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Cytokines That Promote Nerve Regeneration

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After axonal damage, regeneration occurs spontaneously in the mammalian peripheral nervous system (PNS); however, this is extremely rare in the central nervous system (CNS). Nerve regeneration is generally thought to depend on two types of factors: intrinsic and extrinsic (Ferguson and Son, 2011; Bosse, 2012). Study of the extrinsic factors that limit CNS regeneration has a long history (Caroni and Schwab, 1988; Galtrey and Fawcett, 2007; Lee and Zheng, 2012); however, study of what regulates the intrinsic growth capacity of central neurons has received attention much more recently (Liu et al., 2011). Part of the intrinsic mechanism has long been believed to involve changes in gene transcription, a part of a set of changes referred to as the “cell body response” (Watson, 1974; Grafstein, 1975; Skene and Willard, 1981b; Smith and Skene, 1997) and triggered either by factors induced by axotomy or by target derived factors whose influence is removed by axotomy or by both (Cragg, 1970; Zigmond, 2012).

In a recent paper entitled “IL-6 promotes regeneration and functional recovery after cortical spinal tract injury by reactivating intrinsic growth program of neurons and enhancing synapse formation”, Yang et al. (2012) reported that infusion of the cytokine interleukin (IL)-6 into the spinal cord following a dorsal hemisection promoted regeneration and functional recovery of the corticospinal tract (CST), a nerve pathway considered to have a particularly low intrinsic growth capacity (Liu et al., 2010; Blackmore et al., 2012). IL-6 is a member of a family of cytokines referred to as the glycoprotein (gp) 130 family or the IL-6 family (Bazan, 1991). The first of these names comes from the fact that these cytokines act on a set of heteromeric receptors all of which contain the signaling subunit gp130 (Taga, 1996). Activation of gp130 leads to phosphorylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) by the enzyme Janus kinase (JAK),

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dimerization of the factor and its translocation to the cell nucleus, and activation of a set of responsive genes (Heinrich et al., 2003). In addition, gp130 can trigger other signaling pathways including the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway (Ernst and Jenkins, 2004). These cytokines are pleiotropic and produce many overlapping effects in the nervous system and elsewhere. Several of them, including IL-6, are induced in the nervous system after injury (Kiefer et al., 1993; Banner and Patterson, 1994; Sun et al., 1994; Bolin et al., 1995; Murphy et al., 1995).

Discovery that exogenous gp130 cytokine actions are involved in PNS development

It is striking that the earliest evidence establishing neurons as targets for gp130 cytokines both involved the peripheral sympathetic nervous system and examples of neurotransmitter plasticity, one occurring in normal neonatal development and the other occurring in adult neurons after injury (Landis, 1996; Zigmond et al., 1996). The first of these was discovered serendipitously in studies on neonatal neurons from the superior cervical ganglion (SCG) that were co-cultured with heart cells or in heart cell conditioned medium. These studies revealed surprisingly that, while the neurons initially released norepinephrine and formed noradrenergic synapses as expected, with time they began to release acetylcholine and to communicate with their target cells via muscarinic receptors (Furshpan et al., 1976). The biological activity in the conditioned medium that triggered this change was referred to as the cholinergic differentiation factor (CDF), and the phenomenon was referred to as “the cholinergic switch”. A very similar phenomenon was later shown to occur *in vivo* during the first 2-3 weeks of life in restricted subpopulations of sympathetic neurons, namely those innervating sweat glands (Schotzinger and Landis, 1988; 1990) and the periosteum, the connective tissue that surrounds bone (Asmus et al., 2000; 2001).

