Review

Degenerative and regenerative mechanisms governing spinal cord injury

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Spinal cord injury (SCI) is a major cause of disability, and at present, there is no universally accepted treatment. The functional decline following SCI is contributed to both direct mechanical injury and secondary pathophysiological mechanisms that are induced by the initial trauma. These mechanisms initially involve widespread haemorrhage at the site of injury and necrosis of central nervous system (CNS) cellular components. At later stages of injury, the cord is observed to display reactive gliosis. The actions of astrocytes as well as numerous other cells in this response create an environment that is highly nonpermissive to axonal regrowth. Also manifesting important effects is the immune system. The early recruitment of neutrophils and at later stages, macrophages to the site of insult cause exacerbation of injury. However, at more chronic stages, macrophages and recruited T helper cells may potentially be helpful by providing trophic support for neuronal and non-neuronal components of the injured CNS. Within this sea of injurious mechanisms, the oligodendrocytes appear to be highly vulnerable. At chronic stages of SCI, a large number of oligodendrocytes undergo apoptosis at sites that are distant to the vicinity of primary injury. This leads to denudement of axons and deterioration of their conductive abilities, which adds significantly to functional decline. By indulging into the molecular mechanisms that cause oligodendrocyte apoptosis and identifying potential targets for therapeutic intervention, the prevention of this apoptotic wave will be of tremendous value to individuals living with SCI.
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Introduction

Spinal cord injury (SCI) occurs in most countries at an annual rate of 20–40 persons per million (Tator and Fehlings, 1991). In the United States, this sums up to approximately 10,000 new cases a year, which continuously adds to the nation's estimated 200,000 quadriplegics (Ropper, 2001; Tyor et al., 2002). The main causes of trauma to the cord are motor-vehicle accidents, sports and recreational activities, work-related accidents and falls at home. More importantly, the majority of SCI victims are young and otherwise healthy, who in addition to costing society up to $200,000 each per year, suffer the impingement of life long disability (Tyor et al., 2002). At present, there is no universally accepted treatment for this condition (Beattie et al., 2002b).

The greater part of spinal cord injuries in civilian life arise from fracture or dislocation of the vertebral column. Most commonly this arises due to compression with flexion in the thoracic cord and hyperextension or flexion in the cervical cord. Indicators increasing the risk of SCI as a result of minimal trauma are preexisting spondylosis, a congenital spinal canal stenosis, hypertrophied ligamentum flavum and instability of apophyseal joints due to rheumatoid arthritis (Ropper, 2001).

Manifestation of SCI has varying degrees and is wholly dependent on the severity and level of injury to the cord. The rule of thumb is that the higher the level of the lesion, the more severe the consequences. In the case of high cervical injury, patients require artificial respiration to stay alive. This type of injury also leads to tetraplegia, impairment of function in pelvic organs and loss in motor and sensory function of the arms, trunk and legs. Injury to lower cord levels, depending again on the exact level, may leave function in the upper limbs with impairment limited to lower limbs. This phenomenon of paraplegia is restricted to injuries of the thoracic or lumbar cord (Maynard et al., 1997; Ropper, 2001).

Neuropathology of SCI

The pathological sequelae following acute SCI are divided into two broad chronological events: the primary injury and the secondary injury (Tator and Fehlings, 1991). The primary injury encompasses the focal destruction of neural tissue caused by direct mechanical trauma. This initial insult then instigates a progressive wave of secondary injury, which via the activation of a barrage of noxious pathophysiological mechanisms exacerbates the injury to the spinal cord. As this leads to the destruction of axonal tracts left

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intact by the initial trauma it is a major impediment to functional recovery after SCI (Beattie et al., 2002b; Schwartz and Fehlings, 2002; Tator, 1995; Tator and Fehlings, 1991).

Primary injury

Macroscopic considerations

To mimic the majority of mechanical events that lead to various forms of human SCI, several experimental models have been developed (Beattie et al., 2002b). The most commonly used model is the contusion model (Tator, 1995; Young, 2002). This model induces instantaneous mechanical deformation of the spinal cord by dropping either a weight (Noble and Wrathall, 1985), an impactor rod (Gruner, 1992) or an impounder with computer-guided assistance (Bresnahan et al., 1987). Another model employed in SCI research is the compression model; in this model, injury is induced by applying either a weight or an aneurysm clip to the spinal cord (Tator and Fehlings, 1991). This model aims to add to that of the contusion model by replicating the persistence of cord compression that is commonly observed in human SCI (Tator, 1995).

The shortcoming of both the contusion and compression models, however, is that the devices they utilise are restricted for use in large animals such as rats and felines (Young, 2002). As a result, to utilise the abundance of genetic mutations engineered in mice, mouse models of SCI are restricted to the use of central nervous system (CNS) axotomy and extradural clip compression (Joshi and Fehlings, 2002a,b; Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). The shortcoming of the CNS axotomy model is that the injury induced is not an accurate portrayal of mechanical events leading to human SCI.

Microscopic considerations

Mechanical forces applied at the site of primary injury shear neuronal and endothelial cell membranes. This leads to a haemorrhagic zone of necrosis that predominantly localises to the gray matter due to this region's soft consistency and highly vascular nature (Tator, 1995). Central localisation of haemorrhage also arises due to uneven movement of tissue after injury. Tissue moves predominantly rostral and caudal to the injury epicentre, with the greatest movement and therefore shearing of neural cell membranes and connections due to apposing forces is experienced at the centre of the cord (Blight, 1988). As this leads to relatively little movement at the surface of the cord, axons localised near the pia experience minimal disturbance and usually survive to form a subpial axonal rim. This is in great contrast to axons localised near the gray matter, which are severely injured after SCI (Young, 2002).

Axonal durability after SCI also depends on the presence of myelin. A contusion injury brought about by deformation of cells beyond their capacity through distortion of their natural form can cause diffuse axonal damage with large myelinated fibres being most vulnerable (Blight, 1988). Myelinated axons are more vulnerable than unmyelinated ones because the longitudinal forces stretching the fibres are concentrated at the nodes of Ranvier. This is supported by the observation that axonal microtubule disruption after SCI tends to be localised at the nodal regions (Maxwell, 1996). In experimental replication of SCI using the transient clip compression model (15 s per compression), a greater amount of myelin destruction per mm^2 compared with gray matter destruction was observed with an increasing extent of injury (larger area of

cord compressed, Gruner et al., 1996). This suggests a distinct vulnerability of the white matter to the extent of compression injury to the spinal cord. However, it has been clearly demonstrated that under slow compression injury, there is a distinct sparing of the large myelinated fibres with small unmyelinated fibres being vulnerable (Blight and Decrescito, 1986). Furthermore, with the persistence of compression on the spinal cord, there occur specific molecular and cellular events that evolve into secondary injury mechanisms.

Secondary injury

There exist striking pathophysiologic similarities between clinical SCI and experimental models of SCI. As a result, the following account is based primarily on findings in rat SCI models, which are justifiably extrapolated to the human form of injury (Tator, 1995). However, there are striking differences between the regulation of the secondary events after SCI between animal strains and species (Hausmann, 2003). Therefore, extrapolation from animal experimental data to human pathophysiology requires caution.

Macroscopic considerations

Apart from gray matter haemorrhage, the primary injury causes no gross damage to the spinal cord despite the direct cell death occurring at the site of the lesion (Beattie et al., 2002b). However, with the advent of secondary injurious mechanisms this picture changes drastically. The earliest sign of gross change (within 2 h) is expansion of the haemorrhagic front at the site of trauma and appearance of numerous diffuse petechial haemorrhages (Tator, 1995; Tator and Fehlings, 1991). Shortly thereafter (6 h), penumbra surrounds the primary lesion and oedema ensues predominantly in the white matter (Guth et al., 1999). In the first day following trauma (12–24 h), haemorrhagic fronts continue to enlarge and become more confluent. Furthermore, at the vicinity of injury, the gray and white matter lose their definition, become softer and swell due to increasing oedema (Tator, 1995). Haemorrhage is still evident by 3 days; however, by the eighth day post-injury it is totally absent and the now much-expanded site of primary injury is affluent in cellular debris (Beattie et al., 2002b). At 21 days post-injury, the spinal cord has evolved clearly visible cavitations, which by 14 weeks coalesce to form large cystic regions. These regions are surrounded by scar tissue originating mainly from glia and to a lesser extent from the PNS (see The glial scar) (Beattie et al., 2002b). In 30% of rats with a compression injury and in 10% of clinical SCI cases (Wallace et al., 1987), the cystic regions expand for considerable distances rostral and caudal to the primary injury. This phenomenon, over many years, produces the long-term syndrome of posttraumatic syringomyelia, which enhances neurological deficit (Tator, 1995).

At more chronic stages of SCI, evolvement of pathophysiological sequale leads to atrophy of the cord at the site of primary injury. However, due to Wallerian degeneration in ascending and descending tracts (see Axonal disruption and Wallerian degeneration), cord atrophy can also be seen both rostral and caudal to the site of initial trauma (Tator, 1995).

Microscopic considerations

A barrage of pathophysiological events that causes both necrosis and apoptosis govern the biology of secondary injury after acute SCI (Beattie et al., 2002b).

Necrosis. Secondary injury mechanisms initiate a centripetal and rostro-caudal necrotic wave that originates at the site of primary injury. This wave, which is irreversible by 8 h, may spread for up to two vertebral levels both above and below the initial lesion (Ropper, 2001).

Infarction. Infarction propagates necrosis in CNS tissues, which particularly affects neurons through various mechanisms (Fig. 1). From the onset of primary injury vascular damage to arterioles, capillaries and venules limits the blood flow to cord tissue (Young, 2002). Shortly thereafter, infarction is intensified by secondary injury mechanisms such as vasospasm (Koyanagi et al., 1993), thrombosis (de la Torre, 1981) and neurogenic shock which causes bradycardia, hypotension, decreased peripheral resistance and decreased cardiac output (Guha and Tator, 1988). At the cellular level, this manifests as a loss of both oxidative phosphorylation and the glycolytic pathway. This ultimately cripples cellular energy metabolism causing necrosis through adenosine triphosphate (ATP) depletion. ATP depletion triggers a barrage of necrotic mechanisms, which include loss of cell membrane permeability, release of lysosomal contents and activation of calcium-dependent auto-destructive enzymes. These enzymes include proteases, phospholipases, ATPases and endonucleases which degrade plasma membranes, the nucleus and cytoskeletal components (Cotran et al., 1999; Tator, 1995).

Excitotoxicity. Following SCI, extracellular glutamate concentration rises continuously as a result of self-amplifying glutamatergic loops. These loops function due to compromised reuptake of glutamate, exocytosis of calcium-dependent glutamate synaptic vesicles and release of intracellular glutamate as a result of cell lysis (Panter et al., 1990). This abundance of extracellular glutamate, especially in a hypoxic environment (Choi, 1996), over-stimulates its ionotropic glutamate receptors, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate, to trigger waves of excitotoxic cell death (Doble, 1999).

