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Autism-Relevant Behaviors Are Minimally Impacted by Conditional Deletion of *Pten* in Oxytocinergic Neurons

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Abstract

Germline heterozygous mutations in *Pten* (phosphatase and tensin homolog) are associated with macrocephaly and autism spectrum disorders (ASD). *Pten* germline heterozygous ($Pten^{+/-}$) mice approximate these mutations, and both sexes show widespread brain overgrowth and impaired social behavior. Strikingly similar behavior phenotypes have been reported in *oxytocin* (Oxt) and/or *oxytocin receptor* (OxtR) knockout mice. Thus, we hypothesized that the behavioral phenotypes of germline $Pten^{+/-}$ mice may be caused by reduced *Pten* function in Oxt-expressing cells. To investigate this, we tested mice in which *Pten* was conditionally deleted using *oxytocin-Cre* (*Oxt-Cre*$^{+}$*;* $Pten^{loxP/+}$*, Oxt-Cre*$^{+}$*;* $Pten^{loxP/loxP}$) on a battery including assays of social, repetitive, depression-like, and anxiety-like behaviors. Minimal behavioral abnormalities were found; decreased anxiety-like behavior in the open field test in *Oxt-Cre*$^{+}$*;* $Pten^{loxP/loxP}$ males was the only result that phenocopied germline $Pten^{+/-}$ mice. However, Oxt cell size was dramatically increased in *Oxt-Cre*$^{+}$*;* $Pten^{loxP/loxP}$ mice in adulthood. Thus, conditional deletion of *Pten* using *Oxt-Cre* has a profound effect on Oxt cell structure, but not on ASD-relevant behavior. We interpret these results as inconsistent with our starting hypothesis that reduced *Pten* function in Oxt-expressing cells causes the behavioral deficits observed in germline $Pten^{+/-}$ mice.

Keywords

phosphatase and tensin homolog; autism spectrum disorder; oxytocin; social behavior; anxiety-like behavior; hypertrophy

Introduction

Deficits in social behavior and communication are one of the hallmarks of autism spectrum disorder (ASD), along with restricted, repetitive interests and behavior patterns [American Psychiatric Association, 2013; World Health Organization, 1992]. ASD is a neurodevelopmental disorder with high sexual dimorphism (~80% male) present in more than 1% of the population [Perou et al., 2013]. Up to 20% of the subset of individuals with ASD and macrocephaly (head circumference >2 standard deviations above the mean) have mutations in the gene *Pten* (phosphatase and tensin homolog) [Butler et al., 2005; Buxbaum

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Supporting Information

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et al., 2007; Klein, Sharifi-Hannauer, & Martinez-Agosto, 2013; McBride et al., 2010], which encodes a negative regulator of the PI3K–Akt–mTOR pathway [Sulis & Parsons, 2003]. *Pten* haploinsufficiency results in *Pten* hamartoma tumor syndromes, involving benign tumor-like malformations and brain overgrowth, which are often comorbid with ASD [Butler et al., 2005; Buxbaum et al., 2007; Matson & Shoemaker, 2009; Mester, Tilot, Rybicki, Frazier, & Eng, 2011]. The genomic lesions found in individuals with ASD and macrocephaly associated with germline heterozygous *Pten* mutations, which are generally missense mutations that reduce protein levels [Frazier et al., 2015], are approximated in *Pten* germline heterozygous (*Pten*$^{+/-}$) mice. In addition to widespread brain overgrowth [Chen, Huang, Sejourne, Clipperton-Allen, & Page, 2015; Clipperton-Allen & Page, 2014; Page, Kuti, Prestia, & Sur, 2009a], these mice show social behavioral deficits and several sex-specific behavioral phenotypes related to ASD and comorbid disorders, including repetitive behavior, aggression, mood and anxiety phenotypes in males [Clipperton-Allen & Page, 2014, 2015; Page et al., 2009a; Sejourne, Llaneza, Kuti, & Page, 2015].

There are some striking similarities between the observed behavioral phenotypes of germline *Pten*$^{+/-}$ mice and those of *oxytocin* (Oxt) and/or *oxytocin receptor* (OxtR) knockout (KO) mice. Specifically, germline *Pten*$^{+/-}$, OxtKO, and OxtRKO mice all show deficits in social recognition [Clipperton-Allen & Page, 2014; Ferguson et al., 2000; Ferguson, Aldag, Insel, & Young, 2001; Lee, Caldwell, Macbeth, Tolu, & Young, 2008; Macbeth, Lee, Edds, & Young, 2009; Page et al., 2009a; Takayanagi et al., 2005], and are less social than controls [Clipperton-Allen & Page, 2014, 2015; Lazzari et al., 2013; Pobbe et al., 2012a; Pobbe, Pearson, Blanchard, & Blanchard, 2012b]. Germline *Pten*$^{+/-}$ and OxtRKO mice fail to show a social preference in the three chamber social approach test [Amico, Mantella, Vollmer, & Li, 2004; Clipperton-Allen & Page, 2014; Page et al., 2009a; Pobbe et al., 2012a, 2012b; Sala et al., 2011, 2013; Sejourne et al., 2015], while OxtKO and OxtHT mice show normal social interest in this task [Crawley et al., 2007]. Germline *Pten*$^{+/-}$ and OxtKO mice both show increased repetitive behavior [Clipperton-Allen & Page, 2014, 2015; Lazzari et al., 2013], as well as decreased anxiety [Clipper-ton-Allen & Page, 2014; Mantella et al., 2003; Sala et al., 2011, 2013; Winslow et al., 2000]. Additionally, in a free social interaction or resident-intruder test, decreased aggression is observed in germline *Pten*$^{+/-}$ males [Clipper-ton-Allen & Page, 2015] and in OxtKO males born to Oxt heterozygous (OxtHT) females [Lazzari et al., 2013; Nishimori et al., 2008; Takayanagi et al., 2005], while OxtRKO males, and OxtKO males born to OxtKO females, show increased aggression [Dhakar, Rich, Reno, Lee, & Caldwell, 2012; Sala et al., 2011; Takayanagi et al., 2005; Winslow et al., 2000].

On the basis of these results, we hypothesized that the behavioral phenotypes of germline *Pten*$^{+/-}$ mice may reflect a role of *Pten* in the development or function of Oxt-expressing cells. Thus, we mated mice expressing Cre recombinase under control of the *oxytocin* promotor (*Oxt-Cre*) [Wu et al., 2012] with a floxed *Pten* line [Lesche et al., 2002], resulting in mice with conditional homozygous (*Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$) or heterozygous (*Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$) *Pten* mutations in Oxt cells. These mice, and littermate controls, were tested on a battery of behavioral assays, chosen based on observed phenotypes in germline *Pten*$^{+/-}$ mice and those of OxtKO and OxtRKO mice in the literature. These included social (three-chamber social approach and social novelty, social recognition, social aspects of resident-

intruder), repetitive (marble burying, digging measure in resident-intruder), anxiety-like (dark-light emergence, open field), depression-like (tail suspension test) and motor learning (rotarod) tests. Additionally, as *Pten* mutations are known to alter cell number and size [Backman et al., 2001; Chen et al., 2015; Fraser et al., 2004; Goberdhan, Paricio, Goodman, Mlodzik, & Wilson, 1999; Groszer et al., 2001; Kazdoba et al., 2012; Kwon et al., 2001], we assessed the number and size of Oxt cells in the paraventricular nucleus of the hypothalamus (PVN) of mutant mice in adulthood, and developmentally at post-natal day 7 (P7) and P14.

