear localization [34]. Furthermore, we generated the $Pten^{Y68H/+}$ mouse model by introducing a single missense mutation into exon three of mouse $Pten$ (i.e., $Pten$ c.202 T>C), thus converting tyrosine residue 68 into histidine (Fig. 1a). To assess the subcellular localization of Pten in our $Pten^{Y68H/+}$ mouse, we performed nuclear-cytoplasmic fractionation of cortical tissue from six-month-old mice and assessed protein localization via Western blot (Fig. 1b). We observed a decrease by visual inspection in Pten in the cytoplasmic fraction of $Pten^{Y68H/+}$ hemibrain relative to $Pten^{+/+}$ (N = 4 mouse/genotype, Fig. 1b). Additionally, quantitative assessment showed that the ratio of nuclear-to-cytoplasmic Pten is increased in the $Pten^{Y68H/+}$ mice compared to $Pten^{+/+}$ (Median$_{\Delta N/CRatio} = 0.20$; 97% CI: 0.15 - 0.34; $P = 0.029$; Fig. 1c). Next, we performed immunofluorescence staining for Pten in the brains of six-month-old $Pten^{Y68H/+}$ mice, which also indicated an increase in the nuclear-to-cytoplasmic ratio of Pten expression by visual inspection (Fig. 1d). These observations are consistent with our Western data (Fig. 1b), both of which show enrichment of nuclear Pten expression relative to cytoplasmic Pten expression in the $Pten^{Y68H/+}$ brain (Fig. 1d).

Macrocephaly is a hallmark of PHTS individuals and all ASD in this setting is accompanied by macrocephly [29,40,41]; therefore to determine if a similar overgrowth phenotype exists in $Pten^{Y68H/+}$ mice, we performed a gross examination of $Pten^{Y68H/+}$ brains at six-months-of-age. We found a significant increase in brain mass as measured in grams in $Pten^{Y68H/+}$ mice compared to $Pten^{+/+}$ littermate controls.
(Median$_{\text{ΔBrainMass}} = 0.15; 97\% \text{ CI}: 0.090 - 0.21; P < 0.0001) with no change in overall body weight (Fig. 1f). These differences in brain mass are also apparent by visual inspection and displayed in Fig. 1e.

**Pten$^{Y68H/+}$ mutant mice exhibit decreased sociability and repetitive behavior**

Next, we sought to examine the possibility that nuclear-predominant Pten expression in the central nervous system (CNS) may alter social behavior. Several studies have demonstrated that Pten loss, whether constitutional or conditional to the CNS, can have deleterious consequences on social behavior, anxiety, learning, memory, and/or repetitive behavior; phenotypes that are associated with ASD in humans [16,29]. In order to assess if there were any changes in sociability at six-week-old Pten$^{Y68H/+}$ mice compared to Pten$^{+/+}$ littermate controls, we employed the three-chamber test (Fig. 1g). We found that Pten$^{Y68H/+}$ mice spent less time in the chamber containing the social target than the empty chamber (Mean$_{\Delta\text{Seconds}} = 162; 95\% \text{ CI}: 121 - 203; P < 0.0001; N_{+/+} = 6; N_{Y68H/+} = 6; \text{Fig. 1h})$. Then, utilizing the same three-chamber test model, we assessed changes in preference for social novelty by placing a familiar mouse in one chamber, and a novel social target in the other. We found a significant shift for Pten$^{Y68H/+}$ mice toward a reduced preference for a novel versus familiar social target compared to Pten$^{+/+}$ littermate controls (Mean$_{\Delta\text{DSeconds}} = 101; 95\% \text{ CI}: 56.5 - 145; P < 0.0001; N_{+/+} = 6; N_{Y68H/+} = 6; \text{Fig. 1i})$. In order to assess repetitive behavior, since it is defined as one of the two core behavioral domains of ASD [42], we performed the marble burying test with six-week-old Pten$^{Y68H/+}$ mice. We found that the cages of Pten$^{Y68H/+}$ mice had more marbles buried compared to Pten$^{+/+}$ controls (Median$_{\Delta\text{Marbles}} = 4.68; 97\% \text{ CI}: 2.12 - 7.23; P = 0.002) and increased displacement of the marbles from their original positions compared to the cages of wildtype mice as assessed by visual inspection (Fig. 1j, k). In addition, the appearance of bedding surface in the cages of Pten$^{Y68H/+}$ mice was indicative of repetitive burying and digging behavior (Fig.1j). All together these behavioral data demonstrate that Pten$^{Y68H/+}$ mice exhibit decreased sociability and increased repetitive behavior, fitting the two core behavioral domains of ASD.

