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TITLE

Targeting Cell Surface Receptors for Axon Regeneration in the CNS

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PERSPECTIVE

After injury, axon regeneration in the adult central nervous system (CNS) is a challenging task. In addition to facing a plethora of growth inhibitory molecules such as chondroitin sulfate proteoglycans (CSPG), myelin-associated glycoprotein (MAG) and Nogo in the environment, injured neurons also lack intrinsic regenerative ability. As evident in neural development, the appropriate response to guidance cues in the environment is crucial for axonal growth. With the correct cell surface receptors, growing axons can respond to these guidance cues by regulating their intracellular signalling pathways and in turn modifying growth cone behaviours. By translating this concept, we propose that cell surface receptors can be a target for CNS axon regeneration after injury. In this Perspective, we highlight a few receptors which have been demonstrated to be successful for axon regeneration (Figure 1).

The first promising receptor type we discuss here are integrins. Structurally, an integrin is a heterodimeric transmembrane cell adhesion receptor consisting of an α and β subunit which dimerise to form interactions with neighbouring cells or extracellular matrix (ECM) molecules. Integrins are bidirectional signalling molecules. The activation of integrin from a bent low-affinity conformation into a stable extended high-affinity conformation intracellularly by an activator such as kindlin or talin is termed as 'inside-out' signalling. For example, kindlin contains a FERM (4.1/ezrin/radixin/moesin) domain containing a phosphotyrosine binding site serving as the integrin binding site of the membrane-distal NxxY motif on the β subunit cytoplasmic tail. This results in a higher affinity binding of extracellular ligands to integrin and triggers a series of intracellular signalling cascades termed as 'outside-in' signalling. The recruitment of various intracellular pathways results in a variety of cellular responses, ranging from short-term responses such as adhesion and motility, to long-term responses such as proliferation, differentiation and neurite outgrowth which include gene regulation.

Recently, we published a study that shows the potential of an activated integrin to be a treatment strategy for functional long-distance sensory axon regeneration in the spinal cord (Cheah et al., 2016). In both developing and adult nervous systems, integrins are required for

neuronal migration and axonal outgrowth or extension. Due to spatial-temporal regulation, many integrin subunits are downregulated after development, affecting the ability of adult neurons to form specific cell-matrix interactions and reducing their ability to respond to certain extracellular ligands. More than a decade ago it was demonstrated that forced expression of a specific integrin subunit in adult dorsal root ganglion (DRG) neurons increases their neurite outgrowth on substrates containing the ligand for the overexpressed receptor; for example α1 integrin was required for growth on laminin and α5 integrin on fibronectin (Condic, 2001). Conversely, in the same study, embryonic DRG neurons grew robustly on these substrates due to their endogenous expression of integrins (Condic, 2001). In addition to showing the importance of the presence of a specific integrin subunit for correct ligand binding, the study was also one of the first to show that integrin manipulation in adult neurons can promote successful neurite outgrowth. Although successful peripheral nerve regeneration after injury is correlated with an upregulation of some integrin subunits, such as α4, α5, α6, α7 and β1, other subunits such as α9 integrin remains downregulated after CNS injury (Andrews et al., 2009). Alpha9 integrin is a receptor for the main extracellular matrix glycoprotein tenascin-C. In the injured CNS, tenascin-C is upregulated at the lesion site by astrocytes, however its receptor α9 integrin is not re-expressed after injury. The lack of α9 integrin-tenascin-C interaction in the injured CNS is likely to contribute to the inability of axons to regenerate through tenascin-C rich regions. In verifying this hypothesis, we showed that overexpression of α9 integrin in DRG neurons can indeed promote a modest amount of sensory axon regeneration through the tenascin-C rich dorsal root entry zone (DREZ) following a dorsal root crush injury and through the lesion cavity after spinal cord injury (Andrews et al., 2009). Despite the positive results, the amount of axon regeneration observed *in vivo* was not as robust as the amount of neurite outgrowth observed in cell culture assays, questioning whether the ectopically-expressed α9 integrin had been inactivated in the injured spinal cord by inhibitory molecules such as CSPGs in the lesion milieu (Tan et al., 2012).