Yamamori et al. (1989) isolated the CDF from heart cell conditioned medium and identified it as leukemia inhibitory factor (LIF), which, like IL-6, is a gp130 cytokine. They pointed out, however, that the role of this molecule in normal development was unknown. Nevertheless, it still came as a surprise when later the cholinergic switch was shown to occur normally in animals in which the gene for LIF had been knocked out (Rao et al., 1993). In line with that finding, it turns out that LIF is not actually expressed by the exocrine cells in the sweat gland, the targets of the cholinergic sympathetic innervation (Rao et al., 1993; Stanke et al., 2006). Regulation of the cholinergic phenotype is a good example of the pleiotropy of gp130 cytokines as each of the following cytokines triggers a cholinergic switch when added to cultured sympathetic neurons: LIF (Yamamori et al., 1989), ciliary neurotrophic factor (Saadat et al., 1989), oncostatin M (Fann and Patterson, 1994), cardiotrophin-1 (Habecker et al., 1995) and a hybrid molecule containing IL-6 and the soluble IL-6 receptor (Marz et al., 1998). Studies with microdissection followed by PCR and experiments with a conditional knockout of the receptor gp130 have now established that several members of this cytokine family are present in the sweat gland and that cholinergic differentiation is almost totally blocked in the receptor knockout animals (Stanke et al., 2006).

Evidence that endogenous gp130 cytokines are involved in the response of adult PNS neurons to injury: Induction of regeneration-associated genes and stimulation of the conditioning lesion response

The first *in vivo* demonstration of a neural response to an endogenous gp130 cytokine came from studies on the regulation of neuropeptide expression in adult SCG neurons. Following axotomy, these neurons begin to express certain neuropeptides that intact neurons do not express, and these changes are seen at both the mRNA and protein levels (Hyatt-Sachs et al., 1993). Similar changes are found when neurons from control animals are placed in explant or dissociated cell cultures (Sun et al., 1992; Zigmond et al., 1992). This type of phenotypic plasticity is not restricted to sympathetic neurons but is seen also in sensory and motor neurons. Thus, all three classes of neurons begin to express three neuropeptides after axotomy: galanin, vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase activating polypeptide (PACAP) (Zigmond et al., 1996), and these genes and proteins can be considered regeneration-associated genes (RAGs) or growth-associated proteins (GAPs), respectively (Zigmond, 2001). Experiments in culture using a neutralizing antibody to LIF established that the induction of VIP was due, at least in part, to the action of LIF released by ganglionic nonneuronal cells (Sun et al., 1994). While LIF is largely undetectable in sympathetic ganglia under normal conditions, its expression is increased within hours after axotomy both *in vivo* and *in vitro* (Sun et al., 1996; Sun and Zigmond, 1996). *In vivo*, the axotomy-induced up regulations of VIP and galanin were significantly, though not totally, blocked in LIF -/- mice (Rao et al., 1993), raising the possibility that other signaling molecules might be involved in addition to LIF. This hypothesis has now been proven by the demonstration that multiple gp130 cytokines are expressed in these ganglia and that the inductions of VIP, galanin, and PACAP are completely abolished in the conditional gp130 knockout (Habecker et al., 2009).

The first involvement of gp130 cytokines in PNS regeneration was a study on the IL-6 -/- mouse (Zhong et al., 1999). The sciatic nerve was crushed and animals were followed for 30 days using the footprint assay of sciatic nerve function. By that time, the change in behavior of both mutant and wild type animals had leveled off with the control animals back to normal and the knockouts only recovered by about 50%.

Stephen Thompson's laboratory was the first to show that endogenous gp130 cytokines (LIF and IL-6) are required for the normal increase in neurite outgrowth following a conditioning lesion (Cafferty et al., 2001; 2004). This response in which the outgrowth that occurs *in vivo* after a "test" lesion is enhanced by a prior "conditioning" lesion was discovered in the sciatic nerve by McQuarrie and Grafstein (1973). Subsequently it was found that a conditioning lesion to the sciatic nerve also promotes regeneration of the lesioned central processes of DRG neurons (Chong et al., 1999; Neumann and Woolf, 1999). Also it was discovered that a conditioning lesion *in vivo* of either sensory or sympathetic neurons leads to increased neurite outgrowth when the neurons are subsequently placed in culture (Hu-Tsai et al., 1994; Edstrom et al., 1996; White et al., 1996; Shoemaker et al., 2005).

