Multiple autism risk genes converge on the regulation of mTOR signalling, which is a key effector of neuronal growth and connectivity. We show that mTOR signalling is dysregulated during early postnatal development in the cerebral cortex of germ-line heterozygous Ptene (Ptene+/−) mutant mice, which model macrocephaly/autism syndrome. The basolateral amygdala (BLA) receives input from subcortical-projecting neurons in the medial prefrontal cortex (mPFC). Analysis of mPFC to BLA axonal projections reveals that Ptene+/− mice exhibit increased axonal branching and connectivity, which is accompanied by increased activity in the BLA in response to social stimuli and social behavioural deficits. The latter two phenotypes can be suppressed by pharmacological inhibition of S6K1 during early postnatal life or by reducing the activity of mPFC–BLA circuitry in adulthood. These findings identify a mechanism of altered connectivity that has potential relevance to the pathophysiology of macrocephaly/autism syndrome and autism spectrum disorders featuring dysregulated mTOR signalling.
The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that integrates environmental cues (for example, growth factors and energy status) to control cell growth and proliferation. mTOR interacts with several proteins to form at least two distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). When activated, mTORC1 promotes protein synthesis mainly by phosphorylation of ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (eIF4E-BP)2. Phosphorylation of eIF4E-BP by mTORC1 releases eIF4E from binding to eIF4E-BP, forming the eIF4F complex to initiate cap-dependent protein translation. Phosphorylation of ribosomal protein S6 (rpS6) by S6K correlates with the translational efficiency of messenger RNAs containing a tract of oligopyrimidine in the 5'UTR, named 5'TOP messenger RNA3. Phosphorylated rpS6 (p-S6) is a readout for mTORC1 signalling, and has been shown to regulate cell size. Moreover, studies in the mammalian nervous system have shown that mTOR-S6K signalling regulates neuronal soma size, dendritic arborization, axonal growth and connectivity.

Numerous lines of evidence implicate dysregulated mTOR signalling in the pathogenesis of autism spectrum disorder (ASD) and related neurodevelopmental disorders. Genes impinging on the PI3K-Akt-mTOR pathway, for example, phophatase and tensin homologue (PTEN), TSC1/2, NFI and FMR1, are highly represented amongst ASD risk genes identified to date. Elevated mTOR signalling in the cerebral cortex has been reported in postmortem samples from individuals with autism, and several animal models of risk genes acting in this pathway also show altered mTOR signalling in the brain.

PTEN encodes a phosphatase that is a negative regulator of the PI3K-Akt-mTOR pathway. Germ-line heterozygous mutations in PTEN are found in ~7–17% individuals with autism and macrocephaly (head circumference >2 s.d.’s above normal), generally reduce PTEN protein levels, and cause macrosomia, cognitive delay and autistic features. Numerous lines of evidence implicate dysregulated mTOR signalling in the pathogenesis of autism spectrum disorder (ASD) and related neurodevelopmental disorders. Genes impinging on the PI3K-Akt-mTOR pathway, for example, phophatase and tensin homologue (PTEN), TSC1/2, NFI and FMR1, are highly represented amongst ASD risk genes identified to date. Elevated mTOR signalling in the cerebral cortex has been reported in postmortem samples from individuals with autism, and several animal models of risk genes acting in this pathway also show altered mTOR signalling in the brain.

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we detected increased soma size in layer V neurons, but not in layers II–IV or VI neurons, in P14 Pten+/− mice as compared with WT mice (Fig. 1d,e). This indicates that the growth of cell types enriched for p-S6 (for example, layer V neurons), as reflected by cell soma size, is desynchronized relative to neurons with lower levels of p-S6 (for example, layers II–IV or VI neurons) in Pten+/− mice.

As we observed enrichment of p-S6 in developing subcortical-projecting layer V neurons, amongst other cell types, and we detected a pronounced change in cell soma size in layer V
neurons in Pten<sup>+/−</sup> mice, we decided to examine these as a representative of cell types whose growth is impacted by Pten haploinsufficiency. To understand whether overgrowth of layer V neurons is restricted to the cell soma or also occurs in neurites, we examined dendritic structure. We made use of a genetic reporter for layer V neurons (Etv1-CreERT2; Ai14<sup>30,31</sup>) and measured dendritic tree size of these cells in P14 Pten<sup>+/−</sup> mice (Etv1-CreERT2; Ai14; Pten<sup>+/−</sup>). We found that, relative to controls, layer V neurons in Pten<sup>+/−</sup> mice exhibited increased cell soma volume and dendritic complexity (Fig. 1f–l). Thus, changes in neuronal soma size in layer V neurons caused by Pten haploinsufficiency correlate with changes in neurite growth.

To explore whether dysregulated neurodevelopmental mTORC1 signalling might be a point of convergence across multiple mouse models of autism risk genes, we performed these analyses in another mouse model of an autism risk gene, Fmr1<sup>+/−</sup>. We found that, similar to Pten<sup>+/−</sup> mice, the spatiotemporal distribution of p-S6 in the cerebral cortex is not grossly altered, and p-S6 levels are elevated at P14 as compared with WT, but not other ages (Supplementary Fig. 2A–C), indicating that cortical mTORC1 signalling is transiently elevated during early postnatal life. Measuring cell soma size across cortical layers, we found that layer V neurons are enlarged in P14 Fmr1<sup>+/−</sup> mice as compared with WT mice (Supplementary Fig. 2D,E). Consistent with overgrowth in layer V neurons, dendritic tree size is also slightly greater in P14 Etv1-CreERT2; Fmr1<sup>+/−</sup> mice as compared to control littersmates (Supplementary Fig. 2F–L). This suggests that multiple autism risk genes may converge on dysregulated neurodevelopmental mTORC1 signalling and desynchronized neuronal growth.

