Regulation of PI3K signalling by the phosphatidylinositol transfer protein PITPα during axonal extension in hippocampal neurons

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Summary

Phosphatidylinositol transfer proteins (PITPs) mediate the transfer of phosphatidylinositol (PtdIns) or phosphatidylcholine (PtdCho) between two membrane compartments, thereby regulating the interface between signalling, phosphoinositide (PI) metabolism and membrane traffic. Here, we show that PITPα is enriched in specific areas of the postnatal and adult brain, including the hippocampus and cerebellum. Overexpression of PITPα, but not PITPβ or a PITPα mutant deficient in binding PtdIns, enhances laminin-dependent extension of axonal processes in hippocampal neurons, whereas knockdown of PITPα protein by siRNA suppresses laminin and BDNF-induced axonal growth. PITPα-mediated axonal outgrowth is sensitive to phosphoinositide 3-kinase (PI3K) inhibition and shows dependency on the Akt/GSK-3/CRMP-2 pathway. We conclude that PITPα controls the polarized extension of axonal processes through the provision of PtdIns for localized PI3K-dependent signalling.

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Key words: PITP, Axonal elongation, Phosphoinositide 3-kinase, Hippocampal neurons

Introduction

The establishment of neuronal morphology is essential for the functioning of the nervous system and is regulated through the integration of intracellular signalling pathways activated by different extracellular cues. Among these, the phosphoinositide 3-kinase (PI3K) pathway is particularly important for early neuronal events, including the specification of immature neuronal processes into axons and the control of their extension behaviours necessary for navigation toward appropriate targets (Cosker and Eickholt, 2007). One of the best-characterized cellular models used for studying neuronal morphogenesis is the in vitro culture of hippocampal neurons, in which morphological events during neuronal maturation have been divided into stages (Dotti et al., 1988). Shortly after plating, hippocampal neurons extend several short processes of equal length. Restricted PI3K activation in only one of the processes accelerates elongation and initiates the specification of this process to become an axon (Menager et al., 2004; Shi et al., 2003), a response mediated by the selective recruitment and activation of specific downstream effectors to the future axonal process (Arimura and Kaibuchi, 2007). Different extracellular factors, including components of the extracellular matrix (ECM), axon guidance molecules and growth factors, have been shown to alter axonal growth behaviour through changes in PI3K signalling (Adler et al., 2006; Chadborn et al., 2006; Chang et al., 2006; Eickholt et al., 2002; Yoshimura et al., 2005; Zhou et al., 2004). Much progress has been made in understanding the downstream components required during PI3K-mediated axonal remodelling; however, far less is known concerning the molecular machinery that ensures substrate availability during sustained activation of PI3K.

The substrate of PI3K, PtdIns(4,5)P2 (PIP2), is synthesized from phosphatidylinositol (PtdIns) by sequential phosphorylation steps that are mediated by PI4K and PI(4)P5-kinase. As PtdIns is synthesized in the endoplasmic reticulum, mechanisms for the transport of PtdIns to its sites of use at the membrane have been proposed. Phosphatidylinositol transfer proteins (PITPα and β) possess the unique ability to specifically bind PtdIns or phosphatidylcholine (PtdCho) (Tilley et al., 2004; Yoder et al., 2001), and to facilitate their transfer between membrane compartments (Cockcroft, 1998; Wirtz, 1991). The PtdIns transfer activities of PITPs have been shown to be required for both PI3K- and PLC-mediated signalling, and during PLC signalling, PITPs are thought to function by replenishing the plasma membrane pool of inositol lipids with PtdIns from its intracellular site of synthesis as PIP2 is consumed (Kular et al., 1997; Thomas et al., 1993). Thus, it can be speculated that PITPs may play a role in the development of the nervous system, given the evidence that both PI3K and PLC activity can modulate neurite outgrowth responses (Ming et al., 1999). In this context, PITPα has indeed been shown to be an essential component during netrin-1-induced PLC signalling and neurite outgrowth (Xie et al., 2005; Xie et al., 2006) – by contrast, whether PITPs also controls PI3K-dependent signalling events during neurite remodelling is currently unknown.

Results

In mice, an essential requirement for PITPα for nervous system development and/or function is indicated in the *vibrator* mutation, which causes neurodegeneration due to a five-fold reduction in PITPα levels (Hamilton et al., 1997). Although neural phenotypes have been reported in PITPα knockout mice (Alb Jr et al., 2003; Alb Jr et al., 2007), surprisingly little detailed information is available concerning the tissue distribution of PITP proteins in the brain and/or the molecular function of PITPα in neurons. Analysis of PITPα expression by western blotting revealed the highest expression in the brain (Fig. 1A; see Fig. S1 in the supplementary material), supporting previously reported mRNA distributions (Imai et al., 1997; Utsunomiya et al., 1997). PITPβ, by sequence and structure a closely related PITP, is also expressed in the brain, although levels are substantially lower than levels of PITPα (Fig. 1A; see Fig. S1 in the supplementary material). Histochemical analysis in the adult mouse cerebellum showed restricted PITPα expression in the molecular layer and punctuate immunoreactivity in the granule cell layer (Fig. 1B). Although low signals are detected in the white matter tract of the adult mouse cerebellum, an axonal distribution in this region at P15 suggests a requirement for PITPα in developmental events contributing to the establishment of neuronal connectivity (Fig. 1C). In the hippocampus of adult (Fig. 1D,E) and P15 mice (Fig. 1F), PITPα was broadly expressed; again, specific synaptic contacts were enriched in the stratum radiatum (Fig. 1E).

We examined the distribution of PITPα during neuronal maturation in embryonic day 18 (E18) rat hippocampal neurons that were cultured in vitro for up to 21 days. At day 2, the majority of hippocampal neurons acquired the characteristic stage 3 morphology with one long process (axon) and several minor processes (immature dendrites) (Dotti et al., 1988). At this stage, the anti-PITPα antibody labelled the axonal processes and their growth cones (Fig. 2A-C). Little or no staining was present in the remaining shorter processes, including their growth cones. This distribution was observed in approximately 70% of hippocampal neurons that had acquired a stage 3 morphology (Fig. 2D). The axonal accumulation was specific to PITPα, as PITPβ appeared to be enriched in the perinuclear Golgi region of the neuronal soma (Fig. 2E). At day 5 (stage 4 neurons), PITPα immunostaining was detected in both axons and dendrites (Fig. 2F), and at day 14, strong, punctate PITPα signals showed a degree of co-localization with the pre-synaptic marker synaptophysin (Fig. 2G). These results manifest a dynamic distribution and a steady increase in expression of PITPα during neuronal maturation, which we also observed by western blot analysis (Fig. 2H).

To examine whether PITPα is required for neuronal development, we perturbed the levels of endogenous PITPα by overexpression