In neurons, glutamate binds initially to AMPA receptors and causes depolarisation. This activates voltage-dependent sodium channels leading to further depolarisation and thus a marked increase in intracellular sodium. Chronicity of this response goes on to release NMDA receptors from their magnesium block, which leaves them available for activation by glutamate and thus adds to the increase of intracellular sodium. The intracellular ionic imbalance imposed by this sodium flood is amended by an influx of chloride ions. However, this ultimately upsets the osmotic balance of the cell and leads to influx of water and cell lysis (Doble, 1999). Alternatively, excitotoxicity may kill neuronal cells via calcium-dependent mechanisms. Through this means, chronic depolarisation leads to massive influx of intracellular calcium via voltage-
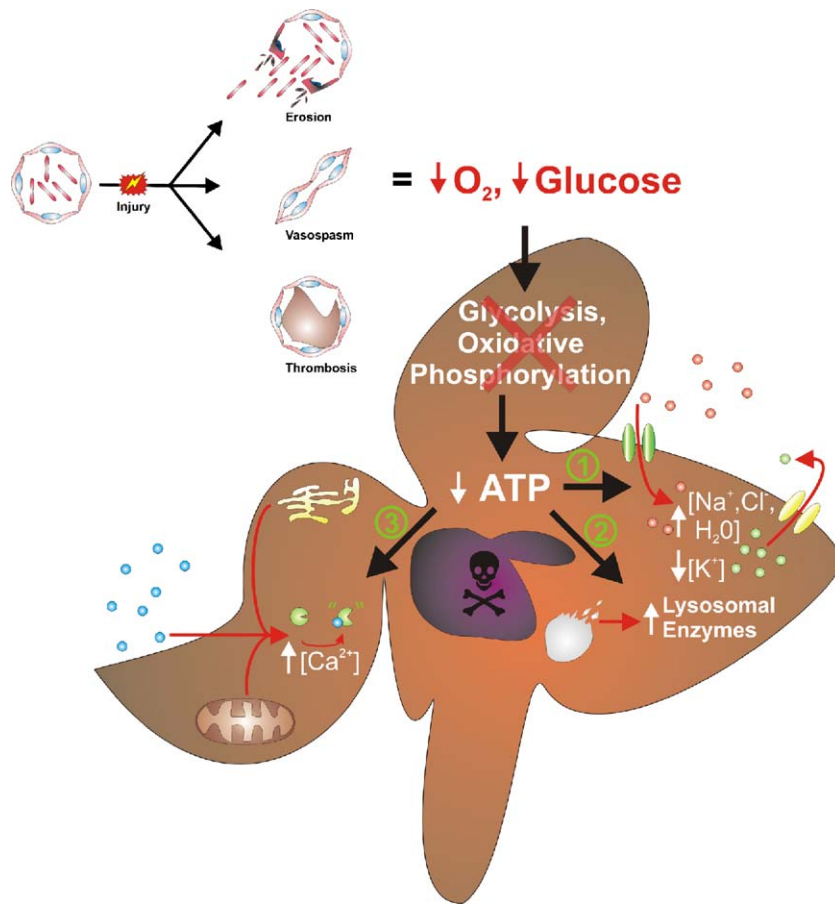

Fig. 1. Mechanisms of ATP depletion after ischaemia within the spinal cord after injury. The cell death cascades which are mediated through these mechanisms involve: (i) loss of membrane permeability; (ii) release of lysosomal contents intracellularly initiating autophagy and (iii) the increase in intracellular calcium levels triggering calcium-dependent protease activation.

dependent calcium channels and opening of NMDA receptor channels. This influx is further fortified by calcium mobilisation from intracellular stores and reverse operation of the membrane sodium/calcium exchanger. Consequently, this leads to activation of auto-destructive calcium-dependent enzymes (see Infarction), which in due course trigger cell death (Doble, 1999).

Following SCI, excitotoxic cell death also arises in CNS glia, of which oligodendrocytes are the most vulnerable (McDonald et al., 1998). As glial cells are void of NMDA receptors (Steinhauser and Gallo, 1996), excitotoxicity in glia is prompted via AMPA and kainate receptors. Importantly, both AMPA and kainate receptors in differentiated oligodendrocytes are more permeable to calcium, than those of neurons, due to alterations in their subunits (Puchalski et al., 1994). This, together with the poor capacity of oligodendrocytes to buffer calcium is the reason why these cells are highly susceptible to excitotoxic cell death (Mattson et al., 1991; Matute et al., 2001).

Reperfusion injury. Reperfusion of tissue in the first few days following SCI exacerbates tissue damage within the cord. During ischaemia, xanthine dehydrogenase within endothelial cells undergoes limited proteolysis. The resulting modified enzyme, xanthine oxidase, unlike its original form, transfers electrons to molecular oxygen. As a result, reexposure of endothelial cells to oxygen leads to an enzymatic reaction that gives rise to reactive oxygen species (ROS) (Cotran et al., 1999; Guth et al., 1999). This source of ROS together with ROS from neutrophils (Carlson et al., 1998) and necrotic cells (Tator, 1995) creates a potent stimulus for cell death.

ROS can induce damage to CNS cells by modifying their lipids, proteins or nucleic acids. In plasma and organelle membranes, free radicals cause peroxidation of lipids by attacking the double bonds of unsaturated fatty acids. This lipid–radical interaction yields peroxides, which due to their own reactivity can intensify membrane damage. Protein modification is another means through which ROS can induce death. This creates havoc within cells by oxidising amino acid side chains, causing fragmentation of proteins by backbone oxidation or forming protein–protein cross-linkages. Finally, ROS can also react with thymine in both nuclear and mitochondrial deoxyroribonucleic acid (DNA) to produce single strand breaks (Cotran et al., 1999).

Apoptosis. Apoptosis is an important mediator of secondary damage after SCI (Beattie et al., 2002b). It incurs its affects through at least two phases: an initial phase, by which apoptosis accompanies necrosis in the degeneration of multiple cell types and a later phase, which is predominantly confined to white matter and involves oligodendrocytes (see Oligodendrocyte apoptosis and survival) and microglia (Beattie et al., 2000). Chronologically, apoptosis initially occurs 6 h post-injury at the lesion centre and for several days thereafter the number of apoptotic cells in this region rises steadily. However, by 1 week the apoptotic count decreases and there is now an increase in apoptotic death away from the site of primary injury. This new apoptotic wave is predominantly localised in the white matter and can arise at large distances from the lesion centre (Crowe et al., 1997).

Under certain conditions, such as ischaemia and excitotoxicity, apoptosis may arise as the emerging cell death pathway at the time where necrosis is currently the most predominant degenerative event following SCI (Zipfel et al., 2000).

Axonal disruption and Wallerian degeneration. Axonal pathology following SCI is profuse. As early as 15 min after injury there exists periaxonal swelling with the myelin laminations peeling away from each other. At this time point, there also exists myelin

rupture, and by 24 h, axonal contents can be observed in the extracellular space. Neuronal axoplasms undergo transition as well; they display a granular appearance with disarray of their neurofilaments and in many axons there is an unusual abundance of intracellular organelles. As time progresses, other pathologic features of injured axons, such as those of widespread demyelination and abortive growth cones, are increasingly observed. In fact, by 24 h, a common phenomenon is the appearance of giant axons (greater than 40 μm), which exhibit a combination of all the aforementioned pathological features (Anthes et al., 1995).

These axonal changes are ultimately accompanied by Wallerian degeneration, which in rodents lasts for several months and in humans for years (David, 2002). Wallerian degeneration describes the withering of axonal segments separated from their neuronal soma. The process commences with the degeneration of separated fibres, which is accompanied by fragmentation of their associated myelin. It continues with the accumulation of resultant debris and culminates with the phagocytosis of this debris by macrophages and microglia (Sanes and Jessell, 2000b).

As the primary injury severs both ascending and descending axonal tracts, Wallerian degeneration is present both rostral and caudal to the initial lesion (Quencer, 2002). Furthermore, the sites of degeneration have a strong correlation with areas of delayed apoptosis. This has important implications, as most of these dying cells are oligodendrocytes that have lost trophic support from severed axons with which they were associated (see Trophic support deprivation) (Warden et al., 2001).

The glial scar. As in any other CNS injury, SCI initiates reactive gliosis. In this response, glial cells and non-CNS cells invade the site of primary injury. By invading this site, they clear up debris and wall it off to prevent secondary pathophysiological injurious mechanisms from spreading. However, with this comes the unfortunate burden of massive deposition of molecules that inhibits axonal regrowth (Fawcett and Asher, 1999).

The glial orchestra. The earliest component that makes up the glial scar is debris from myelin and oligodendrocytes, as well as oligodendrocytes that survive the primary injury. This initial component is followed by the activation and migration of microglia (by 48 h), which is also accompanied by invasion of blood borne macrophages (see Axonal regeneration). Up to this point, the cellular components of the glial scar with the exception of myelin debris are permissive to axon regrowth (Fawcett and Asher, 1999). In fact, phagocytosis by microglia of myelin debris, which is highly inhibitory to axon regrowth, is postulated to enhance axonal sprouting (David, 2002).

Following these initial events, the glial scar begins to be dominated by components that block axonal regrowth (Fawcett and Asher, 1999). After the intrusion of phagocytes, oligodendrocyte precursors (35 days post-SCI) with the surface markers NG2—an inhibitory chondroitin sulphate proteoglycan (see Axonal regrowth inhibitory molecules)—and platelet-derived growth factor receptor α (PDGFRα) intrude into the glial scar (Fawcett and Asher, 1999). As the differentiation of these cells is inhibited by CNS myelin, they may potentially differentiate into mature oligodendrocytes at 2 weeks after injury, in areas were axons are void of myelin (McTigue et al., 2001; Miller, 1999). At this same time point, meningeal cells from the CNS surface also migrate into the glial scar. These cells go on to re-form the disrupted glia limitans that surrounds the CNS by making contact with astrocytes walling off the primary lesion (Fawcett and Asher, 1999). Shortly

thereafter, multipotential progenitor cells from the spinal central canal also invade into the site of primary injury; however, this is not localised to the primary lesion as these cells can be observed throughout the cord (Takahashi et al., 2003).

The end point in the evolution of the glial scar is migration and proliferation of astrocytes. These cells up-regulate their production of glial fibrillary acidic protein (GFAP), become hypertrophic and appose many of their processes via gap junctions to the processes of neighbouring counter partners. Upon their infiltration, astrocytes isolate the site of primary injury by delineating the necrotic area and enveloping it with a dense glial lining and basal lamina (Bignami and Dahl, 1994). As astrocytes also fill the empty space produced by the primary lesion they eventually form the bulk of the glial scar (Fawcett and Asher, 1999). More importantly, astrocytes attempt to reestablish the integrity of the lesioned microenvironment to retain the function of nondamaged circuits. They facilitate this by regulating the blood–spinal cord barrier (BSCB), concentrations of excitatory amino acids (EAA), ionic concentrations and secreting trophic factors and cytokines (Rabchevsky and Smith, 2001). Importantly, these cells are also the producers of the majority of extracellular inhibitory axonal regrowth molecules (see Axonal regrowth inhibitory molecules) (Fawcett and Asher, 1999).

As a result of BSCB disruption, fibroblasts may also migrate into the glial scar (Grimpe and Silver, 2002). At more chronic stages (about 3 weeks), another non-CNS cell, the Schwann cell, may migrate into the lesion as well. Interestingly, Schwann cells have the ability to remyelinate denuded axons (Li et al., 1999b).

Axonal regrowth inhibitory molecules. The glial scar and surrounding environment is awash with molecules that are potent in making growth cones of injured neurons collapse (see Growth cone collapse). These molecules together with the rigid structure of the glial scar impose both a molecular and mechanical barrier to axonal regrowth. In general, the inhibitory molecules that are up-regulated after SCI can be divided into two categories: (1) myelin-associated inhibitory molecules (see Myelin-associated inhibitory molecules) and (2) molecules synthesised by cellular components of the glial scar, which either remain on the surface of these cells or are secreted in the extracellular matrix (Fawcett and Asher, 1999; Morgenstern et al., 2002).

Inhibitory molecules synthesised by cellular components of the glial scar are dichotomous (Grimpe and Silver, 2002). The first category involves molecules that are solely inhibitory to neurite outgrowth. Such molecules are proteoglycans, which are characterised by unbranched repeating disaccharide units termed acid glycosaminoglycans (AGAGs) connected via ether links to specific amino acid residues or a protein core (Bannister, 1999). The chondroitin sulphate proteoglycans (CSPGs), whose AGAGs are of chondroitin sulphate, is the major inhibitory proteoglycan subgroup. The members of this subgroup, which include NG2, neurocan, versican, brevican and phosphocan, are believed to inhibit neurite outgrowth by binding to the growth-promoting molecule laminin and thereby preventing it from interacting with its growth cone receptor integrin (Burg et al., 1996; Morgenstern et al., 2002; Sanes and Jessell, 2000a). The negative affects of CSPGs in SCI have been recently confirmed in vivo by Bradbury et al. (2002). By administering an enzyme that degrades the AGAGs from CSPGs, they were able to demonstrate both axonal regeneration and behavioural improvement in their lesioned animals.