**Early inhibition of S6K1 corrects deficits in Pten mutants.** The observation that p-S6 levels are transiently dysregulated in the cerebral cortex of P8 Pten<sup>+/−</sup> mice led us to speculate that this could represent a critical period where normalization of p-S6 levels may suppress the overgrowth of layer V neurons and behaviour deficits in these animals. To test this possibility, we treated Pten<sup>+/−</sup> mice and WT controls with the S6K1 inhibitor PF-4708671 (ref. 32), injected daily from P4 to P14 (Fig. 2a). We found that systemic PF-4708671 treatment reduced elevated p-S6 in the cerebral cortex and suppressed the increased soma size of layer V neurons in the somatosensory cortex of P14 Pten<sup>+/−</sup> mice (Fig. 2b,c). The most robust and consistent behavioural phenotype we have observed in Pten<sup>+/−</sup> mice is deficits in the three-chamber social approach test in adult females<sup>20–22</sup>. Adult male Pten<sup>+/−</sup> mice, in contrast to females, display variable phenotypes in this assay that are highly sensitive to testing conditions<sup>20,21</sup> (Supplementary Fig. 3), so three-chamber social approach testing in this study focused on female animals. We found that PF-4708671 treatment in the P4–P14 developmental window is sufficient to reverse the three-chamber social approach deficit present in vehicle-treated adult female Pten<sup>+/−</sup> mice (Fig. 2d). We also observed that WT mice receiving PF-4708671 treatment displayed social approach deficits, consistent with a
previous report of reduced social interaction in WT mice receiving rapamycin treatment beginning at 5–6 weeks of age39. Thus, we speculate that an optimal level of mTORC1 signalling is required during development for normal social behaviour, with too much or too little activity resulting in similar social behavioural deficits, in line with a previously proposed model of bidirectional control of protein synthesis and connectivity in ASD23. Taken together, these results point to a developmental window in early postnatal life in which social behaviour is sensitive to modulation of mTOR/S6K1 signalling and connectivity in ASD23. Implied in these two areas is the processing of the three-chamber social approach deficits in Pten+/− mice.

In addition to the core symptoms of ASD, some individuals with germ-line heterozygous Pten mutations also have deficits in working memory17. To explore whether germ-line Pten+/− mice also show working memory deficits and whether this phenotype may be sensitive to modulation of mTOR/S6K1 signalling and connectivity in ASD23. Considering that layer V neurons in the cerebral cortex are hypertrophic in Pten+/− mice (Supplementary Fig. 5A–C), consistent with our observation of decreased synaptic function and c-fos activation in the mPFC and BLA of both sexes showed normal performance in this test (Supplementary Fig. 4B,C), indicating that the particular aspect of working memory probed by the non-matching to sample T-maze test is not altered in these animals.

Neuronal hyperconnectivity in Pten mutants. Considering that layer V neurons are the primary output neurons of the cerebral cortex, we speculated that corticol–subcortical connectivity between areas important for social behaviour might be altered in these animals. A circuit that is particularly interesting in this context involves projections from the mPFC to the BLA (see Fig. 3a). Both of these areas are implicated in the processing of social information23 and autism pathophysiology24. Thus, we formulated the hypothesis that dysregulated mTORC1 signalling during a critical period of development alters neuronal connectivity, including mPFC–BLA projections, and social behaviour.

To test this hypothesis, we first confirmed that BLA-projecting mPFC neurons are hypertrophic in Pten+/− mice (Supplementary Fig. 5A–C), consistent with our observation of layer V neuron hypertrophy in somatosensory cortex (Fig. 1e). Furthermore, we found that protein synthesis and p-S6 levels are dysregulated in the prefrontal cortex of Pten+/− mice, indicating dysregulated mTORC1 activity (Supplementary Fig. 5D–G). We next injected reporter-expressing (tdTomato) adeno-associated virus (AAV) into the mPFC of adult female mice and visualized axon terminal branching and synaptic boutons within the BLA (Fig. 3a). We found that axons originating from the mPFC in vehicle-treated Pten+/− mice displayed excessive branching and synaptic boutons within the BLA, as compared with vehicle-treated WT mice, and that PF-4708671 treatment during development (P4–P14) was sufficient to reverse this phenotype in Pten+/− mice (Fig. 3b–e). These results indicate that mPFC–BLA circuitry is hyperconnected in Pten+/− mice, and that this phenotype is sensitive to early postnatal PF-4708671 treatment.

As an initial exploration into the molecular mechanism by which dysregulated mTORC1 signalling might lead to altered axon branching and structural connectivity, we measured levels of CRMP2 (collapsin response mediated protein 2) and Tau (microtubule associated protein), both of which are regulated by mTORC1 signalling and have been shown to influence axon growth35,36. We observed a significant increase in CRMP2 levels and a trend towards increased Tau levels in P8 Pten+/− cerebral cortex (Fig. 3f,g). Actin remodelling is a regulator of axon growth, and homozygous Pten mutations can alter actin levels37. Thus, we measured β-actin levels and did not observe any differences between WT and Pten−/− cortex (Fig. 3g), indicating that this is a valid loading control and that heterozygous Pten mutations do not grossly alter β-actin levels, unlike homozygous Pten mutations. Since the excessive axon branching in Pten+/− mice was suppressed by pharmacological inhibition of S6K1 during early postnatal life, we suspected that this effect could be due to the inhibition of S6K1-dependent translation of CRMP2 (ref. 36). To test this possibility, we measured CRMP2 levels in the P8 cerebral cortex of Pten+/− and WT control mice that received PF-4708671 treatment from P4 to P8, by western blot. We found that PF-4708671 treatment suppressed elevated CRMP2 in Pten+/− mice (Fig. 3h,i). Thus, normalisation of excessive axon branching in Pten+/− mice by PF-4708671 treatment correlates with normalization of CRMP2 levels in these animals.