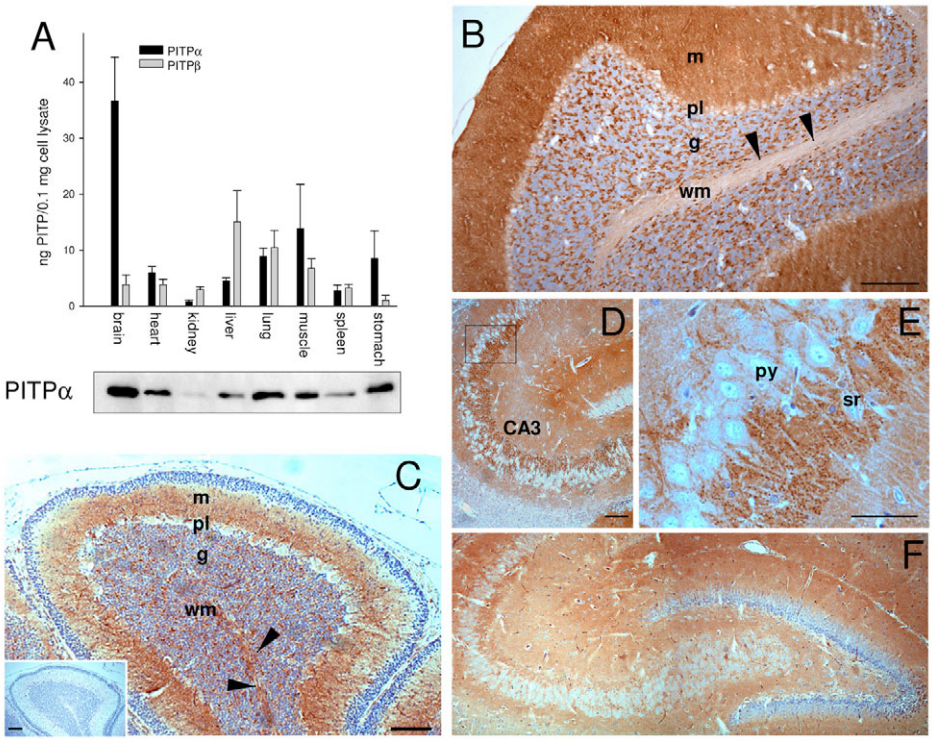

Fig. 1. PITPα is highly enriched in the adult and developing brain. (A) Comparison of PITPα and PITPβ expression in the adult rat. Equal protein amounts of indicated tissue extracts were separated by SDS-PAGE and probed using anti-PITPα or anti-PITPβ antibodies. For quantification of PITPα and PITPβ expression see Fig. S1 in the supplementary material. Bars show the mean of three independent analyses ± s.e.m. (B) In the adult mouse cerebellum, anti-PITPα labelling was detected in the molecular layer and the granule cell layer, whereas low signals were associated with the Purkinje cell layer and the white matter tract. (C) In the P15 mouse cerebellum, PITPα was expressed in the molecular layer and in the granule cell layer. In addition, afferent fibres in the developing white matter tract were positive for PITPα immunoreactivity. In control experiments performed in parallel, no signal was detected in the presence of control serum (see insert). (D) PITPα was broadly expressed in the CA3 cells of the adult hippocampus. A higher magnification of the outlined box in D is shown in E. (E) Particularly enriched labelling was seen in the stratum radiatum. The cell bodies of the pyramidal neurons were devoid of PITPα. (F) PITPα was broadly expressed in the P15 hippocampus. Scale bars: 200 μm in B; 100 μm in D,F; 50 μm in E. m, molecular layer; g, granule cell layer; pl, Purkinje cell layer; py, pyramidal neurons; sr, stratum radiatum; wm, white matter tract.

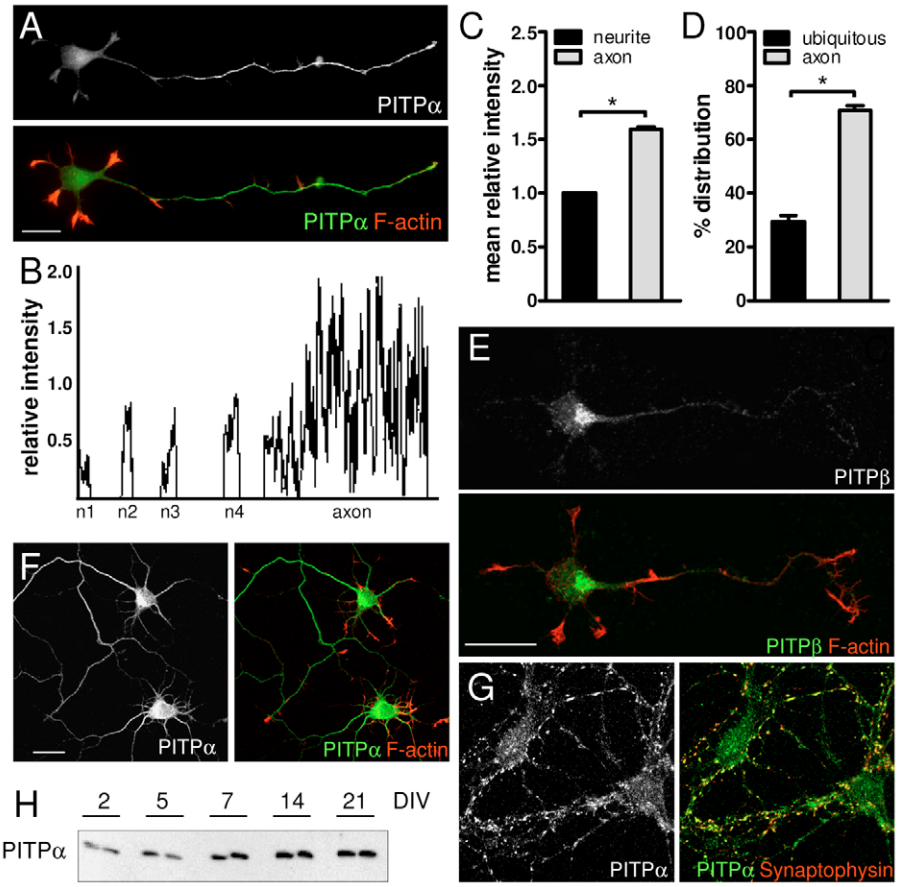

Fig. 2. Localization of PITPα during neuronal maturation in hippocampal neurons. E18 hippocampal neurons were cultured for 2, 5 and 14 days before fixation and antibody labelling. (A) At day 2 (stage 3 neurons), PITPα mainly localized to axonal processes (arrowhead). No or little staining was present in the remaining shorter processes clearly visible in the Phalloidin staining visualizing F-actin (red). (B) Line scan of a stage 3 hippocampal neuron demonstrating the relative intensity of PITPα fluorescence along each minor neurite (n1-n4) and the axon. (C) Mean relative PITPα fluorescence intensity in neurites and axonal processes. Each data point is the mean ± s.e.m., n=7. (D) Percentage of hippocampal neurons that showed PITPα fluorescence in all processes (ubiquitous) or in the axon, only. Each data point is the mean ± s.e.m., n=106, *P<0.0001. (E) PITPβ was enriched in the Golgi region of stage 3 hippocampal neurons. (F) At day 5 (stage 4 neurons), anti-PITPα antibody labelled both axonal and dendritic processes. (G) At day 14, anti-PITPα immunostaining appeared punctate and showed some co-localization with the pre-synaptic marker synaptophysin (red). (H) Hippocampal neurons were cultured for indicated days in vitro (DIV), lysed and 10 μg protein was separated and analysed by western blotting using anti-PITPα. Scale bars: 20 μm.

and by reduction of PITPα levels using siRNA. Overexpression of PITPα was achieved by nucleofection, which resulted in an approximately twofold increase in PITPα protein levels at day 2 (see Fig. S2 in the supplementary material). GFP alone localized throughout the neuron (Fig. 3A), whereas PITPα-GFP showed similar localization to endogenous PITPα with an enrichment in axonal processes (Fig. 3B; see Fig. S3A,B in the supplementary material). We assessed neuronal morphology at 2 days after nucleofection. Although neuronal polarization was not affected (see Fig. S4 in the supplementary material), overexpression of PITPα-GFP led to a significant increase in axonal, but not dendritic, length (Fig. 3C,E). PITPα can bind to either PtdIns or PtdCho and facilitate lipid transfer. PtdIns binding to PITPα is dependent on four amino acid residues that form specific hydrogen bonds with the hydroxyl groups on the inositol ring of PtdIns. Previously, we have shown that a PITPα-K61A mutant is unable to bind PtdIns and to facilitate its transfer, without affecting its ability to bind and transfer PtdCho (Tilley et al., 2004). Here, overexpression of PITPα-K61A-GFP in hippocampal neurons did not cause an increase in axonal length (Fig. 3E), demonstrating a dependency on PtdIns binding and transfer during PITPα-induced axonal elongation. In addition to PITPα, the related protein PITPβ is effective for PtdIns transfer (Allen-Baume et al., 2002). Following nucleofection of hippocampal neurons, levels of PITPβ-GFP expression were comparable to PITPα-GFP (Fig. 3D); however, the beta isoform did not cause an increase in axonal length (Fig. 3E).

