Electrical activity suppresses intrinsic growth competence in adult primary sensory neurons - Implications for spinal cord regeneration -

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Vorgelegt von Joana Enes
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Erstgutachter: Prof. Dr. Benedikt Grothe

Zweitgutachter: Prof. Dr. Alexander Borst

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München, 20 Januar 2009

Joana Enes

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ABBREVIATIONS

AP	action potential
BBB	blood-brain barrier
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
$Ca_v1.2$	pore-forming subunit of L-type calcium channel
cAMP	cyclic AMP
CNS	central nervous system
CREB	cAMP response element binding protein
CSPG	chondroitin sulfate proteoglycan
CTB	cholera toxin β-subunit
Da	Dalton (g/mol)
DAB	3, 3´diaminobenzidine
DCL	dorsal column lesion
DRB	5,6-dichlorobenzimidazole riboside
DRG	dorsal root ganglia
E	embryonic day
GC	growth cone
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
h	hour
H_2O	water
HBSS	Hank´s balanced salt solution
HEPES	N-2-hydroxyethylpiperanzine-N´-2-ethanesulfonic acid
K^+	potassium ion
IL-6	interleukin-6
IP_3	inositol 1,4,5 triphosphate
Kb	kilo bases

KCl	potassium choride
KO	knock-out
LIF	leukemia inhibitory factor
MAG	myelin-associated glycoprotein
min	minutes
mRNA	messenger ribonucleic acid
Na^+	sodium ion
NGF	nerve growth factor
NT-3	neurotrophin-3
Omgp	oligodendrocyte myelin glycoprotein
ON	overnight
P	postnatal day
PBS	phosphate buffer solution
PFA	paraformaldehyde
pH	*potentium hydrogenii*
PNL	peripheral nerve lesion(ed)
PNS	peripheral nervous system
RAGs	regeneration-associated genes
RB	retraction bulb
rpm	revolutions per minute
RyR	ryanodine receptors
SCI	spinal cord injury
Sec	seconds
TTX	tetrodotoxin
VGCC	voltage-gated calcium channel
wt	wild-type
µ	micro

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Abstract

The ability of neurons to regenerate in the adult mammalian central nervous system (CNS) is often poor, leading to persistent deficits after injury. Failure of axon regeneration in the CNS has been attributed to the presence of an extrinsic inhibitory environment and to an intrinsic limitation to support growth. Remarkably, in adult primary sensory neurons of the dorsal root ganglia (DRG), a peripheral lesion primes neurons to grow and to override the inhibitory environment. Under this condition not only their peripheral axons regrow, but also their injured central axons coursing in the spinal cord regenerate. However, the nature of the signal that is sensed by the cell upon peripheral lesion to initiate the regenerative response is poorly understood.

This study started from the hypothesis that electrical silencing caused by peripheral deafferentiation is an important signal to trigger axon regrowth in adult DRG neurons. I first examined the effect of electrical activity on axon growth of cultured DRG neurons. I found that either chronic depolarization or electrical field stimulation strongly inhibits axon outgrowth in cultured DRG neurons. The inhibitory effect depends on Ca^{2+} influx through L-type voltage-gated calcium channels and involves transcriptional changes. Consistently, after a peripheral lesion, L-type current is diminished and the L-type pore-forming subunit $Ca_v1.2$ is downregulated. To determine whether the lack of L-type channels is sufficient to promote axon growth, mice lacking the pore-forming subunit of L-type channel, $Ca_v1.2$, in the nervous system were generated. Neurons isolated from adult $Ca_v1.2$ knockout (KO) mice grew more extensively than those from their control littermates.

Taken together, these data provide evidence that electrical activity is a limiting factor for axon growth in adult DRG neurons and that releasing this "brake" is sufficient to induce axon growth. My results further suggest that electrical silencing might promote axon regeneration *in vivo*. Consequently, I have

attempted to apply this knowledge to a model of spinal cord injury. However, these *in vivo* experiments have been so far hampered by technical limitations. Further endeavors are currently in progress.

1. Introduction

All nervous system functions such as motion, sensation and cognition depend on the rich diversity of nerve cells and their interconnections via axon pathways that form complex functional neuronal circuits. During evolution, however, circuit complexity seems to have developed at the expense of regenerative ability (Popovich and Longbrake, 2008). Whereas axons in the adult central nervous system (CNS) of "lower" vertebrates regenerate e.g. in optic nerves of fish or in the spinal cords of lampreys, axons do not regenerate in the adult mammalian CNS. Consequently, injury leads to permanent disconnection and loss of function. Therefore, the understanding of the basic mechanisms that hinder regeneration in the adult mammalian CNS is crucial for the development of strategies to promote recovery of circuit connectivity and function.

This study investigates the mechanisms that prevent adult primary sensory neurons to regenerate after spinal cord injury. The following chapters introduce the mechanisms of axon growth and of the failure of regeneration after spinal cord injury. The subsequent chapters are dedicated to the signal we propose to inhibit regeneration, namely electrical activity.

1.1 Axon growth and axon regeneration in the mammalian nervous system

How is the complex mammalian nervous system formed in the first place?

The establishment of the proper connections among nerve cells during development takes place in a period of massive growth and plasticity. A remarkable feature of embryonic neurons is their ability to sustain axon growth

over long distances while searching for their appropriate targets (Figure 1A). Such rapid growth involves the coordinate actions of producing membrane and cytoskeleton elements in great amounts, transporting them to the right location and inserting them into the growing axon (Goldberg, 2003). To keep up with this high demand of cellular elements, longer axons may also rely on local axonal translation, in addition to the import of proteins synthesized in the cell body (Lin and Holt, 2008). In support of this idea, recent studies showed the presence of ribosomes, translation initiation factors and mRNAs in developing mammalian axons. Local axonal translation also seems to mediate axonal response to several stimuli.

At the tip of the growing axon, the growth cone senses the extracellular environment and determines the course and rate of axon growth (reviewed by Guan and Rao, 2003; Wen and Zheng, 2006). Diffusible or substrate-bound molecules present in the environment may either serve as attractants or repellents to influence the direction of growth-cone extension. To decipher the environmental cues, growth cones are enriched in guidance receptors which recognize molecules like netrins, slits, semaphorins, and ephrins, as well as cell-adhesion proteins that respond, e.g. to laminin. To steer axon growth, growth cones have a complex signaling network to translate ligand binding into pathfinding decisions. Although the molecular mechanisms through which these extracellular signals are integrated by the growing axon are not well defined, it is clear that most of the activated signaling cascades converge onto the cytoskeleton. Axon growth and turning involve a coordinated cross-talk between actin filaments and microtubules, the dynamic components of the cytoskeleton (Pak et al., 2008; Wen and Zheng, 2006).

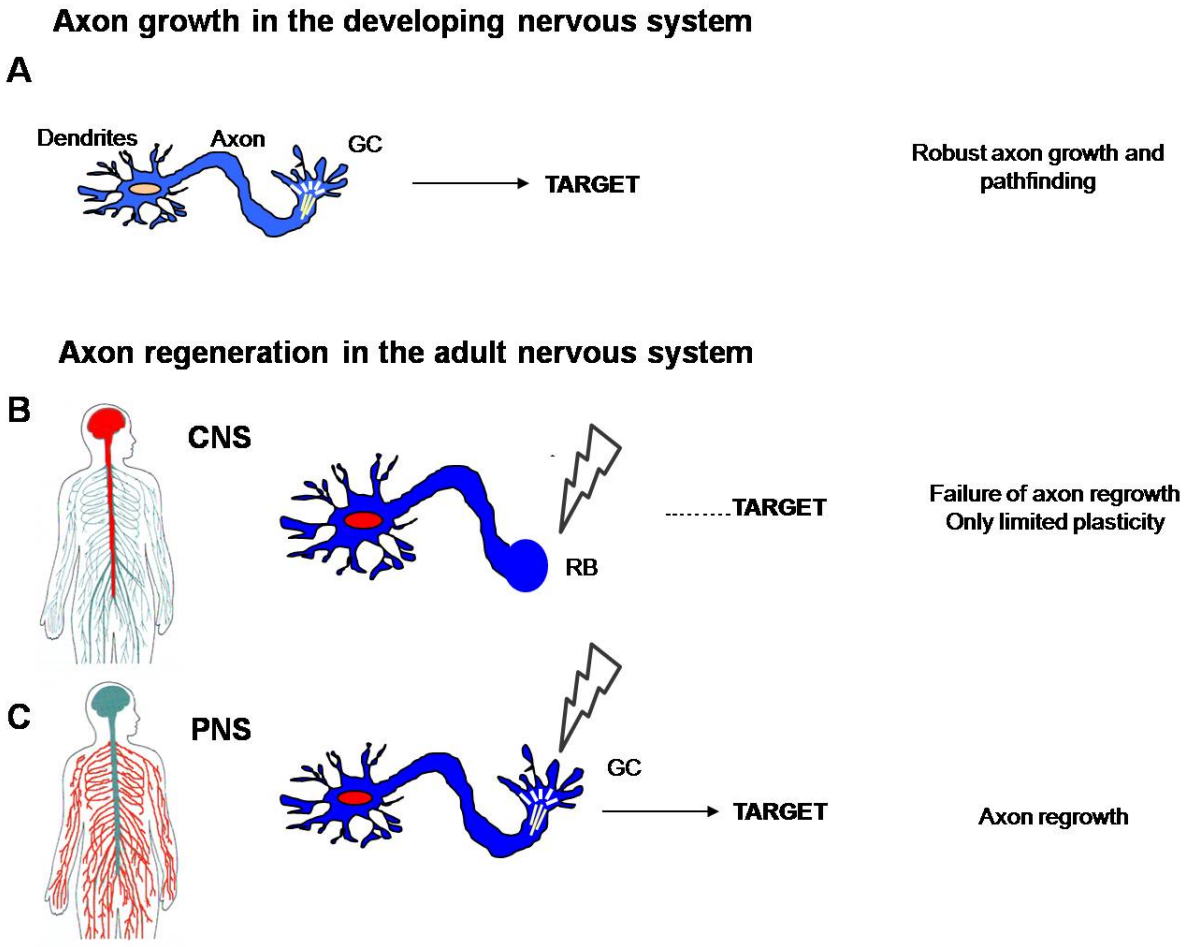

Figure 1 | Axon growth versus axon regeneration in the mammalian nervous system. **A |** During embryonic development, neurons support extensive axon growth to reach their appropriate targets and form a functional neural circuitry. At the tip of the growing axon, the growth cone (GC) explores the environment and responds to various trophic and guidance molecules. **B |** In the adult CNS (in red), severed axons stall at the lesion site and form retraction bulbs (RB) at their distal ends, representing the failure of regeneration. **C |** In the adult PNS (in red), severed axons can re-grow and often re-wire.

It has been suggested that the mechanism of axon growth is not a default pathway but must be specifically signaled (Goldberg, 2003; Goldberg et al., 2002). This signaling pathway is thought to decrease sharply when axons contact their targets, which occurs around birth. Upon growth cone arrival at the targeted

place, contact with the appropriate cell would transform the growth cone from a high motile structure into a stable synaptic terminal. Target contact would thus signal the switch from a "growth mode" to a "functional mode". Consistent with this idea, it was shown that spinal cord axons lose the ability to regenerate between P8 and P20 (Kalil and Reh, 1982), and Purkinje cells show a decline in growth ability at P0 (Dusart et al., 1997).

Therefore, it is not surprising that injured adult CNS neurons fail to revert to a rapidly regenerating mode and their axons stall at the lesion site (Figure 1B). Curiously, the inability to regenerate is a special feature of the central nervous system. Injured axons of peripheral nervous system (PNS) neurons do regenerate and often reestablish contact with their targets (Figure 1C). The reasons for this dichotomy between CNS and PNS regeneration will be addressed in further detail in chapter 1.3, in the context of spinal cord injury.

Although the extent to which axon regeneration represents a recapitulation of axon growth during development is still subject of debate, the high similarities found between both processes strongly suggest that it might be possible to re-apply basic developmental mechanisms to the adult context (Harel and Strittmatter, 2006; Snider et al., 2002; Ylera and Bradke, 2006).

1.2 Spinal cord injury (SCI)

The spinal cord transmits signals between the brain and body through long axonal pathways. The ascending sensory pathways conduct information from peripheral targets, such as skin and muscle, to the central nervous system. The descending motor pathways deliver information from the brain to the peripheral organs and muscles (Figure 2A).

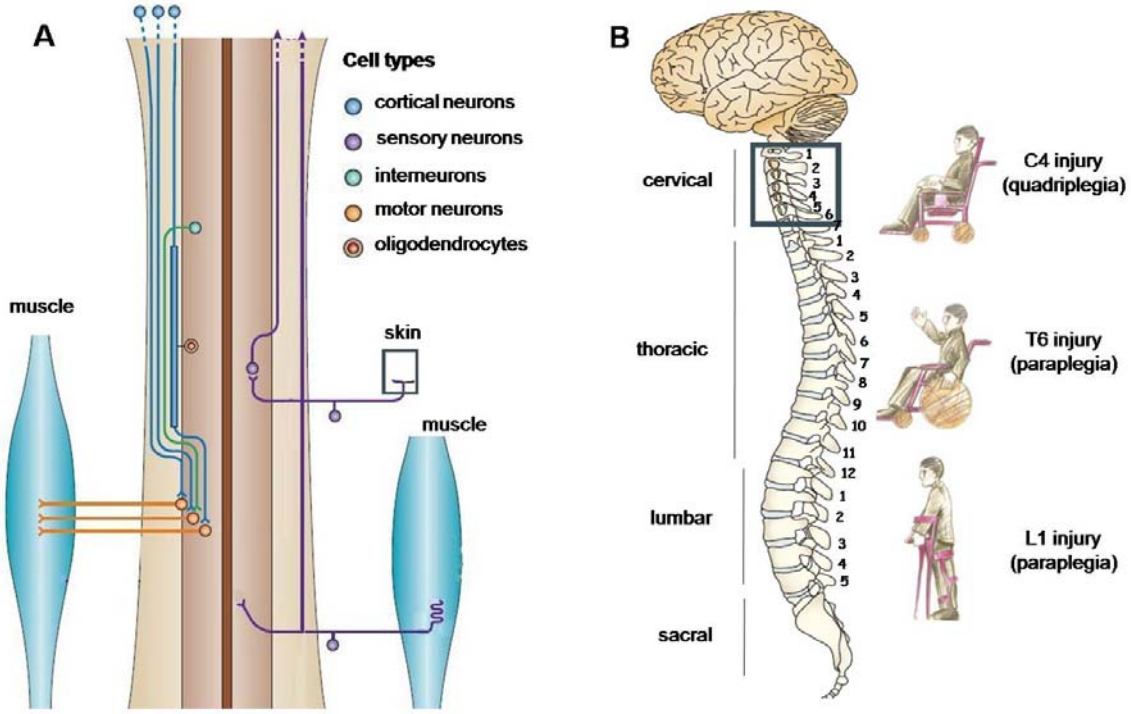

Figure 2 | Anatomy of the spinal cord. A | Primary sensory neurons collect information from noci-, thermo- and mechanoreceptors in the peripheral targets and convey it to the spinal cord and subsequently to the brain (right side). Motor neurons receive information from cortical, brainstem and spinal axons and transmit it to target organs and muscles (left side). **B |** Schematic representation of the human CNS. The spinal cord is organized in 4 major segments: cervical, thoracic, lumbar and sacral. An injury to the spinal cord results in loss of sensation or functional control of areas below the level of injury. Modified from (Thuret et al., 2006) and www.wingsforlife.com.

The spinal cord is segmentally arranged and the function of each segment depends on connections with supraspinal sites for all conscious or voluntary actions. Injury as a result of contusion, compression, or transection disrupts the normal function of the spinal cord below the level of injury (Figure 2B). Common symptoms range from loss of movement or sensation, to the inability to control bladder, bowel or cardiovascular function.

Although some neurons die as a result of injury, those symptoms reflect more the permanent interruption of the flow of information from the surviving neurons. Attempts of the severed axons to regenerate spontaneously can occur, but are often limited to short-distance compensatory sproutings (Blesch and Tuszynski, 2008; Bradbury and McMahon, 2006). No treatment to date can promote substantial functional recovery after spinal cord injury. The standard initial procedure aims to reduce secondary damage and cell death, often involving stabilization of the spine and administration of steroids to minimize inflammation. Afterwards, treatment is limited to physical therapy. A list of the therapies currently under research can be found in (Rossignol et al., 2007; Thuret et al., 2006), or at www.clinicaltrials.gov.

1.2.1 The factors that hinder regeneration after spinal cord injury

In seeking new ways to restore the connections of severed axons, one needs to understand why it fails. Failure of axon regeneration after SCI has been generally attributed to 1) the presence of an extrinsic inhibitory environment and 2) a limited intrinsic growth competence.

The extracellular inhibitory environment develops gradually after injury and ultimately forms a compact glial scar (Figure 3). As a consequence of the blood-brain-barrier (BBB) breakdown, the lesion site is quickly filled with blood and invaded by macrophages, meningeal fibroblasts and vascular endothelial cells

(Popovich et al., 1997; Sroga et al., 2003; Zhang et al., 1997). Around the lesion area, astrocytes become activated, hypertrophic and extend processes into the lesion site, forming a dense network. The injury also affects oligodendrocytes and damages the myelin structure surrounding the axons.

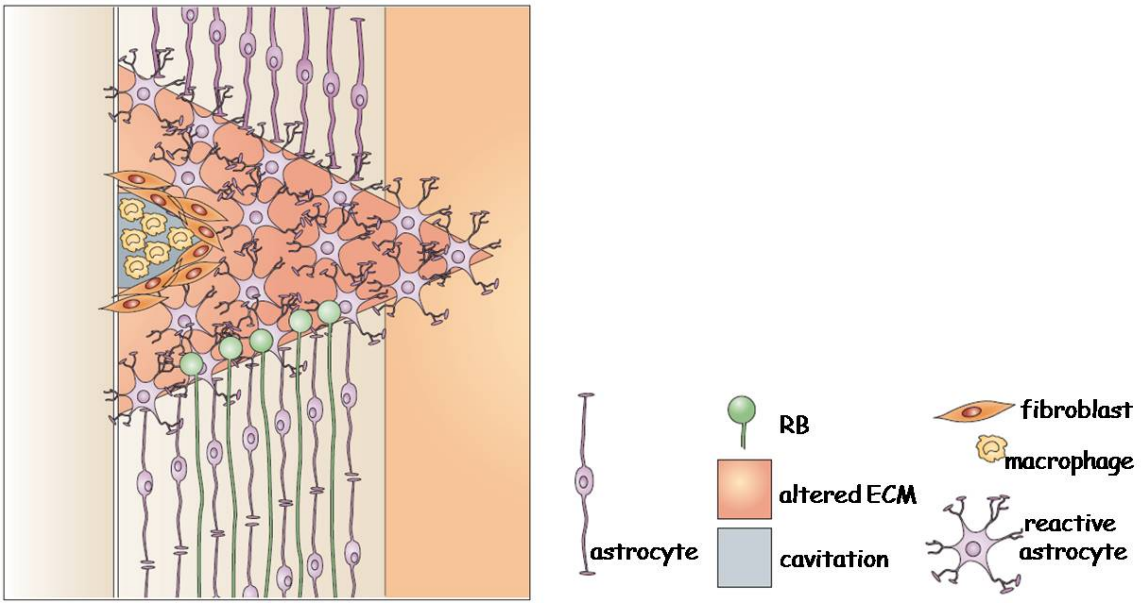

Figure 3 l Schematic representation of the injury site. After injury, a glia scar is formed primarily by reactive astrocytes. Several other cell types invade the lesion site too and secrete extracellular inhibitory molecules. Severed axons are repulsed by the increasing gradient of inhibitory molecules and lacking the ability to support growth and overcome inhibition, they stall at the lesion site forming retraction bulbs. Adapted from (Silver and Miller, 2004).

Besides forming a physical barrier, these altered cells also create a molecular barrier, up-regulating a variety of secreted and transmembrane growth inhibitory proteins. These include Nogo, oligodendrocyte myelin glycoprotein (OMgp), and myelin-associated glycoprotein (MAG) that are present in oligodendrocytes and their myelin debris, as well as chondroitin sulfate proteoglycans (CSPGs), semaphoring 4D and ephrin B3 produced by reactive astrocytes and oligodendrocyte precursor cells (Caroni et al., 1988; Filbin, 2003;

Silver and Miller, 2004). Because neurons express receptor complexes for several of these molecular cues, growth cones collapse and axons are repelled. Among the signaling mechanisms that mediate growth cone collapse are the activation of the RhoA pathway and the elevation in intracellular calcium (Yiu and He, 2006). Although the glial scar serves to stabilize the CNS tissue and to prevent an overwhelming inflammatory response (Okada et al., 2006; Popovich and Longbrake, 2008), it constitutes an impenetrable barrier which hinders long-distance regeneration.