Hyperactivity in the mPFC and BLA of Pten mutant mice. To test if neuronal activity in response to a social cue is altered in Pten+/− mice, we measured the number of c-fos+ neurons in the mPFC and BLA in response to exposure to a novel stimulus mouse (Fig. 4a). Previous work has shown that social exposure induces c-fos activation in the mPFC and BLA, and that the number of c-fos+ cells in these two regions correlates with the amount of time engaged in social interaction38. Consistent with this, we found that exposure to a novel social stimulus results in a robust induction of c-fos+ cells in the mPFC and BLA of both WT and Pten+/− adult female mice (Fig. 4b–d) and that the number of c-fos+ cells in these two regions correlated with the duration each mouse spent sniffing the novel stimulus mouse (Supplementary Fig. 6A). Although the total number of c-fos+ cells in the mPFC and BLA is not altered in Pten+/− mice compared with WT mice (Fig. 4c,d), we found that social interaction time, as reflected by time spent sniffing, varied between and within genotype during the social exposure period (Fig. 4e). Because the induction of c-fos+ cells in the mPFC and BLA is dependent on the amount of social interaction time, we calculated a c-fos activation index (number of c-fos+ neurons/time mouse engaged in sniffing; Supplementary Fig. 6B). We found that the c-fos activation index is elevated in the mPFC and BLA of vehicle-treated Pten+/− mice. Furthermore, we found that the elevated c-fos activation index in the mPFC and BLA is normalized in Pten+/− mice receiving PF-4708671 treatment during early postnatal life (P4–P14; Fig. 4f,g). We confirmed this result in a separate cohort of mice in which the animals were allowed to sniff a novel stimulus mouse to a fixed criterion of 30 s, thus ensuring equivalent amounts of exposure to the social cue across subjects. In this cohort, we found that c-fos+ cells in the mPFC and BLA are elevated in Pten+/− relative to WT mice, and this phenotype is rescued by PF-4708671 treatment during early postnatal life (Supplementary Fig. 7). Thus, with equivalent social exposure, there are more c-fos+ cells in the mPFC and BLA of Pten+/− mice, and this phenotype can be normalized by suppression of S6K1 during development.

Adult S6K1 inhibition does not rescue Pten mutant deficits. We next asked whether mPFC–BLA connectivity and social phenotypes in Pten+/− mice might be responsive to PF-4708671 treatment in adulthood, when cortical circuitry has reached a mature state. To address this issue, we treated adult Pten+/− mice with the equivalent dosage and duration of PF-4708671 as that of developmental experiments and tested social behaviour 7 days after the final injection of PF-4708671 (Fig. 5a). We found that the three-chamber social approach deficit is not
Corrected in Pten⁺⁻ mice treated with PF-4708671 in adulthood (Fig. 5b). This result prompted us to investigate mPFC–BLA structural connectivity and c-fos reactivity in these mice. We found that excessive axon branching and increased density of synaptic boutons in mPFC to BLA projections was not rescued in Pten⁺⁻ mice receiving PF-4708671 treatment in adulthood (Fig. 5c–f). The elevated c-fos activation index in the mPFC and BLA in response to social exposure was also not rescued in Pten⁺⁻ mice treated with PF-4708671 in adulthood (Fig. 5g–k). Taken together, our PF-4708671 treatment results indicate that there is a developmental critical period in which pharmacological suppression of S6K1 can cause a lasting rescue of mPFC–BLA hyperconnectivity, excessive c-fos reactivity and three-chamber social approach deficits in Pten⁺⁻ mice.

**Hyperactivity of mPFC-BLA circuitry in Pten mutants.** To test whether Pten⁺⁻ mice exhibit hyperactive mPFC–BLA circuitry in response to social cues, we used c-fos expression as a measure of neuronal activity in BLA-projecting mPFC neurons in WT and Pten⁺⁻ mice. To label BLA-projecting mPFC neurons, we injected an AAV vector carrying the reporter mCherry, expressed in a Cre-dependent manner (double-floxed inverted open reading frame) into the mPFC, and a retrograde-transporting canine adenovirus carrying Cre recombinase (CAV2-Cre) into the BLA frame) into the mPFC, and a retrograde-transporting canine adenovirus carrying Cre recombinase (CAV2-Cre) into the BLA in a Cre-dependent manner (double-floxed inverted open reading frame). 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neurons) were c-fos+ in Pten+/− mice as compared with WT mice (Fig. 6b,c), suggesting that mPFC–BLA circuitry is hyperactive in response to social exposure in Pten+/− mice.

To test whether decreasing activity in mPFC to BLA projections may normalize three-chamber social approach deficits in Pten+/− mice, we reduced excitability in mPFC neurons projecting to the BLA using retro-DREADD (Designer Receptor Exclusively Activated by Designer Drugs) technology39. An AAV vector carrying hM4Di and mCherry expressed in a Cre-dependent manner was bilaterally injected into the mPFC, and a CAV2-Cre vector was bilaterally injected into the BLA of adult female Pten+/− mice (Fig. 6d,e). Clozapine n-oxide (CNO) or vehicle was injected into these mice, and we then tested social behaviour using the three-chamber social approach assay. We found that the three-chamber social approach deficit present in vehicle injected hM4Di expressing Pten+/− mice or vehicle and CNO injected mCherry expressing Pten+/− mice was corrected in CNO injected hM4Di expressing Pten+/− mice (Fig. 6f).

We next measured c-fos+ cells in the mPFC and BLA of these mice in response to sniffing a novel stimulus mouse to a fixed criterion of 30 s. We found that the number of c-fos+ cells in the mPFC and BLA in response to social exposure was reduced in CNO injected compared with vehicle injected hM4Di expressing Pten+/− mice, and we found no change in the number of c-fos+ cells in either vehicle or CNO injected mCherry expressing