Thompson and colleagues examined outgrowth in LIF -/- and in IL-6 -/- mice both *in vivo* and *in vitro* on a permissive substrate (i.e., laminin) and demonstrated that, under both conditions, the conditioned lesion effect was significantly reduced, though not totally abolished (Cafferty et al., 2001; Cafferty et al., 2004). The induction of GAP-43, a protein that increases in neurons in the DRG after axotomy (Bisby, 1988), was also blocked in IL-6 -/- mice. These workers went on to show that both the *in vivo* and *in vitro* conditioning lesion response and the induction of GAP-43 could be reduced by the inhibitor of JAK2, AG490. In a later study, Cao et al. (2006) reported that IL-6 stimulated neurite outgrowth *in vitro* on a growth inhibiting substrate (i.e., myelin proteins), though not on a permissive substrate; furthermore, they found no decrease in the conditioning lesion effect *in vivo* in IL-6 -/- animals. Thus far, there has been no explanation for these apparent discrepancies.

There is one additional context in which the stimulation of the release of endogenous gp130 cytokines has been implicated in promoting CNS regeneration. This paradigm was also, presumably, discovered serendipitously and involves the triggering of axonal outgrowth after an optic nerve crush when the lens is damaged (Leon et al., 2000). A controversy still exists over the primary mechanism underlying this effect with the Benowitz group proposing that the effects are mediated by the influx of macrophages and their release of oncomodulin (Yin et al., 2009) and the Fischer group proposing alternatively that the effects are mediate by the activation of astrocytes/Müller cells and their release of CNTF and LIF (Leibinger et al., 2009). While this dispute also remains to be resolved, what is most relevant here is that LIF and CNTF double knockout animals do not show the effects of lens injury on neurite outgrowth. Furthermore, in wild type animals, CNTF and LIF mRNA increase in retinal astrocytes after lens injury, and STAT3 phosphorylation increases in retinal ganglion cells (Leibinger et al., 2009). Whether induction of CNTF and LIF by itself is sufficient to mimic the effects of lens injury remains to be determined.

Effects of exogenous gp130 cytokines on CNS regeneration

This brings us back to the paper by Yang et al. (2012). This group first confirmed the findings that IL-6 promotes neurite outgrowth from DRG neurons cultured on myelin and that this effect can be blocked by AG490 (Cao et al., 2006). They next wondered what the molecular changes associated with this outgrowth might be. They examined mRNA for three known RAGs, GAP-43 (Skene and Willard, 1981a), the small proline-rich repeat protein 1A (Bonilla et al., 2002; Costigan et al., 2002; Boeshore et al., 2004), and arginase 1 (Costigan et al., 2002; Boeshore et al., 2004) and found that, when neurons were plated on myelin proteins and stimulated with IL-6, all three mRNAs increased. These inductions are interesting because it has previously been shown that SPRR 1A and arginase 1, when over expressed in cultured DRG neurons, increase neurite outgrowth on myelin proteins (Bonilla et al., 2002; Cai et al., 2002). Overexpression of GAP-43 in cultured DRGs on a permissive substrate and in cerebellar Purkinje cells *in vivo* led to some local sprouting; however, only a few DRG neurons showed the type of elongated growth characteristic of neurons that have received a conditioning lesion (Buffo et al., 1997; Bomze et al., 2001).

Yang et al. next placed a catheter chronically into the spinal cord at the gap between vertebra L4 and L5, and, a few days later, they performed a bilateral dorsal hemisection of

the cord at T9/10. After the lesion was made, animals were injected through the catheter for 7 days with either saline or IL-6 at a dose of 20 ng/day for one week. Five weeks later the CST was labeled anterogradely from the sensorimotor cortex, and two weeks after that the spinal cords were examined. The authors reported that in both groups no axons passed through the site of the lesion, but that, in the IL-6 treated animals, there were more sprouting fibers somewhat rostral to the lesion site. One question these findings raise is whether this growth should really be called regeneration (see recent discussion in Tuszynski and Steward, 2012).

