Neuroscience 163 (2009) 213–221

SCHWANN CELL INFLUENCE ON MOTOR NEURON REGENERATION ACCURACY

R. D. MADISON,[a,b,c]* M. V. SOFRONIEW[d] AND G. A. ROBINSON[a]

[a]*Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA*

[b]*Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA*

[c]*Biological Laboratory Research and Development Service of the Veterans Affairs Medical Center, Building 16, Room 38, 508 Fulton Street, Durham, NC 27705, USA*

[d]*Department of Neurobiology, UCLA School of Medicine, Los Angeles, CA 90095, USA*

Abstract—Extensive peripheral nerve injuries can result in the effective paralysis of the entire limb or distal portions of the limb. The major determinant of functional recovery after lesions in the peripheral nervous system is the accurate regeneration of axons to their original target end-organs. We used the mouse femoral nerve as a model to study motor neuron regeneration accuracy in terms of regenerating motor neurons projecting to their original terminal pathway to quadriceps muscle vs. the inappropriate pathway to skin. Using a variety of surgical manipulations and the selective removal of Schwann cells in the distal nerve via molecular targeting, we have examined the respective roles of end-organ influence (muscle) vs. Schwann cells in this model system. We found evidence of a hierarchy of trophic support that regulates motor neuron regeneration accuracy with muscle contact being the most potent, followed by the number or density of Schwann cells in the distal nerve branches. Manipulating the relative levels of these sources of influence resulted in predictable projection patterns of motor neurons into the terminal pathway either to skin or to muscle.

Key words: femoral nerve, PNS.

Recovery of function following nerve lesions in the peripheral nervous system (PNS) depends on the accurate regeneration of axons to their original target end-organs. A recognized leader in the field of clinical nerve repair once stated that, "[t]he core of the problem is not promoting axon regeneration, but in getting them back to where they belong" (Sunderland, 1991). Regenerating motor axons are often misrouted to sensory targets, and sensory axons formerly innervating skin are often misrouted to muscle. Such misdirected regeneration is a major detriment to functional recovery and rehabilitation. Nevertheless, under certain conditions, axonal regeneration in the mammalian PNS can and does occur with a remarkable degree of accuracy and return of function. The cellular and molecular determinants of this limited, but nonetheless remarkable capacity for re-establishing connections remain largely unknown. An improved understanding of the mechanisms that influence motor neuron reinnervation of distal nerve pathways could have a direct bearing on improving functional nerve repair; since if accurate choices are not made at the terminal nerve branch level progressively finer discriminations (e.g. correct receptor or fiber reinnervation) might be rendered impossible by the lack of appropriate end-organ choices available at the termination of the tributary nerve.

A useful model to study terminal pathway reinnervation is the rodent femoral nerve. Proximally in the leg, motor axons are dispersed across the entire mixed nerve. Distally, the nerve divides into two approximately equal terminal branches; a motor branch to the quadriceps muscle and a purely sensory branch which continues as the saphenous nerve (Brushart, 1988; Franz et al., 2005). Much previous work from several laboratories has shown that following a proximal lesion regenerating motor neurons preferentially, albeit incompletely, reinnervate the terminal quadriceps branch (to muscle) versus the saphenous branch (to skin) (Madison et al., 1996; Al-Majed et al., 2000; Robinson and Madison, 2005; Eberhardt et al., 2006; Uschold et al., 2007). However, there is controversy concerning the degree of influence the terminal nerve pathways alone have on such preferential reinnervation, vs. the influence of the respective end-organs of muscle and skin (Brushart, 1993; Hoke et al., 2006; Robinson and Madison, 2006; Uschold et al., 2007).

Given the vast amount of previous work regarding the general influence of Schwann cells on peripheral nerve regeneration (Hall, 1989, 2005; Chen et al., 2007) it is reasonable to suspect that Schwann cells may be a likely cellular candidate in the terminal nerve pathways to influence regeneration accuracy. Accordingly, it has been suggested that Schwann cells from the cutaneous and muscle pathways of the femoral nerve have distinct sensory and motor phenotypes that can impact the accuracy of axon regeneration of motor neurons (Hoke et al., 2006). Conversely, Madison and colleagues (Robinson and Madison, 2005; Uschold et al., 2007) have proposed that it is not inherent molecular differences between Schwann cells that determine the accuracy of motor neuron regeneration, rather motor neurons can be directed to reinnervate either

*Correspondence to: R. D. Madison, Biological Laboratory Research and Development Service of the Veterans Affairs Medical Center, Building 16, Room 38, 508 Fulton Street, Durham, NC 27705, USA. Tel: +1-919-286-6956; fax: +1-919-286-6811.
E-mail address: madis001@mc.duke.edu (R. D. Madison).

Abbreviations: EO, End Organ group; GCV, ganciclovir; GFAP, glial fibrillary acidic protein; HSV-TK, thymidine kinase gene of the herpes simplex virus; NEO, No End Organ group; PBS, phosphate-buffered saline; PNS, peripheral nervous system; PSA, polysialylated neural cell adhesion molecule; TK, thymidine kinase; WT, wild type.

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doi:10.1016/j.neuroscience.2009.05.073

the muscle or the cutaneous pathway depending on the relative balance of trophic influences from the terminal pathways and end-organs of muscle and skin. The current work was aimed at clarifying the role of Schwann cells in the denervated distal nerve branches in terms of their influence on the accuracy of motor neuron regeneration into their original terminal nerve pathway.