In addition to tenascin-C, CSPGs are also upregulated and widely known for their role in inhibiting axon growth after spinal cord injury. CSPGs affect several signalling pathways such as Rho/ROCK activation, protein kinase C (PKC) activation and epidermal growth factor receptor (EGFR) signalling. In a study using a similar experimental paradigm as the previous α9 integrin study, we demonstrated that overexpression of an integrin activator,

kindlin-1, promotes axon regeneration in the presence of CSPGs and CSPGs have a direct effect on integrin inactivation (Tan et al., 2012). In other words, the presence of kindlin-1 in DRG neurons allows injured axons to overcome CSPG inhibitions resulting in axon regeneration due to the integrin receptors being less susceptible to CSPG-mediated inactivation. The findings from the kindlin-1 study provided possible explanations for the less than expected amount of axon regeneration when α9 integrin was expressed on its own (Andrews et al., 2009). Only tenascin-C was present in cell culture for the α9 integrin study and therefore CSPG-mediated inactivation of α9 integrin was not initially considered. However, both CSPGs and tenascin-C are upregulated after spinal cord injury, thus it is reasonable to suggest that the ectopically-expressed α9 integrin receptors could have been inactivated by CSPGs and also myelin debris, resulting in less than optimum growth-promotion. By combining the findings of these two separate α9 integrin and kindlin-1 studies, we hypothesised that overexpression of both α9 integrin and kindlin-1 would overcome the inhibition of CSPGs and subsequent inactivation of α9 integrin to promote better axon regeneration in the presence of tenascin-C and CSPGs which are highly upregulated in the injured spinal cord (Cheah et al., 2016) (Figure 2). We have successfully verified this hypothesis in our recent study demonstrating that overexpression of both α9 integrin and kindlin-1 resulted in much better recovery as well as more significant axon regeneration in comparison of expression of either molecule alone. By overexpressing both α9 integrin and its activator kindlin-1 in adult rat DRG neurons, axon regeneration of up to 25 mm from the injury site up to the medulla was observed following lower cervical dorsal root (C5-C8) crush injury. Although we did not observe direct connections to one of the terminal targets, the cuneate nucleus in the brainstem, the regeneration was coupled with topographically accurate axonal reconnections of NF200-, CGRP-, IB4-positive fibres in the dorsal horn, behavioural recovery and electrophysiological improvement. In addition, the observed regeneration was time-dependent with regenerating axons not reaching the DREZ until 3 weeks post-injury, entering the spinal cord at 6 weeks and growing along the spinal cord of up 12 weeks.

Given the prolific amount of sensory axon regeneration observed in the α9 integrin-kindlin-1 combination study (Cheah et al., 2016), the question remains as to whether the α9 integrin-kindlin-1 treatment strategy can be applied to cortical neurons to promote motor axon regeneration within the corticospinal tract (CST). Unfortunately, cortical neurons have a very different integrin trafficking mechanism as compared to DRG neurons. Based on our

observation, integrins transport down the corticospinal axons readily during development but as the neurons mature, integrins become restricted to the somatodendritic compartment and are excluded from the axon (Andrews et al., 2016). One suggestion for this trafficking defect may be due to the formation or maturation of the axon initial segment acting to restrict the transport of certain molecules down the axon, potentially resulting in the lack of intrinsic regenerative ability of CNS axons. An extension of the α9 integrin-kindlin-1 approach to cortical neurons would first require a solution to this restrictive trafficking problem. Nevertheless, the use of integrin to promote sensory recovery alone is already extremely useful for spinal cord injured patients in restoring lost sensation and avoiding burns and bedsores.

Although integrin may not yet be a possible treatment strategy for CST regeneration at present, another class of cell surface receptors, Trk receptors, has been shown to be a promising target. Trk receptors are a family of tyrosine kinases which bind neurotrophic factors required for the development, survival, and functioning of neurons. Upon ligand binding at the axonal terminal, the Trk receptor-ligand complex becomes internalised and is transported retrogradely to the soma. Of the three main family members (trkA, trkB and trkC), trkB which is the receptor for brain-derived neurotrophic factor (BDNF) is the most promising candidate for CST regeneration (Hollis et al., 2009). When trkB receptors were overexpressed in corticospinal neurons after a subcortical lesion, regenerating axons were observed growing into a BDNF-secreting graft. Phosphorylation of trkB receptor upon ligand binding activates the intracellular Erk pathway to induce neurite outgrowth, an event which also occurs during development. In fact, neurotrophic factors are crucial guidance cues for directing growing axons to the correct targets and strengthening newly formed synapses during development of the CNS. Many studies trialled a variety of neurotrophic factors such as nerve growth factor (NGF), neurotrophic factor-3 (NT-3) and BDNF to mimic development in order to promote axon regeneration. Although the inherent lack of neurotrophic factors at the lesion site may be a contributing factor in the failure of CNS axon regeneration, it is worth considering if the corresponding neurotrophin receptor is expressed on injured axons as both receptor and ligand expression is required for their growth-promoting function.