In addition to the hostile extrinsic environment, adult neurons are not prepared to support axonal growth, i.e they are growth incompetent. The fact that adult neurons differ substantially from their embryonic counterparts in growth ability was evidenced by cell culture studies. When retinal ganglion cells (Chen et al., 1995; Goldberg et al., 2002) or DRG neurons (Fawcett et al., 1989) are isolated from animals of varying developmental ages and cultured under the same conditions, embryonic neurons extend axons at much higher rates than adult neurons. Moreover, neurons develop responsiveness to Nogo (Bandtlow and Loschinger, 1997) as they mature, and their response to MAG switches from growth-promoting to growth-inhibiting (Mukhopadhyay et al., 1994). These results clearly demonstrate that neurons undergo a profound loss in their intrinsic growth competence and increase responsiveness to inhibitory cues as they mature.

Therefore, therapeutic strategies to successfully promote regeneration must act on both extracellular and intracellular components. Besides finding ways to create a more permissive extrinsic environment, we need to learn more about the molecular mechanisms that can revert adult neurons to their embryonic axon growth ability to "rejuvenate" them. Primary sensory neurons are a good model to study intrinsic growth competence. Because they constitute the focus of this study, they will be presented in more detail in the next chapter.

1.3 Primary sensory neurons and the conditioning paradigm

1.3.1 One neuron, two different responses to injury

Primary sensory neurons of the dorsal root ganglia (DRG) present a pseudounipolar morphology, with two long axonal branches stemming from a single axon (Figure 4A). One axonal branch extends in the PNS and terminates at the skin or muscle, the other axonal branch enters the CNS.

DRG neurons are a diversified population of neurons (Purves et al., 2001). Based on their function, they can be divided into nociceptor-, thermoceptor-, and mechanoreceptor- afferent fibers. Nociceptor- and thermoceptor- afferents provide pain and temperature sensation. Mechanoreceptor- afferents code for touch and proprioception. Proprioceptors (meaning "receptors for the self") are located at the muscle spindles, Golgi tendon organs and joints, and give a sense of position and movement of one´s own limbs and body without using vision. This information is essential for the accurate performance of complex movements. Depending on whether they belong to the mechanosensory system or to the pain and temperature system, the first-order axons carrying information from the receptors have different patterns of termination in the spinal cord (Figure 4A, red and blue lines).

The mechanosensory DRG neurons, whose axons ascend in the spinal cord, provide a useful model to study central regeneration. Whereas the peripheral axonal branch regenerates after injury, the central axon does not regenerate when lesioned (Ramon y Cajal, 1928) (Figure 4B). Initially, this differential regenerative response was attributed solely to the extracellular environment, which is growth encouraging in the PNS but growth inhibitory in the CNS (David and Aguayo, 1981). However, the discovery that a preceeding lesion to their peripheral branches can promote regeneration of the severed axons in the spinal cord

challenged this idea (Neumann and Woolf, 1999; Richardson and Issa, 1984). It proved that neurons can indeed regenerate in the adult CNS if they are primed to do so. This paradigm, named "conditioning", shows that mature neurons can be reprogrammed to support growth. Nowadays, it is considered one of the most robust regeneration paradigms.

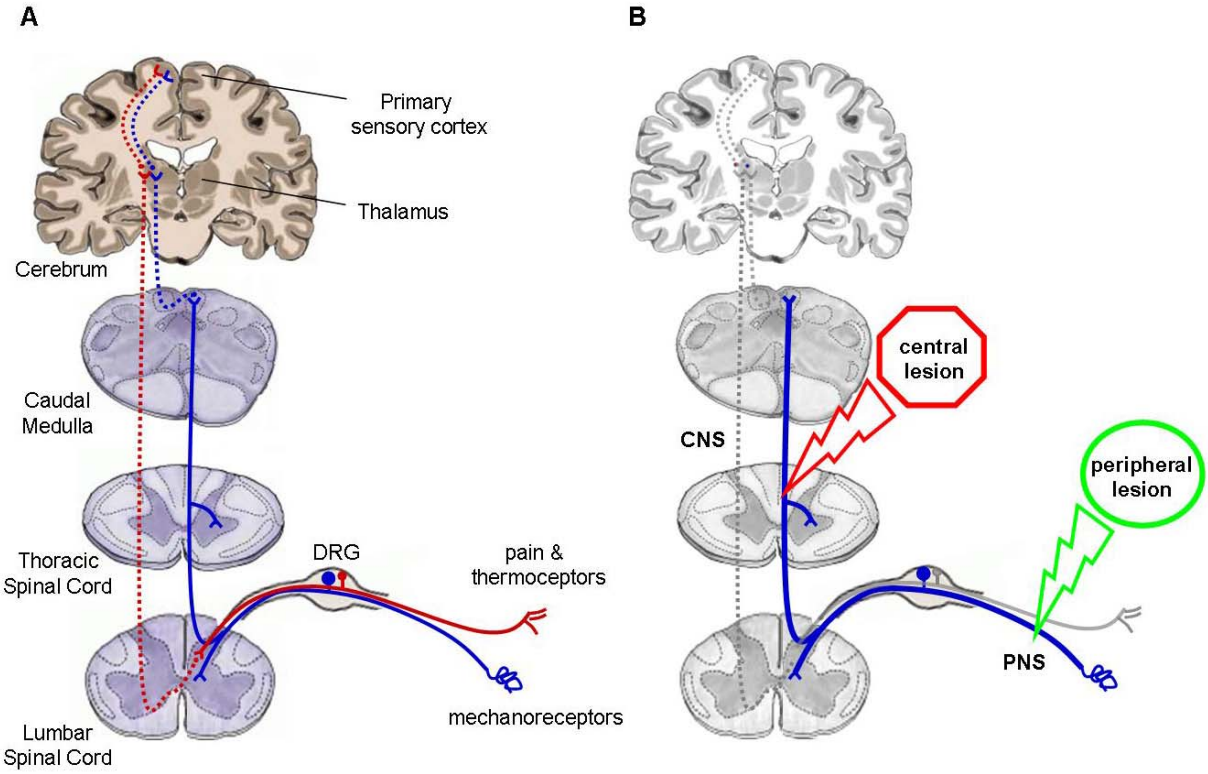

Figure 4 | Mechanosensory DRG neurons and their response to injury. A | Lumbar DRG neuron subtypes and their central pattern projections. Large myelinated, mechanosensory fibers (blue line) ascend in the spinal cord and terminate at the caudal medulla. Second- and third- order neurons (dashed blue line) deliver the information from the medulla to the primary sensory cortex. Collateral branches of mechanosensory fibers are present at the lumbar level, where they synapse onto motor neurons, and at the thoracic level where they communicate to neurons innervating the cerebellum. Small unmyelinated, pain- and temperature- fibers (red line) synapse immediately at the level where they enter in the spinal cord. Second- and third- order neurons (dashed red line) carry the information from there to the thalamus and farther to the primary sensory cortex. Modified from (Purves et al., 2001). **B |** DRG neurons regenerate after a

peripheral nerve lesion, but not after central nerve lesion. Importantly, when a conditioning peripheral lesion precedes a central lesion, axon regeneration is promoted both in the PNS and the CNS tissues.

Although the conditioning paradigm is known for a long time, the underlying cellular mechanisms remain poorly understood. Importantly, the growth-promoting effect can be replicated in cell culture to study the conditioning paradigm. Whereas isolated naïve neurons extend neurites only after 2-3 days, neurons subjected to a peripheral lesion a few days before isolation show robust neurite growth already at 24 h in culture (Hu-Tsai et al., 1994; Smith and Skene, 1997).

1.3.2 The knowns and unknowns about the conditioning paradigm

Since the conditioning effect was first described, there has been an ever growing list of proteins whose expression or activity is reported to be altered in the DRG after peripheral lesion (Raivich and Makwana, 2007; Snider et al., 2002; Ylera and Bradke, 2006). It is important to note, though, that the functional roles of several of those identified molecules remain undefined, not being possible to determine which ones are strictly necessary for the growth response. Here, I summarize all molecules strictly involved in the conditioning paradigm, based on the following criteria: 1) their expression level or activity is altered upon peripheral lesion; 2) they promote axon growth in DRG neurons; 3) they are necessary for the conditioning effect (Figure 5).

It is known that the switch of adult DRG neurons to a growth mode requires *de novo* transcription of genes specific for axon elongation (Smith and Skene, 1997). Accordingly, several transcription factors namely c-Jun, CREB and ATF3

(Herdegen et al., 1992; Seijffers et al., 2007; Tsujino et al., 2000), STAT3 (Qiu et al., 2005) and RARβ2 (Zhelyaznik and Mey, 2006) appear activated in a period of hours to days after injury. They play a key role in changing the gene expression profile in the injured neurons, leading to the transcription of regeneration-associated genes (RAG) that mount the growth response.

Among the RAGs there are cytosolic molecules that regulate the interaction between the cell surface and the cytoskeleton core of the growing axon. For example, the growth cone proteins GAP43 and CAP23 are upregulated after injury eliciting growth (Bomze et al., 2001). They interact with calmodulin, actin filaments and phosphoinositides, and regulate actin cytoskeleton polymerization, organization and disassembly. The Small Proline-Rich Repeat Protein 1A (SPRR1A) also appears upregulated by a peripheral lesion (Bonilla et al., 2002). It mediates the reorganization of the actin and microtubule cytoskeleton.

A modified expression of cell-adhesion molecules on the cell surface of the injured neuron appears to be another essential factor to promote growth and overcome extracellular inhibition. Axotomized neurons upregulate a variety of cell surface molecules which promote neurite outgrowth, namely α7β1 integrins which bind to the extracellular matrix component laminin (Condic, 2001; Werner et al., 2000), ninjurin involved in hemophilic binding (Araki and Milbrandt, 1996), and FLTR3 which can form complexes with FGF receptors and promote FGF signaling (Robinson et al., 2004; Tanabe et al., 2003). Signal integration at the growth cone in response to extracellular inhibitory molecules is also modified after conditioning. Myelin components bind to a receptor complex composed of NgR, LINGO and either p75 or TROY and inhibit growth via activation of the RhoA signaling pathway. However, conditioning leads to an increase of the intracellular cAMP pathway which interferes with the RhoA pathway, thereby preventing growth inhibition (Cai et al., 1999).

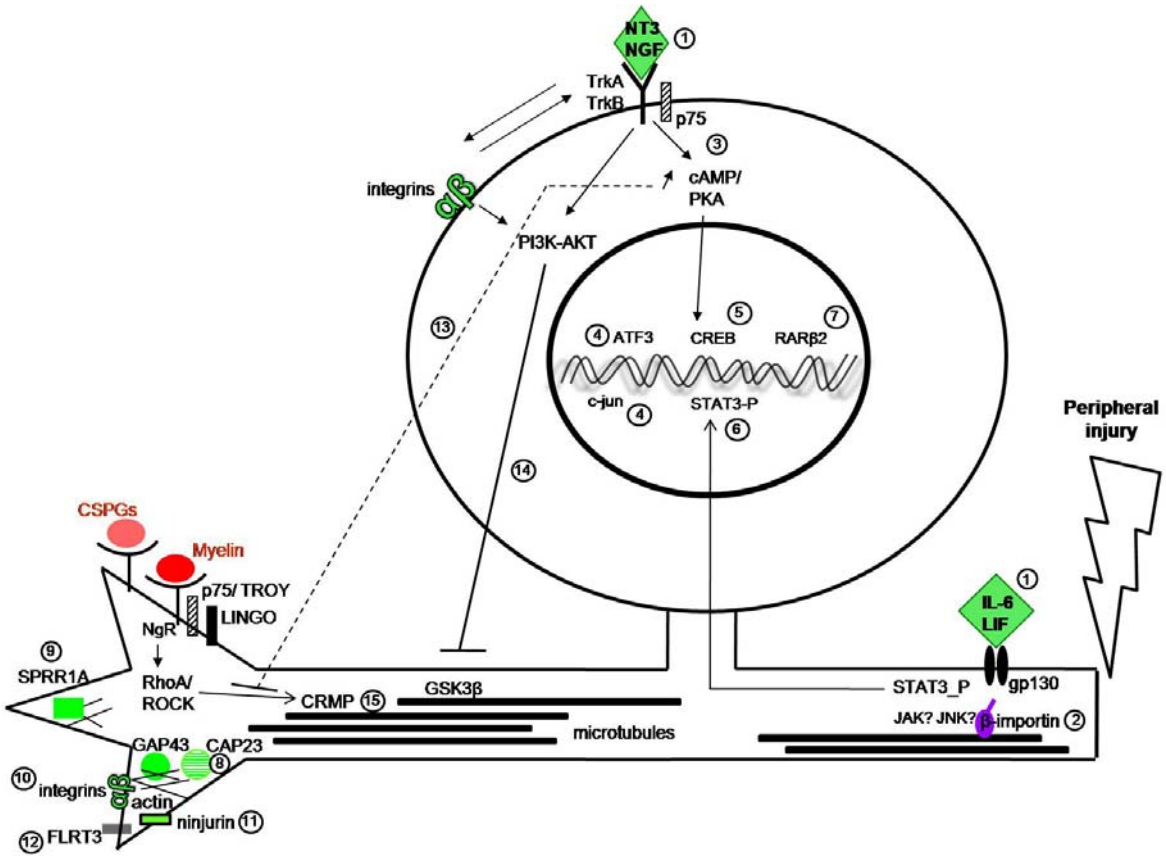

Figure 5 | Signaling pathways involved in peripheral axon regeneration of DRG neurons. A wide variety of molecules has been involved in the conditioning effect, including transcription factors, cytoskeleton proteins, molecular motors, cell-adhesion molecules and membrane receptors. How such coordinated program is brought into action in response to peripheral injury is unclear. In part, the growth program may be elicited by neurotrophins (NT3, NGF) and cytokines (IL-6, LIF), which are overexpressed by peripheral nerves in response to injury. Neurotrophic signals trigger cAMP elevation, blocking inhibition by myelin and activating CREB. Cytokines may activate axoplasmic proteins containing nuclear localization signals, which are then transported to the cell body by fast axonal transport (0.4 m / day) via the β-importin-dynein motor complex. The encircled numbers in the diagram refer to the following publications: 1) (Cafferty et al., 2004; Ramer et al., 2000); 2) (Hanz et al., 2003); 3) (Neumann et al., 2002; Qiu et al., 2002); 4) (Raivich et al., 2004; Seijffers et al., 2007); 5) (Gao et al., 2004); 6) (Qiu et al., 2005); 7) (Wong et al., 2006); 8) (Bomze et al., 2001); 9) (Bonilla et al., 2002); 10) (Condic, 2001; Werner et al., 2000); 11) (Araki and Milbrandt, 1996); 12) (Robinson et al., 2004; Tanabe et al., 2003); 13) (Cai et al., 1999); 14) (Zhou et al., 2004); 15) (Yoshimura et al., 2005).

Despite these major advances, the more elementary questions regarding the conditioning paradigm remain unanswered. What is the nature of the signal that ascends from the peripheral lesion site to the cell body to trigger the growth response? How to explain that growth is induced after peripheral but not after central lesion? It has been proposed that a peripheral lesion may activate the regenerative program either by i) exposing the tip of the injured axon to growth promoting **positive signals** present in the reactive PNS tissue or ii) interrupting the retrograde transport of growth inhibiting **negative signals** from the peripheral target. Molecules such as neurotrophins and cytokines have been identified as extracellular positive signals (Figure 5), as they are upregulated in peripheral nerves as early as 2 h after injury and promote growth of DRG neurons both *in vitro* and *in vivo* (Cafferty et al., 2004; Cafferty et al., 2001; Ramer et al., 2000). However, they do not fully reproduce the growth induced by a conditioning lesion (Cafferty et al., 2004; Smith and Skene, 1997), suggesting that additional signaling mechanisms are required for successful regeneration. No intracellular negative signals have been identified so far.

1.4 Electrical activity

Neurons are excitable cells that generate electrical signals to communicate within the nervous system. Remarkably, electrical activity is not only essential for neuronal function, but it also has a key role in circuitry assembly. Several studies in the past have highlighted the role of electrical activity in a wide variety of developmental processes, ranging from neuronal proliferation, migration and differentiation to synaptic formation and refinement (Hanson and Landmesser, 2004; Hubel and Wiesel, 1970; Spitzer, 2006; Sretavan et al., 1988; Zhang and Poo, 2001). Although the initial neuronal connections are guided largely by intrinsic developmental genetic programs, signaling by electrical activity can intermingle with regulatory mechanisms controlling gene expression and signal transduction. Changes in firing patterns may provide instructive information to the developing neuron regarding the developmental or functional state of the cell (Fields, 1998). Thus, electrical activity seems to be required to implement those hardwired programs in a temporally and spatially appropriate manner (Spitzer, 2006).

Given the prominent role of electrical activity during nervous system development, surprisingly little is known regarding its potential during adulthood. An interesting question is whether electrical activity still plays an important role when the structure of the adult CNS is disrupted, for example after spinal cord injury. In this study, the influence of electrical activity on the regenerative ability of adult sensory neurons is explored.

1.4.1 Effects of electrical activity on axon outgrowth

In cultured embryonic neurons, electrical activity can steer the growth cone and modulate axonal extension in a cell-type dependent manner. For instance, electrical stimulation potentiates axon growth in rat retinal ganglion cells (Goldberg et al., 2002) and sympathetic motor neurons (Singh and Miller, 2005). By contrast, electrical activity stops axon growth in mouse primary sensory neurons (Fields et al., 1990). The effect of electrical activity in adult cultured neurons has not been studied.

Activity-induced depolarization of the cell membrane triggers influx of Ca^{2+} ions, which is often amplified by the Ca^{2+}-induced-Ca^{2+}-release mechanism from internal stores (endoplasmic reticulum and mitochondria). Elevations in intracellular calcium levels ($[Ca^{2+}]_i$) can occur as localized or global events (Figure 6). Voltage-gated calcium channels (VGCC) are present in the cell membrane and open in response to membrane depolarization, constituting an important route of Ca^{2+} entry. Several types of VGCC have been identified, showing different kinetic, functional and pharmacological properties (Catterall, 2000) (table 1 in results).

Changes in $[Ca^{2+}]_i$ translate an electrical signal into biochemical signals (Hille, 2001), and therefore play a central role in regulating the neuronal response to electrical activity (Spitzer, 2006; Zheng and Poo, 2007). Indeed, it was shown that spontaneous as well as elicited Ca^{2+} increases can regulate axon extension in a variety of neuronal types (Bixby and Spitzer, 1984; Gomez et al., 1995; Gomez and Spitzer, 1999; Gu and Spitzer, 1995; Tang et al., 2003). In addition to mediating the effects of electrical activity, calcium signals also mediate the actions of several environmental cues. Extracellular gradients of attractants such as netrin-1 (Hong et al., 2000) and brain-derived neurotrophic factor BDNF (Song et al., 1997), or of repellents such as MAG (Henley et al., 2004; Wong et al., 2002) and Sema3A (Nishiyama et al., 2008) induce growth cone turning in a Ca^{2+}-

dependent manner. Therefore, both attractive and repulsive cues trigger calcium increases to affect axon guidance. Removal of extracellular calcium abolishes the growth cone response. Importantly, Ca^{2+} can also integrate local and global signals, generating different growth cone responses under different environmental conditions. For example, brief electrical pulses that raise $[Ca^{2+}]_i$ can enhance netrin-induced attraction and convert MAG-inducing repulsion into attraction (Ming et al., 2001).

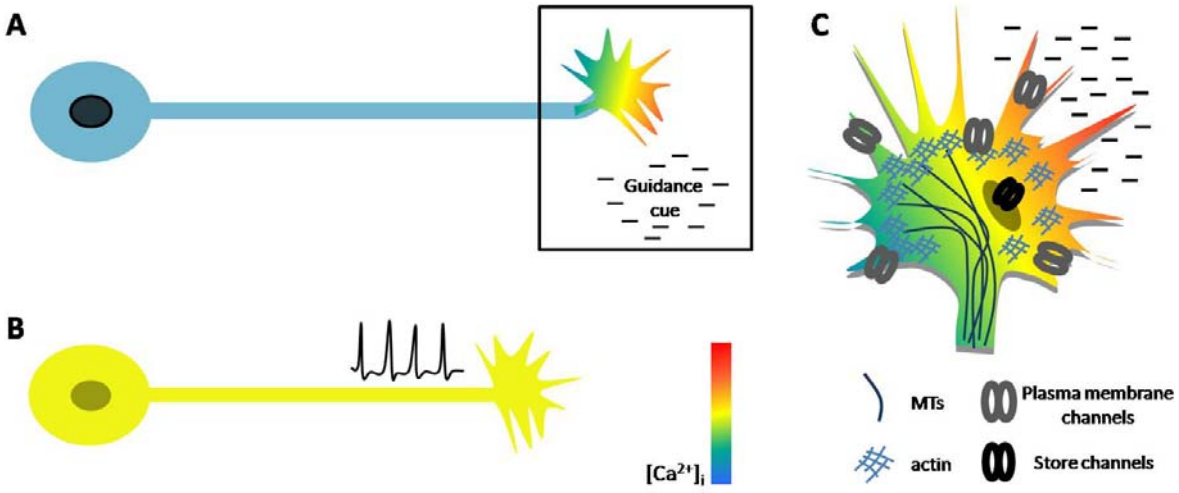

Figure 6 | Types of Ca^{2+} signals in growing neurons. Ca^{2+} signals range from **A |** highly localized signals at the growth cones to **B |** global events involving the entire growth cone or even the whole neuron. Detail of A in C. **C |** When Ca^{2+} signals are localized to one side of the growth cone, they lead to asymmetric activation of effector proteins to steer the axon. Instead, global Ca^{2+} signals are more likely to control axon elongation. The unique combination of the calcium signal properties and the type of neuron dictates the outcome of electrical activity. MTs, microtubules. Modified from (Gomez and Zheng, 2006; Zheng and Poo, 2007).

