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Running title: Timing and nerve reconstruction

The role of timing in nerve reconstruction

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Abstract

The surgeon, who treats nerve injuries, should have knowledge about how peripheral nerves react to trauma, particularly an understanding about the extensive pathophysiological alterations that occur both in the peripheral and in the central nervous system. A large number of factors influence the functional outcome, where the surgeon only can affect a few of them. In view of the new knowledge about the delicate intracellular signaling pathways that are rapidly initiated in neurons and in non-neuronal cells with the purpose to induce nerve regeneration, the timing of nerve repair and reconstruction after injury has gained more interest. It is crucial to understand and to utilize the inborn mechanisms for survival and regeneration of neurons and for activation, survival and proliferation of the Schwann cells and other cells that are acting after a nerve injury. Thus, experimental and clinical data clearly point towards the advantage of early nerve repair and reconstruction of injuries. Following an appropriate diagnosis of a nerve injury, the nerve should be promptly repaired or reconstructed, and new rehabilitation strategies should early be initiated. Considering nerve transfers in the treatment arsenal can shorten the time of nerve reinnervation of muscle targets. Timing of nerve repair and reconstruction is crucial after nerve injury.

Key words

Nerve reconstruction, nerve injury, timing, transcription factor, Schwann cell, neuron, apoptosis, brain plasticity, rehabilitation, plasticity

A nerve injury has a severe impact on the individual patient, who may experience a broad spectrum of symptom after injury, including sensory dysfunction, lack of muscle function, pain, allodynia and cold sensitivity. These symptoms, with a profound impact on the patient's global hand and arm function, do not only cause individual suffer to the patient, but can also reduce the ability of the patient to enjoy leisure activities and particularly perform their work. Therefore, there is a high risk of long sick leave after a peripheral nerve injury, and the costs for lost production may stand for about 80% of the total cost for treatment (Rosberg et al., 2005; Thorsen, Rosberg, Steen Carlsson, & Dahlin, 2012). The final outcome of a nerve injury depends on many factors. The individual surgeon cannot affect the majority of them, but the time at which a nerve injury should be repaired or reconstructed is important. A patient with a trauma to the hand, arm, shoulder or lower leg should be properly examined in the emergency room with the attempt to make an appropriate diagnosis of any nerve injury. Once the diagnosis of a nerve injury has been made, all efforts should be directed to repair or reconstruct the nerve injury as soon as possible, although one should consider the general condition of the patient; i.e. strict medical priority of the patient's injuries. Based on the delicate intracellular signaling pathways that are rapidly initiated in neurons and non-neuronal cells with the purpose to induce regeneration as well as recent advances in the knowledge about brain plasticity in rehabilitation strategies, the factor *timing* of nerve repair and reconstruction will be the key word.

The intrinsic response in neurons and Schwann cells after injury

After a nerve transection, signals are initiated and sent from the site of injury along the proximal segment of the transected axon up to the nerve cell body (Figure 1). These signals may be both positive and negative in nature. The normally transported retrograde signals from

targets or from the microenvironment of the axon may be suppressed by the inhibition of axonal transport of substances from the periphery; i.e. a negative injury signal. The transcription factor, nuclear factor kappa B (NFκB), bears a specific code, which is called a nuclear localization sequence, and allows the entrance in the nucleus. Trauma to the nerve may inactivate this factor and thereby it also is an important negative modulator of the response in the nerve cell body (Raivich & Makwana, 2007). After transection, the proximal tip of the axon is rapidly sealed after extracellular cations, such as Na^{+} and Ca^{2+}, are diffused through the open cell membrane. The entrance of calcium is also reported to be important for local activation of protein kinases at the tip of the axons. One example of such a locally activated protein kinases is extracellular signal-regulated kinase (ERK) that is fundamental for activation both in neurons and in Schwann cells (Mårtensson, Gustavsson, Dahlin, & Kanje, 2007; Raivich & Makwana, 2007; Stenberg, Kanje, Martensson, & Dahlin, 2011). The microenvironment also contains molecules that are produced by the Schwann cells, from invading inflammatory cells as well as from other injured axons. These substances, like Ciliary NeuroTrophic Factor (CNTF) and Leukemia Inhibitory Factor (LIF), can activate the signal transducer and activator of transcription 3 (STAT3) (Wang et al., 2009). Other examples of locally activated molecules are ERK and c-Jun N-terminal kinase (JNK), which are activated by phosphorylation. These complexes are shuttled by retrograde transport with added nuclear localization sequences that allow the translocation to the nucleus. Thereby, these signals are positive injury signals that are generated at or around the tip of the axon with the purpose of translocation to the nucleus for activation of gene transcription (Hanz & Fainzilber, 2004, 2006; Lindwall & Kanje, 2005b) (Figure 1).