The second category of inhibitory molecules has both inhibitory and axonal regrowth-promoting effects. Within this category are the tenascins (tenascin-R, tenascin-C and tenascin-X), large glycoproteins consisting of six subunits that makeup a stellate-like structure. Many of the tenascins, as well as inhibiting neurite outgrowth, are also able to bind most CSPGs and thereby retain secreted CSPGs within the vicinity of the glial scar (Fawcett and Asher, 1999; Grimpe and Silver, 2002). Other members within this category are the netrins and semaphorins, which are molecules that contribute to axonal guidance during development (Grimpe and Silver, 2002).

The immune system in SCI

Inflammatory responses are of central importance in the pathogenesis of the acute and chronic phases of SCI. During these phases, the CNS recruits both the innate and adaptive arms of immunity (Hauben and Schwartz, 2003).

Innate immunity

The injured environment

The injured environment during the acute phase of SCI is dominated by the presence of the pro-inflammatory cytokines tumour necrosis factor α (TNFα), interleukin (IL)-1 and IL-6. IL-1 is released immediately after injury by microglia (Allan and Rothwell, 2001). Furthermore, within 15 min, its mRNA levels as well as those of TNFα and IL-6 are increased in most cellular components of the CNS (Yan et al., 2001). As both IL-1 and TNFα can co-stimulate the expression level of each other, as well as that of IL-6, the levels of all three cytokines increase rapidly (Pan et al., 2002). Their levels peak after several hours, but by 24 h they are barely detectable (Klusman and Schwab, 1997). Despite this drop, the protein levels of TNFα continue to increase during the first week following SCI (Tyor et al., 2002). This is attributed to leukocyte infiltration (24 h post-SCI) and secretion of pro-inflammatory cytokines at the site of primary injury (Popovich and Jones, 2003). Further fortifying the armada of recruited cytokines is the up-regulation of a saturable unidirectional transport system in the blood–brain barrier (BBB) that is specific for TNFα. This allows TNFα produced by peripheral trauma to enter and thus accumulate within the CNS (Pan et al., 1999; Pan and Kastin, 2001).

Binding of both TNFα and IL-1 to their receptors causes them to induce the inflammatory response by signalling through the nuclear factor κB (NFκB) pathway (Allan and Rothwell, 2001). An observed increase of the pathways end product, NFκB, is observed in neurons, microglia and endothelial cells by 30 min post-SCI and persists for at least 72 h (Bethea et al., 1998). This is crucial as active NFκB stimulates the production of inflammatory mediators such as ROS, cytokines, inducible nitric oxide synthase (iNOS), prostaglandin (synthase-2, arachidonic acid, proteases and endothelial cell adhesion molecules (CAMs) (Allan and Rothwell, 2001; Bethea et al., 1998; Kim et al., 2001). Interestingly, the anti-inflammatory agent methylprednisolone, which is the only drug therapy to be approved in SCI treatment, has been shown to inhibit the NFκB pathway (Xu et al., 1998).

SCI also induces the expression of the anti-inflammatory cytokine transforming growth factor-β (TGFβ) (Tyor et al., 2002). In contrast to pro-inflammatory cytokines, the expression of TGFβ is delayed. Although it is found at the lesion site within 24 h, its mRNA levels do not peak until 7 days later (Semple-Rowland et al., 1995). TGF-β counteracts the effects of pro-

inflammatory cytokines by down-regulating iNOS and decreasing endothelial CAMs (Hamada et al., 1996; Kitamura, 1997).

Neutrophil recruitment

Up-regulation of endothelial CAMs [intracellular (I)-CAM, vascular (V)-CAM, platelet endothelial (PE)-CAM, P- and E-selectin] recruits leukocytes to the site of primary injury (Lee et al., 2000b; McTigue et al., 1998; Schnell et al., 1999). In addition to these factors, IL-8 and the CXC chemokine, cytokine-induced neutrophil chemo-attractant 1 (CINC-1), both enhance neutrophil recruitment and activation even further (Tonai et al., 2001). Consequently, neutrophils adhere to postcapillary venules 6–12 h post-SCI and by 24 h they migrate into the lesion site to phagocytose debris (Guth et al., 1999; Taoka et al., 1997). Upon their entry, pro-inflammatory mediators induce neutrophils to generate their own cytokines as well as the production of proteases via the NFκB translocation pathway (McDonald et al., 1997). Such mediators include matrix metalloproteinases (MMPs)—in particular MMP-9—(Noble et al., 2002), and the cytokines TNFα, IL-1, IL-8 and TGFβ (Cassatella, 1995). These mediators loosen the extracellular matrix to enhance extravasation of leukocytes, stimulate leukocyte chemotaxis, activate glia and enhance neuronal damage (Carlson et al., 1998). Furthermore, the activities of the superoxide dismutase and neutrophil myeloperoxidase enzymes, which mediate the respiratory burst, are enhanced. As a result, the attachment of neutrophils to endothelium exacerbates reperfusion injury (see Reperfusion injury) and thereby gives rise to the aforementioned petechial haemorrhages (see Macroscopic considerations). The activity of these cells however, is only transient as by 48 h their recruitment declines (Guth et al., 1999; Taoka et al., 1997).

Macrophage/microglial activation

As neutrophil numbers decline, there is an increasing presence of monocytes in the injured parenchyma, which by 72 h go on to differentiate into macrophages. Their chemotaxis is dependent upon CAMs as well as IL-8 and macrophage inflammatory proteins (MIP) α and β (Bartholdi and Schwab, 1997; Cassatella, 1995). It has been demonstrated that after CNS injury there exist a few haematogenously derived macrophages at the site of transection were the blood–brain barrier is disrupted (George and Griffin, 1994). Following their recruitment, macrophages—and microglia—are activated by TNFα as well as by ligand binding to their receptors complement receptor 3 (CR3) and mannose receptor (Fitch et al., 1999). Interestingly, microglial cells are activated at the site of primary injury at an even earlier time point (1 h) (Dusart and Schwab, 1994). This results due to an elevated concentration of extracellular ATP, which via a calcium-dependent mechanism acutely up-regulates the microglial protein, allograft inflammatory factor-1 (AIF-1), and thereby causes microglial activation (Schwab et al., 2001; Tanaka and Koike, 2002). In addition to their acute activation, microglia are also activated during later stages of SCI (5 days) at sites distant to the primary injury (Koshinaga and Whittemore, 1995; Watanabe et al., 1999). This activation arises predominantly in tracts of white matter undergoing Wallerian degeneration (see Axonal disruption and Wallerian Degeneration) (Frisen et al., 1994).

The activation of macrophages leads to the secretion of glutamate, the pro-inflammatory cytokines TNFα, IL-1 and IL-6, and activation of the respiratory burst enzyme inducible nitric oxide synthase (iNOS) (Leskovar et al., 2000; Satake et al., 2000). In addition, macrophage activation up-regulates cyclooxygenases,

which are key enzymes in the conversion of arachidonic acid—plentiful in the injured cord—to prostanoids. Prostanoids have a wide variety of immunomodulatory properties and have the potential to augment secondary injury (Schwab et al., 2000).

Upon activation, macrophages and microglia, like neutrophils, phagocytose necrotic and apoptotic debris (Guth et al., 1999; Taoka et al., 1997). However, unlike neutrophils, their activity is sustained for longer periods. In fact, macrophage presence in rat models of SCI does not decrease until 7 days post-injury and microglial presence in degenerating tracts plateaus at between 2 and 4 weeks (Popovich et al., 1997). Finally, overactivation of these cells can lead to their degeneration into ghost cells, lipid engorged phagocytes, which remain in the spinal cord for months to years after SCI (Popovich and Jones, 2003).

Due to the lack of specific markers, it has been extremely difficult to differentiate invading macrophages from activated microglia within the injured spinal cord. As a consequence, their individual functional roles remain elusive. Recently, Popovich and Hickey (2001) have used radiation bone marrow chimeric rats to directly address this question. These investigators demonstrated that the initial injury response (onset and plateau) occurring at the 3- and 7-day intervals post-injury, previously thought to be related to haematogenously derived macrophage infiltration, were in fact dominated by microglial-derived macrophages. These cells were prevalent at the injury site and where active Wallerian degeneration was occurring. Haematogenous macrophages were determined to infiltrate the injury site slowly, appearing initially in dorsal gray matter, subpial white matter and around branches of the ventral spinal artery, eventually occupying nearly all of the gray matter by 7 days. These data signify a predominant association of macrophage infiltration and gray matter pathology, as well as a unique association with Wallerian degeneration, oligodendrocyte death and microglial activity.

Attenuation of innate immunity

Given the wide array of noxious mediators that are secreted by neutrophils and macrophages, decreasing their presence has been widely employed as a therapeutic approach in SCI models. Strategies such as inhibition of MMPs (Noble et al., 2002), decreasing the availability of CAMs (Taoka et al., 1997), elastase inhibition (Tonai et al., 2001), inflammatory cytokine down-regulation (Xu et al., 1998) and hampering the production of ROS and lipid peroxidation (Hall, 1992) have all been employed with some success. Despite this success, decreasing the presence of macrophages and microglia at the locus of primary injury may potentially be harmful. In addition to ridding the injured environment of myelin derived axon-growth inhibitory molecules (see Myelin-associated inhibitory molecules), macrophages and microglia can potentiate the release of mediators that promote CNS repair (Nguyen et al., 2002). These mediators, which include ciliary neurotrophic factor (CNTF) (Herx et al., 2000), insulin-like growth factor 1 (IGF-1) (Mason et al., 2001) and the neurotrophins, nerve growth factor (NGF) (Heese et al., 1998) and brain-derived growth factor (BDNF) (Kerschensteiner et al., 1999) are mainly released by astrocytes in response to macrophage and microglial stimulation by TNFα and IL-1 (Nguyen et al., 2002). Hence, after contributing to the acute phase of injury, these phagocytes may potentially aid regeneration at more chronic stages by secreting neuronal survival and regeneration factors. Furthermore, TNFα via binding to TNF receptor 2 (TNFR2) has been shown to promote re-myelination of demyelinated axons (Arnett et al., 2001).

Adaptive immunity

After engulfing debris, macrophages at the site of primary injury function as antigen-presenting cells (Popovich et al., 1993). This is specifically enhanced by the high concentration of IL-1, which promotes major histocompatibility complex class II (MHC II)-mediated antigen presentation (Cassatella, 1995). Although microglia do not constitutively express MHC II, the injury—especially at more chronic stages—evokes them to up-regulate this antigen (Moalem et al., 1999b; Schmitt et al., 2000). However, unlike the blood-borne macrophages, microglia are devoid of the B7 co-stimulatory molecule (Schmitt et al., 2000). Hence, it is likely that the majority of T helper (T_H) cells activated in SCI are activated by macrophages, whereas those that encounter microglia fall into anergy (Schmitt et al., 2000). Interestingly, it has also been reported that astrocytes may mediate antigen presentation (Moalem et al., 1999b).

The CNS contains systems that rapidly counteract the activation of T_H cells to block the recognition of cryptic epitopes by immune cells and thereby prevents autoimmunity. Specifically, these systems involve increased glial surface expression of FAS ligand (FASL) to induce apoptosis of infiltrating lymphocytes (Moalem et al., 1999b) and suppression of auto-reactive T_H cells by regulatory T cells ($CD4^+$ and $CD25^+$) (Yoles et al., 2001). In fact, the importance of immune regulation is underlined by a study showing that T cells isolated from rats 1 week post-SCI and injected into naïve recipients induce experimental autoimmune encephalomyelitis (EAE), whereas T cells isolated at later stages do not (Olivares-Villagomez et al., 1998; Popovich et al., 1996).

Silencing the adaptive immune response may however impede functional recovery after SCI (Schwartz and Hauben, 2002). Adaptive immunity facilitates neuroprotection via T-cell-mediated reduction of nerve activity to prevent ATP depletion (Moalem et al., 1999a). It has the capacity to enhance myelin phagocytosis by macrophages (David, 2002) and triggers secretion of neurotrophic factors. Activated T_H cells secrete significant amounts of the neurotrophins NGF, BDNF, neurotrophin (NT)-3 and NT-4/5 (Ehrhard et al., 1993; Kerschensteiner et al., 1999; Moalem et al., 2000). In fact, SCI performed on rats that are immunised against myelin proteins, hence an enhanced immune response, is associated with improved functional recovery (Hauben et al., 2000). Nevertheless, benefit from immunisation is strain specific as recovery depends upon the individual strains ability to regulate the immune response (Kipnis et al., 2001).

