Peripheral nerve graft architecture affects regeneration

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CHAPTER 10

Conclusions and discussion

Evaluation methods

The inventory of the evaluation methods to qualify peripheral nerve regeneration through synthetic nerve grafts demonstrated that nerve fibre count, nerve fibre density, N-ratio, nerve histological success ratio, Compound Muscle Action Potential, muscle weight, and comprehensive analysis of the walking pattern possess resolving power (chapter 2). We therefore recommend to perform these evaluation methods in future studies.

These evaluation methods, however, yield only information about the morphology of the nerve, about the potential of the nerve to stimulate the muscle, and indirectly, about the function of the muscles. Comprehensive information about the electrophysiological functioning of the regenerated nerve fibres is missing. It was thus obvious that research in that area was considered to be contributing to the evaluation of peripheral nerve regeneration.

A new *in vitro* electrophysiological evaluation method

We adapted a model that was originally used to electrophysiologically discriminate nerve fibre populations in a much smaller nerve and applied it to the much thicker (regenerated) sciatic nerve. As in all conventional nerve electrophysiological evaluation methods, compound action potentials were evoked from isolated sciatic nerves. But because the tests were performed *in vitro*, the signal to noise ratio was much reduced compared to an *in vivo* situation, and small differences between responses could be measured. This allowed us to measure a gradually increasing displacement of charge upon a gradually increasing stimulus voltage, which is informative about the distribution of nerve fibres with respect to the voltage threshold. Likewise, we were informed about the fibre distribution with respect to the refractory period. Thus, additionally to the outcome of the maximal charge displacement at supramaximal stimulation, the tests also yielded information about the mean firing threshold and the mean refractory period. Moreover, mathematical modelling was applied to the curves that resulted from gradually increasing the stimulus voltage and gradually shortening the timeinterval. This allowed discrimination of two populations of nerve fibres. Since the conduction velocities of the measured responses were in the range of the myelinated nerve fibres, the two populations envisioned had to be the Aα- and Aβ-fibre populations (chapter 3).

In the first place this is interesting because the Aα-fibre population represents predominantly the motor nerve fibres and the Aβ-fibre population the sensory fibres. In the future, we would like to couple the outcome of the electrophysiological analysis to an actual functional outcome like motor function or sensibility recovery. Currently, the evaluation method to study the motor function is the Sciatic Functional Index, which we demonstrated to be not discriminative. A new walking track evaluation method that was recently developed by de Ruiter et al. [33], called 'motion analysis', may have resolving power because measurements are not hampered by contractures and autotomy of the rat hind paw. If this method indeed

demonstrates to be able to differentiate between synthetic nerve grafts, our electrophysiological (and morphological) data can be coupled to motion analysis outcome, in order to evaluate the implications of functionality of nerve fibres on motor function after nerve injury. It would also be interesting to couple the results to a sensory parameter, like pain. But since pain is related to functioning of the C-fibres, which need a much higher stimulus voltage and deliver a much smaller response, we would have to adapt our electrophysiological evaluation method.

Secondly, the separation of the Aα- and Aβ-fibre populations is interesting because it allows to look more in detail at the changes that occur during regeneration. The charge displaced is informative about all nerve fibres that regenerated since its value is a summing up of all the charges that are displaced by the individual nerve fibres. But it is even more informative to know the distinctive charge which is displaced over of the population of larger nerve fibres, and separately, to know the charge which is displaced over the population of smaller nerve fibres. We additionally applied mathematical modelling to nerve morphological data and could likewise discern Aα- and Aβ-fibre populations. We could thus assign a charge to a morphological set of nerve fibres and thereby draw conclusions on the (mal)functioning of the nerve fibres in that population. The same holds true for the mean voltage threshold and mean refractory period. For these parameters it is even more important to divide the total nerve fibre population in two, since their value is mainly determined by the thicker nerve fibres in the (sub)population.