The transcription of multiple genes is modulated by retrograde signals, such as the immediate early genes c-Jun and the transcription factor like ATF3 (Hunt, Raivich, & Anderson, 2012);

ATF3 being important for a variety of functions (Rynes et al., 2012). These molecules are rapidly up-regulated, probably depending the retrograde transport of JNK, with the purpose of both preservation of neuronal survival as well as to induce regeneration (Lindwall, Dahlin, Lundborg, & Kanje, 2004; Lindwall & Kanje, 2005a). STAT3 is also a survival-promoting factor that is present in neurons by using alternative pathways. The activation and compensatory mechanisms are complex, since there is a cross-talk between the various signal transection pathways; a phenomenon also seen in Schwann cells (Mårtensson, 2012). Induction of ATF3 may also differ between motor and sensory neurons. The number of neurons that express ATF3 also declines rapidly after an injury, particularly observed in dorsal root ganglia, i.e. sensory neurons, which is important when discussing the timing of nerve repair and reconstruction. Thus, an early repair or reconstruction facilitates neuronal survival. In addition, there seems to be a differential up-regulation of ATF3 after injury in different types of sensory neurons since the up-regulation of ATF3 is particularly important for sensory neurons that project to the skin (Reid, Welin, Wiberg, Terenghi, & Novikov, 2010). Thus, a substantial number of different molecules are activated by different mechanisms in neurons after injury. They are all of importance for the survival of neurons and particularly for redirection to the production of substances needed for nerve regeneration (Abe & Cavalli, 2008; Raivich & Makwana, 2007; Rossi, Gianola, & Corvetti, 2007).

The close interaction that normally occurs between axons and Schwann cells is important for maintenance of the differentiation of the Schwann cells. After injury, the Schwann cells are activated (or de-differentiated) as a preparation before proliferation. In the distal nerve segment, proteases in the axons are rapidly activated for breakdown of the axon and both Schwann cells and the invading and infiltrating macrophages contribute to the breakdown and ingestion of myelin. Myelin-associated genes in Schwann cells are down-regulated during this

degeneration process. Other molecules, like neural cell adhesion molecule (NCAM), which is linked to non-myelinating Schwann cells, are differently expressed depending on the relation to the outgrowing axons (Saito, Kanje, & Dahlin, 2010). p-ERK1/2 and STAT3 expression is also lower in the distal nerve segment compared to just distal to the site of transection, which may be related to the presence of inflammatory cells or apoptotic Schwann cells. Schwann cells also rapidly express the transcription factor ATF3 as a sign of activation after nerve injury, but such an expression is down-regulated in the distal nerve segment in the Schwann cells along with the progress of the outgrowing axons (Kataoka, Kanje, & Dahlin, 2007). Again, these phenomena indicate that there is a close interaction between the outgrowing axons and Schwann cells (Figure 1).