**Microglial activation in the brains of Pten$^{Y68H/+}$ mice**

It has been well established that disrupted Pten expression in mice can lead to increased cellular proliferation, white matter abnormality, astrogliosis, and microglial activation in vivo [16,23,30,31]. Therefore, we sought to determine if there were any clear cellular pathologies in the brains of six-month-old Pten$^{Y68H/+}$ mice, using immunofluorescence staining and Western blotting for markers specific to neurons, oligodendrocytes, astrocytes, and microglia. To our surprise we did not observe any significant abnormalities in gross white matter nor in the populations of oligodendrocytes, astrocytes, or neurons with respect to proliferation or activity (Additional file 1: Fig S2A-H); however, there was a trend showing a slight decline in cell populations of oligodendrocytes and astrocytes in Pten$^{Y68H/+}$ mice compared to Pten$^{+/+}$ controls (Additional file 1: Fig S2B-D).

Despite the unexpected observations made regarding neurons, astrocytes, and oligodendrocytes in the cortex of six-month-old Pten$^{Y68H/+}$ mice, we did observe clear microglial activation in the cortex. We stained microglia for ionized calcium binding adapter 1 (Iba1) in the cortex of six-month-old Pten$^{Y68H/+}$ mice and
observed increased Iba1-positive cells and morphological changes indicative of microglial activation, such as increased cell area (Fig. 2a, b). Thus, we quantified the cell area of individual microglia in \( Pten^{Y68H/+} \) \textit{in vivo} and found a significant increase in the cell area of these microglia (Mean\( _{\Delta\text{CellArea}} = 0.29; \) 95% CI: 0.12 - 0.46; \( P = 0.002; \) Fig. 2c). Moreover, we measured the integrated density of the Iba1 stain and found it to be significantly increased in \( Pten^{Y68H/+} \) compared to \( Pten^{+/+} \) microglia (Mean\( _{\Delta\text{IntDensity}} = 0.37; \) 95% CI: 0.22 - 1.5; \( P = 0.025; \) Fig. 2d).

Next, we validated \( Pten^{Y68H/+} \) microglial activation by quantifying microglia-specific proteins via Western blot. We found a significant increase in Iba1 (\( P = 0.029 \)) and C1q (\( P = 0.029 \)), a secreted complement component, expression; both proteins are only expressed by microglia in the CNS, implicated in synaptic pruning, and involved in phagosome formation (Fig. 2f, g). These data suggest not only that microglia are activated, but also that they may have increased phagocytic capabilities. Together, these data indicate that alterations in Pten localization and expression via the \( Pten^{Y68H} \) mutation leave astrocyte and oligodendrocyte populations unaffected while contributing to a microglial pathology.

**\( Pten^{Y68H/+} \) microglia have increased phagocytic ability and efficiency \textit{in vitro}**

To assess phagocytosis in \( Pten^{Y68H/+} \) microglia, we performed a phagocytosis assay and found the total number of \( Pten^{Y68H/+} \) phagocytic microglia was significantly higher than the number of microglia isolated from \( Pten^{+/+} \) littermate controls (Mean\( _{\Delta} = 0.16; \) 95% CI: 0.040 - 0.27; \( P = 0.01; \) Fig. 2h, i). In addition, phagocytic \( Pten^{Y68H/+} \) microglia were able to engulf more fluorescent beads compared to \( Pten^{+/+} \) littermate controls (Mean\( _{\Delta} = 3.2; \) 95% CI: 2.5 - 4.0; \( P = 0.005; \) Fig. 2j). These data not only demonstrate that \( Pten^{Y68H/+} \) microglia have increased phagocytic ability and efficiency compared to wildtype controls, but also that this pathology arises cell-autonomously as these cellular phenotypes are observed in primary microglial cultures devoid of external influences from neurons, oligodendrocytes, or astrocytes.

