PI 3-kinase delta enhances axonal PIP₃ to support axon regeneration in the adult CNS

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Abstract

Peripheral nervous system (PNS) neurons support axon regeneration into adulthood, whereas central nervous system (CNS) neurons lose regenerative ability after development. To better understand this decline whilst aiming to improve regeneration, we focused on phosphoinositide 3-kinase (PI3K) and its product phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). We demonstrate that adult PNS neurons utilise two catalytic subunits of PI3K for axon regeneration: p110α and p110δ. However, in the CNS, axonal PIP₃ decreases with development at the time when axon transport declines and regenerative competence is lost. Overexpressing p110α in CNS neurons had no effect; however, expression of p110δ restored axonal PIP₃ and increased regenerative axon transport. p110δ expression enhanced CNS regeneration in both rat and human neurons and in transgenic mice, functioning in the same way as the hyperactivating H1047R mutation of p110α. Furthermore, viral delivery of p110δ promoted robust regeneration after optic nerve injury. These findings establish a deficit of axonal PIP₃ as a key reason for intrinsic regeneration failure and demonstrate that native p110δ facilitates axon regeneration by functioning in a hyperactive fashion.

Keywords
axon transport; CNS axon regeneration; optic nerve; p110 delta; phosphoinositide 3-kinase

Subject Categories
Neuroscience; Stem Cells & Regenerative Medicine

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Introduction

Adult central nervous system (CNS) neurons have a weak capacity for axon regeneration, meaning that injuries in the brain, spinal cord and optic nerve have devastating consequences (He & Jin, 2016; Curcio & Bradke, 2018). In most CNS neurons, regenerative capacity is lost as axons mature, both in vitro (Goldberg et al, 2002; Koseki et al, 2017) and in vivo (Kalil & Reh, 1979; Wu et al, 2007). Conversely, peripheral nervous system (PNS) neurons maintain regenerative potential throughout adult life. This is partly because PNS neurons mount an injury response in the cell body (Smith & Skene, 1997; Ylera et al, 2009; Puttagunta et al, 2014), and also because PNS axons support efficient transport of growth-promoting receptors, whilst many of these are selectively excluded from mature CNS axons (Hollis et al, 2009a,b; Franssen et al, 2015; Andrews et al, 2016). Studies into intrinsic regenerative capacity have implicated signalling molecules, genetic factors and axon transport pathways as critical regeneration determinants (Park et al, 2008; Blackmore et al, 2012; Fagoe et al, 2015; Eva et al, 2017; Weng et al, 2018; Hverera et al, 2019). This leads to a model where axon growth capacity is controlled by genetic and signalling events in the cell body, and by the selective transport of growth machinery into the axon to re-establish a growth cone after injury.

To better understand the mechanisms regulating axon regeneration, we focused on the class I phosphoinositide 3-kinases (PI3Ks). These enzymes mediate signalling through integrins, growth factor and cytokine receptors, by producing the membrane phospholipid PIP₃ from PIP₂ (phosphatidylinositol (3,4,5)-trisphosphate from

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phosphatidylinositol(4,5)-bisphosphate). Class 1 PI3Ks comprise 4 catalytic isoforms called p110α, β, γ and δ, with distinct roles for some of these emerging in specific cell populations (Bilanges et al., 2019). The p110α and p110β isoforms are ubiquitously expressed, whilst p110δ and p110γ are highly enriched in leucocytes (Hawkins & Stephens, 2015). p110β and p110γ have not been studied in neurons, but p110α mediates axon growth during chick development (Hu et al., 2013) whilst p110δ is required for axon regeneration of the PNS (Eickholt et al., 2007) and localises to the Golgi in mature cortical neurons, where it controls the trafficking of the amyloid precursor protein (APP) (Low et al., 2014; Martinez-Marmol et al., 2019).

The PI3K pathway is strongly implicated in the regulation of regenerative ability because transgenic deletion of PTEN promotes CNS regeneration (Park et al., 2008; Liu et al., 2010; Geoffroy et al., 2015). PTEN opposes PI3K by converting PIP3 back to PIP2. Additionally, inhibiting negative feedback of this pathway similarly enhances regrowth (Al-Ali et al., 2017). These findings indicate a pro-regenerative role for PIP3; however, this molecule has not been directly studied in adult CNS axons. Axonal PI3K contributes to polarity in developing hippocampal axons (Shi et al., 2003), and PNS axons segregate PI3K at the growth cone to elicit rapid growth (Zhou et al., 2004); however, the neuronal distribution of PIP3 is not known. We reasoned that CNS regenerative failure might be associated with a developmental decline in PIP3 specifically within the axon, and wondered whether individual PI3K isoforms were required to yield sufficient axonal PIP3.

We investigated the class I PI3K isoforms and found that both p110α and p110δ are required for PNS axon regeneration and that p110δ is specifically required within the axon. In CNS neurons, we found PIP3 was sharply downregulated with development, diminishing in the axon at the time when axon transport and regeneration also decline. We attempted to restore PIP3 through overexpression of either p110α or p110δ; however, only p110δ led to elevated axonal PIP3. Importantly, by introducing the hyperactivating H1047R mutation into p110δ, we found that it could mimic the effect of p110δ, with the expression of either p110δ or p110δH1047R facilitating axon regeneration. This suggests that regeneration is hindered by low activation of PI3K in mature CNS axons. We found that transgenic expression of p110δ or p110δH1047R led to enhanced retinal ganglion cell (RGC) survival and axon regeneration after optic nerve crush, whilst viral expression of PIP3 led to stronger regeneration. Importantly, overexpression of p110δ had both somatic and axonal effects, enhancing the axonal transport of growth machinery and signalling through ribosomal S6 in the cell body. These findings demonstrate a deficit of axonal PIP3 as a novel reason for intrinsic regenerative failure, whilst establishing that native p110δ functions in a hyperactive fashion to enable CNS axon regeneration. Our results emphasise the importance of elevating growth-promoting pathways in the axon as well as the cell body to stimulate axon regeneration.

Results

Gene expression of p110 isoforms in the nervous system

Because localised axonal activation of PI3K is essential for rapid PNS axon growth (Zhou et al., 2004), we reasoned that there may be specific PI3K isoforms that support regeneration within PNS axons and that these might be under-represented in CNS neurons. p110δ plays a role in PNS axon regeneration (Eickholt et al., 2007); however, the contribution of other isoforms has not been examined. We investigated the RNA expression of the individual p110 subunits (p110α, β, γ and δ) in four published neuronal RNAseq datasets (Fig EV1), examining expression in dorsal root ganglion (DRG) neurons during axon growth and regeneration (Tedeschi et al., 2016), in developing cortical neurons (Koseki et al., 2017) and in the Brain-RNAseq databases (http://www.brainrnaseq.org/) of individual cell types in mouse and human brain (Zhang et al., 2014, 2016). In DRG neurons, p110α, β and δ are expressed at all stages in vitro, whilst p110γ is expressed at very low levels. p110α is present at the highest levels, whilst p110δ is upregulated in development and further upregulated upon peripheral nerve lesion (Fig EV1A–C). In developing cortical neurons, p110α is again expressed at the highest levels, p110β and δ are present but not abundant, and p110γ is at very low levels. Remarkably, p110α, β and δ are all downregulated as cortical neurons mature (Fig EV1D and E). The Brain-RNAseq database indicates p110α and β are the principal neuronal PI3K isoforms in both the mouse and human adult brain, with p110α again expressed at the highest levels, whilst p110δ and γ are enriched in microglia and macrophages (Fig EV1F–M). These datasets indicate that p110α, β and δ are expressed in regenerative PNS neurons, but are almost absent or downregulated with maturation in CNS neurons. We therefore chose to investigate the contribution of the p110α, β and δ isoforms of PI3K to axon regeneration of DRG neurons.

