Analysis of the C/EBP family of transcription factors in neuronal repair

by

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Abstract

Neurons within the peripheral nervous system (PNS) have a remarkable ability to repair themselves after injury; however, neurons within the central nervous system (CNS) do not spontaneously regenerate. Therefore, understanding the molecular elements responsible for successful regenerative response in the PNS can help us to establish basic principles and strategies for promoting regeneration in CNS structures such as the spinal cord. Nerve repair in the PNS has been suggested to be in part due to the involvement of intrinsic molecules such as transcription factors. In this thesis, I am focusing on the C/EBP family of transcription factors and their potential role in axonal regeneration after PNS injury. I examined the expression of different C/EBP members in PNS after injury after, using the sciatic nerve crush injury model I found that C/EBPδ mRNA is upregulated 4, 24 and 72 hours in dorsal root ganglia (DRG) following injury, whereas C/EBPβ and C/EBPγ expression is transiently upregulated by 4 hours resuming background levels after 72 hours. Conversely, C/EBPα and C/EBPε did not show upregulation following injury. In order to determine the function of C/EBPs in axonal growth in an *in vitro* system I used the ND7/23 cell line where I found that upon neurite growth induced by cyclic adenosine monophosphate (cAMP), the mRNA levels of C/EBPβ and C/EBPδ were upregulated. Furthermore, the conditional expression of a C/EBP total inhibitor or a C/EBPδ antisense construct decreases neurite elongation *in vitro*. Additionally, I found that 24 hours after treatment of ND7/23 cells with trichostatin A (TSA) C/EBPδ expression is elevated. Subsequently, I found that in DRG cultures from C/EBPδ knock-out animals, the lack of C/EBPδ affects the intrinsic growth capacity of dorsal root ganglion neurons which show a

drastically reduced axonal growth *in vitro*. To address the role of C/EBPδ *in vivo,* peripheral nerve repair was assessed in transgenic C/EBPδ knock-out animals following sciatic nerve crush. C/EBPδ knock-outs show, by immunostaining, impaired nerve regeneration 3 days and 14 days after sciatic nerve injury. Furthermore, functional recovery and morphometric analysis indicate that nerve regeneration is delayed in C/EBPδ deficient animals. These data demonstrate that the C/EBPδ gene is involved in neuronal repair after peripheral nerve injury.

Statement concerning co-joint work

Mr Luis Lopez de Heredia, the author of the PhD thesis titled, "Analysis of C/EBPs family of transcription factors in neuronal repair", performed all the procedures, surgery, breeding, genotyping, tissue handling, RNA extraction, reverse transcription, RT-PCR, immunohistochemistry, functional behavioral analysis, cloning, and analysis of neuronal cultures, by himself.

Dr Babis C. Magoulas

(Thesis Supervisor)

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List of abbreviations

Arg-1	Arginase-1
AP-1	Activator protein-1
ATF	Activating transcription factor
Bcl-2	B cell lymphoma leukaemia 2 protein
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop helix
cAMP	Cyclic adenosine3'-5' monophosphate
CAP-23	Cytoskeleton associated protein-23
C/EBP	CCAT-enhancer binding protein
CBP	CREB binding protein
CGRP	Calcitonin gene-related peptide
Chip	Chromatin immunoprecipitation
CHOP	C/EBP Homologous Protein
CNS	Central nervous system
CNTF	Ciliary neurotriphic factor
CRE	cAMP response element
CREB	cAMP responsive element binding protein
CS-PG	Chondroitin sulphate proteoglycans
CT-1	Cardiotrophin-1
dbcAMP	Dibutyryl cAMP
DMEM	Dulbeco's modified eagles media
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglia
EDTA	Ethylene disodium tetra acetate
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GAP-43	Growth associated protein-43
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
gp130	glycoprotein 130
HAT	Histone acetyltransferase
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylases
HNF	Hepatocyte nuclear factor
IFN	Interferon

IL	Interleukin
JAK	Janus kinase
JNK	Jun N-terminal Kinase
kDa	Kilodalton
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTM	Long term memory
MAG	Myelin associated glycoprotein
MAP	Mitogen activating protein
MAPK	MAP kinase
MARCKS	Myristoylated alanin rich C kinase substrate
MBP	Myelin basic protein
MCP-1	Macrophage chemoattractant protein-1
MEKK	Erk kinase kinase
MKK	MAP kinase kinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
ND7/23	Mouse neuroblastoma x rat neuron hybrid cell line
NFIL-6	Nuclear factor Interleukin
NF-κB	Nuclear factor NF-κB
NGF	Nerve growth factor
NgR	Nogo receptor
NPC	Neural precursor cells
NPY	Neuropeptide Y
NT	Neurotrophin
NTF	Neurotrophic factor
Omgp	Oligodendrocyte myelin glycoprotein
p75NTR	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PC12	Pheochromocytoma derived cell
PDE4	Phosphodiesterase
PDGF	Platelet-derived growth factor
PI3-K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLP	Proteolipid protein
PNS	Peripheral nervous system
Reg-2	Regenerating protein 2

RHD	Rel homology domain
RAG	Regeneration associated genes
RGC	Retinal ganglion cells
ROCK	Rho kinase
RT-PCR	Quantitative Real-Time PCR
SDS-PAGE	Sulphate polyacrylamide gel electrophoresis
SPRR1A	Small proline rich repeat protein 1A
STAT-3	Signal transducers and activators of transcription 3
tPA	Tissue plasminogen activator
TGF	Transforming growth factor
TNF-R	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor alpha
trk	Tyrosine kinase receptors
TSA	Trichostatin A
uPA	Urokinase plasminogen activator
UTF	Upstream stimulating factor
VIP	Vasoactive intestinal peptide

Table of contents

List of figures

List of tables

List of publications

1. Magoulas C, Jasim A, Lopez de Heredia L. (2009) Prediction of novel isoforms of the mouse Mpv17l protein. Genome 52(11):968-74.

2. Gushchina S, Leinster V, Wu D, Jasim A, Demestre M, Lopez de Heredia L, Michael GJ, Barker PA, Richardson PM, Magoulas C. (2008) Observations on the function of nuclear factor kappa B (NF-kappaB) in the survival of adult primary sensory neurons after nerve injury. Mol Cell Neurosci. 40(2):207-16.

3. Lopez de Heredia L, Sterneck E, Magoulas C. Severe neuronal regeneration after sciatic nerve injury in C/EBP-delta null mice. *In preparation.*

4. Lopez de Heredia L, Magoulas C. Conditional transgenic targeting of C/EBP activity inhibits neurite outgrowth of neuronal cells *in vitro*. *In preparation.*

Chapter 1 – Introduction

After injury in the central nervous system (CNS) injured axons usually fail to regenerate, whereas following injury to the mature peripheral nervous system (PNS) damage axons regenerate successfully if not to appropriate targets. Two main complications have been identified to account for this poor regeneration in the mature CNS. One is unfavourable glial environment for axonal growth, characterised by the presence of myelin debris, glial scar, cavitation and lack of facilitatory molecules (Bradbury et al., 2002; Silver and Miller, 2004; Fawcett, 2006). The other is failure of damaged CNS neurons to initiate a programme of gene expression which is beneficial for axon regeneration (Bomze et al., 2001; Woolf, 2001). Accumulating evidence indicates that overcoming the inhibitory signals is beneficial but must be combined with activation of the intrinsic growth state of neurons to achieve successful axon regeneration (Schnell et al., 1994; Fischer et al., 2001; Fischer et al., 2000). Key molecules for orchestrating the changes in gene expression that increase the intrinsic growth state of peripherally injured sensory neurons necessary for regenerating an axon are transcription factors. Therefore, the objective of this project is to examine the CAAT enhancing binding protein (C/EBP) family of transcription factors' potential role in axonal regeneration after PNS injury. Specifically, my work aims to addresses the effect of C/EBPδ during nerve regeneration.

1. 1 The peripheral nerve anatomy

The peripheral nerve is composed of connective tissue and neural components. The smallest functional units in the peripheral nerve are the nerve fibres which can be either

unmyelinated or myelinated (Flores et al., 2000). While unmyelinated fibres are composed of several axons, enveloped as a group by a single Schwann cell, myelinated fibres consist of a single axon, enveloped individually by a single Schwann cell. The length of an axon covered by a single Schwann cell is called an internode and the short distance between Schwann cells processes, in which the axon is not myelinated, is known as the node of Ranvier.

The connective tissue structures of the peripheral nerve are composed of three distinct sheaths: endoneurium, perineurium, and epineurium, from innermost to outermost, respectively (Fig. 1). These three structures form a framework that organizes and protects the nerve fibres and axons (Flores et al., 2000). The epineurium made up the connective tissue that surrounds the entire nerve trunk. The perineurium is the middle-level connective tissue sheath around the nerve fibres, and it made up of concentrically arranged, more compact cellular layers. The innermost sheath surrounding the Schwann cells and individual fascicles of longitudinally running nerve fibres is the endoneurium.

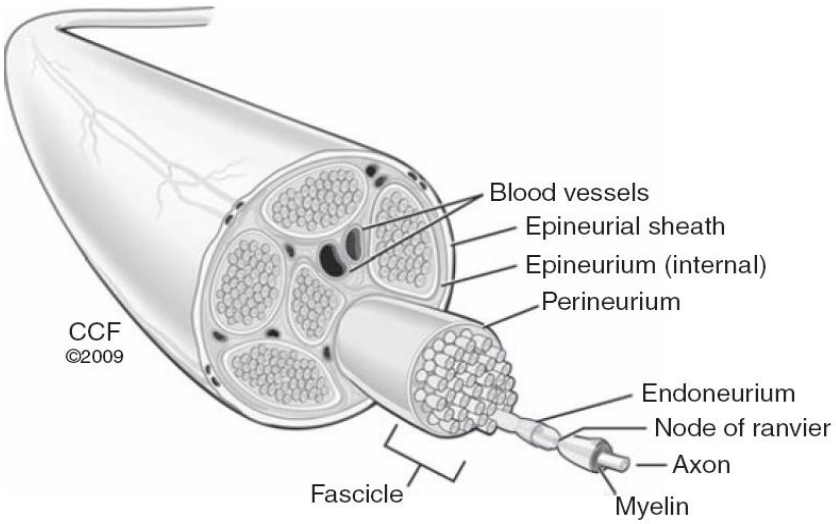


Fig. 1 Peripheral nerve fibre components. From International review of neurobiology, volume 87.

1.2.1 Effects of peripheral axotomy

The study of peripheral nerve regeneration potential has been reported since ancient times (Naff and Ecklund, 2001). However, it is only since the second half of nineteenth century that a body of literature on nerve regeneration and nerve repair strategies began to accumulate, starting with the milestone observations of Augustus Waller in 1850, reprinted in (Stoll et al., 2002).

In his influential paper entitled "Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres" and without the advantage of staining techniques that were later developed by Golgi, Cajal, and others, Waller described for the first time the progressive disorganization of the axons which occurs downstream to nerve transection and which also involved the myelin sheaths. This sequence of pathological changes leading to axonal degeneration distal to the site of the lesion is today known as Wallerian degeneration (Fig. 2), a complex process which is crucial for subsequent regeneration of axons. In mammals, Wallerian degeneration is fast in the PNS, taking about 7–14 days (Griffin et al., 1992; George and Griffin, 1994). The complex histological changes during Wallerian degeneration involve several cell types: neurons, monocyte lineage cells, myelinating and non-myelinating Schwann cells (Vargas and Barres, 2007).

The neuronal response induced by peripheral axotomy of adult PNS neurons has been intensively investigated. Early experiments showed that axotomy changed the cell morphology, the status of metabolism in the nerve cell bodies and axonal transport (Grafstein, 1975). First, axotomy divides the axon into a proximal segment that remains attached to the cell body and a distal segment that has lost that attachment. Because the

capacity for protein synthesis is largely restricted to the cell body, axotomy dooms the distal segment. Additionally, glial cells that ensheath the distal segment are also affected. The myelin sheath, which requires axonal contact for its maintenance as well as genesis becomes fragmented and is eventually enveloped, along with axonal debris, by phagocytic cells.

1.2.2. The distal segment

In Wallerian degeneration the primary histological change involves physical fragmentation of both axons and myelin, a process that begins promptly after nerve injury. Within hours after transection, the axon membrane fuses and seals the ends. Disintegration of the axons starts within the first days. The first stages of this process are characterized by a granular disintegration of axoplasmic microtubules and neurofilaments due to proteolysis (Schlaepfer, 1977). Ultrastructurally, both neurotubules and neurofilaments become disarrayed, and axonal contour becomes irregular, due to varicose swellings. By 48 to 96 hours post injury, axonal continuity is lost and conduction of impulses is no longer possible. Myelin disintegration lags slightly behind that of axons but is advanced by 36 to 48 hours. Myelin debris is cleared rapidly and efficiently in the PNS of mammals over the course of 7–14 days (Griffin et al., 1992; George and Griffin, 1994). However, in humans, myelin debris is still present years after degeneration of CNS axons (Buss et al., 2004; Becerra et al., 1995).

Myelinating Schwann cells respond rapidly to axonal injury, prior to the degeneration of the axon. ErbB2 receptors located in Schwann cells microvilli are

transiently activated within minutes following axotomy (Guertin et al., 2005). Schwann cells play a key role in both the breakdown of the myelin sheath and the clearance of its debris. Schwann cells decrease their synthesis of myelin lipids within the first 12 h after axotomy (White et al., 1989) and halt their production of myelin proteins within 48 h (Trapp et al., 1988). Following peripheral nerve injury, Schwann cells degrade their own myelin, they phagocyte extracellular myelin debris, and they pump myelin debris into the extracellular space for phagocytosis by macrophages (Hirata et al., 1999). During the first few days following injury, Schwann cells clear myelin debris in the absence of macrophages to promote fast clearance of nerve debris in the PNS (Stoll et al., 1989; Perry et al., 1995).

Following axon degeneration, around 3 days after nerve injury, Schwann cells start proliferating organizing themselves into columns, forming bands of Büngner as guideposts for new sprouting axons during regeneration. Interestingly, Schwann cells in contact with injured axons enter the cell cycle, whereas myelinating Schwann cells in contact with intact axons do not enter the cell cycle (Murinson et al., 2005). Not only Schwann cells but also perineural satellite cells which surround nerve cell bodies in sensory and autonomic ganglia, proliferate within a few days after peripheral axon injury. Satellite cells have little cytoplasm with their flattened nuclei closely appose neurons and their processes are involved in metabolic interactions with the neuron (Lu and Richardson, 1991). Interestingly, blocking the satellite cells proliferation does not impede peripheral regeneration (Lu and Richardson, 1991). In cases where the axonal injury results in a significant amount of nerve cell death in the affected ganglia, the subsequent phagocytosis of neuronal debris is also carried out by satellite cells. Additionally, satellite cells are

known to provide trophic molecules such as CNTF to support neurons and protect them from injury.

In the PNS, Schwann cells release a wide variety of chemokines and cytokines after injury; a number of these recruit macrophages to the degenerating nerve (Shamash et al., 2002). Schwann cells secrete leukemia inhibitor factor (LIF) which attracts macrophages *in vitro* (Tofaris et al., 2002). Additionally, LIF and tumor necrosis factor-α (TNFα) induce production of macrophage chemoattractant protein-1 (MCP-1) by Schwann cells (Subang and Richardson, 1999). Macrophages are recruited from the circulation into the degenerating sciatic nerve by 3 days post axotomy (Perry et al., 1987) also as a result of local bleeding and migration of monocytes through the blood vessel endothelium. Macrophage numbers peak between 14 and 21 days after axotomy in the PNS (Avellino et al., 1995). Macrophages in the PNS mediate the final phase of myelin debris removal in the distal nerve stump. Post injury, macrophages enter the endoneural tubes at 5 days, and by 7 days post injury numerous macrophages are found within the basal laminar tubes (Griffin et al., 1992).

1.2.3 The proximal segment and the cell body

The proximal portion of the neuron also suffers the effect of a nerve lesion. In some cases the neuron dies by apoptosis, probably due to restriction in the supply of target derived trophic factors or due to calcium influx when the axonal membrane is disrupted. In those neurons that are not killed immediately by the lesion, there is a well described set of changes that occur, many of which are associated with re-initiating synthesis of the proteins that are needed for axon growth and regeneration. In terms of anatomical appearance of the

neuron, these changes take the form of chromatolysis: Within 6 hours of the injury, the cell body swells, the nucleus migrates to the periphery of the cell and Nissl granules and the rough endoplasmatic reticulum becomes fragmented (Lieberman, 1971).

Metabolic changes also accompany chromatolysis, including overall increases in protein and RNA synthesis as well as changes in the pattern of genes that the neuron expresses. The neurons switch from a signaling mode to a growing mode and the increased RNA synthesis implies some alteration of gene expression. Protein synthesis is upregulated together with increased proteolysis in parallel with an increase of production of material required for restitution of the axon and a decreased production of material associated with synaptic transmission. Lipid synthesis is also significantly increased, possibly corresponding to the stimulated synthesis of membranous components of the nerve cell.

There may be a decreased axonal transport of transmitter-associated materials 1-2 days after axotomy, followed by an increased axonal transport of protein after initiation of axon regeneration (Grafstein, 1975). Changes within nerve cell body are prerequisite for sustained axonal regeneration. The detailed molecular mechanisms have been further explored in recent studies which aim to elucidate a specific growth programme characterized by expression of a series of regeneration-associated genes (Snider et al., 2002; Smith and Skene, 1997; Aubert et al., 1995).

Axotomy also affects postsynaptic neurons. When axotomy disrupts the major inputs to a cell, as happens in denervated muscle, the consequences are severe. Usually the target atrophies and sometimes dies. When targets are only partially denervated their responses are more subtle. Axotomy affects also inputs to the injured neuron. Frequently, synaptic terminals withdraw from neuronal cell bodies or dendrites of chromatolytic

neurons and are replaced by the processes of glial cells, Schwann cells in the PNS and microglia or astrocytes in the CNS. This process is called synaptic stripping, depresses synaptic function and can impair recovery of function.

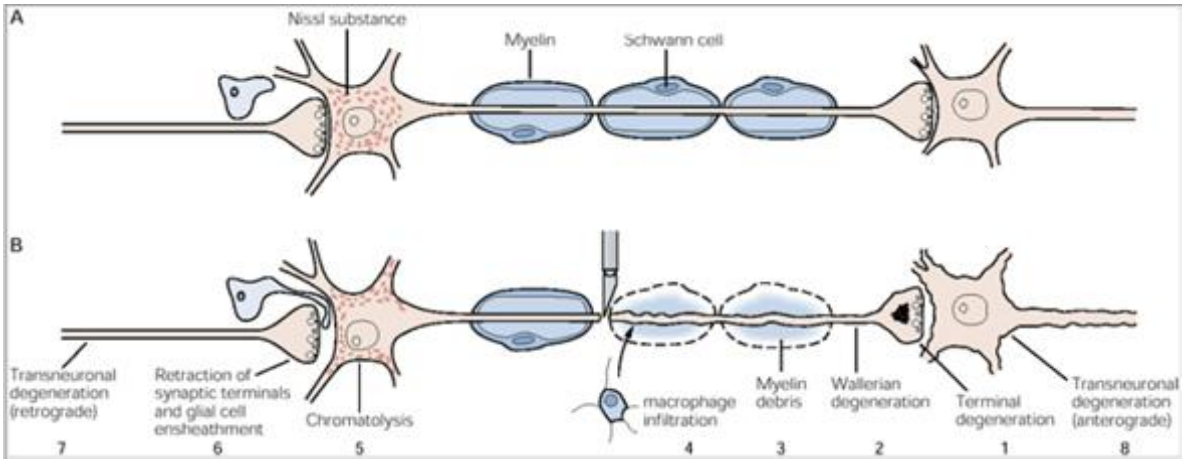


Fig. 2 Wallerian degeneration. A) A normal neuron with a normal functional axon. B) After axotomy the nerve terminals of the injured neuron fail rapidly (1). The distal stump, separeted from the cell body, undergoes Wallerian degeneration (2). Myelin degenerates (3) phagocytic cells invade (4). The cell body undergoes chromatolysis , in which the nucleus moves to an eccentric position (5). Presynaptic terminals on the chromatolytic neuron withdraw and are enwrapped by glial proccesses (6). The inputs to and targets of the injured neuron can atrophy and even degenerate (7,8). From Principles of neural science 4th edition by Kandel, Schwartz and Jessell.

1.3 Differences between the peripheral and the central nervous system following nerve injury

Brain and spinal injuries are one of the largest single causes of severe disability and loss of working days in the United Kingdom. Worldwide over a half million people each year suffer from brain damage and spinal cord injuries and the UK contributes a 1000 of those cases. The majority of patients affected by nerve injury are young adult males, who represent around 70% of all the cases.

The aetiology of nerve injury varies substantially among different countries. However, motor vehicle accidents are the most frequently implicated factor, followed by

falls and sport injuries. In most cases injury to the spinal cord is not fatal and life expectancy is not shorted when compared to the normal population. However, many people become disabled due to full or partial paralysis below the level of injury, resulting in a radical and sudden change in their way of life and becoming in numerous cases dependant on the help of others. Spinal cord injuries present serious consequences for the affected individual and despite major progress in the field cannot yet be repaired.

Most of the harmful outcome of spinal cord injury results from damage to axons that due to their length are in a more vulnerable position than other cell components. Injuries to axons in both the central nervous system (CNS) and peripheral nervous system (PNS) of mammals result in axonal degeneration distal to the site of the lesion: a process known as Wallerian degeneration. Although neurons within the PNS have a remarkable ability to repair themselves after injury neurons within the central nervous system CNS do not spontaneously regenerate. To some extent the difference in regenerative potential between the PNS and CNS can in part be due to the permissive environment in the PNS, in which axons regenerate. This permissive environment consists of Schwann cells and their basal laminae, fibroblasts, collagen, and macrophages (Fawcett and Keynes, 1990; Fawcett and Keynes, 1986). Of all these components Schwann cells are essential for axonal regeneration (Hall, 1986). This is shown by the decrease of axonal growth when viable Schwann cells are absent from the area of injury (Smith and Stevenson, 1988). In the PNS, Schwann cells and macrophages are key players in Wallerian degeneration. Their main role is to pave the way to a permissive environment for axonal growth by helping eliminate the damaged and degenerated axons and myelin debris, also by secreting a diversity of proteins, including neurotrophic factors such as nerve growth factor (NGF) and brain

derived neurotrophic factor (BDNF) which are involved in axonal regeneration (Heumann et al., 1987; Acheson et al., 1991).

In the PNS, Schwann cells release a wide variety of chemokines and cytokines after injury; a number of these recruit macrophages to the degenerating nerve (Shamash et al., 2002). Schwann cells secrete leukemia inhibitor factor (LIF) which attracts macrophages *in vitro* (Tofaris et al., 2002). Additionally, LIF and tumor necrosis factor-α (TNFα) induce production of macrophage chemoattractant protein-1 (MCP-1) by Schwann cells (Subang and Richardson, 1999). As previously mentioned, during the first few days post injury, Schwann cells clear myelin debris in the absence of macrophages to promote fast clearance of nerve debris in the PNS (Perry et al., 1995). Thereafter, macrophages are recruited from the circulation into the degenerating sciatic nerve by 3 days post axotomy (Perry et al., 1987; Stoll et al., 1989), and macrophage numbers peak between 14 and 21 days after axotomy in the PNS (Avellino et al., 1995). Macrophages in the PNS mediate the final phase of myelin debris removal in the distal nerve stump. Post injury, macrophages enter the endoneural tubes at 5 days, and by 7 days post injury numerous macrophages are found within the basal laminar tubes (Griffin et al., 1992).

Conversely in the CNS, most macrophages present in the white matter early post injury are the resident microglia. Microglia numbers rise in response to injury 2–3 days later than do macrophages in PNS injury (Lawson et al., 1994). After DRG rhizotomy, it takes almost two weeks for microglia to express immunoreactivity for ED-1, a marker for activated macrophages (George and Griffin, 1994). Nonetheless these microglia clear disintegrating myelin only extremely slowly (Aldskogius, 1974). Another important difference in the response of the PNS and the CNS to injury is that the blood brain barrier is

disrupted at the site of injury but not along the entire length of the distal nerve tract as occurs in the PNS. The absence of blood brain barrier opening post injury may limit the infiltration of macrophages into degenerating CNS tracks (George and Griffin, 1994).

In addition to the environmental factors exerting a positive effect on the regenerative capacity of neuronal axons in the PNS, it appears that intrinsic factors also play a pivotal role in regeneration. However, the molecular identity of such factors is far from known.

In contrast to the PNS, axons injured inside CNS tracts normally fail to regenerate, independent of whether their cell bodies are located inside or outside the CNS. There is a wealth of data indicating that the CNS environment is inhibitory to axonal regeneration, particularly the non permissive environment generated by the scarring process that occurs around all CNS injuries, most predominant in the spinal cord (Fawcett and Asher, 1999). Additionally, the inhospitable glial environment of the CNS composed of astrocytes and oligodendrocytes results in CNS axons growing defectively (Fawcett et al., 1989a; Fawcett et al., 1989b). Consequently, it is becoming apparent that successful neuronal regeneration depends on signaling events and processes representing an interplay of inhibitory growth signals in the environment and the intrinsic growth capacity of the damaged neuron.

Below I shall review the diverse inhibitory and permissive factors that exert different roles, both intrinsically and extrinsically, in the process of regeneration in the central and peripheral nervous system.

1.4 Inhibitory factors

The inability of axons to regenerate after injury to the adult CNS could be the consequence of several factors including the formation of a glial scar, the absence of neurotrophic factors, the intrinsically reduced capacity for growth, or the presence of growth inhibitory molecules. There are several inhibitory molecules present in the injured CNS which gives rise to a less favourable environment for axonal regeneration, limiting the axon's ability to sprout, growth, reach its target and restore functional synapses. Consequently injury to the CNS is in general irreparable. Axon inhibitory molecules can be categorized in different groups such as myelin associated molecules, components of the extracellular matrix and inhibitory molecules released by the glial scar.

The myelin sheath is a membranous extension which encircles an axon in a multilayered concentric fashion. Myelin is composed of approximately 80% lipid and 20% protein. Some of the proteins that make up myelin are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). Myelin serves as an electrical insulator which increases the speed of conducted action potential impulses via a process called saltatory conduction. In the PNS, myelin is produced by Schwann cells with a single Schwann cell myelinating a single axonal segment. In the CNS, however, myelination is performed by oligodendrocytes which send out projections that upon contact with an axon begin the new process of membrane extension and axonal envelopment. In the CNS a single oligodendrocyte can myelinate many axons. In both systems myelin is crucial for accurate signal propagation.

During the myelination process changes occur in the expression of myelin membrane proteins. The final phase of myelination also correlates with an abrupt drop in axonal sprouting and growth (Savio and Schwab, 1990; Kapfhammer and Schwab, 1994a; Kapfhammer and Schwab, 1994b). This loss of growth potential may be attributed to the expression of the myelin associated inhibitors of regeneration. Since these myelinated axons have already reached their targets prior to the end of the myelination process, it is believed that the myelin associated inhibitors may act to prevent unintentional sprouting and improper synapse formation. While this blockage of axonal growth by the inhibitors is essential following the end of development, it also has the adverse effect of blocking any efforts by damaged adult axons to regenerate after injury.

1.4.1 Myelin associated molecules

1.4.1.1 Nogo

Studies performed by Schwab and co-workers during the mid-late 1980s examined CNS myelin's inhibitory properties and found that the main inhibitory components were membrane bound and associated with the protein fraction of CNS myelin. Subsequently, the inhibitory components of myelin were biochemically separated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as 35-and 250 kDa proteins called initially neurite growth inhibitor NI-35 and NI-250. Interestingly, it has been shown that treatment of axons with NI-250 *in vitro* causes a rapid and large increase in growth cone calcium, which in turn leads to growth cone collapse (Bandtlow et al., 1993; Schwab et al., 1993). Furthermore, the application of the monoclonal antibody IN-1 raised against a

NI-35 and NI-250 showed a reduced inhibitory activity of myelin *in vitro* (Caroni and Schwab, 1988). The IN-1 antibody was then instrumental in the characterization and protein sequencing of its target antigen, which led to the cloning of what is today known as Nogo (Chen et al., 2000a; GrandPre et al., 2000; Prinjha et al., 2000).

There are three Nogo isoforms, Nogo-A, -B and –C, encoded from a single gene by alternative splicing. The three Nogo isoforms contain a 66 amino acid extracellular region (Nogo-66) which displays neuron-specific growth inhibitory activity *in vitro* (GrandPre et al., 2000; GrandPre et al., 2002). During development Nogo-A mRNA is highly expressed in the ventral spinal cord, DRG and autonomic ganglia of both humans and rodents. In the adult, Nogo-A (in addition to the spinal cord and ganglia), is also highly expressed in oligodendrocytes and motor neurons. However, it is not expressed in astrocytes or Schwann cells (Josephson et al., 2001). Schwab and colleagues demonstrated that Nogo-A expressed mainly by oligodendrocytes is highly inhibitory for axonal growth (Savio and Schwab, 1989; Savio and Schwab, 1989; GrandPre et al., 2000; Caroni et al., 1988).

Several knock-out models targeting the three different isoforms have been generated but at the present there is a lack of consensus on the effect of the Nogo deletion. Regeneration studies performed on these animals have shown limited axonal repair (Kim et al., 2003; Zheng et al., 2003; Simonen et al., 2003). It has been shown that neutralization of Nogo-A inhibitory activity with the IN-1 antibody permits some axons to regenerate. Curiously, this effect on regeneration was shown initially to be limited since only 5-10% of the axons regenerate (Schnell and Schwab, 1990; Bregman et al., 1995). Furthermore, the humanized anti-Nogo antibody promotes axonal sprouting and functional recovery

following unilateral transection of the cervical spinal cord in macaque monkeys (Freund et al., 2006; Freund et al., 2007).

1.4.1.2 Myelin associated glycoprotein

Another inhibitory molecule is the myelin associated glycoprotein (MAG) that is expressed in myelin forming cells, oligodendrocytes in the CNS and Schwann cells in the PNS. MAG appears to play a major role in myelination, it has the ability to inhibit the growth of many but not all types of axons and like Nogo, it is capable of causing growth cone collapse (Mukhopadhyay et al., 1994). MAG is a bifunctional protein that intriguingly can promote or inhibit neurite outgrowth depending on the neuronal age (Mukhopadhyay et al., 1994; Sheng et al., 1989; Turnley and Bartlett, 1998). For instance, it has been demonstrated that during development MAG does not impede axonal growth. While at a later stage, the neuron's response to the activity of MAG changes giving rise to an inhibitory reaction (DeBellard et al., 1996). Additionally, the effect of MAG on the growth cone depends on the level of cyclic adenosine monophosphate (cAMP). MAG is inhibitory when cAMP is low, normally in adult neurons, but it allows growth when cAMP is high, which is the case of young neurons (Cai et al., 2002; Cai et al., 2001; Cai et al., 1999) .

In an effort to further elucidate the role of MAG in the inhibition of axonal regeneration, MAG knock-out mice have been generated. Myelin purified from these animals exhibits a reduced ability to inhibit axonal growth from primary neurons *in vitro* (Li et al., 1996; Shen et al., 1998). Additionally, MAG knock-out mice were cross-bred with Wld^{s} mice that have a delayed lesion-induced myelin degeneration following

peripheral nerve injury and which show impaired axonal regeneration (Brown et al., 1991). After peripheral nerve injury, analysis of MAG deficient/Wld^s mice revealed that the number of regrowing axons travelling along intact myelin sheaths doubled as compared to Wld^s mice expressing MAG, suggesting that the absence of MAG may contribute to the improved axonal regrowth in the double mutants (Schafer et al., 1996). The dramatic difference in the clearance of myelin debris could be attributed, at least in part, to the difference in regenerating ability seen between CNS and PNS neurons.

1.4.1.3 Oligodendrocyte myelin glycoprotein

Oligodendrocyte myelin glycoprotein (OMgp) is a 120 kDa highly glycosylated protein and the most recent myelin-associated molecule with inhibitory properties that has been identified (Kottis et al., 2002). OMgp has been found on neurons such as pyramidal cells in the hippocampus and cortical neurons (Mikol and Stefansson, 1988; Habib et al., 1998). OMgp is also expressed during fetal and post-natal brain development and its concentration peaks at the late stages of myelination (Mikol and Stefansson, 1988; Vourc'h et al., 2003a). OMgp induces growth cone collapse and inhibits axonal growth *in vitro* (Kottis et al., 2002; Wang et al., 2002b). OMgp contains a highly conserved leucine-rich repeat (LRR) domain which is required for proper receptor binding. Different studies have shown that the deletion of this LRR domain leads to a total loss of the OMgp inhibitory capacity *in vitro* (Vourc'h et al., 2003b).

1.4.2 Myelin inhibitor receptors

Interestingly, Nogo, MAG and OMgp bind to the neuronal Nogo receptor (NgR) with high affinity (Fournier et al., 2001; Fournier et al., 2002; Wang et al., 2002b) see (Fig. 3). Binding requires the interaction with the p75 neurotrophin receptor (p75NTR) for mediation of the inhibitory signal (Wang et al., 2002a) that activates Rho-A, a GTPase member of the Rho family involved in growth cone collapse and the retraction of neurites (Lehmann et al., 1999). Furthermore, inactivation of Rho-A with C3 enzyme can stimulate axonal outgrowth on MAG and myelin substrates (Lehmann et al., 1999).

The expression pattern of NgR is consistent with a role in the inhibition of axonal regeneration. Transcripts are present mainly in the adult and maturing brain where NgR protein is found in many type of neurons but not in oligodendrocytes (Hunt et al., 2002) . Importantly, blocking studies on NgR and p75NTR independently have shown that the inhibitory effects of Nogo, MAG and OMgp can be reduced (Wang et al., 2002b; Wang et al., 2002a). Furthermore, NEP1-40, a peptide antagonist of NgR competes for binding of Nogo-66 to NgR (GrandPre et al., 2002). NEP1-40 which blocks Nogo, but not MAG/OMgp *in vitro*, has shown significant efficacy in promoting regeneration and recovery after experimental spinal cord injury (GrandPre et al., 2002).

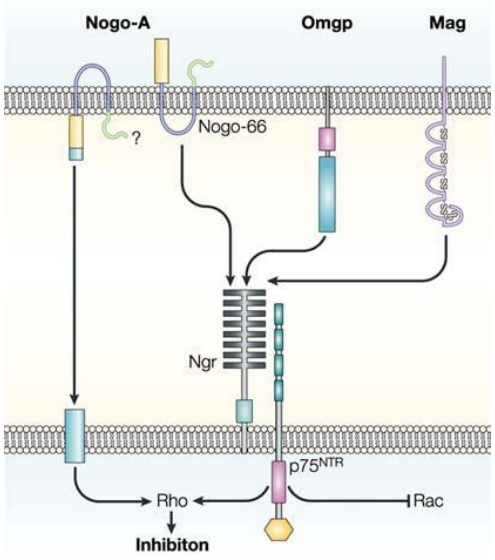


Fig. 3 Nogo, MAG and OMgp all interact with Nogo receptor to bring about inhibition of neurite outgrowth: Modified from Filbin M, Nature Reviews Neuroscience 4, 703-713. NgR contains two regions of LRRs — a cluster of eight together, and a series closer to the carboxyl terminus. NgR interacts with $p75^{NTR}$ to transduce the inhibitory signal across the membrane. Nogo, Mag and OMgp all activate Rho to bring about inhibition. Mag has been shown to inactivate Rac, but it is not known whether this effect is required for inhibition.

NgR does not possess intracellular signalling domains and therefore require co-receptors to transduce inhibitory activity. The low-affinity neurotrophic factor receptor, p75NTR, has been reported to transduce growth inhibitory signals through a membrane complex involving NgR1 (Wang et al., 2002a). During early development p75NTR is expressed in many types of cells within the CNS and PNS (Ryffel and Mihatsch, 1993). Injury to the nervous system induces p75NTR expression in many cells types.