**\( Pten^{Y68H/+} \) mutants do not exhibit activation of Akt, Erk1/2, and S6 in the cortex at six-months-of-age**

Next, we wished to determine if the changes in macrocephaly, behavior, microglial activation, and increased phagocytosis were associated with changes in downstream canonical Pten signaling, namely, Pi3k/Akt/mTor activation. It has been previously reported and well established, including in other Pten mouse models, that disruptions of Pten expression and protein stability can lead to increased downstream phosphorylation and activation of S6, Akt, and Erk1/2 [17,20,23]. Therefore, we used six-month-old cortical lysates and performed Western blot analyses for Pten, Akt, P-Akt (S473), Erk1/2, P-Erk1/2, S6, and P-S6. We found a significant decrease in Pten expression, which is consistent with previous reports regarding human PTEN [33,34,43]. However, we did not observe any significant differences in the phosphorylation of Akt, Erk1/2, or S6, though there was a trend toward increased phosphorylation of Akt and S6 (Additional file 1: Fig S3A-E). To our surprise, these data show that the pathologies found in \( Pten^{Y68H/+} \) mutants are not necessarily associated with global changes in the classic signaling cascades downstream of Pten. Therefore, we sought to determine if nuclear-predominant Pten results in downstream transcriptome-wide changes, which might at least in part explain our behavior and cellular observations \textit{in vivo} and \textit{in vitro}. 
Transcriptomic characterization of the $Pten^{Y68H/+}$ cortex

Given the surprising lack of disruption of canonical signaling downstream of Pten in the context of the striking behavioral and cellular findings, we performed a transcriptomic survey of the cortex of young adult (six-week-old or P40), male mice ($N_{+/+} = 6$; $N_{Y68H/+} = 5$). RNA-sequencing analysis of cortical RNA identified 332 differentially expressed genes (threshold: $P < 0.05$; $\log_2$(Fold Change) $\geq 1.0$ or $\log_2$(Fold Change) $\leq -1.0$; Additional file 2), which are summarized in a volcano plot (Fig. 3a). The volcano plot also illustrates a skew toward overexpression with relatively fewer under expressed genes being observed. Moreover, the changes in gene expression are visualized in a heatmap (threshold: $P < 0.001$), showing a clear separation between genotypes with a general pattern of increased expression in the heterozygous mutant and decreased expression in the wildtype (Fig. 3b).

To gain insight into the biology affected by the expression changes observed in the $Pten^{Y68H/+}$ cortex, we performed Ingenuity Pathway Analysis (IPA), which identified the top “canonical pathways” that show enrichment beyond random chance based on the input gene list, i.e. the genes showing differential expression (threshold: $P$<0.05; $\log_2$(Fold Change) $\geq 1.0$ or $\log_2$(Fold Change) $\leq -1.0$). The top ten pathways are all related to cellular stress and inflammation signaling (Fig. 3c). This signature is driven by the differential expression of $Card10$, $Il1r1$, $Ngfr$, $Tcf7l2$, and $Ttr$, where $Il1r1$, the interleukin 1 receptor type 1 gene, appears in the associated lists of 90% of the pathways (Fig. 3c). The top network showing how the differentially expressed molecules are biologically related implicates $Tcf7l2$ as an important regulatory node given that it has the highest degree centrality (i.e., 12) in the network (Fig 3d). Furthermore, using STRING analysis, an important gene-gene association network was identified from among the differentially expressed genes, implicating oxytocinergic signaling. Differential expression analysis found a roughly five-fold increase in oxytocin ($Oxt$), and network analysis of associated genes showing DE found a small network where $Oxt$ has the highest degree and betweenness centrality (Fig 3e; Additional file 2).

