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Accepted Manuscript

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Please cite this article as: Berry, Martin, Ahmed, Zubair, Morgan-Warren, Peter, Fulton, Daniel, Logan, Ann, Prospects for mTOR-mediated functional repair after central nervous system trauma, *Neurobiology of Disease* (2015), doi: 10.1016/j.nbd.2015.10.002

1

PROSPECTS FOR mTOR-MEDIATED FUNCTIONAL REPAIR AFTER CENTRAL NERVOUS SYSTEM TRAUMA

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ABSTRACT

Recent research has suggested that the growth of central nervous system (CNS) axons during development is mediated through the PI3K/Akt/mammalian target of rapamycin (mTOR) intracellular signalling axis and that suppression of activity in this pathway occurs during maturity as levels of the phosphatase and tensin homolog (PTEN) rise and inhibit PI3K activation of mTOR, accounting for the failure of axon regeneration in the injury adult CNS. This hypothesis is supported by findings confirming that suppression of PTEN in experimental adult animals promotes impressive axon regeneration in the injured visual and corticospinal motor systems. This review focuses on these recent developments, discussing the therapeutic potential of a mTOR-based treatment aimed at promoting functional recovery in CNS trauma patients, recognising that to fulfil this ambition, the new therapy should aim to promote not only axon regeneration but also remyelination of regenerated axons, neuronal survival and re-innervation of denervated targets through accurate axonal guidance and synaptogenesis, all with minimal adverse effects. The translational challenges presented by the implementation of this new axogenic therapy are also discussed.

INTRODUCTION

Recent research aimed at promoting axon regeneration in the injured central nervous system (CNS) has achieved impressive regrowth of long axonal tracts by activation of the phosphatidylinositol/protein kinase B/mammalian target of rapamycin intracellular signalling pathway (PI3K/Akt/mTOR; mTOR is used throughout as an inclusive term for mTORC1 and mTORC2 – abbreviations of all signalling molecules are given in Fig. 1) (reviewed by Liu *et al*., 2011; Kanno *et al*., 2012; Aruni *et al*., 2012; Maiese *et al*., 2013; Maiese, 2014; Lu *et al*., 2014). These and others studies attribute the poor prognosis for functional restitution to suppression, at around birth, of developmental mTOR-mediated neuroprotection and axogenic protein synthesis (Park *et al*., 2008, 2010; He, 2010; Pernet and Schwab, 2014). This proposition predicts that treatments which re-establish the sensitivity of the PI3K/Akt pathway to growth factor activation in maturity augur well for the restoration of function in CNS trauma patients.

During mammalian CNS development, the viability of neurons and growth of axons is supported by neurotrophic factors (NTF) such as: (i), neurotrophins (NT) including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophic factor 3 (NT3), and NT4/5, which activate the PI3K/Akt intracellular signalling pathway after engaging tyrosine kinase receptors (Trk); and (ii), cytokines such as ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) all of which bind to the trimeric gp130 receptor complex and activate PI3K through JAK/SHP2 signalling (Heinrich *et al*., 2003; Müller *et al*., 2007, 2009) - negatively regulated by suppressor of cytokine signalling 3 (SOCS3) (Nicholson *et al.,* 2000) (Fig.1). The PI3K/Akt pathway controls cell survival and

axogenic protein synthesis (reviewed by Fonseca *et al.*, 2014) through mTORC1 and also cytoskeletal growth cone dynamics by modulation of GSK3β activity (Zhou and Snider, 2005; Liu *et al.*, 2012), potentiated through the mTORC2/Akt loop. CNS axon growth declines during maturation, possibly through suppression of NTF responsiveness engendered by a progressive up-regulation of the PI3K antagonist, phosphatase and tensin homolog (PTEN), a decline in IL-6 secretion by astrocytes and neurons (Codeluppi *et al.*, 2014), a rise in the ratio of repressors:enhancers of transcriptional axon growth Kruppel-like factors (KLF) (Arlotta *et al.*, 2005; Moore *et al.*, 2009), and soaring titres of scar- and myelin-derived axon growth inhibitory factors (AGIF), the potency of which is enhanced by a postnatal decline in intracellular cAMP (Shewan *et al.*, 2002; Peace and Shewan, 2011;Cai *et al.*, 2001). Further suppression of mTOR is induced in mature CNS neurons after axotomy (Liu *et al.*, 2010; Park *et al.*, 2010) but *pten* gene deletion re-activates the PI3K/Akt pathway promoting new axon growth and additional NTF/cytokine priming may be required to initiate axon sprouting (Leibinger *et al.*, 2012; Fischer and Leibinger, 2012; Lee *et al.*, 2014).

Because of the cellular omnipresence of the mTOR pathway, many phenotypes in the CNS are affected indiscriminately by mTOR-related treatments unless cell targeting techniques are employed. The cellular functions of mTOR include the regulation of metabolism, growth, proliferation and viability (Laplante and Sabatini, 2009; Dibble and Cantley, 2015) and the uncontrolled dysregulation of any of these responses could provoke potentially deleterious side effects in the CNS. In experimental animals, Cre/loxP recombination and delivery of DNA and si/shRNA PI3K/Akt/mTOR therapies to neurons is achieved using phenotypic promoters and neurotrophic adeno-associated virus (AAV) serotypes with minimal effects on non-

neuronal cells. However, although genes may be successfully targeted to particular neurons using phenotypic promoters (e.g. Thy1 for RGC), AAV vector neurotropism is age-dependent and not absolute (Harvey *et al.*, 2002; Aschauer *et al.*, 2013; Gholizadeh *et al.*, 2013) and may lead to potentially deleterious side effects in non-targeted cells. Neural targeting of conditional gene deletion has employed local injection of specific neurotrophic AAV-Cre constructs into CNS sites where the floxed neuronal phenotype is concentrated (e.g. intravitreal injection of AAV2/Cre for floxed retinal ganglion cells (RGC)) but does not entirely insure against gene deletion in non-neuronal floxed cells and leads to gene knockout in widespread areas of the CNS outside the AAV-Cre injection site (Thévenot *et al.*, 2003; Ahmed *et al.*, 2004; Madisen *et al.*, 2015; Yang *et al.*, 2015). Moreover, a therapy that claims to promote recovery in CNS trauma patients by targeted activation of the PI3K/Akt/mTOR pathway, would need to meet the following criteria: (i), high levels of neuro-protection for axotomised neurons; (ii) axon regeneration for all axotomised neurons; (iii), accurate axon guidance to the original denervated targets; (iv), homologous synaptogenesis within targets; (v), congruent myelination of different calibre regenerated axons; (vi), minimal adverse effects; and (vii) translation into the clinic of the candidate regimen – a tall order for a uni-dimensional treatment?