Figure 4 | mPFC and BLA hyperactivity in Pten mutant mice is corrected by inhibition of S6K1 during development. (a) Diagram showing the workflow of social exposure and c-fos staining. Adult mice were exposed to a novel mouse for 20 min, perfused 2 h later, and then underwent c-fos staining to measure neuronal activity in the mPFC and BLA. (b–d) Representative images (b) and quantification of c-fos staining in the mPFC (c) and the basolateral amygdala (BLA) (d) of control or social exposed WT and Pten+/− mice receiving either vehicle or PF-4708671 from postnatal day 4 (P4) to P14. Scale bar, 100 μm. N = 4 animals in each group. (e) Graph showing social interaction time, reflected as time spent sniffing, in WT and Pten+/− mice receiving either vehicle or PF-4708671 from P4 to P14. N = 4 animals in each group. (f,g) Quantification of c-fos activation index (number of c-fos+ neurons/time spend sniffing) in the mPFC (f) and BLA (g) of social exposed WT and Pten+/− mice receiving either vehicle or PF-4708671 from P4 to P14. Planned comparisons revealed a significant difference between WT and Pten+/− mice receiving vehicle, and a significant difference between vehicle and PF-4708671-treated Pten+/− mice. For (f) two-way ANOVA revealed a main effect on drug, F(1,12) = 3.194, P = 0.099 (interaction), F(1,12) = 7.069, P < 0.05 (drug), F(1,12) = 3.947, P = 0.07 (genotype). For (g) two-way ANOVA revealed main effects on genotype and interaction. F(1,12) = 16.8, P < 0.01 (interaction), F(1,12) = 0.571, P = 0.464 (drug), F(1,12) = 4.901, P < 0.05 (genotype). *P < 0.05 and **P < 0.01. N = 4 animals in each group. All mice used in this figure were female.
Reducing mTOR activity corrects deficits in Pten mutant mice. To determine whether isolating Pten\(^{+/−}\) mutations to the cerebral cortex will lead to three-chamber social approach deficits, we generated conditional Pten\(^{+/−}\) mutant mice using Emx1-Cre,
Figure 6 | Reducing activity in the mPFC to BLA projections corrects social behavioural deficits in Pten mutant mice. (a) Diagrams showing experimental design. To label BLA-projecting mPFC neurons, CAV2-Cre was injected into the BLA, and AAV-DIO-mCherry was injected into the mPFC. Social exposure was controlled for 30 s sniffing time. (b) Representative images of mCherry (red) and c-fos (green) double labelling in the mPFC of WT and Pten+/− mice. Scale bar, 100 μm. (c) Quantification of the percentage of c-fos+ neurons within the mCherry+ neuronal population. Independent-sample t tests were used. *P<0.05. N = 6 animals per genotype. (d) Diagrams showing the injection of CAV2-Cre into the BLA and AAV-DIO-mCherry or AAV-DIO-hM4Di-mCherry into the mPFC of Pten+/− mice. (e) Images showing reporter (mCherry) expression in mPFC. PrL: prelimbic cortex. IL: infralimbic cortex, with magnified images in (e). Scale bar, 500 μm (left and middle) and 100 μm (right). (f) Per cent time spent in each chamber during the three-chamber social approach test. N = 8 mice in Pten+/− (hM4Di) group and 9 mice in Pten+/− (mCherry) group. Two-way ANOVA and Sidak’s post hoc tests were used. For mCherry group, F(1,32) = 0.0569, P = 0.813 (interaction), F(1,32) = 0.02535, P = 0.8745 (treatment) and F(1,32) = 0.1798, P = 0.6744 (chamber time). For hM4Di group, F(1,28) = 32.40, P < 0.001 (interaction), F(1,28) = 0.00068, P = 0.9794 (treatment) and F(1,28) = 63.12, P < 0.001 (chamber time). **P<0.001 and NS indicates not significant difference. (g,h) Images (g) and quantification (h) of c-fos staining in the mPFC and BLA of social exposed Pten+/− mice that expressed either hM4Di plus mCherry or mCherry alone in BLA-projecting mPFC neurons and received either vehicle or CNO injections. Scale bar, 100 μm. N = 4 mice in hM4Di expressing Pten+/− mice receiving either vehicle or CNO, N = 5 mice in vehicle injected, and 4 mice in CNO injected, mCherry expressing Pten+/− mice. Independent-sample t tests were used. *P<0.05. All mice used in this figure were female.
Suppression of mTOR activity corrects cellular and behavioural deficits in Pten mutant mice. (a) Schema showing the brain regions where one copy of Pten is deleted (green colour, primarily in the cerebral cortex). (b) Per cent time control and Emxl-Cre+/Ptenloxp/+ mice spent in each chamber. Two-way ANOVA was used. F(1,56) = 0.8877, P = 0.3502 (interaction), F(1,56) = 0.3242, P = 0.5714 (genotype) and F(1,56) = 11.16, P = 0.0015 (chamber time). Paired t-tests showed that control, but not Emxl-Cre+; Ptenloxp/+, mice exhibited a significant preference for the social chamber. **P<0.01. N = 15 animals in each genotype. (c) Schema showing the brain regions where one copy of Rptor is deleted in Pten+/− mice (red colour, primarily in the cerebral cortex). (d,e) Representative images (d) of western blot for p-S6, total S6 and β-actin in the cerebral cortex of control (Rptorloxp+/−), Pten+/− (Rptorloxp+/−; Pten+/−), Emxl-Cre+; Rptorloxp/+, and Emxl-Cre+; Rptorloxp/++; Pten+/− mice. (e) Quantification of p-S6 levels relative to total S6, expressed as per cent of control. One-way ANOVA and Tukey post hoc tests were used. F(3,25) = 4.153, P < 0.05 (genotype), *P < 0.05. N = 8 control, 8 Pten+/−, 6 Emxl-Cre−; Rptorloxp/+, and 7 Emxl-Cre+; Rptorloxp/++; Pten+/− mice. (f) Representative images of fluorescent Nissl staining in somatosensory cortical layer V, and quantification of layer V cell soma size in control (Rptorloxp+/−), Pten+/− (Rptorloxp+/−; Pten+/−), Emxl-Cre+; Rptorloxp/+, and Emxl-Cre+; Rptorloxp/++; Pten+/− mice. One-way ANOVA and Tukey post hoc tests were used. F(3,16) = 9.183, P < 0.001 (genotype), **P < 0.01. N = 5 animals per genotype. Scale bar, 25 μm. (g) Per cent time mice spent in each chamber. Two-way ANOVA and Sidak’s post hoc tests were used. F(3,60) = 3.902, P = 0.013 (interaction), F(3,60) = 0.0842, P = 0.9684 (genotype) and F(1,60) = 60.65, P < 0.001 (chamber time). *P < 0.05, and **P < 0.01. N = 8 control (Rptorloxp+/−). 8 Pten+/− (Rptorloxp+/−; Pten+/−), 10 Emxl-Cre−; Rptorloxp/+, and 8 Emxl-Cre+; Rptorloxp/++; Pten+/− mice. All mice used in this figure were female.

which is expressed primarily in cortical pyramidal cells throughout development40 (Fig. 7a). We used a Cre driver that is expressed broadly in the cortex, rather than a Cre driver that is restricted to subcortical-projecting pyramidal cells, because we do not know at present if the effects of Pten+/− mutations on the development of this neuronal population are cell autonomous or non-cell autonomous. We have previously shown that Emxl-Cre−; Ptenloxp/+ mice recapitulate the cortical overgrowth phenotype seen in germ-line Pten+/− mice19. Testing social behaviour using three-chamber social approach test, we found that although adult female Emxl-Cre−; Ptenloxp/+ mice did not display a significant difference in allocation of chamber time as compared with controls, a within-group analysis revealed a lack of significant difference in time spent in the stimulus mouse versus object chambers (Fig. 7b).