During later phases of the nerve regeneration process, the myelination by ensheathment of the outgrowing axons is promoted by positive regulators between these axons and the different types of Schwann cells, which includes a radial sorting process involving neuregulin-1 (Jessen & Mirsky, 2008). In addition to the role of ERK1/2, recent data also indicate that the transcription factor Pax-3 has a role in differentiation and proliferation of Schwann cells after a peripheral nerve injury (Doddrell et al., 2012). Finally, and maybe of convincing importance, the transcription factor c-Jun, which is expressed in Schwann cells, seems to be a global regulator of the Wallerian degeneration process, since it may determine the expression of trophic factors, adhesion molecules, formation of regeneration tracks and myelin clearance as well as the control of distinct regenerative potential of the peripheral nerve. If c-Jun is not induced, there is a dysfunction of repair of the cell and with subsequent failure of functional recovery as well as induction of neuronal death. Thus, this single glial transcription factor appears to be of extreme importance in this context (Arthur-Farraj et al., 2012). The c-Jun is also a negative regulator of the myelination (Parkinson et al., 2008). Interestingly, ATF3

expression may be dependent on the c-Jun appearance, particularly observed in sensory neurons (Lindwall, et al., 2004).

The knowledge about single transection pathways that are involved in cell death by apoptosis after nerve injury is incompletely known (Mantuano et al., 2011). Apoptosis does occur in some neurons, particularly among sensory neurons, and appears also in satellite cells and in Schwann cells. Those sensory neurons that projects to the skin seems to be more vulnerable to apoptosis than those sensory neurons that project to a muscle (Welin, Novikova, Wiberg, Kellerth, & Novikov, 2008). Furthermore, the apoptotic mechanism(s) may be different in Schwann cells and satellite cells compared to motor and sensory neurons, since a marker of apoptosis, cleaved caspase 3, is not observed in neurons after injury, but is clearly expressed in Schwann cells and in some satellite cells (Saito, Kanje, & Dahlin, 2009).

The timing of nerve repair and reconstruction

As pointed out earlier there are large numbers of factors that influence the functional outcome after nerve injury and repair and reconstruction; some of them not possible to influence by the surgeon, like the age of the patient (Chemnitz, Bjorkman, Dahlin, & Rosen, 2013). The timing of nerve repair or reconstruction is an important point in nerve regeneration. It is one of the factors that the surgeon can guide and thereby improve functional outcome. In an interesting clinical paper it was clearly demonstrated that the results after reconstruction of a brachial plexus nerve injury in adults were considerably better, with respect to motor functional recovery, if the delay of the nerve reconstruction procedure was shorter than two weeks (Jivan, Kumar, Wiberg, & Kay, 2009). This clinical observation should be put into perspective with the described mechanisms in signal transduction. An injured neuron with its transected axon can probably be reactivated if a secondary nerve reconstruction procedure is

done by “refreshing” the proximal nerve end during surgery through the re-transection of the axon. By such a procedure the described intraneuronal activation mechanisms can probably be reinitiated with a further outgrowth of the axons. However, the obstacle for an efficient axonal outgrowth after delayed nerve repair or reconstruction is the events that occur in the distal nerve segment. The impaired axonal outgrowth after a delayed nerve repair or reconstruction after a transection injury seems to be related to factors like a decrease in p-ERK1/2 in Schwann cells in the distal nerve segment (Tsuda, Kanje, & Dahlin, 2011). Furthermore, the numbers of Schwann cells that express the transcription factor ATF3, which is also associated with an efficient axonal outgrowth, decline with time after injury. This is particularly obvious in experimental models if the delay exceeds 30 days with a resulting impaired nerve regeneration (Saito & Dahlin, 2008). Apoptosis, detected by presence of cleaved caspase 3 in Schwann cells, increases substantially in the distal nerve segment if the nerve repair or reconstruction is delayed (Tsuda, et al., 2011). Furthermore, the contact between the outgrowing axons and the Schwann cells seems to be essential for the number of cells that express cleaved caspase 3 only if the nerve repair is immediately performed (Tsuda, et al., 2011). A delayed nerve repair increases the number of Schwann cells that express cleaved caspase 3 from around 10% of the cells in the distal nerve segment up to around 20% after delayed nerve repair irrespective of the length of the delay (Saito, et al., 2009) (Figure 2).