We examined the requirement of PITPα to support neurite elongation under laminin-stimulated growth conditions, by interference of PITPα protein using specific siRNAs. Nucleofection of siRNAs targeting the transfer protein led to a strong suppression of approximately 70% at day 2 (Fig. 4A) and resulted in reduced axonal length without affecting neuronal polarization (Fig. 4B; see Fig. S4A in the supplementary material). By contrast, scrambled siRNAs did not affect PITPα protein levels or process elongation at any stage (Fig. 4A,B). The reduction in PITPα protein was also reflected in the staining profile using specific antibodies (Fig. 4C).

Our results demonstrate that overexpression of PITPα increases the extension of axonal processes in hippocampal neurons and that PITPα is necessary to support efficient axon growth without displaying noticeable effects on process formation and/or elongation per se. Therefore, we tested whether the effects of PITPα on axon growth can be linked to its established function in delivering PtdIns to membrane domains of specific phosphoinositide (PI)-dependent signalling necessary for axonal elongation. In this context, it has been shown that localized laminin stimulation at the neurite tips of hippocampal neurons induces localized activation of PI3K, which is followed by rapid process elongation (Menager et al., 2004). We cultured GFP- or PITPα-GFP-expressing hippocampal neurons on a substrate consisting of poly-lysine only and determined axonal length as previously. As shown in Fig. 5A, axons are insensitive to PITPα-increased elongation in the absence of laminin. To test whether the effect on axonal elongation involves PI3K, we applied LY294002 to PITPα-GFP-overexpressing neurons at day 1 and incubated further to day 2. Under these culture conditions, PITPα-mediated increases in axonal extension were fully antagonized and indistinguishable from control, LY294002-treated neurons (Fig. 5A).

Axonal extension in hippocampal neurons is also increased by application of growth factors, an effect that is thought to involve PI3K and its pivotal downstream target Akt, which has been shown to mediate axon elongation and morphological responses induced by neurotrophins in sensory neurons (Da Silva et al., 2005; Ip et al., 1993; Labelle and Leclerc, 2000; Markus et al., 2002; Yoshimura et al., 2005; Zhou et al., 2004). Indeed, stimulation of hippocampal neurons with BDNF for 1 hour or 15 hours increases levels of activatory Akt phosphorylation (see Fig. S5A in the supplementary material). We therefore tested whether PITPα is required during BDNF-induced axonal growth in hippocampal neurons. Application of BDNF to hippocampal neurons increased axonal length as previously described (Yoshimura et al., 2005), an effect that was reduced by inhibition of PI3K and by knockdown of PITPα (Fig. 5B). These results indicate an essential requirement of PITPα during growth-factor-induced axon outgrowth. It is interesting to note that the BDNF effect on axonal branch formation in PITPα siRNA-treated neurons was not reduced (Yoshimura et al., 2005) (see Fig. S5 in the supplementary material).

Next, we examined whether overexpression or reduction of PITPα protein directly affects PI3K signalling by analysis of the phosphorylation state of Akt. Overexpression of PITPα-GFP in hippocampal neurons increased pAkt levels by approximately 50%, as confirmed by western blotting (Fig. 4C,D). We additionally examined the consequence of PITPα-GFP overexpression on Akt phosphorylation in hippocampal neurons by immunocytochemistry and found significant increases in relative fluorescence intensity of pAkt labelling in axonal growth cones (Fig. 5E,F,G), whereas overall growth cone morphologies were not affected by overexpression of PITPα-GFP (data not shown). In summary, these results suggest that PITPα is essential during axonal growth responses accelerated by laminin and BDNF through its ability to augment PI3K signalling.

PI3K activity has previously been shown to be required for accelerated growth responses in hippocampal neurons (Menager et al., 2004; Shi et al., 2003; Yoshimura et al., 2006). Localized, PI3K/AKT-dependent signalling reduces GSK-3β activity towards microtubule-binding proteins including CRMP-2 and promotes axon outgrowth (see Fig. 6A) (Jiang et al., 2005; Yoshimura et al., 2006; Yoshimura et al., 2005; Zhou et al., 2004). To investigate the possibility of whether GSK-3β functions downstream of PITPα-

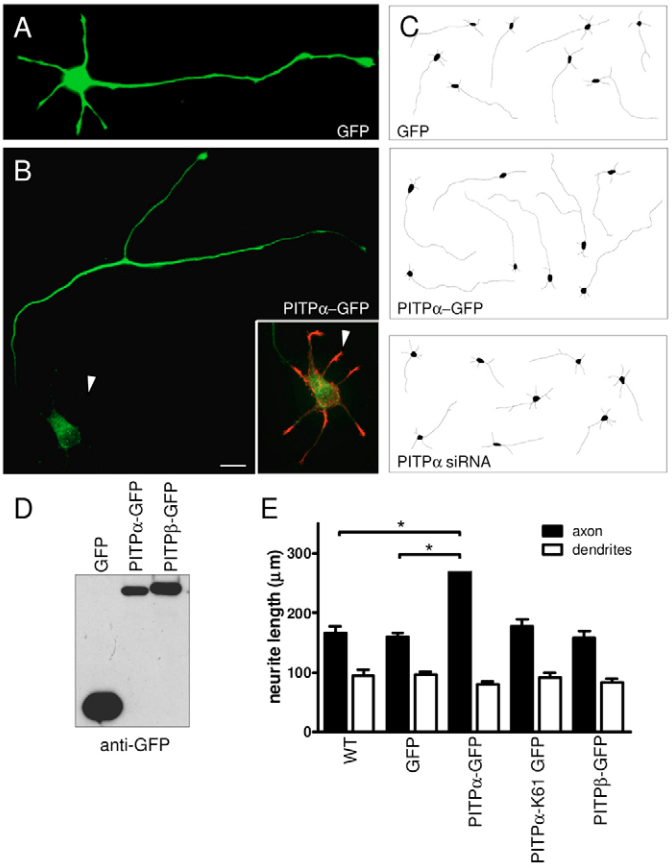

Fig. 3. Overexpression of PITPα increases axonal growth in hippocampal neurons. (A,B) E18 hippocampal neurons were nucleofected with indicated constructs and cultured for 48 hours. (A) GFP distributed throughout the neuron, whereas (B) PITPα-GFP localized to the axons of stage 3 hippocampal neurons. Little signal was detected in immature dendrites (arrowheads); these minor processes, however, were clearly visible in the Phalloidin staining (see insert, red). (C) Representative outlines of hippocampal neurons expressing GFP, PITPα-GFP or PITPα siRNA. (D) Hippocampal neurons expressing GFP, PITPα-GFP or PITPβ-GFP were analysed by immunoblotting using an anti-GFP antibody. (E) Evaluation of process length. Neurites were scored as polarized in the presence of a single, long neurite exhibiting no less than twice the length of the remaining shorter neurites. The length of the longest neurite (axon), and all remaining shorter neurites (immature dendrites) of an individual neuron were measured within each treatment. The asterisk indicates significant increases in axonal length following overexpression of PITPα-GFP in comparison with untransfected (WT) or GFP-expressing neurons (*P<0.0001). This effect was specific to the PtdIns transfer activity of PITPα, as PITPα K61A, a mutant unable to bind and transfer PI, or PITPβ did not induce increased axonal length. All data are the mean ± s.e.m. of at least three independent experiments. Scale bar: 20 μm.