p110α and δ are required for DRG axon regeneration

We used laser axotomy to sever the axons of adult DRG neurons and measured the effect of specific inhibitors of p110α, β and δ on growth cone regeneration (Fig 1). Inhibiting either p110α or δ reduced the percentage of axons regenerating, as did pan-PI3K inhibition (α, β and δ) or targeting p110α and δ together. Inhibition of p110β had no effect, but inhibiting all of the isoforms increased the time taken to develop a new growth cone (Fig 1A and B and Movies EV1 and EV2). Some inhibitors also caused uncut axons to stop growing, so we measured the extension rate of uncut axons. Treatment with p110α inhibitors, pan-PI3K or dual α/δ inhibitors led to a dramatic reduction in the percentage of uncut axons extending in 2 h, whilst they continued to extend in the presence of specific p110δ inhibitors (Fig 1C and D and Movies EV3 and EV4). We next examined the effect of PI3K inhibition using microfluidic compartmentalised chambers, in which axons extend through microchannels into a separate compartment from the cell bodies (Fig 1E). Inhibition of p110α or p110δ showed different effects depending on localisation. Inhibition of p110δ in the axonal compartment reduced the percentage of regenerating axons, but had no effect in the somatic chamber. In contrast, the p110α inhibitor A66 reduced regeneration when applied either to the axonal or somatic compartment, and also increased the time taken to generate a new growth cone (Fig 1F). These data show that DRG neurons require p110α and δ for efficient regeneration. Axon growth and regeneration require p110α activity in both the cell body and axon, whilst axon regeneration further relies on p110δ activity specifically within the axon.
Figure 1.

A

Uncut | Time after growth cone axotomy

**Regenerative**

![Image](50 \(\mu m\) growth cone)

0 hr

1 hr

2 hr

**Non-regenerative**

![Image](50 \(\mu m\) growth cone)

Cut site

1 hr

2 hr

New growth cone

B

% axons regenerating

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C

A66 treated (p110α inhibition)

Static axons

Uncut axon tip

![Image](50 \(\mu m\) static tip)

0 hr

2 hr

Cut axon

Growing axons

Uncut axons

![Image](50 \(\mu m\) growing axons)

0 hr

2 hr

D

% uncut axons growing

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E

Microfluidic compartmentalised DRG neurons

Cell body side

Axon side

![Image](50 \(\mu m\) microfluidic)

F

% axons regenerating

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Cumulative probability

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PI3K is developmentally downregulated in cortical neurons, but present in adult DRG axons

The RNAseq data described above (Fig EV1) suggest that PI3K expression increases with maturation in DRG neurons, whilst it is downregulated as cortical neurons mature. CNS regenerative failure might therefore be due to insufficient PI3K activity within the axon and insufficient PIP3. PIP3 is implicated in axon growth but its developmental distribution has not been examined. PIP3 has previously been localised using fluorescently tagged pleckstrin homology (PH)-domain reporters, such as AKT-PH-GFP; however, the principle readout of these reporters is translocation to the surface membrane, and they do not report on abundance or “steady-state” distribution. To accurately measure PIP3 in neurons developing in vitro, we optimised a fixation technique (Hammond et al, 2009) for antibody-based PIP3 detection on immobilised membrane phospholipids utilising an antibody widely used for biochemical assays. To validate this in neurons, we isolated DRG neurons from transgenic mice expressing AKT-PH-GFP at low levels (Nishio et al, 2007) to avoid inhibition of downstream signalling (Varnai et al, 2005). Live imaging of adult DRG neurons from these mice revealed dynamic hotspots of PIP3 at the axon growth cone (Fig EV2A and Movie EV5), and membrane labelling confirmed these are not regions of membrane enrichment (Fig EV2B and Movie EV6). Phospholipid fixation and labelling with anti-PIP3 revealed colocalisation between AKT-PH-GFP and anti-PIP3 at regions within DRG growth cones (Fig EV2C), as well as at hotspots and signalling platforms in non-neuronal cells from DRG cultures (Fig EV2D). In addition to validating this technique, our data also confirm the presence of dynamic PIP3 in the growth cone and axons of regenerative DRG neurons. To further confirm the specificity of the stain for PIP3, we stimulated N1E cells with insulin and labelled for PIP3 in the presence or absence of the pan-PI3K inhibitor GDC-0941, detecting increased PIP3 staining after insulin stimulation alone, and not in the presence of the PI3K inhibitor (Fig EV2E-G).

We then labelled E18 cortical neurons at 3, 8 and 16 days in vitro (DIV) to detect endogenous PIP3. In immature neurons (at DIV 3), we detected high levels of PIP3 in the cell body, and particularly in the distal axon and growth cone (Fig 2A, D and E). At DIV 8, these neurons have a single rapidly growing axon; at this time, there was a sharp decline in PIP3 at the cell body (Fig 2B and D), but only a small decrease in growth cone PIP3 (Fig 2B and E), indicating that during the period of rapid axon extension, neurons possess high levels of PIP3 in axonal growth cones. By DIV 16, cortical neurons have long axons, which are establishing synapses, with some branches still growing. These mature neurons exhibit a sharp decline in regenerative ability and selective axon transport (Eva et al, 2017; Koseki et al, 2017). At this stage, we detected markedly reduced levels of PIP3 at the growth cone, whilst they remained low at the cell body (Fig 2C-E). This indicates a global reduction in PIP3 as cortical neurons develop and demonstrates that the loss of regenerative ability coincides with a deficiency in axonal PIP3 production.

Expression of p110δ or p110αH1047R elevates PIP3 in the soma and axon

The results above show that PI3K and PIP3 are developmentally downregulated in CNS axons as they lose their regenerative ability. In DRG neurons, which continue to express PI3K and produce PIP3, the p110α and δ isoforms are necessary for efficient axon regeneration (Fig 1). We therefore asked whether we could increase PIP3 levels and restore regeneration to CNS neurons by ectopic
Figure 2.
Figure 3.
expression of p110α, its hyperactive H1047R variant p110αH1047R (Mandelker et al., 2009) or p110δ. Previous work has shown that p110δ and p110αH1047R can sustain downstream AKT activation upon overexpression in fibroblasts, acting independently of active Ras, whilst native p110α does not (Kang et al., 2006). These PI3Ks were expressed in cortical neurons at DIV 16, and PIP3 was measured by quantitative immunofluorescence. We used dual-promoter constructs expressing untagged p110 and GFP to avoid potential interference of PI3K function by a protein tag.

Compared with cells expressing GFP alone, expression of p110α had no effect on PIP3 levels in DIV 16 neurons, either in the cell body or within the axons (Fig 3A–D). In contrast, overexpression of either p110αH1047R or p110δ led to a small but significant increase in PIP3 in the cell body (Fig 3A and C) and a striking increase in PIP3 at axon growth cones (Fig 3B and D). These findings indicate that p110δ and p110αH1047R function in a hyperactive fashion to generate PIP3 in cortical neurons, with the strongest effect in growth cones. However, even overexpressed p110δ does not generate PIP3, suggesting a low level of activation in mature neurons.

Expression of p110δ or p110αH1047R increases axon and dendrite growth

We next investigated the effect of p110δ, p110α or p110αH1047R overexpression on the regulation of axon growth in cortical neurons developing in vitro. Expression of either p110δ or p110αH1047R in immature neurons at DIV 2 led to a moderate increase in axon length by DIV 4, compared with neurons expressing either GFP alone or native p110α (Fig 4A and B). p110α or p110αH1047R expression also led to a small increase in the dendrite length (Fig 4A and C). This led to an increase in axon/dendrite length ratio, with axons approximately 31.5 times longer than dendrites, whilst control neurons have axons 21.8 times longer than dendrites. None of the PI3K isoforms therefore affected neuronal polarisation (Fig 4A and D). We also examined the effects of PI3K overexpression on dendrite length and branching at a later developmental stage (transfecting at DIV 10, and analysing at DIV 14). Again, p110δ and p110αH1047R behaved similarly, expression of either construct leading to increases in both the number of dendrite branches and the total dendrite length, compared with control-transfected neurons. In contrast, expression of native p110α had no effect (Fig 4E–G). The PI3K pathway is a well-known regulator of cell size, so we also measured hypertrophy. Overexpression of either p110δ or p110αH1047R led to an increase in cell body size, compared with either GFP or p110α (Fig 4H).

