Engineering an artificial nerve graft for the repair of severe nerve injuries

X. Navarro F. J. Rodríguez D. Ceballos E. Verdú

Group of Neuroplasticity & Regeneration, Institute of Neurosciences and Department of Cell Biology, Physiology & Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain

Abstract—*Nerve repair with tubes has a limit to regeneration depending upon the length of the gap. The characteristics of the guide, in terms of permeability, durability and adhesiveness, also influence regeneration. Considering the importance of the cellular component in regeneration, the development of artificial grafts, composed of a biocompatible nerve guide filled with a neurotropic matrix and seeded with Schwann cells (SCs), is an interesting option to enhance nerve regeneration and provide an alternative to the classical autologous nerve graft. We evaluated the ability of SCs transplanted into a nerve guide to improve regeneration after sciatic nerve resection, leaving a 6-mm gap, in the mouse. Syngeneic, isogeneic and autologous SCs were suspended in Matrigel and seeded in resorbable guides, and compared to acellular guides and to nerve autografts. The immunogenicity of the transplanted SCs clearly influenced the outcome. Transplants of autologous SCs resulted in only slightly lower levels of reinnervation than autografts, but higher recovery and number of regenerated axons than transplants of isologous and syngeneic SCs, and than acellular guides. Thus, by combined developments on nerve guides, extracellular matrix components and cell transplantation, an artificial graft has been designed that allows axonal regeneration across long gaps to levels comparable with an autograft.*

Keywords—*Axonal regeneration, Cell transplants, Graft, Peripheral nerve, Schwann cell, Tube repair*

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1 Introduction

INJURIES TO peripheral nerves result in loss of the neural functions conveyed by the involved nerves, manifested by partial or total impairment of motor, sensory and autonomic functions in the denervated segments of the body, owing to the interruption of axon continuity, degeneration of nerve fibres distal to the lesion and eventual death of axotomised neurons. The structural and functional deficits can be compensated by reinnervation of denervated targets by means of two basic mechanisms: regeneration of injured axons and collateral branching of undamaged axons in the vicinity. However, clinical and experimental evidence usually shows that these mechanisms do not allow for a satisfactory functional recovery, especially after severe injuries (SUNDERLAND, 1991; KLINE, 2000; LUNDBORG, 2000).

After injuries that cause rupture of peripheral nerve fibres, axons and myelin sheaths distal to the lesion site are degraded by Wallerian degeneration. The degenerative end products are eliminated by the co-operative action of Schwann cells (SCs) and infiltrating macrophages. Wallerian degeneration serves to create a micro-environment distal to the injury that is favourable for the axonal regrowth of surviving neurons, and retrograde reaction and chromatolysis represent metabolic changes necessary for regeneration and axonal elongation (for reviews see FAWCETT and KEYNES, (1990) and FU and GORDON (1997)). SCs of the distal nerve segment, stimulated by the axonal–glial disjunction and by cytokines secreted by macrophages, proliferate during the first days after injury and form Büngner bands within the endoneurial tubes. Growth cones emerge from the severed axons that elongate if they find a favourable terrain within the distal endoneurial tubes, in association with SC membrane and basal lamina, to reach synaptic loci at peripheral tissues. However, the regenerative process cannot usually reconstitute a normal nerve structure nor allow for a normal distal reconnection, particularly when the lesion was severe and nerve continuity was initially lost. After nerve injury and repair, the diameter of regenerated axons, their conduction velocity and excitability remain below normal levels for a long time (FIELDS and ELLISMAN, 1986; GÓMEZ *et al.*, 1996), and, consequently, functional recovery of reinnervated organs is incomplete and often inadequate (NAVARRO *et al.*, 1994; GRAMSBERGEN *et al.*, 2000). The limitation to nerve regeneration is more marked when the lesion causes a loss of continuity in the nerve and the outcome is dependent upon the interstump gap length (KIM *et al.*, 1991; BUTÍ *et al.*, 1996).