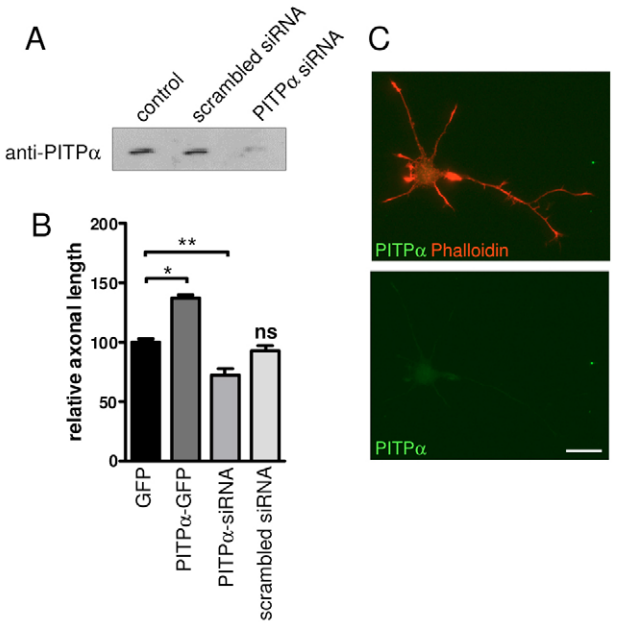

Fig. 4. Knockdown of PITPα by siRNA decreases axonal growth in hippocampal neurons. E18 hippocampal neurons were nucleofected with indicated constructs or siRNAs, and cultured for 48 hours. (A) PITPα siRNA, but not control scrambled siRNA, leads to a reduction in endogenous PITPα levels, as validated by western blotting. (B) Knockdown of PITPα by siRNA significantly decreased axonal length (*P<0.0001, **P<0.0002). All data are the means ± s.e.m. of at least three independent experiments (n>125). (C) Knockdown of PITPα was monitored in each experiment by immunocytochemistry using anti-PITPα antibody. Scale bar: 20 μm.

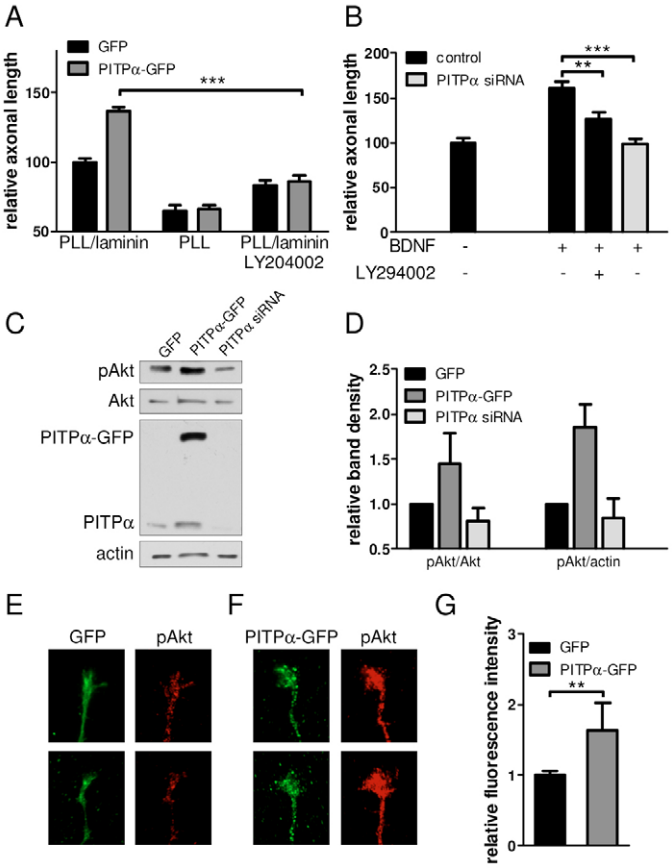

Fig. 5. PITPα-induced increases in axonal length are mediated by PI3K signalling. Hippocampal neurons were nucleofected with GFP, PITPα-GFP or PITPα siRNA, and cultured for 48 hours on poly-lysine/laminin or poly-lysine substrates. (A) Neurons expressing PITPα-GFP did not exhibit increases in axonal length when cultured on poly-lysine alone. In addition, PI3K inhibition by LY294002 (at 10 μM) antagonized PITPα-GFP-mediated increases in axonal length on poly-lysine/laminin substrate. Each data point is the mean ± s.e.m. of at least three independent experiments, n=120. (B) Application of BDNF (50 ng/ml) to hippocampal neurons induces an increase in axonal length, which was partially blocked by LY294002 and fully inhibited by knockdown of PITPα by siRNA. Data points show the mean ± s.e.m. of three independent experiments (n>60). (C) Cell lysates of hippocampal neurons expressing GFP, PITPα-GFP or PITPα siRNA were analysed by western blotting using indicated antibodies. (D) Normalized relative band density of pAkt/Akt and pAkt/actin reveal increased activity of PI3K signalling following overexpression of PITPα-GFP (pAkt/actin), n=5. (E) GFP and (F) PITPα-GFP expressing hippocampal neurons were fixed after 48 hours and stained using anti-pAkt antibody (red). (G) The relative fluorescence intensity of pAkt labelling in axonal growth cones of PITPα-GFP expressing neurons is significantly increased when compared with GFP expressing neurons. Each data point is the mean ± s.e.m. of seven independent experiments (n>120). $^{**}P$<0.005, $^{***}P$<0.0001.

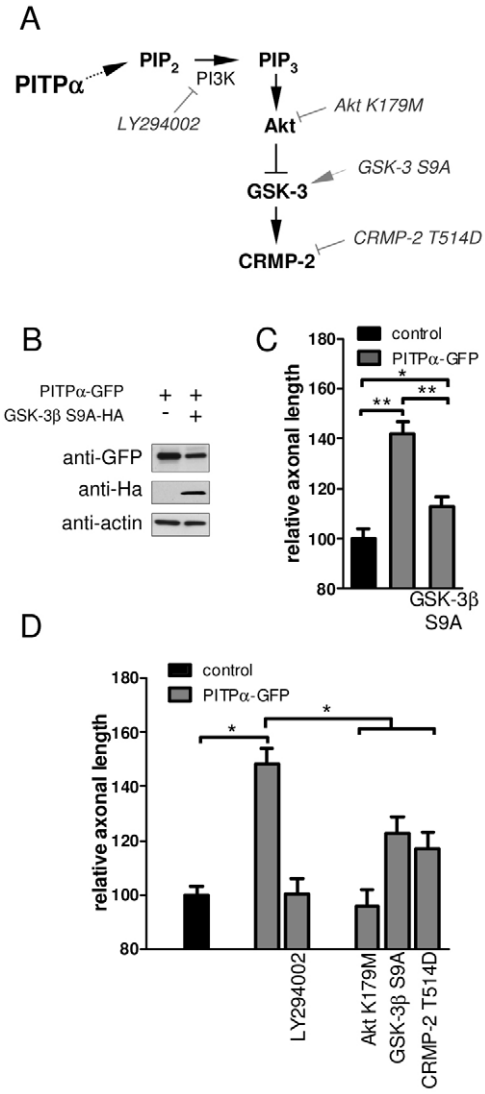

Fig. 6. PITPα-induced axonal elongation is dependent on Akt/GSK-3/CRMP-2 downstream of PI3K. (A) Schematic representation and signalling relationship of the PI3K/Akt/GSK-3/CRMP-2 pathway previously shown to control axon specification and elongation. In grey, the different mutant constructs and pharmacological inhibitors (LY294002) that were tested in their ability to alter PITPα-induced axonal elongation downstream of PI3K signalling. (B) E18 hippocampal neurons were nucleofected with indicated constructs, cultured for 48 hours and analysed by immunoblotting using indicated antibodies. (C) Nucleofection of constitutively active GSK-3β (GSK-3β S9A) with PITPα-GFP antagonise PITPα-induced increases in axonal length. (D) E18 hippocampal neurons were transfected with indicated constructs by Lipofectamine. Following 48 hours, cultures were fixed and analysed as previously. LY294002 (10 μM) was applied after 8 hours. Data points show the mean ± s.e.m. of at least three independent experiments (n>60), $^{*}P$<0.02, $^{**}P$<0.005.

