Perisomatic inhibition of pyramidal neurons is established by fast-spiking, parvalbumin-expressing interneurons (PV cells). Failure to assemble adequate perisomatic inhibition is thought to underlie the aetiology of neurological dysfunction in seizures, autism spectrum disorders and schizophrenia. Here we show that in mouse visual cortex, strong perisomatic inhibition does not develop if PV cells lack a single copy of \textit{Pten}. PTEN signalling appears to drive the assembly of perisomatic inhibition in an experience-dependent manner by suppressing the expression of EphB4; PV cells hemizygous for \textit{Pten} show an ~2-fold increase in expression of EphB4, and over-expression of EphB4 in adult PV cells causes a dismantling of perisomatic inhibition. These findings implicate a molecular disinhibitory mechanism driving the establishment of perisomatic inhibition whereby visual experience enhances Pten signalling, resulting in the suppression of EphB4 expression; this relieves a native synaptic repulsion between PV cells and pyramidal neurons, thereby promoting the assembly of perisomatic inhibition.
Perisomatic inhibition in cortex is established by fast-spiking interneurons called basket cells that express the calcium binding protein parvalbumin (PV). The establishment of this inhibition during postnatal development is centrally important to the opening of critical periods and the establishment of neural gain control, and its failure is linked to a number of disorders of cortical development, including autism and schizophrenia. Remarkably little is known about how PV neurons establish strong synaptic input to local pyramidal neurons during development.

By comparison, a great deal more is known about the postnatal growth of pyramidal neurons. In mice, the growth of axons and dendrites is most pronounced during the first few weeks of postnatal life. Thereafter, growth is slowed as neurons begin to express PTEN. Deleting Pten from adult pyramidal neurons is sufficient to re-start dendritic and axonal growth.

Embyronic PTEN deletion from inhibitory neurons impacts the establishment of appropriate numbers of parvalbumin and somatostatin-expressing interneurons, demonstrating a central role for Pten in inhibitory cell development. To examine how Pten signalling impacts PV cells specifically, we used loxP-mediated recombination to generate mice in which PV copy gene deletion maintains elevated EphB4 expression, thereby repelling the establishment of PV cell synapses onto pyramidal neurons.

Results

Pten in PV cells regulates perisomatic inhibition. All studies were carried out using PV-Cre knockin mice obtained from The Jackson Laboratory (B6;129P2-Pvalbtm1(cre)Arbr/J). To validate the expression of Cre recombinase in these mice, we crossed them with the Ai9 line of reporter mice expressing a flexed tdTomato knocked in to the Rosa26 locus to generate mice expressing tdTomato in cells expressing Cre; the fraction of PV cells that also expressed tdTomato, and thus Cre recombinase in visual cortex, was then measured at three ages (postnatal days 28, 40 and 60) by immuno-staining against PV in fixed, frozen coronal sections. At P28, 92 of 93 PV cells counted in three sections (99%) were co-labelled; at P40 this ratio was 166/168 (99%), and at P60 it was 166/184 (90%), indicating that the PV-Cre line of mice reliably express Cre recombinase and tdTomato in virtually all PV cells (Fig. 1). These values are similar to those reported in ref. 12.