NEURO-PROTECTION

The numbers of neurons surviving axotomy determines the prospective number of axons available for regeneration. However, since mature neurons are not mitotic, those lost after injury are not replaced. Axotomy is often lethal because the constant supply of vital NTF, transported retrogradely along axons from innervated targets, is discontinued (reviewed by Bähr, 2000; Cellerino *et al.*, 2000);

nonetheless, those neurons with axon collaterals preserved proximal to transection survive and this probably accounts for the viability of axotomised dorsal root ganglion neurons (DRGN) and corticospinal tract neurons (CSTN). However, because only a few RGC have collaterals within the retina, and none have collaterals in the optic nerve (ON), RGC are especially sensitive to ON crush (ONC) when, in rats, 50% die by 7d post lesion (dpl) and >90% by 14dpl (Berkelaar *et al.*, 1994). The 10% of RGC that survive axotomy are mostly melanopsin 1 (M1) and M4 (equivalent to αRGC – Estevez *et al.*, 2012) intrinsically photosensitive (ip) RGC, i.e. >80% and ~25% of M4 and 70% and 15% of M1 ipRGC survive at 14dpl and 28dpl, respectively (Duan *et al.*, 2015). There are five classes of ipRGC (M1-M5) (Fig. 2), of which M1 and M4 subtypes comprise ~3% and ~6% of all RGC, respectively. The properties and connections of ipRGC are reviewed by Schmidt *et al.* (2011), Estevez *et al.* (2012) and Cui *et al.* (2015). PTEN promotes cell death by suppressing PI3K signalling and activating pro-apoptotic forkhead transcription factors (Nakamura *et al.*, 2000) and AMPA type glutamate receptor-mediated excitotoxicity (Liu *et al.*, 2013), but deletion of *pten* does not affect the numbers of M1 and M4 ipRGC (Fig. 2) surviving ONC (Duan *et al.*, 2015), although other RGC subtypes are neuro-protected, i.e., ~42.5% survive after *pten* deletion (Park *et al.*, 2008), increased to ~60% after co-deletion of *pten* and *socs3* (Sun *et al.*, 2011). RGC death after ONC correlates with rising REDD2/RT801 activity (Fig. 1) and plummeting mTORC1 levels; whereas REDD2/RTP801 deletion restores mTORC1 activity and rescues RGC from death (Morquette *et al.*, 2014, Morgan Warren *et al.*, 2015), although it has been suggested that Akt/GSK3β rather than Akt/mTORC1 signalling may be more important for RGC neuro-protection (Chin *et al.*, 2005). M1 ipRGC spontaneous survival is correlated with the existence of axon collaterals within the retina, resistance to glutamate

toxicity and possible neuro-protection mediated by melanopsin photo-transduction (reviewed by Cui *et al*., 2015); similar characteristics may prevail for M4 ipRGC which also constitutively express pS6 (Duan *et al*., 2015). The latter indicates endogenously active mTORC1 in these cells (Duan *et al*., 2015) capable of mediating neuro-protection by autophagic degradation and clearance of both damaged organelles and abnormal protein aggregates (Chen *et al*., 2013; Dunlop and Tee, 2014; Heras-Sandoval *et al*., 2014), and also by suppression of caspase (CASP) activation (Hanada *et al*., 2004).

Inhibition of both CASP2 by siRNA, and by dominant negative knock-down, neuro-protects >95% RGC at 14d after ONC (Ahmed *et al*., 2011; Vigneswara *et al*., 2014) (Fig. 3). To sustain neuronal viability after axotomy, pharmacological neuro-protection is required for the duration of a regenerative response until target re-innervation re-instates the target-derived NTF supply. Although the effects of CASP knockdown on M1 and M4 ipRGC are unknown, direct CASP2 suppression promotes better pan-RGC survival than *pten* deletion, implying that an anti-CASP2-based anti-apoptotic therapy may be the optimal pan-RGC neuro-protective treatment. Combining mTOR with stem cell therapy may offer an alternative strategy for increasing neuron numbers after trauma by either replacing lost neurons, providing NTF support for surviving axotomised neurons, or promoting the release of stem cell-derived neuron-differentiating growth factors (reviewed Mead *et al*., 2015). For example, after spinal cord injury, mTOR-dependent differentiation of transplanted neural stem cells into glia and neurons and the subsequent growth of axons establishes functional connections above and below the lesion (Lu *et al*., 2014; Peru *et al*., 2008).

AXOGENESIS

The reliance of regenerating axons on PI3K/Akt/mTOR activation may vary across the spectrum of neuronal phenotypes and even within the axon trajectories of a single neuron (Lee *et al.*, 2014). A small population (~10%) of RGC regenerate axons after NTF/cytokine administration (Berry *et al.*, 1996; 1999; Pernet *et al.*, 2013b) and knockdown of *pten* (Park *et al.*, 2008; Sun *et al.*, 2011; de Lima *et al.*, 2012a, b; Kurimoto *et al.*, 2010) (Fig. 4A, B). Paucity in number is explained by the finding that RGC axon regeneration is exclusively restricted to M4 ipRGC (Fig. 4C) that have high titres of constitutively active mTORC1 (i.e. are pS6[+]), express osteopontin and insulin-like growth factor receptor (IGFR) (Duan *et al.*, 2015) and project to the dorsal lateral geniculate nucleus (DLGN) (Estevez *et al.*, 2012; Brown *et al.*, 2010; Ecker *et al.*, 2010; Schmidt *et al.*, 2014), although the full extent of their innervation fields is yet to be defined. Interestingly, similar numbers of M4 ipRGC axons regenerate after osteopontin plus IGF-1 treatment as after *pten* deletion, indicating that both regulate PI3K/Akt axogenic activity in M4 ipRGC. Paradoxically however, although osteopontin and IGF expression is induced in reactive Müller glia and microglia after ONC by ischaemic and excitotoxic retinal injury and by glia derived neurotrophic factor (GDNF) administration (Kermer *et al.*, 2000; Morimoto *et al.*, 2005; Chidlow *et al.*, 2008; Del Rio *et al.*, 2011), RGC axons do not regenerate in these paradigms. Many ipRGC connect with multiple brain centres outside the visual thalamus and subserve the non-image-forming visual functions of circadian photo-entrainment (suprachiasmatic nucleus – SCN and intergeniculate leaflet - IGL), pupillary reflexes (olivary pretectal nucleus – OPN), visual masking (ventral sub-paraventricular zone - VSPZ) and sleep patterns (ventrolateral preoptic nucleus – VLPON) (Estevez *et al.*, 2012; Hattar *et al.*, 2006; Schmidt *et al*, 2011; Duan *et al.*,

2015; Cui *et al.*, 2015) but M4 ipRGC driven by rod and cone bipolar cell inputs may also contribute to pattern vision through their DLGN projections (Estevez *et al.*, 2012). Consistently, <10% RGC axons regenerate irrespective of either the axogenic or neuro-protective stimuli employed (reviewed by Berry *et al.*, 2008). The explanation for restricted RGC axon regeneration may rest with the observation that only M4 axons regenerate after mTORC1 activation (Duan *et al.*, 2015) and thus increasing the survival of other RGC subsets would not improve axon regenerative success; an observation that forecasts limited recovery of sight in blind patients after *pten* deletion, since rod/cone-mediated conscious perception of pattern colour vision may not be restored (Cui *et al.*, 2015).