The factors that stimulate and control axonal regeneration originate from multiple sources, but the most important influences derive from the local environment of the lesion. Axonal elongation requires an adequate substrate of trophic and tropic factors, provided by reactive SCs, macrophages and the extracellular matrix within the degenerated nerve. SCs play a key role in nerve regeneration, because they constitute a favourable

Correspondence should be addressed to Dr X. Navarro; email: xavier.navarro@uab.es

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substrate over which axons regrow and are the main source of a variety of trophic factors (BUNGE, 1993; VERDÚ and NAVARRO, 1998). The proliferating capacity of SCs is lost in the mature peripheral nerve; however, after nerve injury, SCs re-express capabilities for proliferation and secretion of different factors that support axonal regeneration (MIRSKY and JESSEN, 1996).

2 Repair of peripheral nerve injuries

After peripheral nerve injuries, the capability of severed axons to regenerate and recover functional connections is dependent on the site and type of lesion and the distance over which the axons must regrow to span the injury. After nerve crush, regeneration is usually successful, because the continuity of the endoneurial tubes is preserved (SUNDERLAND, 1991; NAVARRO *et al.*, 1994). This is in contrast to the limited growth across a gap imposed after complete severance or resection of a nerve, when nerve continuity is completely disrupted.

The use of biochemical or metabolic factors to support and enhance regeneration still remains speculative, and thus the management of most nerve injuries is limited to surgical repair (KLINE, 2000). The objective is to provide axons growing from the proximal stump, with an appropriate substrate supporting regeneration, such as the distal degenerating nerve. If left unrepaired, the regenerative sprouting of fibres in the proximal stump forms a neuroma, because growing axons do not find a favourable terrain to elongate through the extraneural ambiance to reach the distal stump (Fig. 1). When the lesion is a clean cut, mobilisation of the nerve followed by epineurial suturing of proximal and distal stumps, attempting correct matching of individual fascicles of the nerve trunk, is the usual repair (LUNDBORG, 2000).

When the length of the gap created by tissue destruction and nerve retraction is too long to allow apposition and suture without tension, nerve grafts are usually employed (MILLESI, 1981; KLINE, 2000). The purpose of introducing a graft between the stumps of a transected nerve is to offer mechanical guidance, as well as a stimulating environment for the advancing axons. The SCs of the graft and their basal lamina play an essential role in promoting neurite growth (HALL, 1986). It is generally agreed that gaps in injured nerves are most successfully bridged by the use of autologous nerve grafts, which behave in the same way as does the distal segment of the severed nerve (POVER and LISNEY, 1989; KIM *et al.*, 1991; SUNDERLAND, 1991; GÓMEZ *et al.*, 1996). However, autograft repair involves some problems, such as the need for a second surgical step, elimination of the donor nerve function, a limited supply of donor nerves and the mismatch between nerve and graft dimensions.

The alternative use of allografts has not been encouraging, because the specific tissue immunity rejection, mainly directed against the SCs and myelin sheaths of the graft, precludes axonal regeneration (SUNDERLAND, 1991; ANSSELIN *et al.*, 1992; EVANS *et al.*, 1994). Immunosuppressive therapy is needed for reducing graft rejection, but its secondary complications can overcome the benefits (EVANS *et al.*, 1994; MIDHA *et al.*, 1994). With the evolution of new principles for immunosuppression, however, allografts are coming into clinical application for selected cases in which the nerve gap exceeds the length that can be reconstructed with available autograft material (MACKINNON *et al.*, 2001).

The development of an artificial nerve graft, composed of a conduit filled with exogenous elements that promote axonal regeneration, would help to solve the secondary problems of

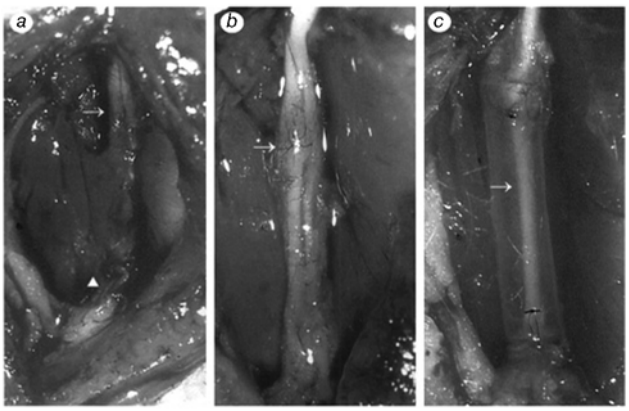

Fig. 1 *Micrographs taken 4 months after resection of 6 mm gap in mouse sciatic nerve. Regeneration has failed if nerve is left unrepaired. (a) Neuroma was formed at proximal stump (arrow), disconnected from distal stump (arrowhead), in contrast with (b) effective axonal regeneration, when both stumps are reconnected by autologous nerve graft. Note enlarged and highly vascularised tissue around suture lines due to scar reaction (arrow). (c) Nerve regeneration and re-establishment of continuity can also be achieved by bridging nerve stumps with silicone tube, in this example prefilled with collagen gel. Newly regenerated nerve is located at centre of tube*