1.4.3 Components of the extracellular matrix

The extracellular matrix represents the environment for numerous inhibitory molecules that exert their effect on axons during elongation processes immediately after injury. One of the

most prominent members in this category is Tenascin R, present in the basement membrane and in white matter. It is produced by astrocytes during development and with important roles in mediating axonal-glia interactions (Lochter et al., 1991; Faissner and Kruse, 1990). Tenascin R is upregulated after axonal injury (Laywell et al., 1992) and it has been shown to be inhibitory for axonal growth (Pesheva et al., 1989).

Damage to the CNS results in an injury response mechanism that ultimately after several weeks results in the formation of a glial scar, this process is also known as gliosis. This glial scar formation represents a major obstacle to the regeneration of axons. Several studies indicate that glial scars can inhibit both axon growth and myelination. Throughout gliosis, different cell types become involved and recruited to the injury site at different time points presenting a variable environment for growing axons. This can be supportive during early stages post-injury or inhibitory by the end of the glial scar formation (Kreutzberg, 1996). Initially, within hours after injury microglia cells and macrophages from the bloodstream will enter the CNS lesion and 3-5 days after injury oligodendrocyte precursors are recruited particularly to demyelinated areas of a CNS injury. These cells correspond to a major source of inhibition for axonal growth in the injured CNS since they express a wide variety of inhibitors such as NG2 proteoglycans (Dou and Levine, 1994), neurocan (Asher et al., 2000) and versican (Asher et al., 2002). If the injury penetrates the meningeal surface of the brain or spinal cord, meningeal cells will migrate to the injury site immediately after oligodendrocyte precursor cells arrival. Meningeal cells have been described also as inhibitory since they produce the non-permissive molecules NG2 proteoglycan (Levine and Nishiyama, 1996) and tenascin (Faissner, 1997).

Ultimately, the glial scar's final organization consists principally of tightly packed and interconnected astrocytes which are also inhibitory to axon growth. Astrocytes and oligodendrocyte precursors account for the production of the inhibitory chondroitin sulphate proteoglycans (CS-PGs), neurocan, brevican, and NG2 (Rhodes and Fawcett, 2004; Friedlander et al., 1994). Furthermore, it has also been suggested by electron microscopy studies that the structural organisation of the astrocyte processes are tightly interlinked, making the astrocytic scar impenetrable and physically impossible for axons to cross. CS-PGs consist of a core glycoprotein (neurocan, versican, NG2, brevican, phosphacan, aggecan and biglycan) attached to sulphated glycosaminoglycan (GAG) sugar chains. CS-PGs become highly upregulated in the CNS lesion site (Fitch and Silver, 1997; Snow et al., 1990). Interestingly it has been demonstrated that axonal re-growth in the injured CNS is prevented around the areas where CS-PG is expressed (Davies et al., 1997).

Additionally CS-PGs have been implicated in axonal regenerative failure after injury *in vitro* (Niederost et al., 1999) and *in vivo* (Davies et al., 1999). A related series of observations showing the inhibitory effects of CS-PGs on axonal growth has been made during neuronal development where chondroitin sulphate GAGs (CS-GAG) function as repulsive cues for elongating axons (Kubota et al., 1999). Conversely, inhibition of CS-PG's negative effect on axonal growth can be partially overcome by using chondroitinase, an enzyme that digests CS-GAG sulphate chains. This treatment on gliotic tissue in CNS lesions has been demonstrated to be favourable to some extent for axonal regeneration and recovery of function (Bradbury et al., 2002; Moon et al., 2001). However, this beneficial effect on axonal regeneration is not nearly equivalent with axonal regeneration observed in the PNS. Even though the mechanism by which CS-PGs exert their inhibitory action on

axonal growth is still not well understood, some indications demonstrate that GAG chains are responsible in great part for this effect which is dependent on GAGs sulfation pattern (Clement et al., 1998). *In vitro* studies indicate that if GAG sulfation is avoided then most of its inhibitory effect is reduced (Smith-Thomas et al., 1995). However, CS-PG protein cores are also involved in axonal growth inhibition. Interestingly, some evidence suggests that the growth inhibitory effects of CS-PGs in the glial scar appear to be mediated by activation of protein kinase C (PKC) and via a signalling pathway which, like NgR-p75NTR pathway utilizes the small GTPase Rho and its downstream effector, ROCK (Dergham et al., 2002; Borisoff et al., 2003; Sivasankaran et al., 2004). Rho in its active form has a negative effect on the actin cytoskeleton, thereby inhibiting axonal elongation and mediating growth cone collapse (Borisoff et al., 2003).

1.5 Neurotrophins and cytokines

One common effect of axonal insult is the variation in the accessibility of target-derived neurotrophic growth factors. Following injury, the axotomized neurons lose connection with their targets and consequently they no longer receive any target-derived neurotrophic factors. Interestingly, Schwann cells in the peripheral damaged area continue making some of these factors. However, this compensatory production of neurotrophic factors is not sufficient to replace normal factors derived from their targets (Heumann et al., 1987). These neurotrophic factors include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT 4/5). This group of neurotrophic factors exert their effects through binding specifically to tyrosine kinase receptors (trk). It is known

that NGF interacts with trkA, BDNF and NT 4/5 with trkB and NT-3 with trkC (Fig. 4). All these three high-affinity receptors are expressed within DRG (McMahon et al., 1994; Kashiba et al., 1995; Wright and Snider, 1995). Additionally, both NGF and its receptors are produced during development, adult life, and aging by many cell types in the CNS and PNS, immune and inflammatory system. In addition to trk A, B and C, a low affinity cell surface receptor, p75 neurotrophin receptor, binds and mediates the cellular response to NGF and BDNF (Lindsay, 1994; Chao and Hempstead, 1995; Friedman and Greene, 1999). During embryonic development sensory neurons require a sufficient supply of these factors in order to survive and maturate. However, after birth these factors are no longer needed for neuron survival.

In vitro overnight treatment, "priming", of neurons with NGF and BDNF is another way to increase cAMP levels. Neurotrophins can initiate an intracellular signalling cascade that prevents cAMP degradation. Neurotrophin binding to trk receptors leads to autophosphorylation of the receptor and activation of extracellular signal-regulated kinase (ERK) resulting in the inhibition of the phosphodiesterase (PDE4) (Kaplan and Miller, 2000). As mentioned before, inhibition of phosphodiesterase leads to cAMP elevation by preventing its degradation. Subsequently, by blocking the activation of ERK, the improved regeneration observed after cAMP increase *in vitro* is abolished (Gao et al., 2003).

NGF signalling has also been demonstrated to regulate the expression of CREB through the cAMP-PKA pathway (Finkbeiner, 2000) which has been previously reported to be involved in neuronal regeneration. Additionally it has been demonstrated *in vitro* that NGF expression by Schwann cells is upregulated by cytokines and other inflammatory mediators (Lindholm et al., 1987; Mirsky and Jessen, 1999).

In the adult nervous system neurotrophic factors are suggested to have a neuroprotective activity on severely injured or degenerating neurons. For instance, in adults, NGF expression is undetectable in Schwann cells, but after nerve insult, Schwann cells distinctly upregulate expression of NGF *in vivo*. Additionally, following nerve injury retrograde axonal transport of NGF is transiently downregulated 10-fold for nearly 48hrs (Raivich et al., 1991). NGF withdrawal induces axotomy-like effects in the uninjured, NGF sensitive sensory and sympathetic neurons, predominantly when it comes to the expression of transcription factors such as c-jun (Gold et al., 1993) and different neuropeptides including galanin, vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) (Shadiack et al., 2001).

However, unlike the traditional view of NGF effects *in vitro* and in developmental studies where treatment with NGF has been shown to promote neurite outgrowth (Levi-Montalcini and Angeletti, 1968) the effects of NGF *in vivo* remains controversial.

On one side intriguing observations suggest that treatment with NGF, NT-3, or glial cell derived neurotrophic factor (GDNF) can be successful in promoting sprouting of axons by regulating the intrinsic capacity of neuronal regeneration in the damaged spinal cord (Ramer et al., 2000). In addition, functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy found that adenoviral gene transfer of NGF or FGF2 promoted functional regeneration of sensory axons into the spinal cord following dorsal root injury (Romero et al., 2001). Similarly, intrathecal administration of BDNF in conjunction with transplantation of embryonic spinal cord tissue into damaged spinal cord resulted in marked enhancement of axon growth from descending tracts beyond the lesion, leading to partial functional recovery (Coumans et al., 2001).

On the other hand, it has been shown that the application of NGF to axotomized neurons causes a delay in axonal regeneration (Hirata et al., 2002; Gold, 1997). Additionally, some experiments associate the NGF treatment with development of hypersensitivity to nociceptive stimuli in form of allodynia and hyperalgesia (Petty et al., 1994; Shu and Mendell, 1999). Interestingly, NGF deprivation by the use of specific antibodies prevents hyperalgesia (McMahon et al., 1995).

These reports from different sources indicate that treatment with NGF although may be beneficial after nerve injury for the survival of neurons and sprouting of axons may have limited potential to translate into human trials due to its influence on pain.

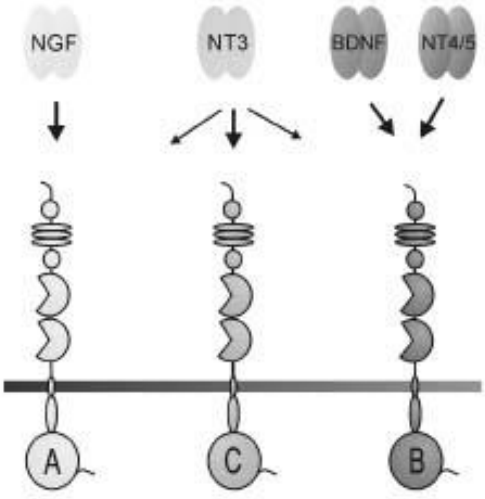


Fig. 4 The trk family of neurotrophins receptors: All neurotrophins bind to one or more receptor of the Trk protein family. (Tyrosine receptor kinase, TrkA, B, C) Trk receptors are a small subfamily consisting of three members out of the large superfamily of several hundred proteins with tyrosine kinase enzymatic activity that also includes receptors for epidermal growth factor (EGF), insulin, fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and numerous other growth factors. The Trk receptor tyrosine kinases are characterized by a specific combination of structural motives: In their extracellular domains: three tandem repeat leucine-rich motives are flanked by two cysteine clusters. The main contacts between the Trk receptors and their ligands occur within two Ig-like C2 type domains. The protein sequences of the intracellular, enzymatically active, tyrosine kinase domains are highly conserved in the three receptors. The neurotrophins NGF, NT-3, BDNF and NT 4/5 bind to the Trk receptors with strong (thick arrows) and weak interactions (thin arrows). From (Dechant, 2001).

Interleukin – 6 (IL-6), LIF, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) are structurally and functionally related cytokines which have been defined as neuropoietic cytokines (Heinrich et al., 2003). Previously, it has been shown that neuropoietic cytokines promote survival of various populations of embryonic and newborn neurons, including sensory and motor neurons (Sendtner et al., 1996; Simon et al., 1995; Horton et al., 1998).

Both LIF and IL-6 are strongly upregulated after sciatic nerve transection (Murphy et al., 1995) and some injury responses are lost in LIF -/- or IL-6 -/- mice (Kurek et al., 1996; Zhong et al., 1999; Cafferty et al., 2004; Cafferty et al., 2001). For example, both LIF -/- and IL-6 -/- mice display reduced peripheral nerve regeneration (Zhong et al., 1999; Cafferty et al., 2001). Additionally, without IL-6 or LIF the intrinsic growth capacity of DRG neurons is significantly reduced *in vitro* and *in vivo* (Cafferty et al., 2004; Cafferty et al., 2001; Inserra et al., 2000).

IL-6 upregulation following peripheral axotomy has been correlated with accelerated peripheral nerve regeneration (Hirota et al., 1996). Additionally, IL-6 expression is also increased after CNS injury (Gadient and Otten, 1997), however, high levels of IL-6 could also exacerbate the inflammation that occurs after spinal cord injury and lead to increased cell death (Gadient and Otten, 1997).

CNTF is an abundant cytosolic molecule in myelinating Schwann cells of adult rodents and after nerve lesion CNTF mRNA and protein levels are reduced in distal regions of the sciatic nerve and then increase concomitantly with regeneration (Sendtner et al., 1992b). In response to nerve injury, CNTF is transported axonally at an increased rate to

the neuronal cell body (Curtis et al., 1993). CNTF supports survival and/or differentiation of a variety of neuronal cell types including sensory, sympathetic, and motoneurons. Also, non-neuronal cells, such as oligodendrocytes, microglial cells, liver cells, and skeletal muscle cells, respond to exogenously administered CNTF, both *in vitro* and *in vivo*. Due to the fact that CNTF -/- mice appear remarkably normal and display only mild motor neuron problems and that the expression of CNTF is altered by nerve injury in the CNS or PNS, suggest that CNTF does not play a critical role in development of the nervous system but does play a role in the injury response of the nervous system (Ip, 1998; Seniuk et al., 1992; Sendtner et al., 1997; DeChiara et al., 1995). Additionally, *in vivo*, CNTF rescues several kinds of neurons from axotomy-induced death (Richardson, 1994).

1.6 Intrinsic factors involved in axonal regeneration

Axonal regeneration is influenced not only by inhibitory cues, but also by permissive neuronal factors such as second messengers, regeneration associated genes, transcription factors, growth associated proteins, cytoskeletal proteins, growth factor, their different receptors and cytokines. Therefore, the final outcome in nerve regeneration reflects an interaction between the growth ability of the neurons and different signalling pathways responsive to the non-permissive environment. These pathways are fully exemplified by regeneration of the somatosensory system.

Each segment of the spinal cord has a sensory dorsal root and motor ventral root (Fig. 5). The prime function of the spinal dorsal roots and their cranial equivalents is to serve as afferent conduits to the central nervous system. The primary afferent fibres are

sensory neuronal peripheral branches, which terminate in the skin or other peripheral tissues. Primary sensory neurons also have central branches, which end in the dorsal horn of the spinal cord. The cell bodies of the neurons that innervate the head and body areas are located in the trigeminal and dorsal root ganglia (DRG), respectively.

Sensory axons have their cell bodies in the DRG, which have an axon which divides into two branches, the first targeting the PNS and another branch entering the CNS at the dorsal root entry zone (DREZ). Due to this particular anatomical organization of the DRG, it is possible to perform studies in order to directly compare CNS and PNS regeneration and also to determine whether it is the inability of the axon or the inhibitory surrounding of the CNS environment that inhibit regeneration. Nerve injury leads to a vigorous axonal regeneration when the injury is elicited in the peripheral branch; in contrast a nerve lesion on the central branch extending into the dorsal column of the spinal cord results in a failure in regeneration. Interestingly, if the peripheral branch is injured at a time point before the central branch, regeneration of the central axons can be enhanced (Neumann and Woolf, 1999; Richardson and Issa, 1984; Richardson and Verge, 1986). This effect is called the conditioning lesion.

The capability of a conditioning peripheral lesion to induce regeneration of central dorsal column axons has previously been documented. The first studies described improved growth after a conditioning lesion of dorsal column axons into a peripheral nerve graft (Richardson and Issa, 1984). Recent studies have shown that a conditioning lesion can induce regeneration of dorsal column axons in the absence of a peripheral nerve graft (Neumann and Woolf, 1999).

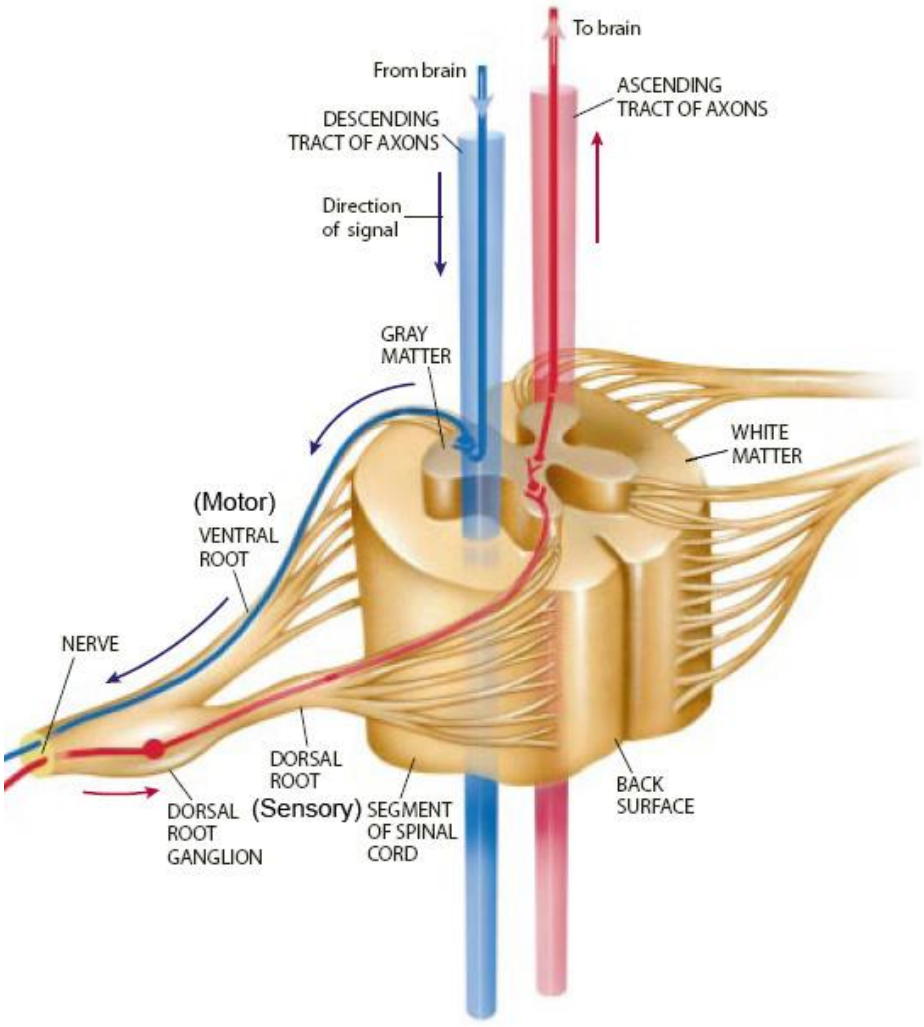


Fig. 5 Anatomical organization of the spinal cord and the dorsal root ganglion: Red colour illustrates the sensory tract whereas blue colour illustrates the motor pathway. Taken from McDonald J, Scientific American, September, 1999.

1.6.1 Cyclic AMP

The molecular mechanisms responsible for the conditional lesion effect were not well understood until it was discovered that the levels of a second messenger, cAMP become elevated following a conditioning lesion, allowing neurons to grow on MAG and myelin (Qiu et al., 2002; Neumann et al., 2002). Twenty four hours after a sciatic nerve injury,

cAMP levels in DRG neurons are upregulated three-fold. This effect depends on protein kinase A (PKA), a downstream effector of cAMP. By 1 week post lesion, cAMP levels in DRG neurons return to control, but growth on MAG/myelin improves and is now PKA independent. To address if the ability of mature DRG neurons to grow on MAG and myelin after a conditioning peripheral lesion is dependent on cAMP, an inhibitor of protein PKA, was injected into DRGs at the same time as the conditioning lesion. PKA inhibition blocks completely the improved growth on MAG and myelin 1 day later and attenuates growth after one week (Qiu et al., 2002). Additionally, dibutyryl cAMP (dbcAMP), a non-hydrolyzable cAMP analogue, was injected into DRGs 1, 2 or 7 days before plating the neurons on myelin. The result showed that neurite outgrowth was significantly increased, demonstrating that cAMP has the capacity to mimic the conditioning lesion effect *in vitro* in a PKA-dependent manner that becomes PKA independent (Qiu et al., 2002; Neumann et al., 2002) see (Fig. 6). Additionally, administration of dbcAMP overcomes inhibition by MAG and myelin in different neuronal subtypes, including cerebellar, cortical, and hippocampal neurons (Cai et al., 1999).

The therapeutic potential of cAMP has also been assessed in studies of spinal cord injury. This has been done by elevating cAMP levels through the application of rolipram, a phosphodiesterase inhibitor that blocks cAMP degradation and induces an increase in cAMP levels *in vitro* and post-injury *in vivo* improving regeneration and functional recovery (Nikulina et al., 2004; Pearse et al., 2004). Rolipram treated animals also showed a decreased expression of glial fibrillary acidic protein (GFAP) indicating a reduced glial scarring (Nikulina et al., 2004). As mentioned before the glial scar is another major contributor to regenerative failure, forming both a physical and biochemical barrier to

regenerating axons. These results suggest that astrogliosis can be reduced through elevation of cAMP, which could enhance axonal regeneration by making the CNS environment more permissive.

Rolipram has the added advantage of being able to cross the blood-brain barrier, which makes subcutaneous and oral administration possible (Krause and Kuhne, 1988) alleviating the need for invasive surgery that could cause further damage to the spinal cord. This makes Rolipram a logical attractive drug for potential therapeutic intervention.

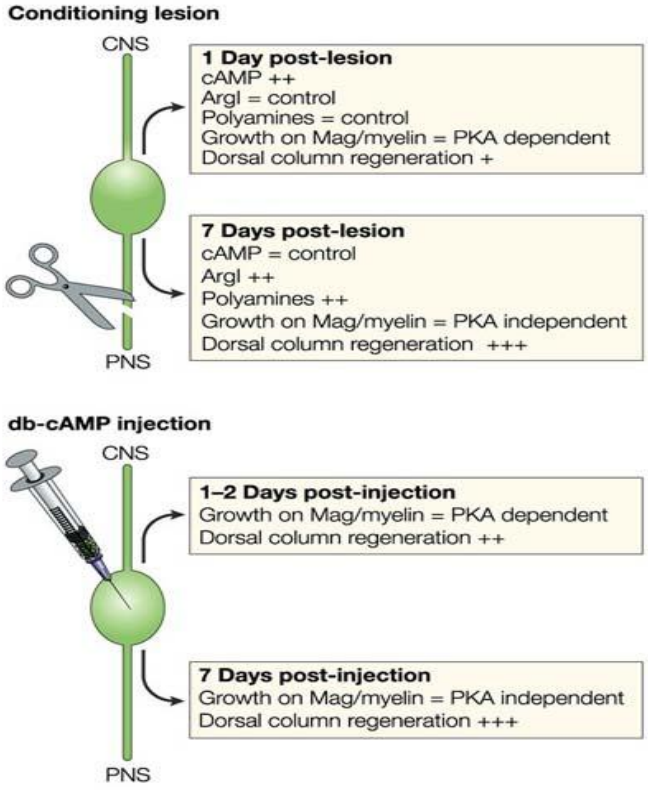


Fig. 6 Elevation of cAMP mimics the effects of a peripheral conditioning lesion on dorsal column regeneration: From Filbin M, Nature Reviews Neuroscience 4, 703-713.

In a conditioning lesion the peripheral branch of the DRG is lesioned 1, 2 or 7 days before carrying out the measurements as described. To elevate cAMP, dibutyryl cAMP (db-cAMP) was injected directly into the dorsal root ganglion cell bodies in the absence of a peripheral lesion, and the animals were left for 1, 2 or 7 days before carrying out the assays/measurements as described. By day 7 cAMP-induced neurite outgrowth becomes PKA-independent post-lesion and post-injection. ArgI, Arginase I; CNS, central nervous system; MAG, myelin-associated glycoprotein; PKA, protein kinase A; PNS, peripheral nervous system. + indicates expression or regeneration relative to untreated/unlesioned. Control indicates background levels.

It has also been shown by other groups that the increase of cAMP levels is involved in a variety of neuronal processes including memory and learning (Alberini et al., 1995), neuronal survival (Hanson, Jr. et al., 1998), neurite outgrowth (Kao et al., 2002), axonal regeneration (Teng and Tang, 2006), axonal growth cone turning and developmental guidance (Song et al., 1997).

It is known that MAG can induce a low-level gradient of intracellular calcium within the growth cone leading to a repulsive turning response (Henley et al., 2004). Interestingly, by administration of cAMP analogues calcium levels can be further increased. This can subsequently convert MAG-mediated repulsion to attraction (Henley et al., 2004; Henley and Poo, 2004).

It is normally accepted that embryonic neurons are capable of extending long axons, both during development or following axotomy at the embryonic stages. Consequently, cAMP studies have revealed that the cAMP levels of embryonic neurons are significantly higher than that of their adult counterparts, and that this elevation can account for the ability of these neurons to extend processes in the inhibitory CNS environment (Cai et al., 2001). Furthermore, the loss of regenerative capacity which occurs postnatally correlates with a decrease in endogenous cAMP levels. Even in neuronal types which retain their regenerative abilities in the neonatal stages, cAMP levels appear to remain high until the developmental switch occurs (Cai et al., 2001).

While cAMP has many different roles in a variety of signalling pathways in neuronal cells, the artificial increase of cAMP alone may not be a satisfactory therapeutic approach. Therefore, it is necessary to understand the entire complexity of the cAMP

signalling pathway; particularly the downstream effectors and cAMP-regulated genes that might mediate this improved regenerative capacity. Understanding these events may present yet another potential target for therapeutic intervention, which in combination with other approaches could hopefully encourage regeneration in adults.

The intracellular second messenger cAMP is produced close to the cytoplasmic surface of the plasma membrane in response to a variety of extracellular signals, including hormones, growth factors, and neurotransmitters. These signal molecules bind to a surface receptor to activate adenylyl cyclase through a trimeric stimulatory G protein. Active adenylyl cyclase catalyses the synthesis of cAMP from ATP, which upon formation binds to the regulatory subunits of PKA (Fig. 7). PKA is normally inactive as a tetrameric holoenzyme, consisting of two catalytic and two regulatory units (C_2R_2), with the regulatory units blocking the catalytic centres of the catalytic units. cAMP binds to specific locations on the regulatory units of the protein kinase, and causes dissociation between the regulatory and catalytic subunits, thus activating the catalytic units and enabling them to phosphorylate substrate proteins.

The activated catalytic subunits of PKA are imported to the nucleus due to a putative nuclear localization signals in their amino acid sequence that facilitate passage through nuclear pores. In the nucleus, the activated kinase subunits phosphorylate cAMP response element binding protein (CREB) and other transcription factors. CREB binds to the 5′ untranslated regions of genes that contain a cAMP-response element (CRE) and, upon phosphorylation, initiates a cascade of gene expression. Normally the action of the kinase ceases when the cAMP is removed by enzymatic degradation by phosphodiesterase.

In the absence of cAMP, the catalytic subunits again become inhibited by re-association with regulatory subunits (Schwartz, 2001).

It has been demonstrated that cAMP acts on the downstream effector PKA to mediate its biological function. A possible mechanism of cAMP-induced activation of PKA on the ability to overcome MAGs inhibitory effect on neurite outgrowth is the phosphorylation and the subsequent inhibition of Rho GTPase signalling pathway, which is involved in growth cone collapse (Lang et al., 1996; Lehmann et al., 1999).

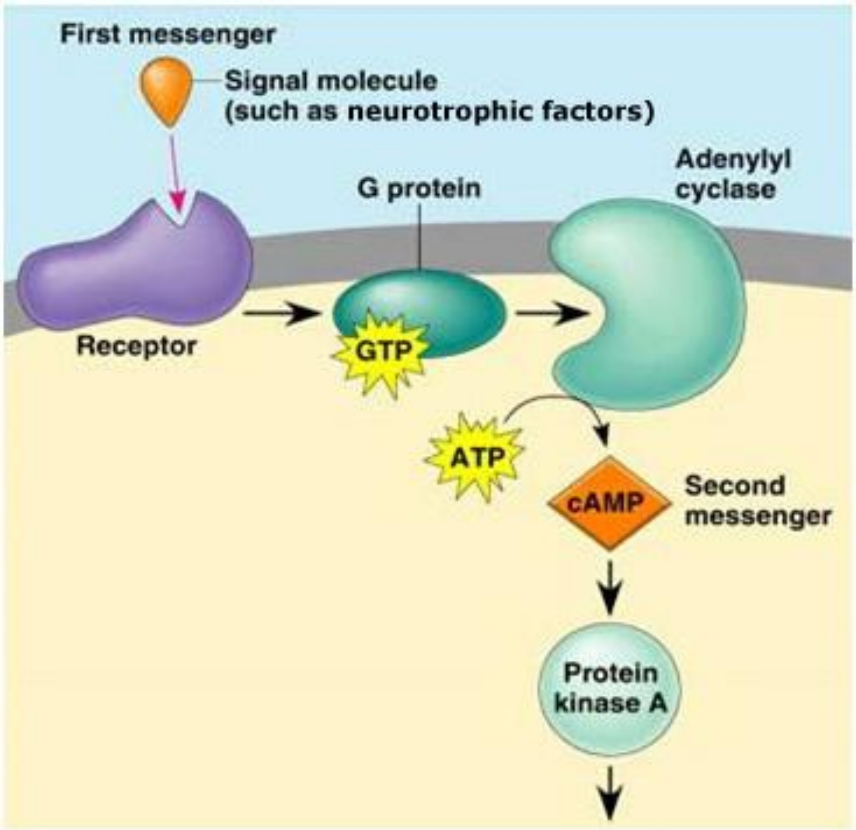


Fig. 7 cAMP signalling cascade leading to the activation of PKA: cAMP is a second messenger, used for intracellular signal transduction, such as neurotrophic factors, which cannot get through the cell membrane. cAMP is used by a major class of G proteins. cAMP is synthesised from adenosine triphosphate (ATP) by adenylyl cyclase which is embedded in the plasma membrane with the enzymatic activity in the cytoplasm. Adenylyl cyclase is activated by binding a subunit of the Gs G-protein (GTP-Gs). Protein kinase A (PKA), a cAMP-dependent kinase, is the main intracellular target of cAMP. Active PKA can further phosphorylate a number of proteins. Taken from Biology (Fifth Edition) by Campbell, Reece, and Mitchell.

Another signalling component that has been suggested to be involved in the regeneration promoting signal is the transcription factor CREB which is activated by elevated levels of cAMP (Lonze and Ginty, 2002). Indeed, recent evidence indicates that the phosphorylation and activation of CREB is a vital part of cAMP-mediated axonal growth (Gao et al., 2004). Upon treatment of cerebellar neurons with dbcAMP, CREB phosphorylation occurs within 5 minutes, and peaks by 1 hour after treatment (Gao et al., 2004). CREB activity is essential for overcoming inhibition by MAG and myelin. Expression of dominant negative CREB in DRG and cerebellar neurons blocks the ability of dbcAMP to overcome inhibition, whereas constitutively active CREB promotes neurite outgrowth on MAG in the absence of elevated cAMP *in vitro* (Gao et al., 2004). Furthermore, adenoviruses expressing constitutively active CREB have been injected into DRG neurons of adult rats prior to a dorsal column lesion 4 days later. The results showed a significant regeneration of axons into the lesion site in animals expressing constitutively active CREB (Gao et al., 2004). These findings suggest that activation of CREB is sufficient to promote axonal regeneration, indicating that the activation of CREB by cAMP leads to the transcription of genes that are involved in overcoming myelin inhibition.

One cAMP regulated gene that has been involved in overcoming inhibition by myelin is arginase I (Arg I). It has been shown that exogenous application of Arg I is neuroprotective against apoptosis-inducing stimuli in cortical neurons *in vitro* (Esch et al., 1998). During post-natal development, Arg I expression level in DRG neurons drops spontaneously between P3 and P5 and remains at a low level coinciding with cAMP decline and the onset of myelin inhibition (Cai et al., 2002). The elevation of cAMP leads to an upregulation of Arg I and the subsequent synthesis of polyamines, which overcome

inhibition of axonal regeneration by MAG/myelin (Fig. 8). Priming with polyamines and overexpression of Arg I using an adenoviral construct carrying the Arg I cDNA can block MAG/myelin-induced inhibition of regeneration (Cai et al., 2002). Conversely, blocking Arg I/polyamines abrogate the cAMP mediated regenerative increase (Cai et al., 2002).

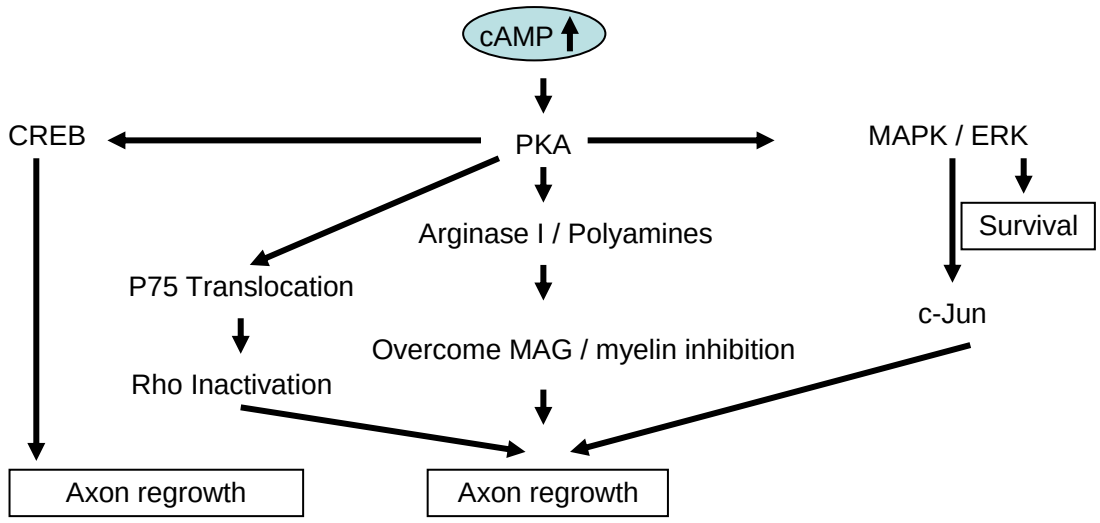


Fig. 8 Possible cAMP pathways involved in axon regrowth and neuronal survival: Modified from (Cui and So, 2004). Elevation of cAMP can influence neuronal survival and axonal regrowth via multiple signaling cascades in the nervous system. The primary signaling transduction executor is PKA, which acts on various pathways, including MAPK/ERK upregulation of c-Jun, Rho inactivation by p75 translocation into lipid rafts and upregulation of CREB and Arginase I/polyamines. These actions result in enhanced neuronal survival and axon regrowth potential and/or overcome axon growth inhibition.

1.6.2 Regeneration associated genes

The ability of neurons to regenerate an axon after injury is determined by both the surrounding environment and factors intrinsic to the damaged neuron. It is known that different neurons respond in various ways to the same environment and that injury induces changes in the axonal growth capacity of an injured neuron. Presumably, this is attributable to the induction of neuronal regeneration associated genes by peripheral axotomy.

To elucidate which regulated genes are involved in axonal regeneration *in vitro* and *in vivo* functional examination as well as cellular localisation and distribution of gene products has been performed. The majority of these experiments have involved conditioned DRG neurons where the regenerative propensity of DRG neurons after peripheral nerve injury is associated with changes in expression of numerous genes. Here below I shall discussed four regeneration associated genes, which are upregulated following nerve axotomy (Bonilla et al., 2002; Mason et al., 2002; Skene and Willard, 1981; Livesey et al., 1997).

1.6.2.1 GAP-43 and CAP-23

One of the most prominent regeneration associated genes involved in axonal regeneration is the growth associated protein of 43kDa (GAP-43), an acidic membrane protein that, along with cytoskeleton-associated protein of 23 kDa (CAP-23) and MARCKS (myristoylated alanin-rich C kinase substrate), belong to the MARCKS family and code for components of the axonal growth cone which are major protein kinase C (PKC) substrates. These three molecules, sharing similar structural and functional features, can regulate actin dynamics through modulating the accessibility of phosphatidylinositol 4,5-bisphosphate (PIP_2) at plasmalemma rafts (Laux et al., 2000).