It is not known if a similar diversity in growth factor requirements and limited mTORC1-mediated neuro-protection seen in RGC (Duan *et al.*, 2015) applies to other groups of neurons, e.g. DRGN and CSTN. Rheb activation of mTOR (Fig. 1) combined with a chondroitinase ABC anti-scarring treatment promotes CST axon regeneration in the injured cord (Wu *et al.*, 2015). However, after *pten* deletion by AAV-Cre injection of the motor cortex of floxed adult mice, CSTN axons regenerate through acute spinal cord lesions (Fig. 4D) (Liu *et al.*, 2010; Zukor *et al.*, 2013) and surprisingly also through chronic lesions after AAV-Cre cortical injections are delayed by 1 month and 1 year after injury (Du *et al.*, 2015) without supplementation with an anti-fibrotic regime. These latter findings indicate that acute and chronic scar tissue does not impede the transit of regenerating axons through a CNS lesion and that neurons have an extended axogenic potential after axotomy – good news for chronic spinal patients. The peripheral (Abe *et al.*, 2010; Christie *et al.*, 2010) but probably not central projections of DRGN also regenerate after *pten* deletion. However, spontaneous sprouting of spared CSTN axons after pyramidotomy is

independent of mTOR activation (Lee *et al.*, 2014), while *pten* and *nogo* (a CNS myelin-derived *agil* gene) co-deletion enhances CSTN axon regeneration but not sprouting (Geoffroy *et al.*, 2015), supporting the contention that mTOR regulates axon elongation rather than the initiation of axon growth (Leibinger *et al.*, 2012; Fischer and Leibinger, 2012, Morgan-Warren *et al.*, 2015). PI3K/Akt stimulated peripheral axon regeneration is insensitive to rapamycin, as is DRGN neurite outgrowth (Christie *et al.*, 2010), suggesting that growth of both central and peripheral DRGN projections is not mediated by the Akt/mTORC1 but by the Akt/GSK3β pathway enhanced by mTORC2/Akt activity (Fig. 1) – although this assertion is controversial since DRGN axon/neurite outgrowth has been reported after both GSK3β inhibition and activation (Zhou *et al.*, 2004; Dill *et al.*, 2008; Saijilafu *et al.*, 2013, Gobrecht *et al.*, 2014). Even so, the possible failure of large phenotypic groups of axotomised neurons to regenerate their axons after mTOR treatment could give rise to neurological sequelae that exacerbate the already poor quality of life of CNS injured patients. For example, the demonstration that CSTN and DRGN have differential sensitivities to mTOR predicts that, although paraplegic patients may become ambulatory after mTOR activation, they will be left with anaesthesia, paraesthesia, neuralgia and without proprioception (as a consequence of failed dorsal column/spinothalamic/spinocerebellar tract regeneration), possibly equivalent to the sensory deprivation and dyskinesia seen in *tabes dorsalis*.

AXON GUIDANCE

Axon regeneration alone will not re-establish useful function without restoration of the original topographically organised connections. In the adult mammalian CNS, re-innervation of former targets is a poorly executed rare event

(Luo *et al.*, 2013; Diekmann *et al.*, 2013). In the visual system, only M4 ipRGC axons regenerate after *pten* deletion (Duan *et al.*, 2015) and appear to establish homologous connections in the contralateral DLGN (Kurimoto *et al.*, 2010; de Lima *et al.*, 2012a, b), other presumptive M4 axons project ectopically into the hypothalamus (characteristic of M1 ipRGC projections, which do not regenerate), ipsilateral optic tract, and centrifugally within the ipsilateral and contralateral ON (Luo *et al.*, 2013; Yungher *et al.*, 2015) (Fig. 5). Similar aberrant RGC trajectories of presumptive M4 ipRGC axons may occur after NTF/cytokine stimulation and inflammation, independent of targeted up-regulation of mTOR (Berry *et al.*, 1999; Pernet *et al.*, 2013a, b) and could be mediated by osteopontin/IGF released from reactive Müller cells, retinal astrocytes and microglia (Kermer *et al.*, 2000; Morimoto *et al.*, 2005; Chidlow *et al.*, 2008).

Ontogenetic guidance of axon growth and disinhibition of adult regenerating axons in the CNS have much in common; they both exploit growth cone collapse and some developmental repellent guidance ligands are also axon growth inhibitory ligands (AGIL) in the mature CNS (e.g. Sema 3A). In the adult, AGIL derived from incipient scar tissue and from myelin (reviewed by Sandvig *et al.*, 2004), bind to their cognate receptors and activate signalling pathways which converge on RhoA and mediate growth cone collapse by depolymerisation of actin/microtubule filaments through the ROCK/LIMK/Cofilin pathway (reviewed by Ahmed *et al.*, 2005). For instance, myelin-derived AGIL bind to the Nogo receptor (NgR) complex comprised of AGIL-binding, membrane anchored, extracellular NgR, Lingo/Amigo co-receptors and transmembrane signalling p75^{NTR}/TROY components. It was quickly realised that axons blinded to AGIL after knock-down of either NgR (Zheng *et al.*, 2003), RhoA or ROCK (Ichikawa *et al.*, 2008; Lingor *et al*, 2007, 2008; Ahmed *et al.*, 2009)

will not regenerate unless growth cone advance is also stimulated by NTF/cytokines (Ahmed *et al.*, 2005, 2009) and intracellular axogenic pathways are activated (Liu *et al.*, 2011; Geoffroy and Zheng, 2014). Additionally, the discovery that NTF (including a combination of CNTF/BDNF/NT-3/FGF-2) induce regulated intramembranous proteolysis (RIP) of the p75^{NTR} signalling NgR co-receptor (Ahmed *et al.*, 2005; 2006, 2009) revealed the axiom that the coupling of axogenesis with growth disinhibition is mandatory for NTF-induced axogenesis in the AGIL-rich adult CNS.

