CHAPTER 2

Review on misdirection of regenerating axons after experimental nerve injury and repair

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ABSTRACT

Misdirection of regenerating axons is one of the factors that can explain the limited results often found after nerve injury and repair. In the repair of mixed nerves innervating different distal targets (skin and muscle), misdirection may for example, lead to motor axons projecting towards skin, and vice versa, sensory axons projecting towards muscle. In the repair of motor nerves innervating different distal targets, misdirection may result in reinnervation of the wrong target muscle, which might function antagonistically. In sensory nerve repair, misdirection might give an increased perceptual territory. After median nerve repair, for example, this might lead to a dysfunctional hand.

Different factors may be involved in the misdirection of regenerating axons and there may be various mechanisms which can later correct for misdirection. In this review, we discuss these different factors and mechanisms that act along the pathway of the regenerating axon. In addition, we review recently developed evaluation methods that can be used to investigate the accuracy of regeneration after nerve injury and repair (including the use of transgenic fluorescent mice, retrograde tracing techniques, and motion analysis).

INTRODUCTION

Functional recovery after nerve injury and repair is often disappointing, despite the capacity of the peripheral nervous system to regenerate. Several factors can explain this incomplete recovery. First of all, the timing of surgery is an important factor. The best chances for recovery are when nerve repair is performed directly after the injury, because (1) the capacity of regeneration has been shown to decrease with time (fewer neurons from which axons regenerate), and (2) changes occur in the distal nerve and targets due to the prolonged period of denervation (such as fragmentation of the basal lamina tubes and decrease in the number of Schwann cells [1-3]). The type of injury and possibilities for repair may also influence the functional outcome: the recovery following graft repair, for example, is reduced compared with direct coaptation repair. If the patient is older, the chance of functional recovery will be decreased [4].

Another factor that can explain poor recovery after nerve injury and repair is misdirection or misrouting of regenerating axons. Misdirection can explain the difference in recovery for different types of nerves (mixed, motor and sensory nerves). In the repair of mixed nerves that innervate different distal targets (skin and muscle), misdirection may lead to motor axons projecting towards skin, and sensory axons projecting towards muscle. In the repair of a motor nerve that innervates different target muscles, motor axons may be misdirected to antagonistic muscles. As for example following repair of the sciatic nerve, that distally divides into the tibial and peroneal nerve branches involved in ankle plantar and dorsiflexion, respec-

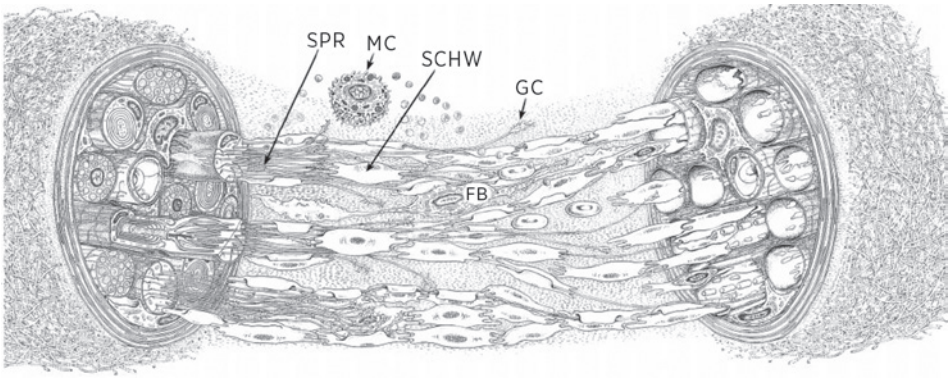

Figure 1

Local cellular response to nerve transection. Sprouting occurs at the cut axonal ends in the proximal nerve segment (left). Sprouts (SPR) arising from one myelinated axons form a regenerating unit surrounded by common basal lamina. At the tip of each sprout there is a growth cone (GC). Sprouts advance over the zone of injury in immediate association with Schwann cells (SCHW). In the injury zone there are macrophages, fibroblasts (FB), mast cells (MC), and blood corpuscle elements. In the distal segment sprouts attach to the band of Bügner and become enclosed in the Schwann cell cytoplasm. Axonal misdirection is frequent. (Figure obtained with permission from article by Lundborg [54]).