GAP-43 is expressed at high levels in both central and peripheral neurons in the embryo but levels decline with maturation and aging (Skene, 1989a; Biffo et al., 1990). GAP-43 is also highly expressed on the surface of the growth cones of growing and successfully regenerating axons and it is expressed at low levels in neuron cell bodies (Chong et al., 1994; Meiri et al., 1988).

Peripheral nerve injury induces robust re-expression of GAP-43 but this upregulation is only seen in CNS neurons when the axon is injured close to the cell body (Tetzlaff et al., 1994). The upregulation of GAP-43 is associated with the activation of the interleukin-6 (IL-6)/ glycoprotein 130 (gp130)/Janus kinase signalling cascade, whilst IL-6 induces GAP-43 in rat pheochromocytoma cells (PC12) (Marz et al., 1997). While null deletion of IL-6 or sustained perineural infusion of the Janus kinase (JAK) inhibitor AG490 blocks upregulation of GAP-43 and compromises neurite outgrowth in the PNS (Qiu et al., 2005; Cafferty et al., 2004). Overexpression of GAP-43 in adult neurons enhances nerve sprouting with growth cones (Caroni 1997). However, GAP-43 alone is not sufficient to elicit regeneration of CNS axons. These results can be interpreted to imply that GAP-43 is important in the generation of growth cones but this is not enough for a successful induction of nerve repair (Mason et al., 2000).

CAP-23 also known as BASP1 and NAP-22 is expressed in several tissues but is particularly abundant in neurons during brain development, accounting for approximately 0.5% of the total amount of brain protein. The expression of the protein is maintained in selected brain structures throughout adulthood (Mosevitsky et al., 1994; Carpenter et al., 2004; Van den Bogaerdt et al., 2004) and up-regulation is observed in neurons during nerve regeneration (Mosevitsky, 2005). Furthermore, overexpression of CAP-23 in PC12E2 cells and primary hippocampal neurons stimulates neurite outgrowth in both cell types (Frey et al., 2000). Interestingly, GAP-43 co-expressed together with CAP-23 can induce axonal elongation by DRG neurons *in vitro* and it can enhance regeneration of central DRG axons into a peripheral nerve graft in adult mice after spinal cord injury *in vivo*(Bomze et al., 2001). This regenerating effect in the CNS is about one-third of what is normally seen with

a conditioning peripheral nerve injury (Bomze et al., 2001). In contrast, mice that are GAP-43 deficient show defects in axonal pathfinding and suppression of GAP-43 expression via antisense oligonucleotides disrupt growth cone formation, spreading, branching and adhesion (Strittmatter et al., 1995; Aigner and Caroni, 1995).

1.6.2.2 SPRR1A

Small proline-rich repeat protein 1A (SPRR1A) is an 18kDa protein consisting of 30% proline residues that belongs to the multigene SPRR family of keratinocyte differentiation markers which also include S100C and p21/wildtype p53 activator 1 (Gibbs et al., 1993; Starkey et al., 2009). Neuronal expression of SPRR1A was first detected in a microarray analysis of genes induced during successful sciatic nerve regeneration in the mouse (Bonilla et al., 2002). Unlike GAP-43, which is expressed during development and constitutively by some cells in the adult (Skene, 1989b), there is no expression of SPRR1A during development or in naïve uninjured cells (Bonilla et al., 2002).

SPRR1A induction is slightly upregulated in DRG neurons 1 day after peripheral sciatic nerve crush (Bonilla et al., 2002) and sciatic nerve transection (Starkey et al., 2009) but is drastically upregulated by 3-4 days, reaching maximal levels of expression at 7-14 days. However, while SPRR1A expression was reduced to negligible levels by 30 days after sciatic crush injury, SPRR1A was still expressed by a significant proportion of DRG neurons following transection (Starkey et al., 2009; Bonilla et al., 2002). Although there is a minimal expression following injury to the centrally projecting branches of DRG neurons, SPRR1A expression has also been detected following peripheral sciatic nerve injury in the

spinal cord by 1 week and in the terminals of DRG neurons in the dorsal horn and in motoneurons in the ventral horn (Starkey et al., 2009). These observations are similar to GAP-43 expression in the adult rat following injury, where virtually all injured DRG neurons are known to upregulate GAP-43 following peripheral nerve injury, but levels do not change following injury to central DRG projections (Schreyer and Skene, 1993; Bradbury et al., 2002). By using double immunofluorescence to show the extent of co-expression of SPRR1A with three markers for DRG subpopulation CGRP, IB4, and NF200, it was found that SPRR1A is expressed in all three DRG cell populations following either crush or transection of the sciatic nerve (Starkey et al., 2009).

SPRR1A localizes selectively to filamentous actin (F-actin)-rich structures and not others. The selective localization suggests that SPRR1A stimulates axonal extension by regulating actin-based motility in growth cone ruffles. In ruffles but not stress fibres, SPRR1A is co-localized with one another and F-actin, indicating that these co-induced proteins co-ordinately modulate actin dynamics. Overexpression of SPRR1A can induce large increases in neurite outgrowth in adult DRG neurons, SPRR1A shifts adult sensory neurons from a branching to an elongating mode of growth, and blockade of SPRR1A decreases the outgrowth potential of preconditioned adult neurons (Bonilla et al., 2002).

Pr**e**vious work has shown that SPRR1A is regulated by the gp130 signalling pathway in the heart. SPRR1A is upregulated by cytokines such as LIF and CT-1 that act upon the receptor signalling subunit gp130 which transduces the signal through, janus kinases (JAKs) into 3 different pathways MAP kinases (MAPKs), signal transducers and activators of transcription 3 (STAT3), and phosphatidylinositol 3-kinase (PI-3K) pathways.

Additionally binding sites for C/EBP and c-Jun were identified in the SPRR1A promoter region (Pradervand et al., 2004) see (Fig. 9).

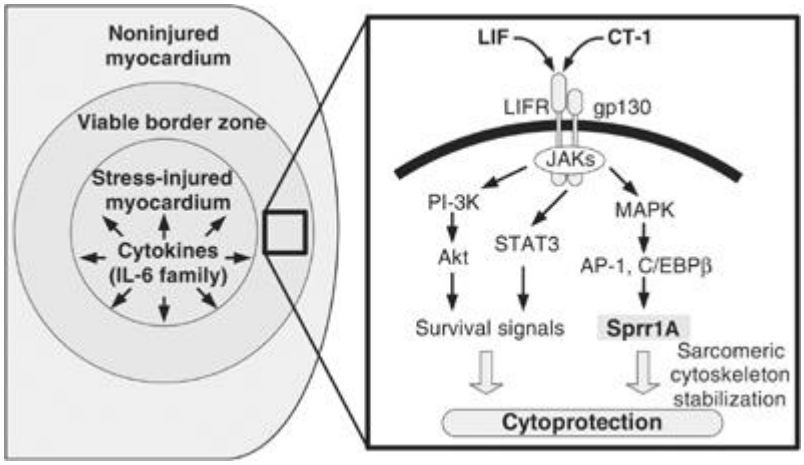


Fig. 9 SPRR1A activation in the myocardium: In response to myocardial injuries, IL-6 cytokines are released and act on the surrounding cells (left panel). The binding of IL-6 cytokines (CT-1, LIF) to gp130/LIFR receptor complex activates STAT3, PI-3K, and MAPK signalling pathways (right panel). A MAPK signalling pathway activates the transcription factors C/EBPβ and (activator protein -1) AP-1. In turn, they bind to SPRR1A promoter and induce SPRR1A gene transcription. Taken from (Pradervand et al., 2004).

1.6.2.3 Reg-2

Regenerating protein 2 (Reg-2) is a 16kDa secreted protein that is expressed in a cytokine-dependent manner in developing rat motor and sensory neurons. However, Reg-2 is normally not expressed in the adult rat central and peripheral nervous system. Following nerve axotomy, Reg-2 is expressed de novo within 24 hrs, in motoneurons, sympathetic neurons and DRG neurons. Reg-2 is upregulated in small IB4 positive DRG neurons 1 day after peripheral injury and its expression shifts from small neurons to medium-large (IB4 negative) neurons 5-7 days later (Averill et al., 2002) . In addition, a recent study showed that in a mono-arthritis inflammation model, Reg-2 is selectively upregulated in IB4

positive DRG neurons and that Reg-2 expression in these cells might be induced by LIF treatment (Averill et al., 2008) .

Reg-2 is axonally transported toward the injury site, it can act as a Schwann cell mitogen *in vitro* and its mitogenic capability can be enhanced by cAMP elevation (Livesey et al., 1997; Namikawa et al., 2005; Averill et al., 2008; Averill et al., 2002). The expression of Reg-2 in both sensory and motor neurons during development is driven by cytokines which include IL-6, CNTF, LIF and CT-1 (Livesey et al., 1997). These cytokines have been shown to prevent motor neuron cell death that follows neonatal axotomy (Cheema et al., 1994; Sendtner et al., 1990; Pennica et al., 1996) and to prolong motor neuron survival in strains of mice that show spontaneous motor neuron cell death (Sendtner et al., 1992a; Nishimune et al., 2000; Mitsumoto et al., 1994; Lindsay, 1996; Winter et al., 1996; Bordet et al., 1999).

Reg-2 inhibition in motoneurons by using antisense adenovirus cancels the survival effect of CNTF on cultured motoneurons, indicating that Reg-2 expression is a required step in the CNTF survival pathway (Nishimune et al., 2000). Direct injection of Reg-2 antibody into the crushed nerve significantly slowed regeneration of peripheral nerve after nerve crush (Livesey et al., 1997).

1.6.3 Retrograde signals

Activation of intrinsic growth capacity in neurons following injury allows regeneration to take place in the PNS. This event, truncated in the CNS is one of the mechanisms that contribute to the difference in the success of regeneration between the PNS and CNS.

Normally, the cell body of an injured neuron must collect accurate and timely information about the site and extent of axonal damage in order to boost its intrinsic growth capacity and successfully regenerate. Specific mechanisms must therefore exist to transmit such information along the length of the axon from the lesion site to the cell body. One postulated mechanism responsible for this event is the retrograde transport of injury signals where molecules activated at the injury site send information to the cell body giving rise to the upregulation of regeneration associated genes and thus increased growth capacity of the neuron. As previously mentioned a conditioning lesion to the peripheral branch prior to injury to the central branch promotes regeneration of central axons suggesting that retrograde injury signals travel from the peripheral injury site back to the cell body to increase the intrinsic growth capacity of the neuron. An increased intrinsic growth state may be one of the factors that could enable centrally injured axons to regenerate.

There are various injury signals functioning in a temporal sequence, including the interruption of the normal supply of retrogradely transported target-derived factors and retrograde injury signals emanating from the injury site travelling back to the cell body, also known as positive injury signals. Target derived signals, also known as negative injury signals, such as the lack of retrogradely transported NGF. This type of signal represses the intrinsic neuronal growth activity during embryonic to adult transition, once a neuron is connected with its target allowing proper synaptic development.

Positive injury signals triggered by axonal injury induces the local activation and retrograde transport of several mitogen activated kinases (MAPKs), including Erk (Perlson et al., 2005) , the c-Jun N-terminal kinase (JNK)(Cavalli et al., 2005). Inhibition of JNKs blocks neurite outgrowth in cultured adult neurons (Lindwall et al., 2004) and it has been

suggested that the activation of these kinases, in particular JNK and Erk and their interaction with the injury activates several downstream genes through the local release of cytokines which include LIF, IL-6, and CNTF.

Among these downstream genes several transcription factors have been shown to become phosphorylated and localized in the nucleus following activation of the different MAP kinase pathways. These genes and others regeneration associated genes may be regulated by transcription factors playing a pivotal role in changing the expression of genes and proteins pattern in the injured neurons that might be associated with axonal regeneration.

1.6.4 Transcription factors

Regulation of mRNA gene expression belongs to one of the most complex types of control in eukaryotic genes, which can be regulated at several steps, including transcriptional initiation, elongation, mRNA processing, transport, translation, and stability. Perhaps, the most crucial control of regulation is at the level of transcriptional initiation (Maston et al., 2006). Among the factors involved in the control of accurate transcription of protein coding genes by RNA polymerase II, transcription factors play a crucial role as a result of their interaction with regulatory sequences present in the promoter and enhancer regions of target genes. Different types of transcription factors, characterized by different DNA binding domains, have been described, each associating with their own class of specific DNA sequences. Examples of transcription factor families include those containing a cysteine rich zinc finger, homeobox, helix-loop-helix and bZIP domains (Pabo CO, 1992).

Additionally to a sequence specific DNA binding domain, a traditional transcription factor also contains an activation domain necessary to promote transcription.

The lack of CNS regeneration in adult mammals is largely attributable to the presence of an inhibitory environment. Another important factor is the absence or limited upregulation in the expression of transcription factors in injured CNS neurons in marked contrast to the robust increase found in neurons whose peripheral axons are injured (Plunet et al., 2002; Mason et al., 2003).

Transcription factors are central to any coordinated molecular programme and several transcription factors are upregulated following peripheral nerve injury and during axonal regeneration, including c-Jun, c-Fos and the activator transcription factor (ATF) subfamily which are member of the AP-1 family of transcription factors. The transcription factors NFIL3/E4BP4, SYR-box containing gene 11 (S0X-11), signal transducer and activator of transcription-3 (STAT-3) and nuclear factor-κB (NF-κB), are also upregulated after nerve injury (Table 1).

Table 1 Transcription factors up or down regulated after nerve injury

Gene	Expression	Reference
ATF-3	↑	(Seijffers et al., 2007)
c-jun	↑	(Jenkins and Hunt, 1991)
junD	↑	(Herdegen et al., 1991)
NF-κB	↓ ↑	(Doyle and Hunt, 1997) (Ma and Bisby, 1998)
NFIL3/E4BP4	↑	(Nilsson et al., 2005)
p 202b	↑	(Nilsson et al., 2005)
P311	↑	(Fujitani et al., 2004)
Sox11	↑	(Boeshore et al., 2004)
STAT-3	↑	(Schwaiger et al., 2000)

1.6.4.1 C-jun

Jun proteins in combination with Fos proteins, form the AP-1 early response family of transcription factors. The Fos proteins (c-Fos, FosB, Fra-1, Fra-2) can only heterodimerize with members of the Jun family whereas the Jun proteins (c-Jun, JunB, JunD) can both homo- and heterodimerize with Fos members to form transcriptionally active complexes (Jochum et al., 2001). Additionally Jun proteins can also heterodimerize with other transcription factors such as ATF, CREB (Hai et al., 1999) and C/EBP (Hsu et al., 1994).

In different cell types, c-Jun has been shown to participate in regulation of the cell cycle, cellular differentiation, organogenesis, tumor transformation and apoptosis. c-Jun is activated through double phosphorylation by the c-Jun N-terminal kinases (JNK) pathway but has also a phosphorylation-independent function. c-Jun knockout is lethal, due to defects of the interventricular septum in the heart and incomplete separation of the aorta and the pulmonary artery. Additionally c-Jun deficient foetuses present abnormalities in the liver (Eferl et al., 1999).

c-Jun is expressed during neurogenesis and in the adult brain, it is highly expressed in regenerating PNS neurones; it promotes neuronal differentiation and neurite outgrowth. Axotomized retinal ganglion cells (RGCs) that regenerate through a peripheral nerve graft co-express c-Jun and the major growth-associated protein GAP-43 for several weeks (Herdegen et al., 1997).

The expression of c-Jun is upregulated in fetal tissue transplanted into adult striatum for up to six months following grafting, and this upregulation correlates with the ongoing regenerative efforts of the grafted neurons (Aznar et al., 1995). Conditioning ischemia

selectively induces c-Jun (but not JunB or c-Fos) in hippocampal CA1 neurons, and many of the cells positive for c-Jun not only survive the initial ischemia, but become protected from cell death following a subsequent ischemic insult (Sommer et al., 1995). After hypoglycemia, seriously damaged but surviving hippocampal neurons selectively express c-Jun (Gass et al., 1995).

By using cre/loxP technology, conditional mice lacking c-Jun in neuronal cells have been generated. They display slight defects during neurogenesis. However, after transection of the facial nerve, c-Jun deficient animals suffered from severe defect in the axonal response. Additionally c-Jun deficiency blocks post-traumatic neuronal cell death, but it leads to severe neuronal atrophy (De Felipe and Hunt, 1994; Raivich et al., 2004).

1.6.4.2 STAT-3

STAT-3 is a transcription factor phosphorylated by Janus kinases (JAKs) in response to cytokine activation of a cell surface receptor tyrosine kinase. Upon activation, STAT-3 becomes dimerized and localized to the nucleus where it activates transcription of cytokine-responsive genes (Ihle, 2001; Heinrich et al., 2003). It is known that JAK/STAT pathway is activated through neuropoietic cytokines signalling via gp130 receptor, which dimerises and associates with JAKs. JAKs phosphorylate gp130 and then recruit STAT-3, leading to tyrosine 705 phosphorylation, dimerization and nuclear translocation to initiate target gene transcription (Fig. 10).

Immunohistochemical evidence indicates that in axotomized DRG neurons, STAT-3 is phosphorylated and activated (Qiu et al., 2005). A conditioning lesion has been shown

to activate a robust neurite outgrowth of adult DRG neurons *in vitro* (Smith and Skene, 1997). Conversely, conditional knockout studies by using cre-mediated gene ablation in motoneurons suggest that STAT-3 is involved in supporting survival of injured neurons and the lack of STAT-3 downregulates the expression of anti-apoptotic genes Reg-2 and Bcl-xl in axotomized motoneurons (Schweizer et al., 2002).

In order to study signalling mediators that underlie regenerative axon growth in the conditional model, Snider and colleagues used chemical inhibitors in axotomized sensory neurons *in vitro* to block the activation of Erk kinase (MEKK), phosphatidylinositol-3 kinase (PI3-K), and JAK signalling. The results indicated that unlike JAK, neither MEK nor PI3-K inhibitors blocked elongation of adult sensory neurons after a conditioning lesion, suggesting that JAK/STAT-3 signalling is required for the upregulation of gene expression for cytoskeletal elements normally observed after axotomy (Liu and Snider, 2001). Similar results have shown that inhibition of JAK2 by continuous perineural infusion of inhibitor AG490 to the proximal nerve stump after nerve injury blocks STAT-3 phosphorylation, leading to a compromised neurite outgrowth *in vitro* and a diminished axonal regeneration of the dorsal column in the adult spinal cord after a preconditioning sciatic nerve transection (Qiu et al., 2005).

Recently, it has been suggested that in response to the chronic insults or stress of the pathogenesis of multiple sclerosis (MS), cortical neurons of MS patients upregulate a CNTF-mediated neuroprotective signalling pathway involving STAT-3 and B cell lymphoma leukaemia 2 protein (Bcl2). Induction of CNTF signalling, the phosphorylation of STAT-3 and the expression of anti-apoptotic molecule, Bcl2, thus represents a

compensatory response to disease pathogenesis and a potential therapeutic target in MS patients (Dutta et al., 2007).

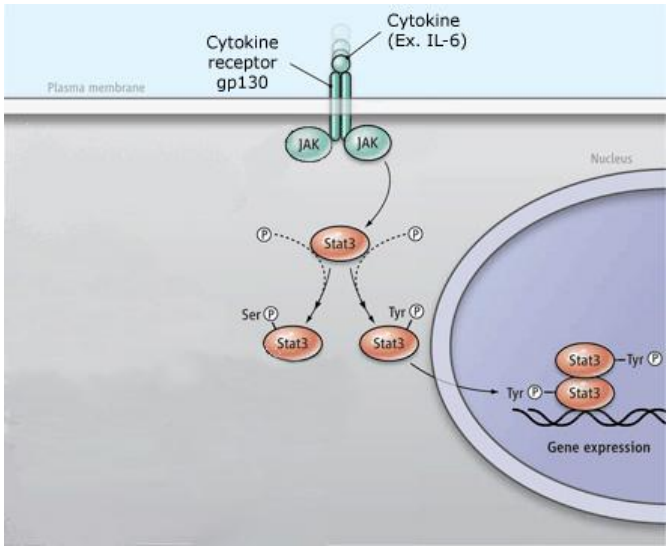


Fig. 10 STAT-3 activation pathway: IL-6 leads to dimerization of gp130 receptors. Associated JAKs become activated and phosphorylate the cytoplasmic part of gp130, thereby creating docking sites for STAT-3. STAT-3 becomes phosphorylated form homo- or hetero-dimers and translocate into the nucleus, where they regulate gene transcription. Modified from Suzuki T, Nature Genetics 32, 166 – 174.

1.6.4.3 CREB

The regulation of cAMP has been shown to be crucial in nerve regeneration. Therefore, the regulation of gene transcription by cAMP has been studied extensively. Even though there may be several transcription factors capable of mediating this response, the most widely studied so far has been CREB. CREB belongs to the basic leucine zipper (bZIP) superfamily of transcription factors. CREB binds to certain DNA sequences called cAMP response elements (CRE) and thereby increases or decreases the transcription, and thus the expression, of certain genes (Fig. 11). CREB is closely related in structure and function to cAMP response element modulator (CREM) and ATF-1.

CREB mediates the positive effects of cAMP on axonal regeneration (Lonze et al., 2002; Sheng et al., 1991) and it is also responsible for the mediation of neuronal survival responses to neurotrophins (Finkbeiner, 2000). It is well-established that CREB has both constitutive and cAMP-inducible activities, with distinct domains within the protein contributing to these activities, like the palindromic binding site 5′-TGACGTCA-3′ for CRE. The cAMP-inducible activity of CREB is activated via phosphorylation of the specific serine residue ser133 by PKA, and the phosphorylated form of CREB then is able to bind the co-activator, CREB-binding protein (CBP) and recruit it to the promoter to provide further bridging to the pre-initiation complex (Mayr and Montminy, 2001) (Fig. 11).

CREB activity has also been implicated in learning, memory formation and synaptic plasticity in experiments using the marine snail *Aplysia* (Kandel, 2001). Related experiments in rodents indicate that CREB may be required for long-term memory (Bourtchuladze et al., 1994). Furthermore, previous studies found that inducible expression of a dominant inhibitor of all CREB family members in the dorsal hippocampus produces spatial memory deficits (Pittenger et al., 2002). Since the formation of long-term memory and axonal growth both require cellular changes, it is perhaps not unexpected to think that both processes would have overlapping requirements for diverse genes downstream of CREB.

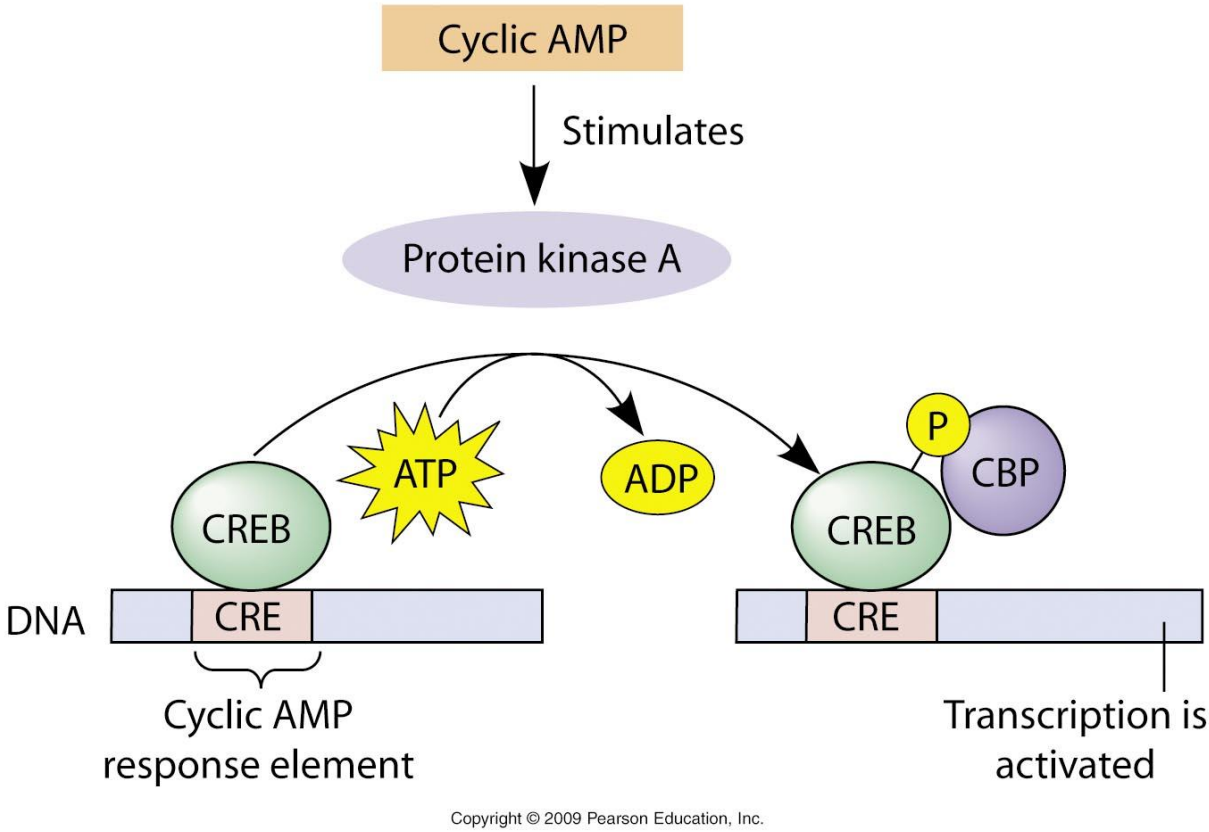


Fig. 11 CREB activation pathway: CREB controls gene expression when cAMP levels increase. Genes activated by cyclic AMP possess an upstream cyclic AMP response element (CRE) that binds CREB protein. In the presence of cyclic AMP, cytoplasmic PKA is activated and its activated catalytic subunit then moves into the nucleus, where it catalyzes phosphorylation of the CREB protein, thereby stimulating its activation domain. The recruitment of the co-activator CREB-binding protein (CBP), allows CREB to regulate the transcription of certain genes. From The World of the Cell, 7th Edition, Chapter 23 by Wayne M. Becker, Lewis J. Kleinsmith, Jeff Hardin & Gregory P. Bertoni, 2009.

1.6.4.4 ATF-3

Activating transcription factor-3, is another important transcription factor upregulated in Schwann cells and injured DRG neurons after peripheral injury and is downregulated after reinnervation of peripheral targets (Seijffers et al., 2007). ATF-3 is induced in sensory neurons of large diameter after spinal cord injury (Huang et al., 2006). Upregulation after injury is preceded by phosphorylation of c-Jun and JNK activation (Pearson et al., 2003).

ATF-3 is normally induced by stress stimuli and cellular damage and has both apoptosis-promoting and survival functions in different tissues (Allen-Jennings et al., 2001; Perez et al., 2001; Hartman et al., 2004). In the nervous system, ATF-3 appears to have a survival role (Herdegen et al., 1997). After delivery to the hippocampus, ATF-3 protects CA3 pyramidal neurons from kainic acid-induced apoptosis (Francis et al., 2004) and rescues neonatal superior cervical ganglion neurons from apoptosis due to NGF withdrawal (Nakagomi et al., 2003). In a transgenic animal model that constitutively expresses ATF-3 in non-injured adult DRG neurons; peripheral nerve regeneration was enhanced in the transgenic mice to a level comparable to that produced by a preconditioning nerve injury (Seijffers et al., 2007). The expression of some growth-associated genes, such as SPRR1A, but not others like GAP-43, was upregulated in the non-injured neurons. However, even though ATF-3 increased DRG neurite elongation on permissive substrates *in vitro*, it did not overcome the inhibitory effects of myelin or promote central axonal regeneration in the spinal cord *in vivo* (Seijffers et al., 2007).

1.6.4.5 NF-κB

The transcription NF-κB exists in all cell types and has been implicated in the early control of the neuronal response to injury and regeneration. NF-κB is present in the cytoplasm in an active form bound to its inhibitor IκB. Upon stimulation by an extracellular signal, NF-κB is released from its inhibitor, translocates to the nucleus and participates in many regulatory processes.

NF-κB is active as early as 30 minutes after CNS injury, is still present at 72h, and is expressed by macrophages, microglia and neurons (Bethea et al., 1998). Activation of NF-κB in neurons occurs in response to pro-inflammatory cytokines that are elevated after injury. NF-κB is also activated during the excitotoxin-induced apoptosis of striatal neurons (Qin et al., 1998). NF-κB can be activated by Rho (Montaner et al., 1999), raising the possibility that the abnormal Rho activation after spinal cord injury (Dubreuil et al., 2003; Madura et al., 2004) may lead to activation of NF-κB and neuronal death.

The function of NF-κB in the nervous system remains unclear and its role in neuroprotection or neurodegeneration is open to debate. NF-κB in brain neurons promotes their death in an ischemia animal model (Herrmann et al., 2005), while neuron-restricted ablation of NF-κB driven gene expression increases neurodegeneration suggesting that NF-κB expression in forebrain neurons is required for neuroprotection (Fridmacher et al., 2003). Furthermore NF-κB expression studies in DRG neurons after peripheral nerve injury have shown that the activated form of NF-κB, the heterodimer p65, is expressed in a sub-population (32%) of mixed diameter sensory neurons in L4 and L5 DRG, indicating that this transcription factor is involved in intracellular signalling in sensory neurons under physiological conditions. Four hours after crushing the sciatic nerve, ipsilateral p65 staining was abolished in a subgroup (60-70%) of these neurons. The contralateral side was unaffected by the injury and loss of NF-κB activity was not observed following sham surgery. Within 24 hrs of sciatic injury, ipsilateral p65 staining resumed control levels (Doyle and Hunt, 1997). This downregulation in the expression of NF-κB in DRG neurons after nerve injury contrasts with another study where upregulation of NF-κB in DRG was observed 2 weeks following partial sciatic injuries, possibly in response to cytokines and

neurotrophins produced by endoneurial cells in the partially injured nerve during Wallerian degeneration (Ma and Bisby, 1998). More recently our group has shown that that NF-κB does not contribute to the intrinsic resistance of adult DRG neurons to apoptotic death after axotomy. Additionally, NF-κB is unlikely to have an involvement in DRG neurons processes such as axonal regeneration and neuropathic pain, which are responsive to nerve injury (Gushchina et al., 2009). NF-κB has been proposed to be important in the axonal growth of developing sensory neurons (Gallagher et al., 2007). However, it has also been suggested that axonal outgrowth is not mediated by NF-κB in adult DRG cultured neurons (Gushchina et al., 2009). These discrepancies could be explained by a recent study demonstrating that NF-κB can either stimulate or inhibit neurite growth in developing neurons depending on the phosphorylation status of p65 (Gutierrez et al., 2008).

1.7 The CAAT enhancer binding protein family of transcription factors

The CAAT enhancing binding proteins (C/EBP) are a family of transcription factors consisting of six members α, β, γ, δ, ε and ζ (genetic nomenclature *CEBPA, CEBPB, CEBPG, CEBPD, CEBPE* and *CEBPZ*, respectively) (Fig. 12). To avoid confusion since several independent groups have characterized and given C/EBPs different names, a systematic nomenclature was proposed by Steve MacKnight and colleagues, in which members are designated as C/EBP followed by a Greek letter indicating the chronological order of their discovery (Table 2). The C/EBPs have been involved in the regulation of multiple aspects of cell function including inflammation, differentiation, proliferation, stress response and neuronal development (Ramji and Foka, 2002). The C/EBPα gene was first cloned in 1988, in Steve McKnight's laboratory (Landschulz et al., 1988a) where

further studies led to the discovery of the leucine zipper as well as the basic helix loop helix groups of transcription factors (Landschulz et al., 1988b), and to the identification of an optimal canonical binding site, RTTGCGYAAY (R = A or G, and Y = C or T). The C-terminal of the C/EBPs contains the bZIP domain, highly conserved among all the members of the family, which consists of a basic amino acid rich DNA binding region followed by a dimerization motif called the leucine zipper (Fig. 12). Due to high sequence identity of over 90% in the C- terminal 55-65 amino acid residues between the six members of the family, the C/EBPs can form homodimers and heterodimers in all possible interfamilial directions (Roman et al., 1990; Williams et al., 1991). Additionally, the C/EBPs can also give rise to protein-protein interactions with other bZIP and non-bZIP families of transcription factors. For instance, C/EBPs can bind to transcription factors of the NF-kB, CREB/ATF and AP-1 families (Vallejo et al., 1993; Vinson et al., 1993; Lekstrom-Himes and Xanthopoulos, 1998). Dimerization is a prerequisite for DNA binding and is mediated by the basic region (Landschulz et al., 1988b).

Besides the vast number of possible heterodimer formations between the six different members of the family, the number of C/EBP proteins present in an organism may be even higher due to a leaky ribosomal scanning mechanism where different size polypeptides can be generated. Two isoforms of C/EBPα can be generated from its mRNA. The full-length protein is 42 kDa and contains three activation domains which mediate cooperative binding of C/EBPα to TATA box-binding protein and transcription factor IIB (TFIIB), two components of the RNA polymerase II basal transcriptional apparatus (Nerlov and Ziff, 1995). The second C/EBPα isoform is a shorter 30-kDa protein that retains its dimerization and DNA-binding domains; however, it possesses an altered transactivation

potential compared with the 42-kDa isoform (Fig. 12). C/EBPβ mRNA can produce at least three isoforms, 38 kDa (LAP*), 35 kDa (LAP) and 20 kDa (LIP), the last two being the most common. However, LIP, unlike LAP, does not contain any activation domain (Fig. 12) (Descombes and Schibler, 1991). C/EBPε mRNA can generate at least four isoforms (32 kDa, 30 kDa, 27 kDa and 14 kDa), of which the 32 kDa has the highest activation potential and the 14 kDa the lowest since it lacks an intact transcriptional activation domain (Fig. 12) (Lekstrom-Himes, 2001; Yamanaka et al., 1997).

In contrast to the C-terminal, the N-terminal does not share high sequence identity among the members of the C/EBP family; there is less than 20% homology, with the exception of three small subregions conserved in almost all the members but C/EBPγ (Fig. 12) (Cooper et al., 1995). These regions are activation domains responsible for the interaction with the components of the basal transcription apparatus which can promote transcription, repression and autoregulatory function. (Johnson, 2005; Williamson et al., 1998; Tang and Koeffler, 2001). Since C/EBPγ lacks the transcriptional activation domains present in all the other members, it acts as a transdominant negative regulator by forming inactive heterodimers with other members leading to the repression of gene transcription (Cooper et al., 1995). C/EBPε can generate 4 isoforms of 32 kDa, 30 kDa, 27 kDa and 14 kDa with the first having the highest activation potential (Yamanaka et al., 1997; Lekstrom-Himes, 2001).

The functional properties of C/EBPs has been investigated extensively in the past years using different methods such as analysis of promoter regions of target genes, overexpression or inhibition, and studies on knock-out mice (Ramji and Foka, 2002). It has been demonstrated that C/EBPs are involved in a variety of different cellular events for

instance the control of cell differentiation especially during adipogenesis (Darlington et al., 1998), and myeloid-cell differentiation and maturation (Yamanaka et al., 1998; Zhang et al., 1997). Furthermore, it has been determined that C/EBPs also have an important role in the response to inflammatory insult. C/EBPα, β and δ expression levels can be regulated by an array of different cytokines such as interleukin -1 and -6 and TNF-α (Poli, 1998; Poli, 1998; Alam et al., 1992; Akira et al., 1990). In addition, numerous binding sites have recently been identified for several C/EBPs in regulatory regions of multiple genes involved in the inflammatory response, including those coding for cytokines and their receptors, acute-phase plasma proteins, and components of signal transduction pathways present in adipocytes, hepatocytes, myeloid cells and keratinocytes, among others (Ramji and Foka, 2002).