Binding of Sema 3 family of axon guidance molecules to the neuropilin/plexin receptor complex inhibits PI3K/Akt signalling disrupting mTOR1/2 dependent activation of RhoA (Nakayama *et al.*, 2015). Axon trajectories may also become disordered when PI3K/Akt activation inhibits ephrin-induced growth cone collapse after disturbed Rheb signalling in *tsc2* haplo-insufficiency mice (Nie *et al.*, 2010), and to altered growth cone sensitivity to AGIL/axon guidance cues after moderation of GSK3β signalling (Dent and Gertler, 2003; Zhou and Snider, 2005). GSK3β controls axon outgrowth *de novo*, guidance and branching by regulating multiple transcriptional factors and also growth cone actin filament/microtubule assembly through cytoskeletal binding proteins, including collapsing response mediator protein (CRMP) and adenomatous polyposis coli (APC) (Fig.1) in response to inhibition/attraction guidance ligands (reviewed by Dent and Gertler, 2003; Goold *et al.*, 2004; Zhou and Snider, 2005; Hur and Zhou, 2010; Liu *et al.*, 2012). GSK3β is constitutively active and either inhibited by Akt phosphorylation (Arevalo and Rodriguez-Tébar, 2006; Dill *et al.*, 2008) or activated by repulsive guidance cues and AGIL (Zhou and Snider, 2005; Ito *et al.*, 2006; Shen *et al.*, 2011; Eickholt *et al.*, 2002). However, the finding that inhibition of GSK3β can also block axon growth (Alabed *et al.*, 2010) has led to the concept that differential priming of substrates by

phosphorylation determines whether GSK3β promotes the advance or collapse of growth cones (Kim *et al.*, 2006). Activated GSK3β regulates the sequential phosphorylation of cyclin-dependent kinase 5 (Cdk5) as well as both primed CRMP (Uchida *et al.*, 2005) and APC (Zumbrunn *et al.*, 2001), causing growth cone collapse by abrogating binding to the cytoskeleton (Fukata *et al.*, 2002). By inhibiting GSK3β, the PI3K/Akt axogenic pathway acts to disinhibit by default AGIL-mediated inhibition of axon growth (Uchida *et al.*, 2005; Zhou *et al.*, 2005). Accordingly, CST and serotonergic axons regenerate in the injured cord after lithium-induced inhibition of GSK3β (Dill *et al.*, 2008), DRGN axons regenerate through AGIL-rich dorsal column glial scars after GSK3β-deletion (Liz *et al.*, 2014) and the neurites of cortical neurons grow on an AGIL-coated substrate after over-expression of phospho-Akt and deletion or knockdown of *pten* (Perdigoto *et al.*, 2011). Growing axons are steered through CNS neuropil by collapse and counter-collapse in different regions of their growth cones and thus AGIL/repulsion guidance cue-activation of GSK3β may not completely arrest axon growth (Zhou *et al.*, 2004; Hur *et al.*, 2010). Hence, assuming that ontogenetic path finding maps are retained in mature CNS neuropil (discussed by Koeberle and Bähr, 2004), misguidance of regenerated adult axons after *pten* deletion, shRNA *pten* silencing (Yungher *et al.*, 2015) and activation of the PI3K/Akt pathway may be attributed to an inability of growth cones to respond to AGIL and repellent guidance cues that define the boundaries of the original axon trajectories, implying that CNS axogenic treatments are incompatible with functional recovery, since NTF-induced RIP- and GSK3β-mediated disinhibition (essential for growth cone advance in the mature CNS) inevitably fashions disorganised connectivity. This disinhibited axon growth paradox may become an impediment for researchers attempting to achieve functional recovery after CNS injury, although the

preservation of axon fasciculation mechanisms may compensate for poor path finding when attraction/repulsion cues are not detected (Marcos *et al.*, 2015).

SYNAPTOGENESIS

Connectivity is controlled by mTOR-mediated regulation of dendritic stability (Morquette *et al.*, 2014), synaptic protein production, synaptic plasticity (Hoeffer and Klann, 2009) and the morphology and possibly the density of dendritic spines (Lai *et al.*, 2006; Li *et al.*, 2010; Haws *et al.*, 2014; Di Polo, 2015). After spinal cord injury, *pten* deletion stimulates transected CST axons to regrow through the lesion (Fig. 4D) and form new synapses in homotopic regions of the caudal cord, suggesting re-engagement of former post-synaptic membranes (Liu *et al.*, 2010; Zukor *et al.*, 2013). In the visual system after *pten/socs3* co-deletion, new synaptic connections are formed in the SCN by regenerating axons (Li *et al.*, 2014, Yungher *et al.*, 2015), presumed to be heterotopic contacts of misguided regenerating M4 ipRGC axons since, after *pten* deletion/silencing, MI ipRGC axons (that normally innervate the SCN) do not regenerate (Duan *et al.*, 2015). In adult mice after *pten* deletion combined with cAMP and zymogen administration, homotopic DLGN and SC re-innervation by presumptive regenerating M4 ipRGC axons may explain the partial return of both depth perception and visually guided behaviours (de Lima *et al.*, 2012a, b), but the return of circadian photo-entrainment, normally mediated by M1-SCN connections (Estevez *et al.*, 2012; Hattar *et al.*, 2006; Schmidt *et al*, 2011; Duan *et al.*, 2015), is unexpected and more difficult to explain. Thus, aberrations in axon guidance (see above) together with ectopic synaptogenesis after *pten* deletion may cause gross CNS dysfunction (Ebrahimi-Fakhari and Sahin, 2015), although the capacity of the CNS to preferentially process information from the original,

denervated centres after suboptimal re-innervation, while filtering out misinformation generated in rogue connections, may restore limited function after activation of mTOR (Weidner *et al.*, 2001). However, in CNS with raised mTOR activity, imbalance in excitatory and inhibitory synaptic activity and disordered transmission of action potentials through abnormally myelinated regenerated neural circuits are all likely to cause seizures and deterioration in intellect, memory and cognition (Garcia-Junco-Clemente and Golshani, 2014; Williams *et al.*, 2015).

MYELINATION OF REGENERATED AXONS

Return of function after CNS injury is critically reliant on remyelination of regenerated axons to re-instate the temporal integrity of axon conduction patterns between centres and to preserve the viability of axons (reviewed by Doring and Yong, 2011; Franklin *et al.*, 2012). The prevalence of psychiatric and neurological conditions in myelin disorders, including the Tuberous Sclerosis Complex (TSC) genetic disorder, is probably explained by such desynchronisation of propagated axon potentials caused by changes in conduction velocities and refractory times as a consequence of abnormal 'g' ratios (diameters of axon:myelinated fibre), variable inter-nodal lengths and abnormal nodal physiology (Fields, 2008; Bartzokis, 2012). Schwann cells invade penetrant lesions of the CNS, myelinate axons and often become replaced by remyelinating oligodendrocyte precursors (OPC – reviewed by Crawford *et al.*, 2014) and oligodendrocytes (Jasmin and Ohara, 2002). A few regenerating axons in the transected ON become myelinated after *pten* deletion (de Lima *et al.*, 2012a, b), but whether by oligodendrocytes or Schwann cells is unknown. In demyelinating diseases, like multiple sclerosis, remyelination is limited, transient and often fails, probably because of impaired recruitment of OPC and their