To confirm downstream signalling through the PI3K pathway, we employed phosphorylation-specific immunolabelling of ribosomal S6 protein, a transcriptional regulator routinely used as a reporter of somatic signalling through the PI3K/AKT/mTOR pathway. p110δ or p110αH1047R expression led to a strong phospho-S6 signal compared with GFP-expressing controls, whilst expression of p110α again had no effect (Fig 4E and I). These findings confirm that hyperactive p110αH1047R behaves like p110δ to trigger downstream signalling through the PI3K pathway in neuronal soma, with effects on size, dendrite branching and axon length. The results demonstrate that p110δ and p110αH1047R enhance both axonal and dendritic growth, whilst native p110α does not.

p110δ and p110αH1047R promote axon regeneration of CNS neurons in vitro

To determine whether activation of p110 can facilitate CNS regeneration, we used an in vitro model of regeneration in mature cortical neurons, comparing overexpression of either p110δ, p110α or p110αH1047R. In this model, axon regeneration ability is progressively lost with maturity, and molecules that promote regeneration may differ from those that promote developmental outgrowth (Koseki et al., 2017). In vitro laser axotomy was used to sever the axons of E18 cortical neurons cultured to DIV 15–18, at which stage they have a limited capacity for regeneration (Eva et al., 2017; Koseki et al., 2017). Expression of either p110δ or p110αH1047R led to a sharp increase in the percentage of axons regenerating after axotomy compared with GFP-expressing controls. p110α expression again had no effect (Fig 5A and B and Movies EV7 and EV8). Expression of p110δ or p110αH1047R also led to an increase in the length of regenerated axons and a trend towards shorter time of onset to regeneration compared to controls (Fig 5C and D).

We further investigated the translational potential of p110δ by examining axon regeneration in human neurons maturing in vitro, derived from human embryonic stem cells (hESC). We have previously demonstrated that these lose their regenerative ability in vitro when cultured beyond 50 days (Koseki et al., 2017). Overexpression of p110δ fully restored the regenerative ability to a level observed for younger neurons (Fig 5E and F). We observed no difference in the length of regenerated axons compared with GFP-expressing control neurons (Fig 5G), but there was a tendency for p110δ expressing neurons to initiate regeneration faster (Fig 5H). Overexpression of p110δ therefore enables CNS axon regeneration in both rat and human neurons, and this is mimicked by hyperactive p110αH1047R, whilst overexpression of p110α is ineffective.

p110δ functions through multiple downstream pathways

The data presented so far demonstrate that p110δ expression elevates axonal PIP3 and enhances axon regeneration. We performed a series of experiments to investigate the downstream pathways involved, focusing on mTOR, CRMP2 and ARF6. Deletion

**Figure 3.** p110δ or p110αH1047R expression elevates PIP3 in the soma and axon, whilst native p110α does not.

A PIP3 immunofluorescence in the soma of DIV 16 cortical neurons expressing p110 isoforms and GFP. Spectrum heatmap shows fluorescence intensity.

B PIP3 immunofluorescence at the axonal growth cone of DIV 16 neurons expressing p110 isoforms and GFP. Spectrum heatmap shows fluorescence intensity.

C Quantification of PIP3 immunofluorescence in the soma. Data are shown as the mean ± SEM. P-values indicate significance measured by ANOVA with Tukey’s post-hoc analysis. n = 3 experiments, 60 neurons.

D Graph showing PIP3 quantification of immunofluorescence at the axon growth cone. Data are shown as the mean ± SEM. P-values indicate significance measured by ANOVA with Tukey’s post-hoc analysis. n = 3 experiments, 60 growth cones.
Figure 4.
of PTEN and PI3K activity promotes regeneration by signalling through mTOR (Park et al., 2008) and through GSK3 and CRMP2 (Leibinger et al., 2019). Deletion of PTEN or elevation of PI3K activity could also affect signalling through many molecules with PH, PX or FYVE domains (Vanhaesebroeck et al., 2012). One molecule of particular interest is the small GTPase ARF6, which regulates regenerative ability in both PNS and CNS neurons by controlling the axonal supply of integrins in Rab11-positive endosomes, governed by its activation state (Eva et al., 2017). In CNS neurons, axonal ARF6 activation is maintained by two ARF6 GEFs, ARNO and EFA6, which results in predominant retrograde transport away from the axon (Franssen et al., 2015; Eva et al., 2017). EFA6 and ARNO interact in a complicated fashion to sustain active ARF6, regulated by their PH domains (Malaby et al., 2013; Padovani et al., 2014). We reasoned that elevated PI3K activity might be associated with a decrease in axonal ARF6 activation, and an increase in the anterograde transport of integrins in Rab11 endosomes.

We used laser axotomy and pharmacological inhibitors on mature cortical neurons to test the contribution of mTOR and CRMP2 to the regenerative effects of p110δ, and to confirm that p110δ enabled regeneration through its kinase activity. Overexpression of p110δ again led to a robust increase in the percentage of axons regenerating after laser injury; however, this was prevented by the presence of the specific p110δ inhibitor idelalisib, whilst the p110α inhibitor A66 had no effect (Fig 6A). To investigate the role of mTOR and CRMP2 in p110δ-mediated axon regeneration, we applied rapamycin, an inhibitor of mTOR, or lacosamide, a specific inhibitor of CRMP2, which has been previously used to study CRMP2 and regenerative growth in vitro (Leibinger et al., 2019). Both drugs inhibited the regenerative effects of p110δ expression to similar degrees (Fig 6A), confirming that both pathways are functioning downstream of p110δ activity to mediate regeneration. The increase in phospho-S6 observed in p110δ expressing neurons (Fig 4E) further supports the involvement of the mTOR pathway.

We also examined the localisation of overexpressed p110δ. Endogenous p110δ has been observed at the Golgi, where it controls the trafficking of cytokines and APP (Low et al., 2010, 2014; Martinez-Marmol et al., 2019). We did not find overexpressed p110δ enriched at any specific subcellular structures, but instead found it distributed throughout the neuron (Fig 6B) in the somatodendritic domain (upper panel), axon (middle panel) and axon growth cone (lower panels), whilst being excluded from the nucleus. Overexpressed p110δ may therefore exert its effects throughout neurons, including at the Golgi, with the exception of the nucleus.

To investigate ARF6 activation, we visualised activated ARF6 in the axons of DIV 16 neurons by using the ARF-binding domain (ABD) of GGA3 fused to a GST tag, which was previously used to measure ARF6 activation in neurons (Eva et al., 2017). Overexpression of p110δ led to a reduction in ARF6 activation compared to control neurons expressing GFP alone, whilst we detected no change in the amount of total ARF6 protein (Fig 6C and D). We next investigated whether the reduction of ARF6 activation was accompanied by an increase in integrin or Rab11 transport. ARF6 controls directional transport in a complex with Rab11 (Montagnac et al., 2009), a marker of recycling endosomes that controls neuronal integrin traffic, as well as other growth receptors (Ascano et al., 2009; Eva et al., 2010; Lazo et al., 2013), and contributes to the regenerative ability of cortical neurons (Koseki et al., 2017). We used spinning disc microscopy to image integrin dynamics in the distal axon by visualising α9-integrin–GFP, in the presence of mCherry (control) or mCherry plus p110δ. The presence of mCherry allows visualisation of the entire axon. Anterograde transport was almost undetectable in control-transfected neurons where we observed predominantly retrograde and static vesicles (Fig 6E and F), in keeping with previous studies (Franssen et al., 2015). Expression of p110δ triggered anterograde movement of integrins, leading to an increase in static integrin vesicles in the distal axon, and an overall increase in the total number of integrin vesicles (Fig 6G). We also analysed the dynamics of Rab11-GFP vesicles. These moved throughout axons in an oscillatory fashion displaying mostly bidirectional and retrograde movements, in keeping with previous findings (Eva et al., 2017); however, expression of p110δ initiated anterograde transport and caused an increase in static and bidirectional movements. This led to an overall increase in Rab11-GFP vesicles in the distal part of p110δ expressing axons, compared with controls (Fig 6H–J). Expression of p110δ therefore enhances the anterograde transport of α9-integrins and Rab11-positive endosomes. These transport changes may occur as a result of a reduction in axonal ARF6 activation, although it is also possible that signalling through CRMP2 may also contribute (Rahajeng et al., 2010). Additionally, p110δ may also be functioning at the Golgi to regulate anterograde transport, as has been shown previously (Martinez-Marmol et al., 2019). Together, our findings demonstrate that p110δ functions to support regeneration through multiple downstream pathways, including the increased transport of regenerative machinery.