Thirdly, empirical relations between electrophysiological and morphological parameters were used to predict values for the electrophysiological parameters based on the morphologically determined values. It was thus possible to compare the electrophysiologically predicted and measured values. Inequality of those values was interpreted as a change in the electrophysiological properties of the nerve fibres, i.e. a change in the ion channel composition. Remarkably, several times we demonstrated that the changes in the electrophysiological properties were different in the Aα-fibres compared to the Aβ-fibres. Therefore we consider it very likely that the ion channel composition changed in a different way in the Aα- and Aβ-fibre populations. In the future, hypotheses on changes in ion channel compositions can be verified by applying ion channel stainings to the nerve fibres, as we have demonstrated successfully in crushed nerves [39]. Unfortunately, we are not able yet to differentiate the Aα- and Aβ-fibre populations in the ion channel staining experiments. We have to consider that even ion channel stainings may not be conclusive, since the composition and (para)nodal organisation of the several types of ion channels can change simultaneously [15]. As a consequence, this does not have to result in a change of the electrophysiological response, and vice versa.

Properties that a synthetic nerve graft should preferably have

At this point, information was gathered on which evaluation methods should and could be performed to successfully evaluate nerve regeneration. The next step was to gain information on the properties a synthetic nerve graft should preferably have. Although a lot of papers were published on the implantation and subsequent evaluation of synthetic nerve grafts, papers concerning preferable properties of those nerve grafts were remarkably scarce.

From our studies it was concluded that synthetic nerve grafts should be both porous and degradable, in order to obtain regenerating nerve fibres with more preferable electrophysiological properties (chapter 4, 5 and 6). Moreover, it was demonstrated that the newly introduced electrophysiological evaluation method indeed had the power to successfully resolve differences between grafted nerves.

Porosity of nerve grafts

Using synthetic nerve guides of biodegradable ε-caprolactone (CL), it was demonstrated that microporous nerve grafts performed much better than non porous and macroporous grafts (chapter 4). This result was strongly dependent on the presence of a tissue bridge in the graft. A tissue bridge with a considerable diameter containing a lot of myelinated nerve fibres was present in the majority of microporous nerve grafts in strong contrast with the non porous graft group. It was considered only logical that subsequently nerve and muscle morphological and electrophysiological data were in favour of the microporous nerve grafts.

In nerve grafts made of a copolymer of CL with trimethylene carbonate (TMC) the formation of a tissue bridge was not hampered; in 18 of 18 implanted TMC/CL grafts (with variations in the inner layer) a tissue bridge of considerable size was present. This was in contrast to the CL nerve guides, which lacked a tissue bridge in several grafts, even in the microporous group. TMC/CL was therefore judged to be more favorable in the stimulation of the formation of a tissue bridge and thus better suitable to judge the influence of micropores in a synthetic nerve graft on newly regenerated axons.

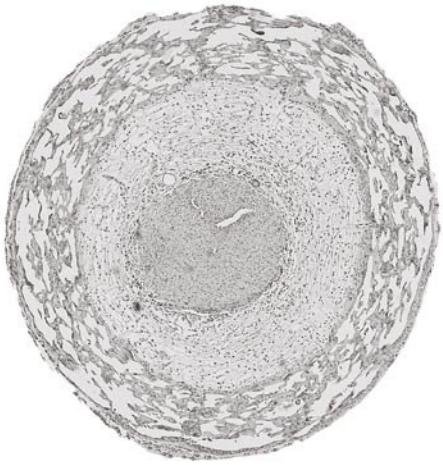
When nerve regeneration was compared through TMC/CL guides with an inner layer of either *non porous* TMC or *porous* TMC, the values of most parameters were comparable (chapter 5). But in porous nerve grafted nerves the refractory periods were shorter, and the voltage thresholds were higher. Since a shorter refractory period enables the axon to follow the firing frequency of the neuron more effectively and thereby allows a more adequate target organ stimulation, we preferred the *porous* over the *non porous* nerve graft. The higher value of the voltage threshold was not considered as a drawback. Apparently, the voltage threshold was not that high that it was impossible to elicit action potentials altogether. The higher value of the firing threshold can even be considered as beneficial, since it prevents the axon from ectopic firing.