Table 2 Nomenclature of C/EBP genes

Gene	**Alternative name**	**Source**
C/EBPα	C/EBP, RcC/EBP-1	Rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Rana catesbeiana*, fish
C/EBPβ	NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP	Rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Aplysia*, fish
C/EBPγ	Ig/EBP-1	Rat, mouse, human, chicken, fish
C/EBPδ	NF-IL6b, CRP3, CELF, RcC/EBP2	Rat, mouse, human, *Rana catesbeiana*, bovine, ovine, fish
C/EBPε	CRP-1	Rat, mouse, human, ovine, fish
C/EBPζ	CHOP-10, GADD153	Mouse, rat, human, hamster

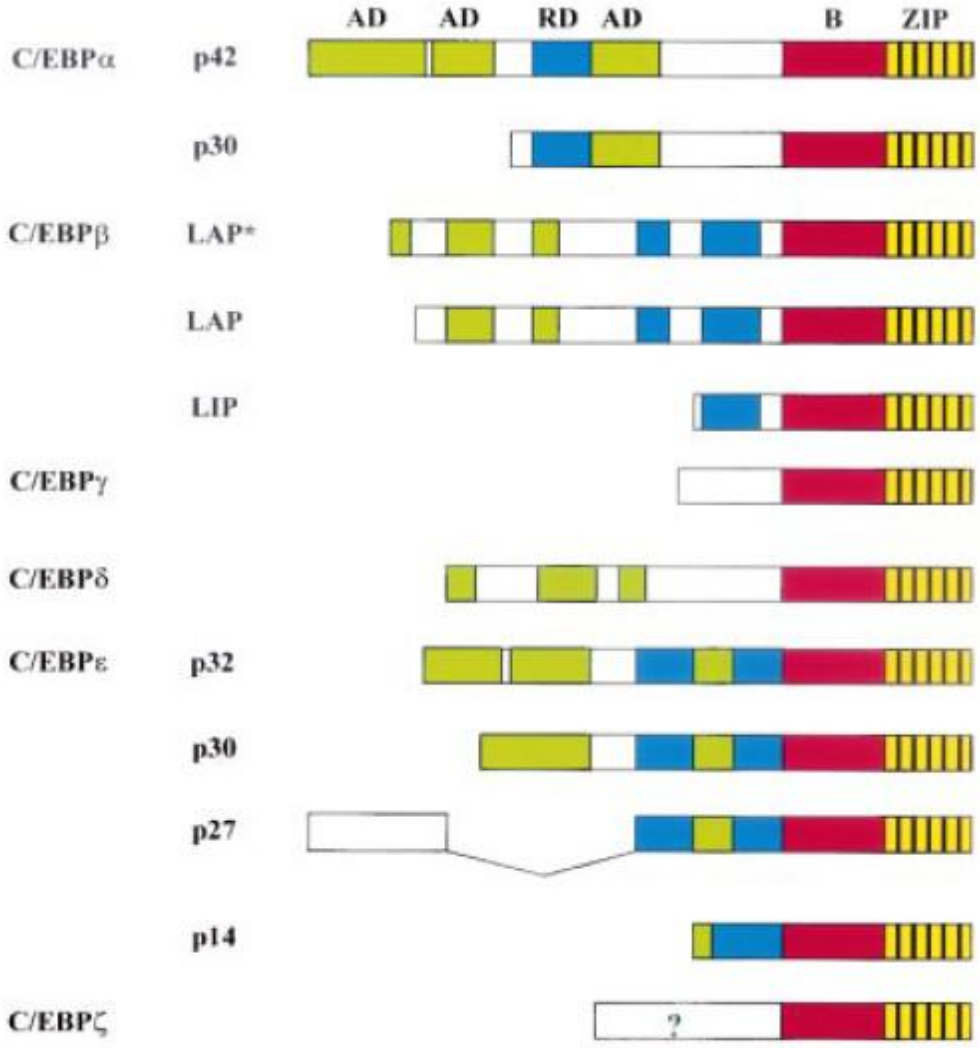


Fig. 12 The C/EBP family of transcription factors: C/EBPα, β, γ, δ, ε, and ζ and their different isoforms. The N-terminal consist of the activation domain (AD) represented in green and the negative regulatory domain (RD) shown in blue. The basic leucine zipper domain in the c-terminal consists of the basic region (red) and the leucine zipper domain (yellow with black lines). Taken from (Ramji and Foka, 2002).

1.7.1 Regulation of the C/EBP family

The expression of the C/EBPs is regulated by the actions of a number of factors, including cytokines, mitogens, hormones, nutrients and agents that cause cellular stress (Table 3). The C/EBPs have been found to be regulated at a number of different levels, including gene transcription, translation, protein–protein interactions and phosphorylation-mediated changes in DNA- binding activity, activation potential and nuclear localization (Table 3). Additionally, there is a potential effect of tissue, cell and species specific differences in the mechanisms of regulation. For example, TNFα induces C/EBPβ mRNA expression in astrocytes and renal mesangial cells, but modulates nuclear–cytoplasmic translocation in hepatocytes (Yin et al., 1996; Buck et al., 2001; Cardinaux et al., 2000; Tengku-Muhammad et al., 2000; Granger et al., 2000b).

1.7.1.1 Transcriptional regulation

Transcriptional regulation is the main point of control in the regulation of mRNA (Table 3). The promoter regions of C/EBPα, -β, -δ, -ε, -ζ, have been analyzed. The role of the different sequence elements in the regulation of gene expression during adipogenesis, inflammation and hepatocyte proliferation is described below under global gene regulation, with other aspects addressed here.

The proximal promoter region of the mouse C/EBPα gene was initially characterized and shown to contain potential binding sites for C/EBP, Sp1, nuclear factor (NF)-1, NF- Y, upstream stimulating factor (USF), basic transcription element-binding

protein (BTEB) and NF-kB (Christy et al., 1991; Flodby et al., 1993; Legraverend et al., 1993). This promoter can be activated by C/EBPα and -β by specific binding to the C/EBP recognition sequence (Christy et al., 1991; Legraverend et al., 1993). The proximal promoter region of the rat and mouse C/EBPα gene are almost identical and can be auto-activated through the C/EBP-binding site (Rana et al., 1995). However, the promoter regions of the human, chicken and *Xenopus* C/EBPα genes present only partial sequence identity with the mouse/rat promoter (Kockar et al., 2001; Timchenko et al., 1995). In the case of the human promoter, it lacks a C/EBP recognition sequence, due to a single base substitution but it can still be auto-activated, although only by C/EBPα (Timchenko et al., 1995). All this evidence indicates that the C/EBPα gene is auto-activated in a species specific manner.

The promoters of the mouse, rat, chicken and *Xenopus laevis* C/EBPβ genes which are also capable of autoregulation have been identified (Foka et al., 2001; Chang et al., 1995; Niehof et al., 2001a; Mink et al., 1999). The transcription factor CREB also controls C/EBPβ expression by interacting with two sites near the TATA box called the cAMP-response element (CRE) (Niehof et al., 1997). The CRE sequences are also required for the IL-6-mediated induction of C/EBPβ transcription during the acute-phase response (APR) through a signalling pathway involving tethering of STAT-3 to a DNA-bound complex (Niehof et al., 2001b), and in combination with an Sp1 site for the activation of gene transcription during macrophage differentiation (Berrier et al., 1998).

C/EBPδ is expressed constitutively in osteoblasts, where it activates the synthesis of insulin-like growth factor I by the binding site for the runt domain factor 2 present in the 3´

proximal region of the C/EBPδ gene promoter (McCarthy et al., 2000). The transcription of the C/EBPδ gene in growth-arrested mammary epithelial cells and in the mammary gland is activated by STAT3 which binds to a recognition sequence in the promoter region (Sabatakos et al., 1998). This STAT3 site is also required for the IL-6-mediated induction of C/EBPδ transcription in hepatocytes (Cantwell et al., 1998). Analysis of the mouse, rat and ovine C/EBPδ promoters suggests the potential existence of species-specific mechanisms for autoregulation (Davies et al., 2000; Yamada et al., 1998; O'Rourke et al., 1999).

The C/EBPε gene promoter is transcribed by two alternative promoters, Pα and Pβ, which contains no TATAAA box, but through a combination of differential splicing and alternative use of promoters generates four mRNA isoforms (Yamanaka et al., 1997). The promoter also contains a functional retinoic acid-responsive element that can be induced by the corresponding ligand (Park et al., 1999).

1.7.1.2 Translational regulation

A key aspect of translational control of C/EBPα and -β is the alternative use of initiation codons (Calkhoven et al., 2000). As mentioned previously, two distinct isoforms of C/EBPα, 42kDa and 30kDa, can be produced from a single mRNA (Ossipow et al., 1993; Lin et al., 1993). The 30kDa polypeptide as opposed to the 42kDa form has a lower transactivation potential and lacks antimitotic activity (Ossipow et al., 1993; Lin et al., 1993). The ratio of the two isoforms changes during both adipocyte differentiation and

hepatocyte development (Ossipow et al., 1993; Lin et al., 1993). Similarly, the C/EBPβ ratio of the activator LAP form to the repressor LIP polypeptide has been shown to increase under a number of conditions, and such changes are likely to be functionally important (Descombes and Schibler, 1991). For example, LIP is elevated in breast cancer which is consistent with its ability to induce epithelial cell proliferation and the formation of mammary hyperplasia (Zahnow et al., 2001). The C/EBPα and C/EBPβ mRNA contains a conserved short upstream open reading frame (uORF) that is located seven bases from the initiation codon that specifies for the full-length protein (Calkhoven et al., 1994). Previously it has been shown that the integrity of the uORF is essential for the leaky ribosome scanning mechanism that leads to the production of different sized polypeptides (Calkhoven et al., 2000).

1.7.1.3 Regulation by phosphorylation

Phosphorylation plays a key role in the modulation of C/EBPβ function. C/EBPβ is normally repressed and negative regulatory regions mask its transactivation domains (Williams et al., 1995; Kowenz-Leutz et al., 1994). This repression is abolished by phosphorylation of the repression domain (Kowenz-Leutz et al., 1994). Transactivation of C/EBPβ can be induced by phosphorylation of Thr^{235} through a Ras/MAPK pathway (Zhu et al., 2002), by phosphorylation of Ser^{105} via protein kinase C (PKC) (Trautwein et al., 1993), and Ser^{276} by Ca^{2+}/calmodulin-dependent protein kinase (Wegner et al., 1992). However, phosphorylation can also suppress the activation potential of C/EBPβ. For example, insulin inhibits transactivation by C/EBPβ via the PI 3-kinase pathway (Guo et

al., 2001). In contrast, the PKA-mediated *in vitro* phosphorylation of a region between Ser^{173} and Ser^{223}, and Ser^{240}, suppresses C/EBPβ DNA-binding activity (Trautwein et al., 1994). Similarly, a PKC-mediated phosphorylation of Ser^{240} leads to a decrease in DNA binding (Trautwein et al., 1994). On the other hand, phosphorylation of PKA in rat PC12 cells results in the translocation of C/EBPβ into the nucleus, where it then activates the transcription of the c-fos gene (Metz and Ziff, 1991). Translocation of C/EBPβ in a colorectal cancer cell line DKO-1 is also mediated by a PKA-dependent phosphorylation, but of Ser^{299} (Chinery et al., 1997).

Under certain conditions a potential role of phosphorylation in the regulation of C/EBP isoforms α, -δ has been shown. For example, PKC can phosphorylate C/EBPα at several sites (Ser^{248}, Ser^{277} and Ser^{299}), and leads to a weakening of its DNA-binding activity (Mahoney et al., 1992). In addition, insulin can reduce the expression of genes in adipocytes, in part, due to a dephosphorylation-mediated degradation of C/EBPα (Ross et al., 1999). Studies on the a_1-acid glycoprotein regulation and the serum amyloid A genes during the APR have shown that dephosphorylation of C/EBPδ results in an inhibition of its DNA binding activity (Ray and Ray, 1994). Additionally, the transactivation potential of C/EBPδ was also found to be increased when hepatocytes were treated with cellular phosphatase inhibitors, such as okadiac acid and sodium orthovanadate (Ray and Ray, 1994; Lekstrom-Himes and Xanthopoulos, 1998).

1.7.1.4 Protein–protein interactions

Protein-protein interactions are important for the regulation of the C/EBP family since the different members can form heterodimers under all interfamilial conditions and such interactions are likely to have a profound influence on the regulation of gene transcription. Furthermore, the C/EBPs can form protein–protein interactions with other bZIP and non-bZIP factors. For example, C/EBPβ has been shown to interact with the p50 subunit of NF-kB, CREB/ATF, AP-1, glucocorticoid receptor, hepatitis B virus X protein, and the retinoblastoma (Rb) protein (LeClair et al., 1992; Vallejo et al., 1993; Lekstrom-Himes and Xanthopoulos, 1998; Hsu et al., 1994; Hsu et al., 1994; Wedel and Ziegler-Heitbrock, 1995). Such heterodimers often have different transactivation potentials and/or DNA binding specificities or affinities compared with the corresponding homodimers. Additionally, the transactivation potential of the different C/EBP members differs; for example, C/EBPα is a stronger transcriptional activator than C/EBPβ.

Table 3 Regulators of the C/EBP family. Level abbreviations: DBA, DNA-binding activity; NL, nuclear localization; AP, activation potential.

Gene	Level	Activator	Repressor
C/EBPα	mRNA	Thyroid hormone, thiazolinediones	EGF, glucocorticoid, growth hormone, Interferon-gamma(IFN-g), IL-1, IL-6, insulin, LPS, TNF-a
	DBA		EGF, PKC activators
C/EBPβ	mRNA	cAMP, glucagon, glucocorticoid, growth hormone, IFN-g, IL-1, IL-6, LPS, noradrenaline, NGF, VIP	
	NL	cAMP, LPS, TNF-a, TPA	TNF-a
	AP	Ca^{2+}, IL-6, MAPK activators, PKC activators	Insulin
	DBA	Transforming growth factor beta (TGF-b), EGF	
C/EBPδ	mRNA	Glucocorticoid, growth hormone, IFN-g, IL-1, IL-6, insulin, LPS, noradrenaline, PDGF, TNF-a, VIP	
	NL	cAMP, TNF-a	
C/EBPε	mRNA	Retinoic acid	
C/EBPζ	mRNA	Amino acid deprivation, agents that cause ER-, nutrient- and oxidative stress, Ca^{2+}, nitric oxide, LPS, prostaglandin A_2, UV light	
	AP	Cellular stress	

1.7.2 C/EBP in the nervous system

Although it is known that C/EBPα, β and δ are expressed in the brain (Sterneck et al., 1998; Sterneck and Johnson, 1998) and methodical analysis on the function and properties of C/EBP proteins has been performed in a variety of tissues such as liver and adipose, much less is known about the role of these transcription factors in the nervous system, especially in nerve regeneration after adult nerve injury. Nevertheless, some evidence suggests a potential function and possible implication of C/EBPs in gene expression mechanisms that underlie neuronal functions such as neuronal repair. This is supported indirectly by recent studies showing that members of this family are involved in the control of biological processes critical to neuronal development and survival, including cell fate determination, apoptosis (Paquin et al., 2005; Marshall et al., 2003; Menard et al., 2002), synthesis of trophic factors (Takeuchi et al., 2002; McCauslin et al., 2006), response to neurotrophic factors (Sterneck and Johnson, 1998; Calella et al., 2007), response to brain injury and ischemia (Kapadia et al., 2006; Soga et al., 2003) and neural plasticity in the adult (Sterneck et al., 1998; Sterneck and Johnson, 1998).

There are multiple approaches to enhance regeneration following nerve injury. For instance, by overcoming the MAG/myelin inhibition of regeneration through the upregulation of permissive factors like cAMP, CREB and Arg I. Also by the upregulation of several cytokines, neurotrophic factors and regeneration associated genes. Therefore, it wouldn't be unexpected to discover a pivotal role of the C/EBPs during nerve repair given the numerous signalling pathways and molecules involved in this event. In addition, the fact that computational predictions show over 30 million C/EBP binding sites in the rat genome (Falvey et al., 1996), which is not surprising given the accepted variation in the

C/EBP consensus sequence, leads to the hypothesis that functional C/EBP sites are defined not just by their sequence but also by adjacent binding sites for transcription factors that interact with and stabilize C/EBP binding (Friedman et al., 2004; Calella et al., 2007). Moreover, the variety of DNA binding and activation domains available through heterodimerization enables C/EBPs to interact with a broad range of DNA sequences, transcription factors, co-activators and chromatin remodelling complexes which presumably increases the number of genes that are regulated by C/EBPs

1.7.3 Potential signalling pathways of C/EBPs

Several genes and signalling mechanisms are known to be upregulated following nerve injury and have also been implicated in nerve repair. Although, much less is known about the expression and function of C/EBPs after nerve injury and during nerve repair, some indirect evidence showing different interactions between C/EBPs and these signalling pathways associated with nerve repair, suggests a possible implication of C/EBPs during neuronal regeneration. Some of the interactions between C/EBPs and nerve regeneration associated genes and signalling pathways are discussed below.

1.7.3.1 C/EBP interaction with cAMP and CREB

Structure and function analysis of C/EBPs have shown that this family of transcription factors possesses constitutively active domains, suggesting that C/EBPs can be induced in response to cAMP (Pelletier et al., 1998; Wilson et al., 2001; Roesler et al., 1998). In non-

neuronal tissue, it has been shown that cAMP responsiveness is mediated by a cAMP response unit (CRU) (Roesler, 2000). This CRU consists of five cis-elements, and includes a typical CRE to which CREB can bind. However, the CRE on its own has weak enhancer activity, and a strong response to cAMP is obtained only when all five cis-elements are in use. C/EBPs bind to several of the cis-elements that make up the CRU, and mutations in these sites which disrupt C/EBP binding also affect the fold-responsiveness to cAMP (Liu et al., 1991; Roesler et al., 1994). In addition, overexpression of a dominant negative C/EBP inhibits cAMP responsiveness in liver-derived cells (Roesler et al., 1996). In the nervous system, cAMP elevation, has also been shown to regulate the expression of C/EBPs. In hippocampal neuron cAMP stimulation enhances mRNA expressions and DNA binding activities of C/EBPβ and -δ (Yukawa et al., 1998). In pheochromocytoma PC12 cell line cAMP elevation, following forskolin treatment, phosphorylates and translocates C/EBPβ to the nucleus (Metz and Ziff, 1991). In rat cerebral cortex, the activation of β-adrenergic receptors (BAR) increases cAMP levels leading to an increase of C/EBPδ-binding activity resulting in the upregulation of NGF (Colangelo et al., 1998).

Furthermore, C/EBPs are also known for having a role in transcriptional programs underlying more complex brain functions, such as learning and memory**.** The first evidence that C/EBPs are expressed in neurons and involved in long-term synaptic plasticity underlying memory formation came from studies of the invertebrate Aplysia californica, which is used for *in vitro* modelling of the short- and long-term synaptic responses that occur during simple formation of memory. Experiments in coculture systems were instrumental in determining the fundamental role of C/EBP in long-term synaptic plasticity.

Indeed, this in vitro system pioneered the identification and characterization of several molecules and molecular mechanisms underlying memory formation.

C/EBPβ and C/EBPδ were co-localized with phosphorylated CREB in synaptic plasticity for long term memory (LTM) consolidation in Aplysia models (Guan et al., 2002) and in mice (Taubenfeld et al., 2001). The Aplysia C/EBP (ApC/EBP) is found to be increasingly induced in response to cAMP and CREB signals (Alberini et al., 1994).

Over the past 20 years, several studies have demonstrated that CREB holds an essential role in LTM formation (Dash et al., 1990; Yin et al., 1994; Bartsch et al., 1995; Bartsch et al., 1998; Guzowski and McGaugh, 1997; Taubenfeld et al., 2001). Phosphorylation of CREB can in turn regulate the transcription of more downstream target genes required for LTM formation such as the expression of late response genes and other inducible transcription factors (e.g., Arc, c-Fos, C/EBP) (Lonze et al., 2002; Alberini et al., 1994). Additionally, it has been shown in Aplysia and Drosophila models and in different areas of the rodent brain that cAMP and PKA signalling pathway, working upstream of CREB, are involved in synaptic plasticity or learning (Kandel and Pittenger, 1999). Similarly, as mentioned before, this signalling cascade is also involved in axonal regeneration. Formation of LTM requires mRNA and protein synthesis, (Davis and Squire, 1984; DeZazzo and Tully, 1995) whereas inhibition of protein synthesis completely blocks LTM even after spaced training without affecting learning and early memory (Tully et al., 1994; Davis and Squire, 1984). Enormous research efforts have been used to identify the regulatory mechanisms of learning-induced gene transcription and translation.

There are different groups of genes which contain CRE sites in their promoters, including growth factors, structural proteins, cellular metabolism, transcription, and signal transduction factors (Lonze et al., 2002). Among these elements in Aplysia models, it has been shown that ApC/EBPs become induced upon binding to the CRE element on its promoter, indicating a direct sequential interaction between C/EBP and CREB. Furthermore, studies in Aplysia indicate that following neural stimulation the activation of C/EBP is induced by the Aplysia LAPS18-like protein (ApLLP) through a direct binding to CRE (Kim et al., 2006) which in turn leads to the consolidation of long term facilitation (LTF) (Kim et al., 2006; Alberini et al., 1994). Interestingly, it has also been demonstrated in Aplysia that axotomy induces the ApC/EBP mRNA expression (Alberini et al., 1994).

Analogous results were demonstrated in mice where C/EBPβ and C/EBPδ are induced in the hippocampus after learning, correlating with CREB activation (Taubenfeld et al., 2001). Conversely, disruption of CREB activation in the hippocampus blocks the induction of C/EBPβ and C/EBPδ (Taubenfeld et al., 2001).

1.7.3.2 C/EBP and STAT-3

After STAT-3 activation by cytokines, dimerised STAT-3 translocates to the nucleus where it binds to specific enhancer sequences and regulates the transcription of various target genes, including C/EBPδ (Yamada et al., 1997). STAT-3 binding sites are often located proximal to binding sites for other transcription factors including C/EBPβ (Schumann et al., 1996), suggesting that these factors cooperate in gene regulation. Additionally, in epithelial cells, co-immunoprecipitation and chromatin immunoprecipitation (ChIP) assays

demonstrated that pSTAT3 and Sp1 interact and bind to the transcriptionally active C/EBP δ promoter (Zhang et al., 2007) and in hepatocytes DNA binding experiments and transactivation assays demonstrated that STAT-3 binds to C/EBPβ and-δ promoter sequence. Moreover, the suggested CNTF-mediated neuroprotective response through the activation of STAT-3 signalling led to an upregulation of C/EBPβ and C/EBPδ which was detected in microarrays of MS patient's cortex (Dutta et al., 2007).

1.7.3.3 C/EBP and Arginase I

Previously, it has been demonstrated that Arg I has neuroprotective properties in cortical neuronal cultures (Esch et al., 1998). Other studies conducted by Filbin and colleagues have shown that Arg I overcomes MAG/myelin inhibition and it is involved in the cAMP regeneration pathway (Spencer and Filbin, 2004; Filbin, 2003; Filbin, 1996; Filbin, 1995). Additionally, polyamines, a group of arginase downstream products with widespread biological effects, are also involved in neuronal growth and development (Slotkin and Bartolome, 1986; Slotkin et al., 1982), axonal regeneration after injury (Gilad and Gilad, 1988; Gilad et al., 1996) and in wound healing outside of the CNS (Witte and Barbul, 2003). Several studies also implicate a protective role for polyamines. For instance, all 3 polyamines (putrescine, spermine, and spermidine) prevent apoptotic cell death induced by high potassium chloride concentrations in cultured cerebellar granule neurons (Harada and Sugimoto, 1997). Furthermore, the neuroprotective role of spermine has been demonstrated in several models of neurodegeneration by Morris and colleagues (2002) who showed that L-arginyl-3,4-spermidine significantly reduced cell death in the hippocampal subregion

CA1 when administered prior to, or immediately after, global ischemia *in vivo*. L-arginyl-3,4-spermidine also reduced cell death in excitoxicity models, as well as in superoxide-mediated cell injury (Morrison, III et al., 2002).

C/EBPs are known to regulate the expression of Arg I by binding to Arg I promoter region –90 to –55 relative to the transcription start site (Lange et al., 2008; Takiguchi and Mori, 1991; Morris, Jr., 2002). In the Arg I promoter region there are 2 binding sites for C/EBPs, 1 at position around –90 bp and the other around –55 bp (Gotoh et al., 1994; Chowdhury et al., 1996). Binding of C/EBP family members to the region around –55 bp stimulates the promoter activity. Hepatocyte nuclear factor-4 (HNF-4) represses the promoter activity without directly binding the promoter region and the region overlapping with the C/EBP binding site at ~55 bp is responsible for the HNF-4 repression. Other studies linking C/EBPs with the regulation of ArgI have shown that the transcription of the rat Arg I gene is induced by glucocorticoids. The glucocorticoid response of the Arg I gene appears to be mediated by C/EBPβ. In C/EBPα-deficient mice, mRNA and protein levels for arginase I decrease markedly (Kimura et al., 1998). Additional evidence indicates that Arg I expression is induced in macrophages by cytokines such as IL-4 which require a responsive element containing STAT6 and C/EBPβ sites located ~3 kb upstream of the transcription start site (Gray et al., 2005).

1.7.3.4 C/EBP and Neurotrophic factors

C/EBP-binding elements have been found to be present in the proximal region of the mouse BDNF gene which is conserved in the rat and it is suggested that C/EBPs might regulate

BDNF expression (Hayes et al., 1997). Moreover, in neuronal cells NeuroD a basic helix loop helix transcription factor, forms a complex with C/EBPα and β to modulate the signalling response of BDNF/Trk receptor, which regulates the expression of the immediate early genes Fos, Egr1 and Egr2 (Calella et al., 2007). Additional evidence suggesting that C/EBP has a potential role in neuronal plasticity and in nerve repair has been proposed previously. Sterneck and colleagues identified the C/EBPβ protein and gene as direct downstream targets of the NGF receptor, proposing a role for C/EBPβ in neurotrophin signalling in the brain (Sterneck and Johnson, 1998). It has been reported that C/EBPδ interacts with a sequence spanning nucleotides –90 to –59 of the NGF promoter (Colangelo et al., 1998). Recent evidence indicated that C/EBPδ, in combination with CREB, contributes to NGF gene regulation in the glioma cell line and in rat brain (McCauslin et al., 2006; Colangelo et al., 1998).

1.7.3.5 Others

During regeneration several cytoskeletal proteins become involved. One particular member of this group is Tα1 tubulin, which is vigorously expressed in neurons during early differentiation (Miller et al., 1987) and following axonal injury (Miller et al., 1989) where it is likely to function as building blocks for the formation of nascent and regenerating axons.

Another important cytoskeletal protein is mitogen activated protein (MAP) which in contrast to Tα1 tubulin does not recapitulate the embryonic pattern of expression after nerve injury in the adult (Fawcett et al., 1994). MAPs are important for the stabilization of the structure of growing axons, preventing their depolymerisation. It is possible that the

failure of adult neurons to mimic embryonic MAP expression after nerve injury could be an important factor leading to slower and less robust regenerative axon growth in the adult than axon growth in embryos.

C/EBPβ has been shown to regulate the growth-associated gene Tα1 α-tubulin following axonal injury (Nadeau et al., 2005). In the same study it was also demonstrated that the injury-induced upregulation of the endogenous mouse Tα1 α-tubulin mRNA and GAP-43, was inhibited in C/EBPβ -/- animals (Nadeau et al., 2005). Further support of the involvement of C/EBPβ in the nervous system was showed by (Cortes-Canteli et al., 2002) who found that C/EBPβ induces neuronal differentiation in the nervous system. It still remains unknown whether any other member of C/EBP are associated with neuronal repair in the PNS.

As previously mentioned, C/EBPs are involved in the response to inflammatory insults in non-neuronal systems and in the brain (Cortes-Canteli et al., 2004; Lekstrom-Himes and Xanthopoulos, 1998). This regulation is induced through a wide range of different cytokines in the same way that peripheral nerve injury elicits cellular and molecular inflammatory signals, some components of which might contribute to axonal regeneration (Lu and Richardson, 1991). Inflammatory cytokines, such as TNF-α and interlukin-1 (IL-1β), appear to constitute at least some of the injury-signals in DRG (Murphy et al., 2003). All these data indicates that C/EBPs might also be involved in the regulation of an inflammatory response upon axonal injury in the nervous system, suggesting a potential role in neuronal regeneration analogous to its role in regeneration of other tissues.

1.8 Knockout mice

A knockout mouse is derived from embryonic stem (ES) cells that have integrated a knockout construct by homologous recombination. This construct is cloned by using recombinant DNA technology. The targeting construct specifically replaces a segment of genomic DNA in the ES cell. The knockout ES cells are then microinjected into normal mouse blastocysts where they mix with cells from the normal embryo to form a chimeric mouse which contains cells derived from both the host embryo and knockout ES cells. Up to 100% of the resulting mouse chimera can be formed from cells derived from knockout ES cells. The chimeric mice are bred to produce mice that are heterozygous for the knockout gene. Thereafter the knockout allele can be transmitted in a Mendelian fashion.

1.8.1 Mice breeding

Breeding laboratory mice is never as simple as placing male and female together and waiting for the pups to be born. The genetic background of the mice selected for breeding is always important, and for many purposes it is critical, especially in the case of knockout and transgenic mice. For genetic experiments, the criteria for selection of breeders are quite specific and may vary from one generation to the next. Even for more general purposes, however, failure to choose breeders of the appropriate genetic background will eventually result in unwanted although often subtle changes in the characteristics of the mice.

A strain is defined as inbred if it was produced by sibling matings for more than 20 generations, after which all mice are considered genetically identical. To remain inbred, a strain must be maintained by sibling matings or, if necessary, by parent-off spring matings. The main pedigree line should be derived from a single sister-brother breeding pair at each

generation. With inbred mice, the goal is to preserve genetic uniformity in the colony. With most strains, this involves nothing more than mating males and females of the same strain. The only precaution is to avoid creation of a substrain or genetic drift**,** which occurs when a closed colony of inbred mice is isolated for many generations. A substrain may differ genetically from the original inbred strain and may yield different results when used in research. To avoid this, it is important to introduce outside animals from the same inbred strain on a regular basis.

C57BL/6 is the most widely used inbred strain. It is commonly used as a general purpose strain and background strain for the generation of knockouts mice. Although this strain is refractory to many tumours, it is a permissive background for maximal expression of most mutations. C57BL/6 mice breed well, are long-lived and are used in a wide variety of research areas including cardiovascular biology, developmental biology, diabetes and obesity, genetics, immunology, neurobiology, and sensorineural research. For all the reasons previously mentioned C57BL/6 mice were used for the production of C/EBPδ -/- by Esta Sterneck's group and the production of C/EBP luciferase transgenic mice. These two strains were used in this thesis.

1.8.1.2 Breeding strategies

Heterozygous mutant (-/+) x homozygous mutant (-/-)

This breeding scheme is normally used when only one gender of a mutant is a viable and fertile homozygote. The other gender may be infertile or have reduced fertility, embryonic lethal, die in utero, or die before reaching sexual maturity. Unless they can be recognized by a visible phenotype, all mutant mice must be genotyped to differentiate homozygotes

and heterozygotes. If the mutants are on a mixed genetic background, the off spring should be backcross to F1 hybrids about every 10 generations, to avoid producing recombinant inbred lines. If the mutants are on an inbred background, genetic drift must be avoided by backcrossing them to the appropriate inbred strain every 10 generations (for example, backcross a mutant on a C57BL/6 background to the standard C57BL/6 strain).

Suitable controls if a knockout's genetic background is inbred would be either the inbred strain (C57BL/6) or heterozygous siblings with normal phenotypes.

Heterozygous mutant (-/+) x heterozygous mutant (-/+)

This breeding scheme is used when homozygous mutant mice are severely impaired, infertile, embryonic lethal, die in utero, or die before reaching sexual maturity. This breeding strategy is ideal for studies of knockouts and for experimental comparison with their respective +/+ littermates to ensure that the differences between them are limited to their differences in genotype and not other factors such as how the dams interact with their pups or potential *in utero* differences between knockout and wildtype dams. Thus, it is ideal to be comparing littermates to one another that are raised as similarly as possible. However, since this breeding scheme produces off spring of multiple genotypes: 25% wildtype, 50% heterozygous and 25% homozygous, a lot of effort and resources are used for genetic testing to distinguish those that carry the mutation from those that do not, as all of the pups may look the same. Additionally, most of the heterozygous progeny must be discarded.

Suitable controls for the homozygous are the inbred strain and either wildtype or heterozygous siblings.

Homozygous mutant (-/-) x homozygous mutant (-/-)

This breeding strategy is used when homozygous mutants of both sexes are viable and fertile. Cost-efficacy is one main advantage of this breeding scheme since only homozygous knockouts are generated. No heterozygous are generated and less time and money is used for genotyping. Although all off spring produced are homozygous mutants, breeder genotypes should be verified at least every two generations.

If the knockouts are inbred, genetic drift must be avoided by backcrossing them to the appropriate parental inbred strain every 10 generations (for example like in my case, backcross a knockout on a C57BL/6 background to a standard C57BL/6 inbred mouse). Alternatively, if the mutants are on a mixed genetic background, backcross the off spring to appropriate F1 hybrids about every 10 generations, to avoid producing recombinant inbred lines.

If a mutant's genetic background is an inbred strain, a suitable control for the homozygous knockout would be the inbred strain used to generate the knockout (In this thesis C57BL/6). However, a disadvantage is the risk for subtle genetic differences that might have arisen between knockouts (e.g. C/EBPδ -/-) and the wildtype inbred strain (e.g. C57BL/6).

1.9 Hypothesis

The C/EBP family of transcription factors has six different members and multiple isoforms, they can form homo and heterodimers and they can bind to other transcription factors and proteins. Due to the C/EBPs molecular versatility it is not surprising that they have the ability to regulate the expression of a broad variety of genes with seemingly unrelated

biological function. By taking into account these properties and the C/EBP studies mentioned above, we hypothesised that C/EBPs are involved in the neuronal response after axonal nerve damage. Moreover, we suggest that C/EBPs may be a possible downstream target of regeneration within signalling pathways such as cAMP. This regeneration signalling cascade might work in a similar way to the memory formation pathway, which has several signalling molecules in common with the up to date known axonal regeneration signalling cascade, including cAMP, PKA and CREB (See Fig. 13, a proposed model in long term facilitation and nerve repair). C/EBP, are activated downstream of the cAMP-CREB pathway indicating a possible involvement of the C/EBP, in the synaptic plasticity events of axonal regeneration and LTM formation.

1.10 Aims

- To determine whether injury in the peripheral nervous system elicits a response in the expression of the C/EBP family of transcription factors.
- To assess the effect of C/EBPs in axonal regeneration by modifying their expression *in vitro* and *in vivo*.