To test whether reducing mTORC1 signalling in the cerebral cortex of germ-line Pten+/− mice is sufficient to correct three-chamber social approach deficits, we generated Emxl-Cre−; Rptorloxp/++; Pten+/− mice (Fig. 7c). We found that dysregulated p-S6 in P8 Pten+/− cerebral cortex was normalized in Emxl-Cre−; Rptorloxp/++; Pten+/− mice (Fig. 7d,e). We measured cell soma size as a marker for layer V neuron structural changes in Pten+/− mice and found that layer V neuronal hypertrophy was
suppressed in Emx1-Cre⁺; Rptorloxp/+; Pten⁺/− mice in adulthood (Fig. 7f). Moreover, the three-chamber social approach deficit present in adult female Pten⁺/− mice was also corrected in Emx1-Cre⁺; Rptorloxp/+; Pten⁺/− mice (Fig. 7g). Taken together, our results indicate that dysregulated mTOR signalling in the developing cerebral cortex contributes to social behavioural deficits in Pten⁺/− mice. Future experiments will examine whether conditional Pten⁺/− mutation or modulation of mTORC1 signalling specifically in subcortical-projecting pyramidal cells is sufficient to alter three-chamber social approach behaviour.

Discussion

The genetic causes of ASD are heterogeneous. Studying a signalling pathway regulated by multiple autism risk genes at the level of circuit function will provide insights into common biological mechanisms underlying ASD pathogenesis and pathophysiology. Here we have presented evidence that dysregulated mTORC1 signalling impacts the assembly of circuitry underlying social behaviour in germ-line Pten⁺/− mice, a mouse model for macrocephaly/autism syndrome.

We have mapped the spatiotemporal activity of mTORC1 signalling in developing mouse brain, and found that a subset of cells, including subcortical-projecting layer V neurons in the cerebral cortex, exhibit enrichment for p-S6, a readout for mTORC1 signalling. We further investigated cortical–subcortical connectivity, which is largely mediated by subcortical-projecting layer V neurons, between two brain areas that are implicated in the processing of social information and ASD pathophysiology, the mPFC and BLA. We show that Pten⁺/− mice exhibit hyperconnectivity and hyperactivity of mPFC–BLA circuitry, excessive c-fos reactivity in response to social cues, and three-chamber social approach deficits. Correcting structural connectivity by treatment with a pharmacological inhibitor of S6K1 during early postnatal life or modulating neuronal activity by DREADDs in adulthood can suppress these phenotypes.

We find that Pten⁺/− mice display non-uniform overgrowth across neuronal cell types in the developing cerebral cortex, indicating that the growth of some neuronal populations (for example, subcortical-projecting layer V neurons) is desynchronized relative to others. This finding has implications for information processing within cortical microcircuitry. Fmr1−/− mice, which model fragile X syndrome, display a similar phenotype. One possible explanation for this finding is that mutations in genes that encode negative regulators of mTORC1 signalling, such as Pten and Fmr1, have the greatest effect on growth in cell types with high baseline levels of mTORC1 activity (for example, layer V neurons) because these cells are more dependent on negative regulation by repressive factors. It is worth contrasting the effects of heterozygous and homozygous Pten mutations in light of this explanation. Studies from Pten conditional knockout mice have shown that even relatively small neural cell types, such as hippocampal granule cells, exhibit overgrowth on conditional homozygous mutation of Pten (ref. 11). Indeed, we have found that conditional homozygous mutation of Pten leads to massively increased p-S6 levels and decreased cell density throughout the newborn cerebral cortex, consistent with widespread hypertrophy, whereas we did not observe this phenotype upon conditional heterozygous mutation of Pten (ref. 19). We attribute this to an effect of Pten dosage: homozygous mutation of Pten shifts a wide variety of neural cell types towards a pathological state, with strong upregulation of mTORC1 activity and hypertrophy. In contrast, most neural cell types appear to maintain more or less normal physiological levels of mTORC1 signalling upon heterozygous mutation of Pten.

The normal developmental trajectory of mTORC1 activity in the brain may help to explain our finding of transient changes in cortical p-S6 levels in early postnatal life in germ-line Pten⁺/− and Fmr1−/− mice. One study that examined mTORC1 signalling in the cerebral cortex of WT mice in both early postnatal life and adulthood found that mTORC1 signalling is most active during early postnatal life, especially the first two postnatal weeks41. Just as cell types with high baseline levels of mTORC1 signalling may be particularly vulnerable to the effects of dominant-acting mutations in genes that encode negative regulators of mTORC1 signalling, neurodevelopmental time windows of peak mTORC1 activity (for example, early postnatal life) might represent critical periods of vulnerability to such mutations, as well as targets for therapeutic intervention.

We found that in the mature brain, axonal projections from the mPFC to the BLA have excessive branches and synaptic boutons. Given that elevated cortical p-S6 and CRMP2, and rescue by S6K1 inhibitor treatment, all occur during the early postnatal life, we predict that the initial growth of subcortical-projecting neurons is over-exuberant in Pten⁺/− mice. This would be consistent with reports of Pten and mTOR-S6K signalling acting as regulators of axonal growth7,11. Another factor that could be at play is reduced axonal pruning of subcortical-projecting neurons in Pten⁺/− mice. This is an interesting possibility given that a recent study has reported reduced dendritic spine pruning in cortical layer V cells of individuals with ASD and in Tsc2 mutant mice, with this phenotype resulting from a disruption of mTOR-dependent macroautophagy9. The contribution of mTORC1-mediated axonal branching to the white matter abnormalities reported in individuals with ASD17,42,43 will be an interesting area of future research.