Molecular mechanisms in SCI

The complex pathophysiology of SCI leads to activation as well as inhibition of numerous molecular mechanisms involved in secondary injury. The following paragraphs will deal with mechanisms more specific to oligodendrocytes and the precedence for administering leukaemia inhibitory factor (LIF) as a possible therapeutic measure.

Growth cone collapse

Following SCI, the proximal ends of cut axons reseal and form a growth cone (Ng and Tang, 2002). The growth cone is a sensory and motor structure that upon elongation gives rise to an axon. At its midpoint is a surrounding central core that is rich in micro-tubules, mitochondria and myosin. From here, processes that are long and slender, highly motile, abundant in actin projections, termed filopodia, project outwards. These membrane-limited extensions have scores of receptors—integrins, cadherins, immunoglobulins, receptor tyrosine kinases and plexins—that probe the surrounding environment and deduce if axonal extension is feasible. In the case that extension is feasible, new actin components will be added to the extending front of the filopodia. Conversely, if the environment deters axonal elongation, as in the case of SCI, actin synthesis ceases and existing actin filaments contract against the pull of myosin to cause growth cone collapse (Sanes and Jessell, 2000a).

Myelin-associated inhibitory molecules

The principle axonal regrowth inhibitor in the acute phase of SCI is CNS myelin (Ng et al., 1996). The inhibitory components of myelin include myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002). All three of these proteins are predominantly localised on the innermost lamella of the myelin sheath and make direct contact with axons (Fig. 2) (McKerracher and Winton, 2002).

Of the three inhibitors, Nogo-A (1192 a.a.) has the most interesting structure as it has two domains that can individually inhibit axonal growth (Fig. 2). These attributes have led to the establishment of the following model: constitutive inhibition by Nogo-A is mediated by Nogo-66 and in the case of trauma, amino-Nogo. Membrane disruption permits Nogo-66 to access the extracellular space contributing to inhibition (Huber and Schwab, 2000). However, the amino domain of Nogo-A, through conformations that localise it extracellularly, is also capable of inhibiting neurite outgrowth in the absence of injury (Buffo et al., 2000).

Membrane and intracellular signalling

The lipid raft. Signalling by myelin-associated inhibitory molecules is dependent upon their binding to appropriate receptors on growth cones. Localisation of these receptors and their intracellular signalling machinery is specific to neuronal lipid rafts (Vinson et al., 2003). Lipid rafts are plasma microdomains enriched in cholesterol and sphingolipids that provide an ordered platform for signal transduction (Tsui-Pierchala et al., 2002). Concentrated within these growth cone rafts is the Nogo-66 receptor (NgR), low-affinity neurotrophin receptor ($p75^{NTR}$), gangliosides GD1a and GT1b as well as RhoA a member of the Rho guanine triphosphatases (GTPase; Fig. 3) (Vinson et al., 2003).

Within the lipid rafts, NgR plays a central role due to its ability to bind to all three myelin-associated inhibitory molecules (Fournier et al., 2001; Josephson et al., 2002; Liu et al., 2002; Wang et al., 2002b). As it has no intracellular domains (Fig. 2) that would allow it to transduce the signal of myelin inhibition to the cells interior, upon ligand binding it forms a heterodimer with $p75^{NTR}$ (Wang et al., 2002a). This complex between the two receptors requires the interaction of their entire extracellular portions. Although all myelin-associated growth inhibitors signal through NgR, signalling by MAG is somewhat more ubiquitous. In addition to binding NgR, MAG also signals by directly binding to $p75^{NTR}$ or by first binding to sialic acid residues of the ganglioside GT1b, which then itself associates with $p75^{NTR}$ (Yamashita et al., 2002). Never-

A V-set Ig D. / C2-set Ig D.

MAG (626 a.a.) is a member of the Siglec (sialic acid binding immunoglobulin (Ig)-like lectins) family that has the majority of its sequence localised extracellularly (Kelm et al., 1994). At its N-terminus is a V-set Ig domain, which has high avidity for sialic acid residues (large family of 9 carbon sugars derived from N-acetyl-neuraminic acid). Trailing this domain are 4 C2-set Ig domains that span the majority of sequence and eventually culminate as an intracellular C-terminus (May et al., 1998).

B Nogo-66 / C-terminus / N-terminus

Nogo-A has a C-terminal domain (188 a.a.) that it shares with the other two Nogo splice variants, Nogo-B and Nogo-C. This domain is homologous to members of the reticulon family and contains an endoplasmic reticulum (ER) retention motif, which keeps the majority of Nogo in the ER. Furthermore, within the C-terminal locus are two hydrophobic transmembrane domains that are separated by a 66 a.a. extracellular hydrophilic domain (Nogo-66). It is this domain, together with that of the intracellular N-terminal domain (1024 a.a.) that mediate inhibition of axonal regrowth (GrandPre et al., 2000).

C LRR / Ser/Thr Rich D. / GPI

OMgp (440 a.a.) is a cell adhesion molecule that is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Commencing at its N-terminus are 8 leucine-rich-repeat (LRR) domains that towards the C-terminus are followed by a serine/threonine rich domain. This domain provides a site for potential O-linked glycosylation (Mikol et al., 1993).

D LRR / LRR (cys) / GPI

NgR (473a.a.) has 8 LRR domains that commence from its N-terminus. These are followed by another LRR domain, which differs however, for it is also rich in cysteine; NgR finally culminates at its C-terminus with a GPI anchorage site (Fournier et al., 2001).

E Cys Rich D. / Death D.

P75[NTR] (399 a.a.) has 4 cysteine rich domains that commence near its N-terminus. Following these is a transmembrane segment that gives way to an intracellular module, which contains a type II death domain. This module also has a palmitoylation site at cysteine 279, 2 potential TRAF-binding sites and a PDZ binding motif (not shown) [Roux and Barker, 2002].
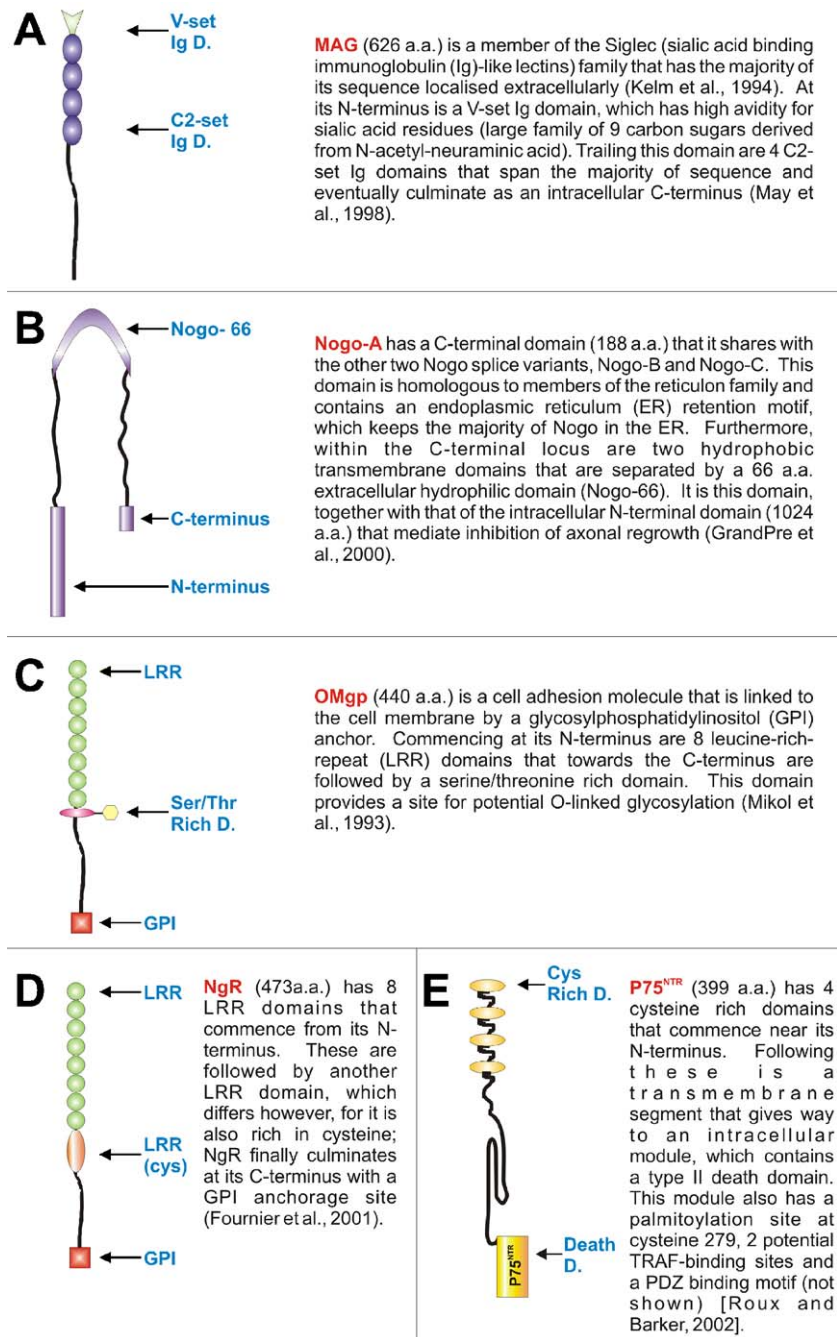
Fig. 2. Structural features of myelin-associated inhibitors and their receptors. The molecular structure of MAG, with its extracellular interactive domain (A) differs considerably with the other major myelin inhibitory molecule of regeneration, Nogo-A (B), which both C- and N-terminus domains are internalised, yet a short extracellular 66-residue segment (Nogo-66) can signal growth cone collapse by binding to the Nogo receptor (NgR). The third member of this group of myelin inhibitory molecules of regeneration is the oligodendrocyte myelin glycoprotein (OMgp) with an eight leucine-rich repeat (LRR) domain, which also binds to NgR producing growth cone collapse. The receptors, which these molecules interact with on the progressive growth cone of regenerating axons, are NgR (D) and p75[NTR] (E). p75[NTR] is a co-receptor of NgR interacting at the C-terminal end of the LRR and mediates growth cone collapse by small GTPase adapter proteins and activation of RhoA. These adaptor proteins associate with the cytoplasmic death domain of p75[NTR].

theless, regardless of the transduction mechanism employed by myelin-associated inhibitory molecules, signalling ultimately converges at the level of p75[NTR] (Yamashita and Tohyama, 2003).