As mentioned above, inhibitory CSPGs are highly upregulated at the lesion site predominantly by reactive astrocytes after CNS injury. Treatments targeting CSPGs such as chondroitinase ABC which digests the CSPG glycan side chains have been demonstrated to reduce the growth inhibitory effect of CSPGs and hence promoting axonal outgrowth. The molecular mechanisms whereby CSPGs mediate their inhibitory effect have only recently begun to be defined and remain poorly understood. The transmembrane protein tyrosine phosphatase σ (PTPσ) has been proposed to be a receptor for CSPGs and it has a direct role in mediating growth inhibition (Shen et al., 2009). By modulating PTPσ to reduce the inhibitory effect of CSPGs, axon regeneration in the spinal cord may be achievable (Lang et al., 2015). In that study, although sprouting of serotonergic fibres together with variable behavioural recovery was observed as opposed to lengthy regeneration of CST fibres (Lang et al., 2015), the discovery of receptor PTPσ and its potential modulation nevertheless highlights our proposal that targeting cell surface receptors can promote axon regeneration in the CNS.

In summary, we have reviewed three types of cell surface receptors: integrins, trk receptors and PTPσ for their potential as targets for enhancing axon regeneration (**Fig 2**). The field of axon regeneration is separated into two main groups: the extrinsic advocates who study the limiting factors found in the lesion environment, while the intrinsic advocates who focus on factors found within the neurons. Perhaps cell surface receptors can serve as a link between these two groups as the presence of a correct receptor on the cell surface is required to relay an extracellular signal intracellularly. An important note although beyond the scope of this review, recent evidence suggests that matrix metalloproteinases (MMPs) play a key role in enhancing intracellular and extracellular interactions after CNS injury. They assist in many of the processes that lead to enhanced regeneration such as proteolysis and/or activation of both ECM and certain cell surface receptors including integrins and TrkB (Reviewed by Andries et al., 2016). Therefore, the appropriate balance and response to cues in the extracellular matrix can determine the regenerative ability of neurons and their axons whereas manipulation of these cell surface receptors could bring upon successful axon regeneration in the CNS. Although manipulation of receptors by transgene expression is therapeutically challenging in human patients based on our current knowledge, this potential strategy can be part of a combinatorial therapeutic approach in achieving CNS axon regeneration in the future.

FIGURES AND LEGENDS

Figure 1. Schematic of transmembrane receptors related to axon regeneration found on CNS neurons. Schematic of a typical axonal growth cone showing a standard distribution of receptors around the surface of the membrane **(A)**. **B-D** Enlarged views of growth cone surface within typical CNS lesion ('extracellular' with myelin debris, and CSPGs) showing the various transmembrane receptors discussed in the current review article. Enlarged view of integrin receptors (α and β subunits) at the surface of a growth cone interacting with ECM glycoproteins such as tenascin C, as shown **(B)**. Enlarged view of Trk receptors (with extracellular cysteine-rich domain and leucine-rich region) at the surface of a growth cone interacting with neurotrophic factors (TrkB receptor, shown with BDNF) **(C)**. Enlarged view of PTPsigma (PTPσ) receptors (with internal D1 and D2 components, and extracellular FNIII domain and Ig-like domains) at the surface of a growth cone interacting with CSPGs **(D)**. See Figure Legend for symbol definitions.

Figure 2. Schematic of spinal cord injury along with activated/inactivated integrin receptors found in response to injury. Schematic of a typical spinal cord lesion site showing disrupted degenerating axons, a glial scar, along with myelin debris, microglia/macrophages, and CSPGs **(A)**. Schematic of regenerating growth cone showing activated and inactivated $\alpha9\beta1$ integrin **(B)**. When kindlin-1 is present and linked intracellularly to the cytoplasmic tail of $\beta1$ integrin, $\alpha9\beta1$ integrin is activated and binds tenascin-C, with neurite outgrowth/axon regeneration occurring despite the presence of CSPGs and myelin debris **(top half of B)**. When CSPGs and myelin debris are present, without kindlin-1, $\alpha9\beta1$ integrin is largely inactivated with only low levels of axon

regeneration occurring in the presence of tenascin-C **(bottom half of B)**. See Figure Legend

for symbol definitions.

REFERENCES

Andrews MR, Czvitkovich S, Dassie E, Vogelaar CF, Faissner A, Blits B, Gage FH, ffrench-Constant C, Fawcett JW (2009) Alpha9 integrin promotes neurite outgrowth on tenascin-C and enhances sensory axon regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:5546-5557.

Andrews MR, Soleman S, Cheah M, Tumbarello DA, Mason MR, Moloney E, Verhaagen J, Bensadoun JC, Schneider B, Aebischer P, Fawcett JW (2016) Axonal Localization of Integrins in the CNS Is Neuronal Type and Age Dependent. eNeuro 3:0029-0016.2016.

Andries L, Van Hove I, Moons L, De Groef L (2016) Matrix Metalloproteinases During Axonal Regeneration, a Multifactorial Role from Start to Finish. Molecular Neurobiology DOI 10.1007/s12035-016-9801-x

Cheah M, Andrews MR, Chew DJ, Moloney EB, Verhaagen J, Fassler R, Fawcett JW (2016) Expression of an Activated Integrin Promotes Long-Distance Sensory Axon Regeneration in the Spinal Cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 36:7283-7297.