Transgenic p110δ and p110αH1047R support RGC survival and axon regeneration in the optic nerve

To confirm whether p110δ and p110αH1047R support regeneration in the adult CNS in vivo, as we found in vitro, we used the optic nerve
Figure 5.
crush model to examine the effects of PI3K activation on regeneration after a crush injury. We used transgenic mice, which conditionally express either p110δ or p110δH1047R from the Rosa26 locus in the presence of Cre recombinase (Fig 7A), and delivered AAV2-Cre-GFP via intravitreal injection. Rosa26 allows expression of a transgene at moderate levels (Nyabi et al., 2009). Two weeks after viral injection, optic nerve crush was performed, and retinal ganglion cell (RGC) survival and optic nerve regeneration were examined 28 days later (Fig 7B). We tested AAV2-Cre-GFP activity using a Cre-reporter mouse, which expresses tdTomato from the Rosa26 locus (Fig EV3A) and confirmed expression in p110δ and p110δH1047R mice by examining GFP expression (from the viral vector) in the retina (Fig EV3B). Activation downstream of PI3K was confirmed by phospho-S6 immunofluorescence. Expression of either p110δ or p110δH1047R led to an increase in the number of cells labelling positive for phospho-S6; however, p110δH1047R expression led to a slightly larger increase than p110δ expression (Fig 7C and D). p110δ and p110δH1047R behaved similarly with respect to their effects on RGC survival. The presence of transgenic p110δ or p110δH1047R led to a doubling of the number of cells surviving after 28 days (from 5.5 to 11%), demonstrating a strong neuroprotective effect (Fig 7E and F). We then measured axon regeneration in the optic nerve and found that p110δH1047R and p110δ again behaved similarly, both enabling a moderate increase in axon regeneration compared with control mice injected with AAV2-Cre-GFP (Fig 7G and H). The results confirm that p110δ and p110δH1047R behave similarly in injured RGC neurons in the CNS in vivo, enhancing RGC survival and axon regeneration.

**AAV2-p110δ facilitates axon regeneration in the optic nerve**

The moderate effects of transgenic p110δ expression on axon regeneration (described above) were surprising, given the robust effects of p110δ or p110δH1047R expression on CNS axon regeneration in vitro. We reasoned that p110δ enabled axon regeneration may be dose-dependent and that the moderate expression generated from the Rosa26 locus (Nyabi et al., 2009) might explain these limited effects. To test whether regeneration could be enhanced using a viral gene transfer approach, we produced an AAV2-p110δ construct for viral transduction of RGC neurons via intravitreal injection. We compared this with a similar AAV2-mediated shRNA approach to silence PTEN, the phosphatase responsible for opposing the actions of PI3K by dephosphorylating PIP3 to PIP2. Transgenic suppression of PTEN is another means of stimulating regeneration in the CNS, although virus-mediated shRNA silencing has not proved to be as effective as transgenic deletion (Yungher et al., 2015). We therefore compared viral vector-based delivery of p110δ versus viral delivery of a PTEN targeting shRNA. We first confirmed that AAV2-shPTEN-GFP transduction resulted in silencing of PTEN in RGCs compared with AAV2-scrambled-GFP control, PTEN levels being measured by quantitative immunofluorescence (Fig EV4A and B). We confirmed transduction of RGCs by AAV2-p110δ immunofluorescence, comparing p110δ with RGC neurons transduced with AAV2-GFP (Fig EV4C and D). We examined downstream signalling by labelling for ribosomal phospho-S6. Transduction with AAV2-shPTEN-GFP led to an increase in the percentage of transduced cells labelling positive for phospho-S6 compared with AAV2-scrambled-GFP-transduced neurons (27% for controls to 48% for PTEN silenced; Fig 8A and B). Due to the lack of a tag on AAV2-p110δ (due to potential effects on activity), we measured the total number of TUJ1 labelled neurons that also labelled for phospho-S6. Transduction with AAV2-p110δ led to an increase in phospho-S6-positive neurons compared with AAV2-GFP-transduced controls (1140 cells for p110δ transduced, compared with 650 cells for GFP transduced) (Fig 8A and C).

Having validated the two approaches, we next compared their effects on RGC survival and axon regeneration after optic nerve crush. Silencing of PTEN led to a robust increase in the survival of RGC neurons compared with controls at 28 days after crush (14.7% of PTEN-silenced neurons survived compared to 4.9% of control neurons), whilst transduction with p110δ led to a smaller effect on survival (11.3% of p110δ-transduced neurons compared 6.3% of GFP control neurons; Fig 8D and E). similar to the amount observed due to transgenic expression (Fig 7F). We examined CTB-traced RGC axon regeneration in the optic nerve and found that both AAV2-mediated silencing of PTEN and AAV2 delivery of p110δ enhanced axon regeneration. Transduction with p110δ had the most robust effects on regeneration, with 180 axons counted at 0.5 mm into the optic nerve, compared with 25 axons in control-injected mice (Fig 8F). Injection of AAV2-shPTEN-GFP led to 97 axons at 0.5 mm, again compared with 25 axons for controls (this compares with 60 axons for p110δ, 70 for p110δH1047R with transgenic expression). AAV2-p110δ also enabled axons to regenerate further into the optic nerve reaching a maximum distance of 3 mm, whilst silencing of PTEN enabled axons to reach a maximum of 1.5 mm (transgenic expression of p110δH1047R and p110δ also gave 1.5 mm regeneration). These data demonstrate that the PI3K pathway can be targeted to stimulate RGC survival and axon regeneration either by
Figure 6.
expressing p110δ or by silencing PTEN, but that expression of p110δ has the most robust effects on axon regeneration. Together, these data show that enhancing PI3K activity in CNS neurons greatly enhances their ability to regenerate their axons and indicate viral delivery of p110δ to CNS neurons as a novel approach to boost signalling through this regenerative pathway.

**Discussion**

Our study aimed to find a new method of stimulating axon regeneration based on manipulation of PIP3 levels in neurons. In previous work, the approach to this has mainly been to knock down the PIP3 dephosphorylating enzyme PTEN (Park et al., 2008; Liu et al., 2010; Lee et al., 2014; Geoffroy et al., 2015). However, this approach depends on generation of PIP3 by PI3K, and if there is little PIP3 being produced, PTEN knockdown will have little effect. We reasoned that limited PIP3 production in mature neurons would explain the observation that PTEN knockout is effective at promoting axon regeneration in immature neurons, less so in the fully adult CNS. We used a new method to visualise PIP3 levels in PNS and CNS neurons at different levels of maturity. In sensory axons, which regenerate readily, we observed high levels of PIP3 in the growth cones, dynamically changing with growth cone movements. In cortical CNS neurons, developing immature neurons exhibited intense PIP3 levels in their axons and growth cone during the period of rapid axon growth. However, PIP3 levels decreased to a much lower level at a time when transport declines and axons lose their capacity for regeneration. We also investigated the role of PI3K isoforms in regeneration in PNS neurons using isoform-specific inhibitors. In sensory axons, which regenerate successfully and quickly, growing in microfluidic chambers to separate axonal and cell body compartments, inhibitors of p110α blocked both growth and post-axotomy regeneration when applied to either compartment, but a p110δ inhibitor blocked regeneration without affecting normal growth. This supports a previous study that links p110δ to sensory regeneration (Eickholt et al., 2007) and suggests that p110δ is uniquely linked to regeneration. Overexpression of p110α isoforms was therefore tested for the ability to increase PIP3 levels and to promote regeneration.

Overexpression of p110α in mature CNS neurons partially restored PIP3 levels, particularly in axon tips but p110α was ineffective. However, introducing the hyperactivating H1047R mutation to p110α had the same effect on PIP3 levels as p110δ.