A relevant prior study is the similar attempt to promote CST regeneration with a gp130 cytokine reported by Blesch et al. (1999). These workers made a comparable lesion of the spinal cord, and, at the same time, they grafted fibroblasts that had been engineered to secrete LIF. Subsequently, they observed axonal extension of anterogradely labeled corticospinal axons. Interestingly the growth was through the gray matter of the spinal cord. They also observed an increase of the expression of mRNA for the neurotrophin NT-3 in the area near the lesion. This observation is noteworthy because in an earlier paper they had shown that fibroblasts expressing NT-3 can produce a similar type of regeneration (Grill et al., 1997), raising the issue of whether LIF (or IL-6 in the case of the study of Yang et al.) acts directly or indirectly by stimulating the expression of another growth factor. Finally, Grill et al. noted that, using a motor grid task, the behavioral deficit they observed after dorsal hemisection was not seen after just a lesion of the dorsal columns that would be expected to lesion 95% of the CST axons. They, therefore, pointed to the fact that the behavioral deficits after dorsal hemisection involve other fiber systems in addition to the CST.

In an attempt to assess whether the sprouting fibers made synaptic contacts in the cord, Yang et al. examined the colocalization of the anterogradely labeled fibers with synapsin 1, a presynaptic marker. The problem here is that no postsynaptic marker was used, for example, as was recently done immunohistochemically in a paper on optic nerve regeneration by de Lima et al. (2012) or electron microscopically in a study on the CST by Liu et al. (2010). Therefore, one would have to assume that synapsin 1 is only expressed presynaptically after a synapse is formed, but no evidence for this assumption is cited.

Another question of considerable interest is onto what neurons synapses were made if, in fact, they were made. Yang et al. demonstrate a clear and impressive improvement in BBB scores in the IL-6 treated rats, which includes measurement of hind limb movement and coordination; however, since no axons were found to traverse the lesion, which was made in the thoracic cord, presumably restoration of function would have to involve synapses onto neurons that eventually contact lumbar motor neurons. What neurons might these be? A study by Bareyre et al. (2004) is very pertinent here. These authors also made a dorsal hemisection at the level of the midthoracic cord and showed that CST axons sprouted into the cervical gray matter where these contacted long propriospinal neurons, which themselves contacted lumbar motor neurons. Next, they did three telling experiments which corroborate the idea that the previously axotomized corticospinal neurons had been able to reconnect to muscles in the hind limb through a multi-synaptic pathway. First they injected hind limb muscles with a pseudorabies virus labeled with green fluorescent protein. This

virus has two important properties: it is carried by retrograde axonal transport, and it crosses synapses in a retrograde direction. Subsequently, Bareyre et al. detected dramatically that the virus was present in neuronal cell bodies at three different levels: lumbar motor neurons, long propriospinal neurons in the cervical cord, and corticospinal neurons projecting to the hind limb. Next, they electrically stimulated this area of the cortex and recorded an electromyographic response from the hind limb muscles. Finally, they showed that the partial recovery of the hind limb placing response that occurred in their hemisected animals was significantly, though not totally, reduced if the CST was relesioned. Perhaps, this new pathway of CST to long propriospinal neurons to lumbar motor neurons is at least in part responsible for the recovery seen by Yang et al.