We also evaluated the differences between measured and predicted values, as we did in unoperated and autografted nerves (chapter 3). We demonstrated that in *porous* nerve grafts the firing threshold and refractory period were lower than predicted. In *non porous* nerve grafts no such compensation could be demonstrated. We hypothesized that an adaptation in the ion channel composition was responsible for the favorable values in the *porous* grafted nerves. The ability to change the ion channel composition to achieve more favorable results, was considered to be an expression of the maturity of the regenerated nerve fibres. It was hypothesized that porosity of the nerve graft positively influences maturation of the nerve fibres, proposedly by speeding up the formation of a tissue bridge. Proposedly, pores can influence this by allowing inward and outward diffusion and also because their presence influences the microgeometry of the inner layer of the synthetic nerve graft. Maturity of the nerve fibres does in this respect not mean the size of the nerve fibre, but the ability of the neuron and axon to adapt the ion channel composition to its needs. Plasticity of ion channel composition of unoperated and axotomized nerve fibres was frequently described [1, 11, 15, 25, 36].

Midgraft morphology demonstrated that the regenerated nerve cable was no free floating structure. It appeared as if a fibrin bridge was formed through the nerve guide and that in an early stage of regeneration the nerve graft modelled itself around this bridge. TMC degrades much faster than CL [31], which may have caused the inner layer of the TMC nerve guide to model itself easily around the fibrin bridge.

It was presumed that this encapsulation did not exert compression on the nerve fibres, because the nerve and muscle morphological and electrophysiological results in alle 18 grafts were satisfactory. However, it may be that some compression was exerted or that expansion of the tissue bridge was prevented and that the results would have been even better if the

Figure 1.



Transverse (4 µm) cross-section of midgraft sciatic nerve grafted with a non porous TMC/CL synthetic nerve graft, immersion fixed after electrophysiological evaluation, and stained with haematoxylin/eosin.

tissue bridge would have been free floating. In this respect, it is interesting to shortly discuss the results of one of the *non porous* TMC/CL nerve grafts that kept its original shape after 12 weeks. Through this graft the nerve bundle regenerated as a more or less free floating tissue bridge, since the space between the tissue bridge and the nerve graft was filled with very loose connective tissue (fig. 1).

The size of the tissue bridge was comparable to those in the other grafts. Remarkably, the charge that was displaced over this nerve bundle was approximately five times as high as the mean charge displaced over the other TMC/CL nerve grafted nerves. Like in the other *non porous* nerve grafts, the mean threshold was low, and the refractory period was high, which may be due to non porosity. Of course this only concerns one sample, but the result is remarkable enough to suggest that a nerve guide that allows the regeneration of a free floating tissue bridge is even better than a nerve guide that enfolds the tissue bridge.

Biodegradability of nerve grafts

If however, the nerve guide would disappear within reasonable time, the tissue bridge would be free floating anyway, and no compression could be exerted on the regenerating nerve fibres. We thus also evaluated the influence of biodegradability of the nerve graft on regenerating nerve fibres. The same TMC/CL copolymer was used to fabricate *fast* and *slowly* degradable synthetic nerve grafts (chapter 6). Twelve weeks after surgery, the *fast degradable* graft had indeed disintegrated and demonstrated a lower refractory period. Particularly based on the short refractory period, we favoured the *fast* over the *slowly degradable* nerve graft. Currently, biodegradability of nerve grafts is observed additionally to nerve transection, and since the influence of nerve transection is much larger than compression and/or inflammation, it should not be unexpected that the influence of compression and/or inflammation is relatively small. For future experiments on the effect of biodegradability on peripheral nerve regeneration it would be advisable to compare uninjured nerves which are encapsulated by a *fast degradable* or a *slowly degradable* synthetic nerve graft and subsequently evaluate their electrophysiological differences.

Moreover, it is important to realise that our evaluation was performed 12 weeks after injury. It is not known whether this is the optimal time to consider changes in compression and/or inflammation. In the future, it is worthwhile to perform this evaluation after longer post operative intervals.