failure to both make contact with axons and differentiate into myelinating oligodendrocytes (Flores *et al.*, 2000; Chari, 2007; Franklin and Kotter, 2008; Franklin and ffrench-Constant, 2008; Barros *et al.*, 2009; Tyler *et al.*, 2009; Bruce *et al.*, 2010; Patel and Klein, 2011). In addition, CNS remyelination efficiency wanes with age as OPC differentiation potential decreases (reviewed by Franklin and ffrench-Constant, 2008) and the frequency of type-M2 microglia declines, suggesting a pro-remyelinating influence of type–M2 microglia on OPC differentiation (Miron *et al.,* 2013). Regenerated axons in the adult CNS may also have defective myelin with related dysfunctional outcomes, because remyelination of regenerated axons is likely to be as aberrant as that of axons demyelinated by disease. Oligodendrocyte myelination is principally regulated by mTORC1 and the PI3K/AKT/GSK3β pathway (Tyler *et al.*, 2009; Narayanan *et al.*, 2009; Azim and Butt, 2011; Normén and Suter, 2013; Dai *et al.,* 2014; Wood *et al.,* 2013; Lebrun-Julien *et al.*, 2014; Wahl *et al.*, 2014; Bercury *et al.*, 2014; reviewed by Bartzokis, 2011), whereas mTORC2 has a modest effect on differentiation with little influence on myelination (Bercury *et al.*, 2014), but the roles of these signalling molecules in remyelination are contentious. Myelin gene transcription is down regulated (Lai *et al.*, 2006) and myelination is retarded (Sachs *et al.*, 2014) after suppression of mTORC1, either by deletion of Akt or treatment with rapamycin, whereas over active mTORC1 promotes remyelination and improves conduction and refractoriness (Moore *et al.*, 2014). Moreover, oestrogen receptor (ER) α and β ligand-activation of PI3K/Akt in oligodendrocytes co-activates Trk and IGF receptors and potentiates IGF1-induced OPC proliferation (Kumar *et al.*, 2013), inhibition of PTEN promotes MBP accumulation and stimulates myelination (De Paula *et al.*, 2014) and over expression of phospho-Akt increases myelin protein production leading to hyper-myelination (Goebbles *et al.*, 2010).

Counter-intuitively however, mTOR activation can cause defective oligodendrocyte differentiation after deletion of Rheb1 (Fig. 1) in murine neural progenitor cells (Zou et al., 2004). GSK3β also has contradictory effects, inducing both hyper-myelination (Carson et al., 1993; Freude et al., 2008) after inhibition by either Akt (Flores et al., 2008; Narayanan et al., 2009; Goebbles et al., 2010; Yu et al., 2011) or IGF (Fig. 1) and oligodendrocyte formation and remyelination through the regulation of Wnt-β-catenin, Notch and CREB signalling (Azim and Butt, 2011). Hyper-myelination induced by mTOR over activity in the mouse ON is correlated acutely with short latencies of visually evoked potentials, which later become delayed as myelin sheaths thicken and decompact (Yu et al., 2011). Although precise unequivocal neuronal targeting of mTOR therapies would protect against possible adverse hyper-myelination effects (Lai et al., 2006; Goebbles et al., 2010; Zou et al., 2011; De Paula et al., 2014), re-myelination of regenerated axons would require a supplementary OPC-oligodendrocyte differentiation regimen (not yet available - Doring and Yong, 2011) to restore normal conduction in regenerated axons.

ADVERSE EFFECTS OF mTOR HYPERACTIVITY IN THE CNS

The use of rapamycin and its analogues as immunosuppressive, anti-cancer, pro/anti-inflammatory and anti-angiogenic agents emphasises the potential for activated mTOR to cause severe pathologies in the CNS. Indeed, the control by mTOR of cell proliferation and growth, food intake and stress reactions makes it unsurprising that over activity is linked to cancer, diabetes, obesity and cardiovascular and neurological conditions. The latter include Huntington's, Alzheimer's, Parkinson's and TSC diseases in which autism, a decline in intellectual and cognitive function, epilepsy, abnormal protein translation and nerve growth are

all prevalent (Inoki *et al.*, 2005; Lai *et al.*, 2006; Tsang *et al.*, 2007; Yang and Guan, 2007; Chong *et al.*, 2010; Don and Zheng, 2011; Don *et al.*, 2012; Alayev and Holz, 2013; O'Neill, 2013). Such adverse side effects of mTOR activation are expected to be largely eliminated in prospective axogenic therapies employing fail-safe neuronal targeting. Nonetheless, in response to perturbed mTORC1 signalling, which frequently occurs during tumour formation and associated angiogenesis, targeted pharmacological suppression of mTORC1, PI3K, Akt and TSC1/2 (Fig. 1) are emerging as effective treatments for multiple forms of cancer (Hay, 2005; Yang and Guan, 2007; Don *et al.*, 2012; Alayev and Holz, 2013; Cargnello *et al.*, 2015), contra-indicating mTOR-based axogenic therapies for CNS trauma patients, particularly those with a high risk of cancer. Moreover, elevated mTORC1 activity after Akt hyperactivity, *pten* deletion, suppression of both PTEN (Hay, 2005; Alayev and Holz, 2013) and TSC1/2 (Crino *et al.*, 2006; Sosunov *et al.*, 2008) (Fig.1) all promote the growth of benign and malignant glia tumours, including the transformation of OPC into malignant glioma (Galvao *et al.*, 2015; Duzgun *et al.*, 2015), accompanied by an astrocytosis (Li *et al.*, 1997, 2015; Fraser *et al.*, 2004; Wullschleger *et al.*, 2006; Codeluppi *et al.*, 2009, 2014; Latacz *et al.*, 2015). The net effect of the latter may also be functionally detrimental, arresting axon growth and generating epileptic foci in the injured CNS, since reactive astrocytes: (i), are a rich source of the AGIL, inhibiting both axon regeneration and synaptogenesis (Sandvig *et al.*, 2004; Li *et al.*, 1997; Fraser *et al.*, 2004; Silver and Miller, 2004; Wullschleger *et al.*, 2006, Liu *et al.*, 2006; Codeluppi *et al.*, 2009; Sofroniew, 2009); and (ii), have impaired glutamate uptake leading to seizures as increasing titres of extracellular glutamate accumulate (Ulmann *et al.*, 2002; Wong *et al.*, 2003). Despite all the above and the predilection of *pten*^{+/-} mice to develop multiple neoplasias (reviewed by Hay, 2005), no tumours

have been observed in the CNS after neuron-targeted *pten* deletion in short duration

axon regeneration studies in which non-neuronal cells may also be affected,

although long term outcomes have yet to be evaluated (Ali *et al.*, 1999).