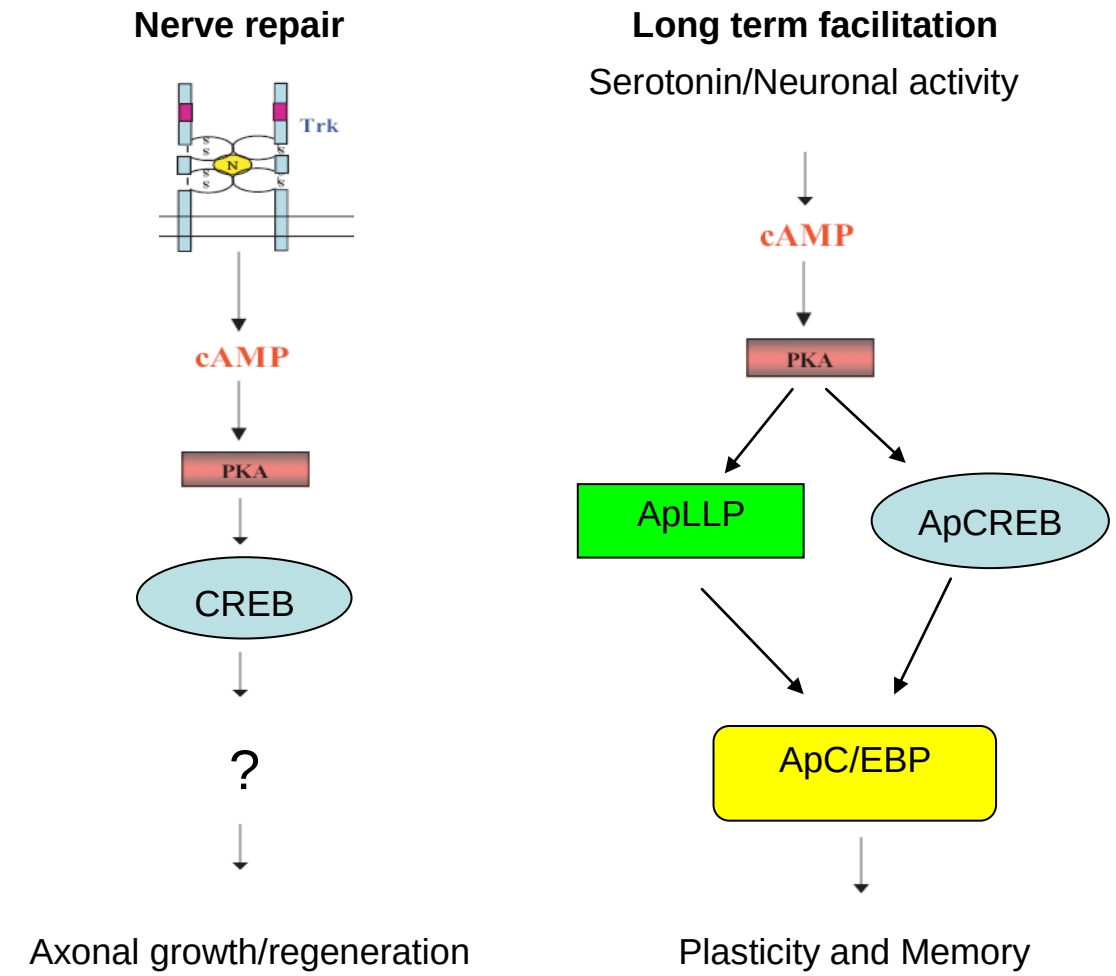


Fig. 13 Signalling pathway in axonal nerve repair in a mammalian model and long term facilitation and memory formation in Aplysia: cAMP elevation leads to PKA activation which phosphorylates CREB. CREB upregulates the expression of certain genes, involved in axonal growth/regeneration in mammals. In a similar signalling cascade in Aplysia, CREB and Aplysia LAPS18-like protein (ApLLP) induce the expression of C/EBP which in turn leads to the consolidation of long term facilitation. The LTF model involving ApC/EBP as a downstream regulator is based on studies by (Kim et al., 2006).

Chapter 2: Materials and methods

Experimental design. Animal care and procedures were carried out in accordance with guidelines and protocols approved by the United Kingdom Home Office. The room was kept at 24°C, with controlled humidity. Small animal rat chow and water were available ad libitum. For the detection of C/EBP isoforms following nerve injury, experiments were performed on 27 adult male Wistar rats weighing 260–320 g. The animals were randomly divided into three main groups representing three different time points, 9 animals into each group. For each one of the three groups, the experiment was repeated 3 times and in each experiment 3 animals were pooled together. Therefore, n=3. Three different early time points were selected following sciatic nerve injury, 4, 24 and 72 hrs.

The animals were anesthetized by halothane inhalation and the left sciatic nerve at level of the sciatic notch was crushed by compression for 30 seconds with fine Dumon No. 5 forceps (World Precision Instruments Stevenage, UK). Rats were sacrificed by decapitation and dissection of ipsilateral and contralateral L4 and L5 DRGs which were frozen in dry ice and stored at -80°C until used. Contralateral DRGs from the same animals served as control samples. Similarly, 1 cm of the axonal injury site, on the ipsilateral site and the corresponding non-injured area on the contralateral site were collected and stored 72 hrs following sciatic nerve injury.

For functional analysis studies, 8 C/EBPδ -/- mice adult female mice (2-4 months) and 8 C57BL/6 wildtype mice (controls) were used. A left sciatic crush injury was performed on all the animals. Function of the sciatic nerve of experimental animals was

evaluated a week before nerve injury and every 4 days until signs of function recovery was observed by day 28 following surgery.

LPS-induced inflammation model. Adult male Wistar rats (400-500 g) were injected intraperitoneally with bacterial lipopolysaccharide (LPS) injection from *Escherichia coli* 055:B5 (2 mg/kg body weight). For control experiments saline was injected intraperitoneally. 24 hours after LPS injection the animals were sacrificed by decapitation and different tissue were carefully harvested, snap frozen in dry ice and stored at −80 °C before processing them. n = 3.

RNA extraction and cDNA synthesis. Frozen tissue such as brain, spleen, L4-L5 DRGs or crush injured sciatic nerve were homogenized independently in Trizol® Reagent (Invitrogen, Paisley, UK) with a mortar and pestle and the samples were incubated for 5 minutes at room temperature (RT) to permit a complete dissociation of nucleoprotein complexes. Subsequently 0.2ml of chloroform was added per 1ml of Trizol reagent. After vigorous shaking for 15 seconds the samples were incubated at RT for 3 minutes and centrifuged at 12,000 x g for 5 minutes. Following centrifugation the colourless upper aqueous phase was transferred into a new tube which was mixed with 0.5ml of Isopropyl alcohol per 1ml of Trizol reagent and 1µl of glycerol. Then after incubation at RT for 10 minutes the RNA samples were centrifuged for 10 minutes at 13,000 x g at RT. Following the phenol-chloroform RNA extraction, isopropanol precipitation and the RNA pellet formation by centrifugation, the RNA samples were washed using 300 ml of 75% ethanol and dissolved in RNase-free water. The RNA concentration was determined spectrophotometrically at 260 nm, and the quality of RNA was assessed based on the ratio of absorbance at 260 and 280 nm with a NanoDrop (ND-1000, NanoDrop Technologies,

USA). 1-2 µg of RNA were treated with RQ1 RNase-free DNase (1µl/µg of RNA) (Promega, Madison, Wisconsin) for 30 minutes at 37°C. After DNase treatment, RNA samples were heated to 75°C for exactly 10 minutes in order to inactivate added DNase and to denature RNA. Reverse-transcription of RNA into cDNA was carried out by firstly adding 1µl of random primers (150 ng/µl) to 1µg of RNA, subsequently the sample were incubated at 70ºC for 5 minutes and then on ice for 3 minutes. Thereafter it was added to each sample a mixture consisting of 4µl 5X reaction buffer, 2 µl of 25 mM $MgCl_2$, 1µl of 10mM dNTP mix, 0.5µl recombinant RNasin ribonuclease inhibitor (40U/µl) and 1µl ImProm-II™ Reverse Transcriptase (Promega, Madison, Wisconsin) followed by double distilled H_2O up to a total of 15µl. The samples were incubated at 25 ºC for 5 minutes, thereafter at 55 ºC for 60 minutes and finally at 70 ºC for 15 minutes. As a negative control the samples were treated similarly but without any addition of 1µl ImProm-II™ Reverse Transcriptase.

Real time PCR. Real-time PCR was performed with TaqMan chemistry (ABgene, Epsom, UK) and with SYBR Green (ABgene, Epsom, UK), only in the LPS-induced inflammation study. The real time PCR analysis was performed on a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). Gene-specific primers (Invitrogen, Paisley, UK) and probes (MWG, Berlin, Germany) were used, (Table 4). Amplification conditions were as follows: 95 °C for15 min followed by 40 cycles of 95 °C for 10 s; 60 °C for 15 s; 72 °C 20 s. Each Real Time-PCR quantitation experiment was performed thrice using triplicate samples from independently generated cDNA templates originated from 1-2 µg of RNA. Expression of genes of interest between different conditions was determined following normalization to the level of a housekeeping gene, ribosomal 18S, in each sample. Due to 18S abundance in

tissues, the 18S cDNAs were diluted 100-fold before measurement of 18S mRNA. A standard curve was generated by real-time PCR analysis from triplicates of five ten-fold dilutions of cDNA generated from 1 ng of spleen RNA. As a negative control mRNA measurements were performed on samples treated with DNase (Invitrogen, Paisley, UK) but not with reverse transcriptase enzyme on each single run. Real time PCR runs showing mRNA expression signals for the negative control samples were discarded and completely new cDNA was generated for a re-run. The relative expression level of the gene of interest was computed from each experimental sample by calculating the ratio between the gene of interest (ng/reaction) and the 18S gene (ng/reaction), and multiplying the result by a 10000. C/EBP fold increase shows the ratio between ipsilateral and contralateral mRNA values.

Cell Culture and neurite outgrowth assay. The ND7/23 cell line was maintained in Dulbecco's modification of Eagle's medium (DMEM) (PAA, Yeovil, UK) complemented with 2 mM glutamine, 100 i.u. ml-1 penicillin, 0.1 mg ml-1 streptomycin and 10% fetal bovine serum (FBS) (PAA, Yeovil, UK) at 37°C, 5% CO2. Stimulation of neurite outgrowth and differentiation towards a neuronal phenotype was induced by reducing the serum concentration to 0.5% and additionally by supplementing the medium with 1 mM dibutyryl cAMP (Sigma, Poole, UK). For epigenetic studies on C/EBPδ activity, ND7/23 cells were treated with 1mM dibutyryl cAMP and with 5μM Trichostatin A (TSA) (Sigma, Poole, UK) for 24 hrs.

For quantification analysis of the neurite length following TSA treatment and transfection with conditional C/EBP inhibitors, the length of the longest neurite for each neuron for TSA experiments and only neurons expressing GFP for conditional inhibitory experiments were measured. Neurons with neurites longer than once cell diameter were

counted as neurite-bearing cells. Image J software (NIH, Bethesda, USA) was used to measure the length of neurites. Culture for each group was performed in triplicate and repeated 3 times.

For RNA extraction cells were grown for 3 days in 6-well plates (Corning, NY) and harvested by adding Trizol® Reagent (Invitrogen, Paisley, UK) directly to the cells.

Primary DRG neuronal cultures and treatments. Dorsal root ganglia dissected from the spinal cord of adult mice were processed for primary cultures basically as previously described by Holmes et al (2000). DRG were placed in Hanks balanced salt solution (HBSS) containing 0.125% of collagenase (Sigma, Poole, UK) for 1h and 30min at 37ºC. Then ganglia were rinsed and placed in fresh Hanks medium containing 0.25% of trypsin (PAA, Yeovil, UK), for 15 min at 37ºC. Medium was then removed and ganglia were placed in F-12 medium (PAA, Yeovil, UK) containing 10% foetal calf serum (FCS) (PAA, Yeovil, UK). Afterwards, ganglia mechanically dissociate into a single cell suspension by trituration with a pipette and syringe in growth medium which was F-12/DMEM medium supplemented with N2, 1% Albumin (Fraction V) (Sigma, Poole, UK) and 1% penicillin-streptomycin. The cell suspension was centrifuged on a 15 % bovine serum albumin cushion for 10 min at RCF of 200g (900 rpm). The supernatant was discarded and the cells were re-suspended in growth medium and plated onto Lab-Tek II 8-well chamber slides (Nunc, Naperville, IL) at a cell density of approximately 1000 cells/well on coverslips coated with 5µg/ml of poly-lysine (Sigma, Poole, UK) overnight and then with 1µg/ml of laminin (Sigma, Poole, UK) for 4h. Cells were incubated in growth medium in a humidified atmosphere at 37°C and 5% CO_2. 48 hrs after plating the neurons were fixed with 4% PFA, washed with phosphate buffered saline (PBS) (PAA, Yeovil, UK) 3 times blocked in 10%

normal goat serum (Jackson Labs, Suffolk, U.K.) for 1 hr at room temperature and immunostained with anti-β(III) tubulin 1:1000 (Sigma, Poole, UK). Neurite/axonal length was measured as previously described in ND7/23 cells. For the quantification analysis, the length of the longest neurite of each neuron was measured with ImageJ software (NHS, USA). Neurons with neurites longer than one cell diameter were counted. Culture for each group was repeated 3 times and the length of approximately 400 neurons per culture was measured.

Reporter system and transgenic animal. The C/EBP-Luciferase plasmid (Stratagene, Amsterdam, Holland) was digested with BamHI (New England Biolabs, Herts, UK) between bp 2670-5361 and the resulting C/EBP-Luc fragment yielded a 2.7-kb product that included the C/EBP enhancer element, the luciferase gene and poly- A tail. The purified 2.7-kb C/EBP-Luc constructs was quantified and microinjected into oocytes and surgically transferred into ovariduct of mice pseudopregnant foster dams previously mated with vasectomised males. Oocyte injection was done by Mr. William Mansfield. Ear biopsies were obtained 5 wk after microinjection of the eggs and analyzed for the presence of the transgenic constructs.

*Genotyping of transgenic founders. T*he screening of transgenic mice was performed with PCR. The primers sense 5'-GATGGAACCGCTGGAGAGC -3', antisense 5'-CATACTGTTGAGCAATTCACGTTC-3' (Invitrogen, Paisley, UK) with a thermal cycler profile of 95°C for 15 min, 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, 35 cycles, generated a 300-bp product of the luciferase gene.

Luciferase assay in transgenic animals. Animals injected with LPS (2 mg/kg body weight) were sacrificed after 4 hrs. Selected tissue was collected and frozen on dry ice. Thereafter the tissue was homogenized on ice with a tissue grinder. For each volume of tissue 3 x Glo Lysis buffer (Promega, Madison WI, USA) was added, mixed and incubated for 10 min at room temperature for cell lysis to occur. Subsequently, the solution was centrifuged and the supernatant was transferred to a new tube. 100 μl of the supernatant was transferred to a well in a 96-well plate (Nunc, New Jersey, USA), thereafter 100μl luciferin substrate (Promega, Madison WI, USA) was added to the well and the luciferase signal was measured in a 1420 VICTOR^2 luminometer (Wallac Co., Turku, Finland).

Generation of conditional constructs. For the conditional C/EBP dominant inhibitor, the A-C/EBP construct a generous gift from Dr. Charles Vinson (National Institutes of. Health) previously described in (Olive et al., 1996) was amplified with primers sense 5' CAGCTGACGCGTGGCTAACTAGAGAACCCACT 3' and primer antisense 5' CAGCTGGCTAGCTGGCTGGCAACTAGAAGGC 3' (Invitrogen, Paisley, UK) and cloned into the tetracycline conditional pBI-EGFP vector backbone (Clontech, Palo Alto, CA, USA) previously digested with MluI and NheI (New England Biolabs, Herts, UK). For the C/EBPδ antisense conditional construct, the IMAGE clone ID: 1528128 was amplified with primers sense.

5' CGCCGCGCTAGCCGCGCGTTACCGGCAGT 3' and antisense 5' CAGCTGACGCGTTTTTCTCGGACTGTGCCGG 3' (Invitrogen, Paisley, UK) engineered with restriction sites MluI and NheI and cloned into the conditional pBI-EGFP vector previously digested with MluI and NheI.

Ligation and transformation. In a 0.5ml Eppendorf tube, vector DNA and insert fragments in a molar ratio of 1:6, 1 µl 10X ligase buffer, 1 u T4 DNA ligase (Promega, Madison WI, USA) and nuclease-free water to a final volume of 10 µl were mixed and incubated overnight at 16°C. Competent cells (Novablue Singles, Darmstadt, Germany) were thawed on ice and mixed gently to ensure suspension. 1 µl of ligation mixture was gently mixed with 30 µl cells in an ice-cold 1.5ml eppendorf tube, kept on ice for 30 minutes, then incubated for 30 seconds in a 42°C water bath, transferred immediately to ice were it was kept for 3 minutes. Immediately after 500 µl of room temperature SOC medium was added to the tube. If selecting for ampicillin-resistant colonies, 100-200 µl cells were spread directly on selective agar plates; if selecting for kanamycin-resistant colonies, cells were incubated at 37°C with shaking at 250 rpm for 30 minutes prior plating on selective agar plates. After air-dry, the plates were inverted and incubated overnight at 37°C.

Plasmid DNA extraction with QIAprep Miniprep. Single colony from a freshly streaked selective agar plates was inoculated in 4 ml LB (Luria-Bertani; Sigma, St. Louis, MO, USA) medium containing selective antibiotic (ampicillin 100 µg/ml or kanamycin 50 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm. 1.5 ml cells were centrifuged at 10000 rpm for 1 min. To extract DNA from the pellet, the QIAprep miniprep kit was used (Qiagen, Crawley, UK). Briefly, the pellet was resuspended in 250 µl buffer P1 (50 mM Tris-HCL, pH 8.0; 10 mM Ethylene disodium tetra acetate (EDTA); 100 µg/ml RNase A) and gently mixed after adding 250 µl buffer P2 (200 mM NaOH, 1% SDS), then 350 µl buffer N3 (guanidine hydrochloride, acetic acid) was added and mixed again by inverting the tube a few times. After centrifugation for 10 min at 13000 rpm, the

supernatant was collected to a QIAprep Spin Column, followed by spin for 1 min. The flow-through was discarded and the column was washed with 0.75 ml buffer PE (High-salt, Ethanol) and spun for 1 min. The plasmid DNA was obtained by centrifugation of the column for 1 min after incubation the column with 50 µl buffer EB (10 mM Tris-HCL, pH 8.5) at room temperature for 1 min.

Transfection. One day before transfection, 0.25-1.0 X 10^6 cells were plated onto Lab-Tek II 8-well chamber slides (Nunc, Napperville, IL, USA) with 500 µl of growth medium so that the cells will be 50-80% confluent at the time of transfection. 150 ng of conditional construct and 150 ng of rtTA plasmid vector were diluted into 50 µl Opti-MEM® I Reduced Serum Medium (Invitrogen Paisley, UK). Thereafter 0.5 µl of Lipofectamine™ LTX Reagent (Invitrogen, Paisley, UK) was added to the diluted DNA and incubated at room temperature for at least 30 min. ND7/23 cells were washed with fresh medium without serum and antibiotics, lipofectamine complex was added to the cells and 300 µl new media without antibiotics was added to each well. The transfection medium was changed after 16 hrs and replaced with fresh culture medium containing antibiotics. The experiment was performed 3 times and the neurite length of 250-300 GFP positive cells was measured every time for each group.

Generation of C/EBPδ null mice. The C/EBPδ null animals were obtained from Dr. Esta Sterneck (National Institutes of. Health) and its generation was previously described (Sterneck et al., 1998)

*Genotyping of C/EBPδ null mice. T*he screening of knock-out mice was performed with PCR. The C/EBPδ primers sense 5' ACG ACT CCT GCC ATG TAC G 3' and antisense 5' GCT TTG TGG TTG CTG TTG AA 3' (Invitrogen, Paisley, UK) yielding a 133 bp fragment were used in combination with the sense 5' AAC AAG ATG GAT TGC ACG CAG 3' and antisense 5' GAA GGC GAT GCG CTG CGA A 3' primers (Invitrogen, Paisley, UK) for the neomycin cassette present in mutant animals yielding a 750bp fragment.

Fixation. The animals were sacrificed by CO_2 asphyxiation until breathing ceased and responsiveness to toe pinch was not detectable. The skin over the sternum was gripped with forceps and incisions made in the abdomen until the diaphragm was fully exposed. The xiphoid process of the sternum was clamped and held up so the diaphragm could be cut. The ribcage was cut bi-laterally and xiphoid process clamped back in a position so as to expose the whole thoracic cavity, with the heart accessible and still beating. A small incision to the left ventricle was made with fine micro scissors and a perfusion-drip needle inserted and clamped into place. The right atrium was identified and an incision made with fine micro scissors, and 0.9% saline was flushed through the hepatic circulation. After 40ml of saline had passed through the circulation, and the exiting fluid from the right atrium and liver was clear of blood, the animal was perfused with 4% paraformaldehyde (PFA) in 0.1M PBS at pH 7.4, until the tissue and limbs were rigid. For morphometric analysis, the animals were perfused with 4% PFA and 1% gluteraldehyde in PBS. Dissection was carried out on the perfused mice extracting the ipsilateral and contralateral sciatic nerves. Nerves for morphometric analysis were sectioned at 6mm distal to the crush site (identified by the

suture) and subsequently stored in phosphate buffer solution (pH 7.4) and labelled appropriately ready for semi-thin sectioning.

Preparation of semi-thin sections. After initial fixation, specimens were post-fixed in 1% Osmium tetroxide (Agar scientific, Stansted, UK) overnight. Subsequently specimens are dehydrated first in distilled water followed by gradual dehydration in alcohol (70%, 90% and 100%). Alcohol was removed from specimens by propylene oxide, followed by embedding sections in epoxy resin, prepared from Araldite (Agar Scientific, Stansted, UK). Sections were then cut using an ultramicrotome and glass knife.

Toluidine staining. 1 µm thick semi thin sections were stained with 1% Toluidine blue while slides were placed on a hot-plate set at 70◦C for 30 seconds after which each slide was washed with distilled water and left for a few minutes to dry, followed by subsequent viewing by oil immersion light microscopy for morphometric analysis using an ocular grid with 50x zoom showing 30 x 30µm squares (900µm²). Semi-thin section preparation and Toluidine staining was performed by Mr. Kawaljit Dhaliwal.

Cryostat sectioning. Fixed nerve specimens and L4-L5 DRGs were prepared for cryostat sectioning, firstly by orientating the nerve longitudinally to identify the proximal and distal ends. After embedding the nerves in embedding medium, 6µm thick sections were cut at -20ºC and subsequently mounted onto SuperFrost Ultra Plus® Slides (VWR, Lutterworth, UK) for warm drying at room temperature. The slides were labelled according to genotype and ipsilateral (injured) or contralateral (uninjured) nerve.

Immunohistochemistry. Nerve sections from 3 day and 2 weeks injured mice were used for analysis of the regeneration associated gene, anti-GAP-43 (Chemicon, Chandlers Ford, U.K.) or the neuronal marker protein gene product 9.5 (anti-PGP9.5). Slides were washed in PBS several times, blocked in 10% normal donkey serum (Chemicon, Chandlers Ford, U.K.) for 1 hour at room temperature and incubated with rabbit anti-GAP-43 antibody (1:2000) overnight or rabbit anti-PGP9.5 antibody (1:1000) (Abcam, Cambridge, UK) overnight. The secondary antibody, donkey anti-rabbit specific tetramethylrhodamine-5 isothyocyanate (1:400 TRITC) (Jackson Labs, Suffolk, U.K.) was applied for approximately 2 hours. After several rinses with PBS, the slides were incubated with a 1:15000 diluted solution of 4,6-Diamino-2-phenylindole dye (DAPI) (Jackson Labs, Suffolk, U.K.) for 10 minutes. Finally, slides were rinsed with PBS and coverslips applied using PBS glycerol (1:8). Immunoreactivity was quantitated using computerized image analysis, Leica Qwin Image Processing and Analysis System, (Leica, Wetzlar, Germany). For L4-L5 DRGs 4 hrs, 1 day and 3 days following sciatic nerve injury, anti-C/EBPδ (Santa Cruz, CA, USA), anti-SPRR1A a generous gift from Professor Stephen Strittmatter and anti-GAP-43 were used at a concentration of 1:500, 1:1000 and 1:2000 respectively. For all experiments negative controls were carried out by omitting the primary antibody from the staining procedure, and incubating with secondary.

C/EBPδ 3,3'-Diaminobenzidine (DAB) staining. Following TSA treatment ND7/23 cells, were incubated with C/EBPδ (1:500) (Santa Cruz, CA, USA) overnight at room temperature, the slides were washed with PBST and then directly incubated in ABC reagents (1:200) (Vector Labs, UK) for 2 h. Sections were then washed in 0.1 M Tris-buffered saline, pH 7.4 and finally developed in DAB solution containing 0.01% H_2O_2 and 0.25% nickel sulfate. For all experiments negative controls were carried out by omitting the primary antibody from the staining procedure, and incubating with secondary.

Imaging. All fluorescence microscopy was undertaken soon after the immunohistochemical incubation, to prevent loss of fluorescence from the fluorochromes. Photographs were taken with a digital camera (Hamamatsu Photonics, Welwyn Garden City, U.K.) connected to the microscope, at a constant exposure time to prevent false variation in staining intensity. For

quantification of immuno reactivity Leica Qwin software was used. For each animal, a fluorescent image of the sciatic nerve section extending 3mm distally from the crush site was captured. Each image was grey-scaled through Adobe Photoshop CS2. The image was subdivided into three different segments, 0-1, 1-2 and 2-3mm distal to the crush site. Three measuring frames (200 x 200 mm) were placed on each segment. A binary image of GAP-43 or PGP9.5 immunoreactive cells was then created within each frame. Both the upper and the lower gray level thresholds for creating the binary image were kept constant across all sections analyzed. The percentage of frame occupied by the binary image was then calculated.

Functional testing. Functional behaviour was tested to compare phenotypic differences between C/EBPδ knockout and C57BL/6 mice before and after sciatic nerve crush injury. The mice were tested at regular intervals for 2 weeks preceding crush injury after which they were tested again at regular periods for a further 4 weeks. Experiments were performed on adult, female mice n=8.

Mechanical sensory test. The baseline withdrawal thresholds of each of the hind paws using von Frey hairs were determined for each mouse prior to surgical manipulation (day 0). A platform containing 1.5 mm diameter holes in a 5 mm grid of perpendicular rows throughout the entire area of the platform was used. This platform is also opaque in appearance so that von Frey hairs are applied from underneath without distracting the mouse. Although objects distal to the surface of the customized platform were not visible, the plantar surface of a hind paw was visible as it was in direct contact with the platform surface. Two different hairs of 1.0g and 1.66g were applied from below the platform in ascending order. Application of the hair was to the central region of the plantar surface

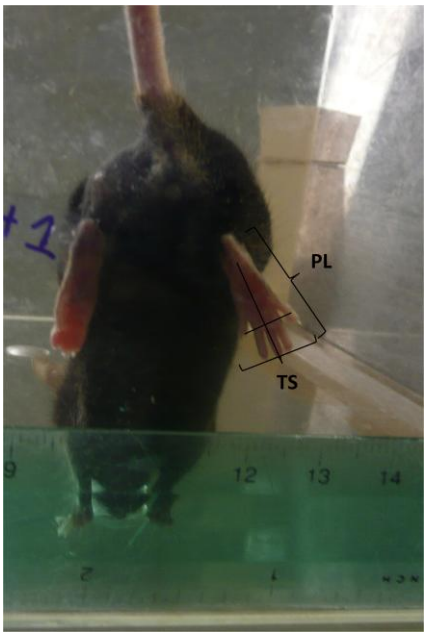
avoiding foot pads of injured compared to uninjured hindlimbs. The hair was applied only when the mouse was stationary and standing on all four paws. A withdrawal response was considered valid only if the animal reacted by lifting the hindlimb from the platform. Although infrequent, if a mouse walked immediately after application of a hair instead of simply lifting the paw, the hair was reapplied. If the animals did not respond with the lightest hair after 5 attempts the next heavier hair (1.66g) was tested.

Gripping and Grip test. Mice were allowed to grasp a rectangular metal bar. Subsequently, the bar was inverted and the number of grasps was measured for each hindlimb before sciatic crush injury. The number of failed attempts to grasp the individual metal bars was recorded out of 10 attempts. Following injury the ability to grasp on the injured side was measured and recorded at regular intervals until 22 days after injury. Before injury a Grip test was also performed using a grip strength meter. The grip strength meter was positioned horizontally and mice were held by the tail and lowered toward the apparatus. Mice were allowed to grasp the smooth, metal, pull bar with all four limbs and then pulled backwards in the horizontal plane. The force applied to the bar at the moment the grasp was released was recorded as the peak tension (g). The test was repeated 5 consecutive times within the same session and the highest value from the 5 trials was recorded as the grip strength for that animal.

Sciatic functional index analysis. Functional motor recovery in mice was quantified by measuring the sciatic static index (SSI). Mice were placed in a transparent acrylic box and pictures were taken from underneath of box while the mice were standing still. Animals were assessed during the testing period from before injury and regularly after injury using a digital camera. They were then analysed with the computer software program Adobe

Photoshop CS3 and the toe spread TS and paw length were measured and computed into the following formula developed by Baptista et al (2007).

SSI = 101.3 (ETS-NTS)/NTS – 54.03 (EPL-NPL)/NPL) -9.5



ETS: toe spread for the injured paw

NTS: Toe spread for the uninjured contralateral paw

EPL: length of injured paw

NPL: length of contralateral uninjured paw

Statistics. Detection of C/EBP mRNA levels after sciatic nerve injury included three measures taken at single time points by 4, 24 and 72 hrs following surgery. These measures of interest represent early time points in the initiation of the regeneration process. Although 9 animals were used on each time point, the sample size was 3, since 3 animals were pooled for each experiment. This sample size was sufficient to find a statistically significant difference between contralateral and ipsilateral DRGs since the results were consistent and the standard error of the mean was fairly small.

Behavioural measures were taken in C/EBPδ -/- or wildtype animals. Such data is typically analyzed using t-tests when only two groups are being compared. Previous

research in our lab has shown that the level of effect of most treatments (both for behavioural and anatomical parameters) along with the relatively small amount of variance of these measures within a group are such that small group sizes (i.e. 5 to 10) are generally sufficient to find a statistically significant effect. The animals were functionally tested

Statistical analysis was performed using SPSS 15.0 software. Statistic significance was determined by Student's t-test, paired t-test. All results in the graphs and tables are indicated as the mean ± standard error of the mean (S.E.M.) where $p<0.05$ is considered as statistical significant.

Table 4 Primer and probe sequences designed to analyze mRNA expression of selected genes by real-time PCR

Genes	Sequence (5'-3')	Accession number
C/EBP Alpha	Forward: GAGTCGGCCGACTTCTAC	NM_012524
	Reverse: GAAAGCCAAAGGCGGCGTT	
Probe	CCCGATGAGCAGCCACCTCCAGA	
C/EBP Beta	Forward: AGCGACGAGTACAAGATGCG	NM_024125
	Reverse: GCTGCTCCACCTTCTTCTGC	
Probe	TCTCCGCCGTCAGCTCCAGCACC	
C/EBP Gamma	Forward: GCAAGCTGTCGCAGCCAG	XM_341842
	Reverse: TGCTTGCTTGGAGGCACAG	
Probe	ACTGCAGGAGTGAACGGGATCAGTGT	
C/EBP Delta	Forward: CTGCCATGTATGACGACGAG	NM_013154
	Reverse: TGATTGCTGTTGAAGAGGTCG	
Probe	TGGCTGCCGTGCCCACCCTAGA	
C/EBP Epsilon	Forward: TTGACCTCTCCGCCTACATC	NM_017095
	Reverse: GGTAGTGAGGAAATGAAGGTG	

Probe	AACAGCTACTTTCTGACCTCTTGCCATGAA	
18S rRNA	Forward: CGGCTACCACATCCAAGGAA	X01117
	Reverse: TGG CAC CAG ACT TGC CCT C	
Probe	CTTTCGAGGCCCTGTAATTGGAATGAGTC	
GAP-43	Forward: AGCCAAGGAGGAGCCTAAAC	M16228
	Reverse: CTGTCGGGCACTTTCCTTAG	
SPRR1A	Forward: TCCATCACCATACCAGCAGA	AA891911
	Reverse: TAGCACAAGGCAATGGGACT	

Chapter 3: Expression of C/EBPs in the sciatic nerve after injury

3.1 Generation of a C/EBP mRNA detection assay

In order to develop a sensitive expression assay to detect and determine the changes in mRNA expression of the different members of the C/EBP family of transcription factors, SYBR Green and TaqMan Real Time- polymerase chain reaction (RT-PCR) analysis were performed 24 hours after LPS-induced inflammation in both brain and spleen of rats. LPS was used in these experiment since it was previously demonstrated that C/EBPs are upregulated following administration of LPS *in vivo* (Alam et al., 1992).

SYBR Green chemistry is a method used to perform RT- PCR analysis. SYBR Green is a dye that binds the Minor Groove of double stranded DNA. When SYBR Green dye binds to a double stranded DNA, the intensity of the fluorescent emissions increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. However, SYBR Green dye will bind to any double stranded DNA molecule in a reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration.

TaqMan Probes are oligonucleotides that contain a fluorescent dye, typically on the 5' base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a non fluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET

no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage. Therefore unlike SYBR Green chemistry, the TaqMan assay is specific to a pre-determined target and it does not generate false positive signals.

In this thesis I initially used SYBR Green RT-PCR, however, due to problems with false negative signals, I subsequently, replaced this method with the TaqMan RT-PCR.

For the TaqMan RT-PCR analysis the mRNA levels of C/EBPα, β, γ, δ and ε were measured 24 hours after injection with LPS or saline as a control. The relative mRNA expression of five different members of the C/EBP family in the LPS versus saline condition was determined following normalization to the level of the ribosomal 18S, housekeeping gene, in each sample.

Both SYBR Green and TaqMan RT-PCR analysis showed mRNA upregulation of the 5 different C/EBP members in the brain after LPS-induced inflammation in comparison to control groups (Fig. 14 and Fig. 15). Similarly, SYBR Green and TaqMan RT-PCR showed mRNA upregulation of C/EBPβ and C/EBPδ and downregulation of C/EBPα, C/EBPγ and C/EBPε in the spleen after LPS treatment (Fig. 14 and Fig 15).

However, these results showed that the magnitude of the C/EBP mRNA changes in the spleen and the brain after LPS treatment is different between the two Real Time-PCR methods (Fig. 14 and Fig. 15).

The two RT-PCR methods indicated that transcriptional mRNA levels of C/EBPβ and C/EBPδ isoforms are upregulated in the brain and spleen after LPS-induced inflammation whereas C/EBPα mRNA is only upregulated in the brain but not in the spleen (Fig. 14 and Fig. 15). Furthermore, these results indicate that LPS treatment also increased

significantly C/EBPε mRNA expression in the brain (Fig 14A and Fig 15A). C/EBPγ mRNA showed a slight upregulation after LPS injection, however, this result was not statistically significant.

Additionally, C/EBPα, C/EBPγ, and C/EBPε mRNA expression was downregulated in the spleen, with the only exception of C/EBPβ and C/EBPδ, which were upregulated after LPS-induced inflammation (Fig 14B and Fig 15B). Notably by using TaqMan RT-PCR, when compared to saline-injected control animals, the C/EBP expression in animals injected with LPS was significantly upregulated in the brain for isoforms alpha ($p=0.009$), beta ($p=<0.001$), delta ($p=0.004$) and epsilon ($p=0.017$). However, in the spleen only C/EBPδ was significantly upregulated ($p=0.001$).

In the brain, LPS injection upregulate C/EBPα, C/EBPβ, C/EBPδ and C/EBPε mRNA expression as compared to mRNA expression after control injections. TaqMan RT-PCR indicated that these mRNA levels in the brain increased by 3-fold for C/EBPα, 3.5-fold for C/EBPβ, 4-fold for C/EBPδ and 5- fold for C/EBPε. Conversely, in the spleen, mRNA expression levels of C/EBPα, -γ and -ε measured by TaqMan RT-PCR, 24 hours after LPS treatment were significantly downregulated (Fig 15B). LPS injection raised dramatically the mRNA levels of C/EBPβ by 26-fold and to a less extent C/EBPδ by 2-fold when compared to control injections. However, SYBR Green RT-PCR indicated that C/EBPβ was increased 2 fold in the spleen after LPS treatment.