We took advantage of advances in molecular targeting technology to selectively remove Schwann cells in the two terminal nerve pathways distal to a femoral nerve lesion. Sofroniew and colleagues have developed a transgenic model system in which specific cellular removal can be targeted genetically *in vivo* (Sofroniew et al., 1999; Morshead et al., 2003; Sofroniew, 2005). The strategy was based on previous demonstrations that promoter sequences specific for particular cell-types can be linked to genes that encode toxic agents, and that such fusion gene constructs will be expressed and function in a cell-type-specific manner in transgenic animals (Sassone-Corsi and Borrelli, 1986; Borrelli et al., 1988). The promoter of glial fibrillary acidic protein (GFAP), an intermediate filament protein (Bignami et al., 1972; Eng and Ghirnikar, 1994) was used to target astroglia and related cells. The thymidine kinase gene of the herpes simplex virus (HSV-TK) was used to achieve cellular removal.

One of the most encompassing classification schemes of Schwann cell types is the myelinating and non-myelinating phenotypes, both of which arise from a common pool of proliferating, immature, GFAP-expressing cells (Jessen and Mirsky, 1992, 1999). Following development GFAP-expression is downregulated. However, if adult Schwann cells lose contact with axons, such as distal to a nerve transection, they de-differentiate and re-enter the cell cycle to become immature, proliferating and GFAP-expressing cells, comparable to immature Schwann cells in neonatal nerve. Schwann cells that have de-differentiated after nerve injury and are proliferating and expressing GFAP can be selectively removed in these transgenic mice that express HSV-TK under the GFAP-promoter simply by the administration of ganciclovir (GCV). Thus, the timing and location of Schwann cell removal can be regulated temporally by application of GCV following lesions of specific nerves. This transgenic strain is identified as TK.

The results of the current studies support the hypothesis of a hierarchy of trophic support from the terminal nerve pathways and muscle that regulate the accuracy of motor neuron regeneration. Within this hierarchy muscle contact is the most potent followed by the number/density of Schwann cells. There is no support for the idea that Schwann cells within the muscle pathway, by themselves, have a unique molecular identity that regulates regeneration accuracy of motor neurons.

EXPERIMENTAL PROCEDURES

Conditional removal of Schwann cells

TK animals were identified by genotyping as previously described (Bush et al., 1998). Adult TK and non-TK wild type (WT) mice were given a unilateral crush lesion of the sciatic nerve and continuous GCV delivery via a minipump (20 mg/kg/day) for the first seven days after surgery. After 14, 21 and 35 days post-injury animals were perfused with paraformaldehyde/glutaraldehyde, processed for plastic embedding, and stained with Toluidine Blue. The number of white blood cells and Schwann cells was quantified using analysis of 1 μm plastic embedded sections. Counting frame fields (8564 μm^2) were selected at random using a computer-driven microscope stage (CAST, Olympus, Center Valley, PA, USA).); at least 15 frames were quantified per tissue sample. The number of positive cells per counting frame was determined, and final counts were expressed as number per mm^2. At least 300 cells were counted for each tissue sample, and counts were carried out by blinded independent observers. Student's *t*-tests were used to compare cell counts within groups. Differences were considered statistically significant when $P<0.05$.

Surgical procedures

All procedures were approved by the Veterans Affairs Medical Center animal use committee. The Durham, NC Veterans Affairs Medical Center is an AAALAC approved facility, and all procedures also conformed to NIH guidelines. General surgical procedures were carried out as previously described in detail (Robinson and Madison, 2003). All of the retrograde labeling studies were carried out using TK mice. Briefly, TK mice (male and female, 20–22 g) were deeply anesthetized for all surgical procedures with a mixture of ketamine, xylazine and acepromazine (100, 6 and 1 mg/kg respectively) in normal (0.9%) saline.

In all groups the femoral nerve was exposed using an inguinal approach and the parent nerve was transected with microscissors ~5 mm proximal to the bifurcation of the nerve into its muscle and cutaneous branches. The nerve stumps were reapposed and repaired with fibrin sealant (Baxter Healthcare Products, Glendale, CA, USA). Two different repair groups were prepared that varied in terms of whether the distal nerve branches remained in continuity with their respective end-organs of muscle and skin.

In the End Organ preparations (EO), the muscle branch and the cutaneous branch remained intact. In the No End Organ groups (NEO), the muscle branch was transected at the quadriceps muscle, ligated and placed in a blind-ended silicone tube to prevent muscle contact by regenerating axons. The cutaneous branch was transected to be the same length as the muscle branch, and was also ligated and placed in a silicone tube. During the initial femoral nerve surgery, all animals also received implantation of osmotic minipumps s.c. on the back (Alzet, # 1007) containing either GCV (20 mg/kg/day) to remove reactive and proliferating Schwann cells distal to the femoral nerve lesion, or saline (control); pumps were removed after the first week. Thus there were four different experimental groups; (1) EO+saline (n=6), (2) EO+GCV (n=8), (3) NEO+saline (n=12), and (4) NEO+GCV (n=7).

Retrograde labeling and counting of motor neurons

Eight weeks after nerve repair, the femoral nerve was re-exposed. The muscle and cutaneous branches were separated by silicone grease dams, trimmed to ~1 mm distal to the bifurcation, and randomly assigned to receive application of crystals of either fluorescein dextran (FD, D-3306, Molecular Probes, Eugene, OR, USA) or tetramethylrhodamine dextran (TD, D-3308, Molecular Probes). After crystal application, each branch was sealed with silicone grease and separated from others by plastic food wrap. Three days later the animal was perfused through the heart with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. The lumbar spinal cord was removed, post-fixed for several hours, and sucrose protected. The cord was frozen on dry ice and stored at −80 °C until being sectioned with a cryostat. Serial 25-μm frozen longitudinal sections were thawed in PBS, mounted onto glass slides, air dried and coverslipped with Prolong (P-7481,