As evidenced from the aforementioned experiments, electrical activity and calcium signals are not universal regulators of axon growth. They have wide-ranging effects on growth cone guidance and axon extension. These seemingly

contradictory results were once unified under the "set-point hypothesis", which suggested that each neuron has an optimal range to support maximal outgrowth, above or under which growth is inhibited (al-Mohanna et al., 1992; Henley and Poo, 2004; Kater and Mills, 1991). Nonetheless, Ca^{2+}-mediated actions are more complex than simple changes in resting intracellular calcium levels. They depend on the combination of several other factors, including the spatiotemporal characteristics of the Ca^{2+} signals, the membrane channels and receptors involved in the process and the intracellular machinery through which the neuron integrates the signals it receives (Bootman et al., 2002; Henley and Poo, 2004; Zheng, 2000). All these factors determine the activation of downstream targets and the consequent response to the stimulus. In this context, it is important to notice that the neuronal properties themselves can vary accordingly to neuronal age, as it is the case of ion channel expression (Spitzer et al., 2002), or intracellular cAMP/cGMP ratio (Nishiyama et al., 2003). Neuronal age is therefore another factor that might alter the response to neural activity.

Eventhough the precise molecular mechanisms that mediate growth cone responses remain largely unknown, some important players have been identified (Gomez and Zheng, 2006). On one hand, Ca^{2+} signals can interact with components of the cytoskeleton to modulate the growth cone behavior. Actin filaments are particularly sensitive to $[Ca^{2+}]_i$. Several cytoskeleton binding proteins are regulated by calcium, namely the small Rho GTPases, Ca^{2+}-activated protease calpain and the actin-binding protein gelsolin. On the other hand, Ca^{2+} signals can act on second-messenger signaling cascades, altering cellular differentiation via gene transcription, or for e.g. increasing the density of trophic receptors on the surface of both RGCs and hippocampal neurons to potentiate growth by trophic factors.

1.4.2 Electrical excitability of primary sensory neurons

In developing DRG neurons, as in other neurons, electrical activity falls into 3 phases: an initial silent period, a spontaneous active phase and a stimulus-evoked active period (Fitzgerald, 1987).

The strong parallelisms found between the action potential (AP) firing patterns and the developmental processes of DRG neurons led to the idea that electrical activity acts as a landmark for the development of these neurons (Fields, 1998) (Figure 7A-C). The idea has been then supported by *in vitro* studies showing that the firing pattern influences a wide range of sensory developmental events. Initially, DRG neurons derived from neural crest cells emerge from the dorsal margin of the neural tube and migrate to the dorsal root ganglia. The period between E10 and E17 is of stunning axon outgrowth with each DRG neuron extending two axonal branches in opposite directions. At this stage, electrical silencing seems to be crucial to support axon growth, as electrical stimulation of embryonic DRG neurons rapidly stops axon elongation (Fields et al., 1990). The central axon terminals reach the spinal cord at E12 and extend collaterals to the gray matter around E15 (Fitzgerald, 1991). The peripheral branch reaches the peripheral target at around E17. At this time, neurons start to fire spontaneously, independent of sensory input and with low frequency (<0.5 Hz) bursts. The process of synaptogenesis begins, and remodeling of the initial connections takes place in an activity-dependent manner. Stimulated afferents from DRG neurons projecting to ventral spinal cord neurons become strengthened, while non-stimulated afferents get weakened (Nelson et al., 1989). Around the time of birth, sensory end terminals are fully differentiated, synapses are established, and neurons start to fire vigorously in response to sensory input. Stimulus-evoked activity is characterized by high frequency (10-20 Hz) bursts and can last as long as 10 seconds. Myelination begins at this stage and continues into the postnatal period. Electrical activity can also affect myelination, either controlling the

expression of cell adhesion molecules (Itoh et al., 1995) or mediating neuron-glia communication (Fields and Burnstock, 2006).

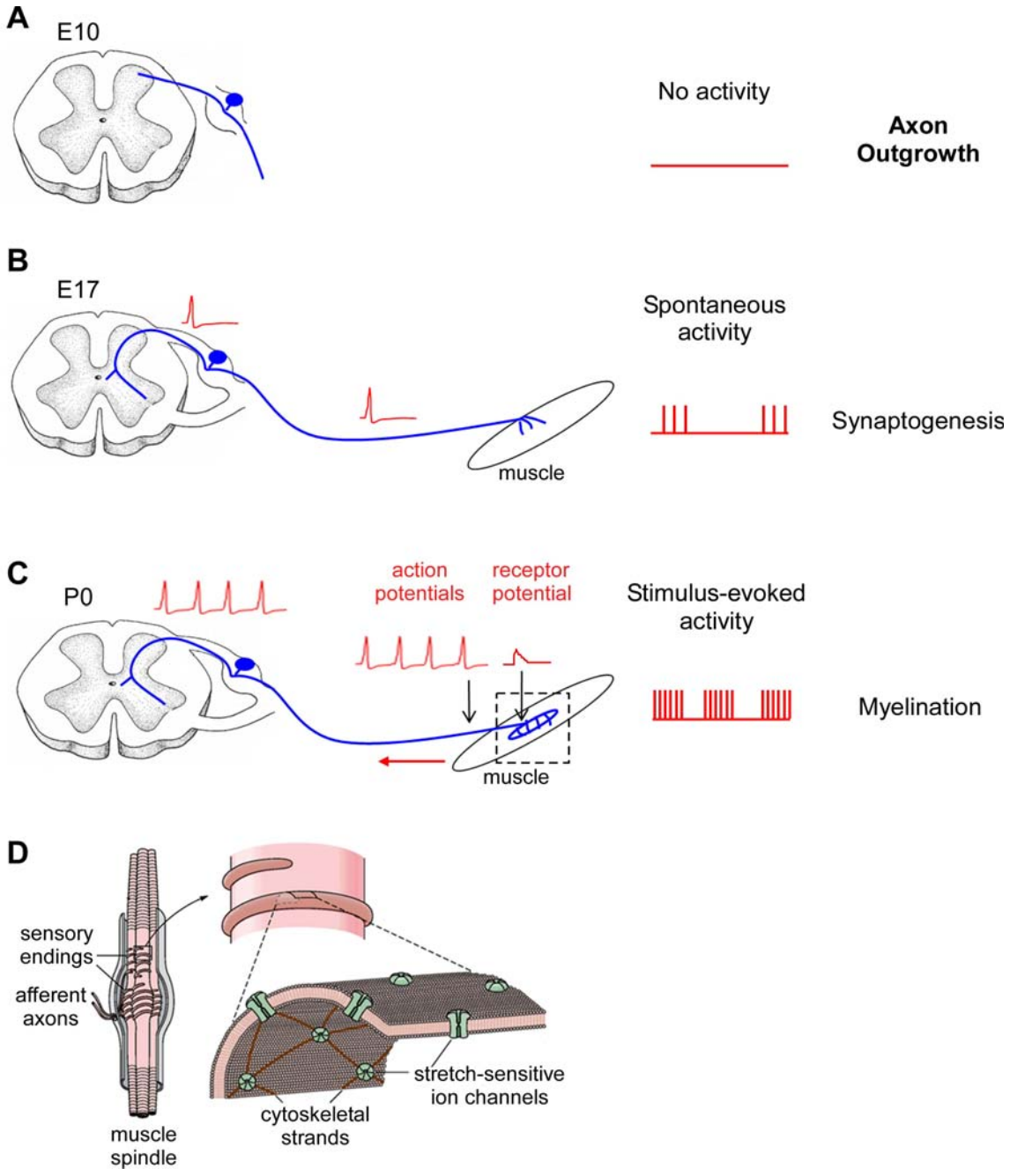

Figure 7 | Morphology meets function in primary sensory neurons (in previous page). **| A-C |** Physiological properties of developing DRG neurons. The different developmental epochs of DRG neurons are accompanied by changes in their firing patterns. **A |** Extensive axon growth takes place when neurons are still electrically silent. **B |** Muscle spindle differentiation and synaptogenesis occurs during a period of spontaneous activity. **C |** Myelination takes place when the contact with the peripheral target is established and neurons fire vigorously in response to sensory input. Red arrow indicates the direction of AP propagation, i.e. information flow. Detail of C in D. Modified from (Fields, 1998). **D |** Proprioception mediated by the spindle organ in skeletal muscle. Stretch of the muscle fibers causes mechanical deformation of the sensory endings membrane. Mechanoreceptors in the nerve membrane are linked to the cytoskeleton and therefore open in response to stretch, allowing the influx of Na^+ and Ca^{2+}. They generate a receptor potential that diffuses passively until the AP-trigger area. Adapted from (Gardner and Martin, 2000).

How are action potentials generated in response to sensory input? Despite the great diversity among the DRG neuronal population, they all transduce sensory information in the same fundamental way (Gardner and Martin, 2000). Sensory stimuli applied to skin or muscle activate specific ion channels present at the nerve endings, which generate a receptor potential. In the particular case of mechanoreception, stimuli such as pressure or muscle tension cause mechanical deformation of the cell membrane of the nerve terminal, which in turn opens stretch-sensitive channels named mechanoreceptors (Figure 7D). The receptor potential then propagates passively for a few μm until it reaches the AP-trigger area where a high density of Na^+ channels generates APs. There, if the receptor potential is sufficiently large, it triggers an AP that propagates actively along the peripheral and central axonal branches.

1.5 The thesis project

As outlined in the previous chapters, adult neurons cannot regenerate in the CNS partly due to a restricted growth competence. A better understanding of the cellular and molecular mechanisms that control growth ability is therefore essential to find new ways to promote repair in the adult nervous system.

This thesis project aims to understand the cellular basis of the conditioning paradigm established in primary sensory neurons, investigating the signals that trigger growth competence after peripheral lesion. The initial hypothesis was that electrical activity still suppresses axon growth in adult primary sensory neurons, and that a key event induced by peripheral lesion is electrical silencing. It is important to note that DRG neurons receive sensory input via their peripheral axon and therefore peripheral deafferentiation renders neurons silent. On the other hand, injury to the central branch leaves neurons electrically active. It thereby also explains why peripheral lesion, but not central lesion, leads to axon regrowth.

To validate our hypothesis, I first set out to examine the effect of electrical activity on axon growth of cultured adult DRG neurons. Having observed that electrical activity strongly inhibits axon growth, I investigated the downstream events mediating growth inhibition. I found that inhibition involves Ca^{2+} influx through L-type channels and *de novo* transcription of growth inhibitory genes.

Next, I analyzed how peripheral lesion changes the electrophysiological properties of DRG neurons. I have shown that peripheral lesion leads to a reduction in L-type current that is accompanied by a downregulation of the L-type pore-forming subunit $Ca_v1.2$ protein. These results suggested that electrophysiological changes might be associated with an increased growth competence, and raised the question of whether lack of the L-type channels would be sufficient to induce growth in adult DRG neurons. To this end, we genetically ablated the $Ca_v1.2$ protein in the nervous system and assessed growth

competence in culture. I found that neurons isolated from adult $Ca_v1.2$ knockout (KO) mice grew more extensively than those from their control littermates.
Further studies were aimed to implement this knowledge in an *in vivo* situation, but were so far inconclusive. They included 1) long-term blockade of AP propagation in the sciatic nerve of rats, and subsequent analyses of growth competence; 2) assessment of sensory fiber regeneration after spinal cord injury in $Ca_v1.2$ KO in comparison to control mice.

2. Results

2.1 Electrical activity inhibits axon growth in adult DRG neurons

I first investigated whether electrical activity affects neurite outgrowth in cultured adult sensory DRG neurons. These neurons are usually electrically silent in culture, as they are deprived of their innervation targets and they do not form synaptic connections with other DRG neurons (Scott, 1977). In order to mimic an electrically active state, I followed two approaches: chronic depolarization by high extracellular K^+ and electric field stimulation.

In the first approach, I depolarized neurons by incubating them in culture media containing 40 mM KCl and compared neurite outgrowth to control neurons 3 days after plating. This treatment alters the physiological electrochemical gradient for K^+ ion - which normally maintains the cell membrane at the resting membrane potential of -70 mV - and thereby depolarizes the cell membrane to about -20 mV (following the Nernst equation) (Lu et al., 2006). I found that membrane depolarization drastically reduces the number of neurons with neurites (Figure 8 A-D and 1I) as well as neurite their neurite length (Figure 8J). While 74 ± 3.4 % of neurons show neurites after 3 days in control conditions, only 29 ± 3.5 % of the depolarized neurons form neurites. Among the neurons forming neurites, a reduction in average neurite length from 209 ± 54.1 μm in control neurons to 70 ± 6.9 μm in depolarized neurons was also observed. To assure that inhibition of growth was not caused by a higher medium osmolarity, I examined neurons cultured in medium with equally high osmolarity achieved by addition of 40 mM NaCl. I observed no differences in growth when compared to control neurons (Figure 8E and 8F). As an additional control, I confirmed that cells cultured under depolarizing conditions remain viable. Depolarized cells returned to control medium for 3 additional days showed extensive neurite

outgrowth (Figure 8 G-H). Thus, these results show that depolarization inhibits axon outgrowth in cultured adult DRG neurons.

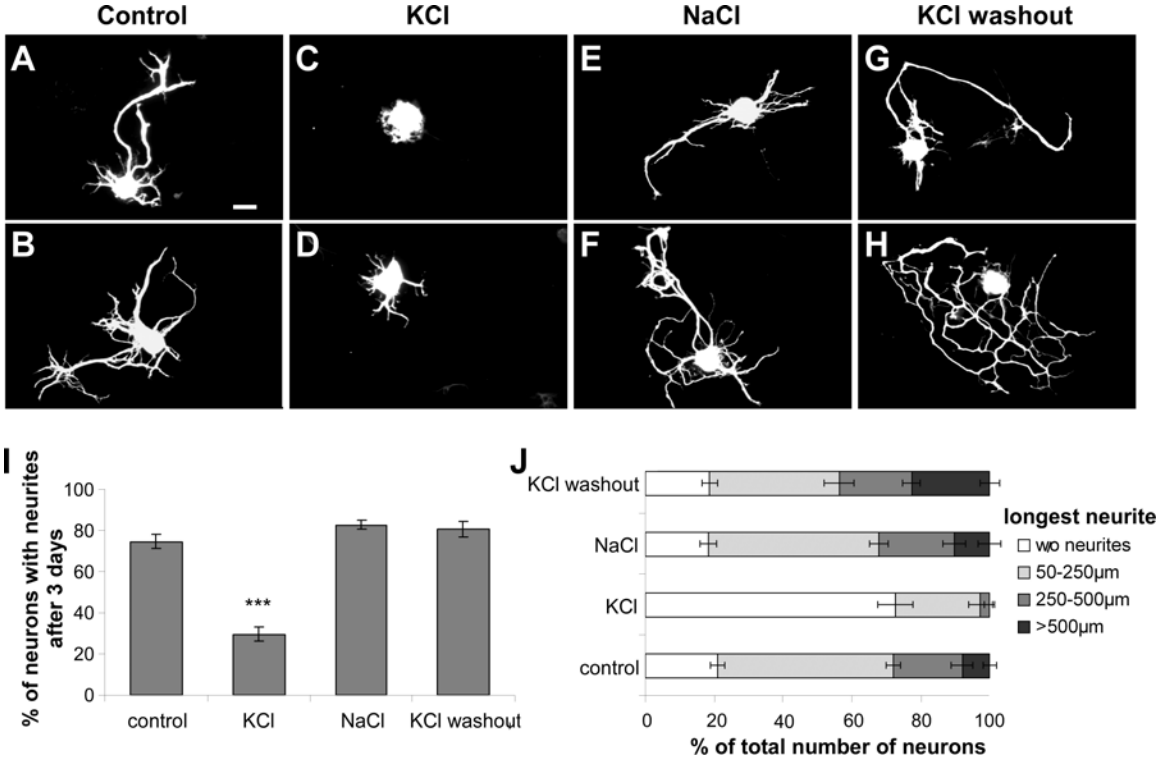

Figure 8 | Depolarization inhibits axon outgrowth on adult dorsal root ganglion (DRG) neurons. Neurons were isolated from adult rats and cultured on poly-lysine for 3 days in **A and B |** control, **C and D |** 40 mM KCl- or **E and F |** 40 mM NaCl- containing media. **G and H |** Neurons treated for 3 days with 40 mM KCl, and subsequently replaced in control media for 3 additional days. Scale bar, 25 µm. **I |** Quantification showing the percent of neurons bearing neurites (mean ± SEM; 3 independent experiments). ***P<0.001. **J |** The longest axon on about 300 neurons per condition was measured (mean ± SEM).

To study whether more physiological levels of activity can also inhibit growth, I next assessed the effect of electrical stimulation in growing neurites. In order to stimulate neurons to fire action potentials (APs), I applied brief trains of voltage pulses intercalated with long off periods (Figure 9A), in a pattern that resembles their sensory stimulus evoked activity (Fitzgerald, 1987). Through separate

patch-clamp experiments, I validated that the stimuli were suprathreshold, i.e. neurons fire APs in response to the stimulation paradigm (Figure 9B).

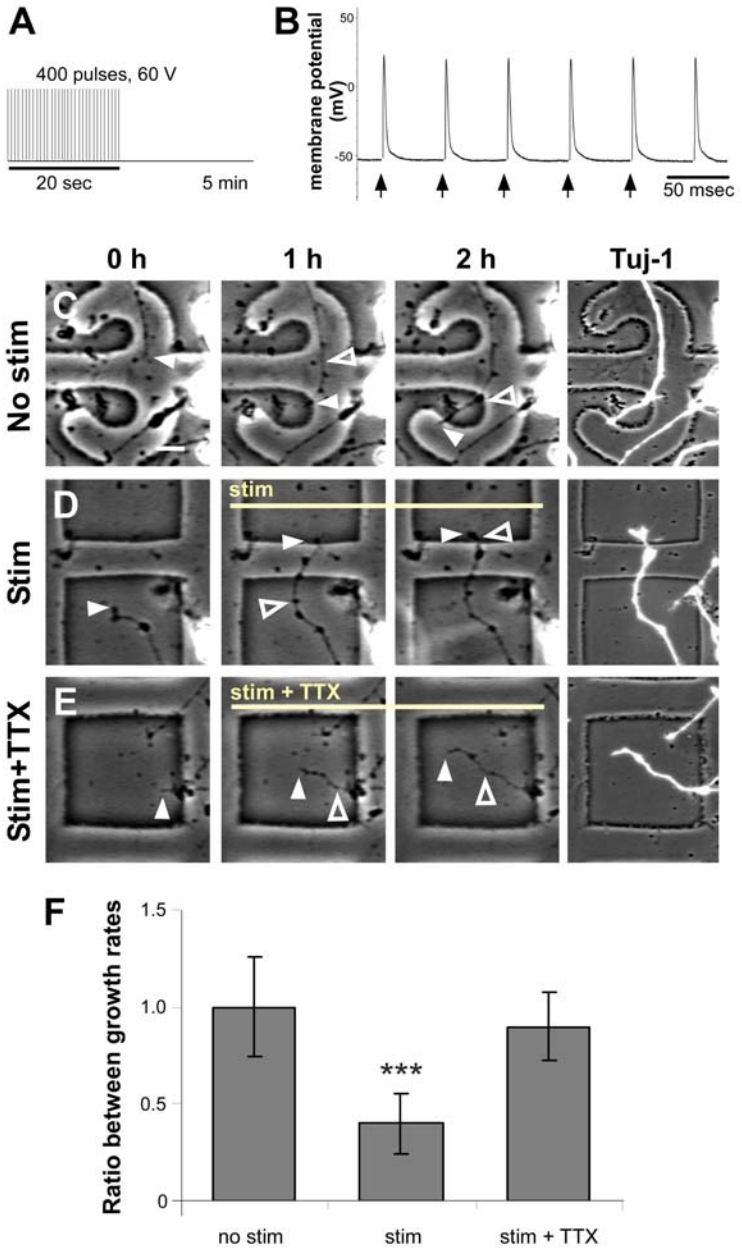

Figure 9 | Electrical activity halts axon elongation. Neurons were cultured on laminin-coated coverslips containing an orientation grid to allow re-localization of the same neurite over time. Brief electric pulses were applied to the neurons through two electrodes placed in the culture dish. **A |** Illustration of the stimulus pattern applied to the cells. **B |** Whole-cell recording, showing that cells fire an action potential in response

to each electric pulse (arrows). **| C-E |** Only neurites growing during the first hour without any stimulus (0 – 1 h) were further evaluated for their growth under **C |** no stimulation, **D |** stimulation, and **E |** stimulation in the presence of 1 µM TTX, for another hour (1 – 2 h). Filled arrow head points to the tip of the process, and its distance to the open arrow heads indicates the growth of the neurite. The identity of the neurite was confirmed in the end of the experiment by fixing and staining the cells with Tuj-1 antibody. Scale bar, 10 µm. **F |** Data analysis comparing axon growth rates on the 2 time periods for individual neurites (mean ± SEM; n > 20 neurites for each condition). ***P<0.001.