Nevertheless, until the above high carcinogenic risks and unreliability of neuronal

targeting are eliminated, potentially carcinogenic mTOR-related therapies are

unlikely to achieve clinical approval. Recent unbiased high-throughput functional

screening of the genome for phosphatase suppressors of axon regeneration has

identified an axogenic pathway independent of the PI3K/Akt/mTOR pathway opening

up the prospect of development of axogenic gene therapies which knock-out non-

pten phosphatases (Zou *et al.*, 2015).

TRANSLATABLE mTOR-BASED THERAPIES

Many of the gene therapy techniques used in experimental animals to achieve

activation of mTOR and axon regeneration (e.g., Flox/loxP recombinant inducible

gene deletion of *pten*) are not translatable possibly because of ethical, toxicity and

anti-mitotic issues compounded by the development of cavitation lesions and

chromosomal aberrations (e.g. Pfeifer *et al.*, 2001; Loonstra *et al.*, 2001; Schmidt *et

al.*, 2000). Refinements in the clinical use of chemically modified synthetic si/shRNA

for knock-down of specific genes (e.g. siRTP801, which has undergone a Pfizer

Phase II clinical trial for the treatment of wet age-related macular degeneration

(AMD) (DDIT4, Quark Pharmaceuticals/Pfizer, Ness Ziona, Israel) have eradicated

the problems of incomplete transient non-specific gene silencing, innate immune

responses, instability, degradation and the need for multiple injections (Ahmed *et al.*,

2011; Guzman-Aranguez *et al.*, 2013). Combinations of AAV-

shPTEN+CNTF+cAMP promote enhanced RGC axon regeneration, compared to

pten deletion alone, with extensive re-innervation of central targets (Yungher *et al.*, 2015), although the caveats relating to poor axon guidance and aberrant target re-innervation apply (see above). Thus, a chemically modified synthetic si/shRNA could be developed for use in the clinic as an alternative to *pten* deletion, although specific unequivocal RGC targeting may not be achievable (Ahmed *et al.*, 2011). For example, intravitreal injections of siRTP801 raise mTOR activity in the retina and induce bystander effects in Müller glia and astrocytes which are partially responsible for the resulting RGC survival and limited axogenesis (Morgan-Warren *et al.*, 2015). Treatments which target elements located higher up-stream in the PI3K/mTOR pathway may cause numerous undesirable down-stream side effects, and more distal down-stream targeting of, for instance, TSC1/2, mTORC1, GSK3β and CRMP/APC reduce but do not eradicate these risks because focal points of activation of alternative regulatory pathways exist, even at these levels. Small molecule kinase inhibitors could qualify as translatable drug candidates but often have a high incidence of unwanted off-target side effects, as well as toxicity, absorption, distribution, metabolism and excretion issues (Meijer *et al.*, 2004; Rosivatz *et al.*, 2006; Douglas *et al.*, 2009). NTF therapies also have a chequered history of success in both laboratory animal (Harvey *et al.*, 2012) and clinical studies (Thoenen and Sendtner, 2002), but the discovery that subtypes within phenotypic groups of neurons may require particular combinations of growth factors to stimulate axon regeneration (Duan *et al.*, 2015) suggests that such therapies may ultimately evolve as cocktails of multiple factors capable of promoting axon regeneration in all axotomised subtypes. However, after spinal cord injury, multiple neuronal subsets become axotomised and thus a plethora of growth factor combinations may be required to promote the regeneration of all transected ascending, descending and

intra-spinal tracts, possibly making growth factor treatment impracticable. Moreover, since NTF induce RIP of the p75[NTR] signalling component of NGR, the disinhibited axon growth paradox complicates the development of growth factor based treatments for CNS axon regeneration.

CONCLUSIONS

The mTOR antagonist rapamycin and its analogues are used to treat cancer and thus the intention to develop a therapy which elevates mTORC1 activity would constitute an anathema to oncologists. Neural targeting might eradicate the carcinogenic risks (since mature neurons rarely, if ever become malignant), but would force the design of a combinatorial therapy to promote, in addition to axogenesis, oligodendrocyte myelination of regenerated axons and more comprehensive viability of axotomised neurons using an anti-CASP regimen. However, neural targeting of mTOR-based therapies does not eliminate the problems of either limited responsiveness within specific phenotypic neuronal groupings or the generation of anomalous axon guidance and synaptogenesis through the disinhibited axon growth paradox. Thus, therapeutic elevation of mTOR activity is unlikely to improve and could possibly lead to deterioration in the quality of life of CNS injury patients. Even so, the demonstration in experimental animals of long tract axon regeneration after PI3K/Akt activation (Park *et al.*, 2008; Liu *et al.*, 2010; Kurimoto *et al.*, 2010; Sun *et al.,* 2011), growth factor administration (Berry *et al.*, 1999; Pernet *et al.*, 2013b; Duan *et al.*, 2015) and inflammation (Leon *et al.*, 2000; Fischer, 20210) demonstrates that CNS axon regeneration is feasible clinically and not an inherent impossibility as once thought, but a therapy to restore function in CNS injury patients is yet to be realised.

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FIGURE LEGENDS

Figure 1. Activators of the mTOR axogenic pathway. Tyrosine receptor kinase (Trk) receptors bind neurotrophic factors (NTF) including nerve growth factor (NGF)/brain-derived neurotrophic factor (BDNF)/neurotrophin 3/4 (NT3/4) which in turn activate the Trk/PI3K/Akt pathway; hypoxia, DNA damage and stress activate the HIF/RTP801/TSC pathway down stream of Akt; and the gp130 receptor complex binds the cytokines leukaemia inhibitory factor (LIF), interleukin 6 (IL6) and ciliary neurotrophic factor (CNTF), and activates the janus kinase (JAK)/signal transducers and activators of transcription (STAT), cAMP and RAS/CREB pathways (Akt - serine/threonine kinase; APC - adenomatous polyposis coli microtubule plus-end–binding protein; CREB - cAMP response element binding protein; CRMP - collapsin response mediator protein; eIF4E - eukaryotic initiation factor 4E; 4E-BP1 - eIF4E binding to protein 1; Epac - exchange protein directly activated by cyclic adosine mono-phosphate (cAMP); ERK - extracellular signal-regulated kinase; GSK3β - glycogen synthase kinase 3β; HIFα - hypoxia inducible factor alpha; IGF- insulin-like growth factor; IGFR-IGF receptor; IRS1-insulin receptor substrate 1; MAPK - mitogen-activated protein kinase; MEK - MAPK/ERK kinase; mTORC1 - mTOR (mammalian target of rapamycin)+Raptor (regulatory association protein to mTOR)+GβL-G (protein β-subunit-like protein); mTORC2 - mTOR+Rictor (rapamycin independent companion of mTOR)+GβL+Sin1; PDK1/2 - phosphatidylinositol-dependent kinase 1/2; PI3K - phosphatidylinositol 3-kinase; PIP2 - phosphatidylinositol (3, 4) bisphosphate; PIP3 - phosphatidylinositol (3, 4, 5) trisphosphate; PKA - protein kinase A; SHP-2, a Src homology 2 (SH2) domain containing non-transmembrane PTP; PTEN - phosphatase and tensin homolog; RAF - proto-oncogene serine/threonine-protein kinases; REDD/RTP801 - regulated in development and DNA damage response protein; RAS - rat sarcoma protein; RHEB

- Ras homolog enriched in brain protein; RSK - 40S ribosomal protein S6 kinase;

S6K1 - p70 ribosomal protein S6 kinase 1; S6 - ribosomal protein S6; S727 -

phosphostat3 serine 727;; SOCS3 - suppressor of cytokine signalling 3; TSCI/2 -

tuberous sclerosis complex 1/2; Y705 - phosphostat3 tyrosine727).