Condic ML (2001) Adult neuronal regeneration induced by transgenic integrin expression. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:4782-4788.

Hollis ER, 2nd, Jamshidi P, Low K, Blesch A, Tuszynski MH (2009) Induction of corticospinal regeneration by lentiviral trkB-induced Erk activation. Proceedings of the National Academy of Sciences of the United States of America 106:7215-7220.

Lang BT, Cregg JM, DePaul MA, Tran AP, Xu K, Dyck SM, Madalena KM, Brown BP, Weng YL, Li S, Karimi-Abdolrezaee S, Busch SA, Shen Y, Silver J (2015) Modulation of the proteoglycan receptor PTPsigma promotes recovery after spinal cord injury. Nature 518:404-408.

Shen Y, Tenney AP, Busch SA, Horn KP, Cuascut FX, Liu K, He Z, Silver J, Flanagan JG (2009) PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. Science (New York, NY) 326:592-596.

Tan CL, Andrews MR, Kwok JC, Heintz TG, Gumy LF, Fassler R, Fawcett JW (2012) Kindlin-1 enhances axon growth on inhibitory chondroitin sulfate proteoglycans and promotes sensory axon regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:7325-7335.
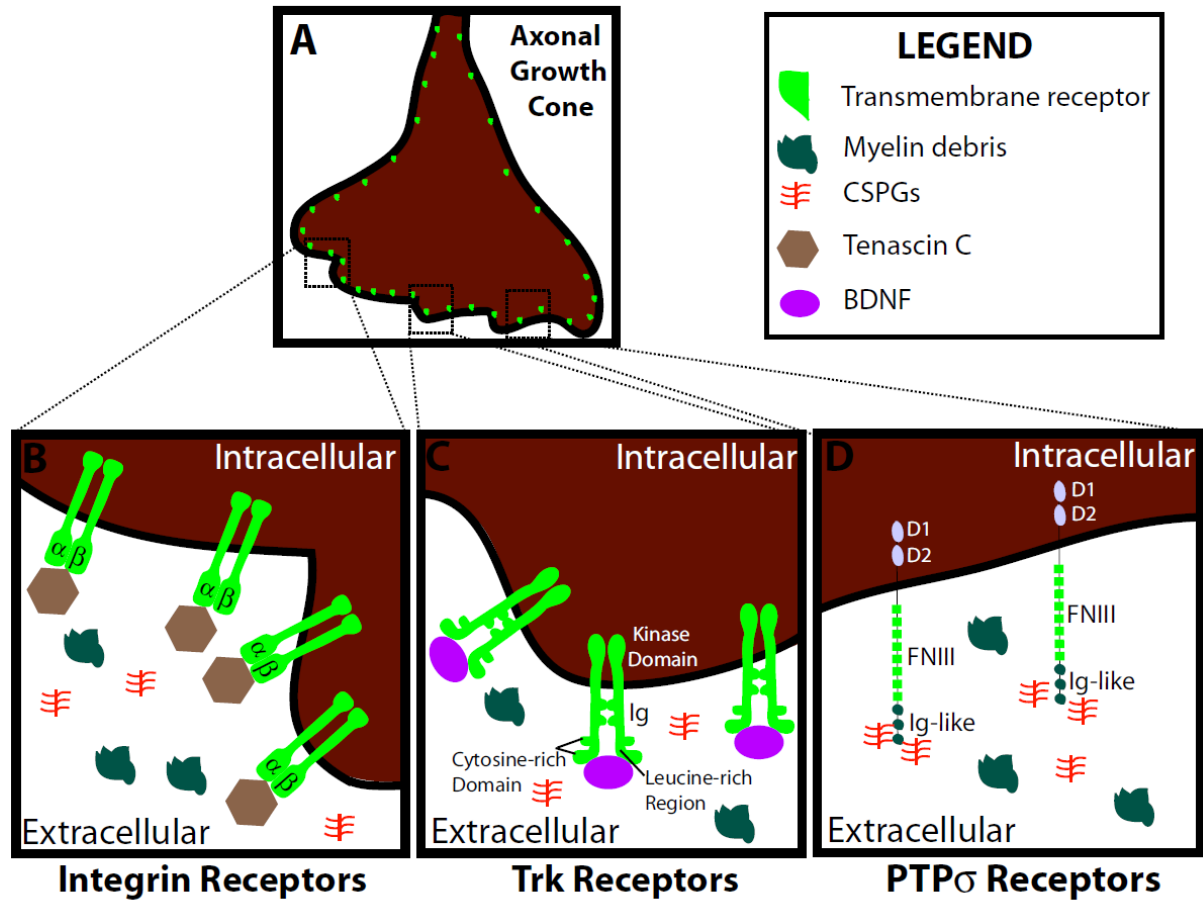
Figure 1

Figure 2