tively [5]. In sensory nerve repair misdirection may limit outcome: after repair of the median nerve at the wrist, patients may experience painful sensations in other median nerve innervated fingers, even years after the repair [6].
Different factors are involved in the misdirection of regenerating axons. Moreover, different biological mechanisms have been shown to exist that can later correct for this misdirection. In this Chapter, these factors and mechanisms are discussed. In addition, several recently developed methods are reviewed that have provided valuable insight into the process of regeneration.

THE COURSE OF THE REGENERATING AXON

After nerve injury and repair, the course of the regenerating axon starts at the *coaptation site*. At this site, multiple cellular events have taken place after the injury, including clearance of the debris of the distal axonal bodies by macrophages and Schwann cells, a process called Wallerian degeneration. The proximal axon extends its course across this injury site by sending out multiple sprouts (Figure 1). At the tip of these sprouts are growth cones that continuously create cell protrusions called filopodia and lamellipodia. Growth cones act as antennae for neurotrophic signals. These signals can attract regenerating axons in a certain direction by stimulating actin dynamics inside the growth cone, which leads to axonal elongation [7]. Different neurotrophic factors have been identified including, for

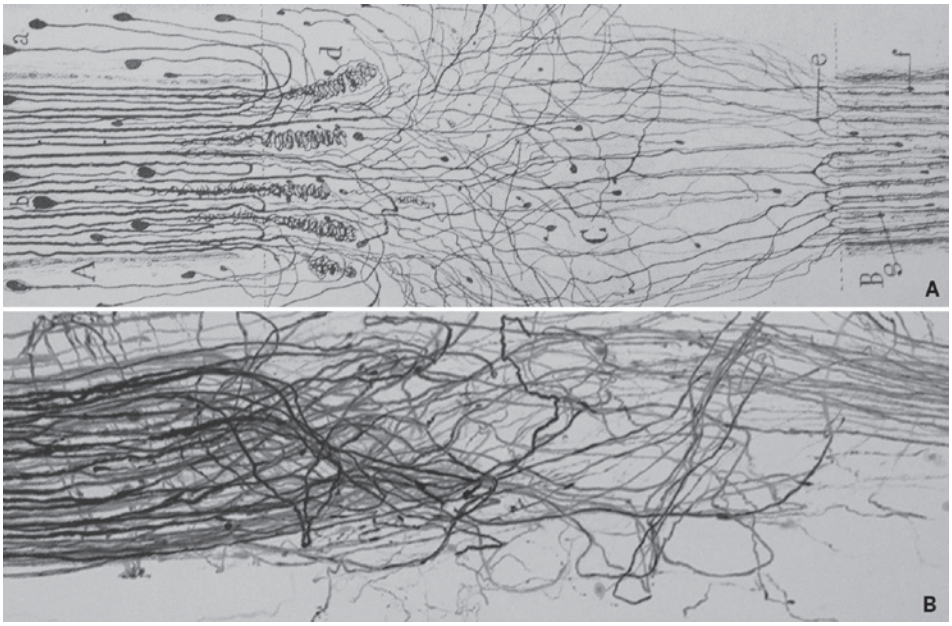

Figure 2

(A) silver staining of Ramon y Cajal demonstrating 'choatic' regeneration across the injury site, (B) similar image obtained using transgenic fluorescent mice. (Figure obtained with permission from article by Pan et al. [55]) In both images the proximal nerve stump is presented left to the injury site and the distal nerve stump right to the injury site.

example, nerve growth factor (NGF), brain-derived neutrophic factor (BDNF) [8] and glial cell-line-derived neurotrophic factor (GDNF) [9]. These factors are produced and secreted by Schwann cells that remain inside the distal basal lamina tubes after Wallerian degeneration. In this way axons are attracted to regenerate towards the distal nerve stump, an effect which has been called *neurotropism* [10]. Besides attractive stimuli, repulsive factors exist that might divert regenerating axons or induce growth cone collapse (for example, semaphorins [11]). In addition, axons might be physically guided. After nerve injury and repair a collagen matrix is formed between the nerve ends. Schwann cells from the proximal and distal nerve stump migrate along this matrix and present specific cell adhesion molecules to guide regenerating axons [12].