Figure 2. Morphology of five types of intrinsically photosensitive retinal ganglion cell
(ipRGC); (**A**) *en face* view of ipRGC dendritic fields (scale bar=100μm), (**B**) dendritic

stratification as viewed in a schematic radial retinal section. Pale blue bands in the

inner plexiform layer (IPL) are the ON and OFF cholinergic bands. There are two

bands of melanopsin dendrites ramifying outside the ON/OFF cholinergic bands; the

outer lying at the margin of the inner nuclear layer (INL) contains M1 and M3 cell

dendrites and the inner broader band is juxtaposed to the ganglion cell layer (GLC)

and contains the dendrites of M2, M3, M4, and M5 cells with subtle differences in

stratification. (from '*Intrinsically photosensitive retinal ganglion cells*', Berson DM,

reprinted courtesy of The MIT Press from The New Visual Neurosciences edited by

John S. Werner and Leo M. Chalupa, with permission).

Figure 3. Comparison of (**A**) CASP2 and (**B**) combined *pten/socs3* deletion on the

survival of axotomised RGC after intra-orbital ONC in rats and mice, respectively. **A**.

Frequencies of FluoroGold back filled rat RGC at 7d post ONC after intravitreal

injection of a control siRNA targeting a random combination of Caspase nucleotides

(siCNL) and after escalating doses of siCASP2 at 0d. Treatment with siCNL resulted

in 60% RGC survival compared with intact controls, while increasing doses of

siCASP2 enhanced RGC survival and, over an optimal dose range of 20–35 mg,

promoted 100% RGC protection compared with intact controls (P>0.001*; from

Ahmed *et al*., 2011 with permission). **B**. Synergistic effects on TUJ1^{+} mouse RGC

survival at 4w post ONC and after deletion of *pten* and *socs3*. Percentages of TUJ1^{+} RGC in wild type (WT), *pten* deleted (PTEN[+]), *socs3* deleted (SOCS3^{+}), and combined *pten/socs3* deleted (PTEN[+]/SOCS3^{+}) groups, compared with intact retinae (*$P>0.001$; from Sun *et al.*, 2011 with permission).

Figure 4. **A**. GAP-43^{+} axons in the non-regenerating and regenerating adult rat ON 20d after ON transaction; (i), non-regenerating ON in which the growth of GAP43^{+} RGC axons is arrested in the proximal lesion (*) margin; (ii), after an intravitreal sciatic nerve implant at 0d regenerating GAP43^{+} RGC axons traverse the lesion (*) and invade the distal ON segment (note there are more GAP43^{+} axons in the proximal regenerating (ii), compared to the non-regenerating ON (i) because more RGC survive in the former; eye to the left, chiasm to the right; magnification bar = 100µm; from Berry *et al.*, 2008 with permission). **B**. Confocal images of the transected adult mouse optic nerve after *pten* deletion showing regenerated Cholera Toxin-B[+] (CTB[+]) axons passing through the lesion site at 14d (i) and 28d (ii) and invading the distal optic nerve segment (scale bar = 100mm; the normal response to optic crush is essentially similar to that of the rat see A(i) above – from Park *et al.*, 2008 with permission). **C**. Selective regeneration of M4 ipRGC (αRGC – KCNG4) axons in the optic nerves of mice after ONC: (i), CTB anterograde axon tracing detects all regenerating RGC axons; (ii), in Kcng4-yellow fluorescent protein (YFP) mice YFP[+] regenerating axons are exclusively M4 ipRGC axons; (iii), the near complete convergence of the images of YFP[+] and CTB[+] regenerating axons demonstrates that most axons derive from M4 (scale bar = 200µm; from Duan *et al.*, 2015 with permission); ipRGC. **D**. Biotinylated dextran amine[+] (BDA[+] - red) regenerating CST axons on the left side of the cord extending for up to 3mm caudal

to the lesion site (*) after *pten* deletion (longitudinal parasagittal sections of the adult

mouse spinal cord crushed at the vertebral level of T8). In these experiments, AAV-

Cre was injected into the right hind limb sensorimotor cortex of *Pten*$^{loxP/loxP}$ mice

aged 4w; 4w later the cord was lesioned and, after a further 4w, BDA was injected

into the right sensorimotor cortex and the mice killed 2w later (scale bar = 500µm;

from Liu *et al.*, 2010 with permission).

Figure 5. Trajectories of (**A)** normal RGC axons in a hypothetical representative

oblique coronal section through the rodent diencephalon (optic nerve (ON); optic

chiasm (OX) – from whence axons invade the suprachiasmatic nucleus (SCN) in the

hypothalamus and where 1-10% RGC axons project ipsilaterally and 90-99%

contralaterally into the optic tracts (OT) connecting with ventral lateral geniculate

nucleus (VLGN) and dorsal lateral geniculate nucleus (DLN) in the thalamus;

superior quadrigeminal brachium (SQB) running to the superior colliculus (SC) in the

midbrain; IIIrd ventricle (IIIV). (**B**) Ventral light sheet fluorescent microscopic view of

regenerated axon projections in an unsectioned mouse brain after ONC and

pten/socs3 deletion. Cholera toxin-B anterograde labelled regenerating RGC axons

are seen in the transected ON and OX. Note: (i), ectopic RGC axons regenerate

centrifugally into each ON; (ii), overgrowth into the hypothalamus; and (iii), equal

numbers of axons regenerating into each OT. (**C**) Lateral view of the 3D

reconstruction showing axons regenerating into the diencephalon (inset shows low

magnification of the whole brain following 3D reconstruction). (**D)** Quantification of