induced axon outgrowth, we co-nucleofected Ha-tagged, constitutive active GSK-3β (GSK-3β S9A) with PITPα-GFP and determined axonal length as previously (Fig. 6B). PITPα-increased axonal growth was significantly reduced in the presence of GSK-3β S9A (Fig. 6C), suggesting that inhibition of GSK-3β is a crucial signalling component involved in mediating PITPα-induced axon growth. We employed a second strategy to elucidate the possible downstream signalling components involved during PITPα-induced axon growth. Hippocampal neuron cultures were established as previously and transfected using Lipofectamine (Shi et al., 2004; Shi et al., 2003). Comparable to the results obtained following nucleofection, overexpression of PITPα substantially increased axonal elongation by approximately 50%, which, again, was sensitive to PI3K inhibition (Fig. 6D). To directly determine the involvement of Akt during PITPα-stimulated axonal elongation, we co-transfected PITPα with dominant-negative Akt (Akt K179M). In the presence of Akt K179M, PITPα-increased axonal length was suppressed, and co-expression of CRMP-2 T514D, a mutant thought

to mimic the GSK-3β-phosphorylated form of CRMP-2 (Yoshimura et al., 2005), reduced PITPα-induced axonal growth similar to co-expression of GSK-3β S9A (Fig. 5D). These results demonstrate that PITPα regulates axonal outgrowth, at least partially by acting through the PI3K/Akt/GSK-3β/CRMP-2 pathway.

Discussion

The establishment of neuronal morphology requires distinct mechanisms to control polarized membrane growth. PITPs are lipid transfer proteins that can bind and transfer PtdIns and PtdCho, thereby regulating PI metabolism and membrane traffic. Detailed information on the tissue distribution of PITPα and PITPβ protein isoforms is not available, although evidence has been presented for an enriched mRNA expression of both isoforms in the brain (Imai et al., 1997; Utsunomiya et al., 1997). Here, we report on the expression of PITPα protein in the nervous system at embryonic and adult stages. PITPα is present in developing neurons concomitant with the extension and guidance of neurites in the central and peripheral nervous systems (Fig. 1). It is generally of note that the expression in axonal tracts decreases to low levels in the adult, at which stage PITPα protein is enriched in dendritic and synaptic regions of the brain. This dynamic expression corroborated the distribution of PITPα we observed in hippocampal neurons cultured in vitro, where PITPα was present early in axons, at later stages in axons and dendrites, and in mature hippocampal neurons in pre-synaptic terminals.

Our results demonstrate that in early hippocampal neurons, PITPα is essential for axonal extension induced in a ligand-dependent manner. PITPα localizes predominantly to the axons of stage 3 hippocampal neurons and overexpression of PITPα increases axonal length, provided that neurons are cultured on laminin. Although increases in elongation of a neuronal process has been shown to lead to its specification into an axon (Bradke and Dotti, 2000; Da Silva et al., 2005; Esch et al., 1999), changes in PITPα expression – either by overexpression or knockdown – never affected neuronal polarization. This is in contrast to a number of axon-specification molecules, which have been characterized by their ability to induce the formation of multiple axons upon overexpression and/or changes in their activity state (Inagaki et al., 2001; Jiang et al., 2005; Nishimura et al., 2004; Schwamborn and Puschel, 2004; Shi et al., 2004; Shi et al., 2003). The effect of PITPα on axonal elongation could be suppressed by PI3K inhibition, suggesting that PITPα might be an important component for PtdIns delivery to plasma membrane domains with increased activity of PI3K. This idea is supported by our observations that absence of PITPα renders neurons insensitive to BDNF, a growth factor known to stimulate axonal elongation in a PI3K-dependent manner (Ip et al., 1993; Labelle and Leclerc, 2000; Yoshimura et al., 2005). Likewise, loss of PITPα reduces axonal elongation on laminin back to non-stimulated outgrowth, masking again an effect that involves PI3K signalling (Menager et al., 2004). Thus, our data suggest that PITPα modulates the receptor-stimulated, PI3K-dependent component of axonal growth only, without affecting the basal machinery of polarization and of axonal development.

The mechanisms underlying the functional interaction between PITPα-mediated PtdIns transfer and PI3K signalling remains unclear. PI3K generates PIP3 from PIP2 and PITPα has indeed been shown to couple PtdIns delivery to the synthesis of PIP2 during cell signalling (Allen-Baume et al., 2002; Cockcroft, 2001). In addition, studies in fMLP-stimulated neutrophils suggest that the generation of PIP3 by PI3K activity requires PITPα (Kular et al., 1997). However, PIP2 is unlikely to be rate-limiting for PI3K activity as it is present in excess. Following overexpression of PITPα we demonstrate increased pAkt levels in the growth cone (as a readout for PI3K activity), which indicates that PITPα has the capacity to function during signalling events upstream of PI3Ks. Conceivably, PITPα might be required as a co-factor of enzymatic activities that control membranous levels of PIP3. An alternative mechanism is that PITPα might interface delivery of vesicles essential during membrane expansion at the growth cone during axonal growth. In either model, PITPα binding to PtdIns is essential, as the K61A mutant is unable to support PITPα-induced increases in axonal elongation.

Downstream of PI3K, PITPα-mediated axonal outgrowth requires an intact Akt/GSK-3β/CRMP-2 pathway. Co-expression of PITPα with dominant-negative Akt, constitutive active GSK-3β or a CRMP-2 mutant that mimics the GSK-3β-phosphorylated form of CRMP-2, attenuated PITPα-induced axonal elongation. At first glance, it seems unlikely that a pathway known to mediate the establishment of neuronal polarization can be a component of the exclusive effect on axonal growth by PITPα. However, although activation of different components of this pathway have indeed been shown to induce the formation of multiple axons (Inagaki et al., 2001; Jiang et al., 2005; Yoshimura et al., 2006; Yoshimura et al., 2005), inhibition is not consistently linked with the reverse effect, i.e. neurons with no axons. In this respect, overexpression of dominant-negative Akt in hippocampal neurons has no effect on either axonal elongation or polarization (Yoshimura et al., 2006), whereas overexpression of constitutively active GSK-3β disrupts elongation of axonal processes, with only 15% of neurons demonstrating defects in axon formation (Yoshimura et al., 2005). In this context it is interesting to note that expression of constitutively active GSK-3β selectively antagonized PITPα-induced axonal growth, without affecting neuronal polarity (now shown here). This indicates that regulation of GSK-3β is a crucial requirement downstream of PITPα-induced axonal growth, but also that PITPα and/or increases in PI3K signalling lie downstream of GSK-3β-dependent changes in neuronal polarization.

Recently, PITPα was shown to be crucial during netrin-1-induced neurite outgrowth responses in cortical explants (Xie et al., 2005). In this study it was demonstrated that the netrin receptors DCC and neogenin interact with PITPα in a ligand-dependent manner and increase both PITPα transfer activity and PIP2 hydrolysis. In addition, cortical explants obtained from mice in which PITPα levels are reduced by approximately 80% (the *vibrator* mice) show decreased extension of neurites in response to netrin-1 (Xie et al., 2005). Similar to our results, there were no apparent differences between neurite elongation in wild-type and mutant explants under basal, non-stimulated outgrowth conditions, which is in line with the idea that PITPα participates during axonal growth in receptor-stimulated downstream signalling only.

In summary, our results demonstrate that PITPα contributes to the molecular machinery underlying axonal extension accelerated by PI3K and provides further evidence for the conserved nature of PITPs to control polarized membrane growth through regulation of PI-dependent signalling.