In an ideal situation this combination of factors results in a straight course of the regenerating axon back towards its original basal lamina tube (as illustrated in Figure 1). In reality however, it has been shown that axons frequently travel laterally before choosing a distal pathway (Figure 2) [13]. This dispersion of axonal regeneration may lead to inappropriate target reinnervation, despite surgically correct alignment of the fascicles during nerve repair, because once the axon has entered

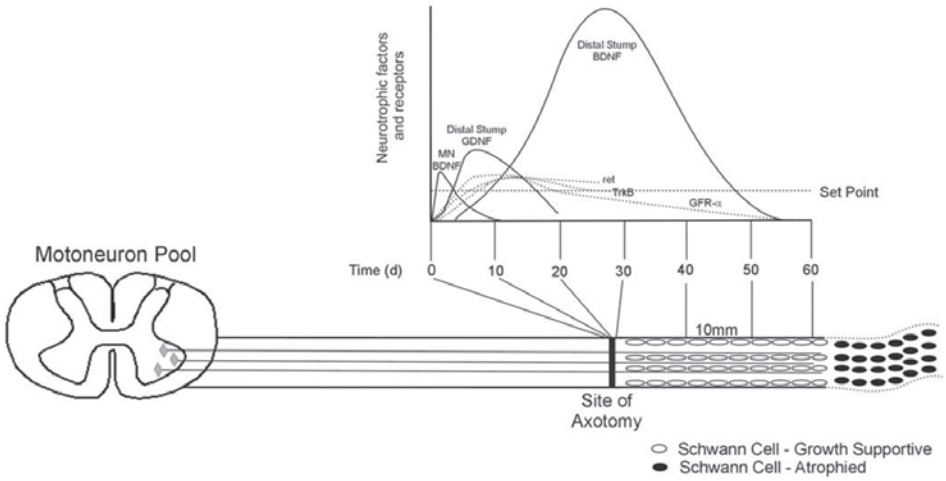

Figure 3

Up-regulation and decline in the expression of growth factors (GDNF and BDNF) and receptors (trkB, ret GFR-α) by motoneurons and in the distal nerve stump 0-60 days after nerve injury. (Figure obtained with permission from article by Furey et al. [56]).

a distal basal lamina tube, the rest of the course is determined by the original pathway of that endoneural tube. Whether or not the axon will eventually also reach the end of the basal lamina tube may still depend on several factors. An important factor is the amount of neurotrophic support, which is again determined by the Schwann cells in the distal nerve stump. As recently demonstrated, Schwann cells only produce these neurotrophic factors for a certain period of time (Figure 3). In addition, the profile of growth factor expression might differ for Schwann cells inside motor versus sensory nerves [14]. Another factor that might determine successful regeneration of the axon across the basal lamina tube is the interaction of the growth cone with specific cell adhesion molecules. Examples of such factors are L2 and HNK-1 [15, 16] and PSA-NCAM [17].

Finally, the regenerating axon has to make a functional reconnection with the target at the end of the basal lamina tube. This last step has mainly been investigated for motor axon reinnervation of the motor endplate. Also in this process selection may occur. After nerve injury and repair, it has been shown that motor endplates are initially polyinnervated, but that later the motor axon to endplate ratio again returns to a normal 1:1 innervation [18-20]. This initial poly- or hyperinnervation may be a mechanism for improving the accuracy of reinnervation. Feedback mechanisms such as tread-mill exercise [21] and manual stimulation may influence this selection process [22].