Materials and Methods

Subjects

All mouse lines used have been described previously. B6;129S-*Oxt*$^{tm+.+(cre)Dolsn}$/J (*Oxt-Cre*$^{+}$, https://www.jax.org/strain/024234) [Wu et al., 2012] was generously donated by Drs. Bradford B. Lowell and David P. Olson. This line arrived on a mixed C57BL/6 × 129S background and was backcrossed to C57BL/6J mice for at least five generations in our facility prior to use. The relevant geno-types used in this study were generated by crossing this line with mice carrying B6.129S4-*Pten*$^{tm+Hwu}$/J (*Pten*^{loxP}, https://www.jax.org/strain/006440) [Lesche et al., 2002] and/or B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (*Ai14*$^{+/+}$, https://www.jax.org/strain/007914) [Madisen et al., 2010], both of which were obtained from the Jackson Laboratory, where they were backcrossed and maintained on congenic C57BL/6J backgrounds. PCR using genomic DNA isolated from tail or ear samples was used to confirm mouse genotypes. In all cases, littermate controls (*Oxt-Cre*$^{+}$; *Pten*$^{+/+}$ or *Oxt-Cre*$^{-}$) were used for experiments.

Groups of three to five mice were housed on ventilated racks (Model No. MD75JU160MVPSHR, Allentown Inc., Allentown, NJ) in clear polyethylene cages (19.1 × 29.2 × 12.7 cm; Allentown Inc., Allentown, NJ) and provided with ¼″ corncob bedding, nestlets and food (Teklad Global 18% Protein Extruded Rodent Diet 2920X) and tap water ad libitum.

Behavioral Tests

At least 1 h prior to testing, mice were moved to a holding room in the behavior area. Automatic scoring of assays used the Ethovision XT video tracking system (Noldus, Leesburg, VA), and manual scoring was performed by a trained observer blind to sex and genotype. Unless specified, 70% ethanol (EtOH; Sigma–Aldrich, St. Louis, MO), 1% Micro-90 (International Products Corporation, Burlington, NJ) and/or quatricide (2oz/gallon; Pharmacal Research Laboratories, Inc., Waterbury, CT) were used to clean apparati between mice.

Experimental (*Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ and *Oxt-Cre*$^{+}$; *Pten*$^{loxP/loxP}$) and control (*Oxt-Cre*$^{-}$ and *Oxt-Cre*$^{+}$*; Pten*$^{+/+}$) mice of both sexes, unless specified, were tested in adulthood (P56–P148) during the dark (active) phase of a 12:12 h reversed light/dark cycle (lights on at 2100 h) under red light conditions, unless otherwise stated. Mice were run through a battery of behavioral tests in the following order: (1) dark-light emergence; (2) three-chamber social approach + social novelty; (3) marble burying; (4) social recognition; (5) tail suspension

test; and (6) open field test. There were at least 3 days between tests. A subset of male mice also underwent the resident-intruder test 7 days after the open field test. Behavior assays are described below.

All research was conducted in accordance with National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care guidelines and approved by The Scripps Research Institute's Institutional Animal Care and Use Committee.

Three-chamber social approach 1 social novelty.—Mice were tested as previously described [Clipperton-Allen & Page, 2014; Page et al., 2009a, 2009b;] under white light conditions. Briefly, test mice were each given 5 min to acclimate to an acrylic arena with black walls and a white floor on each of two consecutive days. On the third day, mice were given 5 min acclimation, followed by 10 min social approach [choice between two clear acrylic tubes (20.25 cm tall, with twelve 14 mm diameter holes drilled in the bottom half of the tube), one containing a same-sex, novel conspecific (location counterbalanced across mice)] and 10 min social novelty (novel, same-sex conspecific placed in the previously empty tube) testing. Time spent in each chamber was automatically scored.

Social recognition.—The social recognition test was performed as described previously [Clipperton-Allen & Page, 2014]. Briefly, test mice were housed alone for 2 h in a home cage-like environment [Takayanagi et al., 2010], then presented with an acrylic tube with 12 holes near the bottom containing a same-sex, juvenile (P21–28) control mouse for 5 min. The first four exposures (habituation trials 1–4) were to the same mouse, whereas the fifth presentation (dishabituation trial) used a novel juvenile stimulus. The duration of investigation was scored manually, and mice spending less than 10 sec investigating the stimulus during the first exposure were removed from the analysis.

Resident-intruder test.—A subset of male mice (P70–P135) underwent this test following 7 days of isolation housing, as previously described [Clipperton-Allen & Page, 2015]. These males had not been housed with females since weaning, and had no sexual experience. Briefly, each resident had a group-housed WT male intruder placed into his home cage. Free social interactions were videotaped from above through clear acrylic lids for 15 min. Using The Observer XT 10 Video Analysis software (Noldus Information Technology, Leesburg, VA), an expert observer, blind to genotype, scored the videotaped interactions for 23 individual behaviors based on Grant and Mackintosh's [1963] ethogram (see Table S1 in Supporting Information) [Clipperton-Allen & Page, 2015]. These behaviors, which focused on the resident (test) mouse, were also grouped into 10 categories to gain overall impressions of the duration, frequency, and latency of the mice's behavior (see Table S2 in Supporting Information) [Clipperton-Allen & Page, 2015]. Because behavior can vary across the 15-min test, the social interaction was divided into three 5-min intervals, and the duration, frequency, and latency were analyzed both by 5 min bins and across the 15 min interaction.

Marble burying.—Mice were placed individually in a home-cage-like environment with 5 cm of ¼″ corncob bedding and 20 black marbles (14.3 mm in diameter) arranged in a 4 × 5 matrix, and left undisturbed for 30 min under white light conditions, as previously described

[Clipperton-Allen & Page, 2014]. The number of marbles that were at least $\frac{2}{3}$ buried at the end of the trial was counted [Thomas et al., 2009].

Dark-light emergence.—Mice were tested as previously described [Clipperton-Allen & Page, 2014]. Briefly, each mouse was placed into the dark chamber and allowed to explore for 5 min. Time spent in each compartment, number of crossings between chambers, and latency to enter the light compartment were manually scored from video.

Open field test.—Each mouse was placed in the center of the open field arena (43.8 × 43.8 × 32.8 cm) under approximately 240 lux for 5 min. Ethovision automatically recorded measures (time and number of entries) of center and thigmotaxis (occupying the corners and sides of the open field), velocity, and total distance moved.

Tail suspension test.—Mice spent 6 min suspended from a hook by medical tape attached approximately 2 cm from the tail tip, and immobility was recorded with Ethovision using a 7% immobility threshold.

Rotarod test.—Mice were placed on a 10.5 cm circumference rotating rod (ENV-577M, MedAssociates Inc., St. Albans, VT), and the rotation speed increased gradually from 4 to 40 rpm over 5 min. Mice received three trials, spaced at least 1 h apart, and their latency to fall, both over time and averaged across tests, was measured.

Neuroanatomical Assays

Immunohistochemistry.—Following transcardial per-fusion, mouse brains were fixed in 4% PFA overnight, incubated in 20% sucrose/PBS solution at 4°C for at least 24 h, then embedded in Tissue-Tek OCT compound (Sakura, Torrance, CA) and frozen at −80°C until sectioned. Twenty-five micrometer thick coronal sections were collected on Superfrost/Plus slides. Female adult (control, *Oxt-Cre*$^{+}$; *Pten*$^{loxP/+}$, and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$), P7 and P14 (control, *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$) brain sections were immunostained with anti-Oxt (1:4000, Immunostar, Hudson, WI, catalog #20068), anti-phospho-S6 (1:2000, Cell Signaling Technology, Danvers, MA, catalog #4858), and/or anti-Pten (1:5000, Cell Signaling Technology, catalog #9556S), and AlexaFluor-488 or 2594 conjugated secondary antibodies (1:2000, Invitrogen, Carlsbad, CA), and then mounted with VectaShield Hard Set mounting media (Vector Laboratories, Burlingame, CA). Nuclear labeling used DAPI (Invitrogen, catalog #D3571) or DAPI-containing mounting media. Coronal sections from *Oxt-Cre*$^{+}$*; Pten*$^{+/+}$; *Ai14*$^{+}$ (control) and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$*; Ai14*$^{+}$ mice were mounted with VectaShield with DAPI. Images were obtained with an Olympus VS120 microscope and processed using the VSDESKTOP (Olympus) and ImageJ (http://imagej.nih.gov/ij/) software.