If a nerve injury is not repaired within three or six months, there is a substantial decline in Schwann cell markers and an increase in fibrosis and proteoglycan scar markers in the distal nerve segment (Jonsson et al., 2013). These changes are similar to those reports that present a decrease in transcription factor, like ATF3, overtime (Saito & Dahlin, 2008). Although it has been suggest that a critical time-point at which outcome of regeneration becomes poor appears to be three months in experimental systems (Jonsson, et al., 2013), even shorter time,

like two weeks, is sufficient to observe an impaired axonal outgrowth. This phenomenon is related to both a less activation of Schwann cells and an increased number of apoptotic cells (Tsuda, et al., 2011).

The cell adhesion molecule NCAM is more abundant in the distal nerve segment if an injured nerve trunk is repaired or reconstructed after a long delay in experimental rat models. After the long delay, when the axonal outgrowth is particularly impaired, NCAM, which is associated with non-myelinating Schwann cells, is predominantly seen in the distal nerve segment. This indicates that particularly non-myelinating Schwann cells are present in the distal nerve segment if the nerve repair or reconstruction is done with a delay (Saito, et al., 2009). Therefore, the myelination of the outgrowing axons may be severely impaired. c-Jun is probably critical in this context, since it reprograms Schwann cells to generate a repair process in the cells after nerve injury (Arthur-Farraj, et al., 2012). In addition, the expression of trophic factors and adhesion molecules is determined by c-Jun. Thus, the regeneration tracks, and thereby the regenerative potential of the outgrowing axons, is influenced by c-Jun. The sprouts that are formed from the tip of the axon, with their filopodia, are orchestrated by extracellular guidance cues for their advancement, the retraction as well as the turning, i.e. direction of growth, which is mediated by the actin filaments within the growth cone (Bloom & Morgan, 2011) (Figure 1).

The permissive cues that guide the advancement of the growth cones are critical restore as soon as possible by the surgeon. The previously described basic concepts of nerve repair include preparation of the nerve ends to create a fresh nerve end without necrotic cells and a careful approximation of the nerve ends without tension (Yi & Dahlin, 2010). Compensation for the mushrooming of the fascicles by a meticulous coaptation, even leaving a minor gap,

that allows formation of a fibrin matrix with macrophages and migration of Schwann cells are one of the steps in the surgical procedure. Finally, the nerve repair is completed by application of sutures or glue. These steps are essentially the same when a nerve reconstruction procedure is done, but it is important that the nerve grafts as well as the proximal and distal nerve ends are handled accurately avoiding drying of the tissues. Hereby, the Schwann cells can be kept viable and the signal transduction mechanisms necessary for proliferation and production of growth factors can be preserved.

Different experimental procedures, including pharmacological treatment (Kvist, Danielsen, & Dahlin, 2003), has been described to improve motor reinnervation after a delayed nerve repair (Sulaiman & Gordon, 2009). There is also a possibility to reactivate the chronically denervated Schwann by treatment with the Transforming Growth Factor ß (TGF-ß) (Sulaiman & Gordon, 2009). Recent clinical experiences also indicate that it is a possibility to overcome the problem with chronically denervated Schwann cells by transferring regenerating axons from another source, by a nerve transfer procedure, closer to the target. A variety of nerve transfers have been described for the upper extremity (Lee & Wolfe, 2012). One of these nerve transfer procedures can be done when an axillary nerve injury is overlooked in young adults after shoulder trauma (Dahlin, Coster, Bjorkman, & Backman, 2012). In nerve transfers, a freshly transected nerve fascicle from an uninjured nerve is transferred, without residual problems from the donor nerve, to the distal nerve segment of a previously injured nerve. It is an advantage if the transfer can be made very close to the target. In this way the regenerating axons from the freshly transected nerve fascicles need only to grow over a limited distance in an environment with suboptimal viable Schwann cells before the axons reach a target, such as a denervated muscle.