METHODS TO INVESTIGATE ACCURACY OF REGENERATION

A number of different methods have been developed to investigate accuracy of regeneration and reinnervation. The experiments by Ramon y Cajal at the beginning of the 20th century are legendary [23] (Figure 2A). His silver staining of regenerated nerve fibers already provided us with most of the insight we have today on the accuracy of regeneration across the coaptation site. Also of historical interest are the experiments on the attractive effect of the distal stump (*neurotropism)*, in which Y-shaped tubes were used with different types of tissue in the distal forks. Based on these experiments, the theory of neurotropism was first rejected by Weiss and Taylor [24], because in their experiment no preference for axonal regeneration to different types of tissue was found. However, later the experiment was repeated by others [25-29], who did find a preference for axons to regenerate towards the outlet containing nerve. Since then this theory has been generally accepted. In this review we will not discuss the results of these experiments in detail. Rather, we present more recently developed methods that in our opinion have provided valuable insight into specificity and accuracy of regeneration, including the use of fluorescent transgenic animal models, retrograde tracing techniques, and functional methods based on motion analysis.

Fluorescent transgenic animal models

An exciting, relatively new evaluation method in research on peripheral nerve regeneration is the development of a fluorescent transgenic animal model (in the beginning only fluorescent mice were use, but more recently a transgenic rat model has been developed [30]). In these transgenic animals, a thy1 promotor construct is used to direct the expression of green fluorescent protein (GFP), or a yellow variant (YFP), selectively in neuronal cells (and not in other cell types such as, for example, Schwann cells, fibroblasts, and muscle fibers).[31]. Subsequently, selection of an transgenic animal line, in which the expression of this fluorescent protein is limited to only a subset of axons, has made it possible to visualize the pathway of an individual regenerating axon *in vivo*. This live imaging can be performed at multiple time intervals, before and after the nerve injury. In this way the accuracy of axon regeneration can be determined. *In vivo* analysis of regeneration towards the platysma muscle (that can easily be accessed and viewed) has, for example, shown that the accuracy of regeneration is high after crush injury, with individual regenerating axons entering their original path and reestablishing branches at nearly every original branching point. This specificity is completely lost after transection injury and repair [32]. In addition, this technique can be used to investigate the accuracy of regeneration at the coaptation site and to investigate reinnervation of the motor endplate at the neuromuscular junction by simultaneous labeling of the acetylcholine receptors (AChRs) with α-bungarotoxin (Btx) [31] (Figure 4). Up to now, experiments using these transgenic animal models have mainly confirmed previous observations, including the relatively chaotic process of

axon regeneration across a coaptation site (as demonstrated by Cajal, Figure 2A) and hyperreinnervation at the motor endplate [20]. In the future, these animals may also be employed to investigate new strategies to improve axonal regeneration and target reinnervation *in vivo*, providing an additional advantage that the same animals could be used for analysis with other evaluation methods (such as those mentioned below).

Retrograde tracing techniques

Retrograde tracing techniques are also useful for analyzing the specificity of regeneration, especially because these methods can be applied to *quantify* the accuracy of regeneration. Retrograde tracing is based on the uptake of a fluorescent dye that is retrogradely transported to the nucleus and/or cell body of the neuron (located in the anterior horn in case of motoneurons, or dorsal root ganglion in case of sensory neurons). This label can be applied anywhere along the course of the nerve or directly into the target muscle, by tracer injection or by cup application to the proximal nerve end (after nerve transection, the proximal end of the nerve is placed in a cup containing the tracer). Different technical issues must be considered in the use of retrograde tracers, including labeling efficiency, possible fading of the tracer, dye interactions (when using multiple tracers), potential toxicity [33] and persistence of the tracer, when multiple tracers are used as in sequential tracing [34]. Sequential tracing is an especially useful technique for investigating the accuracy of regeneration towards a specific nerve branch by application of the first tracer before injury to label the original neuronal pool, and the second tracer at a certain interval after the injury (and possibly repair) to label the neurons from which axons have regenerated towards this branch. In **Chapter 4** we used this technique to investigate the accuracy of regeneration after different types of nerve injury and repair (crush, direct coaptation and autograft repair) in the rat sciatic nerve model.