Taking the results on porosity and biodegradability together, it is not definitively proven that porous and fast degradable nerve grafts are preferable over non porous and slowly degradable nerve grafts, because the relevance of a shorter refractory period to the functional outcome is not proven yet. It is however clear that the electrophysiological properties of the regenerated nerve fibres are influenced by small changes in the constitution of the biomaterial of which the synthetic nerve graft is made. This has not been described before in literature,

and we regard this observation as highly remarkable. Literature has provided us with a lot of papers on hollow synthetic nerve grafts concluding that the nerve guide considered allowed a lesioned nerve to regenerate in a comparable way to an autograft. Together, these papers gave the impression that the composition of a hollow nerve guide was not very important because nerve regeneration was allowed anyway. However, these outcomes were based on results of evaluation methods that did not have much resolving power, as we demonstrated in chapter 2. Applying an evaluation method with more resolving power enables us to actually discern differences between nerves regenerated through different synthetic nerve guides. This makes us believe that there are many more properties of a nerve guide that can influence the outcome of nerve regeneration. It is an option to test a broad range of nerve guides with different properties with the evaluation methods that we used to detect the optimal properties. However, we prefer to do basic research into the changes that take place in regenerating nerves, in order to get insight in the properties that a regenering nerve desires from a nerve graft.

A combination of evaluation methods is essential

The chapters on porosity and degradability not only provided insight in the properties a synthetic nerve graft should preferably have, but the results also established the relevance of using a combination of evaluation methods and the relevance of using evaluation methods with resolving power. The results demonstrated that the most remarkable differences between grafts appeared at times in nerve histology, occasionally in muscle histology, and now and again in nerve electrophysiology, illustrating that it is essential to perform a combination of diverse evaluation methods. The results also demonstrated that performing only a limited number of evaluation methods could easily lead to the conclusion that an autograft and a synthetic nerve graft performed equally well. Adding an evaluation method with much resolving power (the *in vitro* electrophysiological evaluation method) revealed that differences could indeed be demonstrated between autografted and synthetic nerve grafted nerves, and even between synthetic nerve grafted nerves (chapter 5 and 6). This illustrates that it is essential to use evaluation methods with resolving power.

It is regrettable that there are only a limited number of papers that describe execution of a broad range of evaluation methods to test peripheral nerve regeneration, and that many base their conclusions on evaluations without resolving power. If only a minimum of evaluation methods was used, or if only evaluation methods without resolving power were used, it should not be allowed to conclude that synthetic nerve grafts perform equally to each other, and not at all that they perform equally to the current 'gold standard', the autograft. This wrongly judges synthetic nerve grafts to be equal to autografts and thus of acceptable performance.

Currently, three different synthetic nerve guides are commercially available: Neuragen ™, Neurotube® and Neurolac®. Neuragen ™ is a semipermeable biodegradable collagen tube [5, 26]. In monkeys Neuragen™ was reported to be equivalent to direct suture repair with respect to the parameters evaluated: CMAP, CSAP, tactile stimulation, nerve fibre number, diameter, and G-ratio [6]. The CMAPs of the different regenerated nerves were compared with respect to 'the slope of the change of CMAP over time'. In other papers that used CMAP as an evaluation method the amplitude and/or latency was tested, and these parameters were demonstrated to have resolving power, in contrast to the parameter that Archibald used. The CSAP and tactile stimulation tests were not or only very scarcely applied and compared statistically, and it is thus not known whether these tests have resolving power at all. The nerve fibre number is not informative, and the diameter and G-ratio were demonstrated NOT to be discriminative.