Given the importance of oxytocin signaling to social behavior, we sought to understand more about the possible biological effects of increased expression of $Oxt$. Thus, we deployed IPA’s Molecule Activity Predictor (MAP) to understand how the five-fold increase in $Oxt$ may affect downstream interactors. From the top 10 molecules directly downstream of $Oxt$, we found that increased $Oxt$ expression predicts an increase in dopamine, calcium, Prkcz, and Egfr activity and a decrease in Crh and Fos activity (Fig. 3f).

Finally, we sought to confirm the increase in $Oxt$ expression in the brain of $Pten^{Y68H/+}$ mice so we performed immunofluorescence staining for Oxt in six-week-old hypothalamus. By visual inspection alone, we found a dramatic increase in Oxt expression in the paraventricular neurons (PVN) of the hypothalamus of six-week-old $Pten^{Y68H/+}$ mice ($N = 3$; Fig. 4a). We then quantified Oxt expression per individual PVN cell soma and found significantly increased Oxt expression in $Pten^{Y68H/+}$ compared to wildtype controls (Mean$_{\Delta IntDensity}$ = 6630; 95% CI: 4860 – 8400; $P < 0.0001$; Fig 4b). In addition, we plotted the average global expression of Oxt per biological replicate to show these data were not skewed (Median$_{\Delta IntDensity}$ = 7140; 97% CI: 3700 – 10600; $P = 0.0045$; Fig. 4c). Given these data, it is clear nuclear-predominant Pten, $Pten^{Y68H}$, associates with increased Oxt expression in the brain.
Discussion

In this study, we demonstrate that the nuclear-predominant $Pten^{Y68H}$ mutation in mice results in decreased social activity, decreased interest in novel social stimuli, increased perseverative behavior, increased microglial activation, and increased neural oxytocin levels. We show that in addition to deficits in social behavior, $Pten^{Y68H/+}$ mice have macrocephaly from increased brain mass (Fig. 1). Moreover, we show that $Pten^{Y68H/+}$ microglia are activated in vivo, expressing elevated amounts of C1q and displaying enhanced phagocytic activity (Fig. 2). Despite clear organismal and cellular phenotypes, we found that $Pten^{Y68H/+}$ brains do not exhibit disruption of canonical signaling downstream of Pten. Therefore, we resorted to interrogating the biology globally. As such, our transcriptomic survey of the cortex of $Pten^{Y68H/+}$ mice identified broad changes in gene expression much of which implicated neuroinflammatory or other neurological pathways, including the striking finding of increased oxytocin expression (Fig. 3). The oxytocin expression finding was confirmed at the protein level via staining of the hypothalamus (Fig. 4). Together these data implicate steady-state Pten levels as an important cell-autonomous regulator of microglial morphology and activity and suggest that a shift toward nuclear-predominant Pten expression provokes disruption of the oxytocin system in the brain.