This study has focused on connectivity between the mPFC and BLA, as this circuit is implicated in ASD pathophysiology and has relevance for the three-chamber social approach deficit observed in germ-line Pten⁺/− mice. However, we anticipate that altered connectivity is unlikely to be restricted to this circuit, given that we have observed p-S6 enrichment and neuronal overgrowth outside of the prefrontal cortex (for example, in layer V neurons in the somatosensory cortex). Given the relatively selective behavioural deficits in germ-line Pten⁺/− mice20, we predict that the behavioural profile of these animals is a product of altered 'hard-wired' connectivity across multiple circuits, together with the capacity of specific circuits to adapt to these changes during development via synaptic plasticity mechanisms. Indeed, we have found that dopaminergic neurons are enriched for p-S6 during development (Supplementary Fig. 1D). Considering that dopaminergic neurons in the ventral tegmental area encode and modulate features of social interaction44, dopamine signalling plays a key neuromodulatory role in the PFC and limbic system45,46, and conditional deletion of Pten in dopaminergic neurons results in three-chamber social approach deficits20, we speculate that mesocorticolimbic circuitry may be especially vulnerable to the effects of abnormal structural and functional connectivity resulting from Pten haploinsufficiency.

We note that both increased and decreased brain network connectivity has been reported in individuals with ASD47. While we have found evidence for hyperconnectivity in our model of macrocephaly/autism syndrome caused by germ-line heterozygous Pten mutations, it is likely that distinct subgroups of individuals with ASD may display different connectivity endophenotypes that correlate with underlying risk factors. It is possible that either increased or decreased connectivity may cause similar behavioural and cognitive deficits, potentially...
resulting from imbalanced excitation and inhibition across brain circuits and areas critical for processing of social information.

An important problem for developing therapeutic approaches to psychiatric disorders is defining critical periods of phenotypic reversibility. Previous studies have shown that the mTORC1 inhibitor rapamycin, when given to Pten conditional knockout animals at different time points, is able to prevent or arrest, but not reverse, altered neurite projection patterns. Our results show that suppression of S6K1 by PF-4708671 during early postnatal life, but not in adulthood, results in a lasting correction of mPFC–BLA hyperconnectivity, excessive c-fos reactivity to social cues, and three-channel social approach deficits in Pten+/− mice. This indicates that suppression of mTOR-S6K signalling in adulthood is not sufficient to reverse structural connectivity changes (i.e. hyperconnectivity of mPFC–BLA circuitry) in Pten+/− mice. Our experimental design included a washout period for PF-4708671 because we wanted to test whether treatment with this drug during development or in adulthood results in lasting changes in structural connectivity.

We cannot exclude the possibility that overlapping PF-4708671 treatment with behavioural testing may result in modification of behavioural deficits through a mechanism independent of changes in structural connectivity.

While our data indicate that abnormal mPFC–BLA structural connectivity is hard-wired in adulthood, we find that reducing activity in mPFC–BLA projections via DREADD technology is capable of normalizing both c-fos reactivity and the three-channel social approach deficits. Thus, we propose that there are at least two distinct windows and strategies whereby social behavioural deficits in Pten+/− mice can be modulated. The first window is in early postnatal life, when suppression of mTORC1-S6K1 can prevent or arrest the development of altered mPFC–BLA structural connectivity. The second window is in adulthood, where modulation of activity in mPFC–BLA circuitry can offset the consequences of pathological structural and functional connectivity. Future work will explore the specific nature of altered structural and functional connectivity between the mPFC and BLA at the level of cell types within the BLA in Pten+/− mice and whether modulating activity of this circuit is capable of influencing social behaviour in WT animals. It will also be interesting to investigate whether the modulation of amygdala reactivity, habituation and connectivity with the prefrontal cortex that has been associated with genetic variants influencing serotonin signalling might be a substrate for modification of social behavioural endophenotypes in Pten+/− mice by second-site mutations in serotonin transporter (SERT) and pharmacological targeting of serotonin receptor signalling.

An emerging theme in the field of psychiatric disorders is a shift towards stratifying populations based on common underlying risk factors and pathophysiology, and aligning preclinical research to investigate circuit and neurophysiological correlates of these risk factors. We have shown that haploinsufficiency for Pten leads to dysregulation of a disease-implicated signalling pathway (the mTOR signalling pathway) that regulates neuronal connectivity, and altered connectivity in an autism-relevant neural circuit (mPFC-amygdala) affects activity in a key brain region (amygdala) in response to social cue, as well as social behaviour. Given that both mPFC–BLA connectivity and mTOR-S6K signalling are highly evolutionarily conserved between rodents and humans, we anticipate that the mechanism of pathogenesis and corresponding therapeutic strategy presented here may be relevant for individuals with ASD who have been exposed to risk factors associated with dysregulated mTOR signalling.

Methods

Mice. B6.129-Ptenm1Bps (Pten+/−) were obtained from the repository at the National Cancer Institute at Frederick, where they were already backcrossed onto a congenic C57BL/6J background by the Donating Investigator. The line has been maintained by backcrossing to C57BL/6J mice for more than 10 generations. All other mouse lines were obtained from the Jackson Laboratory: Emx1−/− (Cre/+; Emx1-CreERT2, stock number 004907), Gt(ROSA)26Sor (CAG-mTomato)CreERT2 (Ai14, stock number 007914), Rptor−/− DsRed reporter (stock number 013188), Emx1−/− (Cre/Cre); Emx1-CreERT2; Pten+/− mice, stock number 005628), Pten−/− (PtenloxPlox/+), stock number 006440), and Fmr1−/− mice (stock number 003025). To obtain Emx1−/−; Rptor−/− mice; Pten−/− mice, Rptor−/−; Pten−/− male mice were mated with Emx1−/−+ female mice. Rptor−/− mice were used as controls. All animal experiments were conducted in accordance with NIH and AAALAC guidelines and were approved by The Scripps Research Institute’s Institutional Animal Care and Use Committee.