Stimulation of axon regeneration was therefore assessed. p110δ and p110αH1047R transfected into mature cortical neurons strongly enhanced axon regeneration. In the CNS in vivo, transgenic expression led to enhanced neuroprotection in the retina, and regeneration in the optic nerve after a crush injury. Importantly, viral transduction of p110δ (which produces a higher level of expression) into adult RGC neurons led to axons regenerating for a greater distance after injury, demonstrating a novel approach to boost CNS regeneration through the PI3K pathway by gene transfer. Previous work has indicated that the hyperactive H1047R mutation of p110α can behave like p110δ to sustain AKT signalling in fibroblasts, by functioning independently of co-activation by Ras (Kang et al., 2006). We found that expression of native p110α had no effect on PIP3 generation; it was only p110δ or p110αH1047R that enhanced axonal PIP3. (Fig 3). This suggests that receptor activation of PI3K is at a low level in mature CNS neurons and that p110δ can potentiate PI3K signalling in low trophic conditions that are insufficient to activate native p110α. Our findings suggest that p110δ has a lower threshold of activation and that signals normally required to fully activate p110α (such as adhesion and growth factor receptors or Ras) may not be available in the axon. This has important implications for understanding the nature of p110δ beyond the nervous system and may help to explain how T cells utilise p110δ for development, differentiation and function (Okkenhaug, 2013).

The PI3K signalling pathway is a well-known regulator of axon regeneration, based on seminal studies which demonstrated that deletion of PTEN leads to robust regeneration in the CNS through downstream signalling via mTOR (Park et al., 2008). Aside from mTOR, the mechanism through which PTEN deletion stimulates regeneration is not completely understood. One difficulty in understanding its mechanism is that in addition to functioning as a lipid phosphatase (PTEN opposes PI3K by converting PIP3 back to PIP2), it also has protein phosphatase activity (Kreis et al., 2014). It is now also apparent that PTEN not only dephosphorylates PI(3,4,5)P3, but also functions as a PI(3,4)P2 phosphatase, a role linked with cancer...
Figure 7.
invasion (Malek et al., 2017). Our findings argue in favour of PTEN functioning through the regulation of PIP3 and confirm the importance of this molecule in the regulation of axon regeneration.

Most PI3K signalling events rely on more than one isoform (Hawkins & Stephens, 2015), and whilst the p110δ isoform contributes to sciatic nerve regeneration (Eickholt et al., 2007), the contribution of the other isoforms remained unknown. Our findings demonstrate that both p110α and δ are required for efficient axon regeneration and that p110α functions in both the axon and cell body, whilst p110δ is specifically required in the axon (Fig 1). Inhibition of p110α opposed both axon growth and regeneration, whilst the action of p110δ was specific to regeneration, with the p110δ inhibitor blocking regeneration but not growth of uncut axons. This is in keeping with previous studies of the kinase-inactive p110δD910A transgenic mouse, which has normal nervous system development, but defective PNS regeneration (Eickholt et al., 2007). Taken together, these results suggest that p110α mediates the somatic and axonal signalling that is necessary to support adult DRG axon growth (itself a regenerative phenomenon), whilst p110δ is further required within the axon to facilitate the redevelopment of a growth cone after injury. In CNS neurons, overexpression of either p110α or p110δH1047R was sufficient to enable efficient regeneration both in vitro and in vivo, and AAV-mediated p110δ expression in RGCs promoted robust optic nerve regeneration.

Our studies into the downstream pathways mediating the effects of p110δ overexpression suggest the involvement of multiple pathways, including signalling through mTOR and CRMP2, which have previously been implicated in mediating the regenerative effects of PTEN deletion and PI3K activity (Park et al., 2008; Leibinger et al., 2019). Both of these pathways function downstream of AKT, which is recruited to PIP3 by virtue of its PH domain. It is likely that additional pathways could contribute to regeneration downstream of either PIP3 generation or PIP3 reduction, due to the wide variety of proteins with PH domains, including the regulatory molecules of small GTPases that regulate the cytoskeleton, such as Rac1 and Cdc42 (Welch et al., 2002; Yoshizawa et al., 2005; Sosa et al., 2006), or that regulate axon transport such as ARF6 (Gillingham & Munro, 2007; Nieuwenhuis & Eva, 2018). Our findings suggest that p110δ exerts some of its effects by signalling through ARF6, a known regulator of integrin and Rab11 transport (Montagnac et al., 2009; Ghosh et al., 2019). These molecules contribute to the developmental loss of CNS regenerative ability, becoming transported away from axons and restricted to the dendrites and cell body as CNS neurons mature (Franseen et al., 2015; Koseki et al., 2017).

Overexpression of p110δ led to a restoration of anterograde transport of both integrins and Rab11 endosomes. The direction of Rab11 transport is likely regulated in a complex with ARF6, although it may potentially be regulated by activation state or by phosphoinositide generation (Campa & Hirsch, 2017). Previous work has shown that forcing Rab11 vesicles into these mature axons promotes regeneration (Koseki et al., 2017). Rab11 is also associated with the transport of other PI3K activating receptors, which are excluded from mature axons (Hollis et al., 2009a,b). Increased receptor transport due to p110δ expression suggests a feed-forward mechanism, in keeping with previous findings that BDNF stimulation leads to increased axonal transport of TrkB receptors during development (Cheng et al., 2011). Newly transported integrins could potentially signal through p110δ, as has previously been shown in PNS neurons (Eickholt et al., 2007) and cancer cells (Fiorcari et al., 2013).

The exclusion of PI3K-activating receptors from mature axons is probably a reason why the generation of PIP3 is low in mature axons, and why overexpression of p110α in neurons did not increase PIP3 levels. PTEN deletion and p110δ expression also enhance regeneration via mTOR signalling in the cell body; however, mTOR has recently been found to be present at the growth cone of developing axons in the mouse cerebral cortex, suggesting the PI3K-AKT-mTOR pathway may also function locally within the axon (Pouloupolous et al., 2019). Our findings demonstrate the importance of PI3K in the axon as well as the cell body for optimal regeneration. The importance of targeting the axon as well as the cell body is often overlooked; however, there is increased axonal transport as part of the PNS injury response, and in the CNS, increased transport enables regeneration (Petrova & Eva, 2018).

The search for methods for promoting regeneration in the damaged CNS continues. A successful strategy will likely involve multiple interventions. Could manipulation of PI3K be useful? PTEN deletion or p110δH1047R expression can be oncogenic in dividing cells. However, expression under a neuron-specific promoter targets expression to a non-dividing cell type, and it is unlikely that expression of p110δ in CNS neurons would lead to cell transformation, particularly given its usual expression in PNS neurons. Our study puts forward AAV-mediated delivery of p110δ as a novel means of stimulating regeneration through the PI3K pathway. We propose that p110δ should also be considered as an additional intervention with other regenerative strategies that target the PI3K pathway, either through growth factor treatments such as IGF-1 plus osteopontin (Duan et al., 2015; Liu et al., 2017), expression of activated
Figure 8.
integrins (Cheah et al., 2016), or through pharmacological interventions such as insulin (Agostinone et al., 2018).

Materials and Methods

Mouse strains

C57BL/6J mice were used during this study, as well as four transgenic mouse strains: GFP-AKT-PH, Rosa26 p110αH1047R, Rosa26 p110δ and B6;129S6-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (https://www.jax.org/strain/007908). The generation of the PI3P reporter mouse GFP-AKT-PH has been previously described (Nishio et al., 2007) and was a gift from Dr Len Stephens (Babraham Institute, UK). Male and female mice were used dependent on litters available with equal distributions across experiments. Rosa26 p110αH1047R and Rosa26 p110δ were generated according to a previously described protocol (Nyabi et al., 2009). Briefly, cDNA sequences for human p110α or p110δ were cloned into the pENTR D-TOPO vector (Thermo Fisher) to allow cloning into Gateway-compatible vectors. The H1047R point mutation was introduced by site-directed mutagenesis into PIK3CA using the QuickChange II kit (Stratagene #200521) and the primers QuickChange XL kit (Thermo Fisher) to allow cloning into Gateway-compatible pROSA26 destination vector (pROSA26-DV1) was a gift from Jody Haigh (University of Manitoba, Canada; Nyabi et al., 2009). Targeting constructs harbouring sequences for p110αH1047R or p110δ were used to electroporate Bruce4 C57BL/6 ES cells. Selection of targeted clones was undertaken with G418, making use of the neomycin resistance selection cassette. Positively targeted clones were identified by Southern blotting. Positively targeted clones were microinjected into C57BL/6/J-TyrC-2J blastocysts by Babraham Institute Gene Targeting Facility (Babraham Institute, UK). These blastocysts were transferred to the oviducts of time-mated pseudopregnant foster mothers. The progeny were assessed for their degree of chimerism by coat colour and chimeric males mated to white C57BL/6/J-TyrC-2J females. Black progeny were tested for their correct incorporation of the Human PI3K genes at the Rosa26 locus by PCR using the following primers:

<table>
<thead>
<tr>
<th>Rosa26</th>
<th>ROSA26 F1 AMQ</th>
<th>GGCTCAGTTGCGCTTGTGTG</th>
<th>WT allele = 359 bp</th>
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</thead>
<tbody>
<tr>
<td>Hs p110</td>
<td>ROSA26 R2 AMQ</td>
<td>TCTGTGGGAACTCTGTGTGT</td>
<td>KI allele = 603 bp</td>
</tr>
<tr>
<td></td>
<td>ROSA26 loXP AMQ</td>
<td>GTGAGATCGGATGCGC</td>
<td></td>
</tr>
<tr>
<td>Hs p110x</td>
<td>Hs PIK3CA #5 intra fwwd</td>
<td>TGTCTCTTCAATGGTACC</td>
<td>KI allele = 813 bp</td>
</tr>
<tr>
<td></td>
<td>S2rev</td>
<td>CTTGATATGAAATTCGGCCC</td>
<td></td>
</tr>
<tr>
<td>Hs p110δ</td>
<td>Hs PIK3CD #5 intra fwwd</td>
<td>GTACCCTGCTTACACCAT</td>
<td>KI allele = 742 bp</td>
</tr>
<tr>
<td></td>
<td>S2rev</td>
<td>CTTGATATGAAATTCGGCCC</td>
<td></td>
</tr>
</tbody>
</table>

**DRG culture**

Dissociated DRG neuronal cultures were obtained from adult male Sprague Dawley (SD) rats and from the transgenic AKT-PH-GFP adult mouse. DRGs were incubated with 0.1% collagenase in Dulbecco’s modified Eagle’s medium (DMEM) for 90 min at 37°C followed by 10 min in trypsin at 37°C and dissociated by trituration. Dissociated cells were centrifuged through a layer of 15% bovine serum albumin (BSA) and cultured on 1 μg/ml laminin on glass-bottom dishes (Greiner) in DMEM supplemented with 10% foetal calf serum and 50 ng/ml nerve growth factor (NGF). For compartmentalised experiments, DRG neurons were plated in Xona microfluidic devices (Xona SND150) on glass coverslips. For PI3K inhibitor experiments, media were exchanged for serum-free media overnight. Separation of media was achieved by maintaining a pressure gradient between the axonal and somatic sides of the device.
Embryonic cortical neuron culture

Primary rat cortical neuron culture has been described previously (Eva et al., 2017). Cultures were prepared from embryonic day 18 (E18) SD rats. Neurons were dissociated with papain for 8 min at 37°C, washed with HBSS and cultured in MACS Neuro Medium supplemented with MACS NeuroBrew (Miltenyi), and plated on glass-bottom dishes (Greiner) coated with poly-D-lysin. Cells were transfected at 10 DIV, and experiments were performed between DIV 14 and 17. Cortical neurons were transfected with magnetic nano-particles (Franssen et al., 2015).

hESC dopaminergic neuron culture

RC17 hESC cell culture has been previously described in detail before (De Sousa et al., 2016; Koseki et al., 2017). Cells (RRID: CVCL_L206) were sourced from Roslin Cells, Scottish Centre for Regenerative Medicine, Edinburgh, UK. The cell line is free from mycoplasma contamination as determined by RT-qPCR. On d0, hESC were detached and transferred to form embryoid bodies from d0 to d4 in neural induction medium (Neurobasal:DMEM/F12 (1:1), 0.2% P/S, L-glutamine, N2, B27, recombinant Sonic Hedgehog Shh (C24II), recombinant noggin (Ng), SB431542 (SB) and CHIR90021 (CH)). Rock inhibitor (RL) was present in the medium from d0 to d2. At d4, embryoid bodies were plated in poly-L-ornithine laminin and fibronectin (PLF)-coated plates and cultured in neural proliferation medium (Neurobasal:DMEM/F12 (1:1), 0.2% P/S, L-glutamine, N2, B27, supplemented with Shh, Ng, SB and CH from d4 to d7, and with Shh, Ng and CH from d7 to d9). At d11, cells were dissociated with Accutase and 50 mM of 5-lacosamide, 5 mM (IC50 0.1 nM). DMSO was added at a volume corresponding to 10% FBS and incubated for 6 hr. At d14, cells were dissociated with 0.5xN2, 0.5xB27, supplemented with Shh, Ng, SB and CH from d4 to d7, and with Shh, Ng and CH from d7 to d9). At d11, cells were dissociated with Accutase and 50°104 cells per well single-cell suspensions were plated in PLF-coated 8-well chamber slides. Cells were cultured in neuronal differentiation medium (NB with 0.2% P/S, L-glutamine, B27, ascorbic acid, recombinant human BDNF, GDNF, db-CAMP; 50 mM) and DAPT from d14 onwards. Medium was replaced twice weekly up to day 50, after which medium was replaced weekly.

N1E cell culture

N1E-115 cells (ATCC) were plated at a density of 5,000 cells/cm² in DMEM-high glucose with GlutaMAX (Gibco) + 10% FBS and incubated for 6–7 h at 5% CO2 and 37°C.

DNA constructs

Constructs for expressing p110 were generated from pHRSinUbEm, a bicistronic vector expressing EGFP under the control of the SFFV promoter and emerald fluorescent protein cells under the control of the Ubiquitin promoter. p110s (PIK3CD) was a gift from Roger Williams (MRC Laboratory for Molecular Biology, UK). PIK3CD was cloned from pDNA3.1 in place of EGFP. p110z (PIK3CA) or p110x (PIK3CA) were gifts from Jean Zhao (Addgene plasmid #12522 and #12524; http://n2t.net/addgene:12522; RRID:Addgene_12522, http://n2t.net/addgene:12524; RRID:Addgene_12524) (Zhao et al., 2005). AAV.CAG.p110s was generated by cloning the human PIK3CD sequence into an AAV-scCAG vector backbone by Gibson assembly (Gibson et al., 2009).

Antibodies

The antibodies were as follows: P(3,4,5)P: anti-PtdIns(3,4,5)P3 monoclonal antibody (Z-P345b, 1:200, Echelon Biosciences), GFP (rabbit, 1:500 Abcam ab290), phospho-S6 ribosomal protein (Ser235/236) (91B2) (rabbit, 1:200, Cell Signaling 4857S), TUF1 (βII Tubulin) (mouse, 1:400, Promega G7121), PTEN (D4.3) XP (rabbit, 1:100, Cell Signaling 9188S), p110s (rabbit, 1:500, Abcam ab1678), Brn3a (C-20) (goat, 1:200, Santa Cruz sc-31984), ARF6, (rabbit, 1:100, Abcam ab77581), GST (1:400, Abcam ab19256), anti-goat Alexa Fluor 647 (1:1,000, A21447, Life Technologies), anti-rabbit IgG-conjugated Alexa Fluor 568 (A10042, 1:1,000, Thermo Fisher Scientific), anti-mouse IgG-conjugated Alexa Fluor 568 (A11040, 1:1,000, Thermo Fisher Scientific), anti-rabbit IgG-conjugated Alexa Fluor 488 (A-21206, 1:1,000, Thermo Fisher Scientific) and anti-mouse IgG-conjugated Alexa Fluor 488 (A-21202, 1:1,000, Thermo Fisher Scientific).