Murine models of increasing specificity have established that Pten and related downstream signaling participates in the regulation of social behavior, CNS morphology, and neuronal and glial function [23,25,44–61]. Consistent behavioral phenotypes of altered social behavior persist when loss of Pten expression is restricted to mature neurons or neuronal precursors, and these models often have impaired learning/memory, increased anxiety, or altered activity/motor ability [16,44,46,48]. These behavioral abnormalities contrast somewhat with those observed in our germline $Pten^{m3m4}$, cytoplasmic-predominant Pten mislocalization model [23]. $Pten^{m3m4/m3m4}$ mice maintain relatively normal capacities for learning and memory, while showing a sex-specific (i.e., male) increase in social motivation and severely impaired motor coordination [16,23]. $Pten^{Y68H/+}$ mice show decreased sociability and interest in social novelty with increased repetitive behavior, such as marble burying and nesting. It is difficult to assert confidently the sources of variability in the behavioral phenotypes observed in these various Pten mouse models; however, the $Pten^{m3m4/m3m4}$ and $Pten^{Y68H/+}$ models are quite distinct from Pten knock-out or conditional knock-out models in that the mutations are designed to disrupt Pten localization instead of completely eliminating all Pten expression and thus functionality. Moreover, the $Pten^{m3m4}$ and $Pten^{Y68H}$ mutations aim to mimic, or at least simulate, mutations observed in PTEN-ASD individuals, especially the latter mutation. In terms of the behavioral differences between the $Pten^{m3m4/m3m4}$ and $Pten^{Y68H/+}$ models, it is also difficult to confidently ascribe certain behaviors to Pten localization partially because both mutations disrupt Pten stability and phosphatase activity to some extent; for instance, we know that the $Pten^{Y68H}$ mutation likely has a very damaging effect on the stability of Pten [43]. However, it is worth noting that the cytoplasmic versus nuclear localization models appear to associate with contrasting effects on social motivation. It is likely that additional models and elegantly designed experiments will be needed to accurately associate Pten localization changes with specific behavioral changes in mice.
Beyond the behavioral phenotypes, our contrasting Pten models do reveal something about the effects of Pten localization on glial phenotypes. The Pten\textsuperscript{m3m4/m3m4} mouse has aggressive oligodendrocyte and astrocyte pathologies, including increased myelination [23,30], whereas the Pten\textsuperscript{Y68H/+} mouse has no apparent oligodendrocyte or astrocyte pathologies (Additional file 1: Fig S2a-h). However, both the Pten\textsuperscript{m3m4/m3m4} and Pten\textsuperscript{Y68H/+} mice have activated microglia with enhanced phagocytic activity. Comparatively, these findings suggest that the localization affects glial phenotypes, where less nuclear Pten provokes oligodendrocyte and astrocyte pathologies. On the other hand, localization appears to not have an effect on microglia behavior, rather the total steady-state level of Pten appears to regulate microglia function. Both the Pten\textsuperscript{m3m4} and Pten\textsuperscript{Y68H} mutations disrupt Pten stability and both have activated microglia. Glia development and function appear to be tied closely to various aspects of Pten activity, presenting a likely fruitful area of future study.

Possibly the most surprising and important finding of this study is the observation of the lack of disruption of downstream canonical signaling, but instead, increased oxytocin expression in the Pten\textsuperscript{Y68H/+} brain, which occurs in a model with clear behavioral abnormalities. A wealth of research, including extensive animal modeling, demonstrates persuasively that the oxytocin system is important to prosocial cognition [62]. In fact, research has shown that oxytocin may be a viable therapeutic modality for ameliorating social deficits in individuals with ASD [62–65]. Interestingly, many of the models referenced above (e.g., Oxt knockouts) have deficits in the oxytocin system where mice either have lower circulating Oxt or an inability to respond to exogenous Oxt due to knockout (i.e. oxytocin receptor knockout). Our molecular phenotype for oxytocin is quite the opposite. This may seem paradoxical that a mouse with behavioral deficits has an elevated amount of a prosocial neuropeptide. However, it has been shown that high exogenous doses of oxytocin can paradoxically provoke an anxiogenic response due to the excess oxytocin, after oxytocin receptor (OXTR) saturation, acting on vasopressin receptors [65,66]. Moreover, it has also been observed in other models, such as the BTBR mouse, that increased oxytocin expression and social deficits (i.e. increased anxiety) can co-occur [67]. Subsequently, we believe that the increased Oxt expression observed in the RNA-sequencing experiment (Fig. 3) and validated with staining of the PVN (Fig. 4) likely accounts for some of the behavioral abnormalities observed in the Pten\textsuperscript{Y68H/+} mouse. This is important as this is the first time the oxytocin system has been observed as perturbed in a Pten model. Disruption of the oxytocin system may be specific to only a subset of Pten mutations, such as those that cause nuclear mislocalization. More research is required to sort out whether oxytocin system problems are common to all Pten models or only to a specific subset of mutations.