With the recent finding of two novel proteins—NgR homologue 1 and 2 (NgRH1 and NgRH2)—that are homologous both structurally and biochemically to NgR, it is likely that there is an added complexity to membrane signalling by myelin-associated inhibitory molecules (Pignot et al., 2003). This likelihood is rather strong as at present not all the axonal growth restrictive properties of myelin-associated inhibitors can be accounted for by NgR (Yamashita and Tohyama, 2003).
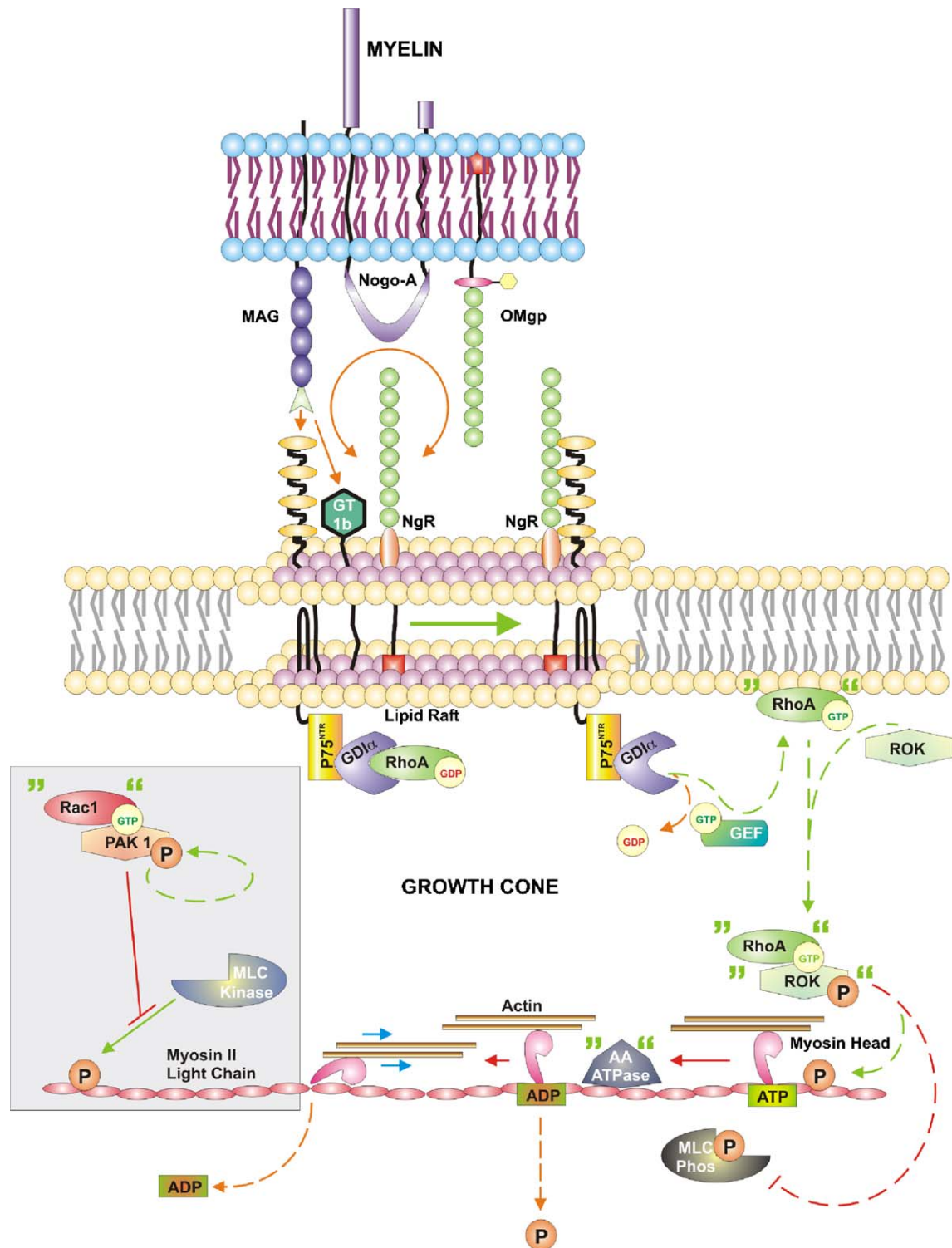
Fig. 3. Molecular mechanisms of growth cone collapse. The responsiveness of p75[NTR] following interaction with myelin-associated inhibitors, the cytoplasmic domain binding RhoA, conformed to RhoA guanine diphosphate dissociation inhibitor α (GDIα), dissociates GDIα from RhoA and thereby allows RhoA to be activated. This is mediated by guanine triphosphate exchange factors (GEFs), which add GTP to RhoA. Following its activation, RhoA associates with the plasma membrane and binds to the serine-threonine kinase Rho-kinase (ROK), which renders ROKs kinase active. Consequently, ROK proceeds to phosphorylate the regulatory light chain of the major cytoplasmic myosin, myosin II, and thereby increases myosin II's activity of actin-activated ATPase. Activation of this ATPase, hydrolyses myosin ATP and thereby initiates contraction between myosin within the growth cone and actin filaments of the filopodia. This contraction pulls the actin filaments towards the centre of the growth cone and away from the direction of protrusion to eventually manifest in growth cone collapse.

Intracellular signalling

Retraction via contractility. The responsiveness of p75[NTR] following interaction with myelin-associated inhibitors induces conformational change of its intracellular domains fifth α helical loop (Yamashita and Tohyama, 2003). In the absence of inhibitory signalling, this domain binds RhoA that is conformed to RhoA guanine diphosphate dissociation inhibitor α (GDIα) (Fig. 3). GDIα-bound RhoA is inactive as GDIα prevents the release of GDP from RhoA and thereby binding of GTP, which is required for RhoAs activation. Once MAG, Nogo or OMgp bind to their targets, however, p75[NTR] dissociates GDIα from RhoA and thereby allows RhoA to be activated. This is mediated by guanine triphosphate exchange factors (GEFs), which add GTP to RhoA (Yamashita and Tohyama, 2003). Following its activation, RhoA associates with the plasma membrane and binds to the serine-threonine kinase Rho-kinase (ROK), which renders ROKs kinase active (Leung et al., 1996). Consequently, ROK proceeds to phosphorylate the regulatory light chain of the major cytoplasmic myosin, myosin II, and thereby increases myosin II's activity of actin-activated ATPase. Activation of this ATPase hydrolyses myosin ATP and thereby initiates contraction between myosin within the growth cone and actin filaments of the filopodia. This contraction pulls the actin filaments towards the centre of the growth cone and away from the direction of protrusion to eventually manifest in growth cone collapse (Amano et al., 1996). Interestingly, ROK also phosphorylates the myosin light chain phosphatase and as a result inhibits its activity. Since this phosphatase is unable to de-phosphorylate the ATPase that ROK phosphorylated in the first place, the likelihood of growth cone collapse is enhanced even further (Kimura et al., 1996).

Whilst active RhoA GTPase is up-regulated during myelin contact-mediated growth cone collapse, another member in the Rho family of GTPases, Rac1, is down-regulated (Niederost et al., 2002). Rac1 is down-regulated as its activities mediate growth cone extension (Fig. 3, gray inset). It accomplishes this by binding to the serine-threonine kinase p21-activated kinase 1 (PAK1), which auto-phosphorylates upon their interaction (Manser et al., 1995). Once activated, PAK1 inhibits the myosin light chain kinase, resulting in decreased phosphorylation of the myosin light chain, whose phosphorylation is a necessity if contractility between myosin and actin is to ensue. Hence, by down-regulation of Rac1, the mechanisms leading to contraction by RhoA remain unopposed (Sanders et al., 1999).

Retraction via aberrant actin polymerisation. Apart from affecting the dynamics of growth cone contractility, the Rho GTPases also influence the kinetics of actin filament assembly. In myelin-mediated growth cone collapse, this assembly is drastically altered by both RhoA and Rac1 (Niederost et al., 2002).

Via the RhoA pathway, Lin-11, Isl-1 and Mec-3 kinase (LIMK) is phosphorylated and activated by ROK (Maekawa et al., 1999). Once active, LIMK has the ability to inactivate, by phosphorylation, the actin de-polymerising protein cofilin. This leads to a shortage of actin monomers and thereby halts the extension of the growth cone (Arber et al., 1998). Furthermore, it has been hypothesised that the initial phosphorylation of cofilin leads to activation via another pathway of a phosphatase that restores cofilin activity. This reactivation however is unremitting and leads to excessive actin depolymerisation causing growth cone collapse (Niederost et al., 2002).

Inhibition of Rac1 activity is instrumental in modulating the kinetics of actin assembly in a manner that causes abortive axon sprouting. Normally, Rac1 enhances the activity of its effector phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), which generates phosphatidylinositol-4,5,-bisphosphate (PIP2) (Tolias et al., 1995, 1998). Once produced, PIP2 localises at lamellipodia and binds the control region of Neural Wiskott Aldrich syndrome protein (N-WASP) to stimulate its association with the Arp 2/3 complex. Once associated, this seven-protein complex nucleates the formation of new actin filaments and is thereby responsible for growth cone elongation (Rohatgi et al., 2000). Remarkably, PIP2 further promotes actin assembly by inhibiting capping protein, which caps filament ends to prevent their elongation. Hence, inhibition of this molecular cascade by down-regulation of the original effector, Rac1, enhances the probability of growth cone collapse (Janmey and Stossel, 1987; Meyer and Feldman, 2002).

Axonal regeneration

Elucidation of the dynamics involved in abortive axonal sprouting has brought about exciting progress in finding new ways to stimulate axonal regeneration. Strategies that block myelin-associated inhibitory molecules and their receptors have been employed with some success. For instance, rats having undergone SCI and then administered antibodies against myelin inhibitors (IN-1: an antibody against Nogo-A, -B and -C) have enhanced functional recovery and considerable axonal regeneration at the lesion site (Thallmair et al., 1998). Other strategies, which aim to promote axonal regeneration by blocking intracellular targets promoting abortive sprouting, have also been employed. In this case, success has come about by blocking the activity of ROK with the analogue Y27632 (Dergham et al., 2002). Although axonal regeneration will not restore the initial neuroanatomical wiring before SCI, it does lead to restoration of lost function possibly by enhancing rewiring of local circuitry (Schwab, 2002).

Nogo mutants and SCI

Considering the success of the antibody studies described above in the enhancement of the regenerative capacities of severed axons with subsequent functional improvements in these animals (Thallmair et al., 1998), it was of some surprise that three recent independent studies generated conflicting data on the regenerative capacities in Nogo-mutant mice following SCI (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). One of these groups generated two lines of mutant mice, one lacking the isoforms Nogo-A and -B but not -C, and the other lacking all three isoforms (Nogo-A/B/C mutants) (Zheng et al., 2003). Despite the Nogo-A/B mutant showing decreased inhibitory activity in neuritic outgrowth assays in culture, anterograde labelling experiments of descending corticospinal fibres in vivo following dorsal hemisection in mouse spinal cords revealed no real increase in regeneration or sprouting (Zheng et al., 2003). Furthermore, CNS myelin from Nogo-A/B mutant mice was still inhibitory in culture. However, when the C isoform of Nogo was deleted from these animals as well, the Nogo-A/B/C mutants on average exhibited slightly better locomotor function without statistical significance among the groups. These data collectively would suggest that animals without expression of Nogo-A and -B do not have an increased ability to regenerate axons following the induction of Wallerian degeneration and that

other myelin inhibitory molecules are as important in blocking axonal regeneration in vivo.

Seemingly contradicting such data, Kim et al. (2003) published their findings in the same issue of *Neuron*, which showed their Nogo-A/B mutant mice to exhibit heavy sprouting rostral to the site of hemisection as well as numerous fibres regenerating into distal cord segments with concomitant recovery of locomotor function. As a confirmatory finding, myelin extracted from wild types or Nogo-A/B heterozygotes potently collapsed DRG growth cones but not those exposed to the myelin of Nogo-A/B knockouts. These data, along with those generated by Simonen et al. (2003), clearly suggest that Nogo-A is the most significant myelin inhibitory molecule of regeneration involved during SCI. Such conflicting data emphasise the concerted effort required to elucidate the various inhibitory components to axonal regeneration following SCI. If in fact the myelin molecules are the most significant of the inhibitory components, then a deliberate focus should be targeted toward the preservation of the myelinating cell, the oligodendrocyte, for the maintenance of lamellated myelin and decrease in myelin protein deposits at the lesion site and beyond.

Oligodendrocyte apoptosis and survival

Oligodendrocyte apoptosis is a widely dispersed phenomenon during SCI that leads to long-term and persistent demyelination (Crowe et al., 1997). Since each oligodendrocyte myelinates multiple axons, their death leads to denudement of many fibres that are left intact by the initial trauma. Consequently, the conductive abilities of these axons are jeopardised and functional recovery of patients is hampered. Importantly however, the delayed appearance of apoptosis in oligodendrocytes provides a therapeutic window for intervention that is way beyond the acute phase of injury (Li et al., 1999a). Furthermore, studies in a contusion model of SCI in the cat have shown that with the preservation of only 5–10% of axons in each individual tract, meaningful recovery with locomotion can be achieved (Blight, 1983; Blight and Decrescito, 1986). As numerous intact demyelinated axons are observed after SCI (Bunge et al., 1993), salvation of oligodendrocytes has the potential to be of great therapeutic value.

Apoptosis

Apoptosis of oligodendrocytes as a result of SCI arises due to their increased susceptibility to insult as a result of trophic support loss (Barres et al., 1992; Raff et al., 1993). Additionally, oligodendrocytes may also undergo apoptosis as a result of apoptotic cascades set off by activation of their surface death receptors (Casaccia-Bonnefil, 2000). In response to these insults, oligodendrocytes employ components of both the intrinsic and extrinsic pathways of apoptosis.