Neurotube® is a bioabsorbable, woven tube made of polyglycolic acid. In primates, nerve regeneration across 3 cm ulnar gaps was compared in conduits and sural nerve autografts, 1 year after surgery. The conduit and autograft group were compared considering mean fiber diameters, nerve fibre densities, EMG amplitudes and conduction velocities . In de conduit group 1 of 8 gaps was not bridged by neural tissue, and in 3 of 8 synthetic nerve grafts the center of the conduit contained some scar tissue with regenerating units located around the periphery of the conduits [13]. Nerve fibre diameter, density and mean conduction velocity did not differ between the two kinds of grafts. However, all of these evaluation methods were demonstrated NOT to be discriminative in comparing grafted nerves as was pointed out in our review (chapter 2).

Neurolac® is a resorbable but non porous nerve graft made of poly(DL-lactide-ε-caprolactone). In laboratory animals this graft demonstrated to have a comparable number of nerve fibres with a comparable diameter as autografts [14], to have a comparable ElectroMyoGram and Sciatic Functional Index, and to demonstrate the same results in a nociceptive test after 15 weeks to one year after surgery [28, 29]. However all of these evaluation methods were demonstrated NOT to be discriminative in comparing grafted nerves as was pointed out in our review.

In conclusion, it is debatable that currently synthetic nerve grafts are implanted in humans, that only have been evaluated in laboratory animals using evaluation methods that were demonstrated to have no resolving power.

Changes in reinnervated muscles

The changes that occurred in reinnervated muscles were also evaluated. In all experiments concerning the effect of porosity or biodegradability of synthetic nerve grafts, the reinnervated target muscles of the sciatic nerve, i.e. the tibialis anterior muscle and the gastrocnemic muscle, were investigated. In both muscles, that consisted mainly of type II muscle fibres, the muscle cross-sectional area halved and the number of muscle fibres remained approximately

the same. It was striking though that in autografted muscles muscle fibres clustered, in contrast to synthetic nerve graft muscle fibres, while the number of (Aα)nerve fibres was equal to or even lower in synthetic nerve grafted nerves in comparison to the number of (Aα)nerve fibres in autografted nerves.

This was explained as follows: regenerating axons branch along their course through the peripheral nerve. It was proposed that the probability of the occurence of clustering of muscle fibre types ('type grouping') is related to the dispersion of sibling branches in the nerve. In the autograft, emerging branches are kept together by Schwann cell basal lamina scaffolds, in contrast to the hollow synthetic nerve grafts where the emerging branches become dispersed. Thus, in muscles reinnervated after autografting, the probability that nerve branches that arrive at a specific muscle territory are sibling branches, is larger than after hollow tube grafting. Consequently, the probability that type grouping will occur is larger. Moreover, typegrouping was visualized in type I muscle fibres, but it is highly likely that it also took place in type II muscle fibres. This means that the architecture of the muscles that are reinnervated by grafted nerves is highly disturbed and deviating from 'normal' muscles.

Thus type grouping seems to be the result of branching along the nerve in combination with the containment of the sibling Aα-motor axon branches within the basal lamina scaffolds along the length of the nerve. In establishing functional relevant muscle reinnervation it is most desirable to lead all branches originating from a single motoneuron to the same muscle, and preferably the same perimysial territory. Bundled branching in autografted nerves establishes such a reinnervation, and typegrouping provided evidence for this. The introduction of a matrix in synthetic nerve grafts, which allows the containment of regenerating nerve fibres, will presumable beneficially influence nerve regeneration through such grafts.

Actually, branching of nerve fibres is a phenomenon that is expected after nerve injury [7, 9, 10, 22, 30, 32]. The phenomenon was described in literature, but still a lot of questions remain [3, 27, 35]. We do not know whether branching of regenerating nerve fibres is stimulated equally, more or less comparing autografts and synthetic nerve grafts. The finding that the branching pattern of DRG neurites were influenced by the presence of laminin [24] is suggestive for a difference in branching pattern in dissimilar nerve grafts.