To examine the impact of Pten haploinsufficiency on PV cell circuitry, we expressed the light-activated cationic channel, channelrhodopsin-2 (ChR2), in PV cells of PV-þ/C0 and littermate control mice; subsequently laser scanning photostimulation of ChR2-expressing PV cells with concurrent whole-cell recordings of layer 2/3 pyramidal neurons was used to measure the strength of PV cell input to pyramidal neurons in primary visual cortex in acute brain slices (P35-P56). We note that mapping studies based on ChR2 expression may be confounded by differences in the optical recruitment of PV cells. With this approach, we found that PV cells hemizygous for Pten produced only half of the total inhibitory drive to pyramidal cells of wild-type PV cells (Fig. 2; Supplementary Figs 1 and 2). This reduced inhibitory current could not be attributed to a reduced expression of ChR2 or responsiveness to blue light stimulation (Supplementary Fig. 3), or to changes in the intrinsic properties of PV cells in the experimental group (Supplementary Fig. 4), as these measures were comparable between PV cells hemizygous for Pten and controls. Nor was it due to a reduction in the number of PV cells in mutants (Cells per field of view visualized using a × 4 objective lens with a field number of 26.5; mean and standard deviation: WT = 354 ± 40; Pten = 367 ± 60; 10 fields of view per group; P = 0.73, Wilcoxon rank sum).
This reduction in PV cell-derived inhibitory current could result from pyramidal neurons receiving fewer PV cell-derived inhibitory synapses; these synapses could be present in normal numbers, but weaker synapses; or it could be some combination of these two. If there are fewer synapses, this would be reflected in a decrease in connectivity. If the synapses are weaker, this would be reflected in a decrease in paired-pulse ratio. To directly measure these, we conducted paired whole-cell recordings from adjacent PV cells and pyramidal neurons and, separately, from adjacent pairs of PV cells (Fig. 3). Recordings were done in L2/3 of the primary visual cortex of young adult mice (P35–56). PV cells were identified by their expression of td-Tomato, high frequency firing pattern, narrow action potentials and sharp after-hyperpolarizations. Pyramidal neurons were identified by their lack of td-Tomato expression and the presence of an accommodating firing pattern (cf. Fig. 3a). Connectivity between the two cells was assessed by stimulating the presynaptic neuron in current clamp and recording from the putative postsynaptic neuron in voltage clamp (cf. Fig. 3b). PV cells hemizygous for Pten exhibited a 32% decrease in probability of connecting to adjacent pyramidal neurons relative to controls (Fig. 3c). This 32% reduction is a measure of connectivity and is distinct from the 50% reduction in total PV cell-derived inhibitory current in pyramidal neurons reported in Fig. 1, which is a measure of total input strength. Notably, the probability of finding the reverse connection, from pyramidal cell to PV cell hemizygous for Pten was no different from control; nor was there any change in the probability of connection between mutant PV cells and between normal PV cells (Fig. 3c).

When we examined the intrinsic excitability of pyramidal neurons in these slices, we found a trend towards increased excitability in PV-Pten+/− mice (Fig. 3d; only comparisons at 150 pA had a P value <0.05, no other comparisons revealed significant differences). This data trend could be due to a true change in the intrinsic properties of these excitatory neurons, despite having normal Pten copy number, or it could simply be an epiphenomenon of the reduction in inhibition in these slices, one result of which would be an increase in recurrent excitatory response following the firing of the recorded excitatory neuron. Supporting this latter view, the data trend towards increased
excitability was erased when all synaptic transmission was blocked by application of AMPAR, NMDAR and GABA\(_A\) antagonists (CNQX, APV, picrotoxin; Fig. 3e; \(P = 0.92\), Kruskal-Wallis one-way analysis of variance (ANOVA)). These paired recordings show that PV cells hemizygous for Pten have normal intrinsic membrane properties, but establish fewer synapses onto local pyramidal neurons.

To determine whether the synapse that are established by the mutant PV cells are of normal strength, we measured response amplitudes and paired pulse ratio of extant connections. We found no significant differences in response amplitudes in any of the types of synapses examined; PV to pyramidal, pyramidal to PV, and PV to PV were all comparable between PV-Pten\(^{+/–}\) mice and controls (Fig. 3f-h). Measures of paired pulse ratio were also equivalent (Fig. 3i). These measures indicate that the synapses established by mutant PV cells onto local pyramidal neurons are as strong as synapses established by normal PV cells.

Taken together, these findings show that single copy Pten loss in PV cells reduces the probability that any given PV cell will establish a connection with a local pyramidal neuron; this, in turn, causes a significant reduction in the strength of perisomatic inhibition. This effect is specific to the connection between PV cells and pyramidal cells.