RGC axon trajectories in 6 different mice (cases #1–6 - values = % total axons

exiting the OX). Note the following abnormalities: (i), near normal numbers of

ipsilateral and contralateral RGC axons in the OT; and (ii), variable ectopic

regeneration of axons into the hypothalamus and contralateral ON (scale bar =
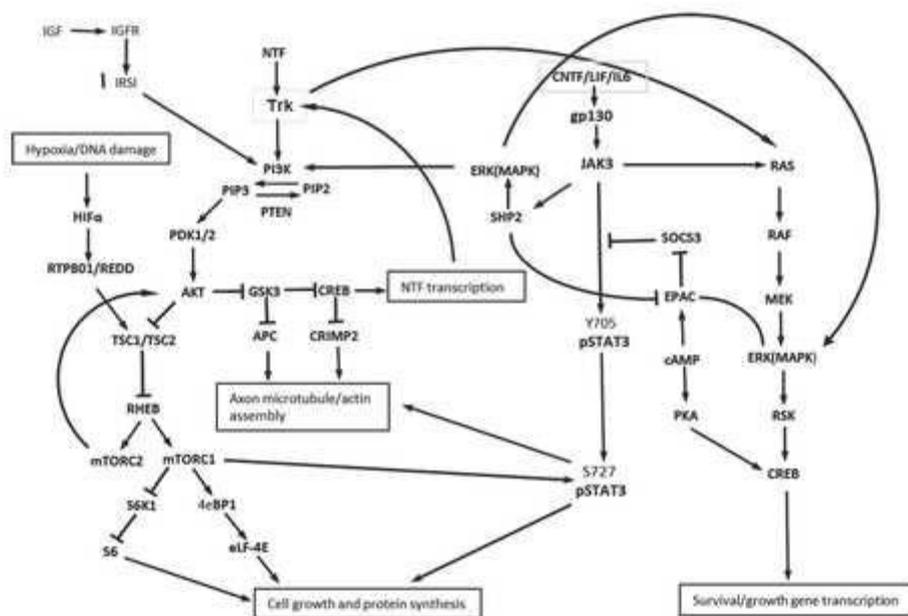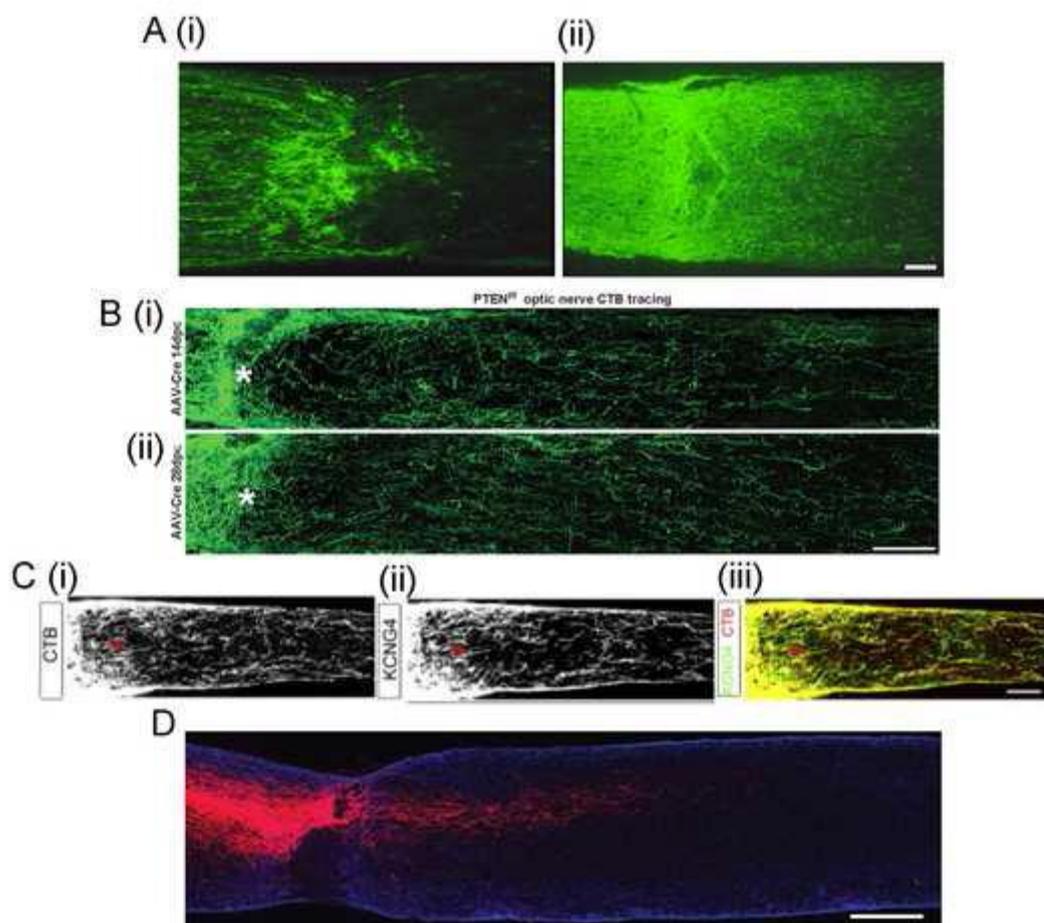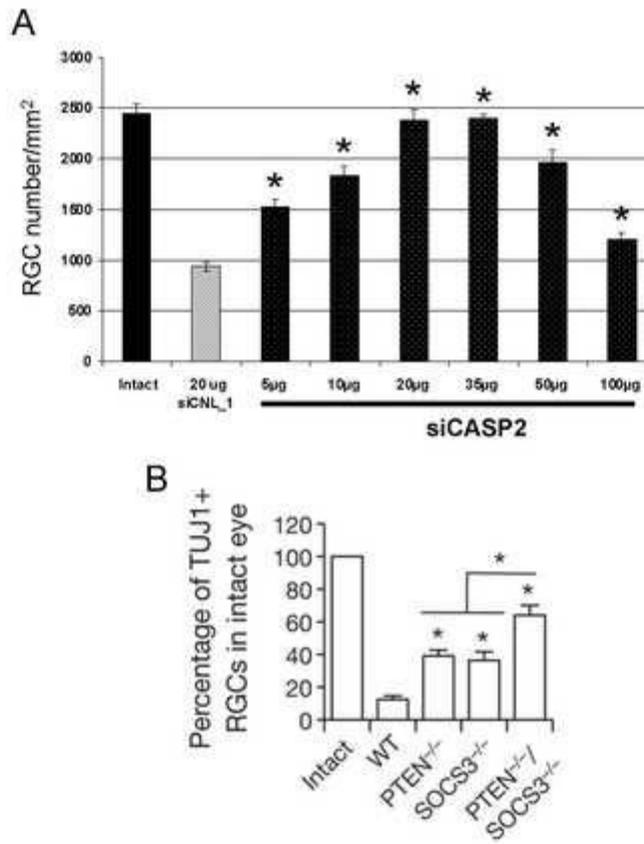
200μm; from Luo *et al.*, 2013 with permission).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5