Oxytocin cell size and number analyses.—Using ImageJ, the medial PVN was delineated in plane-matched sections (Bregma −0.75 mm to −0.95 mm), and the size and mean grey value of Oxt signal in the PVN was measured. Within the region of interest of the PVN, Oxt+ cells were outlined, counted, and the size and mean grey value of Oxt signal for

each cell were recorded. Cell density was calculated by dividing the number of Oxt+ cells by the PVN area.

Statistical Analysis

Three-chamber social approach and social novelty chamber preference, resident-intruder, marble burying, dark-light emergence, open field, tail suspension, and all neuroanatomical data were analyzed using oneway between-subjects analyses of variance (ANOVAs) for genotype. Social recognition [3 (genotype) × 5 (trial)] and rotarod [3 (genotype) × 3 (trial)] data were analyzed with mixed model ANOVAs. If trial × genotype interactions were found, within-subject ANOVAs (effect of trial on each genotype) and/or *t*-tests (effect of genotype on each trial) were used for *post hoc* comparisons. One-sample *t*-tests were used to determine if habituation, dishabituation, or dominance scores were significantly different from zero. Paired-sample *t*-tests were used for within-group comparisons of chamber time in the three-chamber social approach + social novelty (time in mouse chamber vs. time in empty chamber; time in novel mouse chamber vs. time in familiar mouse chamber) and dark-light emergence (time in light chamber vs. time in dark chamber) tests, as well as for center time in the open field test (time in center vs. time in thigmotaxis). Tukey's corrected *t*-tests were used for *post hoc* analysis as appropriate.

Additionally, planned comparisons of mutants to control mice (control vs. *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, control vs. *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$) were performed using independent-sample *t*-tests, as previously described [Clipperton-Allen & Page, 2014, 2015]. In the resident-intruder test, planned comparisons were only performed on behaviors across the whole 15 min trial unless one-way ANOVAs indicated a time bin-specific genotype effect.

For all statistical analyses, which were performed with PASW 18 (IBM Corporation, Armonk, NY), significance was set at $P < 0.05$, and statistics for nonsignificant results are not shown.

Results

Behavior Results: Tests Related to ASD Core Symptoms

Three-chamber social approach 1 social novelty.—Mice were tested on this assay because germline *Pten*$^{+/-}$, OxtRKO, and OxtRHT mice all failed to show a signifi-cant social preference in this task [Amico et al., 2004; Clipperton-Allen & Page, 2014; Crawley et al., 2007; Page et al., 2009a; Pobbe et al., 2012b; Sala et al., 2011; Sala et al., 2013; Sejourne et al., 2015].

All genotypes in both sexes (females: control, $n = 22$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 18$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 19$; males: control, $n = 23$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 19$) spent significantly more time in the chamber containing the social stimulus during social approach (paired *t*-tests, all $t > 2.46$, all $P < 0.025$; Fig. 1a,b), and all groups except the control and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ females spent significantly more time in the novel stimulus chamber during social novelty (paired *t*-tests, all $t > 2.43$, all $P < 0.025$; Fig. 1a,b). No significant genotype differences were found for chamber preference (mouse – object, or novel – familiar; Fig. 1c). Thus, unlike in germline *Pten*$^{+/-}$ mice [Clipperton-Allen

& Page, 2014; Page et al. 2009a; Sejourne et al., 2015], no social approach deficits were found in any of the experimental mice.

Social recognition.—One of the best replicated phenotypes in OxtKO and OxtRKO mice is a social recognition deficit, which we also observed in germline *Pten*$^{+/-}$ male mice. Thus, we examined juvenile conspecific recognition in control, *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice.

One-sample *t*-tests showed that all groups (females: control, $n = 18$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 12$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 15$; males: control, $n = 27$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$) significantly habituated (all $t > 2.35$, all $P < 0.027$) and dishabituated (all $t > 2.57$, all $P < 0.027$) to the stimulus (see Fig. 1d,e). One-way ANOVAs and planned comparisons also found no effects of genotype on habituation or dishabituation. Significant group differences were found for specific trials with planned comparisons: *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ females investigated the stimulus significantly less than controls in habituations 1 and 2 (all $t > 2.10$, all $P < 0.045$; see Fig. 1d), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ males spent less time than controls investigating the stimulus in habituation 4 ($t(45) = 2.40$, $P = 0.021$) and dishabituation ($t(45) = 2.26$, $P = 0.028$; see Fig. 1e). Mixed-model ANOVAs found significant effects of trial in both sexes (females: $F(4,168) = 17.43$, $P < 0.001$; males: $F(4,256) = 13.31$, $P < 0.001$), but no genotype effects or trial × genotype interactions.

Female *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice therefore showed less initial investigation of the stimulus, unlike germ-line *Pten*$^{+/-}$ females, who showed no phenotype in this assay [Clipperton-Allen & Page, 2014]. Males, on the other hand, showed largely normal social recognition and equivalent initial social interest, unlike germline *Pten*$^{+/-}$ males [Clipperton-Allen & Page, 2014].

Resident-intruder test.—In the free social interaction of the resident-intruder test, decreased aggression is observed in germline *Pten*$^{+/-}$ males [Clipperton-Allen & Page, 2015], while OxtRKO, but not OxtRHT, males show increased aggression [Dhakar et al., 2012]. Aggression in OxtKO males differs depending on the genotype of their mother, likely due to the presence or absence of Oxt in utero; when born to an OxtHT female, OxtKO males show normal or decreased aggression, while OxtKO males born to OxtKO females show increased aggression [Dhakar et al., 2012; Sala et al., 2011, 2013; Takayanagi et al., 2005; Winslow et al., 2000]. We therefore tested males of all three genotypes on the resident-intruder test following 7 days of single housing.

There were minimal genotype differences observed among males in the resident-intruder test (control males, $n = 12$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ males, $n = 12$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males, $n = 12$). One-way ANOVAs found significant main effects of genotype for both the duration and frequency (all $F > 3.52$, all $P < 0.042$) of dominance score in the last 5 min of the trial, which planned comparisons revealed to be due to significantly lower dominance scores in both *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (all $t > 2.31$, all $P < 0.031$) and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (all $t > 2.13$, all $P < 0.045$) mice than in controls (see Fig. 2a,b); in fact, only control mice showed significant dominance over the intruder for both frequency and duration in the last 5 min of the trial (all $t > 3.43$, all $P < 0.007$). No other significant differences were found on agonistic

behaviors. However, planned comparisons indicated that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ mice showed a decrease in the frequency of approaching and/or attending to the intruder ($t(22) = 2.15$, $P = 0.043$), particularly in the first 5 min ($t(22) = 2.20$, $P = 0.039$; see Fig. 2c). Unlike germ-line *Pten*$^{+/-}$ mice, no genotype differences were found in social behavior, attacks, or digging (see Fig. 2d–f).

Marble burying test.—Increased repetitive behavior was also observed in germline *Pten*$^{+/-}$ males, expressed as both increased digging in the resident-intruder test and more buried marbles in the marble burying test [Clipperton-Allen & Page, 2014, 2015], and OxtKO males showed a shorter latency to dig during a free social interaction [Lazzari et al., 2013]. To determine if our mutants also show increased repetitive behavior, we administered the marble burying test.

Planned comparison *t*-tests found that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ females buried significantly fewer marbles than controls ($t(44) = 2.09$, $P = 0.042$; see Fig. 1f), but no other genotype differences were found with planned comparisons or one-way ANOVAs (females: control, $n = 25$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 21$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$; males: control, $n = 25$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 19$). This suggests that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ females may show less repetitive, stereotyped behavior than control females, but unlike germline *Pten*$^{+/-}$ mice [Clipperton-Allen & Page, 2014], no differences in repetitive behavior were observed in males.

Anxiety-Like Behavior

In germline *Pten*$^{+/-}$ male mice, we observed decreased anxiety in the dark-light emergence and open field tests [Clipperton-Allen & Page, 2014]. Male OxtKO, but not OxtRKO or OxtRHT males, also showed decreased anxiety on the elevated plus maze [Mantella, Vollmer, Li, & Amico, 2003; Sala et al., 2011, 2013], and female OTKO mice performed more stretched approaches, an indicator of social anxiety [Choleris et al., 2003, 2006]. Thus, we assessed anxiety using the dark-light emergence and open field tests.