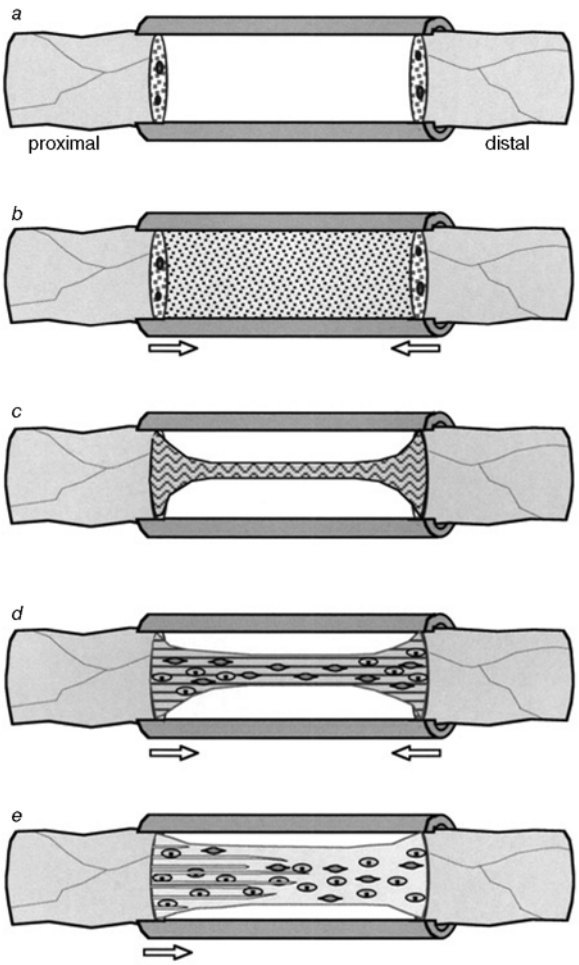

Fig. 2 *Schematic representation of phases of nerve regeneration within synthetic guide used to bridge created nerve gap. (a) Nerve stumps are introduced into ends of guide and sutured at time of repair. (b) During first days, guide is filled by fluid mainly derived from extravasation of plasma from nerve stumps. (c) Fibrin cable is formed within tube, bridging both nerve stumps. (d) Non-neuronal cells (mainly fibroblasts and Schwann cells) migrate from both stumps, enriching connective cable with extracellular matrix and neurotropic factors. (e) Axons grow from proximal stump in association with migrating Schwann cells*

autograft and allograft repairs. Tubulisation, the implantation of a tube to bridge a nerve gap, emerged about 20 years ago as a possible alternative to the repair of transected nerves (LUNDBORG *et al.*, 1982). This experimental paradigm has provided a useful model for studying basic cellular and biochemical events during peripheral nerve regeneration, and it has also been successfully applied for repairing injured nerves in primates (MACKINNON and DELLON, 1990*a*; ARCHIBALD *et al.*, 1995) and human patients (MACKINNON and DELLON, 1990*b*; LUNDBORG *et al.*, 1997).

Nerve guides offer a closed space, where neurotrophic factors normally synthesised by the injured nerve accumulate and facilitate axonal growth (LONGO *et al.*, 1984; DANIELSEN and VARON, 1995). Accordingly, tubulisation allows successful regeneration over longer gaps than in unrepaired nerves. However, a limit to regeneration also exists within nerve guides, depending upon the length of the gap.