The mitochondrion is the most crucial component of the intrinsic pathway of apoptosis as it stores cytochrome *c* to prevent it from escaping into the cytosol. When apoptosis is induced however, cytochrome *c* is released into the cytosol and complexes with Apaf-1 and pro-caspase-9, forming the apoptosome. This structure activates pro-caspase-9 to caspase-9 by proteolytic cleavage, which in turn activates the executioner caspases, caspase-3, -6 and -7. Of these caspases, caspase-3 is of central importance as its activities spur the cleavage of DNA, nuclear lamins, cytoskeletal components and proteins that inhibit apoptosis. In addition to

cytochrome *c*, sequestered within the mitochondrion in an apoptosis-regulated manner is Smac/Diablo. Upon its release to the cytosol, Smac/Diablo enhances the progress of apoptosis by antagonising the activities of cellular inhibitor of apoptosis proteins (cIAP), a protein group that blocks caspase activity (for review, see Hengartner, 2000).

The apoptotic domino effect set off by cytochrome *c* is the reason why its release from the mitochondrion is tightly regulated. Taking on the duty as cytochrome *c* gatekeeper is the Bcl-2 family of proteins. The members of this group are characterised by their ability to bind to the outer mitochondrial membrane and form dimers. If they belong to the pro-apoptotic group (including Bax, Bid and Bad), they will facilitate the release of cytochrome *c*, whereas if they are anti-apoptotic (including, A1, Bcl-2 and Bcl-xL), they will block it. Interestingly, the two groups spend the majority of their time forming heterodimers with one another to counteract each other's function (for review, see Hengartner, 2000). As a result, the relative abundance of pro- or anti-apoptotic Bcl-2 family members is decisive upon the oligodendrocytes fate (Casaccia-Bonnefil, 2000).

The extrinsic pathway of apoptosis is characterised by the binding of ligand to death receptors (see Death receptors). This causes receptor aggregation and conformational change in their death domains that permits adapter protein recruitment. As a result of this interaction, there is the arrival as well as activation of many pro-caspase-8 units. The primary activity of caspase-8 is to cleave caspase-3 and thereby set off apoptosis. Caspase-8 however, can also initiate the intrinsic pathway of apoptosis by cleaving cytosolic Bid and causing it to translocate to the mitochondrial membrane (for review, see Hengartner, 2000).

Trophic support deprivation

Oligodendrocyte survival is conditioned upon stimulation by axolemmal β-neuregulins, a class of epidermal growth factor-like molecules that signal via the tyrosine kinase receptor erbB (Vartanian et al., 1997). In addition to this chief stimulus, their endurance is also dependent upon astrocytic derived insulin like growth factor-1 (IGF-1) and nerve growth factor (NGF), which also signal via tyrosine kinase receptors (Barres et al., 1993). In general, the actions of these survival factors are mediated by the blocking of apoptosis default pathways.

Signalling by tyrosine kinase receptors commences when ligand binds to them and causes their dimerisation. Upon dimerisation, kinase domains of each monomer initially cross-phosphorylate each other on tyrosine residues and then go on to phosphorylate other such residues within the receptors cytoplasmic domain. This mass phosphorylation renders the receptor active and thereby creates high-affinity intracellular docking sites that activate intracellular proteins (Fig. 4) (Schlessinger, 2000).

In the case of the β-neuregulins (Flores et al., 2000) and NGF (Takano et al., 2000), activation of erbB and TrkA, respectively, recruits phosphatidylinositol 3-kinase (PI 3-kinase) to their cytoplasmic domain and causes its activation (Fig. 4). Subsequently, PI 3-kinase phosphorylates membrane inositol phospholipids near the tyrosine kinase receptor and as a result produces $PI(3,4,5)P_3$ and $PI(3,4)P_2$. This leads to membrane docking of the serine-threonine kinases phosphatidylinositol-dependent protein kinase (PDK1) and protein kinase B (PKB, also known as Akt), which is mediated via a domain—Pleckstrin homology (PH)—that conforms tightly to $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Binding to this domain allows PDK1 to change its conformation to an active one and thereby activate Akt
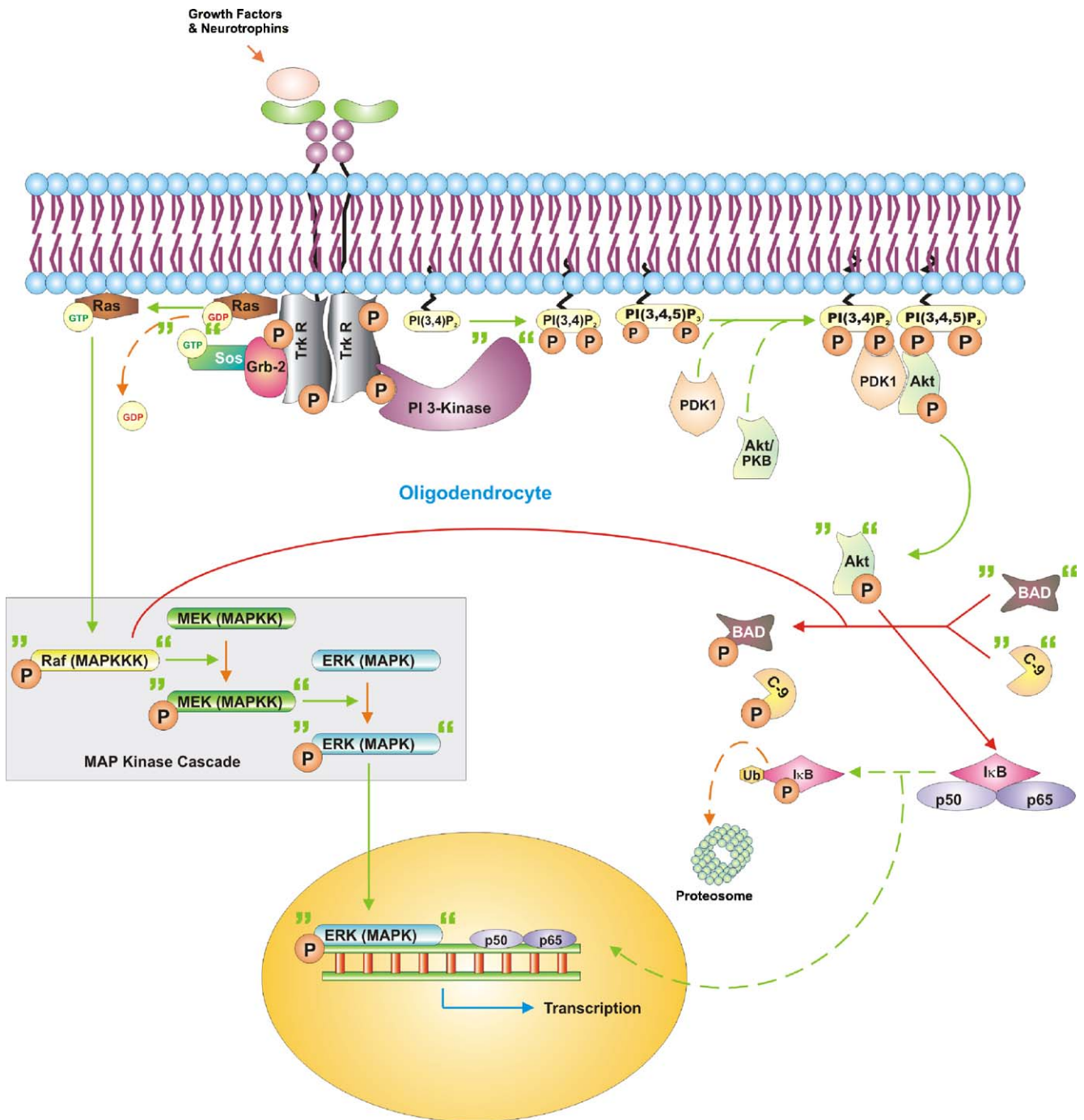
Fig. 4. Molecular mechanisms promoting oligodendrocyte survival. Certain growth factors and neurotrophins (e.g. IGF-1, β-neuregulins and NT-3) mediate their transduction effects in oligodendrocytes through tyrosine receptor kinases (Trk). Following ligand binding, the survival signal begins by recruiting PI-3 kinase and activation of Akt, which can block pro-apoptotic proteins and mediate translocation of NFκB. A subsequent pathway used by this activation is through Ras/MAP kinase activation which can also block pro-apoptotic proteins. This pathway regulates transcription favouring survival.

by phosphorylation. Once active, Akt dissociates from the inositol phospholipids and phosphorylates both BAD and caspase-9 to render them inactive (Flores et al., 2000; Schlessinger, 2000).

NGF, β-neuregulins as well as IGF-1 can block apoptosis by an additional pathway (Casaccia-Bonnefil, 2000). In this pathway, the activated receptor tyrosine kinase recruits to its cytoplasmic domain the adaptor protein Grb-2. Grb-2, which is complexed with a GEF called son of a sevenless (Sos), binds the receptor via

interaction of its Src-homology 2 (SH2) domain with specific phosphorylated tyrosine residues (Fig. 4). This causes conformational change in Grb-2, which in turn opts Sos to change to its active conformer. As a consequence, active Sos prompts the exchange of GDP for GTP of the membrane-bound protein Ras. Once in its active GTP-bound state, Ras activates the mitogen-associated protein kinase-kinase-kinase (MAPKKK) Raf that like Akt inactivates both BAD and caspase-9. Unlike Akt however, Raf

is also able to activate the survival-orientated extracellular signal-regulated kinase (ERK) pathway (Casaccia-Bonnefil, 2000). It accomplishes this by activating the MAP-kinase-kinase (MAPKK), MEK, through phosphorylation of a key residue in its activation loop. As a consequence, this MAPKK then phosphorylates the activation loop of the mitogen-activated protein kinase (MAPK) ERK (Schlessinger, 2000). Upon its activation, ERK quickly translocates to the nucleus where it alters transcription in favour of survival. This is mediated by counteracting the pro-apoptotic actions of other stress-activated MAPKs such as c-Jun NH(2)-terminal kinase (JNK) and p38 (Nakahara et al., 1999; Xia et al., 1995).

Death receptors

TNF receptors. The high concentration of TNFα in the CNS after SCI, together with its ability to induce apoptosis make it a likely instigator of oligodendrocyte apoptosis (Lee et al., 2000a; Yune et al., 2003). TNFα mediates its effects by binding to its cognate receptors TNF receptor 1 and 2 (TNFR1 and 2), of which both belong to the TNFR superfamily. TNFR1 mediates the majority of apoptotic effects as well as cell survival signals, whereas TNFR2 predominantly signals survival (Gupta, 2002).

Binding of trimeric TNFα to TNFR activates the receptor by inducing it to form trimers (Fig. 5). TNFR1 trimerisation induces the release from its death domain of the inhibitory protein, silencer of death domains (SODD), which allows for recruitment of the adapter protein TNFR-associated death domain (TRADD). TRADD then recruits the Fas-associated death domain (FADD) adaptor molecule that in turn causes activation of caspase-8 (see Apoptosis) (Chen and Goeddel, 2002; Gupta, 2002).

TNFα can set off yet another apoptotic pathway following SCI, which can be mediated by both TNFR1 and 2 (Nakahara et al., 1999). The pathway commences with the binding of the adapter protein TNFR-associated factor 2 (TRAF2) to TRADD (Fig. 5). Once TRAF2 binds it recruits and subsequently activates the MAPKKK, apoptosis-stimulated kinase 1 (ASK1). In turn, ASK1, via MAPKs pathways that are similar to the one recruited by Ras (see Trophic support deprivation; Fig. 4, gray inset) activates the pro-apoptotic MAP kinases JNK and p38 (Chen and Goeddel, 2002; Gupta, 2002; Nakahara et al., 1999; Xia et al., 1995). JNK in particular, up-regulates the expression of pro-apoptotic members of the Bcl-2 family and thereby causes cell death (Harris and Johnson, 2001).