Dark-light emergence test.—Both one-way ANOVAs and planned comparisons found no significant differences between genotypes for time in light chamber (see Fig. 3a), latency to enter the light chamber (see Fig. 3b), crossings between chambers, or any other measure (females: control, $n = 19$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 17$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 19$; males: control, $n = 25$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$). Additionally, all groups showed a significant preference for the dark chamber over the light chamber (all $t > 4.10$, all $P < 0.002$).

Open field test.—Planned comparisons revealed that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males spent significantly more time in the center of the arena than control ($t(44) = 2.81$, $P = 0.007$) mice (see Fig. 3c), but no other genotype differences or differences in distance traveled or velocity (see Fig. 3d). Additionally, all groups (females: control, $n = 21$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 19$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$; males: control, n 5 26; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$) spent significantly more time in thigmotaxis than in the center of the arena (all $t > 32.50$, all $P < 0.001$).

Thus, *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males did phenocopy germline *Pten*$^{+/-}$ mice, but only on one of the two tests on which germline *Pten*$^{+/-}$ males showed decreased anxiety-like behavior [Clipperton-Allen & Page, 2014].

Depression-Like Behavior

Tail suspension test.—We observed increased depression-like behavior in germline *Pten*$^{+/-}$ males; while depression-like behavior phenotypes have not been found in OxtKO or OxtRKO mice, Oxt has been shown to be related to depression, with decreased Oxt associated with higher depression scores [Scantamburlo et al., 2007], and Oxt administration having anti-depressant effects [Bakharev, Tikhomirov, & Lozhkina, 1986].

Both planned comparisons and one-way ANOVAs found no significant differences between genotypes (females: control, $n = 22$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 18$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 19$; males: control, $n = 29$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$) for immobility (see Fig. 3e).

Motor Coordination

Rotarod test.—To confirm that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice had no motor deficits, and to ensure differences in motor ability did not lead to the genotype difference in open field center time, we tested motor learning on the rotarod test.

Mixed-model ANOVAs showed a significant main effect of test ($F(2,122) = 17.66$, $P < 0.001$), with all genotypes (males: control, $n = 25$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, $n = 20$; *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$, $n = 20$) showing significant reductions in latency to fall across the three tests (all $F > 3.74$, all $P < 0.034$; see Fig. 3f). Planned comparisons revealed that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ had a significantly longer latency to fall than *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males on test 2 ($t(38) = 2.46$, $P = 0.019$), but no other significant genotype effects on any, or across all, tests (see Fig. 3f).

Neuroanatomical results.—As *Pten* mutations are known to lead to altered cell number and size [Back-man et al., 2001; Chen et al., 2015; Fraser et al., 2004; Goberdhan et al., 1999; Groszer et al., 2001; Kazdoba et al., 2012; Kwon et al., 2001], we examined Oxt cell number and size, as well as PVN size, in conditional Pten mutants and controls following behavioral testing.

Oxytocin Cell Size and Number in Adults

Planned comparisons indicated that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice ($n = 6$) had significantly larger soma in Oxt immunoreactive cells than controls ($n = 6$; $t(5.5) = 5.37$, $P = 0.003$; see Fig. 4a,b,d), and a significantly lower density of these cells in the PVN ($t(10) = 3.20$, $P = 0.010$; see Fig. 4a,b,e). One-way ANOVAs also found main effects of genotype for these measures (all $F > 7.09$, all $P < 0.009$), with *post hoc* tests revealing that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ also had larger cells and lower density than *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ mice ($n = 4$; all $P < 0.020$; see Fig. 4a,b,d,e). A main effect of genotype on PVN area was also found by one-way ANOVA ($F(2,13) = 4.23$, $P = 0.038$; see Fig. 4a,f), with *post hoc* tests indicating

that the PVN of *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice was significantly larger than that of *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ mice ($P = 0.044$; see Fig. 4a–f).

As a way to assess the characteristics of these cells without considering current Oxt levels, we examined the PVN of mice expressing the red fluorescent protein tdTomato in cells in which *Oxt-Cre* had been active (*Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$*; Ai14*$^{+}$). Qualitatively, these mice also show increased soma size and lower density of tdTomato expressing cells (see Fig. 5a,b).

Interestingly, we found that *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice also appeared to lack Oxt immunoreactive projections in anterior hypothalamic area just lateral to the PVN (see Fig. 4c). However, tdTomato positive projections were present in this region (see Fig. 5c), suggesting that the lack of Oxt immunoreactivity in projections may be due to an Oxt trafficking deficit, not defects in Oxt neuronal projections per se.

Oxytocin Cell Size and Number in Juvenile Mice

We first confirmed that *Oxt-Cre* had early developmental activity by examining the PVN in *Oxt-Cre*$^{+}$*; Ai14*$^{+}$ P7 and P0 brains, which displayed robust reporter expression in the PVN (see Fig. 6a,b and Supporting Information Fig. S1a). We next analyzed Oxt cellular characteristics in P7 mice to assess whether the neuroanatomical phenotype was present as the circuitry underlying social behavior is assembled. Unlike adult brains, no significant genotype differences were found for cell soma size, number, density, or grey value, or for PVN area or grey value (see Fig. 6c–h).

We then examined Oxt cell and PVN structure in control and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ brains at P14 (see Fig. 7a,b) and found no gross differences, indicating that conditional deletion of *Pten* induces hypertrophy in Oxt cells predominantly after the circuitry underlying social behavior is largely assembled. Because we did not detect significant phenotypic differences between controls and mutants at P14, we confirmed that *Pten* deletion resulted in elevated mTORC1 activity by immunostaining for phospho-S6, a readout of mTOR Complex 1 (mTORC1) activity [Chen et al., 2015; Zhou & Parada 2012], and found increased phospho-S6 levels in the PVN of *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice (see Fig. 7c,d). To confirm that *Pten* was deleted in Oxt cells, we immunostained for *Pten* and *Oxt* and found that, while Oxt-immunoreactive cells were also immunoreactive for *Pten* in the PVN of control mice, Oxt-immunoreactive cells were not immunoreactive for *Pten* in the PVN of *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice (see Supporting Information Fig. S1B). Taken together, these results are consistent with the progressive hypertrophy and elevated phospho-S6 that have been reported in numerous other *Pten* conditional knockout models [e.g., Backman et al., 2001; Fraser et al., 2004; Gregorian et al., 2009; Gregory et al., 2009; Kwon et al., 2001; Kwon, Zhu, Zhang, & Baker, 2003; Ljungberg, Sunnen, Lugo, Anderson, & D'Arcangelo, 2009; Pun et al., 2012; Takeuchi et al., 2013; Wen et al., 2013].

Discussion

Deletion of *Pten* in Oxt neurons had minimal effects on ASD-relevant behavior. No genotype effects were found in either sex for the three-chamber social approach or social

novelty assay, in habituation or dishabituation in the social recognition assay, or for overall social behavior, social investigation, or related individual behaviors in males in the resident-intruder test. However, both *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males did show a decreased dominance score in the latter part of the resident-intruder test, but no differences in attacking or individual aggressive behaviors, and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ males also spent less time approaching and/or attending to the intruder. Male mutants did not differ from controls in repetitive behavior (marble burying or digging in the resident-intruder test), but *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ females did bury fewer marbles than controls. No genotype results were found for depression-like or motor behavior, and while *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males did show less anxiety-like behavior in the open field test, these differences were not found in a second anxiety test (dark-light emergence).