**Mutant PV cells express high levels of EphB4.** We next examined how a reduction in Pten expression could result in decreased, synapse-specific connectivity between PV cells and pyramidal neurons. Since PTEN is a phosphatase that negatively regulates the AKT signalling pathway, which in turn regulates gene transcription and protein translation\(^{15}\), we screened for...
possible differences in gene expression in Pten+/− and wild-type PV cells. To do so, live PV cells were harvested from the primary visual cortex of intact adult brains via fluorescence-assisted laser cell sorting (Fig. 4a,b). RNA from live PV cells was isolated and amplified, and complementary DNA libraries synthesized. Transcript copy number was quantified using Illumina mouse gene chip arrays, with a total of 26,500 genes screened (Fig. 4c). Seventeen transcripts met established criteria for further examination, as described in ref. 14, namely a > 1.4-fold change in expression (logFC > 0.5) and a P-value < 0.001 (Table 1). Of these, the majority encoded intracellular glycosylation proteins. One standout transcript was Ephb4 (logFC = 0.56, P = 0.00039), which encodes the presynaptic partner of ephrinB2. Quantitative real-time PCR (ddCt = 1.84) confirmed that Ephb4 RNA was in fact increased in PV cells hemizygous for Pten.

These data suggest a model in which Pten expression in PV cells suppresses expression of EphB4 in these same cells. In the central nervous system, ephrinB/EphB signalling mediates cell-to-cell repulsion during development. Supporting this view, RNA sequencing data of adult mouse primary visual cortical neurons, published from the Allen Brain Institute, shows high expression of EphrinB2 in pyramidal neurons; Pten, but not EphB4, is expressed in adult PV cells (see http://casestudies.brain-map.org/celltax). Increased expression of Ephb4 in PV cells could account for the reduction in PV to pyramidal inhibitory synapses via increased repulsion between the presynaptic terminals of PV cells and cell bodies of pyramidal neurons.

**EphB4 overexpression dismantles perisomatic inhibition.** To directly test whether increased Ephb4 signalling in PV cells is sufficient to prevent the normal formation of perisomatic inhibition of pyramidal neurons, we selectively over-expressed Ephb4 in mature, genotypically normal PV cells in the primary visual cortex of mice expressing ChR2 under the PV promoter (PV-Cre/Ai32) via viral transduction of Ephb4 (AAV2/1-Syn. flex.mCherry.D2A.mEphB4; Fig. 5a-c) and then mapped net PV cell-mediated inhibition across all cortical layers onto individual pyramidal neurons via laser scanning photo-stimulation of ChR2 expressing PV cells with concurrent whole-cell recordings of pyramidal neurons as in Fig. 1. Control recordings were obtained from PV-Cre/Ai32 mice that received the same viral injection but failed to express Ephb4. A 67% reduction in local PV mediated inhibition of pyramidal neurons was observed in mice over-expressing Ephb4 (Fig. 5d-f), supporting the view that Ephb4 signalling in PV cells impairs the formation of perisomatic inhibitory synapses.

**Early vision increases Pten and suppresses EphB4 in PV cells.** Since visual experience is required for the formation of perisomatic inhibition in visual cortex, we wondered whether vision also informs levels of Pten and Ephb4 expression in PV cells. To do so, we reared PV-Pten+/− mice and littermate controls in darkness from birth until postnatal day 35. PV cells in these mice also expressed the red fluorescent protein tdTomato. Using fluorescent-assisted laser cell sorting to extract live PV cells and subsequent qrtPCR normalized to GAPDH expression, we found a ~3-fold increase in Ephb4 expression in PV cells from dark reared mice relative to WT mice; these mice also expressed the red fluorescent protein tdTomato expression. Real-time quantitative PCR (ddCt = 1.84) was used to verify this result. Note the increased expression of Ephb4. Real-time quantitative PCR (ddCt = 1.84) was used to verify this result.