Despite the pro-apoptotic potential of TNFα (Lee et al., 2000a; Yune et al., 2003), more compelling evidence by Kim et al. (2001) indicates that TNFRs mediate anti-apoptotic affects following SCI. This group showed that mice with deletions in TNFR1 or 2 presented with a worse functional outcome after SCI than their wild-type counterparts. They correlated this outcome to the ability of both TNFRs to induce anti-apoptotic signals (Kim et al., 2001).

In the case of TNFR1, TRADD has the ability to bind additional adaptor proteins other than FADD, which include the aforementioned TRAF2 and receptor interacting protein (RIP) (Fig. 5). Both TRAF2 and RIP promote cell survival by activating the NFκB pathway. TRAF2 and RIP accomplish this by recruiting a protein kinase complex composed of NFκB essential modulator (NEMO), IκB kinase (IKK) α and IKKβ. Activation of this complex activates the cytoplasmic NFκB heterotrimer composed of p50, p65 and inhibitor κB (IκB). Specifically, the complex phosphorylates IκB and renders it for ubiquitination and thereby

proteosomal degradation. As a result, the remaining NFκB dimer is activated and translocates to the nucleus to transcribe numerous genes of which a subset inhibits apoptosis (Chen and Goeddel, 2002; Gupta, 2002). Such gene products include cIAP1, cIAP2, A1, Bcl-xL, Flice-inhibitory protein (FLIP), TRAF1 and TRAF2. The cIAP1 and 2 block activated caspase-3, -7 and -9; A1 and Bcl-xL prevent the release of cytochrome *c*; FLIP restricts the activation of caspase-8; and finally the TRAF proteins potentiate the activation of the NFκB pathway and thereby survival. Importantly, the cIAP proteins may also be activated by direct interaction with TRAF2 (Gupta, 2002).

TNFR2 initiates most of TNFR1s signalling pathways, albeit that of caspase-8 activation, by directly binding both TRAF1 and TRAF2 to its intracellular domain (Gupta, 2002). Interestingly, in addition to having a reduced capacity to promote apoptosis, it seems that TNFR2 also has a reduced capacity to signal oligodendrocyte survival. This is suggested by the fact that the TNFR2 knockout mice recover better after SCI than their TNFR1 knockout counterparts (Kim et al., 2001).

NGF/pro-NGF/P75^{NTR}. Following SCI, there is acute up-regulation in the expression of both the immature (pro-) and mature forms of NGF as well as oligodendrocyte expression of p75^{NTR}, a member of the TNFR superfamily (Beattie et al., 2002a; Casha et al., 2001). As spinal cord oligodendrocytes are void of the receptor that NGF uses for pro-survival signalling, tropomyosin-related kinase A (TrkA) (Beattie et al., 2002a), NGF signalling in this oligodendrocyte population can only induce apoptosis through its interaction with p75^{NTR} (Casaccia-Bonnefil et al., 1996; Dechant and Barde, 1997; Yoon et al., 1998). Furthermore, the immature form of NGF, which binds p75^{NTR} with an even higher affinity, is a potent inducer of oligodendrocyte apoptosis regardless of TrkA expression (Lee et al., 2001). Consequently, signalling of pro-NGF and NGF via p75^{NTR} is a powerful stimulus for oligodendrocyte apoptosis in SCI. This has been recently confirmed by Beattie et al. (2002a) who showed that p75^{NTR} knockout mice show enhanced recovery after experimental SCI.

The intracellular mechanisms leading to apoptosis after the binding of pro-NGF or NGF to p75^{NTR} have not been fully described (Roux and Barker, 2002). Nevertheless, it is unequivocal that oligodendrocyte apoptosis caused by p75^{NTR} causes activation of the injury-specific JNK3 MAPK and that this is activated by the Rho GTPase Rac (Harrington et al., 2002). Although the link between p75^{NTR} and Rac is unclear, it is possible that Rac may be activated by the protein 14-3-3ε. 14-3-3ε has been shown to associate with the adaptor protein p75NTR-associated cell death executor (NADE) (Kimura et al., 2001), which in turn binds the intracellular domain of p75^{NTR} (Mukai et al., 2000). The basis for this link is supported by the fact that members of the 14-3-3 group can bind Rho GTPases (Kimura et al., 1996). Furthermore, the interactions between p75^{NTR} and NADE, as well as NADE and 14-3-3ε are NGF dependent and stimulate the production of active caspase-3 (Kimura et al., 2001; Mukai et al., 2000).

Remarkably, although p75^{NTR} has a death domain, it does not appear to activate caspase-8 in oligodendrocyte apoptosis. Instead, the caspases that are activated are caspases-1, -2 and -3 (Gu et al., 1999). Activation of these caspases is most likely the result of JNK3-mediated transcriptional activation of pro-apoptotic members of the Bcl-2 family (Gu et al., 1999).

An additional pathway by which p75^{NTR} may activate oligodendrocyte apoptosis is the ceramide pathway. This pathway is
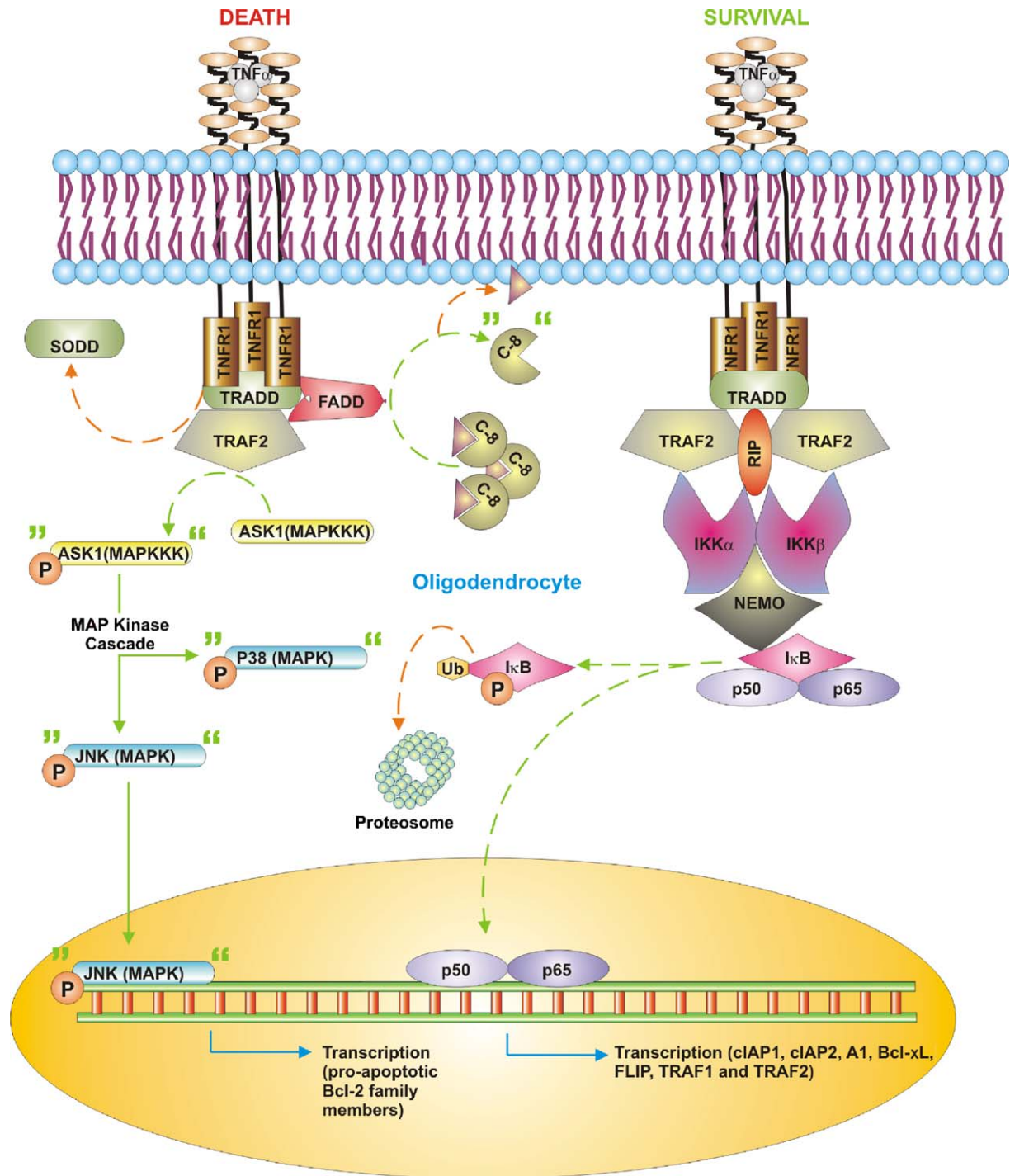
Fig. 5. TNF receptor mediated death and survival pathways reported to occur in oligodendrocytes during injury and disease. Binding of trimeric TNFα to TNFR1 activates the receptor through its trimerisation, releasing the inhibitory protein, silencer of death domains (SODD), allowing for the recruitment of the adapter protein TNFR-associated death domain (TRADD). TRADD then recruits the Fas-associated death domain (FADD) adapter molecule that activates caspase-8. A subsequent pathway of death in oligodendrocytes is through the adapter protein TNFR-associated factor 2 (TRAF2) to TRADD, which via the ASK1 and then the pro-apoptotic JNK and p38 MAPK, up-regulates the expression of pro-apoptotic members of the Bcl-2 family causing cell death.

initiated when ligand binding to p75[NTR] causes the activation of sphingomyelinase. By hydrolysing sphingomyelin, sphingomyeli-nase causes ceramide accumulation which has the ability to activate JNK and thereby apoptosis (Casaccia-Bonnefil et al., 1996).

FASL/FAS. FAS (CD95), which is also a member of the TNFR superfamily, is highly expressed on oligodendrocytes (D'Souza et al., 1996b) and is subject to even higher expression after SCI (Casha et al., 2001). As the majority of apoptotic oligodendrocytes are closely juxtaposed to microglia (Shuman et al., 1997), which

when activated express FAS ligand (FASL) (Vogt et al., 1998), it is likely that microglial stimulation of FAS is an important step in oligodendrocyte death (Casha et al., 2001). Nevertheless, it is also likely that surface changes due to apoptosis, such as movement of phosphatidylserine to the outer membrane, spur microglia to phagocytose dying oligodendrocytes (Savill and Fadok, 2000). In such a scenario, microglial recruitment would be a secondary phenomenon that does not directly contribute to oligodendrocyte apoptosis.

Binding of membrane-bound FASL to FAS causes trimerisation of FAS and recruitment of a complex of proteins to its intracellular domain. This death-inducing signalling complex (DISC) is initiated by binding of FADDs death domain to the death domain of FAS. Once bound, the death-effector domain (DED) of FADD, via another homologous interaction, binds the DED of pro-caspase-8. Recruitment of this pro-caspase leads to its activation and consequently apoptosis (Wajant, 2002).

Targeting apoptosis as a therapeutic strategy

Results from studies that aim to inhibit apoptosis in SCI have been mixed but point towards the notion that inhibiting apoptosis is a therapeutically viable strategy (Beattie et al., 2002a,b; Nottingham et al., 2002; Ozawa et al., 2002). This is confirmed by the study of Nottingham et al. (2002) who showed that inhibition of caspase-3, with the caspase inhibitor FK506, decreased the amount of oligodendrocyte apoptosis after SCI. Nevertheless, the ideal strategy would be to combine apoptosis inhibition with provision of oligodendrocyte trophic support.

Leukaemia inhibitory factor

LIF is a member of the neuregulatory cytokines, a group that also includes CNTF, cardiotrophin 1 and oncostatin M (Fig. 6). It has pleiotropic effects that range from stem cell differentiation and haematopoiesis to bone homeostasis (for review, see Turnley and Bartlett, 2000). Interestingly, recent evidence suggests that LIF also plays a crucial role in oligodendrocyte survival (Butzkueven et al., 2002).

Intracellular signalling

LIF signals by binding and enhancing association of glycoprotein 130 (gp130) with LIF receptor-β (LIFRβ) (Figs. 6 and 7).