Immunohistochemistry. Mouse brains were fixed in 4% PFA, incubated in a 20% sucrose/PBS solution at 4 °C for 2 days, and embedded in Tissue-Tek OCT compound (Sakura). Coronal or sagittal sections were collected on a cryostat (Leica) at 50 μm. Sections were collected from brains of mice that were 3 weeks old at the time of social exposure and from age-matched control mice. Sections were collected from mice that were 3 weeks old at the time of treatment with behavioural testing may result in modification of behavioural deficits through a mechanism independent of changes in structural connectivity.

While our data indicate that abnormal mPFC–BLA structural connectivity is hard-wired in adulthood, we find that reducing activity in mPFC–BLA projections via DREADD technology is capable of normalizing both c-fos reactivity and the three-channel social approach deficits. Thus, we propose that there are at least two distinct windows and strategies whereby social behavioural deficits in Pten+/− mice can be modulated. The first window is in early postnatal life, when suppression of mTORC1-S6K1 can prevent or arrest the development of altered mPFC–BLA structural connectivity. The second window is in adulthood, where modulation of activity in mPFC–BLA circuitry can offset the consequences of pathological structural and functional connectivity. Future work will explore the specific nature of altered structural and functional connectivity between the mPFC and BLA at the level of cell types within the BLA in Pten+/− mice and whether modulating activity of this circuit is capable of influencing social behaviour in WT animals. It will also be interesting to investigate whether the modulation of amygdala reactivity, habituation and connectivity with the prefrontal cortex that has been associated with genetic variants influencing serotonin signalling might be a substrate for modification of social behavioural endophenotypes in Pten+/− mice by second-site mutations in serotonin transporter and pharmacological targeting of serotonin receptor signalling.

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Cell size analysis. Brain sections were stained with NeuroTrace green fluorescent Nissl stain (1:100 dilution). Regions of interest (ROIs) were cropped from primary somatosensory cortex barrel field (coronal sections) using Photoshop (Adobe). Each layer (layers II–V) was further cropped according to the anatomical boundaries as judged by fluorescent Nissl stain. Cell soma size of all neurons in each layer was measured using ImageJ (NIH). For BLA-projecting mPFC neurons, mCherry+ cells were measured. The frehand tool in ImageJ was used to contour the boundary of neuronal soma. Three ROIs were measured in each animal.

Western blot analysis. Whole cerebral cortices or prefrontal cortices were extracted from mice, snap frozen in liquid nitrogen and stored at −80 °C until use. The mTORC1 inhibitor PF-4708671 was dissolved in DMSO. Both vehicle and PF-4708671 were formulated in 10% DMSO, dissolved in DMSO. Both vehicle and PF-4708671 were formulated in 10% DMSO, and vehicle was injected subcutaneously into pups from P4 to P14. For adult female mice (2–4 month of age), intraperitoneal injection of PF-4708671 (75 mg kg−1) was performed for 11 consecutive days. 75 mg kg−1 was selected based on prior studies reporting that doses of PF-4708671 in this range can suppress p-S6 levels in the adult mouse brain.

3D neuronal reconstruction. Emx1-CreERT2; A14+/−; Pten+/− and Emx1-CreERT2; A14+/−; Fmr1−/− mice, as well as controls, received subcutaneous tamoxifen injections (3 mg kg−1) at P7, and were perfused at P14.
Brains were fixed in 4% PFA, incubated in a 20% sucrose/PBS solution at 4 °C for 3 days, and embedded in Tissue-Tek OCT compound. Coronal sections (50 μm thick) from the primary somatosensory cortex were collected and incubated in Scale A2 solution \(^5\) for at least 14 days, making the tissue transparent. Layer V neurons were imaged using a Confocal Laser Scanning Microscope Leica TCS SP8, and reconstructed and analysed using Neuramatic reconstruction software \(^6\), with Sholl analysis performed using NeuroLucida.

Axon tracing of mPFC to BLA projections. Axon tracing in this study followed a previously published method \(^7\). AAV-tTomato was obtained from Penn Vector Core. For injections, mice were anaesthetized under isoflurane and placed onto a stereotaxic frame. Bregma and lambda were used to find the coordinates of the mPFC (1.9 mm anterior to bregma, 0.4 mm lateral to midline and 2.3 mm ventral to bregma). The virus, diluted to 10% with water, was injected by a syringe pump at a rate of 100 nl min \(^{-1}\), with 200 nl volume injected per site in each mouse. Mice were perfused 2 weeks later, allowing for the expression of tdTomato. BLA sagittal brain sections were collected to image mPFC axon terminals in the BLA (based on mouse brain atlas sagittal sections from the Allen Brain Atlas), with setting as z-series (stacks at 1 μm depth interval) using a ×25 Leica objective with constant parameter for laser power and gain. tdTomato expressing axons (300–350 μm long) were characterized as axon branches. Boutons were defined as at least two times wider than the width of axons, with fluorescence intensity at least two times that in axon backbones.

Mouse behaviour tests. For three-chamber social approach test, mice were tested as previously described \(^8\). Briefly, mice were housed under a reversed 12:12 h light:dark cycle for at least 2 weeks before testing. Mice were put into the apparatus for 5 min to habituate on each of the 2 days preceding the test, and then returned to the housing room. During the test, each mouse was put into the three-chamber apparatus for 5 min for habituation, followed by 10 min of testing with a novel stimulus mouse inside a tube of one chamber and an empty tube in the chamber of the other side (the centre chamber was left empty). The per cent time spent in each of the three chambers, containing either a stimulus mouse in a tube, an empty tube or nothing (in the centre) was recorded during the 10-min social approach trial.

For non-matching to sample T-maze task, male mice were food restricted to 85–90% of their body weight to increase motivation for this test. The behaviour protocol consisted of 3 days of habituation, followed by alternation training and then delay testing. During habituation, mice were allowed to explore the maze for 4 min per trial, with a total of 4 trials per day. Mice were trained to obtain food reward from the trough after 3 days of habituation. During the alternation training, each trial consisted of a forced and one non-forced run. In the forced run, the mouse ran down the centre arm of the maze and was directed into one of the arms. The mouse was then returned to the start box. In the free-choice run, the mouse was required to select the arm opposite to that visited in the forced run to receive a food reward. Mice were given daily training of four trials until they reached criterion, defined as performing at least 75% correct per day for 2 consecutive days. Both WT and Pten \(^{+/+}\) mice reached criterion after 7 days of training. During delay testing, each day consisted of two non-delay trials and two delay trials. During delay trials, the mouse was kept in the start box for 60 s between the forced and free-choice runs. Mice received 5 days of delay testing, for a total of 10 trials each for non-delay and delay-testing. Delays were used to determine if the mice maintained criterion performance.