Elongation of individual neurites was evaluated in periods of electrical silence and activity. Whereas neurites grew at a constant growth rate when no stimulation was applied to the cells (Figure 9C), neurite growth rate was reduced to approximately 40% with the onset of electrical stimulation (Figure 9D and 9F). Cessation of growth could be largely prevented by the addition of the Na^+ channel blocker tetrodotoxin (TTX) (Figure 9E), showing that inhibition is dependent on AP firing. Taken together, these findings demonstrate that electrical activity impairs the growth ability of adult DRG neurons. This argues that electrical silencing is necessary for axon growth.

2.2 Axon growth inhibition is mediated by L-type voltage-gated Ca^{2+} channels and involves transcription of growth inhibitors

To gain insight into the mediators of axon growth inhibition, I looked for factors that could counterbalance the effect of electrical activity on DRG neurons. I therefore examined growth of depolarized neurons in the presence of specific VGCC blockers after 40 h in culture. Five different types of VGCC have been described to coexist in DRG neurons, T/P/Q/N and L-type, differing in their kinetics and pharmacological properties as listed in **table 1** (see page 36) (Catterall, 2000; Rusin and Moises, 1995; Tsien et al., 1988). The low-voltage-activated T-type current is rapidly inactivated by keeping the cells at a depolarized membrane potential of about -20 mV, which occurs in the presence of high K^+. Thus, only the high-voltage-activated calcium currents (P/Q/N and L-type) are likely to mediate the effect of electrical activity. Growth inhibition persisted in the presence of *w*-conotoxin MVIIC which blocks P/Q/N-type channels (Figure 10C and 10E), suggesting that these channels are not involved in inhibition induced by high K^+. In contrast, growth inhibition was partly prevented by specifically blocking the L-type channels with nifedipine (Figure 10D). The percentage of neurons bearing neurites was significantly higher in depolarized cells treated with nifedipine (27 ± 3.0 %) than that of non-treated depolarized neurons (8 ± 0.5 %). Conversely, L-type channel activation by the agonist BayK impaired growth (Figure 10A and 10B; 47 ± 5.0 % in control cells versus 30 ± 3.5 % in BayK-treated cells). These data indicate that the inhibitory effect of electrical activity on axon growth is, at least in part, dependent on L-type channel activation.

Previous studies suggest that L-type Ca^{2+} channels regulate different cellular functions by modulating gene expression (Greer and Greenberg, 2008; West et al., 2002). Therefore, I sought to test whether depolarization inhibits axon growth

by inducing the expression of growth inhibitory genes. This possibility implies that growth under depolarizing conditions can be rescued when gene transcription is blocked. Since a discrete initial period of *de novo* gene transcription is itself necessary to support growth, I assessed the role of growth inhibitors at a later time point in culture, when neurons can grow for a period of 12 h in the absence of transcription (Smith and Skene, 1997). Therefore, blockers of gene transcription were added to cells for 12 h after an initial period of 40 h in culture, concomitant to depolarization evoked by 40 mM KCl.

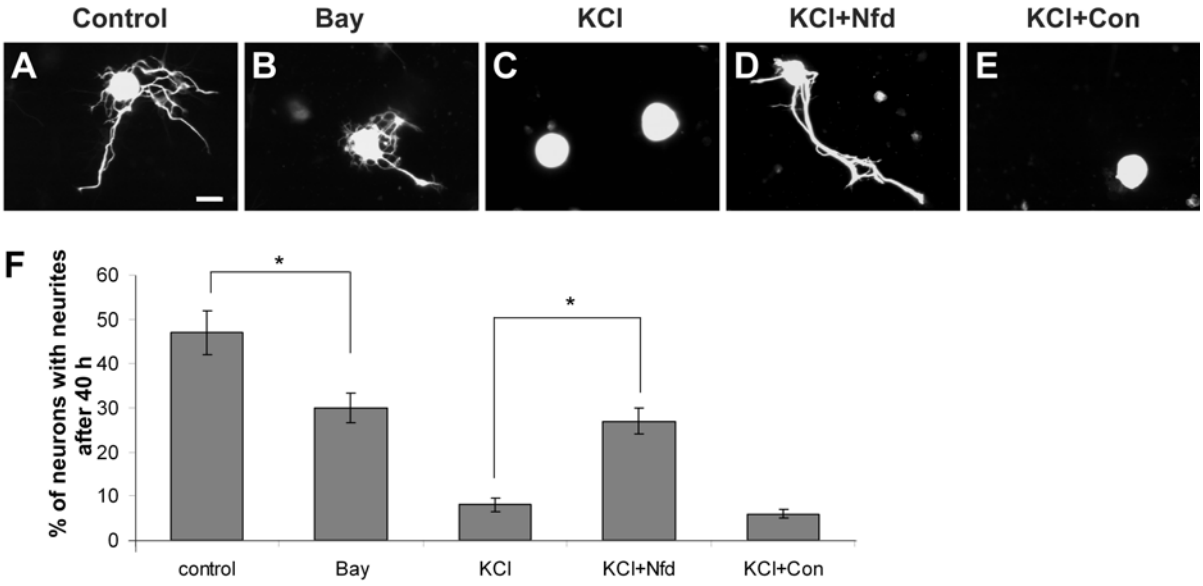

Figure 10 | Axon growth inhibition is mediated by L-type calcium current. Dissociated neurons were grown on poly-lysine for 40 h in the conditions as indicated. To determine the effect of L-type Ca^{2+} current activation, neurons were grown in the **A |** absence or **B |** presence of the L-type Ca^{2+} channel agonist Bay K 8644 (10 µM). The contribution of the different voltage gated Ca^{2+} currents to the inhibitory effect of neuron depolarization was assessed using specific channel blockers. Neurons were depolarized with 40 mM KCl alone **C |** or in the presence of **D |** L-type channel blocker nifedipine (10 µM) or **E |** general blocker for the other HVA Ca^{2+} currents *w*-conotoxin MVIIC (200 nM). Scale bar, 25 µm. **F |** Quantification of neurite formation under the different treatments (mean ± SEM; 3 independent experiments). * $P<0.05$.

When gene transcription was blocked by DRB, an inhibitor of RNA polymerase II, depolarization did not inhibit growth (Figure 11A-C and 11E). A similar result was obtained using another inhibitor of RNA polymerase II, α-amanitin (Figure 11D). The percentage of cells extending neurites in control, depolarized DRB-treated and depolarized amanitin-treated neurons was 59 ± 1.8, 59 ± 4.4 and 58 ± 5.1 %, respectively. These findings suggest that electrical activity triggers the expression of growth inhibitory factors that may interfere with the growth-associated genetic program.

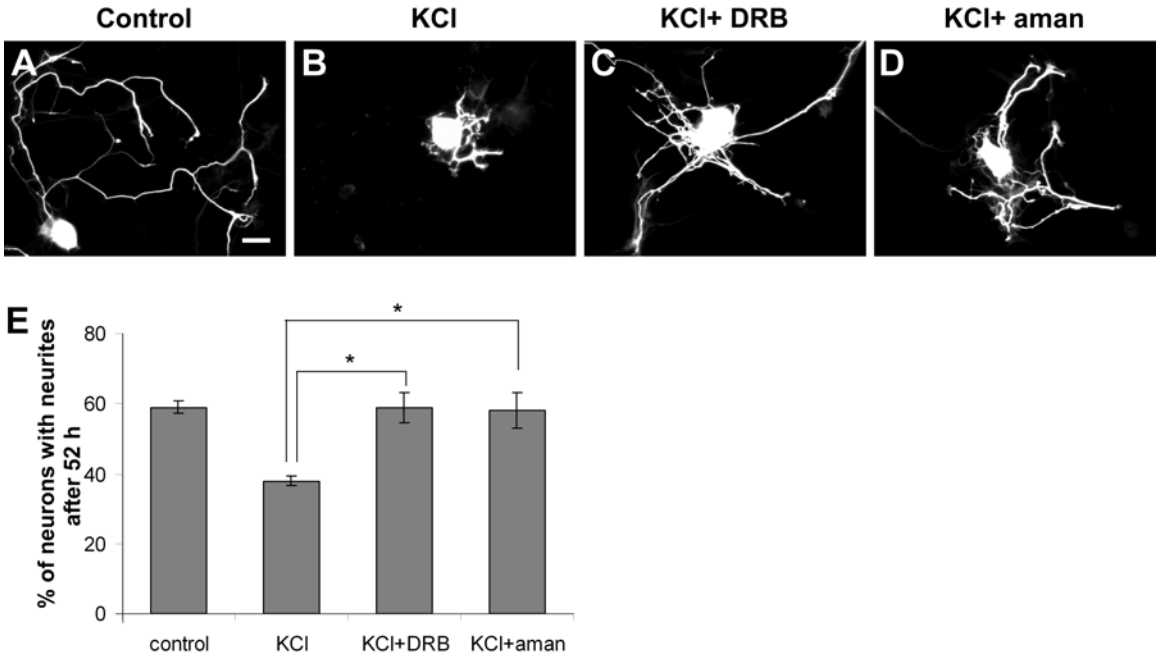

Figure 11 | Axon growth inhibition involves transcription of growth inhibitory genes. Cells were grown for 40 h on poly-lysine in control media, and thereafter in the mentioned media for a period of 12 h. **A |** Neurons cultured for 52 h in control media. **| B-D |** Neurons incubated in 40 mM KCl for the last 12 h in the **B |** absence or presence of gene transcription blockers, **C |** 40 μM DRB (C) or **D |** 500 nM α-amanitin. Scale bar, 25 μm. **E |** Quantification of neurite outgrowth (mean ± SEM; 3 independent experiments).

I next analyzed the response of DRG neurons to peripheral lesion, looking in particular for changes in their electrophysiological properties.

2.3 Peripheral nerve lesioned (PNL) neurons are not inhibited by neuron depolarization

A peripheral lesion strongly enhances the growth ability of the affected DRG neurons. Whereas naïve (unlesioned) neurons are quiescent during the first 24h after plating and require approximately 2-3 days to extend neurites, neurons subjected to a peripheral injury 3-7 days before isolation show robust neurite growth already at 24 h in culture (Neumann et al., 2002; Smith and Skene, 1997).
To further study whether electrical activity is determinant for the enhanced growth ability seen in adult DRG neurons after peripheral lesion, I next explored changes in the electrophysiological properties of PNL neurons that might allow them to circumvent inhibition and promote growth. I observed that unlike naïve neurons (Figure 8A-D), neurite growth on PNL neurons is not impaired by depolarization (Figure 12A, B and 12E). Incubation in 40 mM KCl does not significantly affect the number of PNL neurons extending neurites (58 ± 1.9 % versus 53 ± 1.9 %). Therefore, I next sought to identify the level of the inhibitory pathway at which insensitivity to electrical activity is conferred. A possible cause for insensitivity could be altered plasma membrane properties triggered by the lesion, specifically a reduced calcium influx. To assess this possibility, I asked whether an increase in $[Ca^{2+}]_i$ would inhibit growth of PNL neurons. To this end, I incubated neurons in caffeine to induce Ca^{2+} release from intracellular stores, and thereby resulting in an elevation of $[Ca^{2+}]_i$. I observed that incubation in caffeine substantially decreased outgrowth on PNL neurons (Figure 12C). Whereas 58 ± 1.9 % of the control neurons formed neurites, only 3 ± 0.9 % of the caffeine-treated neurons extended neurites. Because caffeine treated neurons grew neurites after the washout (Figure 12D), the inhibitory effect on axon growth is likely to be associated to an elevation in $[Ca^{2+}]_i$ and not to a decrease in cell viability. Notably, other pathways activated by caffeine, e.g. increase in intracellular cAMP level which was shown to enhance growth of adult DRG

neurons (Neumann et al., 2002), were not sufficient to counteract the inhibitory effect. These findings suggest that insensitivity to electrical activity after peripheral injury is conferred by a reduced Ca^{2+} influx.

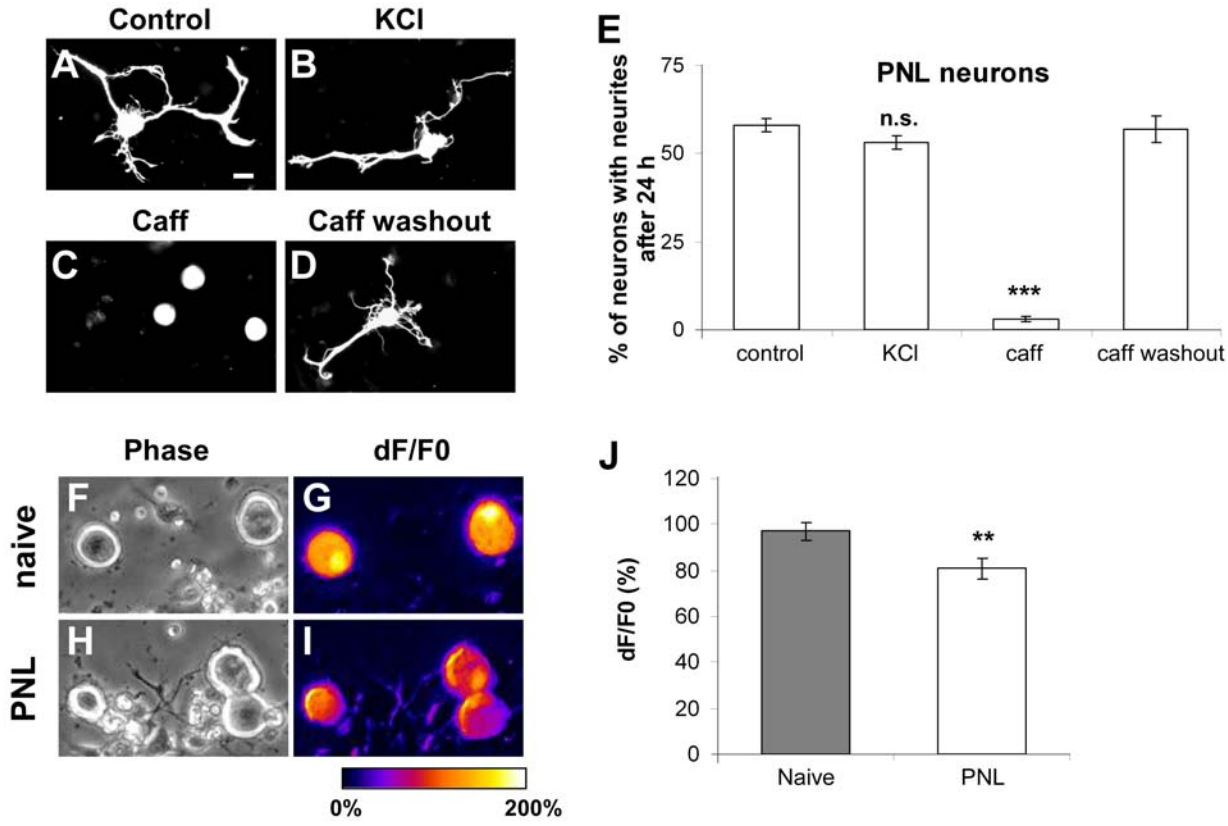

Figure 12 | Peripheral nerve lesioned (PNL) neurons are not inhibited by depolarization. | A-E | Neurons were isolated from rats subjected to peripheral axotomy 3-7 days before culture, and plated on poly-lysine for 1 day. Cells grown in **A |** control and **B |** 40 mM KCl media show extensive neurite formation, whereas cells cultured in **C |** 10 mM caffeine media (caff) exhibit fewer processes. **D |** Neurons cultured in media containing 10 mM caffeine for 1 day, and subsequently in control media for an additional day. Scale bar, 25 µm. **E |** Quantification showing the percent of neurons forming neurites (mean ± SEM; 3 independent experiments). ***P<0.001, n.s. not statistically different. **| F-I |** Increase in $[Ca^{2+}]_i$ upon KCl stimulation was assessed by Ca^{2+} imaging. Representative images of the relative fluorescence changes (ΔF/F0) show a stronger calcium signal on **| G, phase picture F |** naïve neurons than on **| I, phase picture H |** PNL neurons. Color scale represents relative fluorescence intensities. **J |**

Average of the maximum ΔF/F0 values obtained for each control or PNL neuron (mean ± SEM; n > 35 neurons per condition). ** P<0.01. Scale bar, 25 μm.

To test this idea I loaded DRG neurons with the calcium indicator Oregon Green BAPTA-1 AM and measured the elevation in $[Ca^{2+}]_i$ in response to a pulse of high K^+. A small but significant decrease in $[Ca^{2+}]_i$ response was measured in PNL neurons (Figure 12H-J) in comparison to naïve neurons (Figure 12F and 12G). This result shows that intracellular Ca^{2+} levels are altered after a peripheral injury, leading to a smaller Ca^{2+} signal upon depolarization. Overall, these data suggest that peripheral lesion triggers electrophysiological changes that allow adult DRG neurons to escape the growth inhibitory effects of electrical activity.

Table 1 | Types of voltage-gated calcium channels in DRG neurons. HVA, high-voltage activated; LVA, low-voltage activated. Adapted from (Hille, 2001).

	Slow, persistent	**Fast, inactivating**	
	HVA	HVA	LVA
Tsien type	L	P, Q, R	T
Nomenclature	Cav1.x	Cav2.x	Cav3.x
Opens at	-30 mV	-20 mV	-70 mV
Channel openings	continual reopening	long burst	brief burst, inactivation
Inactivation range	-60 to -10 mV	-120 to -30 mV	-100 to -60 mV
Inactivation	very slow (>500 ms)	partial (50-80ms)	complete (20-50 ms)
Blocked by	Nifedipine	Conotoxin MVIIC	

2.4 L-type Ca^{2+} channels are downregulated after peripheral axotomy

Since L-type Ca^{2+} channel activity mediates growth inhibition, I presumed a reduction of this particular current after peripheral lesion. To assess this possibility, I performed voltage-clamp recordings in dissociated DRG neurons to compare the L-type current amplitude in naïve and PNL neurons. Calcium currents mediated by T-type and L-type channels were isolated by recording in an extracellular solution free of Na^+ and K^+ ions, and by additionally blocking P/Q/N-type calcium currents with *w*-conotoxin MVIIC. Currents were evoked by a series of 50 msec voltage steps from a **holding potential of either -90 mV or -40 mV**. I observed the presence of two different cell types in culture, distinguishable by their current profile evoked from a holding potential of -90 mV, as previously described (Baccei and Kocsis, 2000). Type 1 neurons possessed only a high-voltage activated, slowly inactivating L-type calcium current (Figure 13A-C), representing approximately 70% of the neuronal population in cell culture. Type 2 neurons showed both a low-voltage activated, fast inactivating T-type calcium current and the high-voltage activated, slowly inactivating L-type component (Figure 13D-F). They represent approximately 30% of the neuronal population. The L-type current amplitude in type 2 neurons is significantly smaller than in type 1 neurons (Figure 13C and 13F). Therefore, differences in L-type current amplitude between naïve and PNL neurons were evaluated separately for type 1 and type 2 cells. **By holding the neurons at -40 mV** to inactivate the T-type current (present in type 2 cells) and then stepping to the various test potentials L-type currents were isolated (Figure 13A,B and 13D,E, *right traces*). I found that peripheral injury significantly reduced the L-type current amplitude in type 1 neurons (Figure 13A-C, and Figure 13G; -5.0 ± 0.57 versus -3.2 ± 0.54 nA). No difference in current amplitude was observed between naïve and PNL type 2 neurons (Figure 13D-F; -0.93 ± 0.14 versus -1.42 ± 0.43 nA).

However, holding the neurons at -40 mV also partially inactivates the L-type current (compare e.g. left and right traces in figure 13A).