Small-molecule inhibitors

The following small-molecule inhibitors were used to inhibit various isoforms of p110. The indicated concentrations were chosen based on their known IC50 and from previously reported cell culture experiments: Pan-p110 (p110α/β/δ/ε): LY294002, 20 μM (IC50 (in cell-free assays) 0.5 μM/0.97 μM/0.57 μM, respectively); p110α: A66, 5 μM (IC50 32 nM); p110α/ε: XL-147, 5 μM (IC50 39 nM/36 nM); p110β: TGX221, 500 nM (IC50 5 nM); p110β: IC-87114, 10 μM (IC50 0.5 μM); p110δ: idelalisib, 500 nM (IC50 2.5 nM), lacosamide, 5 μM (Leibinger et al., 2019), and rapamycin, 20 nm (IC50 0.1 nM). DMSO was added at a volume corresponding to volume of inhibitor used in the experimental condition, to a maximum of 5 μl (in a final volume of 3 ml culture media).

Virus production and injection

Three viruses were sourced commercially: AAV2.CMV.Cre.GFP (Vector Biolabs, Catalog #7016), AAV2.CMV.GFP (Vigene Biosciences, Catalog #CV10004) and AAV2.U6.shRNA (scramble).CMV.GFP (SignaGen Laboratories, Catalog #SL100815), AAV2.U6.shPTEN.CMV.GFP was a gift from Zhigang He (Boston Children’s Hospital), and AAV2.CAG.p110s delta was produced by Vigene Biosciences. Mice received 2 μl intravitreal injections of AAV. All viruses were injected into the left eye only at 1 × 1013 GC/ml. For validation experiments, mice also received intravitreal injection of the appropriate control into the right eye.

Optic nerve injury

Optic nerve injuries were carried out as previously described (Smith et al., 2009). The optic nerve behind the left eye was exposed intraorbitally and crushed with fine forceps for 10 s, approximately 0.5 mm behind the optic disc. Twenty-six days after the injury, mice received a 2 μl intravitreal injection of cholera toxin subunit β (CTB) with an Alexa Fluor 555 conjugate at 1 mg/ml. Twenty-eight days post-crush, animals were perfused with 4% paraformaldehyde (PFA) and the eyes and optic nerves collected for analysis. Surgical procedures were performed under anaesthesia using intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). This
research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ARRIVE guidelines and ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). We also followed the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Visual Research. Research was carried out on UK Home Office Project Licence 70/8152 under protocols 3 (breeding) and 4 (ocular injections and injury). Mice had unrestricted access to food and water and were maintained on a 12-h light/dark cycle in groups of five. Adult (2–6 months) male and female mice were used dependent on litters available with equal distributions across experiments. The sample size used in each experiment is stipulated in the figure legends.

Immunohistochemistry

Retinas were fixed in 4% PFA for 2 h. Whole-mounts were washed four times with 0.5% PBS-Triton X-100. In between the second and third wash, a permeation step was performed to improve antibody penetration by freezing the retinas in 0.5% PBS-Triton X-100 for 10 min at −70°C, and washing was continued after thawing. Optic nerves were fixed overnight at 4°C in 4% PFA, followed by 30% sucrose overnight at 4°C. Sections were blocked with 2% BSA and 10% donkey serum in 2% PBS-Triton X-100. Primary antibodies were incubated at 4°C overnight and secondary antibodies for 2 h at room temperature.

Standard immunocytochemistry

Cortical neurons were fixed with 3% paraformaldehyde (PFA) in PBS for 15 min and permeabilised with 0.1% Triton X-100 in PBS for 5 min. Cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. After blocking, the cells were incubated with primary antibodies diluted in 3% BSA in PBS at 4°C overnight and secondary antibodies that were diluted in 3% BSA in PBS for 1 h at room temperature.

Phospholipid fixation and immunocytochemistry

We adapted a fixation technique previously used to detect PI(4,5)P₂ and PI(4,5)P₃ (Hammond et al., 2009). Cortical neuron cultures were fixed using pre-warmed (37°C) 3% formaldehyde and 0.2% glutaraldehyde (GA; G011/3, TAAB Laboratories) in PBS for 15 min at room temperature, washed in 50 mM NH₄Cl in PBS and then maintained at 4°C. Cells were incubated with 4°C blocking and permeabilisation solution (0.2% saponin, 50 mM NH₄Cl and 3% BSA in PBS) for 30 min and then with anti-PtdIns(3,4,5)P₃ (Z-P345b, 1:200, Echelon Biosciences) in blocking solution for 3 h before washing three times in 50 mM NH₄Cl for 30 min. The secondary antibody was applied for 2 h at 4°C. After another 30-min wash, cells were post-fixed in 3% formaldehyde in PBS for 5 min at 4°C before being moved to room temperature for a further 10 min.

Axonal ARF6 activation assay

Active ARF protein was detected using a previously established protocol (Eva et al., 2017) by means of a peptide derived from the active ARF-binding domain (ABD) of GGA3 fused to a GST tag (GGA3–ABD–GST, Thermo Fisher Scientific). Neurons were fixed for 15 min in 3% formaldehyde (TAAB) in PBS, permeabilised with 0.1% Triton X-100 for 2 min and incubated with 20 µg/ml GGA3–ABD–GST in TBS and 1 mM EDTA overnight at 4°C. The GST tag was then detected using rabbit anti-GST antibody (ab19256, Abcam, 1:400). Control and experimental cultures were fixed and labelled in parallel, using identical conditions. Axons were analysed at 200–1,000 µm distal to the cell body. Images of axons were acquired by confocal laser-scanning microscopy using a Leica TCS SPE confocal microscope. Detection settings were adjusted so that the pixel intensities of acquired images were below saturation. Settings were then stored and were applied for the identical acquisition of each image using Leica LAS AF software. Lines were then traced along sections of axons to define the region of interest, and mean pixel intensities per axon section were quantified using Leica LAS AF. Images were corrected for background by subtracting an identical adjacent region of interest. The same technique was then used for measuring total levels of ARF6 in axons, after ARF6 immunolabelling.

Insulin stimulation of N1E cells

Cells were starved in DMEM without serum overnight in the presence of either 500 nM GDC-0941 (Selleckchem) or an equal volume of cell culture grade DMSO (AppliChem). Cells were stimulated with 20 µg/ml insulin (Sigma) in DMEM or control treated with equal amounts of DMEM for 1 min prior to fixation in PIP3-Immobilisation fixative and stained with for PIP₃ (anti-PIP₃, Echelon) and F-actin (Phalloidin, Thermo Fisher Scientific).

Confocal and widefield microscopy

Laser-scanning confocal microscopy was performed using a Leica DMi4000B microscope and a Leica TCS SPE confocal system controlled with Leica LAS AF. Fluorescence and widefield microscopy were performed using a Leica DMi6000B with a Leica DFC350 FX CCD camera and a Leica AF7000 with a Hamamatsu EM CCD C9100 camera and Leica LAS AF. Leica AF7000 was used for imaging of axon and growth cone regeneration after axotomy.

TIRF microscopy

Total internal reflection fluorescence (TIRF) microscopy was carried out on a Leica DMi6000B adapted with a dedicated TIRF module from Rapp OptoElectronic. GFP was excited with a 488 nm laser, and images were acquired using a Leica Plan Apo 100×/NA1.47 Oil TIRF objective and Hamamatsu EM CCD C9100 camera and Leica LAS AF. Leica AF7000 was used for imaging of axon and growth cone regeneration after axotomy.

Laser anatomy of DRG neurons

Laser anatomy of DRG neurons was performed as described previously (Eva et al., 2017). Axons were cut directly before a growth cone, to determine the proportion of axons that regenerate rapidly after injury. Cultures were serum-starved overnight, and inhibitors were added at the start of the experiment. Preliminary experiments confirmed there was no effect of DMSO on the rate of regeneration, by comparing DMSO vs. no DMSO conditions. Axons were severed using a 355 nm DPLS laser (Rapp OptoElectronic, Hamburg,
Germany) connected to a Leica DMI6000B, and images were acquired every 15 min for 2 h. Successful regeneration was determined as the development and extension of a new growth cone.

**Laser axotomy of cortical neurons**

Laser axotomy of cortical neurons was performed as described previously (Eva et al., 2017; Koseki et al., 2017). Axons were severed using a 355 nm DPSL laser (Rapp OptoElectronic, Hamburg, Germany) connected to a Leica DMI6000B. Cortical neurons were axotomised at DIV 14–17 at distances of 800–2,000 μm from the cell body on a section of axon free from branches. Images after axotomy were acquired every 30 min for 14 h. Regeneration was classed as the development of a new growth cone followed by axon extension for a minimum of 50 μm.