Nerve regeneration through an empty synthetic tube requires the reconstitution of a new neural structure. During the first week after tube implantation, a fibrin loose matrix is formed within the tube, bridging both nerve stumps. Non-neuronal cells (fibroblasts, endothelial cells, SCs) migrate from both stumps along the connective cable, providing neovascularisation, connective strands and basal lamina and secreting a variety of neurotrophic and extracellular matrix compounds that allow the growth of axonal tips from the severed nerve proximal stump (Fig. 2) (WILLIAMS *et al.*, 1983; FIELDS *et al.*, 1989). The regenerated nerve is usually located at the centre of the tube and comprises a thick fibroblastic outer layer surrounding a central endoneurial cable composed of numerous small nerve fascicles and blood vessels (Fig. 3). The newly formed nerve fascicles contain unmyelinated and myelinated axons, the latter being smaller in diameter and with a thinner myelin sheath than those seen in the intact nerve. Nevertheless, the success of intratubular regeneration depends upon the ability of the injured nerve to provide sufficient humoral and cellular elements to constitute the initial regenerative cable.

The main limitation of tubulisation is the gap length to which it may be applied. The limiting gap depends on the nerve mass and the number of non-neuronal cells present. Several experimental reports have shown that regenerating axons are able to bridge empty tubes made of silicone or other synthetic materials in a gap of up to 4 mm in mice (BUTÍ *et al.*, 1996; GÓMEZ *et al.*, 1996), up to 10 mm in rats (LUNDBORG *et al.*, 1982; WILLIAMS *et al.*, 1983) and less than 30 mm in large primates (MACKINNON and DELLON, 1990*a*; ARCHIBALD *et al.*, 1995), but fail in most cases over longer gaps. The physico-chemical characteristics of the tube, mainly in terms of permeability, durability and cell adhesiveness, influence the chances of regeneration over long gaps, and further advances in biomaterials will probably improve the effectiveness of tubulisation.

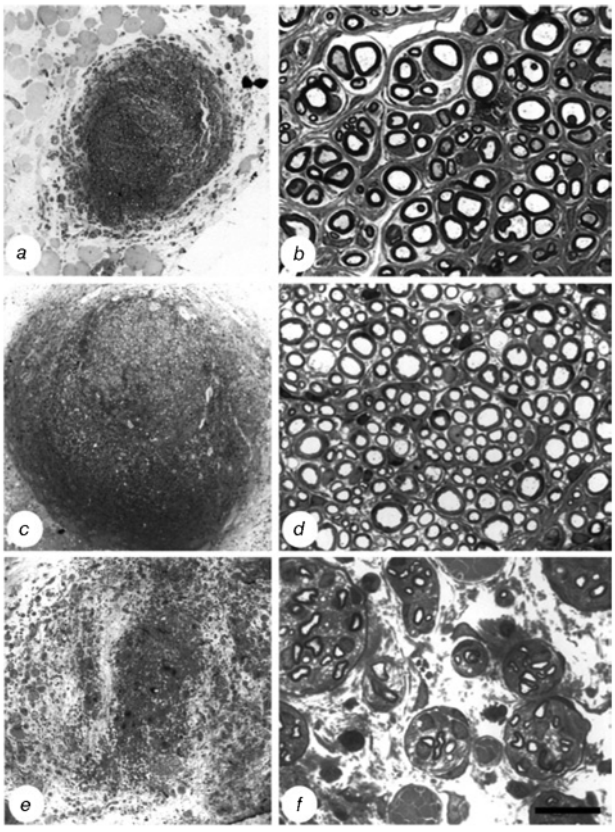

Fig. 3 *Light microscopy of transverse sections of regenerated nerves 4 months after 6 mm resection of mouse sciatic nerve and repair with: (a), (b) autologous graft obtained from peroneal nerve (c), (d); artificial graft composed of PLC guide seeded with autologous Schwann cells (e), (f); silicone tube filled with saline solution. (a), (c) and (e) show low magnification views of regenerated nerve. Note thick epi-perineurial layer surrounding endoneurial circular cable in tube repair in (c), (e), compared with nerve graft in (a), (b), (d) and (f) show higher magnification fields of endoneurial compartment. Density and size of myelinated fibres is comparable between autograft and artificial graft, whereas, in silicone tube, there are significantly fewer axons within dispersed endoneurial fascicles. (b), (d) and (f) Bar = 16 μm*

3 Design of an artificial nerve graft

Over the past decade, we have performed a series of studies to design an artificial nerve graft that enhances nerve regeneration and may become an alternative to the classical autograft repair. Such a graft should mimic the main components of a natural graft: a biocompatible nerve guide that encloses the regenerating ambiance and an inner extracellular matrix that provides physical support and neuritotropic cues, and should be seeded with competent SCs that replace the host cells and secrete a variety of neurotrophic factors.