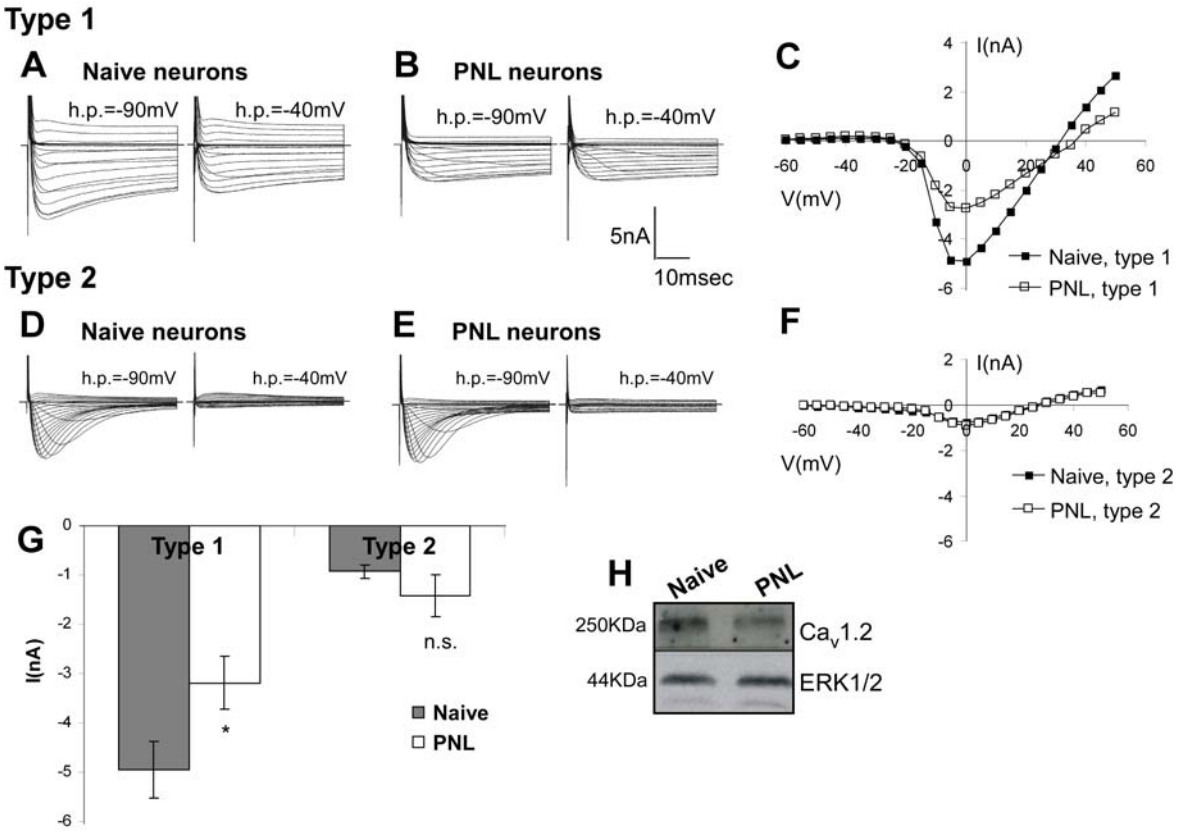

Figure 13 | L-type Ca^{2+} current is reduced after peripheral nerve lesion. Voltage-clamp recordings were performed in control and PNL neurons, in the presence of 200 nM *w*-conotoxin MVIIC. **| A, B and D, E,** ***left traces*** **|** Neurons were classified as type 1 or type 2 based on their current profile evoked from a holding potential of -90 mV. Type 1 neurons present only slow- inactivating currents, whereas type 2 neurons present both slow- and fast-inactivating currents. In control cells, 71% (15/21) were type 1 and 29% (6/21) were type 2. Type frequency was not affected by peripheral injury. To compare L-current amplitude between control and PNL neurons, cells were also recorded from a holding potential of -40 mV, which lacks the fast inactivating T- component. **| A,B,** ***right traces*** **and C |** Representative traces of type 1 neurons, h.p. -40 mV, showing that L-current amplitude is reduced after peripheral lesion. **| D,E,** ***right traces*** **and F |** Representative traces of type 2 neurons, h.p. -40 mV, showing that L- current amplitude is not affected by peripheral lesion. Note that type 2 cells have a characteristic small L-

current. C and F plots show the current-voltage (I-V) relationship for the 4 cells presented here. **G I** Average of L-current peak amplitudes recorded from control and PNL neurons at h.p. of -40 mV. (mean ± SEM; 21 neurons per condition) * P<0.05; n.s. not statistically different. h.p. holding potential **H I** Western Blot showing reduction of $Ca_v1.2$ expression on the DRGs after peripheral lesion. ERK1/2 staining was used as a loading control. A reduction in $Ca_v1.2$ levels was observed in 9 out of 12 analyzed animals.

Considering only type 1 neurons, which present solely the L-type current in the recordings, the comparison between naïve and PNL neurons can be made at a **holding potential of -90 mV**. In this case, a more striking reduction of the L-type current after peripheral lesion is observed (6.0 ± 0.71 versus -3.4 ± 0.66 nA; **P<0.01). These data demonstrate that the L-type current amplitude is significantly decreased in DRG neurons after peripheral lesion.

To test whether a reduction of L-type channel conductance is caused by a reduction in the protein levels, I next compared the amount of the pore-forming subunit of the L-type channel protein in naïve and PNL ganglia. Although two isoforms - $Ca_v1.2$ and $Ca_v1.3$ – can constitute the pore of L-type channels, the $Ca_v1.2$ is predominantly expressed in medium and large diameter DRG neurons (Yusaf et al., 2001) (Figure 14A). Western blot analysis revealed a lower $Ca_v1.2$ protein level in PNL ganglia (Figure 13H), suggesting that peripheral nerve lesion induces a downregulation of L-type channel protein expression in DRG neurons.

Taken together, these findings show a reduction of L-type currents attributed to a decrease in L-type channel protein in the cell membrane of PNL neurons.

2.5. Lack of L-type channels enhances axon growth

Electrical activity inhibits axon growth through activation of L-type channels and leads to the transcription of growth inhibitory genes. Moreover, there is downregulation of L-type channels after peripheral injury, suggesting that loss of L-type channel activity is required to promote axon growth. This raises the question of whether the absence of this channel type is sufficient to promote growth of adult DRG neurons.

2.5.1 Ablation of $Ca_v1.2$ channel subunit in the nervous system

Using the Cre recombinase system, a mouse line with an inactivation of the $Ca_v1.2$ gene (*CACNA1C*) in the whole CNS was generated (Figure 14B). $Ca_v1.2$ knock-out (KO) mice were generated by Nicole Langwieser and Sven Moosmang, at the Institute of Pharmacology and Toxicology, Technical-University-Munich, Germany. $Ca_v1.2$ KO mice are viable and exhibit normal life expectancy, body weight and breeding. Mating with the reporter Rosa 26 line showed that nestin-Cre mice have a strong Cre recombinase activity in DRG neurons (Figure 14C). Using Western Blot analysis, we confirmed that the $Ca_v1.2$ protein is absent from DRGs as well as from the whole brain of conditional KO animals (Figure 14D and 14E). There were no compensatory changes of the $Ca_v1.3$ protein level in the Cav1.2 KO DRG, as demonstrated by semi-quantitative RT-PCR (Figure 14F).

2.5.2 $Ca_v1.2$ KO neurons show enhanced outgrowth in cell culture

I next investigated whether genetic inactivation of $Ca_v1.2$ L-type calcium channels is sufficient to promote axon growth in adult DRG neurons. To this end,

I isolated naïve neurons from control and $Ca_v1.2$ KO animals and evaluated outgrowth after 18h in culture.

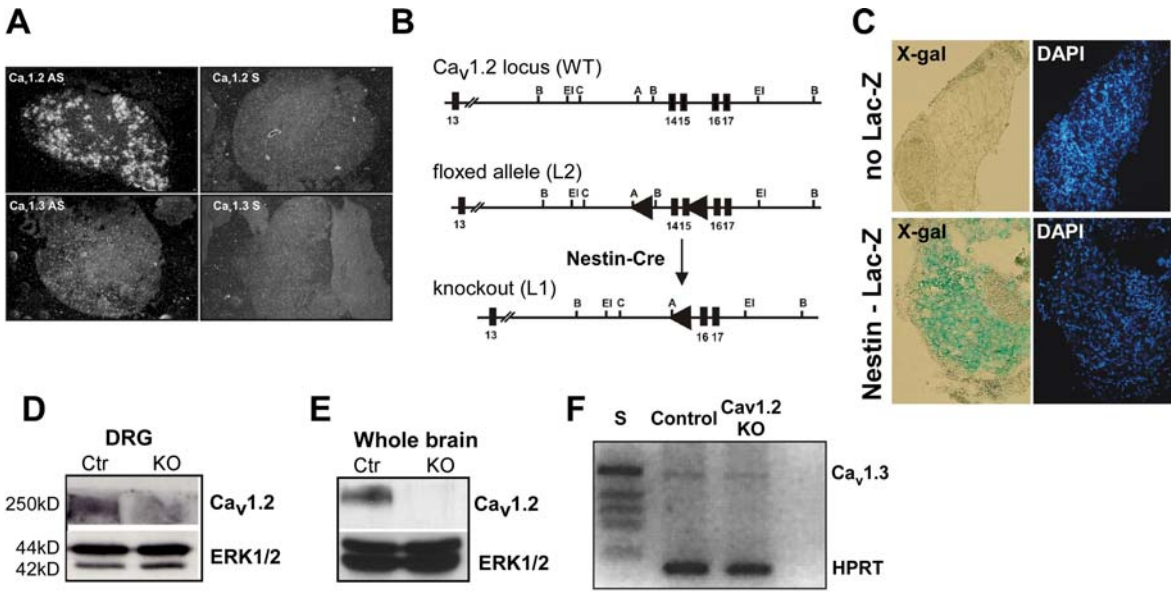

Figure 14 | Genetic inactivation of the $Ca_V1.2$ gene in the nervous system. A | In-situ hybridization showing $Ca_v1.2$ and $Ca_v1.3$ mRNA expression in the dorsal root ganglia. AS, antisense; S, sense. **B |** Schematic representation of the wild type (WT), the floxed (L2) and the knockout (L1) $Ca_v1.2$ alleles. The numbers indicate the exon number. **C |** Reporter stain showing X-Gal expression under the Nestin promoter in the DRG. Western analysis of protein from **D |** DRG and **E |** whole brain, using an anti-$Ca_V1.2$ antibody, shows the loss of the $Ca_V1.2$ protein. ERK1/2 was used as loading control. **F |** RT-PCR demonstrating lack of up-regulation of the $Ca_v1.3$ mRNA in the $Ca_v1.2$ KO mice. Analyses were performed by Nicole Langwieser, at the Technical-University-Munich, Germany.

KO neurons exhibited a more robust outgrowth in comparison to control neurons (Figure 15A, B, E and 15F), indicating that loss of L-type channel activity is sufficient to enhance the growth ability of adult DRG neurons. Moreover, by comparison with neurons previously subjected to a peripheral axotomy, I found that loss of L-type channel activity induces approximately 40% of the growth enhancement seen in PNL neurons (Figure 15 B and 15C).

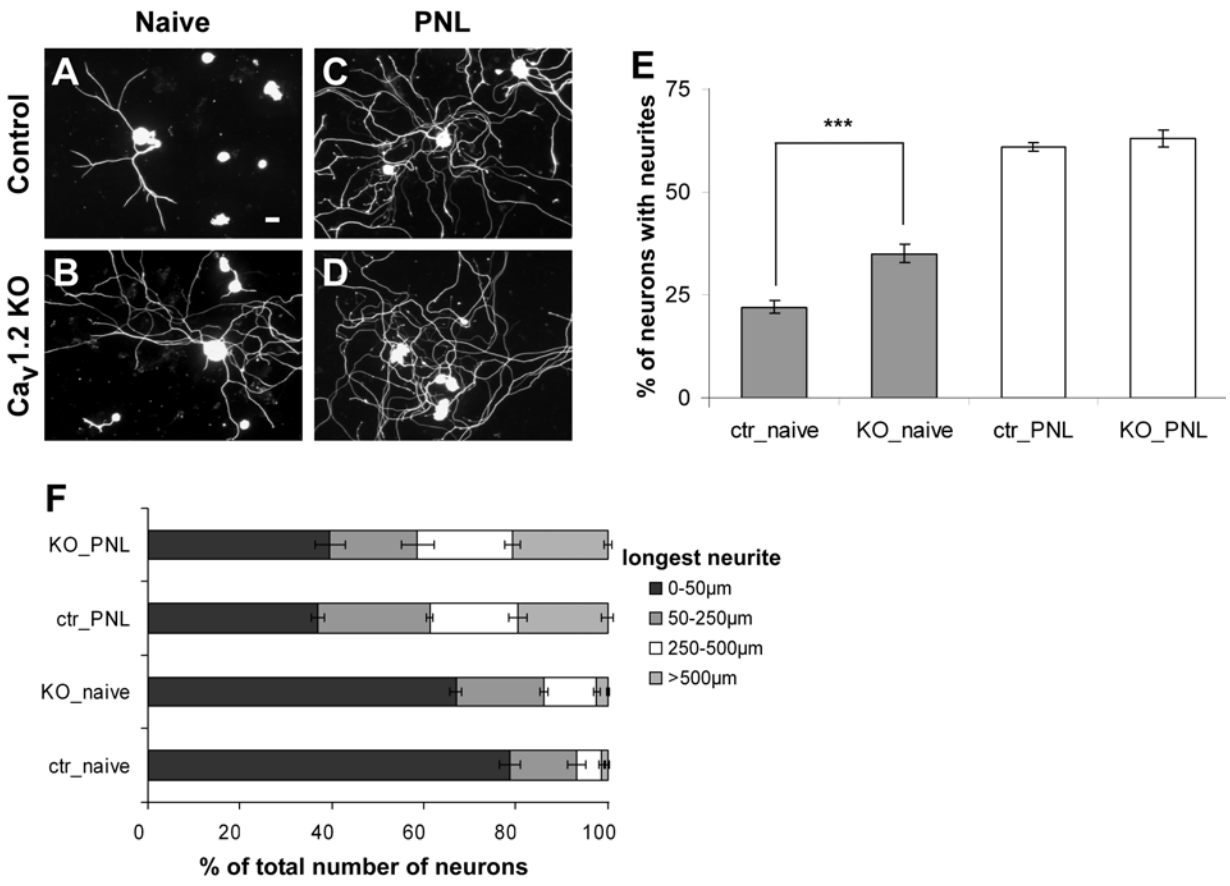

Figure 15 | Lack of L-type Ca^{2+} channels triggers growth competence in adult DRG neurons. | A and B | Neurons were isolated from control or $Ca_V1.2$ knockout adult mice and cultured on laminin for 18 h. **| C and D |** Neurons subjected to peripheral axotomy 5 days before isolation. **E |** Quantification showing that loss of L-current potentiates neurite formation in DRG neurons (mean ± SEM; 9 KO mice per condition) ***P<0.001. **F |** The longest axon on each neuron was measured (mean ± SEM).

An open question was whether the loss of L-type channels is involved in the mechanism enhancing axon growth after peripheral lesion. To address this question, I performed conditioning lesions in the KO animals. In case that L-type channel downregulation and peripheral lesioning are independent events, one would expect their effects to be cumulative. Instead, in case that L-type channel downregulation is part of the conditioning paradigm one would expect that there is no additive effect. In support of the latter possibility, I found that

conditioned KO neurons grew to a similar extent as the conditioned control neurons (Figure 15C and 15D). These findings demonstrate that lack of L-type Ca^{2+} channels is an important component of the conditioning effect, and essential to promote axon growth in adult DRG neurons.

2.6 Translating the knowledge to a spinal cord injury model

From the previous experiments, we conclude that electrical activity suppresses axon growth competence in cultured adult DRG neurons. We argue that *in vivo,* the electrical impulses that continuously arrive at the cell body from the periphery inhibit axon growth in adult DRG neurons. After spinal cord injury, neurons remain connected to their peripheral targets and would thus be suppressed to grow.

How can this information be applied to promote regeneration after spinal cord injury? In the first attempt, I assessed the effect of blocking AP propagation from the peripheral nerve endings to the cell body. In the second attempt, I tested whether the absence of L-type current would be sufficient to promote regeneration after spinal cord injury.

2.6.1 *In vivo* blockade of sciatic nerve transmission

Since spinal cord lesion experiments are long–lasting, extremely laborious and require extensive animal care, an "*in vivo - in vitro"* approach was chosen to first evaluate the potential of the treatment. To this end, I blocked AP propagation in the sciatic nerve of rats and assessed growth competence in culture. Only if sciatic nerve blockade proved to boost the growth competence of adult DRG neurons *in vitro*, I would evaluate axon regeneration after spinal cord injury.

Long-term blockade of AP propagation was achieved by two previously described methods (Xie et al., 2005) (see Figure 21 in Materials and Methods). In the first approach, the sciatic nerve was perfused with 780 µM TTX at a rate of 1 µl/h, following the implantation of a TTX-filled osmotic pump. The local delivery to the sciatic nerve was assured by connecting the pump to a catheter, which in turn connects to a cuff surrounding the nerve. In the second approach, a 200 mg bupivacaine-OH depot was applied around the sciatic nerve. Both TTX

and bupivacaine block sodium channels, although by different mechanisms. Whereas TTX blocks the outer pore of sodium channels, local anesthetics such as bupivacaine cross the cell membrane and bind in the inner pore of sodium channels (Hille, 2001).

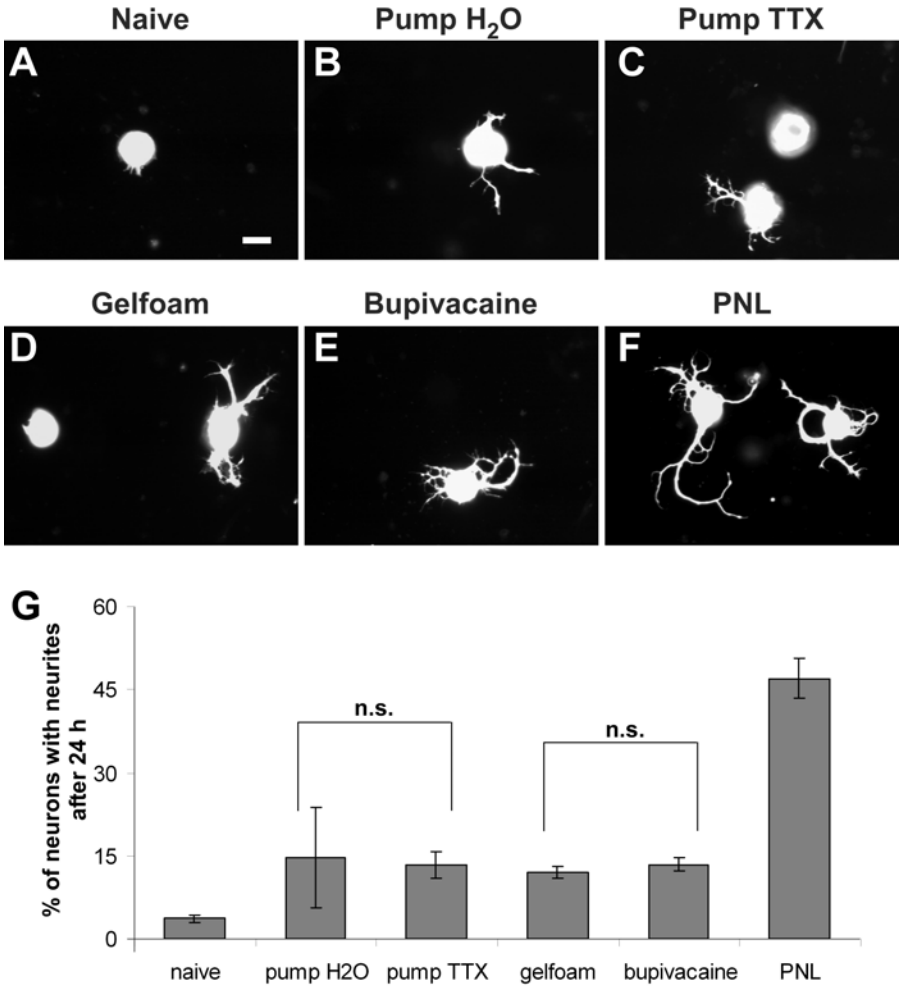

Figure 16 | Sciatic nerve blockade does not improve growth competence of adult DRG neurons *in vitro*. Three days after surgery, neurons were isolated and grown for 24 h on poly-lysine. **A |** Neurons from the contralateral naïve side. **| B and C |** Neurons dissociated from rats where an osmotic pump, filled with either **B |** H_2O or **C |** TTX, was installed. (D, E) Neurons isolated from rats exposed to **D |** gelfoam or **E |** bupivacaine. **F |** Neurons subjected to peripheral nerve lesion (PNL). Scale bar, 25 µm. **G |** Quantification of neurite outgrowth (mean ± SEM; 3 independent experiments). n.s. not statistically different.

