

The Quest to Repair the Damaged Spinal Cord

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Abstract: Spinal cord injuries devastate the lives of those affected. Normally, acute injury leads to chronic injury in the spinal cord, although this has a variable impact on normal sensory and motor functions. Currently the only drug used to treat acute spinal cord injury is methyl-prednisolone, administered in order to prevent secondary inflammatory neural damage. Thus, it is time that alternative and complementary pharmacological, cell and gene therapies be developed. In order to achieve this, several approaches to stimulate spinal cord repair must be considered. Indeed, the main lines of research that have been established in different animal models of spinal cord regeneration are now beginning to produce encouraging results. Several patents have been derived from these studies and hopefully, they will lead to the development of new treatments for human spinal cord injuries. Here is presented a review of the main patents that have been generated by this research, and that can be classified as:

- Patents involving the use of different factors that promote axonal regeneration.
- Patents aimed at overcoming the activity of glial scar inhibitory molecules that hinder axonal regeneration. These approaches can be further subdivided into those that block Nogo and other myelin components, and those that involve the use of chondroitinase against glial scar chondroitin sulphate proteoglycans.
- Patents concerning glial cell therapy, in which glial cells are used to mediate axonal repair in the spinal cord (Schwann cells, olfactory ensheathing cells or astrocytes).

Keywords: Spinal cord repair, central nervous system regeneration, axonal regeneration, neurotrophic factors, glial scar molecules, glial cell therapy.

INTRODUCTION

The successful induction of functional repair in the CNS remains a major challenge for both neuroscientists and clinicians. Since Cajal's historic studies, it has long been known that unlike neurons from the peripheral nervous system (PNS), central nervous system (CNS) neurons do not regenerate [1]. Both intrinsic restrictions preventing adult CNS neurons from expressing the genes necessary to re-initiate the "developmental" program after injury, and modifications of the glial scar microenvironment that are non-permissive to axonal growth, account for the failure to regenerate neurons in the adult CNS after lesion. Over the years, considerable effort has been directed towards achieving repair in the injured CNS, and significant progress has been made in understanding some of the molecular mechanisms underlying axonal regeneration [2-4].

Spinal cord injury is a CNS lesion that has particularly severe physical, psychological and social consequences for the patients and relatives affected. Moreover, it has a profound economic impact on health services. Acute injury leads to chronic spinal cord damage, which hinders sensory and motor functions to a variable degree. Currently, the only drug used to treat acute spinal cord injuries is methyl-prednisolone, which is administered in order to prevent secondary inflammatory neural damage [5]. The diverse

strategies adopted to favor spinal cord repair should generate complementary alternatives to methyl-prednisolone, developing different pharmacological, cell and gene therapies. Several lines of research in different animal models are being pursued in order to achieve axonal regeneration in the CNS, more recently with very encouraging results. Neurotrophic factors, the blockage or removal of axonal growth inhibitors from myelin or glial scar, or cell transplantation [2-4,6-17] have been used to induce axonal regeneration and functional recovery after spinal cord injury. These studies have yielded several patents that hopefully will contribute to the successful development of new treatments for human spinal cord injuries. Here is presented a revision of the main patents currently relevant to this research (Table 1). These can principally be classified as:

- A)- Patents involving the use of different factors that promote axonal regeneration.
- B)- Patents aimed at overcoming the activity of glial scar inhibitory molecules that hinder axonal regeneration. These approaches can be further subdivided into those that block Nogo and other myelin components, and those that involve the use of chondroitinase against glial scar chondroitin sulphate proteoglycans.
- C)- Patents concerning glial cell therapy, in which glial cells are used to mediate axonal repair in the spinal cord (Schwann cells, olfactory ensheathing cells or astrocytes).