The experimental model used was the mouse sciatic nerve, which was submitted to a resection leaving a gap of 6 mm, a limiting distance for regeneration in silicone tubes (BUTÍ *et al.*, 1996; GÓMEZ *et al.*, 1996), it was repaired by the nerve stumps being sutured either to a nerve autograft, as a control for comparison, or to a synthetic guide. The nerve guide was of different materials and filled with saline, with different gels, or with gels seeded with Schwann cells, as detailed in the following sections. After the operation and over a 4 month follow-up, we evaluated, by means of a battery of neurophysiological techniques, the degree of axonal regeneration and reinnervation of distal target organs (NAVARRO *et al.*, 1994).

Reinnervation of plantar muscles by motor nerve fibres and of digital sensory nerves by large sensory fibres was monitored by electrophysiological recordings of compound muscle and nerve action potentials, evoked by electrical stimulation of the sciatic nerve proximal to the graft. Nociceptive reinnervation was estimated from withdrawal responses induced by pinpricking discrete areas of the distal paw. Sympathetic sudomotor function was tested by means of the silicone mould technique.

Values obtained after the operation for each test were expressed as a percentage of the pre-operative values for each mouse. The percentage of reinnervation achieved during follow-up was calculated as the average of the maximum percentage of recovery found for the four functions tested. At the end of follow-up, the regenerated nerve was fixed and embedded in epoxy resin, so that semithin sections could be obtained. Morphometrical assessment of the number and size of

myelinated axons was performed in the regenerated nerve, at midgraft or midtube, and at the distal nerve, under light microscopy. The percentage of successful regeneration in each group was derived from the cases with histological evidence of axons regenerated at the distal nerve stump.

3.1 *Nerve guide*

The type of nerve guide plays a key role in the construction of cellular prostheses. Several physical parameters of the guide used for nerve repair, such as inner diameter, microgeometry of inner surface, thickness, permeability and chemical composition, have all been shown to influence the degree of nerve regeneration (JENQ and COGGESHALL, 1987; AEBISCHER *et al.*, 1990; BUTÍ *et al.*, 1996; NAVARRO *et al.*, 1996). The suitable characteristics of a nerve guide, from a review of previous reports, are:

(*a*) interstump gap: $\leq$4 mm in mice, $\leq$10 mm in rats, $\leq$30 mm in primates
(*b*) tube lumen area: 2.5 times the cross-sectional area of the nerve to be repaired
(*c*) wall: flexible, thin, non-collapsible, translucent
(*d*) inner surface: smooth, homogeneous
(*e*) permeability: impermeable or highly permeable
(*f*) material: biocompatible, resorbable, available.

For attempts to seed a nerve guide with glial cells, silicone and plastic tubes are not the most suitable, as they impede cell adhesion and interchange of molecules with the extraneural milieu. Therefore we compared the outcome of several conduits with variable characteristics in terms of resorption and permeability. In different groups of mice, a 6 mm gap of the sciatic nerve was repaired with guides that were either durable and impermeable (silicone and teflon), durable and permeable (polysulphone), resorbable and impermeable (poly-lactate-caprolactone (PLC)) or resorbable and permeable (collagen and PLC).

The proportion of animals in which we found a regenerated nerve through the tube was significantly higher with resorbable guides made of PLC and of collagen (50–66%) than with durable tubes of silicone, teflon or polysulphone (15–25%). The guide that yielded the best functional and morphological results of regeneration was a highly permeable PLC (PLC-hp) tube (Fig. 4).