### Table 1 | List of non-metabolic genes differentially expressed in PV cells hemizygous for Pten.

| Gene   | P-value | Fold change | Validation |
|--------|---------|-------------|------------|
| CD300A | 5.71E-5 | 1.484       |            |
| E03006KO4RIK  | 0.000182 | 1.641       |            |
| ANLN  | 0.000374 | 0.597       |            |
| EPHB4  | 0.000392 | 1.475       | q-RT PCR   |
| VLDLR  | 0.000577 | 0.730       |            |
| BIN1   | 0.000585 | 0.588       |            |
| GIA5   | 0.000621 | 1.306       |            |
| STMN4  | 0.000661 | 0.544       |            |
| EVII   | 0.000676 | 1.591       |            |
| ADRA2C | 0.000683 | 0.707       |            |
| 9930117H01RIK  | 0.000703 | 1.575       |            |
| TGFB1  | 0.000880 | 0.745       |            |
| CEACAM1 | 0.000885 | 1.351       |            |
| APOE   | 0.000908 | 1.301       |            |
| VLDLR  | 0.000921 | 0.683       |            |
| BCL2L2 | 0.000991 | 0.781       |            |
| OLRF6  | 0.000999 | 1.345       |            |

Criteria for inclusion were a logFC > 0.5 and P < 0.001 (see ref. 28). Note the increased expression of Ephb4.
Discussion

The establishment of perisomatic inhibition progresses through two stages. In the first stage, factors intrinsic to developing cortex guide PV axons to pyramidal cell somas and proximal dendrites. In the second stage, increased neural activity incited by sensory experience drives the proliferation of perisomatic inhibition. Our results suggest a model in which sensory experience suppresses expression of EphB4 in PV cells. This downregulation of EphB4 likely relieves a native synaptic repulsion between PV cells and pyramidal neurons, thereby promoting the assembly of PV-mediated perisomatic inhibition.

The link between increased Pten expression and a reduction in EphB4 levels has been shown in human tumour lines, but has not been studied in the central nervous system. The signalling pathway inducing their growth has recently been examined. This study identifies an essential link between PTEN signalling and the establishment of somatic inhibition.

Methods

Animal research. All experimental procedures were approved by the University of California Los Angeles Office for Protection of Research Subjects and the Chancellor’s Animal Research Committee and by the Office of Research of the University of California, Irvine.

PV-Cre+/-/Ai9+/-/PtenloxP+/- mice. To genetically label PV-positive neurons, PV-ires-cre knock-in female mice (Jackson Laboratories, stock #008069, generated by S Arber, FMI) were crossed with male tdTomato reporter knock-in mice (Jackson Laboratories, stock #007905, ‘Ai9’, generated by H Zeng, Allen Brain Institute). Offspring were hemizygous for both transgenes. These mice were then back-crossed with PV-Cre mice to generate offspring that were homozygous for PV-Cre and hemizygous for Ai9. Using separate breeders, we also generated mice that were hemizygous for both Ai9 and a floxed Pten. PV-Cre+/-/Ai9+/-/PtenloxP+/- mice were then crossed with Ai9+/-/PtenloxP+/- mice to generate offspring that were homozygous for Cre, Ai9 and Pten in PV cells. Littermate controls were hemizygous for Cre and Ai9 in PV cells, but expressed both copies of Pten.

PV-Cre+/-/Ai32+/-/PtenloxP+/- mice. To generate mice in which a single copy of Pten was deleted from PV cells, we first crossed PtenloxP+/- mice with mice homozygous for a conditional knock-in allele of ChR2-YFP (Jackson Laboratories, stock #012569, ‘Ai32’, generated by H Zeng, Allen Brain Institute). Resulting offspring were genotyped and mice hemizygous for ChR2-YFP and PtenloxP+/- were crossed with PV-Cre+/-/Ai32+/- mice to generate PV-Cre+/-/Ai32+/-/PtenloxP+/- mice expressing a YFP-tagged ChR2 in PV-positive neurons.