Cerebral plasticity – the importance of timing in rehabilitation

After a nerve injury, a substantial functional disorganization occurs at the cortical and the subcortical levels in and close to the somatosensory cortex in the central nervous system (Rosen et al., 2012; Taylor, Anastakis, & Davis, 2009). These changes are clearly seen in adults, but younger children have an extensive adaptive cerebral plasticity. Therefore, age is an important factor for the functional outcome after nerve injury and repair and reconstruction. There seems to be a critical time around puberty at which the cerebral plasticity after a nerve injury changes and responds as in adults (Chemnitz, et al., 2013). Before puberty, the functional recovery may return to almost normal after a nerve injury and repair and reconstruction. The most plausible explanation is the mechanisms of cerebral plasticity, where the functional disorganization of the cortical map can be completely restored in young children. Thus, the brain can in young children more easily interpret the new information from the periphery although an extensive misdirection of axonal outgrowth occurs (Rosen, et al., 2012; Taylor, et al., 2009). In addition, there has also been detected a reduction in the cortex after nerve injury. A link between the functional recovery after nerve injury and repair and the disorganization and reorganization of both the gray and white substances has been demonstrated. The described alterations, with a reduced activation of certain brain areas with atrophy of the gray substance as well as the disorganization of reorganization, are rapid. Thus, these changes may also be the target for the factor timing after nerve injury and repair and reconstruction, since they can be considered in the new rehabilitation strategies (Lundborg, Bjorkman, & Rosen, 2007; Weibull et al., 2008). The timing for introduction of training after nerve repair has been highlighted and has focused on the importance of immediate sensory relearning (Rosen & Lundborg, 2007). To provide an alternative afferent inflow from the hand early after nerve repair in the forearm, a “Sensor Glove System” can mediate impulses through the hearing sense. This implies that the

deprivation of one sense, i.e. perception of touch in a denervated skin area, can be compensated by another sense, i.e. sensory-by-pass using the "Sensor Glove System" (Rosen & Lundborg, 2007). By using such a system, initiated early after surgery, with sensory re-education and compared with the conventional sensory re-education, which usually start about three months postoperatively, tactile gnosis can significantly be improved (Rosen & Lundborg, 2007). Thus, even the brain can be utilized in the concept of timing after nerve injury and repair and reconstruction (Rosen & Lundborg, 2004).

In conclusion, the functional outcome after nerve injury and repair and reconstruction is dependent on a broad spectrum of different factors and the results cannot be improved by focusing on one of these factors. Therefore, we have to optimize the whole treatment strategies and improve every single part of the whole treatment chain. One of these components is the timing of nerve repair and reconstruction. This component is dependent on that the surgeon makes an early and proper diagnosis of the nerve injury. If a complete nerve injury is suspected, a prompt nerve repair should be done; a statement that is strongly based on recent knowledge about neurobiology. Any delay of the repair or reconstruction leads to a deterioration of the condition of the different cells in the distal nerve segment, particularly the important Schwann cells. The judgment of the extent of injury can be difficult in some specific cases, like in closed nerve injuries and gunshot and shrapnel injuries, where nerve function is impaired, but the continuity of the nerve is preserved. In such cases, one may consider waiting with any tentative surgical procedure of the nerve trunk, but it is of outmost importance that the surgeon in such cases has an attitude of "active surveillance". Thus, the surgeons should evaluate nerve function often and repeatedly. If no signs of functional recovery are observed, the decision of a nerve reconstruction procedure should not be further delayed. The concept of timing does also include an early rehabilitation of the patient,

utilizing the new treatment algorithm after nerve repair and reconstruction. Timing of nerve repair and reconstruction after nerve injury is one, but only one in a long chain, key factor to improve functional outcome.

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The present chapter is dedicated to my close friend and research colleague Professor Martin Kanje, who passed away in March 2013. Unfortunately, he could not see the present chapter in which he should have been a co-author, but my work is dedicated to him and his memory. Thank you Martin for all our fantastic and stimulating discussions!