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THE QUEST TO REPAIR THE DAMAGED SPINAL CORD

A) Treatments with Different Factors that Promote Axonal Regeneration

i) Levels of cAMP- Inhibitors of Phosphodiesterase [Patents Refs. 23,24]

ii) Neurotrophins Delivery [Patents Refs. 40-47,49,50]

Intrathecally/Viral Delivery/Engineered cell therapy

B)- Treatments Aimed at Overcoming Glial Scar Inhibitory Molecules

i) Blocking Myelin Components

NOGO [Patents Refs. 82-88]

MAG [Patent Ref. 89]

OMgp [Patent Ref. 90]

NgR-p75 [Patent Ref. 91]

Neurotrophins & Antibodies Against [Patent Ref. 50]

Myelin Proteins

ii) Digesting Glial Scar Chondroitin Sulfate Proteoglycans

with Chondroitinase [Patents Refs. 115-118]

C)- Glial Cell Therapy: Glial Cells as Mediators of Axonal Regeneration

Schwann Cells [Patents Refs. 152-154]

Olfactory Ensheathing Cells [Patents Refs.155-158]

Astrocytes [Patents Refs.163, 164]

Genetically Modified Cells [Patents Refs.165, 166]

A) - PATENTS INVOLVING THE USE OF DIFFERENT FACTORS THAT PROMOTE AXONAL REGENERATION

Understanding the key control points in the signal transduction pathways involved in repair may facilitate the design of pharmacological treatments to reactivate axonal growth in the injured spinal cord. Some CNS neurons may be intrinsically, but not necessarily irreversibly, incapable of reassuming axonal growth programs after spinal cord injury. Dorsal root ganglia (DRG) sensory neurons have a peripheral and a central branch and the central branch of large diameter sensory neurons also sends axonal projections to the dorsal columns (DC) in the spinal cord. Sensory neurons are intrinsically incapable of regenerating the central branch and DC sensory projections after injury. This incapacity can be experimentally reverted by a conditioning peripheral lesion, i.e. injuring a nerve containing the axonal peripheral branches of these neurons (for example the sciatic nerve). Under these experimental conditions, CNS axons of DRG neurons regenerate [18-20], accompanied by an important increase in the levels of DRG-cAMP. As a result, these neurons are no longer inhibited by myelin associated glycoprotein (MAG), an effect that is initially PKA dependent [20]. Direct intraganglionic injection of membrane permeable cAMP analogs (di-butyryl-cAMP)

enables DRG neurons to grow on an inhibitory substrate (in culture) and to induce regeneration of dorsal column sensory axonal projections after lesion *in vivo* [19,20]. In an elegant study, Bomze *et al.* demonstrated that central sensory projections of DRG neurons in transgenic mice co-expressing the growth cone proteins GAP43 and CAP23 can regenerate without inducing a conditioning lesion [18]. In agreement with this, the regeneration of central sensory projections dependent on peripheral lesion takes place in conjunction with an increase in the expression of the GAP43 protein by DRG neurons. Indeed, this regeneration is dependent on STAT3 activation and on the presence of interleukin-6 [21,22]. Thus, gene transfer of GAP43 and/or CAP23, or other alternative proteins needed for axonal growth that are no longer expressed in the adult CNS, may be necessary for regeneration of some injured neurons.

In relation to the aforementioned findings, the use of cAMP derivatives or alternative methods to increase PKA signaling merits special attention. Rolipram is an inhibitor of the phosphodiesterase IV that principally increases the levels of the second messenger cAMP and hence, the influence of PKA signaling has been analyzed in experimental models of CNS injury. Rolipram, combined either with embryonic spinal tissue or dibutyryl-cAMP/schwann cell transplantation, has been shown to be useful in promoting serotonergic

axonal regeneration in different models of rat spinal cord injury [15,16]. Although Rolipram has anti-inflammatory properties, a direct effect on the neurons that regenerated their axons in the presence of the respective grafts used was thought to explain its effects [15,16]. Nevertheless, an influence in decreasing glial scar tissue [15], or in promoting axonal sparing and myelination [16], cannot be disregarded. Thus, a combination of different effects must be taken into account. The use of potent and selective inhibitors of PDE IV, used in the treatment of inflammatory diseases or other diseases where cytokine levels are elevated, and also in the treatment of CNS disorders, has led to the corresponding patent applications [23,24].