As many of the behavioral phenotypes observed in germline *Pten*$^{+/-}$ mice [Clipperton-Allen & Page 2014, 2015; Page et al., 2009a; Sejourne et al., 2015] are similar to those found in OxtKO and/or OxtRKO mice [Amico et al., 2004; Clipperton-Allen & Page, 2014, 2015; Ferguson et al., 2000, 2001; Lazzari et al., 2013; Lee et al., 2008; Macbeth et al., 2009; Mantella et al., 2003; Nishimori et al., 2008; Page et al., 2009a; Pobbe et al., 2012a, 2012b; Sala et al., 2011, 2013; Takayanagi et al., 2005; Winslow et al., 2000], we investigated whether conditional deletion of *Pten* in Oxt cells recapitulated these behavioral phenotypes. With the exception of decreased anxiety-like behavior in the open field test in males, germline *Pten*$^{+/-}$ phenotypes were not phenocopied by *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ *or Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice.

Despite the overall minimal impact on ASD-relevant behaviors, we observed increased Oxt cell size in *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice, which more than doubled that of control or *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ mice. This was accompanied by an increase in PVN area and a decrease in Oxt cell density. The number of Oxt immunoreactive cells also trended towards a decrease, although this did not reach statistical significance.

Interestingly, we also observed a reduction in Oxt immunoreactive fibres in the anterior hypothalamic area in both the *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice. We did not detect a decrease in tdTomato positive projections in *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$*; Ai14*$^{+}$ brains. This suggests that there may be defects in the intracellular trafficking of Oxt in these mice. Examining this phenotype is beyond the scope of this study; however, this may provide insight into the mechanisms of Oxt trafficking in future studies.

Given the substantial hypertrophy in Oxt cells, it is somewhat surprising that minimal behavioral phenotypes were observed. However, this may be accounted for by the lack of a developmental neuroanatomical phenotype. Alternatively, Oxt, a long-range, diffuse, and slow-acting neuromodulator, may have been present in sufficient quantities in the PVN and other brain areas to compensate for any local alterations in Oxt signaling.

Despite the lack of behavioral phenotypes, these results do not rule out the possibility that the Oxt system may play a role in germline *Pten*$^{+/-}$ behavioral abnormalities through a non-cell autonomous mechanism in Oxt cells or through an effect in OxtR-expressing cells. Additionally, *Pten* mRNA is expressed throughout the developing hypothalamus as early as

E11.5, prior to the onset of *Oxt* mRNA expression between E13.5 and E15.5 (Allen Brain Atlas, http://developingmouse.brain-map.org/, last accessed 29 January 2016) [Thompson et al., 2014]. Thus, we cannot exclude the possibility that *Pten* may function early in the Oxt cell lineage, prior to *Oxt-Cre* mediated recombination.

In summary, our results indicate that conditional deletion of *Pten* using *Oxt-Cre* has a profound effect on Oxt cell structure, but leaves ASD-relevant behaviors largely unaffected. We interpret these results as inconsistent with our starting hypothesis, that reduced *Pten* function in Oxt-expressing cells causes the behavioral deficits observed in germline $Pten^{+/-}$ mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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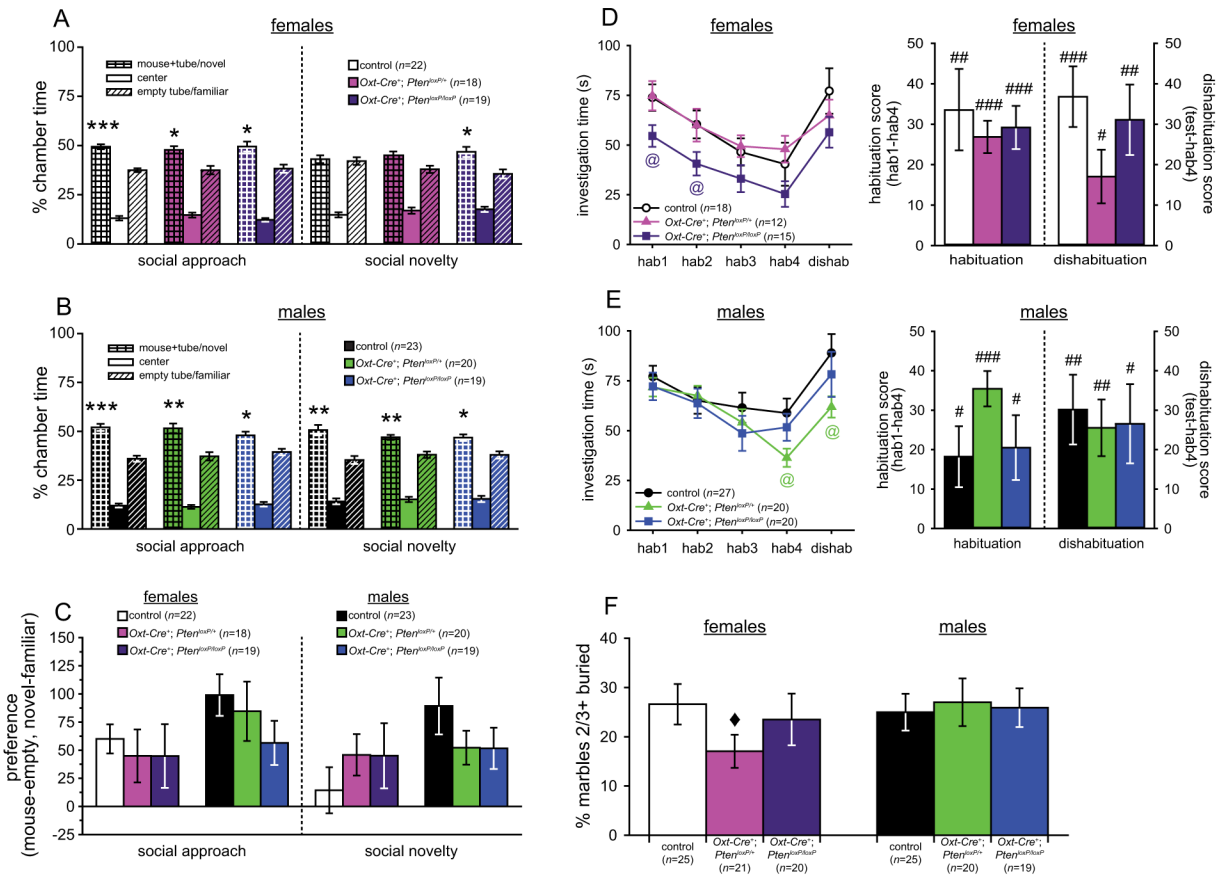

Figure 1.

Results of social approach, social recognition, and repetitive behavior tests in *Oxt-Cre*$^{+}$*; Pten*^{loxP} mice. **(A–C)** Time spent in each chamber **(A,B)** and chamber preference **(C)** during the three-chamber social approach and social novelty in control (n = 22), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 18), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 19) female mice **(A,C)** and in control (n = 23), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 19) male mice **(B,C).** **(D,E)** Investigation of the stimulus mouse in the social recognition assay across trials, and habituation and dishabituation, in control (n = 18), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 12), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 15) female mice **(D)** and in control (n = 27), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) male mice **(E). (F)** Percentage of marbles at least $^{2}/_{3}$ buried after 30 min by control (n = 25), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 21), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) females and control (n = 25), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 19) males. Significant difference between the mouse chamber and the object chamber or between the novel and familiar mouse chambers: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significant difference between controls and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ females **(D)** or between controls and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ males **(E)**: @ $P < 0.05$. Significant habituation or dishabituation: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Significant difference between controls and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ females: ◆ $P < 0.05$.