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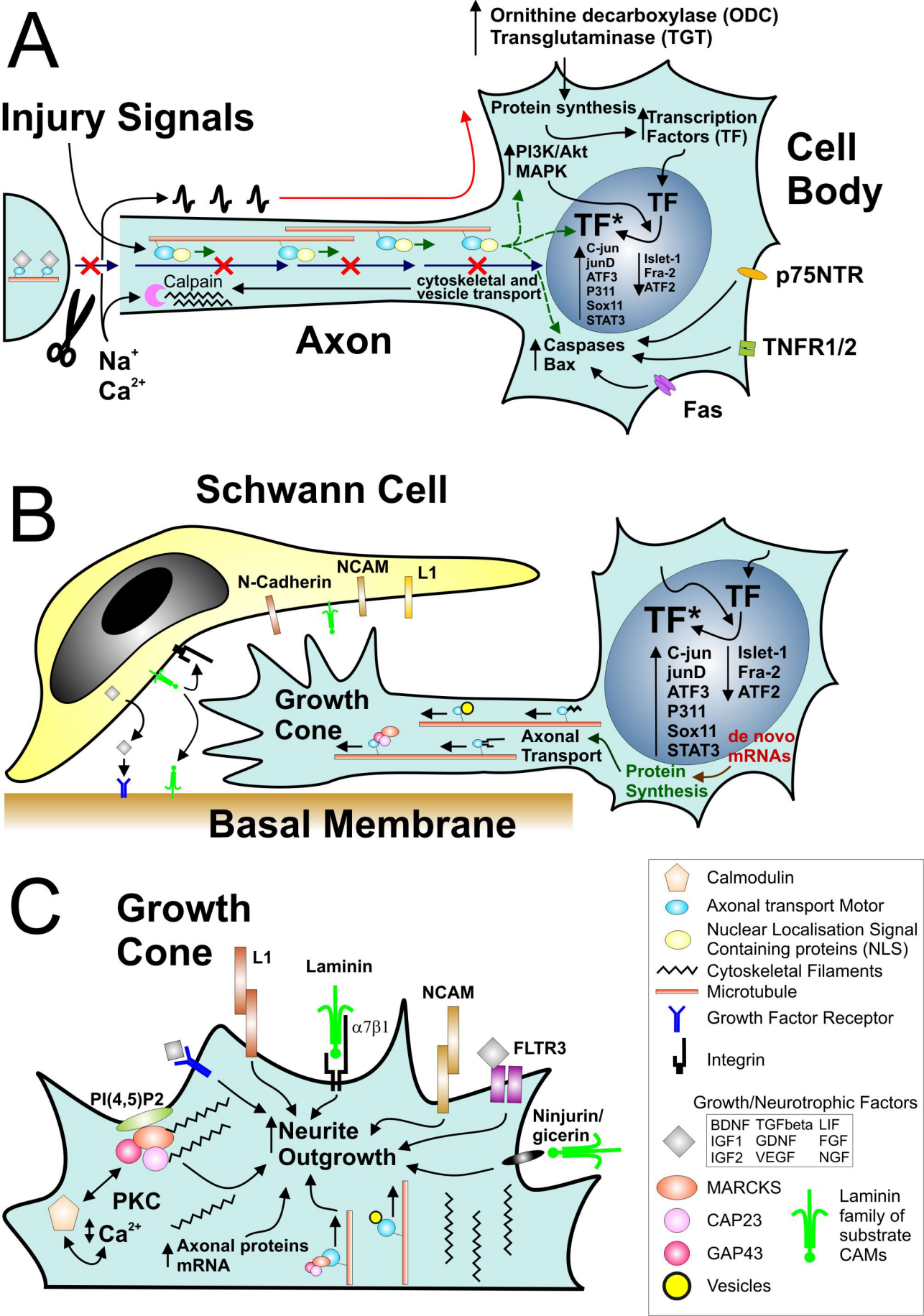
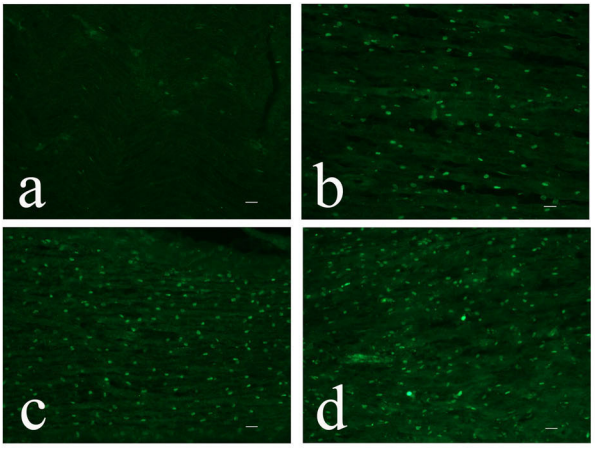
Figure Legends