In considering treatments that permit neurons to reactivate axonal growth programs, it is essential to bear in mind the neurotrophic factors. These will almost certainly play a fundamental role in future spinal cord therapies. In experimental models *in vivo*, it has been demonstrated that NGF, Neurotrophin-3 (NT-3), FGF-2 and glia derived neurotrophic factor (GDNF) were able to promote regrowth of different sensory axons through the dorsal root entry zone (DREZ), after dorsal root lesion, when delivered intrathecally or by adenoviral transfer. They also induced reconnection with dorsal horn neurons and functional recovery [25-29]. Intrathecal NT-3 was also able to promote the regeneration of ascendent sensory projections of the central branch of large diameter sensory neurons (responsive to NT-3) after direct lesion to the dorsal columns in the spinal cord [30]. In addition, injected NT-3 was able to induce regenerative sprouting of the transected corticospinal tract in the spinal cord [31]. Thus, intrathecal administration of neurotrophins might be a viable therapy to foster axonal re-entry and reconnection with the spinal targets after dorsal root avulsion. This approach may also facilitate axonal regeneration of responsive tracts after direct injury to the spinal cord. Viral therapy must still be further developed for human applications, but this will very probably be a future option.

This section shall consider the use of cell/gene therapy to take advantage of different cell types engineered to express neurotrophins and foster axonal regeneration in the spinal cord. Glial grafts to facilitate repair and functional recovery of the injured spinal cord will be dealt with below. It has been demonstrated that the infusion of BDNF and NT-3 into Schwann cell (SC) grafts placed in the transected adult rat spinal cord promotes regeneration of brain stem neurons in the grafts [32]. In experimental models of spinal cord injury, fibroblasts, Schwann cells or olfactory ensheathing glia genetically modified to express or to increase the expression of the neurotrophins NGF, BDNF and NT-3, have been used. Neurotrophin gene transfer increases the regeneration of sensory afferents and several spinal tracts, including corticospinal tract, raphespinal and rubrospinal and putative coeruleospinal tracts [33-39], as well as induces or improves functional recovery [33,37,38]. Nevertheless, the use of neurotrophins in human therapy will have to take into account that different axonal tracts are responsive to different molecules [33-39]. Thus, in extensive lesions of spinal cord, a cocktail of different growth factors would probably be most appropriate. Alternatively, the use of synthetic molecules could be contemplated. Indeed, the

use/delivery of neurotrophins for the treatment of spinal cord or CNS injury has been the focus of several patents and patent applications (see 40-44).

Currently, there is a concerted effort in the pharmaceutical industry to develop synthetic neurotrophins with multiple neurotrophic specificities, with the aim of covering the trophic requirements of several axonal tracts. Antibodies that mimic the actions of neurotrophins are also under development (see the relevant patents to 45-47). The potential to use other neurotrophic polypeptides isolated from glial sheath cells of optic nerve [48] in the treatment of damaged nervous tissue (including spinal cord) is also referred to in the corresponding patent application [49]. Finally, other patents focus on the combination of neurotrophins and antibodies against components of myelin ([50], also see below section: blocking myelin components).

B) - PATENTS AIMED AT OVERCOMING THE ACTIVITY OF GLIAL SCAR INHIBITORY MOLECULES

Glial scar and myelin components are highly inhibitory to axonal growth [3,4]. Different pharmacological treatments neutralizing these molecules have been shown to be useful in different models of CNS lesion, and particularly with respect to spinal cord repair (see below). Here shall be revised the patents concerning: i) antibodies against Nogo and other myelin components; and ii) chondroitinase that digests and thereby neutralizes glial scar chondroitin sulphate proteoglycans, strong inhibitors of axonal growth.

i) Blocking Nogo and other Myelin Components

Under normal conditions, myelin contributes to axonal pathfinding during the growth of certain developing axons along myelinated pathways (e.g. the corticospinal tract, see [51] for discussion). In adulthood, myelin constitutes an axonal sheath fundamental for the normal physiological functioning of the nervous system. However, after CNS lesion, myelin waste proceeding from the severed and destroyed axons constitutes an insuperable barrier to axonal regeneration. Several molecular components of myelin have been shown to inhibit CNS axonal regeneration, such as myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp also referred to as MOG by some authors), and Nogo (the Reticulon family that is composed of three members: Nogo A, B and C, reviewed in [52-55]).