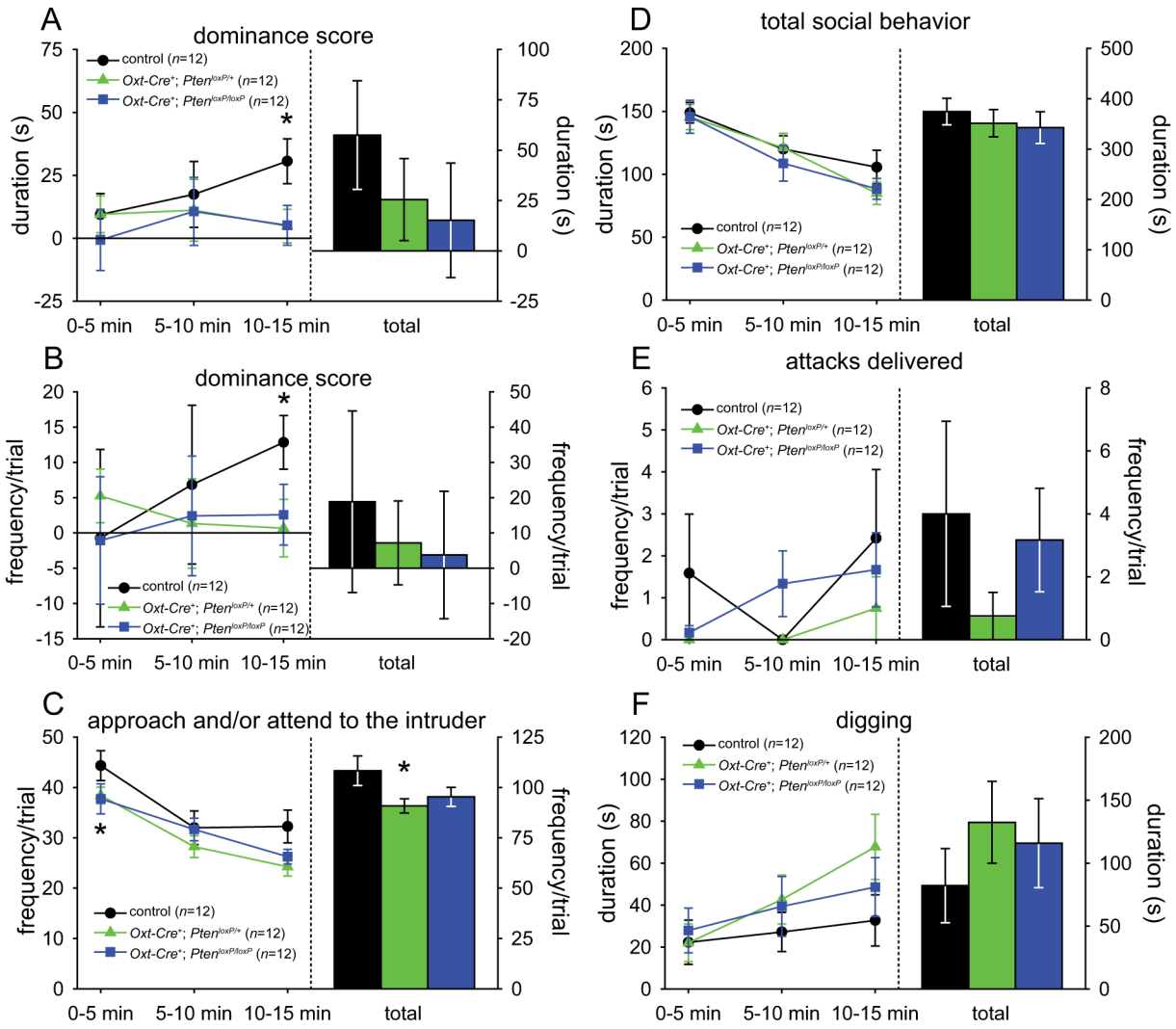

Figure 2.

Resident-intruder test results of male *Oxt-Cre*$^{+}$*; Pten*^{loxP} mice. **(A,B)** Dominance score (agonistic behavior delivered minus agonistic behavior received) duration **(A)** and frequency **(B). (C)** Approaching and/or attending to the intruder. **(D)** Total social behavior. **(E)** Attacks delivered. **(F)** Digging. Significant difference from controls: $*P < 0.05$. Controls ($n = 12$), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ ($n = 12$), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ ($n = 12$).

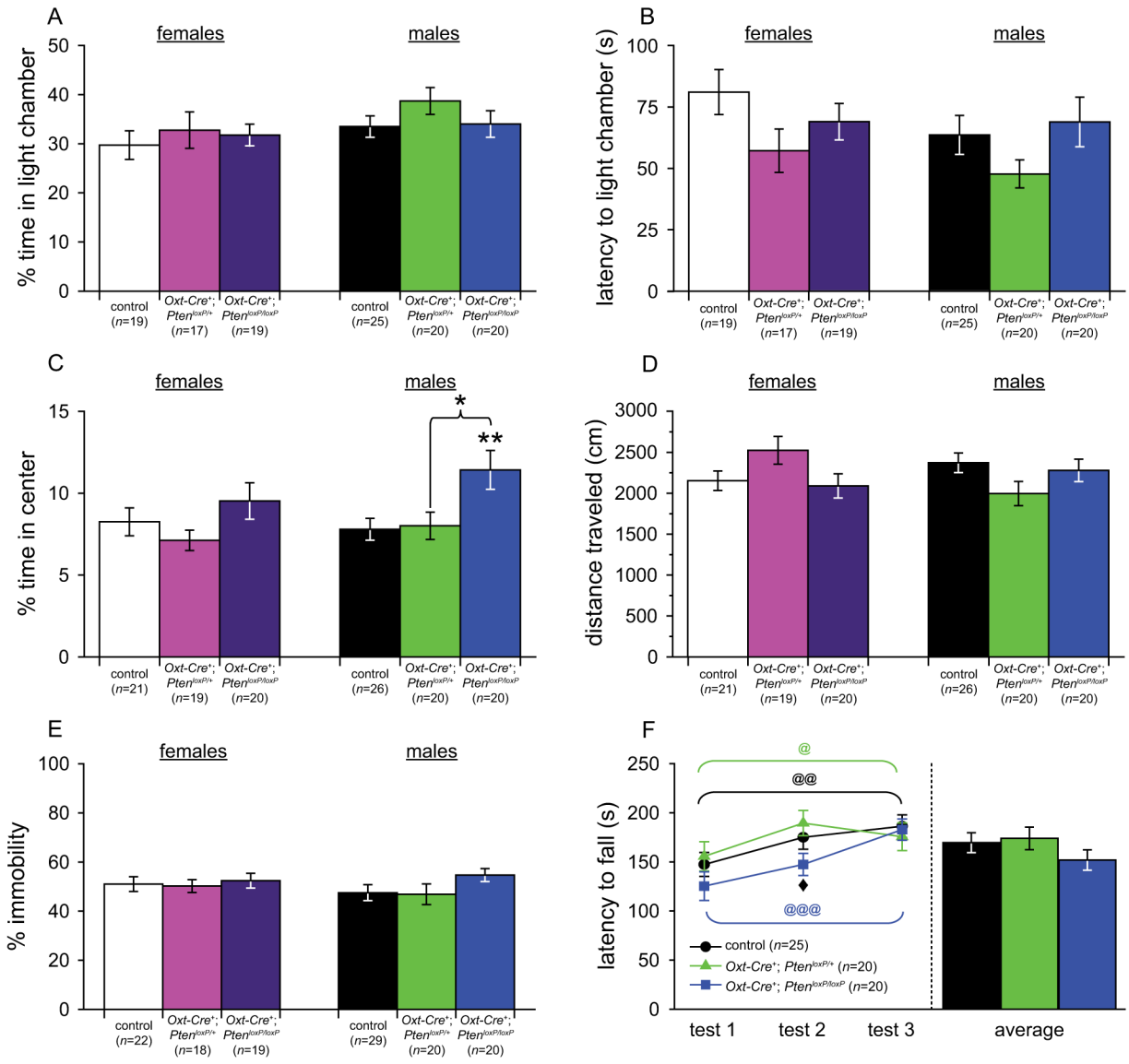

Figure 3.

Tests of anxiety-like, depression-like, and motor learning behavior in *Oxt-Cre*$^{+}$*; Pten*^{loxP} mice. **(A,B)** Dark-light emergence test for anxiety-like behavior in control (n = 19), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 17), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 19) females and control (n = 24), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) males. **(C,D)** Open field test for anxiety-like and locomotor behavior in control (n = 21), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 19), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) females and control (n = 26), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) males. **(E)** Tail suspension test of depression-like behavior in control (n = 22), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 18), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 19) females and control (n = 29), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) males. **(F)** Rotarod test of motor learning and behavior in control (n = 25), *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ (n = 20), and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ (n = 20) males. Significant differences between genotypes: *P < 0.05, **P < 0.01. Significant improvement over trials: $^{@}P$ < 0.05,

@@ $P < 0.01$, @@@ $P < 0.001$. Significant difference between *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ males, ◆ $P < 0.05$.