Branching is favorable from the point of view that the same neuron can send out several neurites that can simultaneously reach different target organs, which theoretically increases the chance that the original target organ is reinnervated. The accuracy of reinnervation may later be improved by means of pruning of mistaken collaterals [16, 21, 37]. The drawback of branching is that the neuron has to make a lot of effort to produce and maintain all branches, which might otherwise have been used to mature the regenerated nerve fibres, in the sense that the ion channel composition of the membrane could be optimized. We thus also do not know whether the quality of the branches decreases with an increase in their number. All of these aspects are very relevant in judging the quality of nerve regeneration. Retrograde tracing studies can be helpful in elucidating the branching pattern of individual neurons, and ion channel staining methods can be helpful in judging the ion channel density on the branches.

Can Schwann cells survive in a TMC/CL nerve guide that contains a matrix?

Nerve fibres that regenerated through autografts demonstrated a much higher charge than nerve fibres that regenerated through hollow nerve grafts. This may be due to the better guiding of the regenerating nerve fibres through the scaffolds in the graft. Moreover, it is conceivable that the containment of branches, as seen in autografts, will lead the branches of one neuron to the same target, and that this results in a better function. Therefore, we consider it a preferable property of a synthetic nerve graft to contain several small tubes instead of one lumen. However, the experimental synthetic nerve grafts with multiple channels that have been implanted so far only contained a limited number of channels [33]. The typegrouping results suggest that a much larger number of channels is more appropriate.

The matrix to construct these small tubes (or to fill the small tubes with) preferably has resemblance with the matrix proteins in the basal lamina scaffolds that remain in the Wallerian degenerated nerves, like laminin, fibronectin and collagen. Another advantage of these naturally occurring biomaterials is that it is unlikely that a foreign body response ensues.

These small tubes will first be filled with extracellular fluid, followed by the formation of a fibrin bridge and subsequently Schwann cells from the proximal and distal nerve stump will enter the graft [38]. Therefore the interaction of Schwann cells with a variety of extracellular matrix proteins and some other matrices was tested. Likewise, the interaction of Schwann cells with the biomaterials 1,3-trimethylene carbonate (TMC) and ε-caprolactone (CL) and its copolymers (TMC/CL) was investigated. Although the interaction of the Schwann cell with the biomaterial itself is of less importance, since preferably the biomaterial is coated, it is important that the biomaterial is not toxic to Schwann cells.

Another consideration to investigate the interaction of Schwann cells with matrices and biomaterials is that it could be stimulatory for nerve regeneration to populate synthetic nerve grafts with Schwann cells [2, 4, 8, 12, 17, 18, 19, 20, 23, 34]. Schwann cells produce growth factors and they interact between the regenerating axon and the extracellular matrix proteins and thereby stimulate adhesion. Schwann cells form bands of Büngner and thus guide the regenerating nerve fibres to a target organ.

Attachment of Schwann cells is a prerequisite for their survival and proliferation. It was demonstrated that human Schwann cells easily attach to almost any culture substratum, and that attachment is not an important consideration in the selection of suitable materials for the fabrication of a synthetic nerve graft or for the coating of their interior (chapter 8 and 9). Proliferation of Schwann cells was acceptable on all biomaterials tested, but it was relative poor on collagen type I and poly-ethylene-imine. Fibronectin, laminin and poly-D-lysine demonstrated the best proliferation rates during the 15 day culture period. Of these fibronectin and laminin have a proven ability to enhance neurite extension, and are therefore the most suitable candidates from our panel to be present in the interior of a (multiple channel) synthetic nerve guide (chapter 8 and 9).

The next step in elucidating mechanisms that play a role in nerve grafting

In this thesis we made several suggestions for the improvement of the architecture of synthetic nerve guides. However, by gaining more insight in the properties such a nerve graft should preferably have, it became clear that there are lots of phenomena concerning peripheral nerve regeneration that are not well understood yet. Research should evolve in that direction. Our electrophysiological evaluation method and the combination of its results with morphological data may be helpful in this respect. We think it valuable to add retrograde labeling studies and ion channel staining methods to our evaluation tools in order to gain insight in the processes that determine the succes or failure of nerve regeneration. In our opinion, such research is of much more importance than the 'trial and error' studies aiming at the development and implantation of a continous stream of new synthetic nerve guides.

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