A better outcome is usually obtained with biodegradable guides than the obtained with durable guides, increasing the gap length that can be regenerated (MADISON *et al.*, 1987; DEN DUNNEN *et al.*, 1993; NAVARRO *et al.*, 1996; RODRÍGUEZ *et al*, 1999*b*). Biodegradable materials offer the advantage of disappearing from the body once regeneration is complete. However, a bioresorbable nerve guide should degrade at a slow rate, in accordance with the rate of axonal growth and maturation, maintaining mechanical continuity and lumen stability for a longer time than to required for the axons to cross the gap. The improvement found with the highly permeable PLC guides is probably due to the inflow of trophic molecules and nutrients from the extratubular ambiance and the infiltration of reparative cells, macrophages and fibroblasts, which contribute to the formation of a richer regenerative cable. PLC guides have also been shown to allow a higher level of reinnervation and to reduce aberrant innervation with respect to silicone tubes in rats (VALERO-CABRÉ *et al.*, 2001). These beneficial effects are attributable to the fact that PLC guides induce a faster regenerative response and earlier maturation of regenerated axons.

3.2 *Matrix components prefilling the nerve guide*

Effective regeneration in nerve guides is dependent on the formation of an initial connective cable between nerve stumps, across the gap. The possibility of securing this initial step by

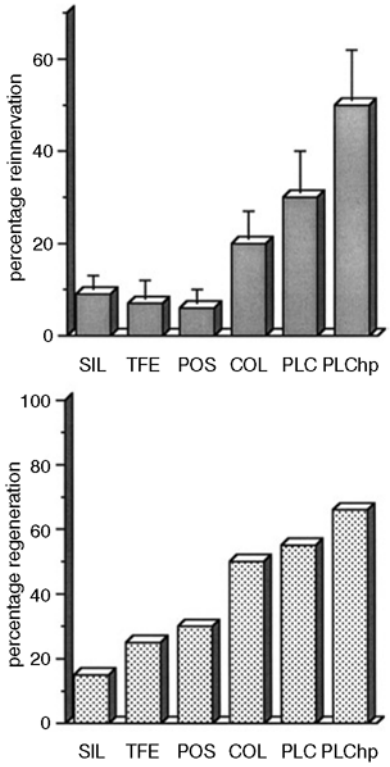

Fig. 4 *Histograms of percentage of target reinnervation (average of degree of reinnervation achieved by large motor and sensory and thin nociceptive and sudomotor fibres with respect to pre-operative control values), and percentage of cases with successful regeneration (demonstrated by presence of regenerated nerve fibres at distal nerve stump) achieved 4 months post-lesion. In different groups of mice, 6 mm gap in sciatic nerve was repaired with guides of: silicone (SIL), teflon (TFE), polysulphone (POS), collagen (COL), impermeable poly-lactide-caprolactone (PLC) and highly permeable PLC (PLC-hp)*

filling the guide with an exogenous matrix at the time of implantation has been investigated. Prefilling the lumen with components of the naturally formed intratubular matrix, such as fibrin, collagen and laminin-containing gels, has been reported to enhance peripheral nerve regeneration with respect to empty guides (WILLIAMS *et al.*, 1987; MADISON *et al.*, 1987; ROSEN *et al.*, 1990; CHAMBERLAIN *et al.*, 1998). However, gel substrates, even those containing neuritotropic factors, can impair regeneration by physically impeding the migration of non-neuronal cells and regenerating axons if they are too dense or provide a network of pores that are too narrow (VALENTINI *et al.*, 1987; LABRADOR *et al.*, 1995).

After defining the most suitable concentration for each one of several constituents of the extracellular matrix, we compared the effects of diluted gels composed of collagen I (1.28 mg ml^{-1}), laminin (4 mg ml^{-1}), hyaluronate (5 mg ml^{-1}) or fibrin (from mouse plasma), prefilling silicone tubes implanted in mice. Regeneration was improved with laminin and collagen gels, resulting in significantly higher levels of reinnervation and proportions of regenerates than those obtained with saline, fibrin and hyaluronate (Fig. 5) (LABRADOR *et al.*, 1998). However, the improvement was limited, and the results were still worse than those found with an autograft; regeneration was successful in less than 50% of mice, and the number of regenerated axons was about 25% of normal counts.