For social exposure without fixed interaction time, female mice were housed individually in a home cage containing a perforated acrylic tube for 7 days before the test. On the test day, a novel conspecific female stimulus mouse was placed in the tube within the home cage of subject female mice. The subject mouse was allowed to explore the stimulus mouse for 20 min (social exposed group) and then the stimulus mouse was removed. The behaviour was video-recorded and manually scored to determine the amount of time that the subject mouse spent sniffing the stimulus mouse. After the stimulus mouse was removed, the subject mouse remained in the home cage for an additional 2 h after being collected and included in the control group, mice were individually housed in a home cage containing a perforated acrylic tube for 7 days and then perfused.

For social exposure with fixed interaction time, female mice were housed individually in a home cage containing a perforated acrylic tube for 7 days before the test. On the test day, a novel conspecific female stimulus mouse was placed in the tube within the home cage of subject female mice. The subject mouse was allowed to explore the stimulus mouse until it reached a fixed criterion of 30 s of sniffing time (manually scored during the social exposure period). In this way, each subject mouse received roughly equivalent social cue exposure.

DREADD experiments. Mice were anaesthetized with isoflurane in the stereotaxic frame for the entire surgery and their body temperature was maintained with a heating pad. To reduce activity in mPFC neurons projecting to the neuromodulatory system, 0.5 μl of cannabidiol carrying Cre recombinase (CAY2-Cre) \(^8\) was bilaterally injected into the BLA (1.45 mm posterior to bregma, 3.3 mm lateral to midline and 5.2 mm ventral to bregma), and 400 nl of AAV carrying Cre-dependent hM4Di and mCherry (AAV-DIO-hM4Di-mCherry) or mCherry alone (AAV-DIO-mCherry) were bilaterally injected into mPFC (1.9 mm anterior to bregma, 0.4 mm lateral to midline, and 2.3 mm ventral to bregma). Injections were performed at a rate of 50 nl per minute. The needle was allowed to sit in the target location for 3 min before the start of viral injection and for 7 min after injection was complete. Social behaviour was tested 4 and 6 weeks after the surgery, with each mouse being tested following vehicle and CNO injections in a counterbalanced design. Mice were given i.p. injections of either vehicle or CNO (3 mg kg \(^{-1}\) body weight) 30 min before social approach test. CNO was dissolved in saline to a working solution of 0.5 mg ml \(^{-1}\).

Laminar plot analysis. After image acquisition, ROIs were cropped from the primary somatosensory cortex across the radial thickness using Photoshop. Cerebral cortex thickness was normalized, and images were then auto-adjusted for each channel and saved. PSD profiles for each channel of each image were obtained using ImageJ. Three brains of WT mice were analysed, and 3 ROIs per brain were used for the laminar plot analysis.

SUnSET method for measurement of protein synthesis. We obtained 400 μm coronal prefrontal cortical slices from postnatal day 8 (P8) WT and Pten \(^{+/+}\) mice. Slices were allowed to recover in artificial cerebrospinal fluid (in mM: 124 NaCl, 2.8 KCl, 2 CaCl\(_2\), 1.3 NaHPO\(_4\), 1.25 MgSO\(_4\), 26 NaHCO\(_3\), 10 Dextrose) for 2 h at 32 °C. Puromycin (5 μg ml \(^{-1}\)) was applied to slices for 40 min at 32 °C to label newly synthesized proteins. Slices were then snap frozen on dry ice and stored at −80 °C until used. Protein (30 μg) was loaded on 4–12% gradient gels, transferred to polyvinylidene difluoride membranes, and probed with anti-puromycin antibody (1:5,000, Millipore, MABE343). Anti-mouse HRP-tagged secondary antibody was used and chemiluminescence was developed using standard ECL reagents. Slices that were not exposed to puromycin were used to control for non-specific labelling. Quantification was done by measuring intensity of each lane from 14 to 191 kDa, subtracting it from the intensity of the unlabelled lane, normalized to loading control and expressed relative to WT.

Statistical analysis. Prior studies using similar experiments or power analysis were used to determine sample size. Planned comparisons between WT and mutant mice were used for all assays using independent-sample t tests. One-way analyses of variance (ANOVA) were used to assess drug and genotype effect in experiments containing control, Pten \(^{+/+}\), Emn1-Cre \(^-\); Rpto\(\text{loxP}\)+/− and Emn1-Cre \(^-\); Rpto\(\text{loxP}\)+/− mice. Tukey’s post hoc tests were used where appropriate. Two-way ANOVA were used to assess drug and genotype effects and their interactions in experiments containing WT and Pten \(^{+/+}\) mice with vehicle or PF-4708671 treatment; Tukey’s post hoc tests were used where appropriate. Planned comparison independent-sample t tests compared WT and Pten \(^{+/+}\) with vehicle treatment, and compared vehicle and PF-4708671-treated Pten \(^{+/+}\) mice. For the three-chamber social approach test, two-way ANOVA were used to assess the interaction between chamber type and genotype; Sidak’s post hoc tests were used where appropriate. Paired t tests were also used within genotype to determine if mice showed a preference for the social chamber. All statistics were performed using Graphpad, with significance set at \(P<0.05\). Throughout the manuscript, values represent mean; error bars indicate s.e.m’s; N values refer to biological replicates. All measurements and testing were performed blind to the genotype or experimental manipulations.

Data availability. All relevant data are available upon request.

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**Author contributions**
W.-C.H. and D.T.P. conceived and designed the experiments. W.-C.H. executed experiments and data analysis, and performed and analysed all the experiments in this study except the three-chamber social approach test on Emx1-Cre /+ Pten+ /− mice, which was performed and analysed by Y.C. Data interpretation by W.-C.H. and D.T.P. wrote the paper. W.-C.H. and Y.C. and D.T.P. edited the paper.
Additional information

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