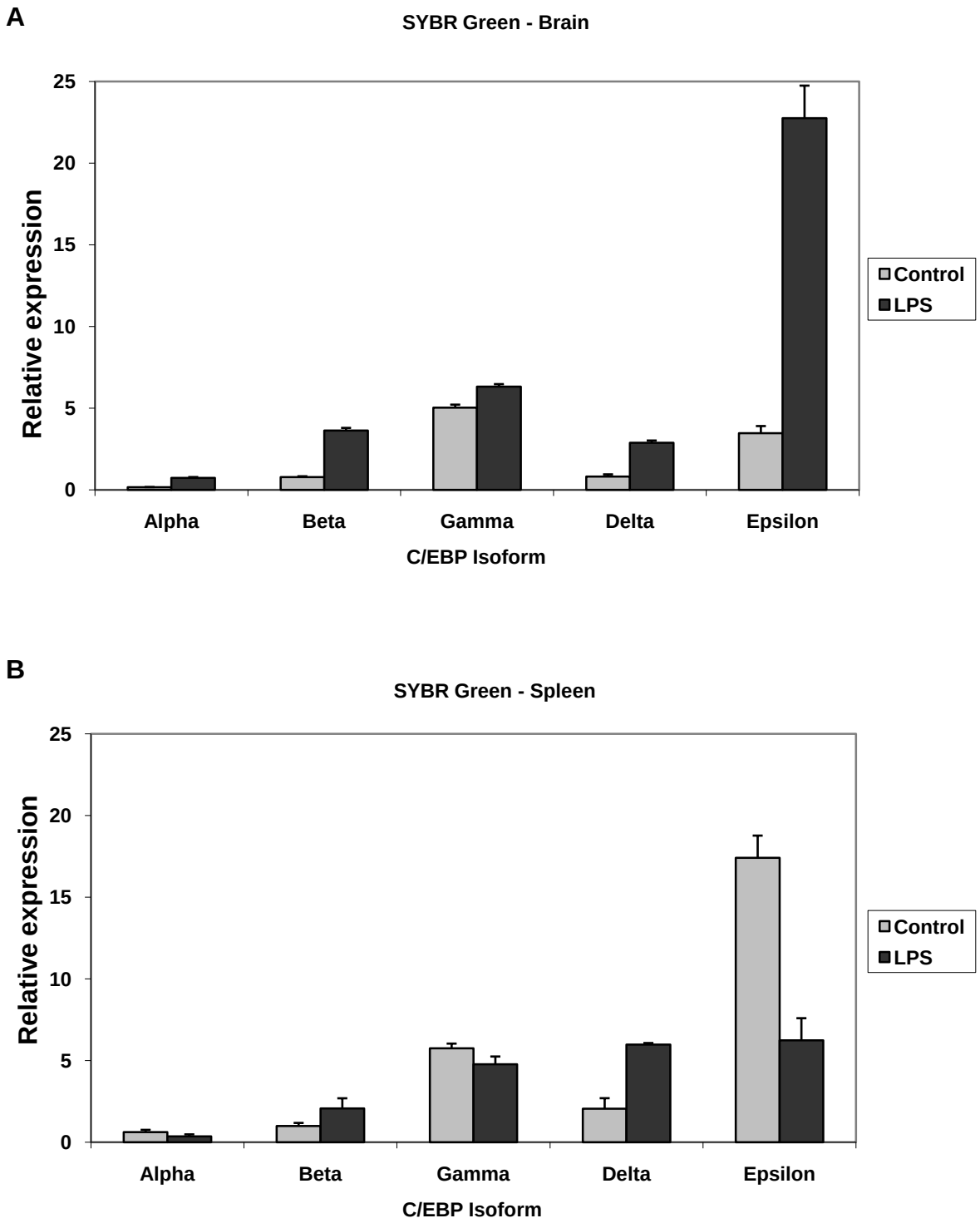


Fig. 14 SYBR Green real time-PCR measurement of C/EBPs mRNA expression 24 hours after LPS-induce inflammation: (A) mRNA expression changes in the brain after LPS treatment. (B) mRNA expression changes in the spleen. Graph showing the normalized relative mRNA expression levels between LPS-injected versus saline-injected animals of five members of the C/EBP family (α-ε). Expression levels are showed as means of the ratio of C/EBPs mRNA to 18S mRNA. Saline injected animals were used as control.

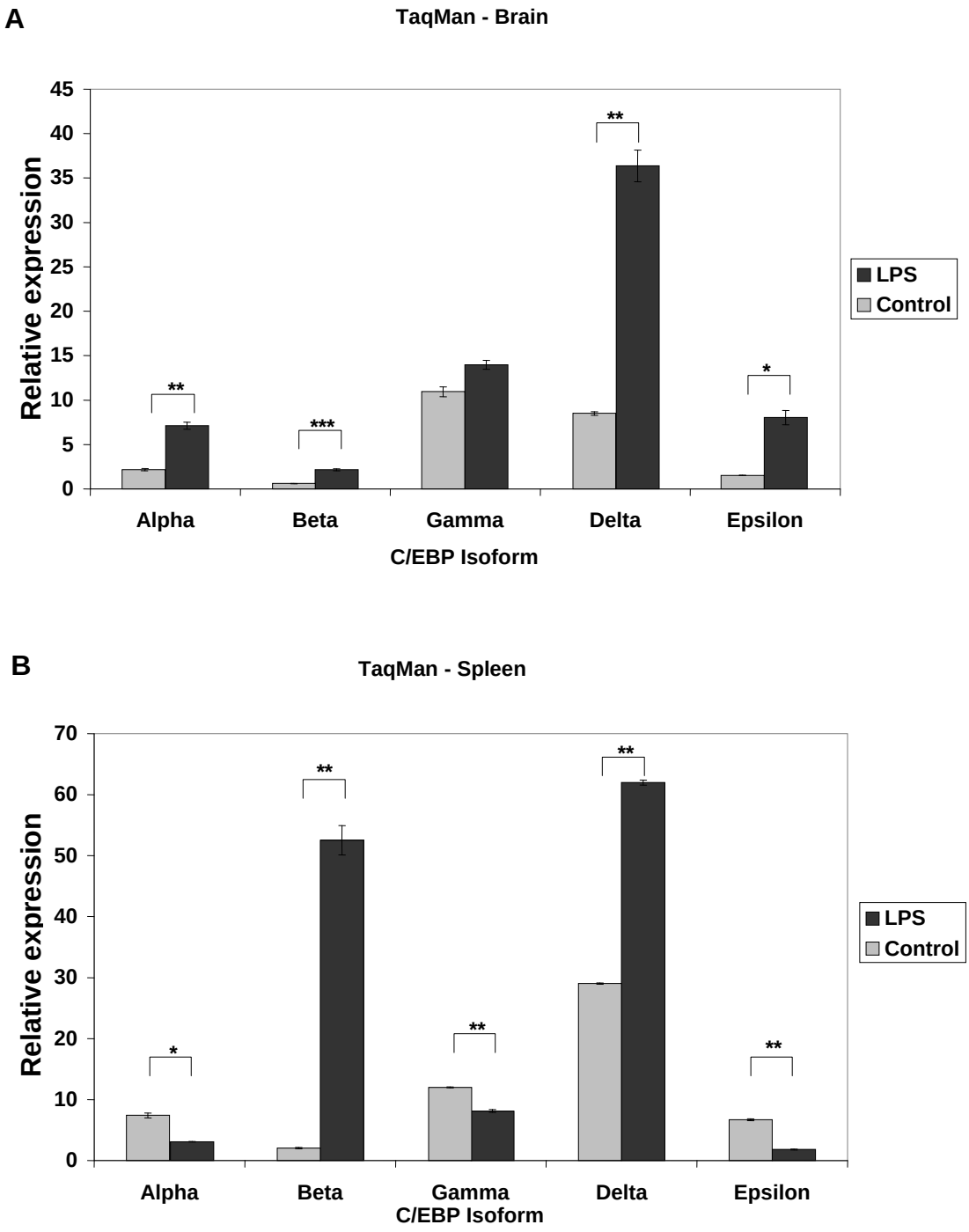


Fig. 15 TaqMan real time-PCR measurement of C/EBPs mRNA expression 24 hours after LPS-induce inflammation: (A) mRNA expression changes in the brain after LPS treatment. (B) mRNA expression changes in the spleen. Graph showing the normalized relative mRNA expression levels between LPS-injected versus saline-injected animals of five members of the C/EBP family (α-ε). Expression levels are showed as means of the ratio of C/EBPs mRNA to 18S mRNA. Saline injected animals were used as control. (Mean ± S.E.M, n= 3 animals, *=P<0.05, **=P<0.01 and ***=P<0.001 by student's t-test).

3.2 C/EBP mRNA expression after crush axotomy of the sciatic nerve *in vivo*

In order to study C/EBPs potential effect and involvement in nerve damage and subsequent axonal regeneration, the first objective was to screen five members of the C/EBP family and determine whether peripheral nerve injury elicits a regulatory response in the mRNA expression of the C/EBP family of transcription factors *in vivo*. The rat sciatic nerve injury model was used to compare the relative C/EBP mRNA expression, between the injured ipsilateral side and the uninjured contralateral side which was used as a control. C/EBP mRNA expression levels were measured in L4 and L5 DRGs.

C/EBP mRNA expression analysis was performed by using a TaqMan RT-PCR assay, a more sensitive method than SYBR green RT-PCR method. In order to correct and compensate for intra-, inter-kinetic variations (sample-to-sample and run-to-run variations), cDNA sample loading variation and differences in the amount of starting material, all the samples were normalized against the housekeeping gene 18S ribosomal mRNA. mRNA expression was assessed in three different groups, in each group L4-L5 DRGs from three animals were pooled together. Therefore, six different DRGs were used in each single experiment. Triplicates were measured in all the experiments.

3.3 C/EBP mRNA expression after sciatic nerve injury in DRGs

Following sciatic nerve crush, three different time points were selected for the *in vivo* analysis of the C/EBP mRNA expression between ipsilateral and contralateral DRGs. The first time point chosen was four hours after injury, representing early events in the neuronal

response to nerve damage, and the second time point was 24 hours after nerve injury and the last time point at a later stage of nerve damage, was 72 hours after injury.

Analysis of the C/EBPs mRNA expression by TaqMan RT-PCR, following sciatic crush injury *in vivo* revealed that C/EBPβ, C/EBPγ and C/EBPδ were upregulated in ipsilateral L4-L5 DRGs compared to contralateral DRGs following sciatic nerve injury peaking at 4 hours (Fig. 16A).

C/EBPβ, C/EBPγ and C/EBPδ mRNA levels increased significantly to approximately 3-fold within 4 hours after nerve injury (Fig. 16B). C/EBPβ mRNA expression resumed background levels by 24 hrs. C/EBPγ mRNA upregulation was maintained 24hrs after injury but returned to normal levels by 72 hrs after injury. C/EBPδ mRNA upregulation remained constant at 4, 24 and 72 hours following sciatic nerve crush in the ipsilateral side compared to the contralateral side (Fig. 16A). Relative mRNA expression shows that C/EBPα and C/EBPε were hardly detected in L4-L5 DRG neurons after sciatic crush (Fig. 16A).

To demonstrate that the surgical procedure was carried out successfully and that the left sciatic nerve was properly crushed by compression, galanin was used as a control. The well known neuropeptide galanin is rapidly and highly upregulated after peripheral nerve injury in the adult (Wynick et al., 2001). Galanin mRNA expression was measured 72 hours after nerve injury on L4-L5 DRG neurons from the ipsilateral and the contralateral side. Measurements of relative mRNA expression indicate that galanin was highly upregulated after nerve crush injury (Fig. 16A), demonstrating that the crush injury of the left sciatic nerve at the mid-thigh level was performed correctly.

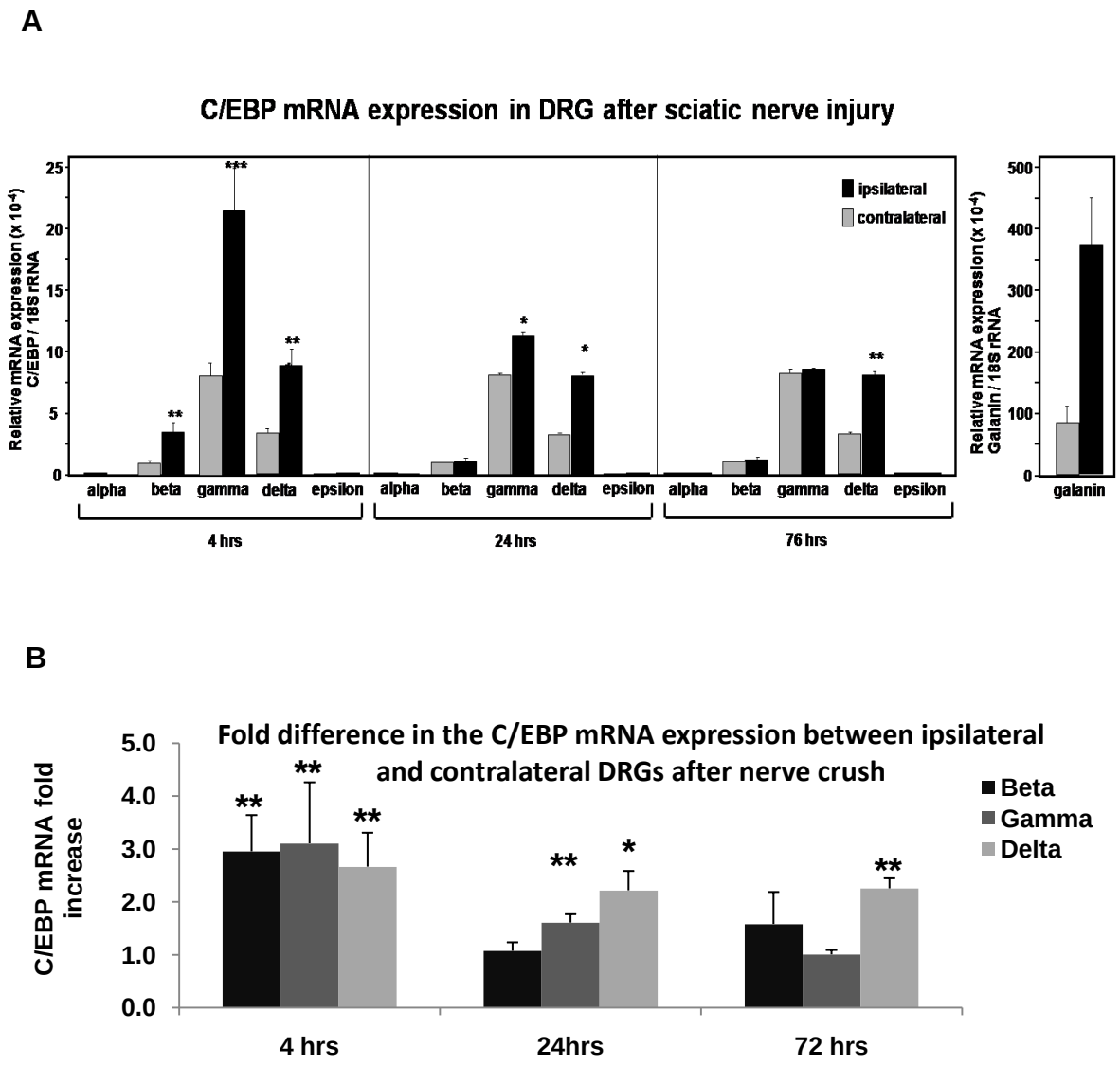


Fig. 16 C/EBPs mRNA expression in DRGs following sciatic nerve injury crush: TaqMan RT-PCR measurements of mRNAs for C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ and C/EBPε in rat L4 and L5 DRGs removed 4, 24 and 72 hours after sciatic nerve injury. Ratios of C/EBP mRNA are normalized to 18S mRNA. (A) Relative C/EBP mRNA expression levels between DRGs from the contralateral and ipsilateral side of the nerve crush. As control for the nerve crush galanin mRNA levels were measured 72 hours after nerve injury. (B) Fold increase indicates the ratio between C/EBP mRNA expression of DRGs from the ipsilateral and DRGs from the contralateral side after nerve crush. C/EBPα and C/EBPε display background levels, therefore no fold increase was calculated. (Mean ± S.E.M, n= 3, *=P<0.05 and **=P<0.01 vs. contralateral DRGs by student's t-test).

3.4 C/EBP mRNA expression in the lesion site of the sciatic nerve after crush

Further analysis of the effect of nerve injury on the transcriptional expression of C/EBPs was carried out on the damaged area of the sciatic nerve (Fig. 17A). C/EBP mRNA expression were measured 72 hours following injury in the lesion site both ipsi- and contralaterally (Fig. 17B).

Relative expression analysis indicated that C/EBPβ mRNA levels were significantly increased on the area were the nerve was crushed in comparison with the untouched contralateral side (Fig. 17B). C/EBPγ mRNA expression was slightly downregulated. However, this was not statistical significant. In contrast, C/EBPδ mRNA expression pattern was significantly downregulated (Fig. 17B). C/EBPα mRNA expression was close to background levels and C/EBPε was not detected. The C/EBPβ mRNA expression on the lesion site 72 hours after nerve injury was upregulated almost 1.4 fold in comparison with the uninjured contralateral side. In contrast, C/EBPδ mRNA concentration level on the ipsilateral side decreased almost 2.4 fold in comparison with the undamaged side (Fig. 17B).

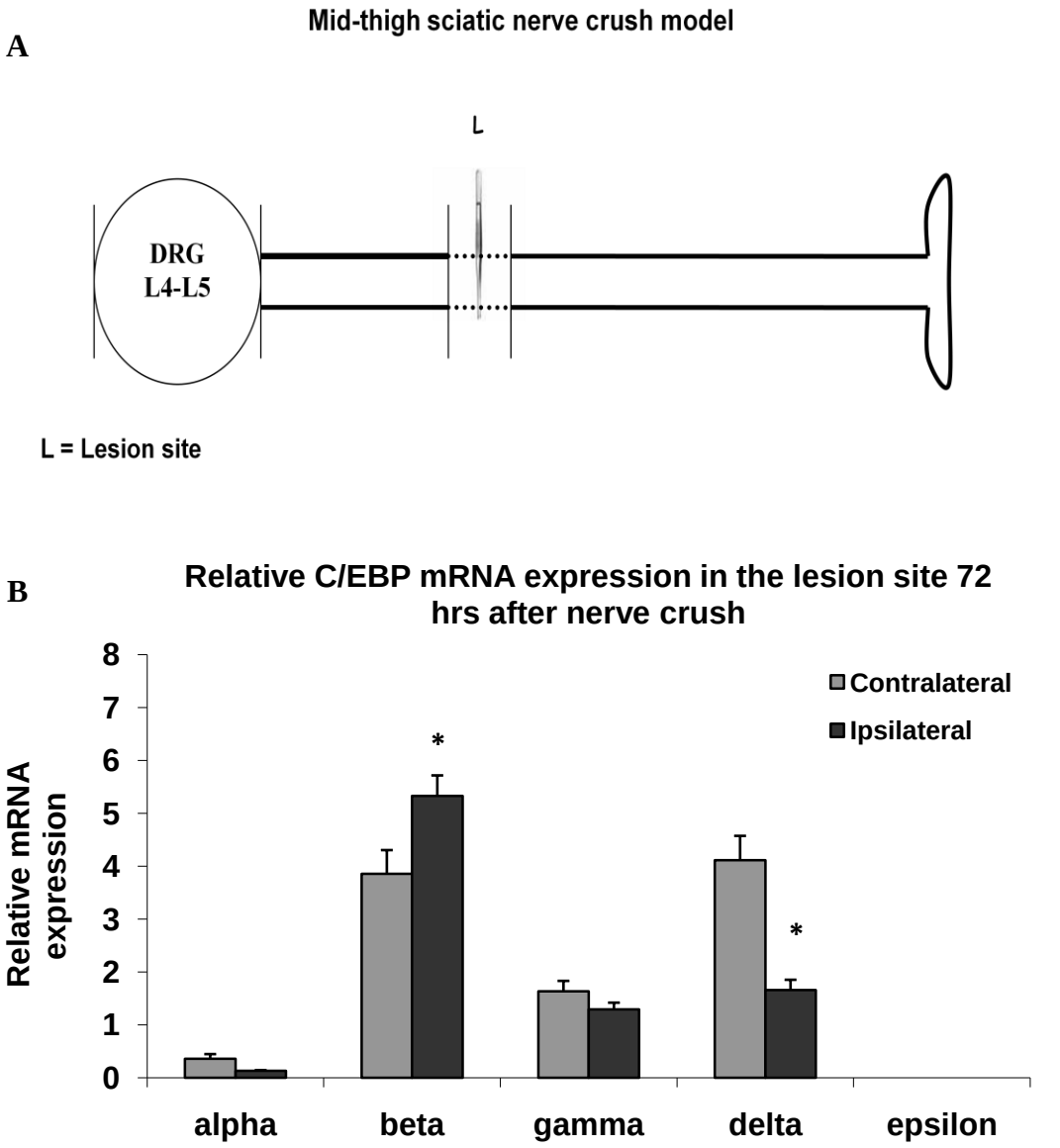


Fig. 17 C/EBP mRNA expression in the lesion site: (A) The left sciatic nerve was crushed by compression with forceps for 30 seconds at the mid-thigh level (L). Following injury L4 and L5 DRGs were taken from the ipsilateral side for mRNA expression analysis and compared to the uninjured contralateral side which was used as control. (B) Relative C/EBP mRNA expression levels between the contralateral and ipsilateral sciatic nerve lesion site 72 hours after nerve crush. (Mean ± S.E.M, n= 3, *=P<0.05 and **=P<0.01 vs. contralateral DRGs by student's t-test).

3.4 Discussion

Generation of a C/EBP mRNA detection assay

Initial experiments were designed to develop a sensitive quantitative RT-PCR assay that could be used in the analysis of small tissues such as DRGs and specifically to be used in the study of C/EBP expression upon different physiological responses. It was previously determined by Northern analysis that C/EBPs respond to inflammatory agents such as LPS (Alam et al., 1992). LPS is a major component of the outer membrane of gram-negative bacteria that contributes in great part to the induction of a strong immunological response upon stimulation. Consequently, the objective of these experiments was to develop a RT-PCR assay by using the known study of the differential expression of C/EBP mRNAs in the LPS-induce inflammatory model.

In order to validate the RT-PCR methodology, comparative results from northern analysis studies provided by Alam *et al.* (1992) were used. In that study, C/EBPβ and C/EBPδ were upregulated in brain and spleen following LPS treatment. C/EBPα mRNA expression was only upregulated in the brain and not in the spleen after LPS treatment. Our results validate the specificity and sensitivity of the TaqMan RT-PCR assay to measure the mRNA expression levels of different members of the C/EBP family, which can be further used for the assessment of the C/EBP transcriptional changes during different physiological states such as after nerve injury and during nerve repair.

The results of this work indicated that LPS-induce inflammation regulates the transcriptional levels of the members of the C/EBP family of transcription factors which exhibit a degree of difference in their expression pattern in diverse tissues. The increase in

mRNA expression levels of C/EBPβ and C/EBPδ correlates with similar results in other studies showing a differential regulation of C/EBP isoforms in response to LPS and different cytokines. This has been shown by RT-PCR analysis of macrophages, where in general, the expression of C/EBPα was reduced and that of C/EBPβ and C/EBPδ was induced in the presence of LPS (Tengku-Muhammad et al., 2000; Bradley et al., 2003). An additional RT-PCR study indicates that C/EBPα, C/EBPβ and C/EBPδ mRNA expression is upregulated in rat glomerular mesangial cells after LPS treatment (Granger et al., 2000a).

Another northern analysis study in mouse primary cortical astrocytes showed that C/EBPβ and C/EBPδ mRNA expression was induced after LPS and other pro-inflammatory cytokines *in vitro* (Cardinaux et al., 2000). However, none of the mentioned studies above examined the mRNA expression levels of C/EBPγ and C/EBPε, which is unique in this study. Furthermore, nuclear factor interleukin 6 (NFIL-6), a human homologue of C/EBPβ, has been shown to be highly upregulated by interleukins and LPS (Akira et al., 1990). In contrast C/EBPα is inhibited in most tissues and cell types (Akira et al., 1990; Poli et al., 1990; Kinoshita et al., 1992; Ramji et al., 1994; Poli, 1998; Tengku-Muhammad et al., 2000; Alam et al., 1992).

In this study, TaqMan RT-PCR results indicated C/EBPα, C/EBPβ, C/EBPδ and C/EBPε were upregulated 3-5 fold in the brain 24 hours after LPS-induced inflammation. C/EBPβ and C/EBPδ were also seen to be upregulated in the spleen by 25 and 2 fold respectively following LPS treatment. In contrast, C/EBPα was downregulated in the spleen. Unique for this study was the analysis of the mRNA expression profile of C/EBPγ and C/EBPε which was also altered after LPS-induced inflammation. Similar results were obtained initially 24 hours after LPS-induced inflammation by SYBR Green RT-PCR

analysis. However, differences in the magnitude of the mRNA response were observed between the SYBR Green and the TaqMan RT-PCR analysis.

Variations between SYBR Green and TaqMan RT-PCR results could be an effect of the different chemistries behind these two methods. SYBR Green assay uses a florescent dye that can bind to any double-stranded DNA even unspecific products. Amplified non-specific products can modify the amplification efficiency for the specific products. Consequently, the amplification of non-specific products, such as primer dimers, results in a systematic error for the quantification independently of whether the non-specific products are detected or not. Additionally, if aggregations of primer dimmer formation take place in the late cycles, they will mask the signal resulting from the target amplification. The sensitivity can be restored by increasing the temperature at which the primer dimers are melted and hence do not contribute to the target signal measured. However, in this case, accurate quantification may be impaired. One major obstacle in the analysis of C/EBP mRNA expression is the fact that most of the C/EBP members are intronless. This makes it troublesome to develop primers for their detection due to the lack of an exon-intron boundary, giving rise in some cases to an unspecific amplification of genomic DNA resulting in high background levels. Additionally, all the members of the C/EBP family have over 90% homology on their basic leucine zipper region at the carboxyl-terminus, making it complicated to discriminate between the different isoforms. Furthermore, a great portion of the N-terminal DNA sequence of the C/EBPs does not fulfil the requirements for the design of an appropriate primer. These technical difficulties gave rise to increased background levels and false positive results observed on negative control samples and on

unspecific bands in a 0.9% agarose gel (data not shown). Therefore, this approach was substituted by the TaqMan assay, which doesn't suffer from the same specificity problems.

The TaqMan assay helps to detect and discriminate between the different C/EBP isoforms by adding an extra stringent factor to the reaction, a probe which is a single stranded oligonucleotide designed to bind only to the target DNA sequence between the two PCR primers. The TaqMan probe has two fluorescent tags attached to it. One is the reporter dye FAM and the other is the quencher TAMRA. The close distance between the reporter dye and the quencher inhibits the fluorescence signal. Therefore, intact probes do not fluoresce since they are quenched. Degradation of the TaqMan probe, by the Taq DNA polymerase, liberates the reporter dye from the quenching activity of TAMRA increasing the fluorescent activity, which is proportional to the amount of PCR product formed. Therefore in contrast to SYBR Green RT-PCR only specific PCR product can generate fluorescent signal in TaqMan RT-PCR.

Another obstacle in the analysis of C/EBP gene expression after nerve injury is the limited amount of tissue which is even more aggravated due to the numerous isoforms present in this family of transcription factors. Therefore, L4-L5 DRGs from three different animals have been pooled together for each single experiment in order to compensate for the restricted amount of RNA extracted from single DRGs.

Importantly, these results helped us to demonstrate that the TaqMan real time PCR assay is a sensitive assay for mRNA measurement of C/EBPs after different physiological conditions. Additionally, TaqMan real time PCR is a convenient method for assaying gene expression in small amounts of tissue such as the DRG.

C/EBP mRNA expression after sciatic nerve injury

Several reports have shown that nerve injury to the CNS and PNS generate different molecular changes in the neuronal cell body (Broude, McAtee et al. 1997; Herdegen, Skene et al. 1997; Herdegen and Leah 1998; Schwaiger, Hager et al. 2000; Sheu, Kulhanek et al. 2000), which is to some degree responsible for diverse regeneration outcomes in the respective nervous system. Peripheral nerve damage alters the expression of a series of genes including regeneration associated genes, in the DRG cell bodies, following with an increased production of several proteins necessary for regeneration (Plunet, Kwon et al. 2002).

One of the aims of my work is to study whether the C/EBP family of transcription factors have a potential role and involvement in axonal regeneration. In order to answer this question I first studied the C/EBPs expression profile following peripheral nerve damage, since upregulation after nerve injury is a prerequisite for involvement in axonal repair. The results showed that the mRNA expression of C/EBPs is regulated after sciatic nerve injury in L4-L5 DRGs. C/EBPβ, C/EBPγ and C/EBPδ mRNA expression levels were upregulated 3-fold, four hours after sciatic nerve injury. In agreement with these results, Nadeau et al. (2005) showed that C/EBPβ mRNA levels are increased in facial motor neurons following axonal injury, suggesting a potential role in neuronal regeneration analogous to its role in regeneration of other tissues. Additionally, they demonstrated that C/EBPβ is necessary for transcriptional induction of the Tα1 α-tubulin promoter following axonal injury and that its lack inhibits regular nerve-injury-induced upregulation of α-tubulin and GAP-43 mRNA, both essential for nerve repair.

On the other hand, it has been shown that C/EBPδ activation by the receptor gp130 in cultures of dissociated Schwann cells of embryonic rat DRG affects the differential mRNA expression of the glial fibrillary acid protein, protein zero and myelin basic protein, suggesting that C/EBPδ might play a role in the switch to a myelinating phenotype, a switch functioning not only in the embryo during development but recapitulated after axonal injury during nerve regeneration (Kamaraju et al., 2004). Furthermore, gp130 receptor has been involved in the activation of STAT-3 which becomes induced in axotomized DRG neurons (Qiu et al., 2005). STAT-3 activation can induce transcription of cytokine-responsive genes (Ihle, 2001; Heinrich et al., 2003) and it has been suggested to be involved in supporting survival of injured neurons by conditional knockout studies (Schweizer et al., 2002). It was unexpected to see an upregulation of C/EBPγ immediately after injury considering that C/EBPγ has an inhibitory effect on other C/EBP members. C/EBPγ can heterodimerize with other C/EBPs, it has a DNA binding domain but it lacks an activation domain.

The results obtained 24 and 72 hrs after sciatic nerve injury indicate that the mRNA upregulation of C/EBPβ and C/EBPγ originally seen during the early events after axonal injury response is a transient effect, since only C/EBPδ mRNA upregulation is maintained 72 hours after injury.

Consistently, previous studies by microarray analysis have shown that C/EBPδ is upregulated 2-3 fold in DRGs 12 and 24 hours after injury (Nilsson et al., 2005) although a 4-fold upregulation has been found in sympathetic ganglia already 6 hours after axotomy and also after 48 hours (Boeshore et al., 2004). However, one study shows that C/EBPδ is upregulated transiently immediately after nerve lesion (5–8 h) (Bosse et al., 2006).

A main disadvantage with the RT-PCR method is the incapability to allow localization of transcripts to specific cells within a tissue. Therefore, it is unclear whether the detected C/EBP mRNA signals come from neuronal or non-neuronal cells within DRGs. This problem could be circumvented by *in situ* hybridization. However, this technique is more complex and comparatively less sensitive than RT-PCR.

RT-PCR analysis showed a significant increase in the C/EBPβ mRNA levels and a significant decrease in the C/EBPδ mRNA levels in the ipsilateral side of the lesion site. These differences in the C/EBP mRNA expression between ipsilateral and contralateral side of the injury could suggest that C/EBP expression might be induced not only in neuronal cells but also in associated glial cells and macrophages surrounding the damaged axons which are normally recruited from the circulation and present in the degenerating sciatic nerve by 72 hrs after nerve injury. Therefore, future studies should include in situ hybridization experiments to analyze the C/EBP expression in non neuronal cells.

Chapter 4: C/EBP axonal outgrowth in cultured neurons

4.1 C/EBP transcriptional activity during neurite outgrowth *in vitro*

In order to determine whether C/EBPs are transcriptionally active during neurite sprouting the ND7/23 cell line was used for this experiment due to its ability to differentiate into a neuronal phenotype and induce neurite outgrowth upon stimulation with cAMP (Fig. 18A-C). Undifferentiated ND7/23 cells (Fig. 18A) were treated with dbcAMP, a permeable analogue of cAMP. Cells untreated with dbcAMP did not differentiate and did not give rise to neurite outgrowth (Fig. 18A). Initial neurite sprouting was first noticed by day 1 following dbcAMP treatment (Fig. 18B) and by three days after dbcAMP addition, fully differentiated ND7/23 cells with extended neurite outgrowth were observed under the microscope (Fig. 18C). Moreover, to assess C/EBP transcriptional activity, a commercial C/EBP luciferase reporter construct was used (Fig. 18D). Expression of the *Photinus pyralis* (firefly) luciferase gene in the reporter plasmid is controlled by a synthetic promoter that contains direct repeats of the transcription recognition sequences for C/EBP. C/EBP activation comprises the dimerization and binding of C/EBP to DNA, in this case, binding to the construct's recognition site in the promoter region. C/EBP binding to the promoter stimulates the luciferase reporter expression which can be measured and used as a direct approach to monitor the C/EBP transcriptional activity.

C/EBP activity was assessed in ND7/23 cultures 1 and 3 days following dbcAMP addition. C/EBP activation levels were compared to cells untreated with dbcAMP which showed no neurite outgrowth. Initial neurite sprouting in ND7/23 cells, 1 day after dbcAMP addition (Fig. 18B), gave rise to a dramatic 157-fold increase in C/EBP protein

activation (Fig. 18E). A 24-fold increase of C/EBP protein activity was observed in fully differentiated ND7/23 cells, 3 days after dbcAMP treatment (Fig. 18E). As a control cells transfected with the C/EBP luciferase reporter construct but untreated with dbcAMP were used. Control cells showed similar luciferase expression levels as dbcAMP untreated cells (Fig. 18E). Altogether these results indicate that neurite outgrowth as a result of dbcAMP treatment increases the C/EBP protein activity.

4.2 C/EBP mRNA expression during neurite outgrow

In order to determine which members of the C/EBPs are upregulated during neurite outgrowth, their relative mRNA expression was measured. mRNA expression levels for C/EBP different members were measured on ND7/23 cells treated with cAMP for 1 day, during differentiation, and 3 days which were fully differentiated cells. C/EBP mRNA expression levels after cAMP were compared to a control group consisting of undifferentiated cells, untreated with cAMP, which did not show neurite outgrowth. As a control for development of neurites, GAP-43 was measured in fully differentiated ND7/23 neuronal cells and also in undifferentiated cells (Fig. 18F). Relative expression studies were performed by TaqMan RT-PCR as described before, during and post differentiation. Cells during differentiation treated with cAMP for 1 day showed mRNA upregulation compared to cAMP untreated/undifferentiated cells. This upregulation was significant for both C/EBPβ ($p=0.02$) and C/EBPδ ($P=<0.0001$). Furthermore, in differentiated cells with long neurites on day 3 following cAMP treatment compared to cAMP untreated/undifferentiated cells, RT-PCR analysis indicates that the mRNA expression was significantly upregulated

for C/EBPβ and C/EBPδ as well as for GAP-43 (Fig 18F). C/EBPα, C/EBPγ and C/EBPε mRNA were not detected in cells during and after differentiation or in the cAMP untreated group. Furthermore, C/EBPβ mRNA level was increased almost 5 fold 1 day after cAMP treatment when the cells were still differentiating and 15 fold after neurite outgrowth whereas C/EBPδ mRNA concentration was increased approximately 35 fold during differentiation and 26 fold after neurite outgrowth. These results indicate that C/EBPβ and C/EBPδ expression is highly upregulated during neurite elongation in ND7/23 cells and it appears to be responsive to cAMP in this particular neuronal system.