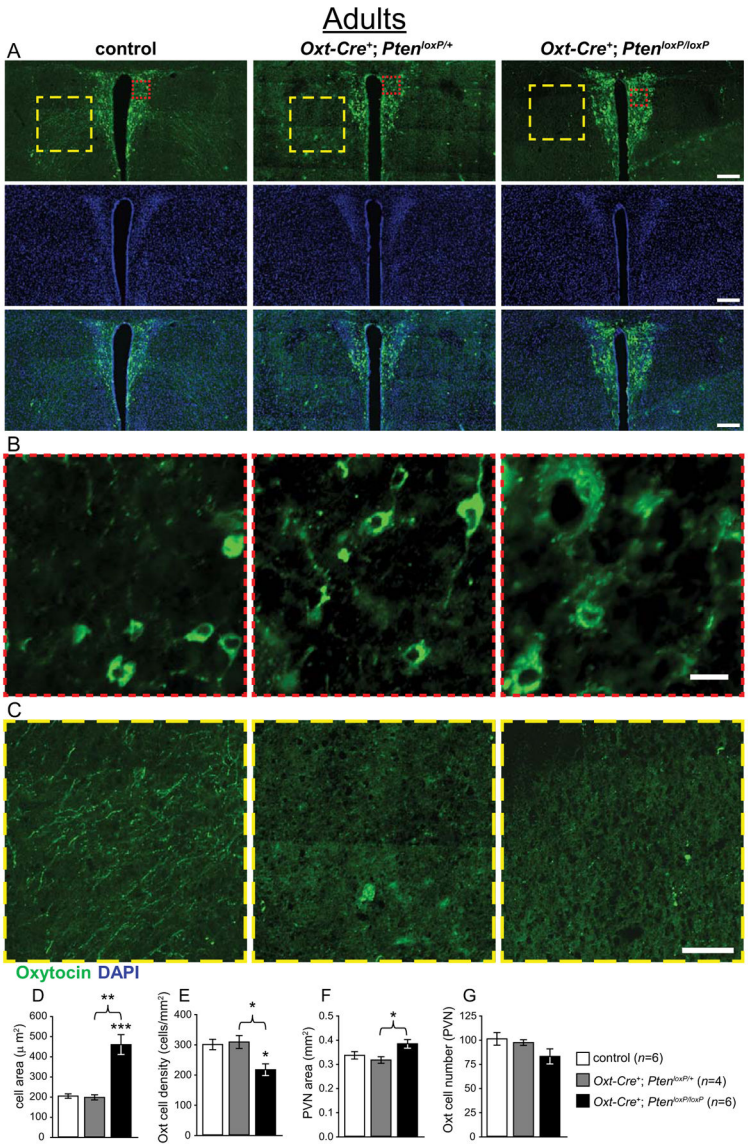

Figure 4.

Neuroanatomical analysis of Oxt immunoreactive cells in the paraventricular nucleus of the hypothalamus (PVN) of adult female *Oxt-Cre*$^{+}$*; Pten*^{loxP} mice. **(A–C)** Representative images of control, *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$, and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ PVN immunostained with anti-Oxt (green) and DAPI (blue), with enlargements showing increased cell size (red square, **B**) and decreased immunoreactivity in the region lateral to the PVN (yellow square, **C**). **(D)** Increased Oxt cell soma area in *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice. **(E)** Decreased Oxt cell density in the PVN of *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice. **(F)** *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice have a larger PVN than *Oxt-Cre*$^{+}$*; Pten*$^{loxP/+}$ mice, with a trend to a larger PVN than controls. **(G)** Non-significant trend to a decrease in Oxt cell number in *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice. Significant differences between genotypes: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bars: 200 μm **(A)**, 20 μm **(B)**, 100 μm **(C)**.

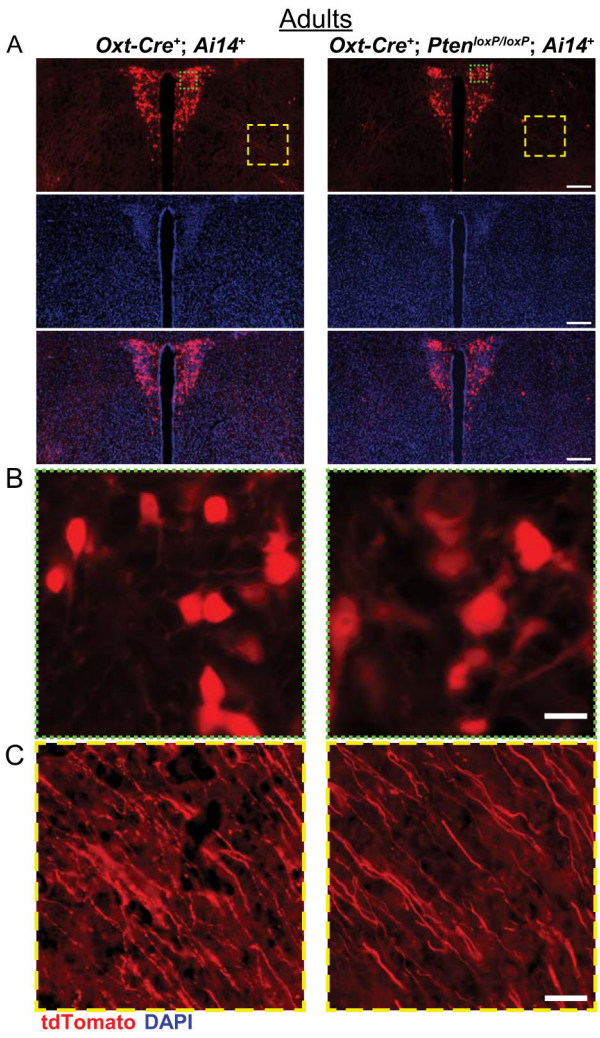

Figure 5.

Neuroanatomical assessment of Oxt cells in the PVN of adult female *Oxt-Cre*$^{+}$*; Pten*^{loxP}; *Ai14*$^{+}$ mice. **(A)** Representative images of control (*Oxt-Cre*$^{+}$*; Pten*$^{+/+}$*; Ai14*$^{+}$) and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$*; Ai14*$^{+}$ PVN with tdTomato (red) expressed in cells in which *Oxt-Cre* has been active, stained with DAPI (blue). **(B,C)** Enlargements showing increased cell size (green square, **B**) and the presence of projections in the region lateral to the PVN (yellow square, **C**) in control and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$*; Ai14*$^{+}$ mice. Scale bars: 200 μm (A), 20 μm (B), 50 μm (C).