Recent disappointing results regarding spinal cord regeneration in a K.O. mice, lacking different Nogo members seemed to indicate that the efficacy of treatments aimed at blocking these components of the myelin to achieve axonal regeneration may be limited [14,56-58]. However, it remains clear that components of myelin are inhibitory to axonal growth. Nogo, MAG and OMgp bind to the Nogo-66 receptor (NgR) and this is coupled to LINGO 1 and the p75-NGFr or TAJ/TROY components, constituting the complete receptor complex [59-66]. This receptor complex mediates the signaling of its ligands, in turn modulating the activity of Rho GTPases [67-69]. However, the p75 receptor seems to be a key component in controlling the transduction of the inhibitory signal. This receptor might fulfill the role of the fulcrum when considering the balance between neurotrophin versus Nogo/MAG inhibitory signaling [70,71]. This may be

fundamental in spinal cord regeneration as seen from studies of a rhizotomy model in mice that lack the extracellular domain of p75 (and that therefore functionally lack this receptor), and that were treated with neurotrophins. In these mice, sprouting of neurons in the sensory and spinal tracts increases when compared to wild-type mice [70]. However, greater complexity is added by the fact that Nogo-A can also signal through its exclusive NiG domain for which no receptor is known, and that is independent of NgR/Lingo-1/p75 [67,72,73].

Myelin components of a lesion are certainly key elements that hinder recovery after spinal cord injury. This is particularly evident if it is considered that intrathecal and intracerebral delivery of monoclonal antibodies to Nogo-A (IN-1 and derivatives) promotes regeneration of the CST and brain stem-spinal axons, and this favors functional recovery after partial transection of rat spinal cord or CST transection [74-76]. In addition, after spinal cord injury, CST regeneration/sprouting can be observed in mice lacking Nogo-A [57]. In fact, some regeneration of raphespinal and rubrospinal tracts and a degree of functional recovery was reported in mice lacking NgR [77]. Furthermore, treatments based on immunization against components of the myelin: myelin basic protein (MBP), peptides MBP-derived or Nogo-A-derived, provide protection against neurodegeneration after spinal cord trauma (contusion model), and thus significantly promote recovery in spinal cord models of injury [78-81].

Several patent applications have been submitted, focused on the results of recent research into myelin components. These involve the use of treatments aimed at blocking or modulating the expression and function of Nogo, MAG, and OMgp, to relieve the inhibition of axonal growth by a central myelin component in CNS diseases or following injury (e.g. spinal cord injury). As a result, numerous patents have been submitted, for example: NOGO [82-88]; MAG [89]; OMgp [90]; reducing NgR-p75 mediated inhibition of axon regeneration [91]; and the combined use of neurotrophins and antibodies directed toward myelin-associated neurite growth inhibitory protein in CNS regeneration [50].

ii) Chondroitinase to Combat Glial Scar Chondroitin Sulphate Proteoglycans

Reactive astrocytes and activated microglial cells are major cellular components of gliotic tissue, one of the most serious obstacles to axonal regeneration in the mammalian CNS [92-94]. The glial scar contains several extracellular matrix molecules, including chondroitin sulphate proteoglycans (CSPGs). Several CSPGs, including neurocan, phosphacan and NG2, are upregulated after CNS injury [94-98] and CSPGs are strong inhibitors of neurite outgrowth and axonal regeneration [92,99-107]. By eliminating CSPG glycosaminoglycan (GAG) chains, CSPG inhibitory activity is attenuated [103,106,108-110]. A recent important study has drawn attention to the possible therapeutic use of chondroitinase to treat spinal cord injury. It was shown that intrathecal delivery of chondroitinase ABC (an enzyme that digests CSPG-GAG chains) after spinal cord lesion in rat (DC crush) facilitated axonal re-growth through the highly inhibitory territory of the lesion site [11]. Chondroitinase treatment promoted regeneration of both the ascending

sensory projections and descending corticospinal tract, and it restored post-synaptic activity below the lesion after electrical stimulation of corticospinal neurons. Very importantly, chondroitinase promoted functional recovery of locomotor and proprioceptive abilities [11]. Chondroitinase treatment alone or in combination with lithium chloride or Schwann cell/OEG grafts has also been reported to produce regeneration of other spinal tracts after injury, including the rubrospinal and raphespinal tracts [111-114]. The treatment of CNS damage by using an agent that reduces the inhibitory properties of CSPGs has been registered in a patent by McMahon, Bradbury and Fawcett [115]. The use of recombinant chondroitinase ABC protein and DNA to promote neurite regeneration (among other uses), as well as the use of other chondroitinase variants in the CNS, is covered by patents such as [116-118].