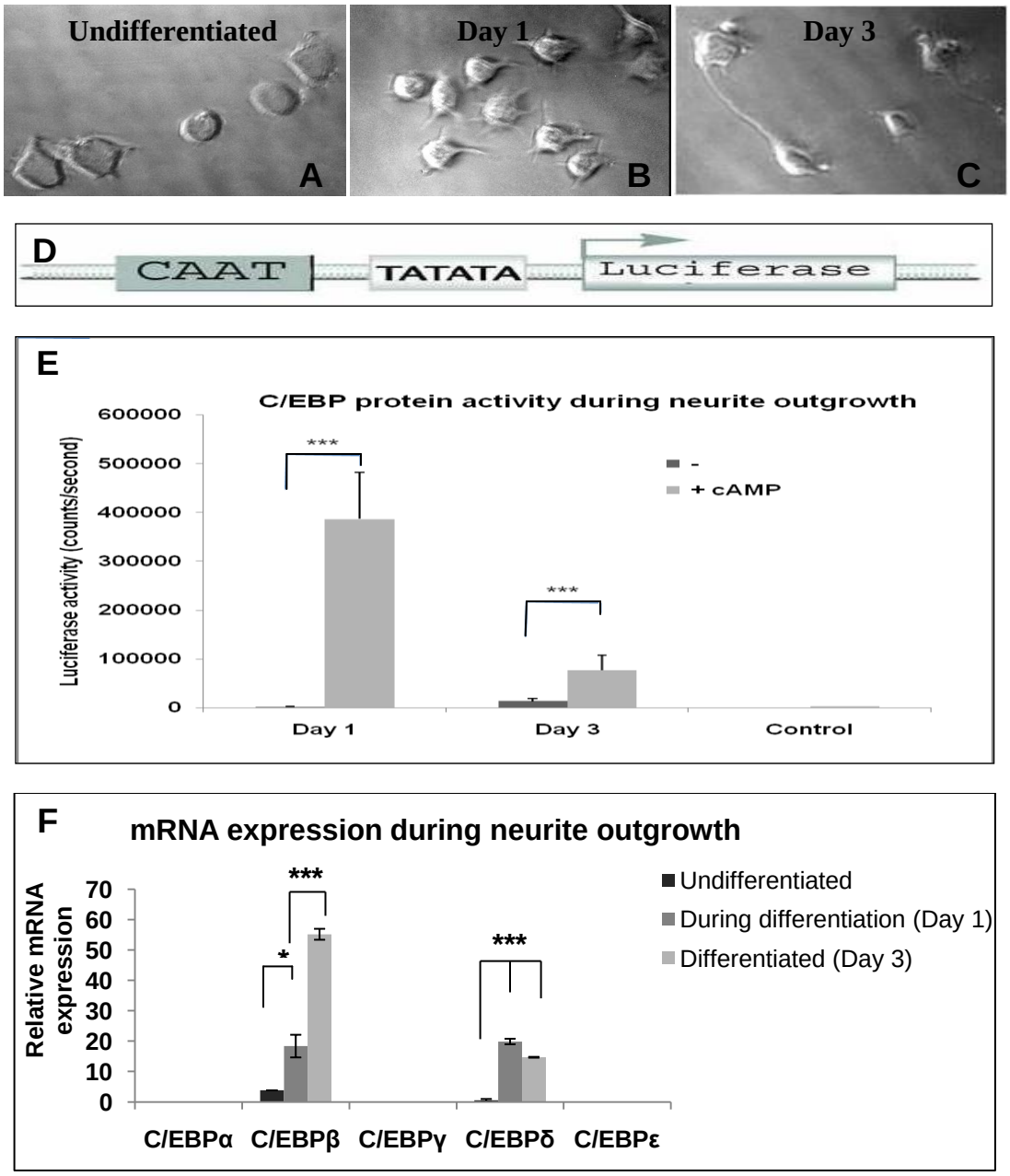


Fig. 18 Neurite outgrowth on ND7/23 cells induced by cAMP exhibits increased C/EBP expression: (A) Normal undifferentiated ND7/23 cells where treated with 1 mM dibutyryl cAMP in order to induce neurite outgrowth. (B) Differentiation of ND7/23 cells by means of neurite elongation was initially observed 1 day following cAMP. (C) Further neurite elongation was seen three days after treatment on fully differentiated cells. (D) Characterisation of the reporter construct transfected into ND7/23 cells and used to monitor the activation of C/EBPs. The components of the construct include a recognition site (C/EBP), a basic promoter element (TATA box) and a reporter gene (luciferase). (E) C/EBP-luciferase activity (counts/second) 1 and 3 days following cAMP addition to ND7/23 cultures compared to cAMP untreated cells. As a control untransfected cells treated with cAMP were used. (F) Relative C/EBPβ and C/EBPδ mRNA expression in ND7/23 cells untreated with cAMP (undifferentiated), treated with cAMP for 1 day (during differentiation) and treated with cAMP for 3 days (differentiated). Expression levels are showed as means of the ratio of C/EBP mRNA normalized to 18S mRNA. As a control for neurite outgrowth GAP-43 was used (Mean + S.E.M., n= 3, *=P<0.05 and ***=P<0.001 by student's t-test).

4.3 Conditional downregulation of C/EBPs in ND7/23 cells

To assess the involvement of C/EBPs on neurite outgrowth in differentiated cells; two conditionally regulated constructs were engineered by using the TetON system to inhibit the expression of the C/EBPs once the ND7/23 cells have acquired a neuronal phenotype.

The constructs were transfected into ND7/23 cells and after induced differentiation with cAMP; the constructs were conditionally activated by addition of doxycycline, a tetracycline derivative. Due to the bidirectional promoter of the constructs, capable of also expressing green fluorescent protein (GFP), transfected cells were readily detected (Fig. 19A). Construct expression was assessed in culture before (Fig. 19B) and after addition of doxycycline (Fig. 19C). Transfected ND7/23 cells without doxycycline did not show GFP expression (Fig. 19B) whereas transfected cell treated with doxycycline displayed GFP expression indicating construct activation (Fig. 19C).

A total dominant inhibitor construct, was designed to target all the C/EBP members (Fig. 20B) whereas a C/EBPδ antisense construct downregulates only the expression of C/EBPδ (Fig. 20C).

Three days after transfection, the ND7/23 cells were fixed and stained with the neuronal marker PGP9.5 to indicate neuronal differentiation (Fig. 20A-C). The longest neurite of green GFP positive cells for each group (Fig. 20A-C) were measured and compared with a control group consisting of cells transfected with a construct expressing only GFP (Fig. 20A).

Total inhibition of all the C/EBPs by using a dominant negative construct showed a statistically significant decrease in the average neurite length of neuronal ND7/23 cells by

41%, in comparison with GFP control group (Fig. 21A). Cells transfected with C/EBPδ antisense showed a similar effect, reducing the average neurite length by 33% in comparison with GFP control group (Fig. 21A). In cells transfected with GFP alone, the percentage for the longest neurites <30 μm, 30-100 μm, or >100 μm was 60%, 36% or 4% respectively (Fig. 21B). C/EBP dominant inhibitor resulted in increased percentage of cells with neurites <30 μm (87%) and decreased percentage of cells with neurites 30-100 μm and >100 μm (11% and 2% respectively). Similarly, after transfection with C/EBPδ antisense, the percentage of cells with neurites <30 μm increased to 75% and the cells with neurites 30-100 μm, or >100 decreased to 23% and 2% respectively. These results indicate that total inhibition of all the C/EBPs and downregulation of C/EBPδ in ND7/23 cells decreases overall neurite growth and specifically increases the percentage of cells with small neurites <30 μm and decreases the percentage of cells with medium and long neurites 30-100 μm and >100 μm.

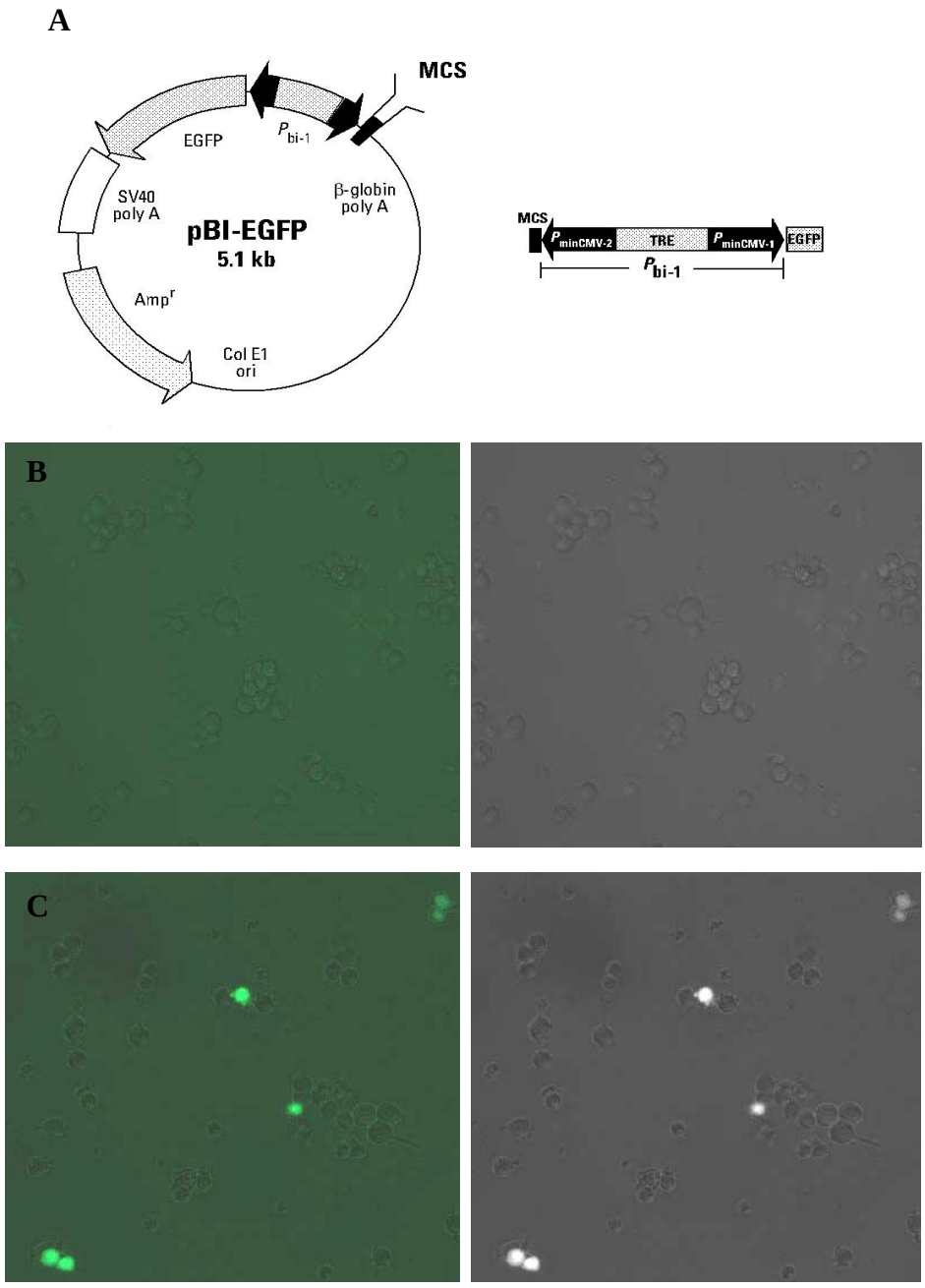


Fig. 19. The bidirectional conditional TetON system: Tetracycline conditional backbone plasmid (A) used to generate C/EBP conditional inhibitory constructs (Fig. 17). The pBI-EGFP bidirectional Tet expression vector allows the simultaneous expression of gene of interest (in our case C/EBP inhibitory constructs) and enhanced green fluorescent protein (EGFP) under the control of a bidirectional tetracycline-responsive promoter (P_{bi-1}) which is responsive to rtTA regulatory proteins. P_{bi-1} contains the tetracycline responsive element (TRE), which consists of seven copies of the 42bp tetracycline operator sequence (tetO). The TRE element is in between two identical minimal CMV promoters (P_{minCMV}), which lacks the enhancer that is part of the complete CMV promoter. Consequently, P_{bi-1} is silent in the absence of binding of rtTA to the tetO sequences. $P_{minCMV-1}$ controls the expression of EGFP and $P_{minCMV-2}$ controls the expression of the gene of interest. The vector backbone of the vector contains an ampicillin resistance gene and a Col E1 origin of replication to allow selection and propagation in *E. coli*. (B) Plasmid transfected into ND7/23 cells (C) was conditionally activated with doxycycline.

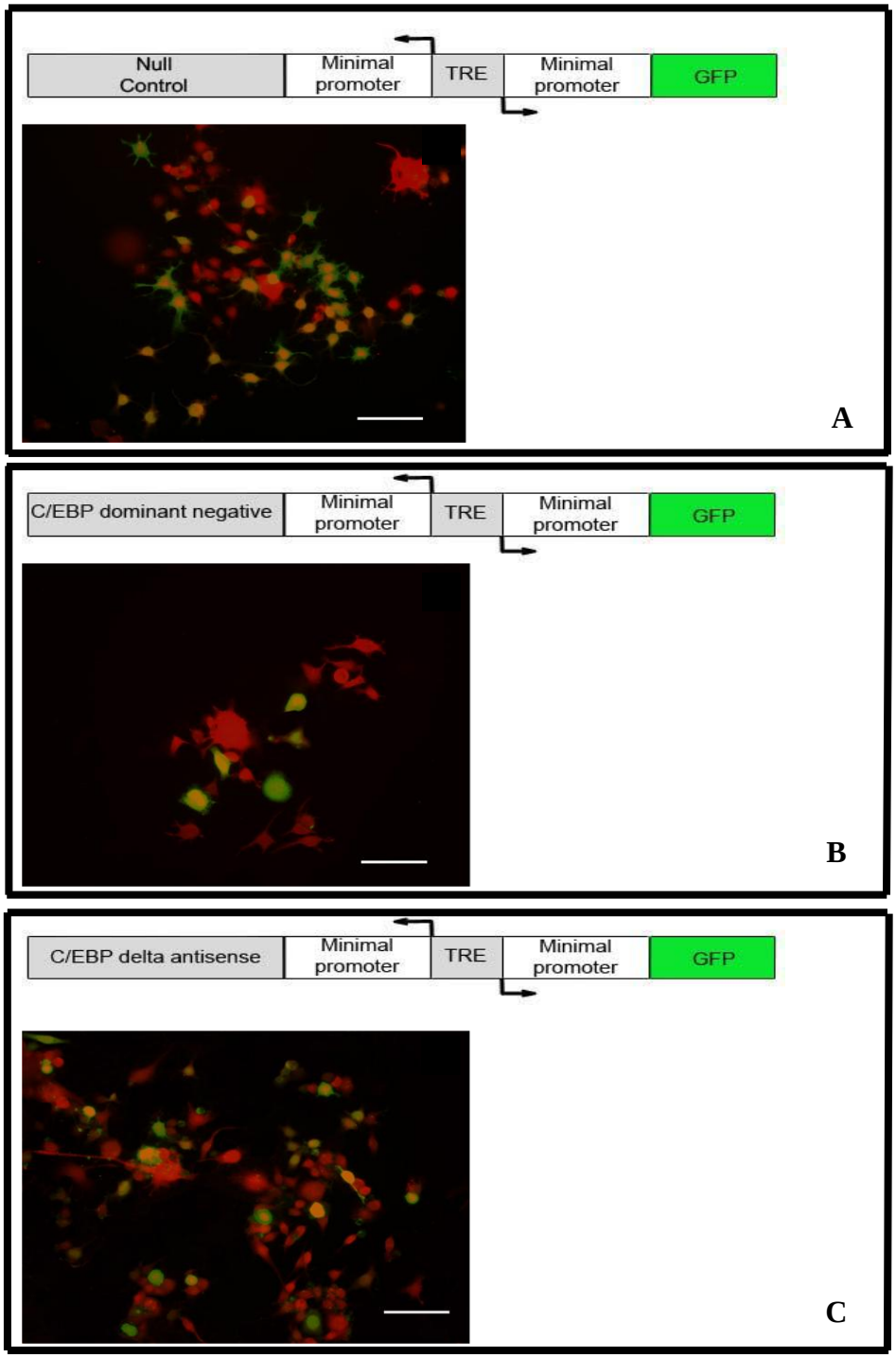


Fig. 20 Neurite elongation on ND7/23 cells transfected with conditionally-regulated constructs: ND7/23 cells treated with cAMP and induced into a neuronal phenotype were thereafter transfected with three different constructs and stained with the neuronal marker PGP9.5 (red) and GFP (green). Tetracycline conditionally regulated C/EBP inhibitory constructs transfected into ND7/23 cells were designed by using the pBI-EGFP vector backbone (Fig. 18A). Control construct expressing only GFP (A), construct expressing a C/EBP dominant inhibitor and GFP (B) construct expressing C/EBPδ antisense and GFP(C). Compared to GFP control (A), total inhibition of C/EBP expression (B) and specific inhibition of C/EBPδ (C) decrease neurite elongation. Note the neurite length of the green fluorescent cells for each group. Scale bar, 100 µm.

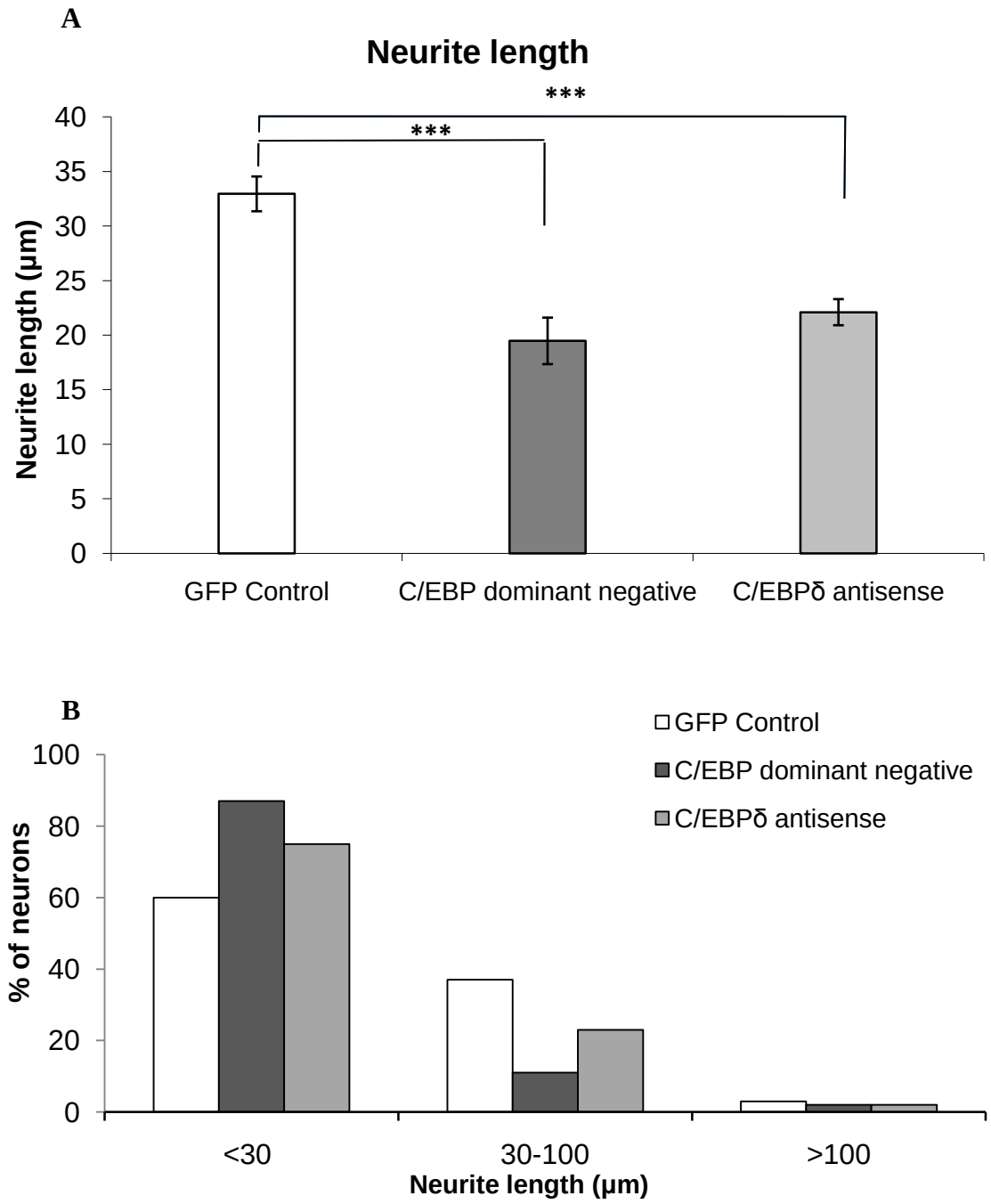


Fig. 21 Suppression of C/EBPs and down-regulation of C/EBPδ impairs neurite outgrowth in differentiated neuronal ND7/23 cells: (A-B) The length of neurites of only green GFP positive cells was measured. (A) Inhibition of all the C/EBPs reduced the neurite length of differentiated ND7/23 cells previously treated with cAMP by 41% and down-regulation of C/EBPδ reduced the neurite length outgrowth by 33% compared to cells transfected with GFP control. (n= 3 cultures, mean ± S.E.M; $^{***}p < 0.001$ Student's *t* test). (B) Frequency histogram shows the percentage of cells with neurites in each of the three length ranges.

4.4 TSA increases C/EBPδ activity in ND7/23 cells

To further examine whether epigenetic modifications can influence the transcriptonal activity of C/EBPδ, ND7/23 cells were treated with cAMP followed by stimulation for 24 hrs with the histone deacetylase inhibitor, Trichostatin A (TSA) and compared with TSA untreated cells. Strinkingly, only 25% of the cells were immunostained for C/EBPδ after 24 hrs in culture (Fig. 22A) whereas approximately 50% of the cells treated with TSA showed C/EBPδ staining (Fig. 22B). This significant increase in the C/EBPδ expression after stimulation with TSA (Fig. 22C) did not show any increase in neurite length after 24 hrs compared with untreated cells (Fig. 22D). However, a statistically significant higher percentage of cells treated with TSA displayed neurite processes compared to TSA unstimulated cells (Fig. 22E).

These results indicate that histone deacetylase inhibition by TSA augments C/EBPδ expression *in vitro*.

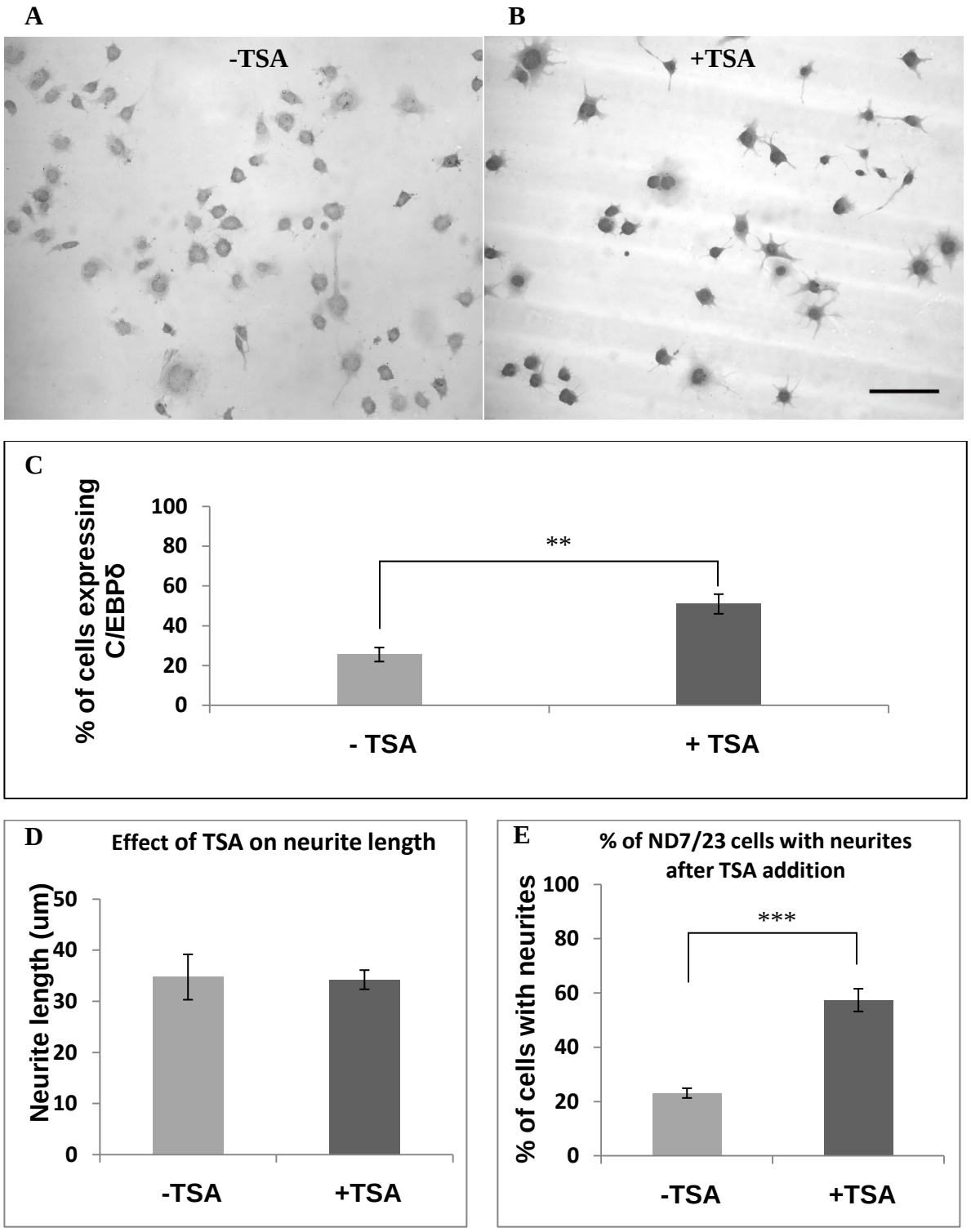


Fig. 22 ND7/23 cells stimulated with TSA showed an increase C/EBPδ activity: ND7/23 cells immunostained for C/EBPδ after cAMP treatment (A) followed by stimulation with TSA for 24 hrs (B). After TSA treatment, a higher percentage of cells express C/EBPδ in comparison with TSA unstimulated cells (C). TSA treatment does not increase the neurite length in cultured cells (D). Nevertheless, 24 hrs after TSA treatment, a higher percentage of ND7/23 cells show neurite processes (E). (Mean ± S.E.M., n= 3 cultures, **=p<0.005 ***=P<0.001 by student's t-test). Scale bar = 100 um.

4.5 Lack of C/EBP delta affects the intrinsic growth capacity of DRG neurons

To assess the influence of C/EBPδ on the intrinsic growth capacity of sensory neurons, DRG neurons from C/EBPδ null mice were cultured for 2 days. Neurons then were fixed, DAB immuno-stained for β(III) tubulin and the length of the longest neurite was recorded and compare to wildtype DRG neurons (Fig. 23A-B). DRG neurons from wildtype exhibited an average neurite length of 540 μm (Fig. 23A) whereas DRG neurons from C/EBPδ deficient animals failed to extend long neurites with an average of 167 μm (Fig 23B). In DRG neurons from wildtype animals, the percentage of neurons with longest neurites <30 μm, 30-200 μm and >200 μm was 2%, 9% and 89% respectively (Fig. 23D). In DRG neurons from C/EBPδ null animals, the distribution of neurite lengths for <30 μm, 30-200 μm and >200 μm was 41%, 29% and 29% respectively (Fig. 23D). These results show that C/EBPδ is required for initial DRG neurite outgrowth indicating that lack of C/EBPδ affects the intrinsic growth capacity of sensory neurons *in vitro*.

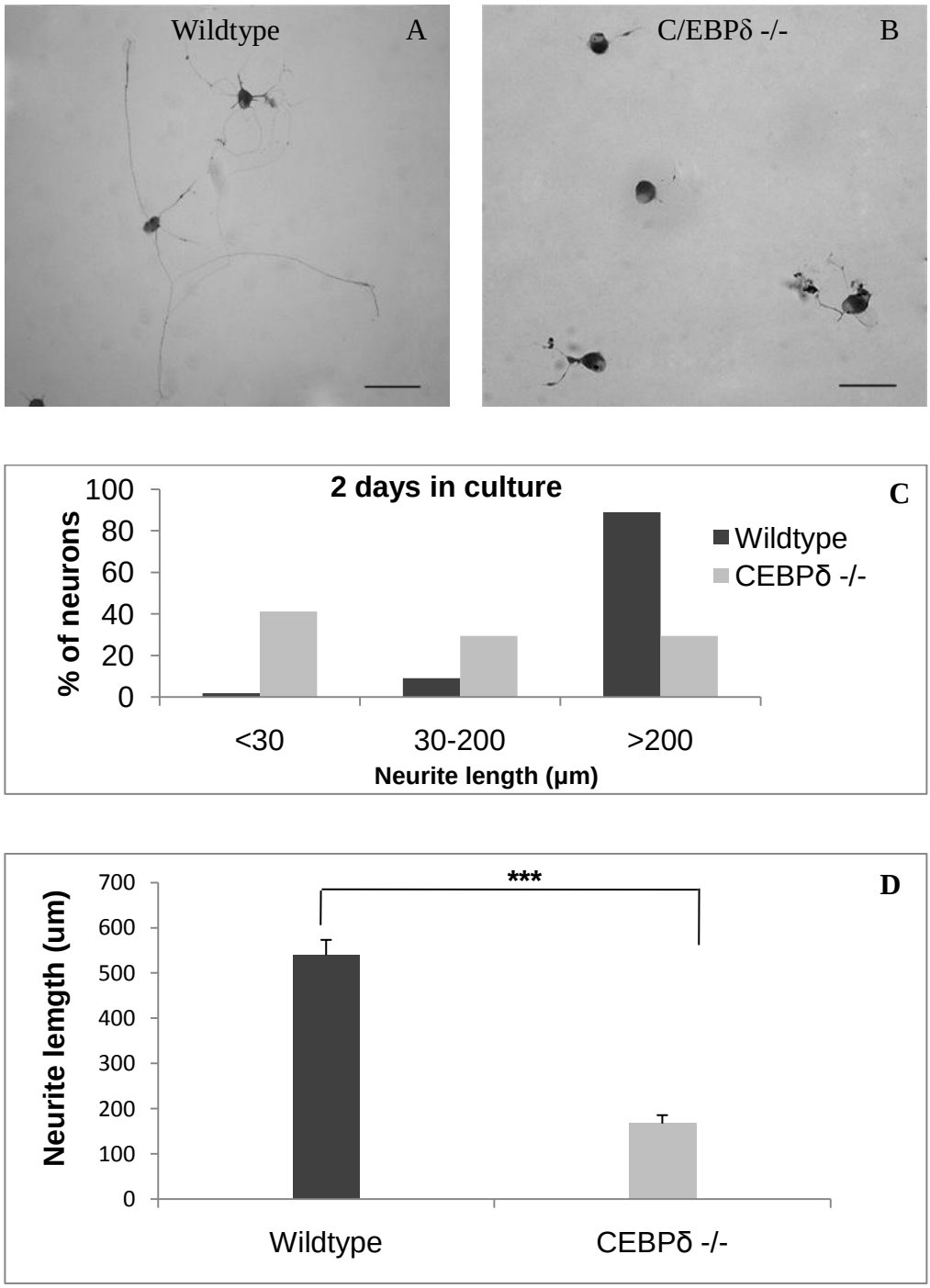


Fig. 23 The lack of C/EBPδ inhibits neurite outgrowth in DRG cultures: (A) DRG neurons from wildtype showing DAB staining for β(III) tubulin (B) Neurite elongation in DRG neurons from C/EBP delta -/- mice is significantly reduced compared with wildtype growth after 2 days in culture. Scale bar 100 µm. (C) Frequency histogram for wildtype and C/EBP delta -/- DRG neurons cultured for 2 days. n=3. (D) Mean± S.E.M. neurite lengths for wildtype (540 ± 34 µm) and C/EBP delta -/- (167 ± 19 µm) ***=P<0.001 by student's t-test.

4.6 Discussion

cAMP induced neurite outgrowth and C/EBP activity

Several signaling pathway cascades and intrinsic factors of the neuron have been implicated to have a role in neuronal outgrowth and regeneration. A line of investigation demonstrated that among these factors, the second messenger cAMP is a prominent and important one. Previously, it has been shown that cAMP is involved in the modulation of growth cone response to a variety of different diffusible and non-diffusible factors (Song et al., 1997), influencing guidance of growth cones towards chemoattractive signals (Ming et al., 1997), enhancing neurite outgrowth (Kao et al., 2002), overcoming the MAG inhibition effect on adult nerve cell extension of neurites (Cai et al.,2001) and promoting neuronal survival (Hanson, Jr. et al., 1998; Rydel and Greene, 1988). Importantly, some recent reports have indicated that elevation of cAMP promotes axonal regeneration in the central nervous system (Qiu et al., 2002; Pearse et al., 2004).

To examine a possible involvement of the C/EBPs in neurite extension induced by cAMP, we used in this study, the sensory neuronal cell line, ND7/23, a neuroblastoma fused with a DRG cell line, which retains the same characteristics of normal DRG but not the properties from parental neuroblastoma and with the unique feature of not being post mitotic like regular DRG neurons. This specific hallmark makes the ND7/23 cell line ideal for *in vitro* experiments given that they can be kept in culture indefinitely. Due to the ability to divide and proliferate, they can be readily transfected which is another important feature that allows the study of the effect of gene manipulation.

Here I examined the C/EBP activity during neurite outgrowth in the ND7/23 *in vitro* model by using a generic C/EBP luciferase reporter construct. C/EBP luciferase activity was dramatically increased during the initial step of neuronal differentiation and neurite outgrowth 1 day after cAMP treatment. The C/EBP activity was also increased 3 days after cAMP treatment, in fully differentiated, ND7/23 cells, expressing long neurites. However, the increment in C/EBP activity observed 3 days after cAMP treatment was substantially less than the activity observed after 1 day, indicating that member of the C/EBP family are more active during the differentiation process distinguished by initial neurite sprouting than post differentiation, which could suggest that some C/EBPs are associated with certain growth cone elements.

Previous studies have shown that C/EBPβ is active in neural precursor cells (NPC) following differentiation into neurons, but not following differentiation into astrocytes (Cheeran et al., 2005). C/EBPs have been shown to couple growth factor induced signal transduction to cellular differentiation in numerous tissues (Lekstrom-Himes and Xanthopoulos, 1998). Activation of an MEK-C/EBP pathway in cortical precursors *in vivo* biases them to become neurons and against becoming astrocytes, thereby acting as a growth factor-regulated switch (Menard et al., 2002; Paquin et al., 2005). Additionally it has been shown that basic helix-loop helix (bHLH) transcription factors are important intrinsic regulators of neural fate determination and differentiation. Retroviral transduction of various neural bHLHs is sufficient to determine neuronal or glial fate in the developing mouse cortex (Cai et al., 2000). Furthermore, *In vitro* studies have shown that C/EBP luciferase is activated in Caco-2 cells after treatment with cAMP (Hershko et al., 2002) and

Neuro 2a cells during neurite outgrowth activate GADD153, which is a C/EBP homologous protein (Dikshit et al., 2006).

In view of the fact that the C/EBP luciferase construct detects the activity of all the C/EBP members, further studies are required to determine which C/EBPs isoforms are active during neurite elongation. Perhaps by using luciferase constructs specifically designed for each C/EBP isoform.