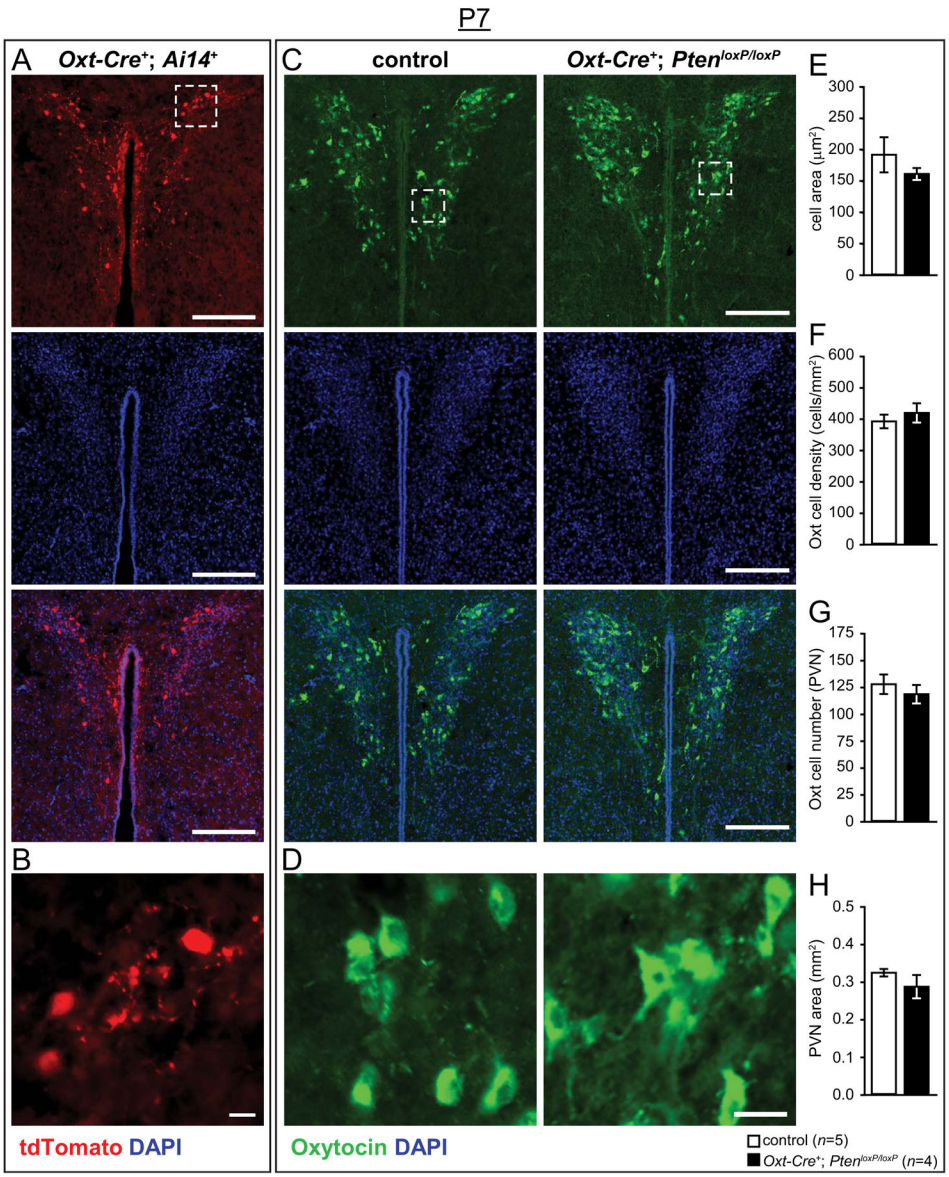

Figure 6.

Neuroanatomical analysis of Oxt immunoreactive cells at post-natal day 7 (P7) in the PVN of *Oxt-Cre*$^{+}$*; Pten*^{loxP} mice. **(A,B)** Representative images of control (*Oxt-Cre*$^{+}$*; Ai14*$^{+}$) and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$*; Ai14*$^{+}$ PVN **(A)** with tdTomato (red) expressed in cells in which Oxt-Cre has been active, stained with DAPI (blue), and enlargements showing cellular resolution (white square, **B**). **(C,D)** Representative images of control and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ PVN **(C)** immunostained with anti-Oxt (green) and DAPI (blue), with enlargements showing cellular resolution (white square, **D**). (**E–H**) Normal Oxt cell soma area **(E)**, Oxt cell density **(F)**, Oxt cell number **(G)**, and PVN size **(H)** in P7 *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ mice. Scale bars: 200 µm **(A,C)**, 20 µm **(B,D)**.

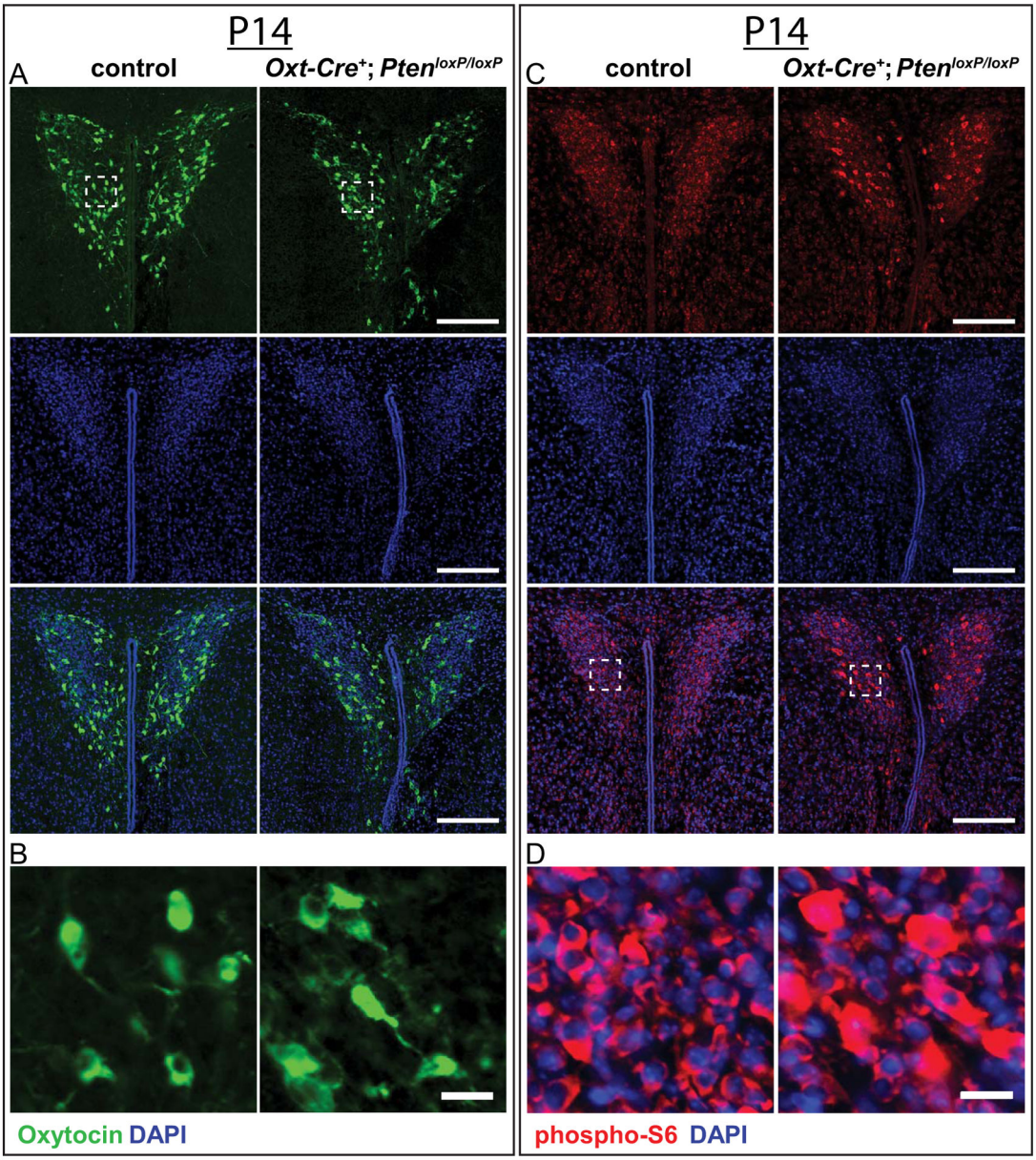

Figure 7.

Assessment of Oxt immunoreactive cells and phospho-S6 enrichment in the PVN at P14. **(A,B)** Representative images of P14 control and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ PVN **(A)** immunostained with anti-Oxt (green) and DAPI (blue), with enlargements showing cellular resolution (white square, **B**). **(C,D)** Representative images of P14 control and *Oxt-Cre*$^{+}$*; Pten*$^{loxP/loxP}$ PVN **(C)** immunostained with anti-phospho-S6 (red) and DAPI (blue), with enlargements showing cellular resolution (white square, **D**). Scale bars: 200 μm **(A,C)**, 20μm **(B,D)**.