C) - PATENTS CONCERNING GLIAL CELL THERAPY, IN WHICH GLIAL CELLS ARE USED TO MEDIATE AXONAL REPAIR IN THE SPINAL CORD (SCHWANN CELLS, OLFACTORY ENSHEATHING CELLS OR ASTROCYTES)

Transplantation of different types of glial cells into the spinal cord lesion site has been studied as a mechanism to try to bridge the lesion gap and to overcome the inhibitory environment of the glial scar. It has been shown that this is a valid strategy to achieve axonal repair/regeneration and functional recovery in the spinal cord. To date, mainly Schwann cells and olfactory ensheathing glia have been used to promote axonal regeneration after spinal cord injury and here shall be considered the patents developed from research findings in this field.

CNS neurons have very little capacity to regenerate [1] but in contrast, neurons from the peripheral nervous system (PNS) display a notable capacity to regenerate. This regenerative capacity may be due, among other factors, to the peculiar nature of the Schwann cells that ensheath the PNS axons. From the pioneering studies of Aguayo, it is clear that some CNS neurons can extend their axons through peripheral nerve grafts that are permissive to axonal growth [119,120]. This finding has stimulated the use of peripheral nerve and SC grafts to foster regeneration in the CNS, so far with promising results (reviewed in [8]). Some groups have favored this approach to achieve axonal regeneration in the injured spinal cord. SC grafts promote axonal regrowth inside the graft [121-123] (for a review see [8]), although the efficiency of re-entry of the regenerating axons from the graft into the spinal cord remains a problem [122]. The fact that CNS regeneration achieved with SC is incomplete has made it necessary to search for more effective mediators of regeneration.

Over the last few years, the special properties of OEG have drawn much attention with respect to their use in CNS regeneration [6,10,12]. Olfactory neuronal axons are ensheathed by a special type of glial cells, OEG, that are generally thought to derive from the olfactory placode during development [124]. In the mammalian olfactory system, olfactory sensory neurons are continuously replenished from progenitor cells present in the olfactory neuroepithelium throughout adult life. These newborn neurons must extend axons to the glomerular layer of the olfactory bulb and

establish appropriate connections with their targets [125-128]. Since OEG usually surround olfactory axons growing and entering into the adult mammalian CNS, it was reasonable to hypothesize that they might facilitate axonal regeneration in the injured CNS [129,130]. In fact, several studies have confirmed their regenerative capacity in the injured spinal cord [131-134], and also when used in combination with SC grafts and chondroitinase [114,133]. However, in some models of spinal cord injury, SC grafts promote axonal regeneration and improve functional recovery of certain axonal tracts almost as efficiently [135] or even more efficiently than OEG [136].

The reparative capacity of OEG is probably due to a combination of several factors (see for review [12]). They are able to preserve the function and morphology of the spinal cord after injury by dampening the inflammatory response and/or maintaining blood vessels [135,137]. These cells are capable of mixing with reactive astrocytes [138] and of modifying the glial scar environment [135,139]. Finally, the molecular composition of their cell membrane is thought to have a strong influence on these effects, as well as the capacity to secrete neurotrophic factors [140]. However, the exact properties and mechanisms underlying the influence of the OEG on regeneration still remain to be fully elucidated [6,10,12,141].