**Laser axotomy of hESC-derived neurons**

Laser axotomy of hESC-derived neurons was performed as described previously (Koseki et al., 2017). Axons were severed using a 365 nm laser (MicroPoint, Andor) connected to an Andor spinning disk confocal microscope. Neurons were axotomised at d45–65 at distances of >500 μm distal to the cell body on a section of axon free from branches. A single axon cut was made per neuron. Images after axotomy were acquired every 20 min for 16 h. Regeneration was classed as the development of a new growth cone followed by axon extension for a minimum of 50 μm.

**Quantification of PIP3 in cortical neurons**

E18 cortical neurons were fixed at DIV 3, 8 or 16, and PIP3 was detected as described above. All cultures were fixed and labelled using identical conditions. Images were acquired by confocal laser-scanning microscopy using a Leica TCS SPE confocal microscope. Identical settings were used for each image using Leica LAS AF. Z-stacks were acquired for each image, spanning the entire depth of either the cell body or the growth cone. PIP3 fluorescence intensity was measured using Leica LAS AF. Immunofluorescence intensities were calculated by measuring the region of interest (ROI) and subtracting the intensity of a control region adjacent to the ROI.

**Quantification of PIP3 in N1E cells**

Imaging was performed on a Leica SP5 confocal system with a 40× objective with identical settings across experiments. Stacks covered the complete height of all cells with. Areas with comparable cell densities were selected in the F-actin channel. Mean PIP3 intensity in N1E cells was quantified in Fiji (ImageJ 1.51n) on max projections. Intensities were compared in 15 images per treatment from three independent cultures.

**Analysis of neuronal morphology**

Images were captured on a Leica DMI6000B, with a 40X-oil objective using Leica LAS AF. Semi-automated and standardised analysis was performed using MATLAB platform version 2017 and SynD (Schmitz et al., 2011). The output of SynD was used for data analysis of dendritic length, fluorescent intensities, sholl analysis and soma size.

**Neurite outgrowth assay**

Cultured cortical neurons were transfected at 2 DIV and fixed at 4 DIV. Images for neuronal morphology were acquired using a Leica DMI6000B microscope, with a 40X-oil objective using Leica LAS AF. Neurite lengths were measured using the ImageJ plugin “simple neurite tracer” (Longair et al., 2011).

**Axon transport analysis**

Axon transport analysis of integrins and Rab11 has been described in detail before (Franssen et al., 2015; Eva et al., 2017). Briefly, cortical neurons were transfected at DIV 10 with α9 integrin-GFP, or Rab11-GFP together with either mCherry (control) or mCherry plus p110δ, and imaged at DIV 14–16 DIV using spinning disc confocal microscopy. Sections of axons were imaged at a region in the distal part of the axon (>800 μm from the cell body). Vesicles were tracked for their visible lifetime and analysed by kymography to classify the proportion of vesicles classed as anterograde, retrograde, bidirectional or immobile per axon section. Vesicles with a total movement less than 2 μm during their visible lifetimes were classed as immobile. Vesicles moving in both directions but with net movement of <2 μm (during their visible lifetimes) were classed as bidirectional. Vesicles with net movements >5 μm in either direction by the end of their visible lifetimes were classed as anterograde or retrograde accordingly.

**Optic nerve regenerating axon counts**

To measure regenerating RGC axons after optic nerve crush, longitudinal sections of optic nerves were serially collected. Regenerating RGC axons were quantified as described previously (Smith et al., 2009), by counting the number of CTB-labelled axons at the indicated distances beyond the crush site from four sections per optic nerve. Axonal sections were imaged using a Zeiss AxioScan Z1 at ×40 magnification.

**RGC survival counts**

For retinal whole-mounts, two images were taken from each of the four retinal quadrants at ×20 magnification, sampling both the more central and the more peripheral region of each quadrant. Images were then analysed in ImageJ Fiji using Image-Based Tool for Counting Nuclei (ITCN) Plugin (University of California, Santa Barbara, CA, USA) to count Brn3A-labelled cells. The number of RGCs in the left injured eye was expressed as a percentage survival value compared to the mean number of RGCs of the contralateral control eyes.

**RGC fluorescence analysis**

To confirm successful viral transduction, eye cup images were stained for GFP or PI3KDelta counterstained with DAPI and imaged using a Zeiss AxioScan Z1 at ×20 magnification. Retinal sections immunolabelled for pS6 were examined by fluorescence
Problem
Young neurons in the central nervous system (CNS) can regrow their axons after injury, but this ability is lost as they mature. Axonal injury or disease in the adult brain, eyes, and spinal cord therefore has devastating consequences, and can result in neurological impairment, vision loss, or paralysis. Conversely, neurons of the peripheral nervous system (PNS) maintain the ability to regenerate their axons into adulthood. Comparing PNS and CNS neurons is one approach to identifying new ways of promoting injured CNS axons to regenerate after injury.

Results
Our study found that adult PNS neurons use two versions of the enzyme PI 3-kinase to regenerate their axons, p110δ and p110x. These enzymes generate the phospholipid PIP3. Visualisation of PIP3 in maturing CNS neurons revealed that PIP3 is strongly downregulated at the time when these neurons lose their regenerative ability. Overexpression of p110δ elevated axonal PIP3 and regeneration after laser injury, but p110x required the activating H1047R mutation to behave similarly, demonstrating that native p110δ functions in a hyperactive fashion. The study found that p110δ functioned through multiple mechanisms, including the enhanced transport of regenerative machinery into axons. Importantly, viral delivery of p110δ to the retina promoted axon regeneration after an optic nerve crush injury and was accompanied by enhanced survival of RGC neurons in the retina.

Impact
These findings suggest that signalling through PI 3-kinase-linked receptors is limited in adult CNS axons, contributing to their weak regenerative ability. Exogenous expression of the p110δ subunit elevates axonal PI 3-kinase activity by functioning in a hyperactive fashion, leading to enhanced regeneration in human and rodent models of CNS injury, and enhanced neuroprotection and regeneration after a mouse optic nerve crush injury.

The paper explained

Statistical analysis was performed throughout using GraphPad Prism. Fisher’s exact test was calculated using GraphPad online (https://www.graphpad.com/quickcalc/contingency1.cfm). Percentage of regenerating axons was compared by Fisher’s exact test. Sample size calculations using data from previous experiments and pilot studies were used to minimise the group sizes required to demonstrate clinically relevant (as opposed to simply statistically significant) effect sizes. Animals of both sexes were chosen randomly, different viral treatments were assigned to animals randomly, and analysis and quantification were blinded. Normality was tested for with GraphPad Prism software, using the D’Agostino–Pearson normality test, when appropriate. Kruskal–Wallis and Fisher’s exact test were used as indicated in the manuscript. Variation was tested using the Brown–Forsythe test using GraphPad Prism. Comparisons between more than two experimental groups (e.g. expression of various PI3K isoforms) and one measured variable were done using one-way ANOVA to test for variance and Tukey’s multiple comparison test.

Data and software availability

This study includes no data deposited in external repositories.

Conflict of interest
KO received consultancy payments and/or research funding from Karus Therapeutics, Gilead Sciences and GlaxoSmithKline.

For more information/relevant links

This study focuses on regeneration in the injured optic nerve, as part of projects aimed at repairing the damage that can occur in the optic nerve due to degenerative conditions such as glaucoma. It is also part of major research projects aimed at stimulating axon regeneration in the injured spinal cord. Below are links to author websites, charities and patient associations relevant to these conditions.

(i) https://www.researchgate.net/profile/Bart_Nieuwenhuis3
(ii) https://www.researchgate.net/profile/Amanda_Barber
(iii) https://www.researchgate.net/profile/Rachel_Evans45
(iv) https://www.researchgate.net/profile/Richard_Eva
(v) International Glaucoma Association: www.glaucoma-association.com
(vi) Centre for Eye Research Australia: www.cera.org.au
References


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