In a subsequent assay, we showed that by aligning the fibrils of a collagen gel under a magnetic field, regeneration was improved in the short term (CEBALLOS *et al.*, 1999). More recently, in a long-term *in vivo* study, we also found that reinnervation reached higher levels (Fig. 5), and the density of

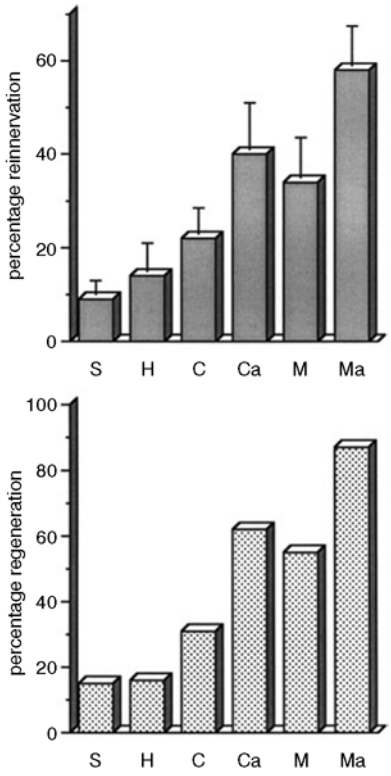

Fig. 5 *Histograms of percentage of target reinnervation and percentage of successful regeneration achieved 4 months post-lesion. In different groups of mice, 6 mm gap in sciatic nerve was repaired with silicone tube prefilled with: saline solution (S), hyaluronate gel (H), type I collagen gel (C), magnetically aligned collagen gel (Ca), laminin-containing gel (Matrigel, M) or magnetically aligned Matrigel (Ma)*

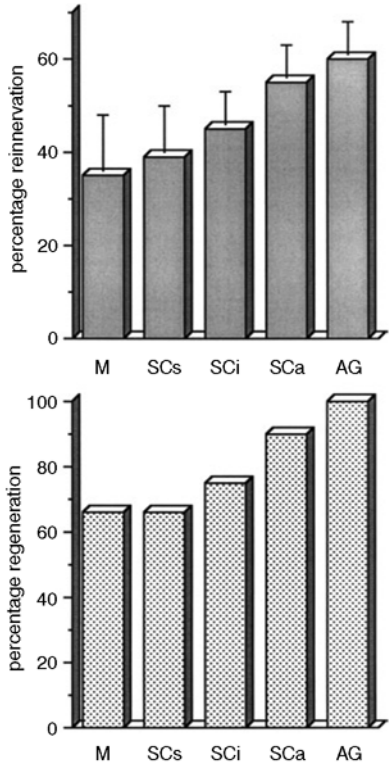

Fig. 6 *Histograms of percentage of reinnervation and percentage of successful regeneration achieved 3.5 months after 6 mm gap resection of mouse sciatic nerve and repair with poly-lactide-caprolactone guide prefilled with Matrigel (M), with Matrigel seeded with Schwann cells from syngeneic (SCs), isogeneic (SCi) or autologous (SCa) donor nerves, in comparison with repair by autologous nerve graft (AG)*

regenerated fibres was higher, with magnetically aligned collagen and laminin gels than with control gels prefilling silicone tubes (VERDÚ *et al.*, 2002). Thus, the composition, density and structural organisation of the exogenous intratubular matrix all influence the fate of axonal regeneration over tubulised gaps. Further *in vitro* and *in vivo* experiments are still needed to investigate the combination of an aligned matrix with embedded SCs.

3.3 *Schwann cell transplants in nerve guides*

Once we had selected an optimum guide for long-gap repair, we approached the construction of a cellular prosthesis comprising a highly permeable PLC guide filled with a laminin-containing gel seeded with SCs. The SCs had previously been isolated and expanded in primary cultures from adult peripheral nerves, using a defined medium (VERDÚ *et al.*, 2000). Previous degeneration of the nerve is required to achieve SC proliferation *in vitro* (MORRISEY *et al.*, 1991; CASELLA *et al.*, 1996). Within a degenerating nerve, SCs deprived of axonal contact proliferate and upregulate the synthesis and release of a variety of neurotrophic factors and basal lamina components (BUNGE, 1993; VERDÚ and NAVARRO, 1998). Furthermore, preactivation of the SCs to be transplanted largely increases their regeneration-promoting effect (GULATI, 1996; RODRÍGUEZ *et al.*, 1999*a*).