The ability to study OEGs in culture facilitates the characterization of the molecular basis of their regenerative activity. Many *in vivo* studies will be necessary to define the optimal therapeutic use (if any) of OEGs for spinal cord injuries. Indeed, continuous preparation of primary OEG cultures is labor-intensive, and is subject to batch-dependent variability as well as being limited in terms of cell numbers. It has been proposed that the heterogeneity of mixed populations, such as occurs in primary cultures of olfactory cells, is important for transplants to effectively promote CNS repair [10,141]. However, it is desirable to have access to pure homogeneous OEG populations that conserve the regenerative properties of primary OEG and that can be more completely characterized. Immortalized OEG clonal lines constitute a feasible alternative to overcome the limiting problems inherent to OEG primary cultures, both for basic and applied research, and for future human therapeutic use. Several such lines have been established and characterized [142-146] providing an unlimited supply of homogeneous OEG cells [142,143,145,146]. Our group has described several immortalized clonal cell lines (TEG1-20), some of which appear to perform, as well as primary OEGs in promoting neuritogenesis and axonal regeneration in culture. The most potent of these is TEG3 [145,146], which has been demonstrated to be derived from a single-cell. This line exhibits morphological plasticity both in culture and *in vivo*, and shows both long term survival and integration into the spinal cord (without forming tumours). Moreover, TEG3 cells mediate axon repair and recovery of motor and sensory function in animals with severe spinal cord injury [147]. This finding indicates that a homogeneous clonal cell line is able to produce repair and functional recovery in a model of spinal cord injury without needing any other accompanying cell [147].

These properties [142,143,145-148] have important implications for human therapy. One practical consideration is that in order to be able to offer OEG transplants to humans after spinal cord injury with a view to achieving functional repair, an accessible and unlimited source of cells must be available. Until now, autologous transplantation with cultured OEG proceeding from the patient was the only available possibility. Primary OEG from the patient can be obtained either from the olfactory bulb, which entails obvious difficulties and risks, or from the olfactory mucosa lamina propria. Given the size of a human spinal cord, to obtain enough OEG cells for transplantation exclusively from the olfactory mucosa lamina propria may be problematic [149]. Human embryos have been shown to be a viable source of OEGs [150] but obtaining them, even if it were possible, may in some cases be restrictive. Libraries of non-tumoral human OEG clonal lines, with unlimited capacity to divide in culture and that maintain the regenerative properties of primary OEG would be a convenient alternative (see [12], for discussion). Immortalized clonal cell lines tested in different animal models do not generate tumors *in vivo* [142,143,147,148,151]. Even so, it will be necessary to perform further biosafety analyses before considering human transplantation, especially in reference to tumor development. Indeed, reversible immortalization systems to obtain human OEG clonal lines for clinical purposes may be a promising approach in this respect. Thus, considering that the limiting number of primary OEG cells would be a key issue for human self-transplantation, these novel findings open the possibility of using reversibly immortalized human OEG clonal lines, alone or in combination with other therapeutic approaches, for clinical purposes [11,15,16].

The research for spinal cord regeneration using:

- i) Schwann cells alone and in combination with other treatments, has produced different patents [152-154].
- ii) OEGs, has produced the patent applications [155-158]:

A small mention must be given to the use of astrocytes in spinal cord regeneration. Immature astrocytes have been reported to reduce scar formation and to favor axonal regeneration in the CNS [159-162]. The use of astrocytes for transplantation to treat CNS injury/diseases is also covered by patents:

- i) "Activated" immature astrocytes and methods of utilizing the activated immature astrocytes to reduce secondary necrosis and scar formation in central nervous system tissue as well as to promote axon and/or blood vessel growth or regeneration [163].
- ii) Inflammation-associated cytokines treated astrocytes to treat CNS injury [164].

Finally, the use of genetically modified cells to treat defects and diseases caused by damage to the CNS have generated patents such as [165,166].

CURRENT & FUTURE DEVELOPMENTS

In summary, human spinal cord repair after injury must be considered from an integrated point of view. Different tracts are affected to a greater or lesser degree, depending on

the extension of the lesion. Several approaches and combinations of therapies must be considered to achieve maximal functional recovery after acute injury, but also in chronic dysfunction of the human spinal cord:

-Treatments with trophic factors to foster axonal regeneration together with treatments to overcome glial scar inhibitory molecules.

-Cell therapy must be fully developed, particularly using glial cells as mediators of axonal repair in the spinal cord and engineered glial cells to foster their regenerative potential.

All these possibilities are open to be developed and tested in currently available animal models of spinal cord injury. However, in the near future, it will be necessary to develop strategies to evaluate the combinatorial possibilities of all these therapies to achieve maximal efficiency in repairing the spinal cord and the corresponding translation to treatments of human central nervous system injuries. The time has come to define the reparative and safety parameters for human therapy. This requires a combined and unreserved effort of all researchers and clinicians, without disregard to any potential therapeutic possibility.

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