A final aspect of the study by Yang et al. was the immunohistochemical examination of three proteins that have recently received considerable interest with regard to both CNS and PNS regeneration: mammalian target of rapamycin (mTOR), suppressor of cytokine signaling 3 (SOCS3) and phospho-STAT3 (pSTAT3). Somewhat surprisingly the only CNS area that was examined was the region “around the lesion site”, and there was no examination of the axotomized CST neuronal cell bodies. Whereas mTOR was found in control animals primarily in non-neuronal cells, after IL-6 treatment, some labeled neurons were seen near the lesion site. mTOR stimulates protein translation initiation and is negatively regulated by another protein, phosphatase and tensin homolog (PTEN) (Dowling et al., 2010). Interest in mTOR and regeneration began with the findings that mTOR activity stimulates axonal protein synthesis (Campbell and Holt, 2001) and that its inhibitor rapamycin inhibits growth cone formation after axotomy (Verma et al., 2005). Zhigang He and colleagues found that a conditional knockout of PTEN in retinal ganglion cells promotes extensive regeneration after optic nerve crush (Park et al., 2008). This dramatic effect was largely blocked by rapamycin. Interestingly, in a study on cultured DRG neurons, pharmacological blockade of PTEN also led to enhanced outgrowth, but, in this case, the effect was not blocked by rapamycin, suggesting that other signaling pathways can be involved (Christie et al., 2010).

In their discussion, Yang et al. cite the findings of Liu et al. (2010) as also showing a relationship between stimulation of the mTOR pathway and increased sprouting and regeneration of the corticospinal axons. However, these two studies are very different. Most importantly, Liu et al. increased mTOR activity in the corticospinal neurons themselves by a conditional knockout of PTEN, while Yang et al. observed increased mTOR immunoreactivity in interneurons within the spinal cord around the lesion site. A second difference is that while Yang et al. stained for mTOR itself, Liu et al. stained for a downstream product of increased mTOR activity, namely, phosphorylated ribosomal protein S6.

The finding by Yang et al. of increased mTOR staining raises several questions. Perhaps, most importantly is what is the target(s) of IL-6 in this preparation? One possibility is that IL-6 acts both on the sprouting corticospinal neurons and on mTOR-positive interneurons. It should be noted that IL-6 was injected into the **lumbar** spinal cord a week before the lesion of the CST at the **thoracic** level. Although LIF and CNTF have been found to be retrogradely transported by axons in the sciatic nerve and in fact to be transported more

extensively after injury (Hendry et al., 1992; Curtis et al., 1993; Curtis et al., 1994), IL-6 was not transported by these axons (Kurek et al., 1996). Given the site of the catheter placement, one wonders how IL-6 would impact the mTOR expressing neurons around the lesion site. An additional point about IL-6's action is that this cytokine requires a specific IL-6 receptor subunit in addition to gp130. Some neurons have been shown to have the latter but not the former. A hypothesis has been proposed that a form of trans-signaling can occur whereby a soluble IL-6 receptor can be released by one cell and, in concert with IL-6, can stimulate another nearby cell that expresses only gp130 (Marz et al., 1999; Rose-John, 2003). Therefore, knowledge of the distribution of the IL-6 receptors in this region of the spinal cord would be of interest. Finally, it is important that neurons are not the only IL-6 responsive cells in the CNS. In particular, astrocytes have IL-6 receptors, and the well known dramatic increase in glial fibrillary acid protein that occurs after injury is significantly reduced in IL-6 -/- mice (Klein et al., 1997; Penkowa et al., 1999). While microglial cells were not found to have IL-6 receptors, a decreased microglial response was seen in IL-6 -/- mice (Klein et al., 1997; Penkowa et al., 1999), perhaps as a result of trans-signaling or by some indirect effect. Obviously, there are many possibilities for sites of action of IL-6 in the Yang et al. paper.