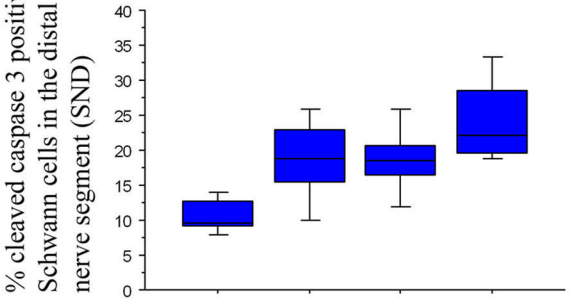
Figure 1. Schematic drawing of some of the signals that occur after injury in neurons and in the growth cone at the tip of the axon. Reproduced by kind permission of Raivich and Makwana.

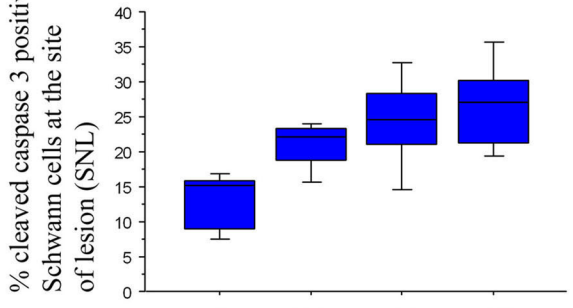
After injury there are both positive and negative injury signals that elicit a number of different events in the nerve cell body and in the nucleus of the neuron; all with the purpose to turn the neuron into a regenerative state instead of transmitting state (A). There is a close contact between the sprout with the growth cone and philopodia and the surrounding Schwann cells during the regeneration which occur along important basal membrane that contains laminin and fibronectin (B). In the growth cone actin filaments are assembled depending on local signal transduction mechanisms that stir the direction of the outgrowing axons (C).

Figure 2. Expression of cleaved caspase 3 which is a marker of apoptosis in Schwann cells after transection of a rat sciatic nerve. If a nerve injury is repaired with a delay [30 days delay in (C) and 180 days delayed repair in (D)] compared to an immediate nerve repair (B). The number of Schwann cells that express cleave caspase 3 increase close to the site of injury (lower diagram) and in the distal nerve segment (upper diagram). Staining of an uninjured nerve is attached in (A) as a comparison.


A
Ornithine decarboxylase (ODC)
Transglutaminase (TGT)
Injury Signals
Protein synthesis
Transcription Factors (TF)
PI3K/Akt
MAPK
Cell Body
TF
TF*
C-jun
junD
ATF3
P311
Sox11
STAT3
Islet-1
Fra-2
ATF2
p75NTR
Calpain
cytoskeletal and vesicle transport
Axon
Caspases
Bax
TNFR1/2
Fas
Na+
Ca2+
B
Schwann Cell
N-Cadherin
NCAM
L1
TF
TF*
C-jun
junD
ATF3
P311
Sox11
STAT3
Islet-1
Fra-2
ATF2
Growth Cone
Axonal Transport
de novo mRNAs
Protein Synthesis
Basal Membrane
C
Growth Cone
L1
Laminin
α7β1
NCAM
FLTR3
PI(4,5)P2
Neurite Outgrowth
Ninjurin/ gicerin
PKC
Ca2+
Axonal proteins mRNA
Calmodulin
Axonal transport Motor
Nuclear Localisation Signal Containing proteins (NLS)
Cytoskeletal Filaments
Microtubule
Growth Factor Receptor
Integrin
Growth/Neurotrophic Factors
BDNF TGFbeta LIF
IGF1 GDNF FGF
IGF2 VEGF NGF
MARCKS
CAP23
GAP43
Vesicles
Laminin family of substrate CAMs







Transection and delayed nerve repair (days)