Figure 5 | EPHB4 over-expression in PV cells reduces perisomatic inhibition of pyramidal neurons. (a) Representative image of a cortical slice from a PV-Cre/Ai32 mouse showing YFP-tagged ChR2 expression in PV cells. White arrows in this panel and the next show the positions of PV cell somas. Scale bar for panels a–c is 50 μm. (b) mCherry expression in the same field of view as in a. Here, mCherry is expressed in cells that also express Ephb4, as the same viral vector expressed both genes: AAV2/t-Syn.flex.mCherry.D2A.mEphB4. Note that the white arrows in a,b show that YFP and mCherry, and by extension ChR2 and EphB4, are co-expressed in PV cells. (c) The black arrow and white cell body identify the position of a L2/3 pyramidal neuron patched in this slice and recorded during ChR2-assisted circuit mapping of PV cell inputs. (d,e) Aggregate heat map of the spatial spread and strength of PV cell input to L2/3 pyramidal neurons in wild-type mice (d, n = 5 cells, 2 mice) and mice over-expressing Ephb4 in PV cells (e, n = 5 cells, 2 mice). Scale bar is 200 μm. (f) Laminar distribution of average PV cell input derived from the heat maps in d,e. Separation between dashed lines is 20 pA. Points are mean +/− s.e.m. Kruskal-Wallis one-way ANOVA.

Rooted in deficits in inhibition. The data we present here show that in PV cells, Pten deletion promotes the disassembly of their synaptic input to local pyramidal neurons. This effect is specific to these synapses, as we found no evidence for changes in excitatory input to mutant PV cells or in inhibitory connections between mutant PV cells. These results, along with those of other investigators, identify an essential link between PTEN signalling and the establishment of somatic inhibition.
Fig. 6 | Visual experience regulates Pten and EphB4 mRNA levels in PV cells. (a) Schematic overview of the approach taken to isolate live PV cells from primary visual cortex in mice raised with normal vision and in mice raised in the dark from postnatal day 8 to postnatal day 35. PV cells expressing tdTomato were fluorescently sorted, live cells were selected, RNA libraries were constructed, cDNA libraries were made, and the expression of Pten and EphB4 message was quantified using real-time qPCR. (b) Plot of log2 fold change in expression of Pten and EphB4 message was quantified using real-time PCR. All values were normalized to the wild-type values. Note the decrease in Pten and increase in EphB4 in the absence of vision. Mean values are Pten dark reared: 0.56, EphB4 dark reared: 2.65. Orange circles are values of individual mice (n = 3 mice, >40,000 PV isolated from each mouse).

PV cells that were either hemizygous for Pten (experimental) or expressed both copies of Pten (littermate controls).

ChR2-assisted circuit mapping. Briefly, whole-cell recordings were obtained from pyramidal neurons in layer 2/3 of the primary visual cortex of PV-Cre

#### Fluorescent-activated cell sorting.

Fluorescent-activated cell sorting was performed at the UCLA Flow Cytometry Core using a FacsARIA cell sorter. In brief, 500 μm thick coronal cortical slices containing the visual cortex were cut in standard artificial cerebrospinal fluid (ACSF). A Worthington Biochemical Papain Dissociation kit was used to dissolve the extracellular matrix to obtain individual cells, which were then filtered through a 70 μm diameter mesh and labelled with the dead cell stain 7AAD. Cells were laser sorted for peridin-chlorophyll protein complex (PerCP) signals (7AAD) and phycoerythrin (PE) signals (td-Tomato). Cells from wild-type animals with no fluorescent proteins were used to calibrate PerCP and PE signals each time. Cells with high PE signals and low PerCP signals were collected. We looked to obtain greater than 60,000 live PV cells per sorting session.