Materials and Methods

Hippocampal neurons

Neuronal cultures were prepared from hippocampi dissected from embryonic day 18 (E18) rat embryos. They were dissociated by trypsin and trituration, plated onto glass coverslips coated with poly-L-lysine alone or poly-L-lysine and laminin (both 20 μg/ml; Sigma), and cultured in Neurobasal medium (Gibco) supplemented with 2% B27, 2% fetal calf serum (FCS), 1% Glutamax (Gibco) and PenStrep for indicated times. For

long-term cultures, the medium was changed after 48 hours to Neurobasal medium, 2% B27, 1% Glutamax (Gibco), PenStrep. For biochemical analysis, hippocampal neurons were cultured on 3 cm glass coverslips coated with poly-L-lysine and laminin as described above and incubated for indicated times. For nucleofection, dissociated hippocampal neurons were spun down at 900 rpm and one million cells were mixed with 100 μl nucleofector solution (Amaxa Biosystems) and 4 μg expression plasmids. In siRNA experiments, either duplex siRNA against rat PITPα (target sequence: AATGCCTACCCTTACTGCAGA) or non-silencing scrambled control siRNA (target sequence: AATTCTCCGAACGTGTCACGT) (Qiagen) was added to neuron-nucleofector solution mixture. The neuron/DNA (siRNA) mixture was then nucleofected using program O-03 (Nucleofector, Amaxa Biosystems). For transfection by lipofection, cells were plated as previously described, and after 6 hours in culture the medium was replaced and cells left for 30 minutes to recover. The conditioned medium was kept and sterile filtered. The DNA/Lipofectamine mix was added to neuronal cultures and incubated for 30 minutes before being replaced into the conditioned media for continued incubation. The PITPα-GFP, PITPβ-GFP and PITPα K61A-GFP constructs were developed in the Cockcroft lab (Tilley et al., 2004); the Akt construct was a gift from J. Downward; the CRMP-2 constructs were from Kozo Kaibuchi (Department of Cell Pharmacology, Nagoya University, Nagoya, Japan) and the GSK-3β constructs from James Woodgett (Samuel Lunefeld Research Institute, Toronto, Canada). Neurites were scored as polarized in the presence of a single, long neurite exhibiting no less than twice the length of the remaining shorter neurites or by the presence of a single, Tau-positive process. The length of the longest neurite (axon), and all remaining shorter neurites (immature dendrites) of an individual neuron were measured within each treatment (Shi et al., 2003). Detailed comparison of the percentage of polarized neurons in PITPα-GFP-expressing neurons using morphological criteria and Tau staining revealed no differences (74.7±1.78 as shown in supplementary material Fig. S4, compared to 76±2.69).

Immunostaining

Rabbit anti-PITPα polyclonal antibody and mouse anti-PITPβ monoclonal antibody were raised in-house and characterized using recombinant proteins for isoform specificity. Anti-synaptophysin antibody was obtained from ICN. Fluorophore-conjugated Phalloidin was from Molecular Probes. Anti-pAkt antibody was obtained from Cell Signalling Technologies, the anti-Tau antibody from Chemicon and the anti-βIII-tubulin antibody from Covance. Neuronal cultures were fixed with 4% paraformaldehyde and permeabilized for 10 minutes using 1% Triton X-100 in PBS. Cells were then blocked for 0.5 hours in blocking buffer (2% BSA, 0.5% Triton X-100 in PBS) and incubated with the primary antibody diluted in blocking buffer overnight at 4°C. After three washes in PBS, cultures were incubated with the appropriate secondary antibody diluted in blocking buffer for 1 hour at room temperature. For quantitative immunocytochemistry, axonal growth cones were selected at random from stage 3 hippocampal neurons stained with Phalloidin for F-actin. Growth cones were imaged at ↔60 on a Nikon inverted fluorescent microscope, taking care to avoid pixel saturation. The growth cone outline was traced using the actin image and superimposed on the fluorescent image. The fluorescence intensities of individual growth cones were measured digitally using SimplePCI software, yielding values of pixel intensity per unit area. The background fluorescence was measured by placing the outline of the growth cone in an adjacent area devoid of cellular material and subtracted from the growth cone values to give a background-corrected intensity value. For presentation of the data, the fluorescence intensity values were normalized to the respective control experiments that were performed in parallel. For paraffin wax sectioning, paraformaldehyde-fixed mouse tissue was embedded in wax, cut into 6 μm sections and processed as described by Bancroft and Stevens (Bancroft and Stevens, 1996).

Western blot analysis

Neuronal cultures in each treatment or rat tissue were lysed in cold 1% NP-40 and the lysate was pelleted and the soluble extract was used for western blot analysis. Equal amounts of proteins from neuronal extracts were separated on 10% polyacrylamide gels and proteins were transferred to nitrocellulose or PVDF membranes. These were blocked for 1 hour in 5% milk/TBST and incubated with primary antibody in blocking buffer for 3 hours. The primary antibodies used were: anti-PITPα, anti-pAkt (Ser 473) (Cell Signalling Technologies), anti-Akt (Cell Signalling Technologies), anti-pGSK-3β (Ser 9) (Cell Signalling Technologies), anti-GSK-3β (Transduction Laboratories), anti-GFP (Molecular Probes), anti-Ha (Roche) and anti-β-actin (Chemicon). After three washes with TBST, the blots were probed with HRP-conjugated secondary antibody (Vector Labs) in blocking buffer for 1 hour. Membranes were then developed using an ECL Advance system (Amersham Pharmacia). In order to quantify protein phosphorylation levels, band density was analysed using Phoretix software and normalized to total protein levels or actin loading control.