An important requirement for these experiments is that the treatment leaves the sciatic nerve intact, which could otherwise mimic a peripheral lesion and lead to a misinterpretation of the results. This was verified by Wenrui Xie and confirmed by us, based on the histological analysis of control and treated sciatic nerves.

The efficacy of both methods in blocking AP propagation was evident in the animal behavior: rats would drag the affected hind limb during the full duration of the treatment. When assessing the effect of sciatic nerve blockade in axon growth, I found that TTX block does not increase the growth competence of cultured adult DRG neurons (Figure 16A, 16C and 16F). A similar result was obtained after bupivacaine treatment (Figure 16E and 16G). Although there is a small increase in the number of neurons extending neurites in the TTX- and bupivacaine- treated neurons when compared to naïve neurons (Figure 16G), this is not likely to be a direct effect of the blockade. Indeed, the mere manipulation to install the osmotic pump with the cuff or to place powder around the sciatic nerve seems to be sufficient to cause a small increase in the growth competence of the neurons (Figure 16B and 16D, respectively). A similar growth potential is observed in water- and TTX-perfused neurons (15 ± 9.2 versus 13 ± 2.5%), and in gelfoam- and bupivacaine-exposed neurons (12 ± 1.2 versus 13 ± 1.3%). A slight degeneration or inflammation of the nerve due to the manipulation might be the cause of such an effect. Hence, these results suggest that there is no specific effect of blocking AP propagation on the growth competence of cultured adult DRG neurons.

These negative results might reflect either the absence of an effect of AP blockade or technical limitations at different levels. A major caveat of this experiment is definitely the lack of *in vivo* recordings to validate the initial assumption. Additional considerations are made in section 3.4 of the discussion.

2.6.2 Spinal cord regeneration in $Ca_v1.2$ KO mice

The previous data showed that neurons isolated from $Ca_v1.2$ KO mice grow better in culture than those from their control littermates. The growth competence induced by the loss of $Ca_v1.2$ protein is about 40% of that triggered by a peripheral lesion (Figure 15). This raised the question of whether $Ca_v1.2$ KO animals show improved axon regeneration after spinal cord injury.

Since the ascending axons of the DRG neurons are confined to the dorsal columns of the spinal cord (see Figure 22A in Materials and Methods), a dorsal column lesion (DCL) is sufficient to transect all the central axons from the DRG neurons. Therefore, I performed DCL at T9-T10 level in control and $Ca_v1.2$ KO mice, and allowed the animals to recover for 4 weeks. As a positive control for regeneration, I included a third group of conditioned mice whose peripheral nerve had been cut 1 week before sectioning the dorsal column. To be able to visualize the regenerating fibers afterwards, cholera-toxin β-subunit (CTB) was injected into the left sciatic nerve of the injured animals, labeling the DRG neurons from L4 and L5 ganglia. Five days after CTB tracing, mice were perfused with 4% PFA and their spinal cords analyzed by histology (see details in Figure 22B-D, Materials and Methods).

In the majority of control mice (4/5), I observed that the ascending CTB-fibers stop at or behind the center of the lesion (Figure 17A and 17B). In one of the control animals though, some fibers were found approximately 150 μm beyond the lesion site (Figure 17I). According to the conditioning paradigm, it is expected that performing a peripheral lesion before sectioning the dorsal column dramatically increases fiber sprouting into the lesion site. However, in conditioned mice, I found that most of the ascending fibers also stall at the lesion site (Figure 17I). Only half of the conditioned mice (3/6) showed 1-2 fibers growing beyond the lesion site, and never extending farther than 200-300 μm from the centre of the lesion (Figure 17C and 17D).

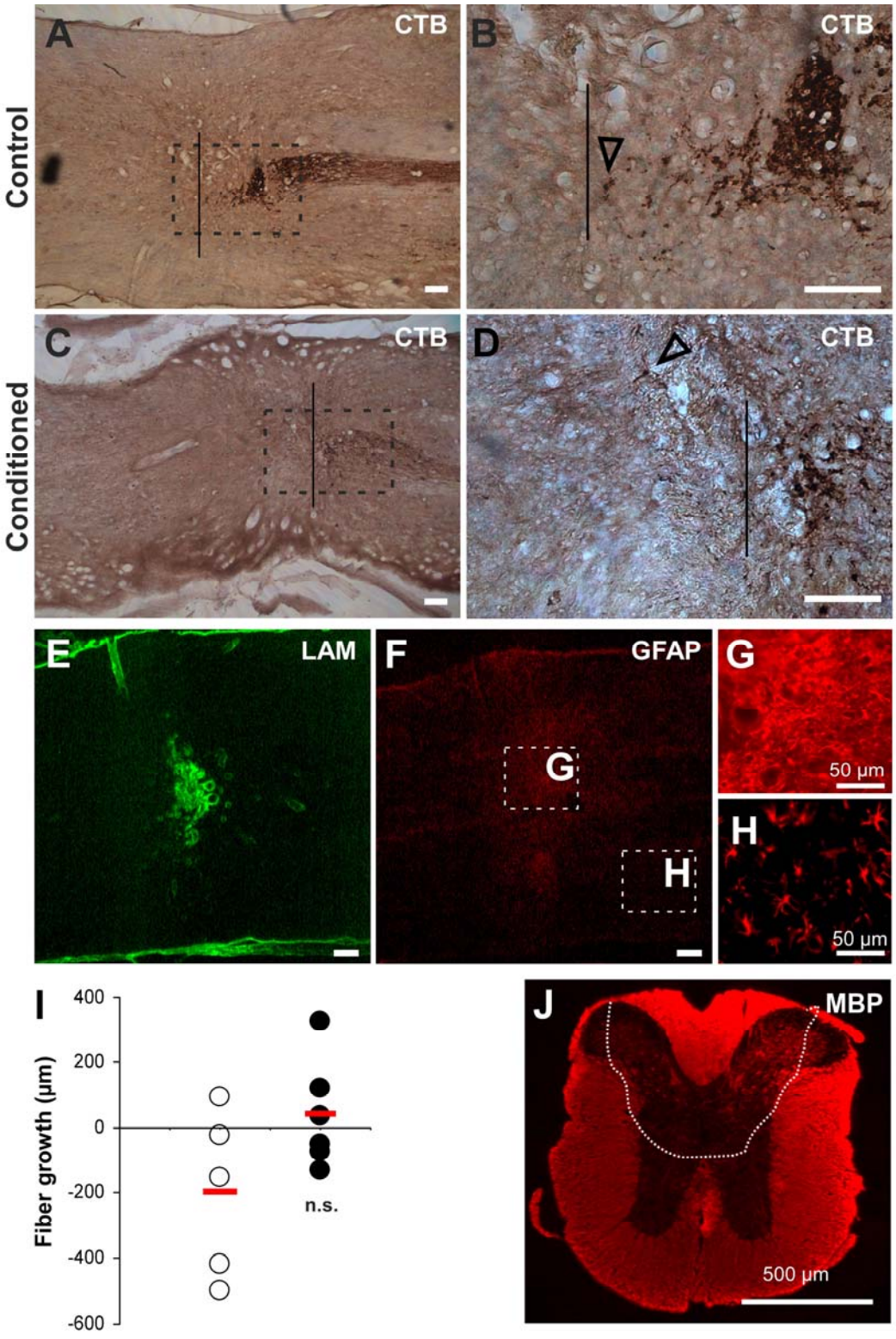
A
CTB
B
CTB
Control
C
CTB
D
CTB
Conditioned
E
LAM
F
GFAP
G
H
G
50 µm
H
50 µm
I
400
200
0
-200
-400
-600
Fiber growth (µm)
n.s.
J
MBP
500 µm

Figure 17 | Spinal cord injury in the C57bl6/sv129 mice. Part I (in previous page).
| A-H | 20 μm horizontal sections of the injured spinal cord; rostral is left and caudal is right. **A and B |** Four weeks after DCL in control animals, CTB-traced axons approach the lesion site but do not cross it (arrow head). Black line indicates the lesion site. Detail of A in B, high magnification. **C and D |** Four weeks after DCL in conditioned animals, CTB-traced axons are also stalled at the lesion site. In some mice, 1 or 2 fibers are seen beyond the lesion site, but never farther than 200-300 μm beyond the lesion (arrow head). Detail of C in D. **| E - H |** Lesion area is identified based on **E |** laminin and **F |** GFAP staining. Replicate of the section shown in A. High magnification of the GFAP staining **G |** in the center of the lesion area and **H |** outside the lesion area. **I |** Quantification of axon "regeneration", showing individual values for control (open circles) and conditioned (filled circles) mice. The bars indicate the average for each group. N= 5 for control and N= 6 for conditioned mice. n.s. not statistically different. **J |** Transversal section of the spinal cord, at the level where the injury was performed (T9-T10), showing the average extent of the lesion. Scale bar, 200 μm, unless otherwise specified.

These results reveal the poor regenerative ability of the ascending sensory fibers in mice, even after peripheral lesion. In addition, they indicate a high intra-group variability in response to injury. Consequently, the average extent of fiber regeneration in control mice is not significantly different from that of conditioned mice (-200 ± 113.7 versus 38 ± 68.2 μm, respectively). Failing to observe a "clear-cut" difference between control and conditioned animals, I concluded that within such a narrow window it is not possible to assess regeneration in $Ca_v1.2$ KO mice. This was also verified experimentally, as no significant difference in regeneration was observed in the $Ca_v1.2$ KO group (data not shown).

To facilitate the identification of the lesion site, replicate sections of the injured spinal cord were also stained for laminin and glial fibrillary acidic protein (GFAP) (Figure 17E-H). Laminin is a component of the extracellular matrix that is overexpressed after injury and accumulates at the lesion site; its staining is also associated with endothelial cells surrounding the lesion zone (Risling et al.,

1993). GFAP allows the visualization of astrocytes and provides a delimitation of the lesion area (Camand et al., 2004; Okada et al., 2006). The center of the lesion contains reactive astrocytes that extend numerous processes, forming a dense network called the glial scar (Figure 17G). Surrounding the lesion area, GFAP-positive astrocytes appear at a lower density than in the glial scar and exhibit a stellate morphology (Figure 17H).

The laminin and GFAP staining also allowed an estimation of the lesion extension. Based on the analyses of sequential sections obtained at different depths of the spinal cord, an average value of deepness and broadness of the lesion was calculated. These are represented in a transversal section of the spinal cord stained for myelin-binding protein (MBP), to better visualize how the lesion affects the white and the gray matter. The lesion does not only affect the white matter where the sensory afferent tracts are, but also causes damage to the surrounding gray matter (Figure 17J). The more extended the lesion is, the more inhibitory the glial scar is. Even if neurons are primed to grow after peripheral lesion, they might not regenerate because the entire surrounding tissue is hostile. Therefore, one possible explanation for the poor regenerative ability seen under these conditions is the large extension of the lesion area.

I next investigated whether a smaller lesion could counteract this problem, allowing a certain extent of axon regeneration. The experiments were performed as mentioned above, except that the dorsal column was sectioned using smaller scissors.

In both control and conditioned mice, I observed fibers extending far beyond the lesion site, some at 1-2 mm from the center of the lesion (Figure 18A and 18B, arrow heads). Moreover, they appear to run in parallel bundles. These features pointed to the presence of unlesioned, spared fibers rather than regenerating fibers.

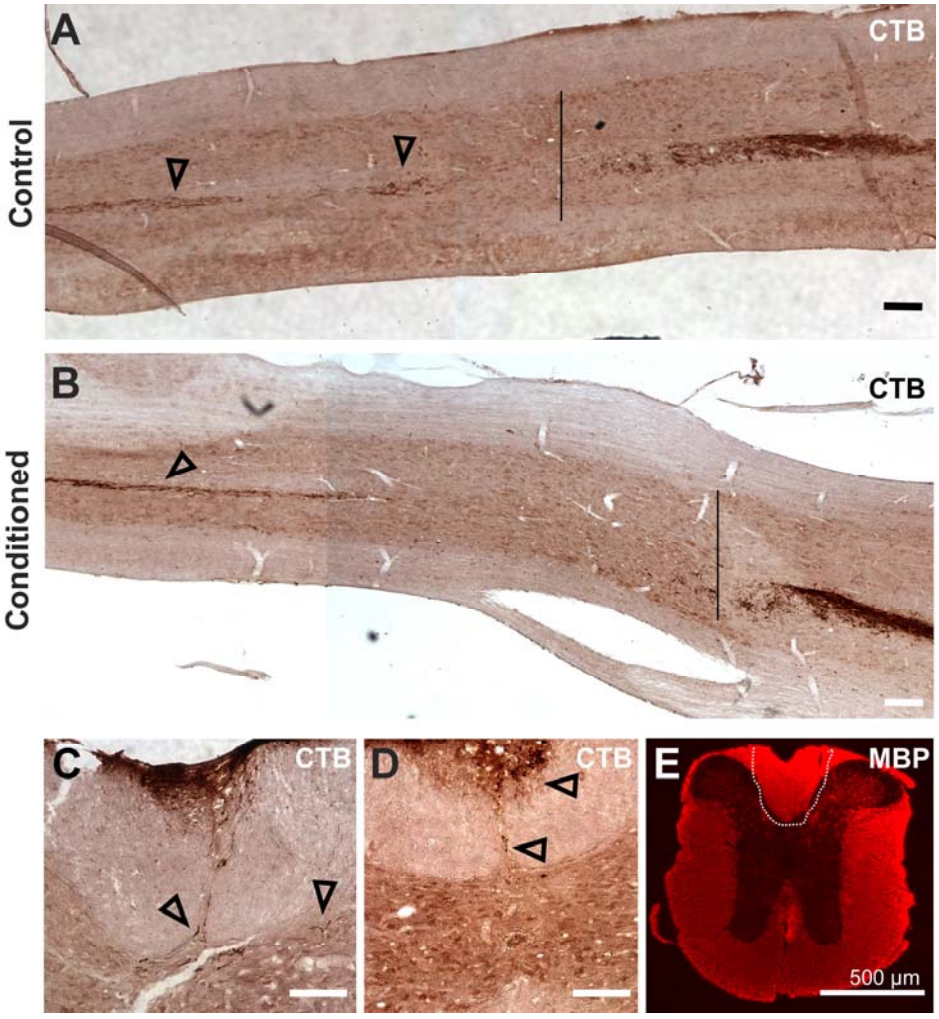

Figure 18 | Spinal cord injury in the C57bl6/sv129 mice. Part II. | A and B | Horizontal view of the injured spinal cord, in **A |** control and **B |** conditioned mice. Rostral is left and caudal is right. Black line indicates the lesion site. In both cases, most of the fibers terminate at the lesion site but some extend far beyond the lesion and in parallel bundles (arrow heads). **| C and D |** Transversal section of the medulla obtained from **C |** control (same mouse as in A) and **D |** conditioned (same mouse as in B) mice, confirming the presence of spared fibers (arrow heads). **E |** Transversal section of the intact spinal cord at the level where injury was performed (T9-T10), showing the average extent of the lesion. Scale bar, 200 µm, unless otherwise specified. N=6 for control and N=7 for conditioned mice.

To analyze this possibility, I checked for the presence of labeled fibers in transversal sections of the caudal medulla. This is where the ascending axons of the mechanoreceptive DRG neurons terminate (see Figure 4 in Introduction).

Because the lesion site is at a distance of many millimeters from the medulla, it is extremely unlikely that injured fibers can regrow that far. Consequently, the appearance of traced fibers at the medullar level indicates that they were spared by the lesion. I observed CTB-labeled fibers in all animals (Figure 18D and 18E), consistent with the idea of spared fibers. The estimated extension of the lesion shows that only the dorsal column is affected (Figure 18C). If the cut is not perfectly centered (the most likely case), it is possible that some fibers are spared by the lesion. Unfortunately, the presence of spared fibers invalidate the quantification of regenerating fibers, as it is impossible to distinguish between these two types of fibers by this method.

Taken together, these results indicate that it is not possible to assess sensory fiber regeneration in mice with conventional methods. Bigger lesions prevent any regeneration or sprouting to take place, smaller lesions leave some fibers spared invalidating quantification. The difference between both lesion extensions is approximately 200 µm, which is beyond the cutting precision that can be achieved by hand in a living animal.

In summary, the work presented here provides *in vitro* evidence that axon regrowth in adult DRG neurons is prevented by electrical activity. The inhibitory effect of electrical activity is mediated by activation of L-type channels and involves changes in gene transcription. Importantly, the absence of L-type calcium channels is sufficient to boost the growth competence of adult DRG neurons. Whether this knowledge can be applied to promote axon regeneration after spinal cord regeneration is an exciting possibility, but remains to be proven.

3. Discussion

Embryonic neurons grow their axons over long distances to connect with their appropriate targets. As the neuron matures, the genetic program associated with axon growth is gradually suppressed. In adult sensory DRG neurons, however, a conditioning peripheral lesion reactivates the genetic program to promote axon regeneration (Ylera and Bradke, 2006). The cellular basis of this switch on growth competence is poorly understood. This study provides evidence for an activity-dependent mechanism regulating the intrinsic growth competence of adult primary sensory neurons (Figure 30). Electrical activity leads to calcium influx through L-type channels and to the transcription of growth inhibitory factors, thereby preventing axon growth. Electrical silencing and the associated lack of L-type Ca^{2+} channel currents are sufficient to promote axon regrowth.

3.1 Electrical activity as the "intrinsic negative signal" for axon growth of primary sensory neurons

I showed that electrical activity significantly inhibits axon growth of cultured adult DRG neurons, as assessed by either chronic depolarization or electric field stimulation. These results are in agreement with those from earlier studies performed in embryonic DRG neurons (Fields et al., 1990; Robson and Burgoyne, 1989), and reveal that the mechanism of axon growth inhibition by electrical activity is conserved in adult neurons. It was important to confirm the effect in adult neurons as the expression of ion channels, which dictate the response to electrical stimulation, can be altered during the developmental process (Spitzer et al., 2002). Because the stimulation protocol used in our experiments was designed to resemble the normal AP firing pattern of adult sensory neurons (Fitzgerald, 1987), our findings support the initial hypothesis that normal levels

of activity experienced by functional adult DRG neurons can indeed inhibit axon growth. Interestingly, cultured adult DRG neurons become growth competent 1-2 days after isolation, a situation which may derive from the fact that these neurons are electrically silent in culture.

Axon growth inhibition by electrical activity seems to be a special feature of primary sensory neurons. It has been previously suggested that electrical activity acts as a physiological sensor of the sensory neuron to be connected to the peripheral target cell (Fields et al., 1990; Peckol et al., 1999). While other neuronal cell types receive input largely via their dendrites, the receiving element in DRG neurons is their peripheral axon. Hence, the onset of electrical activity may bring an important signaling mechanism to suppress further axon growth, representing the transition from a growth phase into a functional, neurotransmission phase. This study supports the idea that in the adult system only a peripheral lesion deprives neurons from electrical activity, setting them in a growth competent state. By contrast, after central lesion neurons remain electrically active and are kept growth incompetent.

Remarkably considering the history of neurobiology, a somewhat similar hypothesis to ours was first formulated in 1897 (Cragg, 1970; Gehuchten, 1897). They observed that adult DRG neurons show marked cell body reaction after peripheral axotomy, but no reaction after central axotomy. As an explanation, they suggested that what causes the cell body reaction is the loss of action potentials! However, instead of interpreting this reaction as a sign of regeneration, they erroneously thought it represented a process of cell death.

3.2 Possible mechanism of axon growth inhibition by electrical activity

This study implicates the L-type Ca^{2+} channel as a mediator of axon growth inhibition by electrical activity, since the block of L-type channels partially prevented axon growth inhibition in depolarized neurons. Furthermore, three lines of evidence for the involvement of the L-type Ca^{2+} channels in the conditioning lesion effect are provided: first, a peripheral lesion leads to a downregulation of L-type channel activity; second, genetic inactivation of this channel type is sufficient to enhance the growth ability of naïve adult DRG neurons in culture; third, genetic inactivation of the $Ca_v1.2$ channels does not further improve growth on PNL neurons, suggesting that lack of L-type channels is a component of the conditioning effect.

Ca^{2+} influx through L-type channels may further stimulates release of Ca^{2+} from internal stores to inhibit growth. Two types of Ca^{2+} stores are known to exist in DRG neurons. One type is rich in ryanodine receptor (RyR) and mainly present in the cell body, and the other is rich in IP_3 receptors and appears in the cell body and cell processes (Thayer et al., 1988). I found that axon growth is inhibited in the presence of caffeine, which is known to mobilize Ca^{2+} from RyR stores. Importantly, it has been previously shown that caffeine triggers $[Ca^{2+}]_i$ oscillations in DRG neurons similar to those originated by the Ca^{2+}-induced-Ca^{2+}-release mechanism from internal stores (Ouyang et al., 2005). Moreover, functional coupling of L-type channels to RyR in neurons has been shown by previous studies (Liljelund et al., 2000; Ouardouz et al., 2003). Although further investigations are required to clearly draw the pathway leading to growth inhibition, our results present the first evidence that Ca^{2+} release from internal stores via RyR is involved in the process.