Initially, LIF associates with the Ig-like domain of LIFRβ and then with the CBM domain of gp130. This triggers homophilic interactions among the fibronectin-type-III-like domains of both receptors and leads to the gp130/LIFRβ complex. The close association of each of the two subunits within this complex leads to cross-phosphorylation of the Janus kinases (JAKs)—possibly JAK1 and 2—that are constitutively bound to both the gp130 and LIFRβ cytoplasmic tails. This then causes activation by phosphorylation
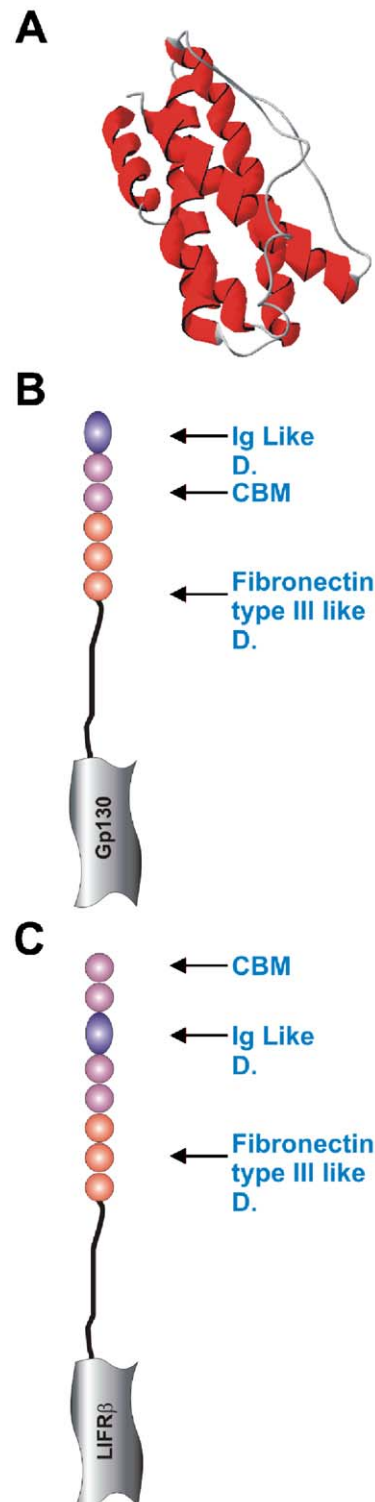

Fig. 6. Structural features of LIF and its receptors. LIF is a 202-amino-acid sequence protein composed of a main chain fold of four long α-helices assorted into a bundle (PDB accession code: 1LKI) (A). The receptor gp130 (918 a.a) belongs to the haematopoietin receptor group of the receptor tyrosine kinase superfamily. It consists of an Ig-like domain at its N-terminus, which is followed by two fibronectin-type-III-like domains that form the four conserved cysteines toward the N-terminus and a WSXWS domain toward the C-terminus, form the cytokine-binding module (CBM) (B). This module is a defining characteristic of all haematopoietin receptors and in the case of gp130 is the sole ligand-binding site. Trailing the CBM are three more fibronectin-type-III-like domains, which give way to a transmembrane segment and a C-terminal cytoplasmic domain that is rich in tyrosine residues. LIFRβ (1097 a.a.) belongs to the haematopoietin receptor group of the receptor tyrosine kinase superfamily. It has two N-terminally placed CBMs that are interspersed by an Ig-like domain; all three domains are involved in ligand association. Trailing thereafter are three fibronectin-type-III-like domains that are also trailed by a transmembrane segment and an intracellular tyrosine rich C-terminus (C). For review, see Heinrich et al. (1998).
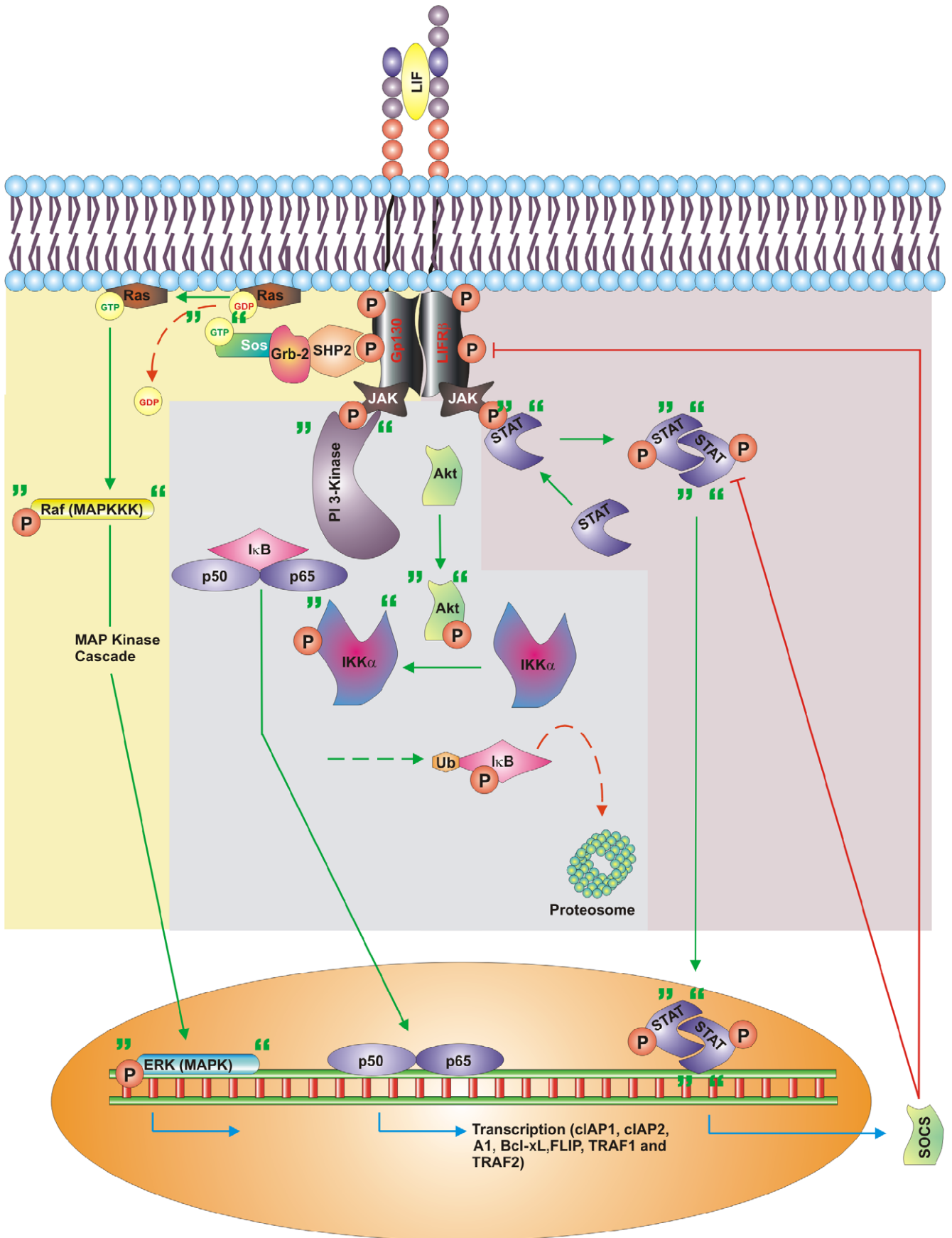
of their tyrosine kinase domains, which leads to phosphorylation of tyrosine residues of the receptor complex. Consequently, this creates docking sites for the SH2 domain of signal transducers and activators of transcription (STAT)—STAT1 and 3—and thereby causes the recruitment of these molecules and their phosphorylation by JAK. Upon activation, STAT is released by JAK, but now its SH2 domain causes it to associate with an SH2 counterpart of another activated STAT. This homophilic dimerisation permits nuclear translocation of STAT where it modulates transcription by binding specific DNA response elements with other regulatory proteins—CREB-binding protein and p300 (for review, see Heinrich et al., 1998; Turnley and Bartlett, 2000). Interestingly, in the context of oligodendrocyte survival, the transcriptional products of the STAT3 pathway include anti-apoptotic members of the Bcl-2 family, which includes Bcl-2 and Bcl-xL (Bowman et al., 2000).

LIF may potentiate oligodendrocyte survival via additional pathways (Fig. 7). A likely pathway is that of the ERK MAP kinase (Segal and Greenberg, 1996). This pathway is activated by association of SHP2, a tyrosine phosphatase, to phosphotyrosine residues on gp130. SHP2 has been shown to associate with Grb2, thus, providing a link between LIF signalling and the ERK MAP kinase pathway (Segal and Greenberg, 1996). LIF may also promote oligodendrocyte survival via the NFκB pathway (Middleton et al., 2000). This pathway may commence when PI 3-kinase associates with the gp130/LIFRβ complex and thereby causes activation of Akt. Activated Akt goes on to activate IKKα by phosphorylation, which in turn phosphorylates IκB to render NFκB active (Middleton et al., 2000).

Signalling via LIF is tightly regulated and subject to feedback inhibition (Fig. 7). The phosphorylation of components in the LIF pathway is only transient. Furthermore, the transcriptional products of JAK/STAT signalling include the protein suppressor-of-cytokine-signalling (SOCS). SOCS inhibits the JAK/STAT pathway by inhibiting tyrosine phosphorylation of gp130, STAT1 and 3. Interestingly, SOCS-1 can also protect fibroblasts from apoptosis induced by TNF-α (Morita et al., 2000). Hence, it is possible that LIF, via SOCS, may increase the durability of oligodendrocytes to the armada of pro-inflammatory cytokines released in SCI. Finally, an extra level of feedback inhibition is accomplished by receptor-mediated endocytosis followed by lysosomal degradation of the LIF-bound gp130/LIFRβ complex (for review, see Heinrich et al., 1998; Turnley and Bartlett, 2000).

Precedent for administering LIF in SCI

There exists a precedent for the pharmacological administration of LIF following SCI, which has been set by work in multiple sclerosis (MS) research by Butzkueven et al. (2002). Based on evidence that oligodendrocyte apoptosis is implicated in certain categories of MS, this group examined the efficacy of LIF in attenuating oligodendrocyte death in the animal model of MS, EAE. They showed that LIF reduced EAE symptoms, reduced oligodendrocyte apoptosis via a JAK/STAT mechanism and that

the therapeutic benefits of LIF were negated when it was administered to mice that were double heterzygous for the LIFRβ-null mutation and a gp130 intracellular truncation. In fact, the LIFRβ and gp130 mutant mice displayed an even worse EAE phenotype than control animals receiving vehicle (Butzkueven et al., 2002).

Corroborative evidence implicating a protective role of LIF in oligodendrocyte survival arises from in vitro data showing that LIF increases the ability of oligodendrocytes to survive in cultured media (Barres et al., 1993; Mayer et al., 1994). It has also been shown that LIF completely abrogates interferon-γ-induced death in rat oligodendrocytes (Vartanian et al., 1995). Interestingly, ciliary neurotrophic factor (CNTF), another neuregulatory cytokine, protects human oligodendrocytes from death by either TNFα or growth factor withdrawal (D'Souza et al., 1996a). These findings, together with the Butzkueven study, imply that LIF signalling is a mechanism that naturally inhibits oligodendrocyte apoptosis and upon enhancement makes these cells more robust to insult. Consequently, it is essential that the role of LIF as a therapeutic measure in experimental SCI be investigated.

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Fig. 7. Transduction mechanisms of LIF signalling. High-affinity interaction with the heterodimeric receptor complex of gp130 and LIFRβ sets off a secondary cascade of survival events within the neural cell. JAK tyrosine kinases bound to the cytoplasmic domains of gp130 and LIFRβ are activated through their phosphorylation which leads to the phosphorylation of tyrosine residues of the receptor complex, providing docking sites for the SH2 domain of signal transducers and activators of transcription (STAT)—STAT 1 and 3—and in turn producing their activation by phosphorylation. This ultimately causes dimerisation of two phospho-STAT molecules and their translocation to the nucleus in which they mediate transcriptional events after binding to specific DNA response elements. This predominantly involves the suppression of cytokine signalling (SOCS) proteins, which negatively regulate this cytokine-induced JAK/STAT pathway. Transcriptional products of STAT3 include anti-apoptotic members of the Bcl-2 family (Bcl-2 and Bcl-xL). Other survival signals are triggered through the phosphorylation of SHP2 binding the adaptor molecule Grb-2, activating the Ras/MAP kinase pathway of survival.

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