Another possibility of retrograde tracing is to apply multiple tracers at the same time to different nerve branches. This technique has also been used in different animal models including the sciatic nerve, femoral nerve and facial nerve model. In the femoral nerve model simultaneous retrograde tracing has been used to investigate accuracy of motor versus sensory regeneration by applying different tracers to the distal cutaneous and motor branches. Experiments using this model have shown that motor axons initially grow equally into both branches, with similar numbers of retrogradely labeled motoneurons at 2 weeks and also a large number of axons innervating both branches. With time (at 3 and 8 weeks) increasingly more motoneurons projected to the motor branch and fewer motoneurons to the cutaneous branch or both branches [35], a phenomenon which was termed *preferential motor reinnervation* (PMR). Several mechanisms may explain this phenomenon of PMR [36], including the *pruning* of misdirected axon collaterals in favor of correctly directed ones.

In the sciatic and facial nerve model, simultaneous tracing has been used to investigate dispersion of axonal collaterals originating from the same motoneuron to different branches [37]. As mentioned above, a regenerating axon may send out multiple sprouts across the coaptation site. These sprouts may often travel laterally before choosing a distal pathway. Sprouts originating from the same motoneuron may, therefore, end up in different distal target branches. Especially after facial nerve repair, a high percentage of multiple projections to the zygomatic, buccal and marginal mandibular branches has been found (15%) [38, 39], compared with 2.2% double projections after sciatic nerve repair [37]. In **Chapter 7 and 8** we used simultaneous retrograde tracing to compare axonal dispersion across single lumen and multichannel nerve tubes for the percentage of motoneurons with double projections. In **Chapter 9** we used simultaneous tracing to investigate preferential regeneration of motor axons towards the peroneal nerve after injection of a lentiviral vector encoding for glial cell line-derived neurotrophic factor (GDNF).
An important advantage of retrograde tracing techniques is the possibility of quantifying accuracy of regeneration. Care should be taken however when interpreting the results of retrograde tracing not only because of the factors mentioned above, but also because it does not evaluate the final step of motor endplate reinnervation.

Motion analysis

Different methods have been applied to investigate accuracy of reinnervation related to function including selective tension contraction measurements [40], selective recordings of compound muscle action potential amplitude (CMAPs) [41] and walking track analysis [42-44]. All these approaches have been used in the rat sciatic nerve lesion model. Although these methods have provided important insight in the recovery of function after nerve injury and repair, they also have several shortcomings, particularly in the analysis of the impact of misdirection on the recovery of function. Muscle contraction measurements and CMAP recordings for example do not account for co-contractions, nor do they measure the actual recovery of function. The most commonly used functional evaluation method, walking track analysis, only looks at the recovery of distal intrinsic foot muscles that often do not recover as well as the more proximally located muscles (such as the gastrocnemius and anterior tibialis muscles), especially after transection injury and repair. Foot print analysis is also limited due to contractures [45] and autotomy [46]. Other gait parameters have therefore been investigated, including analysis of the ankle angle [47-51]. Advantage of ankle motion analysis is that it can also be used to investigate accuracy of reinnervation of muscles involved in ankle plantar (tibial nerve function) and dorsiflexion (peroneal nerve function) [52], especially if used simultaneously with electromyography (EMG) recordings in the tibialis anterior and gastrocnemius muscles [53]. In **Chapter 3** we present a novel evaluation method that we developed to investigate the recovery of ankle motion after different types of sciatic nerve injury and repair (crush injury, direct coaptation and

autograft repair), called *2D digital video ankle motion analysis*. Advantage of this method is that tibial and peroneal nerve function can be determined separately from ankle plantar and dorsiflexion, respectively.

SUMMARY

Several factors can explain the poor recovery of function often observed after nerve injury and surgical repair, such as the interval between nerve injury and repair, the type of injury and possibilities for repair, age of the patient, and, as discussed in this Chapter, misdirection of regenerating axons. As already stated by Sir Sydney Sunderland '*the core of the problem is not promoting axon regeneration, but in getting them back to where they belong*' [4].

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