RNA amplification. RNA from collected PV cells was extracted with the Qiagen RNeasy Mini kit and then stored at −80 °C. RNA quality and quantity were assessed with the Bioanalyzer (Agilent). Only samples with RNA Integrity Number > 8 were subsequently used. cRNA was then generated and amplified with the NuGEN Ultra Low Mass kit (Ovation). Microarray. Microarray experiments were performed at the UCLA Neuroscience Genomics Core. cRNA levels were measured using Illumina mouse arrays (MouseWG-6 v2.0 BeadChip). Identification of differential gene expression was accomplished by first normalizing the gene expression of each sample and then comparing the gene expression of Pten

#### qrt-PCR.

Real-time quantitative PCR of the same cRNA libraries was used to validate hits (here, EphB4). Approximately 300–500 ng of RNA from the amplified control and Pten+/- samples was set aside prior to the microarray experiments. Thus, qrt-PCR was performed on the same samples used in the microarray experiments. Total RNA from each sample was treated with DNase I and converted into cDNA. Assays were performed in triplicate and analysed using an ABI 7700 instrument. The fold change was calculated using both standard curve analysis and the Pfaffl method.

Immunostaining. P16-P42 mice were perfused with 1 x PBS followed by 4% PFA. Brains were removed and stored in 4% PFA overnight and transferred to a 30% sucrose solution with 0.05% Na3Prior to cryosectioning. Brains were
cryosectioned in the sagittal or coronal plane through primary visual cortex at 50 μm thickness per section. Free-floating sections were washed three times with 1 x PBS prior to being placed in a 2 N HCl solution for 17 min for antigen retrieval of EphB4. They were subsequently washed three times in 1 x PBS and blocked with 15% Normal Goat Serum in 0.5% Triton for 1.5 h at room temperature. The sections were then placed in a solution containing a monoclonal mouse anti-EphB4 primary antibody (1:50, Invitrogen) with 0.5% Triton overnight on a shaker in 4°C. The following day, slices were washed and incubated with an Alexa488-conjugated goat anti-mouse secondary antibody (1:1000, Invitrogen) for 2.5 h at room temperature on a shaker.

AAV-EphB4 virus injection. Through a 2.5 mm craniotomy centred on primary visual cortex, we made 3-4 injections of AAV2/1-e-Syn.FlexON.mCherry-T2A.mEphB4 (1.2 x 10^13 GC/ml). Injection sites were roughly 200 μm apart (forming a triangle with three injection sites or a square with four injection sites). Thin walled glass pipettes were pulled to have a long tapered tip with an opening that is <1 μm wide. The glass pipette was filled with ~2 μl of virus and the tip of the pipette was gently broken using a Kimwipe to allow a small drop of virus to push through. The pipette was lowered to a depth of 300 μm below the surface of the cortex. Using a pressure of 15-20 psi, 1 msec puffs of virus were given 40–50 times with 2 seconds separating each puff. Then, the pipette was raised 50 μm towards the surface of the cortex and again 30–40 puffs were injected. This was repeated for every 50 μm up to 100 μm below the pial surface. Mice were euthanized 3 weeks post injection to allow sufficient time for EphB4 expression. Cortical slices prepared and ChR2-assisted circuit mapping was conducted as above.

Statistical analyses and graphs. All data are reported as mean ± standard error of the mean, except for data in Fig. 3c where the probability of connection is reported between patched pairs of cells. Significance in Fig. 3c was determined using Fisher’s exact test. For the remaining data sets, when comparing two independent groups, a Wilcoxon rank-sum test was used. In the case more than two independent groups were compared a Kruskal-Wallis one-way ANOVA was used and followed by post-hoc comparisons when justified (alpha set to 0.05) using Wicoxon rank-sum tests. In the cases where more than two groups were compared and the groups were not independent, a repeated measures ANOVA (Friedman’s test) was used. In all cases, sample size is defined as cell number.

Data availability. All analyses were conducted in Matlab (Mathworks, Natick, MA). The code and data are available on request from the authors.

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Author contributions
A.B., X.X. and J.T.T. designed the experiments. A.B. carried out all of the paired patch recordings and analysed these data sets with J.T.T. A.B. and E.T. carried out the experiments on FAC sorting and rtPCR and analysed these data sets with J.T.T. T.I. carried out the parvalbumin immunostaining in Figure 1 and the ChR2 mapping studies in Figures 2 and 5 and analysed these data sets with X.X. A.B. and J.T.T. wrote the paper with input from X.X.

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