DRG neurons seem to be particularly slow in their ability to buffer intracellular calcium elevations. $[Ca^{2+}]_i$ increases rapidly in response to depolarization and it is maintained at high concentration for a much longer period than in several

other neuronal types (Thayer et al., 1988). This feature may allow Ca^{2+} elevations to have a long-range, determinant effect in DRG neurons.

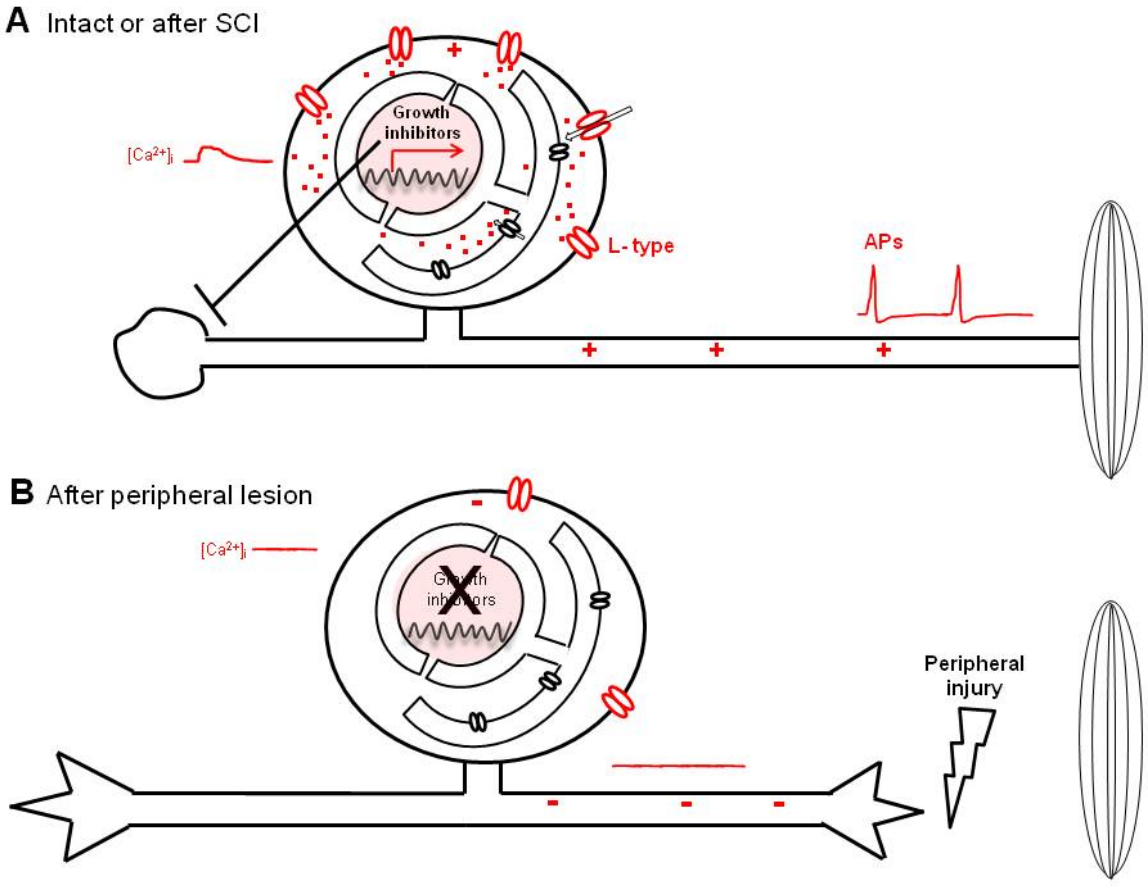

Figure 19 | Electrical activity controls growth competence in adult primary sensory neurons. Proposed model based on the results obtained in this study. **A |** Neurons generate APs in response to sensory input and propagate them through the peripheral and central branches. Activity-induced depolarization activates L-type VGCC present in the cell body, causing a global elevation in $[Ca^{2+}]_i$ in association with Ca^{2+} release from intracellular stores. This leads to the expression of growth inhibitory genes that suppress axon growth. Lesion to their central axons causes disconnection from the central synaptic targets but their cell body still receives electrical signals from the peripheral targets. **B |** Peripheral injury causes loss of sensory input and therefore silences the neurons. As the cell body gets deprived from electrical signals, growth inhibitors are not expressed, and the "brake" is released.

The involvement of L-type channels in this process raises the interesting possibility that electrical activity may interfere with the genetic program controlling growth in sensory neurons, as this particular channel type is known to regulate gene transcription in several ways. Not only can Ca^{2+} influx through L-type channels regulate the activity of several Ca^{2+}-dependent transcription factors and transcriptional repressors (Dolmetsch, 2003; Greer and Greenberg, 2008), but also can the carboxyl-terminus of L-type channels translocate to the nucleus and act itself as a transcription factor (CCAT) (Gomez-Ospina et al., 2006). In this context, our demonstration that transcription blockers abrogate the inhibitory effect of depolarization indicates the expression of growth inhibitory genes. The unanswered question is now whether those genes act in the nucleus to suppress the growth genetic program or if they act locally at the growth cone. Future studies will be necessary to clarify this issue.

In addition to a long-term effect at the gene transcriptional level, electrical activity may also have a short-term effect in the growth of adult DRG neurons in culture. The electric field stimulation experiments show that growth inhibition is visible within 1h of stimulation. Although immediate early genes might account for this fast response, it is possible that other mechanisms operate at this early time period. For instance, short-term effects of electrical activity may involve changes in cytoskeletal structure and dynamic that alter growth cone behavior and axon extension (Zheng and Poo, 2007).

It is noteworthy that the data presented here contrasts with the general idea that axon growth is an instructive pathway, which needs to be specifically signaled. Instead, our data suggest that axon growth is a default pathway at least for primary sensory neurons. After contacting their targets, further growth is actively inhibited by the expression of growth inhibitory genes.

3.3 Reduction in Ca^{2+} influx after peripheral lesion

At the time of axonal injury, there is a large Ca^{2+} influx from the extracellular space through the cut end that is important for rapid membrane resealing and GC formation (Geddis and Rehder, 2003; Gitler and Spira, 1998). However, this acute elevation in $[Ca^{2+}]_i$ turns back to resting levels within a few minutes (Ziv and Spira, 1993). An interesting finding from our studies is that, at a longer term, a peripheral lesion leads to a reduction in Ca^{2+} influx. By semi-quantitative Ca^{2+} imaging analysis, I found that PNL neurons show a smaller elevation in $[Ca^{2+}]_i$ in response to high K^+ than naïve neurons. Our result is based on the assumption that basal levels of $[Ca^{2+}]_i$ are not altered after lesion, which is supported by a previous study (Hayashida et al., 2006) using a ratiometric Ca^{2+} indicator. By voltage-clamp analysis, I showed that L-type channel activity is reduced in PNL neurons when compared to naïve neurons. Our Western Blot analysis on the $Ca_v1.2$ protein content of PNL and naïve ganglia confirmed a downregulation of the protein after lesion. These results are consistent with previous investigations showing that Ca^{2+} entry is downregulated in DRG neurons after peripheral injury in rats (Abdulla and Smith, 2001a; Baccei and Kocsis, 2000; Kim et al., 2001). In addition, our results established the link between a more specific electrophysiological change - the reduction of L-type channel activity - and axon regeneration. It therefore corroborates with the lack of sensory input after peripheral injury, to provide further evidence that electrical silencing is necessary for axon growth.

It is a surprising result though, that L-type voltage-gated Ca^{2+} channels are downregulated at the protein level after a peripheral axotomy, because the lack of sensory input alone would be enough to render these channels inactive. A possible explanation for a coordinated downregulation of Ca^{2+} influx after injury

is the fact that several other pathways converge to Ca^{2+} to inhibit axon regeneration after injury, notably those targeted by extracellular growth inhibitory molecules. For instance, large rises in intracellular calcium are necessary to mediate growth cone retraction upon contact with sulphate proteoglycans (Snow et al., 1994), MAG (Wong et al., 2002) and Nogo (Bandtlow et al., 1993). Therefore, reduction of Ca^{2+} influx through L-type channels might be important to enhance the intrinsic growth competence and to prevent inhibition by extracellular molecules, both relevant factors in the context of spinal cord injury. Another possible explanation is that homeostatic mechanisms could generate spontaneous activity in response to peripheral injury, leading to some extent to L-type channel activation even in the absence of sensory input. Homeostasis is thought to maintain certain levels of activity when electrical input is altered (Turrigiano et al., 1998). Indeed, a small percentage of peripheral deafferentiated fibers does produce ongoing discharges (Abdulla and Smith, 2001b; Michaelis et al., 2000) that may account for the commonly observed symptoms after peripheral injury, such as neuropathic pain.

3.4. The role of electrical activity in spinal cord regeneration

Our results suggest that, after spinal cord injury, electrical silencing or interfering with the aforementioned inhibitory mechanism may promote regeneration of primary sensory neurons. During my thesis project, two attempts have been made in this direction.

First, I assessed the effect of sciatic nerve blockade on the growth ability of DRG neurons. No improvement of growth competence was seen on DRG neurons innervating the silenced sciatic nerves. A clear and final interpretation of these negative results will require *in vivo* recordings to assess the effectiveness of the drugs in preventing AP propagation. Although their effectiveness is supported by the observation that rats drag their foot after treatment, it is important to note that the sciatic nerve is composed of both motor and sensory fibers, and block of motor fibers alone would yield a similar result. Are mechanoreceptive fibers also fully blocked after treatment? What is the contribution of TTX-insensitive Na^+ channels for AP propagation? The literature provides some clues to the answers. A complete blockade of APs in mechanosensory fibers was observed after TTX application (Wall et al., 1982). The presence of TTX-insensitive channels in DRG neurons is known, but it was shown that bupivacaine blocks sodium channels which are insensitive to TTX (Roy and Narahashi, 1992; Scholz and Vogel, 2000). Therefore, the lack of an effect after treatment with either drug rather indicates that blockade of AP propagation does not promote growth in DRG neurons. Still, a major limitation of this approach is that sciatic nerve blockade prevents propagation of APs at the mid-thigh level but does not prevent its generation at the peripheral nerve endings. In case a second messenger signal is generated at that point, it might be able to propagate until the cell body and exert its effects, even in the absence of APs; the highly diffusible IP_3 messenger (Kasai and Petersen, 1994) is a potential candidate. Because sciatic nerve afferents innervate

several targets, such as muscle, skin and tendons in the hind limb of the animal, it is practically impossible to block AP generation in all these nerve terminals.
Next, I assessed sensory fiber regeneration after spinal cord injury in the $Ca_v1.2$ KO mice, in comparison to control mice. The requirement for this experiment though was to observe a significant difference in fiber regeneration between control and conditioned animals, i.e between negative and positive controls. This requirement was not fulfilled. Although several reports exist on the *in vivo* conditioning effect in rats, fewer exist in mice. The major reason lies in the very limited regenerative responses in mice elicited by conditioning after whole dorsal column lesion when compared to rats (Neumann and Woolf, 1999; Seijffers et al., 2007). Regeneration in mice may be visible in experiments that do not affect the whole dorsal column, but only part of it. However, other techniques must be employed, whose analysis is not affected by the presence of spared fibers. One such technique is *in vivo* imaging whereby individual GFP axons can be analyzed at several time points after injury (Erturk et al., 2007; Kerschensteiner et al., 2005). I am currently working to establish the $Ca_v1.2$ KO GFP mouse line.

How does our model fit into the current knowledge?
Some studies in the past have proposed a rather positive role for electrical activity in sensory axon regeneration. Application of weak electrical fields (about 10 mV/mm) at the site of injury has been described to improve recovery of the propriospinal intersegmental reflex in guinea pigs (Borgens et al., 1987). A more recent study suggested that electrical stimulation of the sciatic nerve promotes regeneration of the central injured sensory fibers (Udina et al., 2008). Although seemingly contradictory to our results, such discrepancies may be due to differences in the stimulation protocol. Different spatiotemporal characteristics of the changes in $[Ca^{2+}]_i$ can activate different downstream targets and therefore generate diverse cellular responses (Gomez and Zheng, 2006; Zheng and Poo,

2007). In my experiments, a strong electrical field is applied to DRG cell bodies to mimic the normal physiological activity experienced by adult DRG neurons. The analysis indicates a global Ca^{2+} signal that elicits a nuclear response, leading to long-term modifications of the genetic program of the cell. By contrast, the weak electrical fields are applied to the injured tips. Curiously, their effect is strictly dependent on the positioning of the negative and positive poles, with neurites growing toward the negative pole and retracting when facing the positive pole (Patel and Poo, 1982). The effect of weak electrical fields has been associated with a reduction of the initial cationic current that enters the axonal tip preventing massive retraction (Roederer et al., 1983) and remodeling of actin and microtubule cytoskeleton (Rajnicek et al., 2006a; Rajnicek et al., 2006b). As for the electrical stimulation of the sciatic nerve, it will be important to discard a possible effect of nerve degeneration due to the manipulation and therefore "indirectly" conditioning.

A recent study in lamprey though proposes that a reduction in voltage-gated calcium channels is necessary for axon regeneration after spinal cord injury (McClellan et al., 2008). Unlike higher vertebrates, lamprey shows spontaneous regeneration after spinal cord transection. In this report, it was show that spinal cord neurons display changes in their firing patterns after injury, which is associated with a significant reduction in mRNA levels of voltage-gated calcium channels. At longer recovery times (2-3 months), when reconnection is established, the electrophysiological properties of axotomized neurons resemble those of intact neurons. Hence they suggest that these changes, which are a consequence of injury, are also critical for axonal regeneration. This is the first link between axotomy-induced changes in the electrophysiological properties of neurons and their regenerative ability. Interestingly, this idea finds parallel in our data on the conditioning effect in primary sensory neurons. Perhaps the downregulation of voltage-gated calcium channels is part of a more general regeneration program in vertebrates.

3.5. Concluding remarks

This study focused on one of the most robust regeneration paradigms, the conditioning paradigm, to understand the cellular mechanisms that determine growth competence in adult primary sensory neurons. Our data provide evidence that electrical activity is an "intrinsic negative signal" controlling the growth competence of these neurons. The inhibitory mechanism involves calcium influx through L-type calcium current and leads to transcriptional changes. Absence of L-type calcium channels is sufficient to boost their growth competence.

These data suggest that electrical activity hinders regeneration of sensory neurons after spinal cord injury. In developing strategies for spinal cord regeneration, it may be important to consider electrical activity as an intrinsic factor that can act complementary and synergistically with extracellular factors, to release the pre-imposed "brake" and to prime neurons to grow.

4. Materials and methods

4.1 Materials

4.1.1 Chemicals. High-purity chemicals were purchased from the companies Merck, Invitrogen, Roth, and Sigma-Aldrich. All water used to prepare solutions was filtered with the "Milli-Q-Water System" (Millipore). Special supplies are mentioned in detail with the according material or method.

4.1.2 Pharmacological reagents. Bay K 8644 and *w*-conotoxin MVIIC were obtained from Tocris Bioscience; nifedipine, 5,6-dichlorobenzimidazole riboside (DRB), α-amanitin, caffeine and bupivacaine from Sigma-Aldrich; tetrodotoxin (TTX) from Alomone Labs.

4.1.3 Media, solutions and special preparations

Borate buffer pH 8.5

Boric acid	1.2 g
Borax	1.9 g

Dissolve in 400 mL of distilled water; adjust pH to 8.5.

Complete NeuroBasal media

Neurobasal Medium (Gibco)	48 ml
B-27 Supplement (Gibco)	1 ml
Glutamine (Gibco)	0.5 ml
Pen Strep antibiotic (Gibco)	0.5 ml

Filter sterilize with 22 µm filter.

NeuroBasal media with 5% horse serum

Neurobasal Medium	45 ml
B-27 Supplement	1 ml
Glutamine	0.5 ml

Pen Strep antibiotic	0.5 ml
Horse Serum (Sigma; heat inactivated)	2.5 ml

Filter sterilize with 22 µm filter.

Hank´s balanced salt solution (HBSS), buffered with 10 mM HEPES

Add 5 mL HEPES (1 M) pH 7.25 to 500 mL HBSS (Gibco)

Collagenase type I solution

Dilute 1g of collagenase in HBSS buffer to a final concentration of 3000 U/mL. Sterilize through a 0.22µm filter.

Phosphate-buffered saline (PBS) (immunocytochemistry)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.24 g

Dissolve in 1L of distilled water and adjust pH to 7.4. Sterilize by autoclaving.

16% PFA / sucrose (immunocytochemistry)

PFA powder	160 g
Sucrose	160 g

~3-4 pellets of NaOH

Dissolve in 1L PBS under the fume hood. Adjust pH to 7.4. Filter solution through paper filter.

Synthesis of bupivacaine-OH powder

1 g of bupivacaine-Cl (Sigma) is dissolved in 50 mL H_2O, and 1N NaOH is slowly added to the solution forming a white precipitate. The precipitate is collected by paper filtering, rinsed in fresh H_2O, and dried ON under vacuum and heat.

Phosphate-buffered saline (PBS) (mice perfusion)

NaCl	9 g
Na_2HPO_4	11.5 g
NaH_2PO_4	2.3 g

Dissolve in 1L of distilled water and adjust pH to 7.4.

4% PFA (mice perfusion)

PFA powder	80 g

~3-4 pellets of NaOH

Dissolve in 2L PBS under the fume hood. Adjust pH to 7.4. Filter solution through paper filter.

Tris-Buffered Saline (TBS) (staining for laminin, GFAP and myelin)

Trizma base 6.6 g
NaCl 7.3 g
Dissolve in 1L of distilled water and adjust pH to 8.0.

Tris-Buffered Saline (TBS) (staining for CTB)

Trizma base 12 g
NaCl 9 g
Dissolve in 1L of distilled water and adjust pH to 7.4.

4.1.4 Antibodies

Primary antibodies:

Antibody	Dilution	Type	Supplier
Class III β-tubulin (Tuj-1)	1:1000	mouse	Covance
CTB	1:10.000	goat	List Biological
Laminin	1:100	rabbit	Sigma
GFAP	1:400	mouse	Sigma
myelin-binding protein (MBP)	1:50	rat	Chemicon

Secondary antibodies:

Specificity	Dilution	Fluorochrome	Supplier
Mouse	1:500	Alexa Fluor 555	Invitrogen
Mouse	1:200	Alexa Fluor 568	Invitrogen
Rabbit	1:200	Alexa Fluor 488	Invitrogen
Rat	1:200	Alexa Fluor 568	Invitrogen

Additional for the CTB staining:

Vectastain Elite ABC Kit, goat IgG (Vector Laboratories)

DAB (Sigma)

4.1.5 Equipment

Inverted epifluorescence microscope (Zeiss), equipped with a CCD camera.

Electrical stimulus generator device (*STG1004*, Multichannel Systems)

Electrical stimulus isolator (Hivotronic)

Lid of a 12-well cell culture plate equipped with pairs of platinum wires (home-made by the personnel of the MPI Workshop)

Digital-Multimeter (Conrad)

Osmotic pumps (model 2001, Alzet)

4.1.6 Instruments:

DRG dissection tools:

Scalpel No. 10003-12

Rongeurs curved No. 16000-14

Cutters No. 16140-11

Scissors angled to side No. 15006-09

Forceps Dumont No. 5

Surgery tools:

Forceps with small teeth, No. 11027-12

Scalpel No. 10003-12

Forceps curved, serrated No. 11052-10

Scissors Toughcut No. 14058-09, used for PNL

Needle holder No. 12002-12

Alm retractor No. 17008-07

Forceps with small teeth No. 11071-10

Micro curette No. 10081-10

Rongeurs curved No. 16221-14

Scissors 2.5 mm cutting edge No. 15001-08, used for SCI

Scissors 2 mm cutting edge No. 15000-03, used for SCI

Forceps to apply suture clips No. 12018-12

Forceps Dumont No. 5

All above mentioned tools were purchased from Fine Science Tools.