A total of 150 000 cells, a quantity that could be expanded *in vitro* from only one mouse sciatic nerve, were seeded in the guide suspended in Matrigel at $4\,mg\,ml^{-1}$. To study the influences of the origin and reactivity of the transplanted SCs, we implanted grafts with SCs from syngeneic, isogeneic or autologous donors of an outbred strain of mice. The best outcome, in terms of functional recovery, morphological regeneration and proportion of regeneration success (Figs 3 and 6), was obtained with autologous SCs, followed by isogeneic and finally syngeneic cells (RODRÍGUEZ *et al.*, 2000). Prelabelled transplanted SCs were found to survive in the guide 1–3 months after implantation, in larger numbers if they were autologous than if they were heterologous. These data indicate that transplanted SCs enhance axonal regeneration, but also that immune compatibility between donor and host is an important factor that affects their capability to survive and promote regeneration.

Previous studies (GUÉNARD *et al.*, 1992; KIM *et al.*, 1994; LEVI *et al.*, 1997; ANSSELIN *et al.*, 1997) showed that syngeneic SCs transplanted into nerve guides enhanced axonal regeneration with respect to control guides with saline solution or extracellular matrix components. Although syngeneic SCs have been shown to survive after implantation in unknown proportion, a number of the transplanted cells die because of host rejection (KIM *et al.*, 1994; LEVI *et al.*, 1997). A concentration-dependent effect was reported for syngeneic SCs transplanted *in vivo* (GUÉNARD *et al.*, 1992). Thus a large number of syngeneic SCs should be transplanted initially to ensure that a sufficient number to promote regeneration remain viable after implantation (ANSSELIN *et al.*, 1997). On the other hand, a lower number of autologous cells, as used in our study, may provide better results. Alternatively, results with allogeneic cells may be improved by antigen matching between donor and host or by immunosuppression. Of special interest in this context is that FK506, a drug with strong immunosuppressive action, has been shown to enhance axonal outgrowth in experimental studies after crush or graft repair of peripheral nerves (GOLD *et al.*, 1994; NAVARRO *et al.*, 2001).

Another approach towards treating injuries to the nervous system using glial cell transplants involves the use of cells that

are genetically engineered to secrete certain growth factors. For peripheral nerve repair, the most suitable vehicle is the SC (SORENSEN *et al.*, 1998). SCs regulate the expression and secretion of a variety of neurotrophic factors during Wallerian degeneration (VERDÚ and NAVARRO, 1998), but little is known about their capabilities after culturing and *in vivo* transplantation. The induction of an increased production of neurotrophic factors in the site of the lesion will enhance axonal regeneration when the local environment is poor or inhibitory, such as in spinal cord injuries (MENEI *et al.*, 1998).

In conclusion, through combined research into new materials for nerve guides, extracellular matrix components and glial cell transplantation, we designed an artificial graft that allows axonal regeneration across long gaps, to levels comparable with those achieved with an autologous nerve graft. The demonstration that human SCs isolated in cell culture survive, enhance axonal regrowth and myelinate regenerated axons after transplantation in the peripheral nervous system of immune-deficient rodents (LEVI and BUNGE, 1994; LEVI *et al.*, 1994) is supporting evidence for further clinical research. For clinical applications, small nerve pieces resected from the nerve stumps during an exploratory intervention may be a source of autologous SCs for a cellular graft that can be implanted in a repair after two weeks.

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Authors' biographies

XAVIER NAVARRO received the MD in 1978, and the PhD, in 1985, from the Autonomous University of Barcelona. He completed his specialty training in Neurophysiology at the University of Minnesota while holding a Fulbright fellowship (1986–1988). He returned to the Autonomous University of Barcelona as Associate Professor in 1988, where he is full Professor of Physiology.

F. JAVIER RODRÍGUEZ obtained his degree in biology, in 1993, and PhD at the Autonomous University of Barcelona, in 1999. Since then, he has been a Postdoctoral fellow at the Karolinska Institute in Stockholm.

DOLORES CEBALLOS is a biologist. Having obtained a PhD in this field, she undertook a postdoctoral fellowship at the University of Minnesota. She is currently working as a Research Fellow at the Autonomous University of Barcelona.

ENRIQUE VERDÚ obtained a degree in Biology, in 1989, and his PhD, in 1994. He is presently Associate Professor in Physiology at the Autonomous University of Barcelona.

All four authors are members of the Group of Neuroplasticity and Regeneration, whose research projects are focused on axonal regeneration, functional restitution after neural injuries, and neuroprostheses.