In their immunohistochemical study, Yang et al. found that SOCS3 was expressed in neurons and nonneuronal cells in control animals but was only detectable in nonneuronal cells in the IL-6 treated animals. This finding is somewhat surprising as gp130 cytokines, including IL-6, have been found to induce SOCS3 expression, at least in some systems (Wang and Campbell, 2002; Heinrich et al., 2003). Nevertheless, decreased neuronal expression of SOCS3 in the context of regeneration is of considerable interest because this protein functions by inhibiting the phosphorylation of STAT3 and thereby turns off that branch of the cytokine signaling pathway (Wang and Campbell, 2002; Heinrich et al., 2003). It has been proposed that SOCS3 is one of the key mechanisms that inhibit the intrinsic growth capacity of CNS neurons. This concept is based on an experiment where a conditional knockout of SOCS3 was produced in retinal ganglion cells followed by a crushing of the optic nerve. This produced impressive stimulation of axonal growth, much like what was seen after the PTEN knockout (Smith et al., 2009). This outgrowth was not seen in double knockouts of SOCS3 and either gp130 or STAT3 (Smith et al., 2009; Sun et al., 2011). Furthermore, injection of CNTF into the eye in the SOCS3 knockout animals further enhanced axonal regeneration. In the Yang et al. paper, however, it must be remembered that the down regulation of SOCS3 is not being demonstrated in the corticospinal neurons but in neurons around the lesion site, and we know nothing about whether these neurons were injured and whether they exhibit regeneration.

The final protein examined by Yang et al. near the lesion site is pSTAT3. This protein was only observed in the IL-6 treated rats; however, the cell type in which it was expressed was not identified. Considerable evidence indicates the importance of STAT3 and its phosphorylation in both PNS and CNS regeneration. For example, in a recent broad screen for transcription factors that are involved in regeneration, STAT3 was identified in two independent studies using different approaches (Michaelevski et al., 2010; Smith et al., 2011). The importance of STAT3 phosphorylation was first demonstrated in the PNS. Looking at the increased neurite outgrowth in DRG neurons in culture following a

conditioning lesion, Liu and Snider (2001) showed that this effect could be blocked by adding the inhibitor AG490 to the medium. Interestingly, blockade of the ERK pathway had no such effect. Similarly, Qiu et al. (2005) blocked regeneration of DRG neurons into the dorsal columns after a conditioning lesion using the same drug *in vivo.* Two studies recently over expressed STAT3 and examined neurite outgrowth. When DRG neurons were infected by viral gene transfer with a constitutively active form of STAT3, which led to increased STAT3 phosphorylation, a large increase in sprouting was found in the spinal cord after a dorsal column injury (Bareyre et al., 2011). In the second study, cerebellar granular cells were electroporated with a constitutively active form of STAT3, and a small increase in neurite outgrowth was seen in neurons cultured on a permissive substrate (Smith et al., 2011).

As noted pSTAT3 binds to DNA and leads to changes in neuronal gene transcription (Zigmond, 2012). An example of such a gene that is known to be involved in nerve regeneration is the neuropeptide galanin. Galanin knockout mice show dramatically slow sciatic nerve regeneration after a nerve crush and a reduced conditioning lesion response in DRG neurons (Holmes et al., 2000; Sachs et al., 2007). Infusion of IL-6 into the spinal cord leads to an increase in galanin mRNA in neurons in the DRG (Murphy et al., 1999).

The future

The advances in our understanding of what regulates the intrinsic growth capacity of central and peripheral neurons have been impressive. Nevertheless, at least in the CNS, there is still a ways to go to get more complete regeneration and to restore normal function. One approach that has begun to be used is to activate a combination of the multiple pathways described in this Commentary. So far this has been done in two papers on retinal ganglion cell regeneration after optic nerve crush. In one study, PTEN deletion in the eye was combined with injection of zymosan and a cyclic AMP analogue (the former to trigger macrophage infiltration and the latter to increase oncomodulin binding to its receptor) (de Lima et al., 2012). In the second study, a double knockout of SOCS3 and PTEN was compared with the respective single knockouts (Sun et al., 2011). In both of these studies, extensive regeneration occurred in some cases beyond the optic chiasm and even into the brain. In the case of de Lima et al. (2012) even some recovery of visual function was found. All and all, these are remarkable advances that bode well for the future.

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