4.1.7 $Ca_v1.2$ knock-out (KO) mouse line

$Ca_v1.2$ KO mice were generated by Nicole Langwieser and Sven Moosmang, at the Institute of Pharmacology and Toxicology, Technical-University-Munich, Germany. Two *CACNA1C* ($Ca_v1.2$) alleles were obtained by Cre-mediated recombination in embryonic stem cells, L1 and L2 (Seisenberger et al., 2000). In L1, exons 14 and 15 which encode the IIS5 and IIS6 transmembrane segments and the pore loop in domain II were deleted. Additionally, this deletion causes an incorrect splicing from exon 13 to part of an intron upstream of exon 16, and thereby generates a premature stop codon in exon 16 and a loss of function allele. L2 contains the 'floxed' exons 14 and 15 and encodes a functional *CACNA1C* gene. To generate control and KO mice, the $Ca_v1.2^{+/L1}$ mouse was crossed with a mouse expressing a Cre recombinase under control of the Nestin promoter (Nestin-Cre) (Tronche et al., 1999). The resulting $Ca_v1.2^{+/L1}$, Nestin-$Cre^{+/tg}$ mice were then mated with $Ca_v1.2^{L2/L2}$ mice, to obtain the nervous system specific **knockout** $Ca_v1.2^{L1/L2}$ Nestin-$Cre^{+/tg}$ mice and the **control** $Ca_v1.2^{+/L2}$ Nestin-$Cre^{+/tg}$ mice. The mouse line was maintained in a bl6/sv129 mixed background. $Ca_v1.2$ KO mice are viable and exhibit normal life expectancy, body weight and breeding. There are no obvious morphological abnormalities.

4.2. Methods

4.2.1 Coating of cell culture dishes. Poly-L-lysine (Sigma-Aldrich) was dissolved in Borate Buffer at a concentration of 1 mg/mL and the solution filtered through a 0.22 μm pore syringe. Poly-L-lysine solution was added to slide wells (Nunc) or glass coverslips (Marienfeld) and left at room temperature overnight. Subsequently, poly-L-lysine was aspirated off and each well washed 3 times with sterile water. For an additional coat of laminin, a solution of laminin (Roche) was prepared in complete Neurobasal media at a concentration of 50 μg/mL. A volume of approximately 100 μl of laminin solution was added to the wells or coverslips. They were placed inside the incubator for at least 2h before cell culture.

4.2.2 Dissociated DRG neuronal culture. The dorsal root ganglia projecting peripherally to the sciatic nerve, L4 and L5, were dissected from adult Sprague-Dawley rats (200-250 g). Isolated DRGs were incubated for 90 min with collagenase type I (3000 U/mL, Worthington Biochemical), followed by 15 min with 0.25% trypsin (Sigma-Aldrich), at 37^0C. Enzymatic digestion was stopped by addition of NeuroBasal medium containing 5% horse serum, and neurons were collected by centrifugation at 600 rpm for 5 min. Neurons were resuspended in complete NeuroBasal media and plated onto poly-L-lysine coated dishes. In the specified cases, dishes were additionally coated with laminin. Cells were kept at 37^0C in a humidified atmosphere containing 5% CO_2. All the chemicals were directly added to neuronal culture medium at the indicated concentration and time in culture. DRG neurons from adult C57Bl6/sv129 mice (2-3 months old) were isolated by a similar procedure, except that collagenase treatment was reduced to 45 min.

Our study comprises only mechanosensory medium- to large- DRG neurons (30-50 µm cell body diameter), whose central axons course in the spinal cord and are thus affected by central injury (see Figure 4 in Introduction). They are the majority of neurons under these culture conditions.

4.2.3 Immunocytochemistry. After the indicated time in culture, neurons were fixed with 4% PFA for 15-20 min at room temperature. The remaining PFA was quenched with ammonium chloride solution for 10 min. Following an extraction step with 0.1% Triton X-100 in PBS for 5 min, cells were incubated in blocking solution for 1 h, in primary antibody solution for an additional 1 h and in secondary antibody for 30 min. Slides were mounted using Gel Mount (Sigma).

4.2.4 Quantification of neurite outgrowth *in vitro*. Neurons were stained for neuronal class III ß-tubulin with Tuj-1 antibody. The percentage of neurons forming neurites was obtained by counting the number of cells that showed one or more processes longer than the cell body diameter. To measure neurite length, images of random neurons were collected and analyzed using custom made functions in Scion Image Software.

4.2.5 Electrical field stimulation. Cells were cultured on laminin-coated coverslips containing an orientation grid (*cellocate*, Eppendorf), and used for experiment 18 – 24 h after plating. Because NeuroBasal medium has a sub-physiological concentration of Na^+, 60 mM NaCl was added to the medium at the time of plating and for all conditions. The growth of individual neurites was monitored by collecting images at 1 h intervals, in two subsequent periods. The initial period (0 – 1 h) was sought to confirm neurite growth previously to any of the tested conditions, whereas the second period (1 – 2 h) aimed to assess growth under no stimulation, stimulation or stimulation in the presence of TTX

conditions. In the time between imaging, cells were kept inside the incubator. For electrical stimulation of the neurons, depolarizing voltage pulses (200 pulses at 20 Hz, 60 V) were originated from a pulse generator associated with a stimulus isolator, and delivered to the cells every 5 min through two parallel platinum wires (1 cm long, distance 1.2 cm) placed in the cell culture dish. The rate of axon growth (μm/h) before and after the test condition was compared for individual neurites.

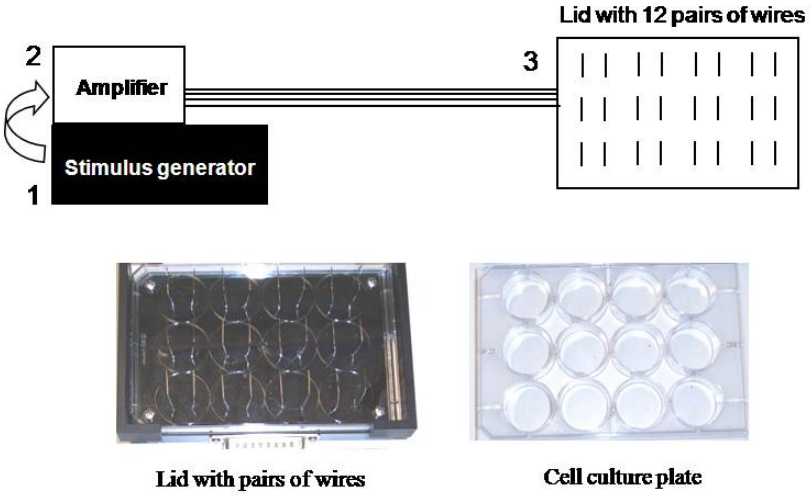

Figure 20 | Set up for electric field stimulation. Electric pulses are (1) originated by a stimulus generator device, (2) amplified by a stimulus isolator and (3) transmitted to the cells via pairs of parallel electrodes. The coverslips with neurons placed in the wells fall between the pairs of electrodes.

4.2.6 Calcium imaging. At 18 – 24 h after plating, cells were incubated with 8 μM Oregon Green BAPTA-1 AM (Molecular Probes, K_d=170nM) for 1 h at 37°C and 5% CO_2. The coverslip was then transferred to a flow chamber with temperature controlled at 35°C, and perfused with HBSS buffer containing (in mM): 137 NaCl, 4.5 $NaHCO_3$, 0.4 NaH_2PO_4, 3.3 $CaCl_2$, 5.4 KCl, 0.4 KH_2PO_4, 0.5$MgCl_2$, 0.4 $MgSO_4$, 5.6 D-glucose. Fluorescence images were acquired at a frequency of 0.7 Hz for 2 min, through a CCD camera controlled with Winview

software (Roper Scientific). Cells were observed with a 32x objective. KCl responses were induced at 30 sec of recording, by changing the perfusion solution from low to high K+ (40 mM). Only medium and large diameter (>30 μm) DRG neurons were included in the quantifications. Values of relative fluorescence change (ΔF/F0) were calculated for the entire cell body area, using custom made applications in Image J. F0 is the baseline fluorescence. Background light levels were determined in an area that did not contain a cell, and subtracted from the measured fluorescent values. The maximum ΔF/F0 value was determined for each cell and averaged. ΔF/F0 images were obtained based on 5 baseline frames and 5 signal frames. Data analysis and image processing were done using macros developed by Christian Lohmann.

4.2.7 Patch-clamp recordings. Medium to large (>30 μm diam) DRG neurons were recorded at 37°C, after 3 – 8 h in culture. Electrodes were pulled to 1.8 - 2.5 MΩ and filled with internal solution containing (in mM): 120 cesium methanesulfonate, 4.5 $MgCl_2$, 9 HEPES, 11 EGTA, 14 tris-phosphocreatine, 4 Na_2ATP, 0.3 tris-GTP, 1 $CaCl_2$. The extracellular solution was based on the previously described (Lu et al., 2006), and contained (in mM): 110 choline chloride, 30 tetraethylammonium chloride (TEA), 0.6 $MgCl_2$, 2.5 $CaCl_2$, 40 glucose, 10 HEPES, 0.4 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate. pH was adjusted to 7.4 with TEA-OH, and osmolarity was approximately 320 mOsm/Kg. In order to block P/Q/N- currents, 200 nM *w*-conotoxin MVIIC was also added to the bath solution. Recordings were performed in voltage clamp mode with a HEKA EPC10 amplifier and using Patchmaster software (HEKA). Series resistance was typically 3-7 MΩ and was compensated for by 75-85%. Cells with more than 10 MΩ of series resistance were not included in the data analysis. Ca^{2+} currents were evoked by 50 msec voltage commands ranging from -70 mV to +50 mV in successive 5 mV increments, from a 200 msec holding potential of -90 mV or -40 mV. Data was analyzed with pClamp software (Axon Instruments). The

peak amplitude of L-type current was determined from a holding potential of -40 mV, whereby the L-current is isolated. The response of naïve DRG neurons to electrical field stimulation was recorded using as extracellular solution HBSS and as intracellular solution (in mM): 120 K-gluconate, 4 $MgCl_2$, 10 HEPES, 5 EGTA, 10 tris-phosphocreatine, 4 Na_2ATP, 0.3 tris-GTP, 0.5 $CaCl_2$.
These experiments were done in collaboration with Achim Klug at the Department of Neurobiology, Ludwig-Maximilian-University Munich, Germany.

4.2.8 Western Blot analysis. The tissue was pulverized under liquid nitrogen and boiled in 2% SDS / 50 mM Tris for 10 min. The resulting homogenates (75 µg of protein) were separated by 10% SDS–PAGE, blotted on a PVDF membrane (Millipore) and probed with a $Ca_v1.2$-specific antibody. Equal loading of slots was ascertained by the use of an ERK1/2 (Upstate Biotechnologies). Antibodies were visualized by the ECL system (NEN). This assay was done by Nicole Langwieser at the Technical-University-Munich, Germany.

4.2.9 Surgery. All animal experiments were performed in accordance with the animal handling laws of the government (Regierung von Oberbayern, No: 209.1/211-2531-115/02). Adult rats (200-250 g) were anesthetized by intraperitoneal injection of a 1:1 combination of ketamine (WDT) and xylazine (Bayer). Adult mice (2-3 months old) were anesthetized by intraperitoneal injection of a mixture containing midazolam (Roche, 5 mg/Kg), medetomidine (Pfizer, 2 mg/Kg), and fentanyl (Hexal, 0.06 mg/Kg). After surgery, mice were woken up by injection of a mixture containing flumazenil (Pfeizer, 2.5 mg/Kg), atipamezole (Roche, 0.5 mg/Kg) and naloxone (Curamed, 1.2 mg/Kg).

Peripheral nerve lesion (PNL), in either rats or mice: the left sciatic nerve was exposed at the mid-thigh level, ligated and sectioned distally to the ligation. Animals were allowed to recover for 3-7 days, before isolating the DRG neurons.

Sciatic nerve blockade, in rats: (i) osmotic pump filled with TTX or H_2O (control) – the osmotic pump was filled with 780 µM TTX or H_20, connected to a self-made catheter from silastic tube (Dow Corning), and incubated ON in saline solution. The left sciatic nerve was exposed, the cuff at the catheter end was carefully placed around the nerve, and the attached osmotic pump was inserted under the skin (Figure 21A) (ii) bupivacaine or gelfoam (control) – the left sciatic nerve was exposed at the mid-thigh level, and retractors were used to keep the muscle cavity wide open while depositing 200 mg of bupivacaine-OH (Sigma) or gelfoam (Pharmacia) powder around the nerve (Figure 21B). Animals were allowed to recover for 3 days, before isolating the DRG neurons (Figure 21C).

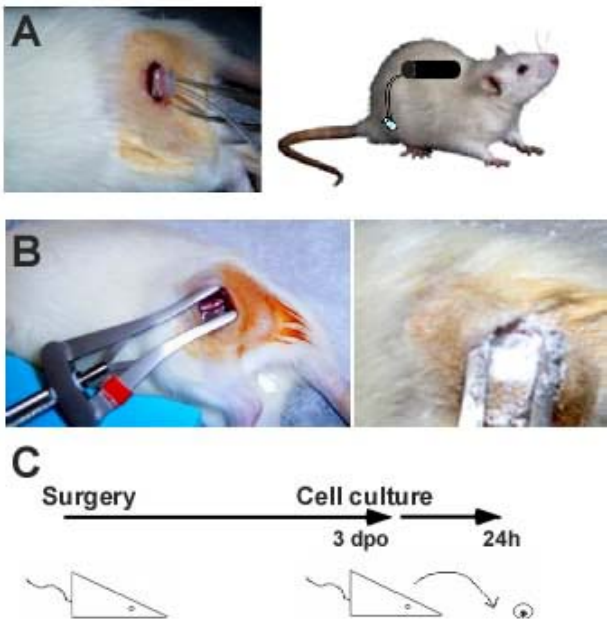

Figure 21 | Surgerical procedure to block AP propagation in the rat sciatic nerve. A | An osmotic pump delivers 1 µl / h of 780 µM TTX to the sciatic nerve, via a catheter connected to a cuff that surrounds the nerve. **B |** 200 mg of bupivacaine-OH are deposited around the sciatic nerve. **C |** Blockade is maintained during 3 days, and its effect on the growth ability of neurons is assessed afterwards in cell culture.

Dorsal column lesion (DCL), in mice: the spinal cord was exposed by a hemi-laminectomy at T_9-T_{10} and the dorsal column was transected bilaterally using fine scissors. The wound was closed and the mice were allowed to recover for 4 weeks. In the conditioning paradigm, mice underwent a left sciatic nerve lesion 7 days before the spinal cord injury (Figure 22). Post-operative care included subcutaneous injection of 15 µl buprenorphin (0.32 mg/mL solution; Essex Pharma) once after surgery and 25 µl antibiotic (7.5% Borgal solution; Hoechst Russel Vet) daily for 5 days.

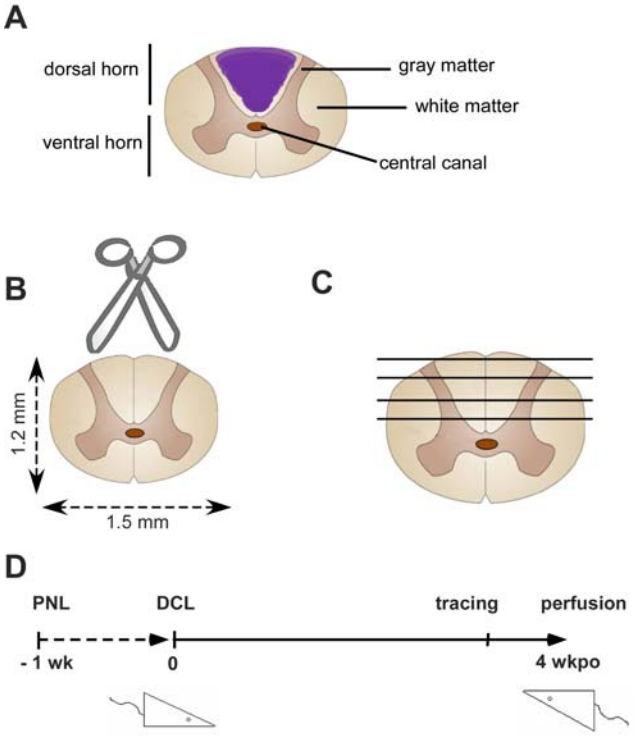

Figure 22 | Details of the spinal cord injury experiments. A | Transversal section of the spinal cord showing the position of the main ascending sensory tracts in violet. Collateral branches are not shown. **B |** Bilateral lesion to the dorsal column is performed with micro scissors **C |** After animal perfusion, the spinal cord comprising the lesion area is sliced in 20 µm horizontal sections. **D |** Time course of the *in vivo* experiment. PNL – peripheral nerve lesion; DCL – dorsal column lesion; wkpo – weeks post-operation.

Neuronal tracing, in mice - Four days before perfusion, DRG neurons were retrogradely labeled by performing a crush in the left sciatic nerve to facilitate uptake, and subsequently injecting 3 µl cholera toxin B subunit (1% CTB dissolved in distillated water; List Biological Laboratories) using a Hamilton syringe with a 31- G needle. CTB is taken up selectively by medium- to large-DRG neurons (LaMotte CC 1991 J. Comp Neurol).

4.2.10 Tissue processing. Mice were anesthetized by peritoneal injection of 8% chloralhydrate and perfused transcardially with 4% PFA in PBS. Spinal cord and brain were carefully dissected out, and the tissues were left ON in 4% PFA, followed by 5 h in 15% sucrose in PBS and ON in 30% sucrose in PBS. Spinal cords and medullas were embedded in optimal cutting temperature (OCT) compound. Tissues were sectioned horizontally or transversally, as specified, at 20 µm on a cryostat. Horizontal sections were collected serially on 5 different slides, such that each section in a single slide represents 100 µm in spinal cord deepness.

4.2.11 Immunohistochemistry. To assess fiber regeneration, tissue sections were incubated in anti-CTB antibody and processed for peroxidase activity using DAB as a substrate. To evaluate the extension of the lesion, tissue sections were stained for laminin, glial fibrillary acidic protein (GFAP), and myelin-binding protein (MBP).

4.2.12 Quantification of axon regeneration. For each animal, four SC sections collected at approximately 100, 200, 300 and 400 µm deepness were analyzed, covering the whole dorsal column. The distance from the lesion center to the fiber extending further rostrally was measured, and the longest fiber for each animal was indicated.

4.2.13 Statistics. For all data sets, the arithmetic average (x), the standard deviation (SD) and the standard error of the mean (SEM) were calculated using Microsoft Excel. The significance of the data was analyzed using Student's T-test, considering significant * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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CURRICULUM VITAE

PERSONAL INFORMATION

Name	Joana Filipa Leitão Pinheiro Almeida Enes
Birth Date	31st October 1978
Nationality	Portuguese
Phone	+49 89 8578 3460
E-mail	enes@neuro.mpg.de

EDUCATION

2004 -	PhD research work at the laboratory of Dr. Frank Bradke
2002 - 2003	Gulbenkian PhD Programme in Biomedicine, Lisbon, Portugal. Courses and lab rotation.
1996 - 2001	Degree in Biology from University of Aveiro, Portugal. Final grade: 16/20
1994 - 1996	High-school at Colegio Horizonte, Porto, Portugal. Qualification for university entry: 17/20.

RESEARCH EXPERIENCE

January 2004 – PhD student

Max-Planck Institute for Neurobiology, Martinsried, Germany. Research project: The role of electrical activity in axon regeneration. Supervisor: Dr. Frank Bradke.

April 2003 – July 2003 Graduated student

Instituto Gulbenkian da Ciência, Lisbon, Portugal. Research project: Effect of a secondary *Plasmodium* hepatic-infection on the pathogenesis of the blood-stage infection - Protection against cerebral malaria. Supervisor: Dr. Maria Mota.

May 2002 – July 2002 Graduated Student

Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Portugal. Research project: MUC1 gene polymorphism in the gastric carcinogenesis pathway. Supervisor: Dr. Leonor David.

February 2001 - November 2001 **Erasmus undergraduate student**

Department of Molecular and Structural Biology, Aarhus University, Denmark. Research project: Cloning and purification of E. coli methionyl-$tRNA_f^{Met}$ formyltransferase enzyme. Supervisor: Dr. Hans Uffe Sperling-Petersen.

CONGRESSES AND COURSES

2008 Axon guidance, synaptogenesis and neural plasticity, Cold Spring Harbor, USA

2008 6th Forum of European Neuroscience (FENS) Meeting, Geneva, Switzerland.

2006 **"***In vivo* Intracellular Recording Techniques" Practical Course, Eilat, Israel

2004 4th Forum of European Neuroscience (FENS) Meeting, Lisbon, Portugal.

FELLOWSHIPS

2002 PhD Studentship in Biomedicine awarded by Fundação Calouste Gulbenkian/Fundação para a Ciência e Tecnologia.

2001 Erasmus/Socrates fellowship awarded by the European Commission.

PUBLICATIONS

Enes J, Langwieser N, Klug A, Hofmann F, Moosmang S, Bradke F (in submission). **Electrical activity suppresses the intrinsic growth competence through $Ca_v1.2$ L-type Ca^{2+} channels in adult primary sensory neurons.**

Ertürk A, Hellal F, Enes J and Bradke F. (2007). **Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration.** *J Neurosci.* 27 (34): 9169-80.