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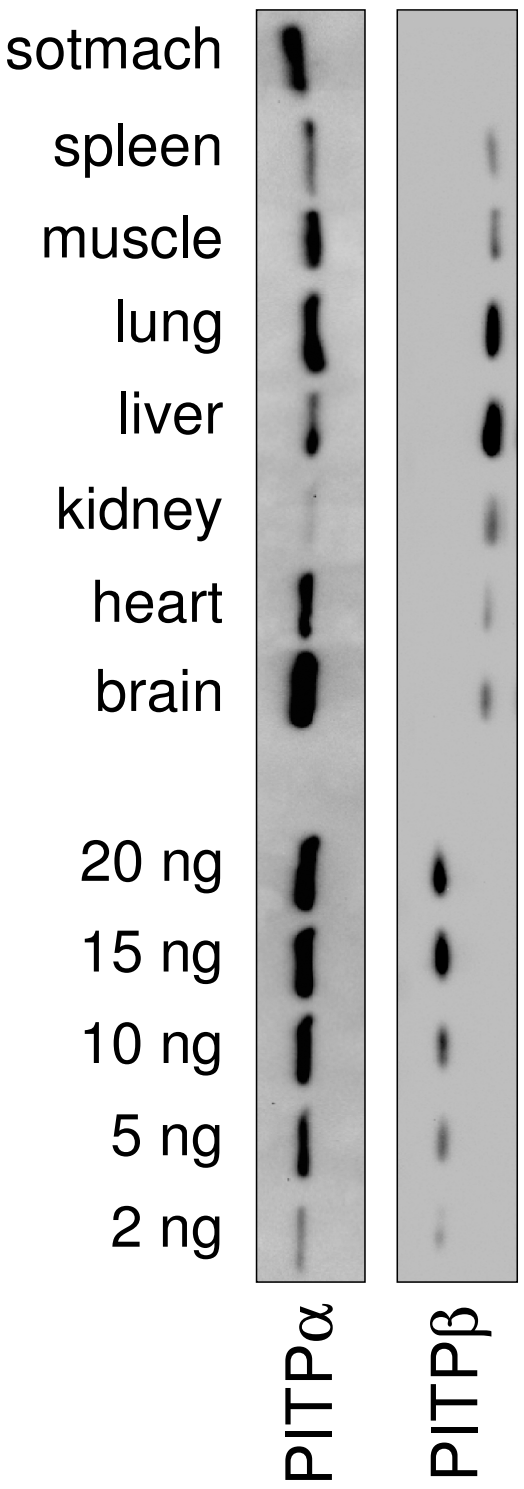
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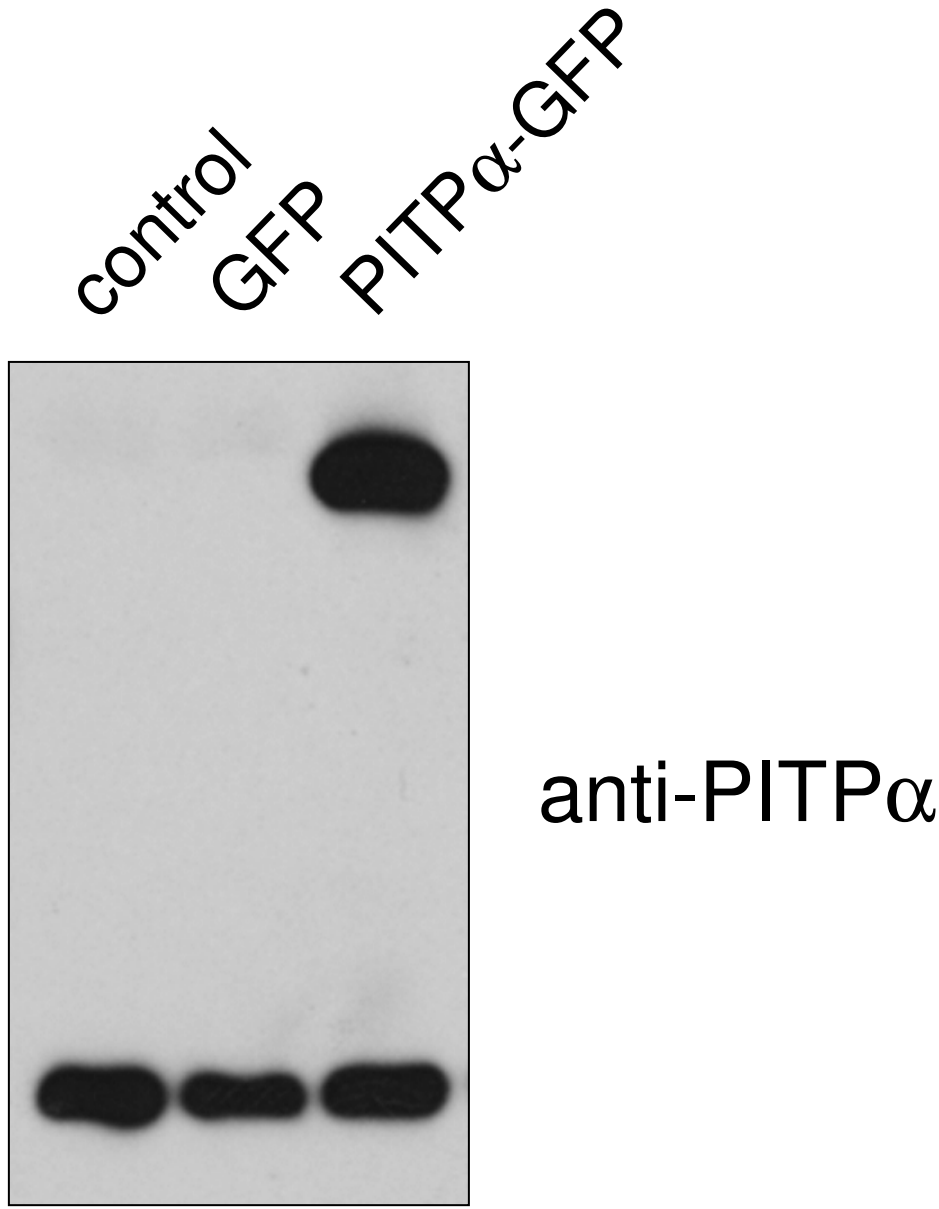
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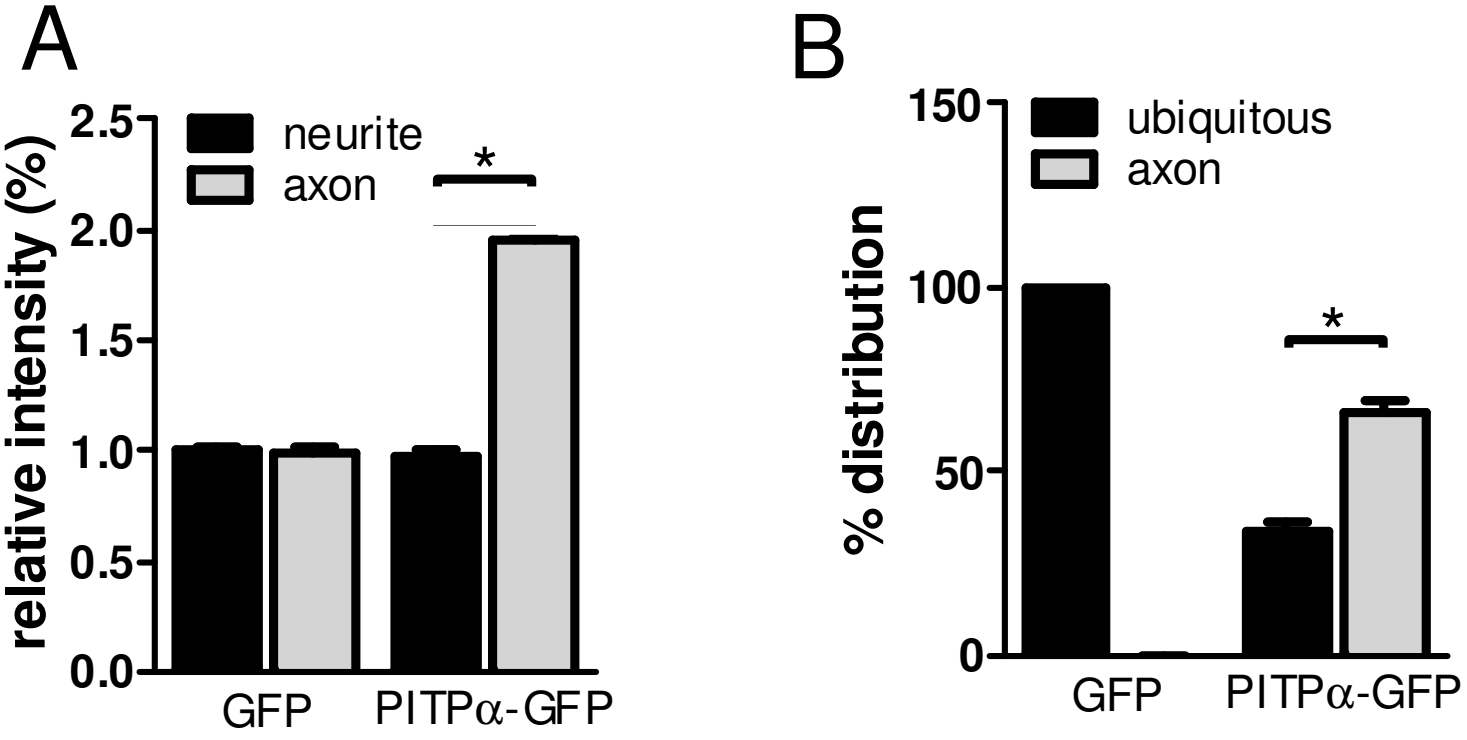
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1.5
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% distribution
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50
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ubiquitous