**Limitations**

As suggested above the strengths of our study are founded on the specificity of the mouse modeling (i.e., a knockin mutation identical to that observed in a PTEN-ASD individual) and the rigorous behavioral, cellular, and molecular phenotyping. However, there are limitations to our Pten\textsuperscript{Y68H/+} model, such as the deleterious effect of the Y68H mutation on the stability of Pten [34,43]. The decreased stability of Pten\textsuperscript{Y68H} makes it difficult to absolutely attribute causality of any phenotype to the localization changes observed. Moreover, the localization changes themselves are not absolute either. As clearly shown in Fig. 1b and c, the change in
localization is relative, and there is still plenty Pten in the cytoplasm; though, the extent to which the cytoplasmic Pten is of wildtype or mutant providence is unknown as the germline nature of the model since the $Pten^{Y68H/Y68H}$ genotype exhibits embryonic lethality, therefore requiring the study of only heterozygous mutants. It is unclear if the Y68H mutation can function as a dominant negative mutant as has been described for other $PTEN$ missense mutations [68], but there is some existing evidence to suggest that it does not function as such [28,43,69]. Future work designed to specifically interrogate questions about the functional effects of Pten localization on social and neurobiological phenotypes will have to utilize more extensive modeling strategies. However, the $Pten^{Y68H/+}$ model is extremely useful for gaining important insights into these challenging and important scientific questions about pathophysiology, which should help inform monogenic ASD risk cases.

Conclusion

Although there, of course, remains much to explore about the $Pten^{Y68H/+}$ mouse and other $Pten$ models, especially in terms of the effects on social cognition and neurobiology, this study is an important step toward understanding how Pten localization in the brain can affect social cognition and neuronal and glial function. Until this study, it was unclear if decreased cytoplasmic expression of Pten would affect the CNS in such a dramatic fashion. Our work on the $Pten^{Y68H/+}$ mouse demonstrates that social behavior can be modulated by mutations that shift Pten to the cytoplasm or nucleus, but the exact social phenotypes can be quite distinct. Contrastingly, certain glial phenotypes seem to be in part dependent on Pten localization as the $Pten^{Y68H/+}$ mouse shows no apparent changes in oligodendrocytes and astrocytes (Additional file 1: Fig S2), whereas its complement, the $Pten^{m3m4}$ model, show aggressive glial pathologies [23,30]. However, microglial dysfunction seems to be entirely independent of Pten localization, instead resulting from decreased Pten expression levels in general. In addition, we find prominent oxytocin overexpression in the hypothalamus of $Pten^{Y68H/+}$ mice, thus linking Pten mutation and the oxytocin system for the first time. Finally, an interesting and potentially significant finding is that all of the molecular and cellular pathologies reported in this study appear to be independent of Pten canonical signaling at least at the cortical level in fairly homeostatic conditions (Additional file 1: Fig S3). In sum, this study demonstrates the importance of nuclear Pten to CNS morphology and function while linking Pten, a prominent ASD risk gene, to a neuroendocrine modulator of social behavior, oxytocin, in a murine model with clear social deficits.

Abbreviations

ASD: autism spectrum disorder; central nervous system: CNS; oxytocin: OXT; PTEN: Phosphatase and Tensin Homolog; PTEN Hamartoma Tumor Syndrome: PHTS

Declarations

Acknowledgements
We are grateful to Qi Yu for helping with mouse breeding and husbandry in the Eng lab, and Peter Bazely for bioinformatics core support. CE is the Sondra J. and Stephen R. Hardis Endowed Chair of Cancer Genomic Medicine at the Cleveland Clinic, and is an ACS Clinical Research Professor.

**Authors’ contributions**

Conception and design: NS, ST, HP, CE

Experimentation and data acquisition: NS, ST, HP

Interpretation of data: NS, ST, HP, CE

Drafting of manuscript: ST, NS

Critical revisions of manuscript: ST, NS, CE

Final approval of manuscript: All authors

**Funding**

This study was funded, in part, by the Ambrose Monell Foundation and the Zacconi Program of PTEN Research Excellence.

**Availability of data and materials**

The datasets for this study are available from the corresponding author on reasonable request.

**Ethics approval**

This study was approved by Cleveland Clinic Lerner Research Institute’s IACUC.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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