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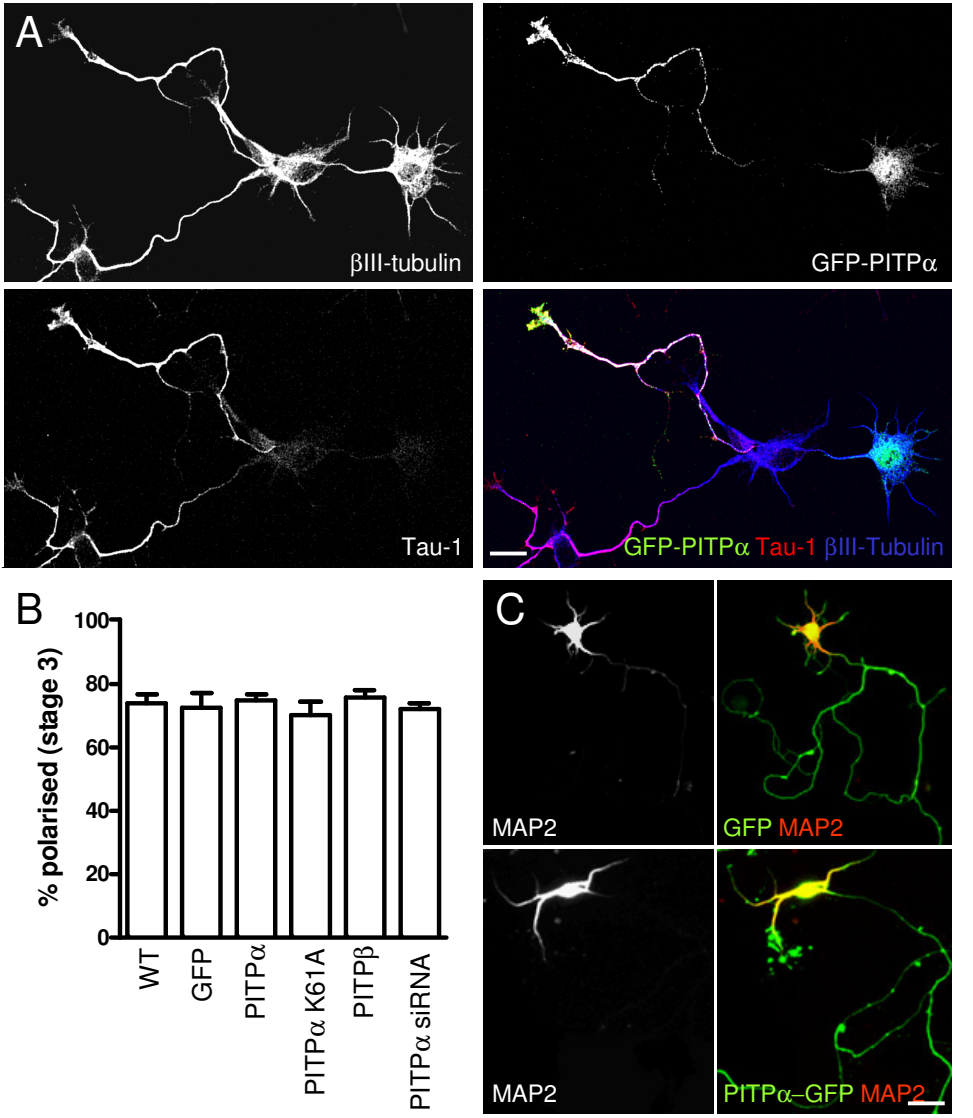
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B
% polarised (stage 3)
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40
20
0
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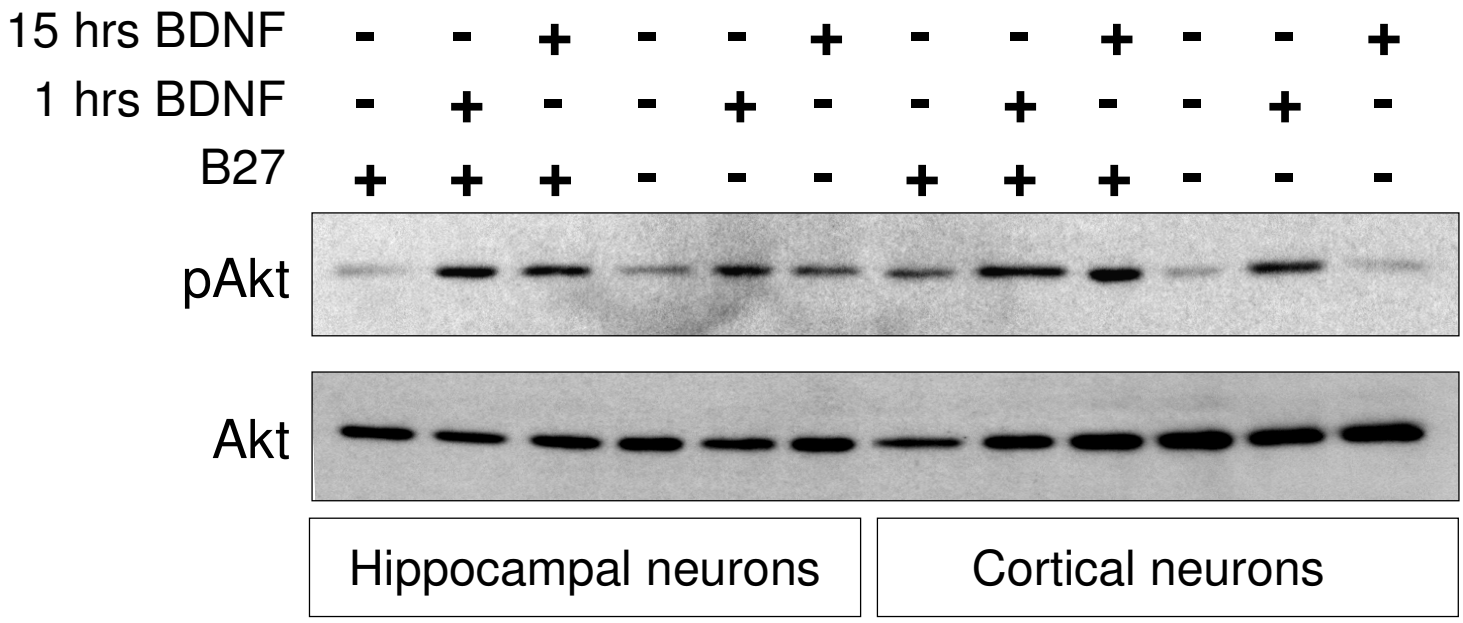
A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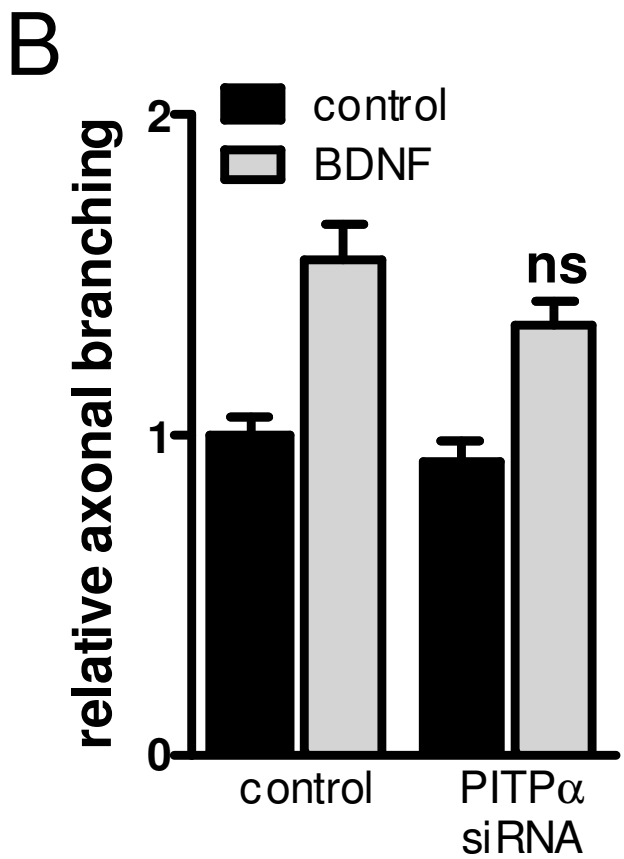
Cortical neurons

B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