Following the detection of increased C/EBP luciferase activity during neurite outgrowth, I measured the C/EBPs mRNA expression in order to determine which members of the C/EBP family are upregulated during neurite elongation. Here we report that the cAMP-induced neurite outgrowth in ND7/23 cells gave rise to an increase in the mRNA expression levels of two of the most prominent members of this family of transcription factors, namely C/EBPβ and C/EBPδ. The upregulation of mRNA expression levels observed here were 5 to almost 35-fold during and after neuronal differentiation and neurite sprouting, 1 day and 3 days following cAMP treatment. This upregulation was almost as high as for the positive control GAP-43 which is an important growth associated proteins involved in axonal regeneration (Bomze et al., 2001; Frey et al., 2000). However no associative studies between GAP-43 and C/EBP role in neurite outgrowth were performed in this work. In previous studies on rat PC12 pheochromocytoma cell line, 4 hours after treatment with the cAMP analogue, 8Br-cAMP, a 6-fold upregulation of C/EBPβ mRNA was seen, while levels of C/EBPδ mRNA were not significantly changed. The different responses of C/EBPβ and δ to cAMP in PC12 cells is surprising given that the promoters of both genes contain cAMP Regulatory Elements (CRE) and are inducible by cAMP in other cell types, including hippocampal neurons (Yukawa et al., 1998). However,

even though PC12 cells have been used for many years to model neuronal functions, these cells are certainly not neurons, unlike the sensory neural cell line ND7/23.

Conditional downregulation of C/EBPs in ND7/23 cell

My previous results indicated that C/EBP-Luciferase and C/EBP mRNA expression is upregulated during neurite elongation. To further address whether C/EBPs are important for neurite outgrowth in neuronal cells, conditional TetON inhibitory constructs were designed. The ND7/23 cells require cAMP in order to acquire a neuronal phenotype (Wood et al., 1990), therefore the use of a conditional approach was necessary in order to activate the construct in post-differentiated cells and not immediately after transfection when the cells are undifferentiated.

A conditional dominant negative C/EBP provides an effective tool for investigating the effect of repression of all the C/EBP members by overcoming the problem of functional redundancy among related gene products. In this case we used A-C/EBP as a dominant negative, which possesses a leucine zipper but lacks functional DNA-binding and transactivation domains and heterodimerizes specifically with C/EBPs. A-C/EBP specificity has been successfully tested and implemented previously by other groups (Greenwel et al., 2000; Olive et al., 1996; Zhang et al., 2004). My results shows that the general effect of a total C/EBP inhibition is unequivocal; it reduces the average neurite length by 41% and decreases the percentage of cells with medium (30-100 μm) and long size neurites (>100 μm), from 36% to 11% and from 4% to 2% respectively when compared to GFP controls. Additionally, since my previous results indicated that C/EBPδ is

upregulated after peripheral injury and during neurite elongation, a C/EBPδ antisense conditional construct was also designed to inhibit C/EBPδ expression specifically during neurite outgrowth. Average neurite elongation is also affected by C/EBPδ downregulation which is reduced by 33% and the percentage of cells with medium (30-100 μm) and long size neurites (>100 μm) decreased from 36% to 23% and from 4% to 2% respectively.

Although, the average neurite length was reduced in cells transfected with the conditional TetON inhibitory constructs, I failed to demonstrate whether the conditional constructs downregulated the expression of the different C/EBP members. Furthermore, the transfection efficiency could have been higher. Therefore, to demonstrate if the C/EBP expression has been successfully targeted, an improved transfection protocol should be developed by combining the co-transfection of the dominant negative conditional construct or the C/EBPδ antisense conditional construct with the C/EBP luciferase plasmid.

Additionally, the transgenic inhibition of C/EBPs suggests that C/EBPs are important factors during neurite outgrowth; however, since C/EBPs can form protein–protein interactions with other bZIP and non-bZIP factors, further studies are required to identify C/EBPs downstream target genes involved in neurite outgrowth. Additionally, overexpression studies should be conducted to elucidate whether the upregulation of C/EBPs gives rise to increased neurite elongation. It has been shown that C/EBPγ is an inhibitor of C/EBP transcriptional activation since it lacks an activation domain and, therefore, represses gene transcription by forming inactive heterodimers with other C/EBP members (Ramji and Foka, 2002). Therefore, combinatorial experiments by inhibition of C/EBPγ and overexpression C/EBPδ could further increase neurite outgrowth.

TSA increases C/EBPδ activity in ND7/23 cells

Here I showed that TSA upregulates the expression of C/EBPδ in vitro. However, this increase in the C/EBP activity does not have an evident phenotypic effect on the neurite length of the ND7/23 cells 24 hours after treatment. The average neurite length for the TSA treated and non-treated group was approximately 34 μm, which is similar to the neurite length observed previously in ND7/23 cells transfected with the conditional GFP control construct and cultured with cAMP for 3 days (Fig. 20A). For quantification analysis, the length of the longest neurite for each neuron was measured and this was done only in cells with neurites longer than one cell diameter. In comparison, the TSA treated group showed a higher number of cells with neurites compared to the TSA untreated group. Since both groups were treated with cAMP for 24 hrs, this difference can be only explained by the stimulation with TSA. It would be interesting to study the effect, especially on the neurite length, of TSA in culture cells 3 days after cAMP treatment since previous observation in the lab has shown that by that day most of the ND7/23 cells express long neurites.

Nuclear histone acetylation has an important role in transcriptional regulation. It is a reversible process regulated by a group of histone acetyltransferases (HATs), which promote acetylation, and histone deacetylases (HDACs), which promote deacetylation. HATs activate transcription by enhancing nucleosomal relaxation. On the other hand, HDACs can stabilize nucleosomal structures and repress transcription. Recent studies have shown that histone acetyltransferases can also acetylate a variety of non-histone proteins, such as transcriptional factors and signalling proteins (Greene and Chen, 2004). TSA induces acetylation of histones by neutralizing positive charges on their tail regions, reducing histones ability to bind to DNA and thus loosening the structure of chromatin,

resulting in a more 'open' chromatin conformation. Therefore, highly acetylated regions are more accessible to transcription factors which are actively transcribed (Toth et al., 2004).

There is emerging evidence indicating that TSA stimulation at various cellular and molecular levels is involved in the regulation of the C/EBPs transcriptional activity. Acetylation of histones by TSA inhibits the degradation of the C/EBP Homologous Protein (CHOP) *in vitro* (Ohoka et al., 2007). Additionally, it has been reported that TSA upregulates C/EBPδ expression in human breast cancer cell line (Tang et al., 2006). Interestingly, DiGiovanni's group previously reported at FENS 2008 (Gaub P, 2008), that the facilitation of HAT activity by using TSA switches the intrinsic neuronal genetic program from a non permissive to a permissive pattern leading to increased neurite outgrowth. Therefore future studies should investigate in detail the processes of histone acetylation/deacetylation on primary sensory neurones, their impact on neuronal repair and the possible participation of the C/EBPs.

Lack of C/EBP delta affects the intrinsic growth capacity of DRG neurons

My data so far indicate that C/EBPs, specially –β and –δ are expressed in response to peripheral nerve injury and neurite outgrowth. However, since previous studies have linked C/EBPβ with a potential role in the neuronal response to injury (Nadeau et al., 2005). I decided from this point to concentrate on the novel analysis of C/EBPδ.

I initially, examined the effects of the lack of C/EBPδ on the intrinsic growth capacity of DRG neurons in culture by using C/EBPδ null animals generated by Sterneck and colleagues (Sterneck et al., 1998). DRG neurons from wildtype animals cultured for 2

days showed an average neurite length of 540 μm whereas DRG neurons from C/EBPδ null mice surprisingly displayed an average neurite length of 167 μm. Additionally, distribution of neurite lengths indicated that in knock-out DRGs the majority of the total number of neurons (41%) has small neurites of less than 30 μm. Simultaneously, only 29% of C/EBPδ null DRGs showed long size neurites of more than 200 μm. On the other hand in wildtype DRGs almost 90% of total number of neurons extended long neurites of more than 200 μm after 2 days in culture.

These results in adult DRG cultures showed almost a 70% reduction of the neurite length from C/EBPδ null mice compared to wildtype. This reduction is much greater than the neurite length reduction oberserved in ND7/23 cells after transfection with a C/EBP dominant inhibitor or a C/EBPδ antisense construct (41% and 33% reduction respectively). A possible explanation could be the differences between the two cell type, one being a hybrid cell line and the other a primary cell line. Another possible explanation could be the fact that the adult DRGs came from knockout animals were the C/EBPδ gene has been totally removed from the whole animal whereas C/EBPδ expression in the ND7/23 cells was downregulated by using constructs which might have not been 100% efficient.

These novel findings clearly indicate that C/EBPδ is an important factor in the intrinsic growth capacity displayed by DRG neurons *in vitro* and may provide an important target for inducing axon regeneration *in vivo*.

It could be suggested that the lack of C/EBPδ affects the cAMP-PKA-CREB signalling pathway which is known to promote neurite outgrowth in DRG sensory neurons and motor neurons (Murray et al., 2009; Aglah et al., 2008; Chen et al., 2007). Activation

of cAMP and CREB is sufficient to overcome inhibition by myelin and to promote spinal axon regeneration *in vivo* (Gao et al., 2004). Therefore if C/EBPδ acts downstream of the cAMP-PKA-CREB, it would broaden the spectrum of possible targets for therapeutic intervention *in vivo*. Future studies should focus on the effect of C/EBPδ overexpression in CNS injuries and to elucidate whether C/EBPδ overcomes inhibition by myelin.

It was previously mentioned that in the nervous system cAMP elevation leads to C/EBPδ upregulation in hippocampal neuron (Yukawa et al., 1998), rat cerebral cortex (Colangelo et al., 1998) and during long term memory consolidation in Aplysia models (Guan et al., 2002) and in mice (Taubenfeld et al., 2001). cAMP is an important intracellular signalling molecule that among other things, leads to the transcriptional activation of a number of genes. cAMP generally exerts its effects in cells via the activation of PKA which catalyses the transfer of the terminal phosphate group of ATP to specific serine and threonine residues within select proteins, such as transcription factors. Within the regulatory region of certain genes exists a short DNA sequence called CRE. This DNA sequence is recognized by the gene regulatory protein CREB. When PKA phosphorylates CREB at a specific serine residue, the CREB protein becomes activated, leading to the transcriptional activation of CREB regulated genes (Alberts et. al., 1994). cAMP mediated regulation of C/EBPδ has been also reported by Cardinaux and Magistretti, (1996) and a CRE site has been identified in the promoter region of C/EBPδ (Cantwell et al., 1998). Therefore C/EBPδ expression may be regulated by cAMP via PKA and CREB proteins and their neurite outgrowth-promoting might be absent in DRG cultures lacking C/EBPδ. However, further studies are required to directly examine the expression of cAMP, PKA and CREB proteins in DRG neurons lacking C/EBPδ.

Chapter 5: Function of C/EBPδ in axonal regeneration

5.1 Evaluation of sciatic nerve axons in C/EBPδ null mice

My *in vitro* experiments have shown that C/EBPδ is upregulated following peripheral nerve injury and that C/EBPδ is required in DRG neurons to extend long neurites. Therefore in order to determine whether C/EBPδ is an important factor for nerve regeneration *in vivo*, we used the sciatic nerve crush model in wildtype and C/EBPδ -/- mice. The sciatic nerve crush model is a well-characterized model of peripheral nerve regeneration. After a crush lesion, nerve fibres in the distal stump degenerate. Myelin and axon debris are removed by the process of Wallerian degeneration. The endoneurial tubes remain intact, enabling fast and qualitatively good anatomical and functional recovery.

First I examined whether the lack of C/EBPδ in C/EBPδ -/- mice had any phenotypic effect on the axons of the sciatic nerve under normal physiological conditions. Thus, semi-thin transverse sections of uninjured sciatic nerve from wildtype (Fig. 24A-B) and C/EBPδ -/- mice (Fig. 24C-D) were compared following staining with Toluidine blue. Initial observations on images taken with a 20X objective (Fig. 24 A and C), showing a transverse section of the entire sciatic nerve, indicated that similar density of axons were present in wildtype as in C/EBPδ -/- mice. Furthermore, axons were analysed quantitatively over a surface of 18,225 μm² area, using a calibrated grid was conducted on the sciatic nerve of wildtype and C/EBPδ -/- mice (Fig. 24 B-D). The sciatic nerve from wildtype animals (Fig. 24B) exhibited a similar axonal density 475 ± 59 vs. 470 ± 55 when compared to knockout animals (Fig. 24E). This finding shows that the lack of C/EBPδ does not affect the morphology of the sciatic nerve.

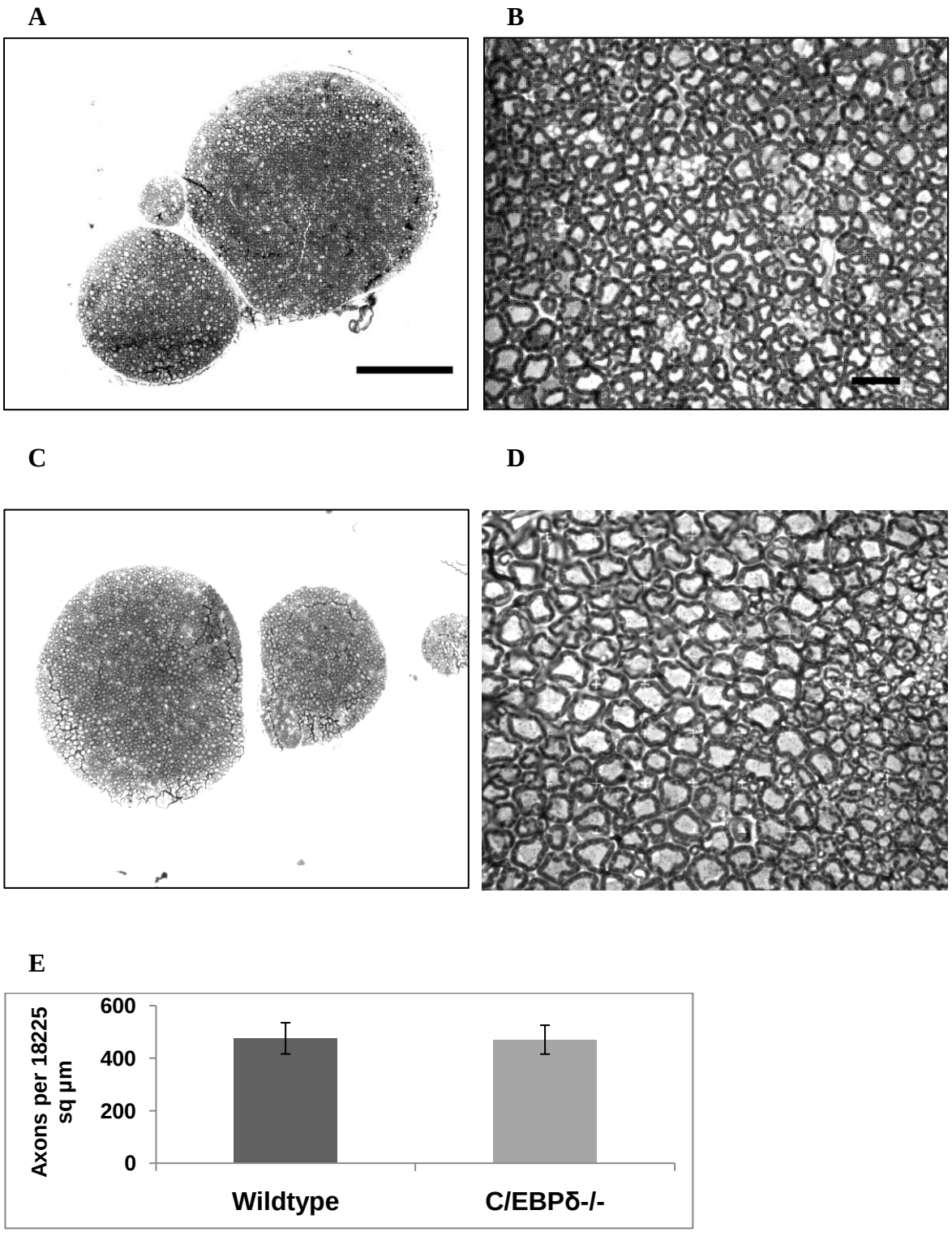


Fig. 24 C/EBPδ -/- animals display equal number of axons than wildtype: Cross-sections of uninjured sciatic nerves stained with Toluidine blue shows the number of axons in (A-B) wildtype compared to (C-D) C/EBPδ -/- . Pictures were taken with 20X magnification A and C and with 100X magnification in B and D. Scale bar in A = 100 µm and in B= 10 µm. (E) Quantification of the data showing equal number of axons in the sciatic nerve of wildtype than in C/EBP δ -/- mice. n=3 animals.

5.2 Muscle strength assessment of C/EBPδ -/- mice

In order to elucidate whether the lack of C/EBPδ affects the neuromuscular function, a grip strength test was performed on uninjured C/EBPδ -/- and wildtype animals. Mice are allowed to grasp a mesh pull bar with the fore and hind limbs and are then pulled backward in the horizontal plane. The force applied to the bar, at the moment the grasp is released, is recorded as the peak tension (force g). The test is repeated 5 consecutive times within the same session and the highest value from the 5 trials is recorded as the grip strength for that animal. Mice were not trained prior to testing and each mouse is tested once (5 trials equal one test session).

The grip strength test shows no difference between wildtype and C/EBPδ null animals (Fig. 25). This indicates that the lack of C/EBPδ has no anatomical and no functional impact on the sciatic nerves.

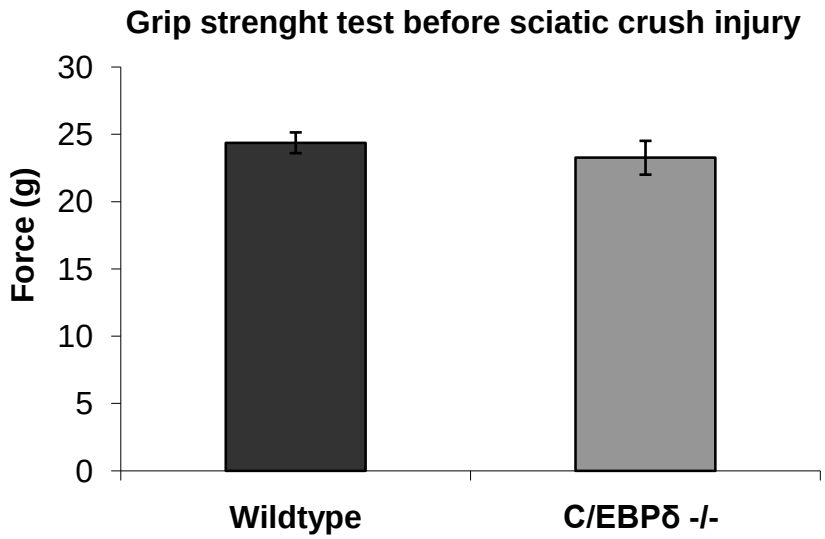


Fig. 25 Grip strength test in wildtype vs. C/EBPδ -/- mice: Forelimb and hindlimb grip strength was assessed by measuring the force (g) applied to a mesh pull bar using a lateralized grip strength meter. No difference between wildtype and C/EBPδ -/- mice was observed in the grip strength test (Mean ± S.E.M., n= 8 animals).

5.3 Sciatic nerve regeneration assessment in C/EBPδ null mice by immunohistochemistry.

Nerve regeneration was assessed 3 days (Fig. 26) and 2 weeks (Fig. 27) following nerve crush by immunostaining with the regeneration associated gene GAP-43 and the neuronal marker PGP9.5. Immunohistochemistry for GAP-43 in wildtype animals identified continuous fibres that extend distally from the crush site for at least 3mm in sciatic nerves 3 days (Fig. 26A) and 2 weeks (Fig. 27A) after injury. In contrast, GAP-43 staining was hardly detected in the sciatic nerve of C/EBPδ -/- mice after 3 days (Fig. 26B) and 2 weeks (Fig. 27B). A similar pattern was observed after staining with PGP9.5 3 days following injury. Sciatic nerve from wildtype animals showed strong PGP9.5 immunoreactivity (Fig. 26C) whereas density of PGP9.5 immunoreactivity was reduced in C/EBPδ -/- mice (Fig. 26D). However, 2 weeks after injury, image analysis indicated that there was no difference in the PGP9.5 staining between wildtype and C/EBPδ -/- null mice. Quantitative analysis of sections 1, 2 and 3mm distal from the injury site of the sciatic nerve, confirmed that GAP-43 was significantly reduced 3 days and 2 weeks after injury in C/EBPδ knock-outs (Fig. 26E and 27E) whereas PGP9.5 was significantly reduced in C/ EBPδ knock-outs after 3 days (Fig. 26F) but not 2 weeks following injury (Fig. 27F).

These data suggest that C/EBPδ is important for peripheral nerve regeneration and the lack of C/EBPδ reduces the expression of GAP-43 and delays the expression of PGP9.5 in nerve regenerating axons.

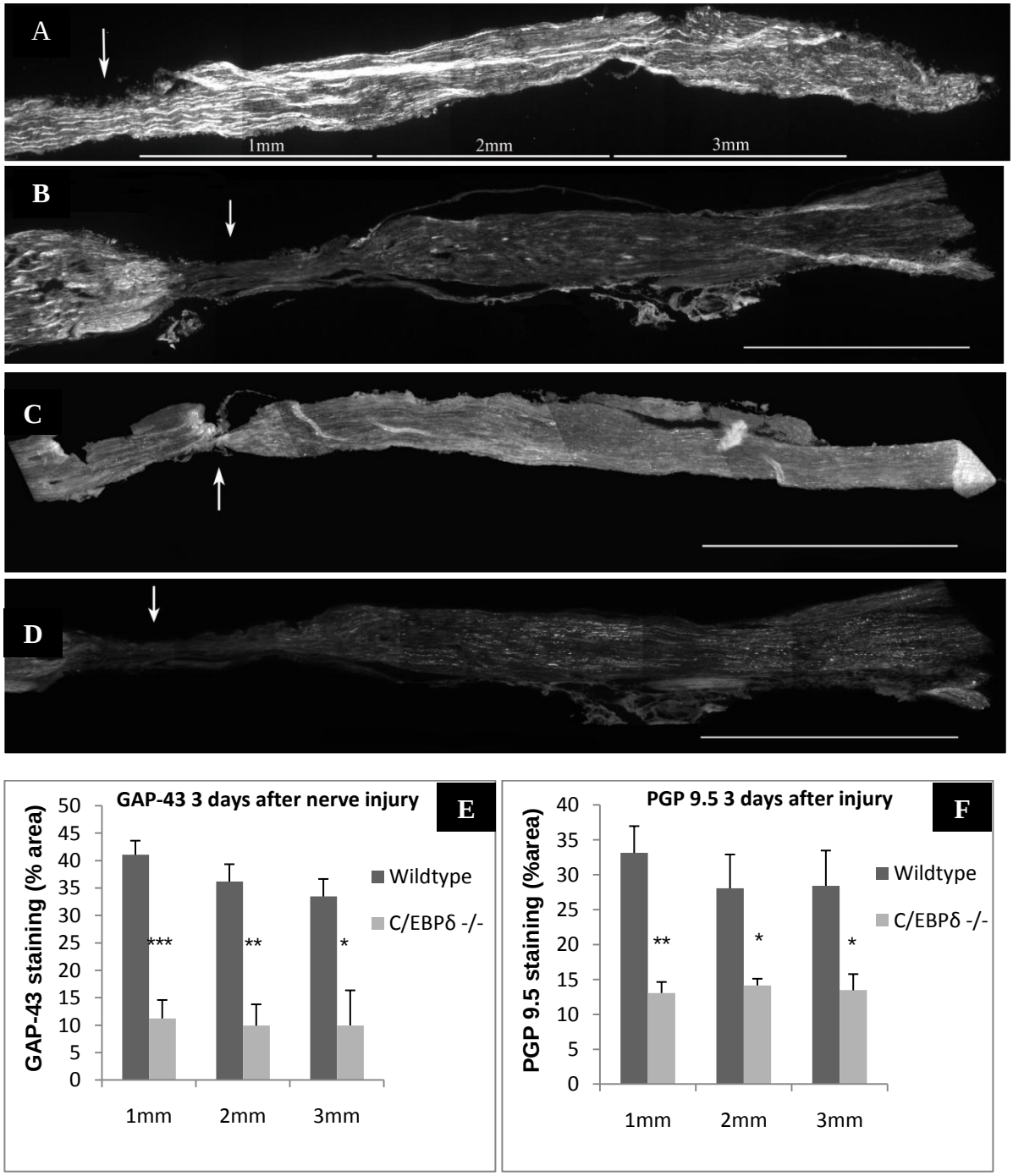


Fig. 26 Regeneration of the sciatic nerve 3 days after injury is impaired in C/EBPδ null mice: Representative microphotographs showing the extent of nerve regrowth detected by the staining intensity of neuronal regeneration markers 3 days after nerve crush (A-D). (A) GAP-43 immunoreactivity in wildtype across the length of the sciatic nerve section (3mm) distal to the crush site (arrow) is much higher than in (B) C/EBPδ -/- mice (C) PGP9.5 immunoreactivity in wildtype is more abundant compared to (D) PGP9.5 expression in C/EBPδ -/- mice. Scale bar = 1mm. (E-F). Quantitative analysis with Leica Qwin software in sections 1, 2 and 3 mm of the sciatic nerve distal from the crush site, expressed as percentage of area stained with (E) GAP-43 and (F) PGP9.5 (Mean + S.E.M., n=4 animals, *=P<0.05, **=P<0.01 ***=P<0.001 by student's t-test).

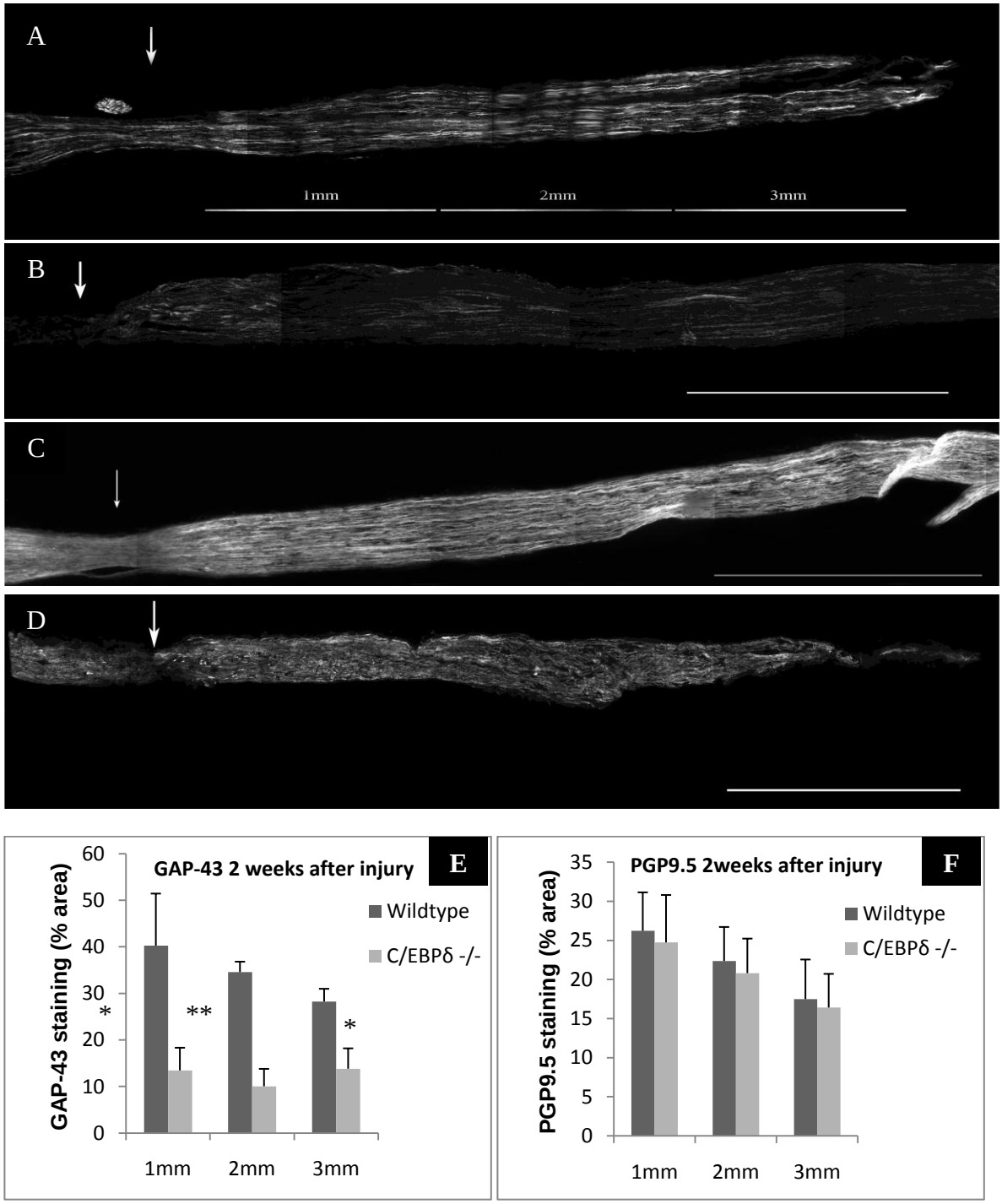


Fig. 27 Regeneration of sciatic nerve 2 weeks after injury: Representative microphotographs showing the extent of regrowth detected by the staining intensity of neuronal regeneration markers 2 weeks after nerve crush (A-D). (A) GAP-43 immunoreactivity in wildtype across the length of the sciatic nerve section (3mm) distal to the crush site (arrow) is much higher than in (B) C/EBPδ -/- mice (C). PGP9.5 immunoreactivity in wildtype is equally abundant compared to (D) PGP9.5 expression in C/EBPδ -/- mice. Scale bar = 1mm. (E-F). Quantitative analysis with Leica Qwin software in sections 1, 2 and 3 mm of the sciatic nerve distal from the crush site, expressed as percentage of area stained with (E) GAP-43 and (F) PGP9.5 (Mean + S.E.M., n=4 animals, *=P<0.05, **=P<0.01 by student's t-test).

5.4 Morphometric analysis of C/EBPδ null mice following sciatic nerve crush

To further characterise the effect of the lack of C/EBPδ *in vivo* during nerve regeneration, morphometric analysis of sciatic nerve cross-sections 6mm distal to crush site (Fig. 28A) was performed to compare the number of regenerating axons in wildtype vs. C/EBPδ null mice. Four sections and ten microscopic fields in each section were randomly selected for quantitative analysis. The number of thinly myelinated regenerating axon were counted in each microscopic field of 30 x 30 μm^2 and analyzed. Two weeks after sciatic nerve injury, a significantly higher number of myelinated axons were observed in the cross sections of wildtype mice (Fig. 28B). A few myelinated axons were also seen in mutant mice (Fig. 28C). The quantitative data showed fewer myelinated axons in the mutant mice 2 ± 0.05 than in the wild-type mice (5 ± 0.05) ($p < 0.001$) (Fig. 28D).

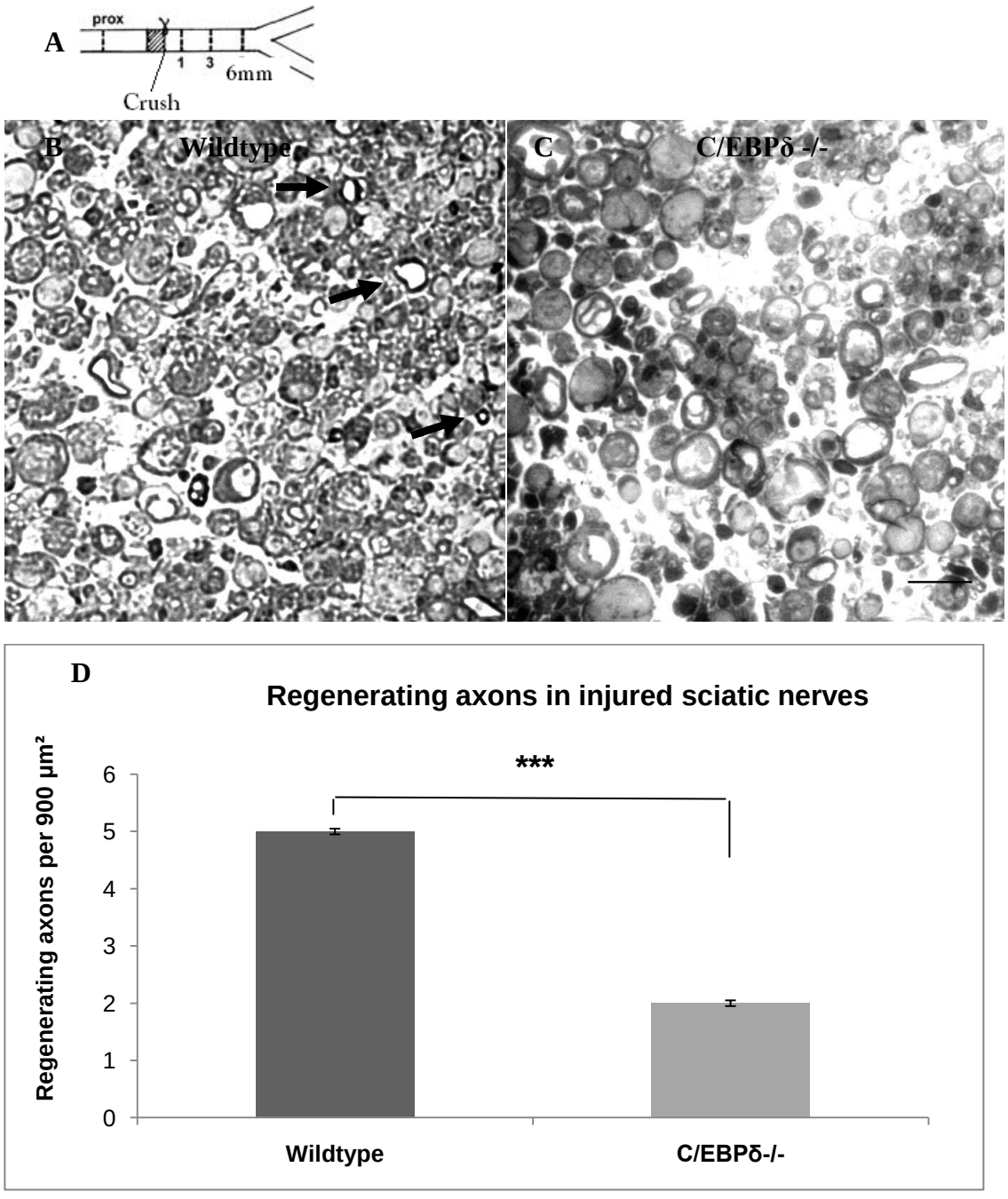


Fig. 28 Regenerating axons 2 weeks following sciatic nerve crush: (A) Diagram illustrating sciatic nerve crush, sections were taken at 6mm distal to injury site. Toluidine blue staining of injured sciatic nerves 2 weeks after crush. (B) Nerve section of wildtype animal showing regenerating axons (arrows). (C) Injured nerve section of C/EBPδ -/- showing high degree of myelin debris and damaged axons. Scale bar =20 um (D) Quantification of regenerating axons in injured sciatic nerves of four mice taken from nerve sections at 6mm distal to the crush site. (Mean ± S.E.M, n= 4 animals, ***=P<0.001 by student's t-test). B-C taken with a 50x objective.

5.5 Functional analysis in C/EBPδ null mice

5.5.1 Mechanical sensory test

Mice lacking the C/EBPδ gene have less regenerating axons than wildtype counterparts, as I showed by immunohistochemical and morphometric approaches in the sciatic nerve crush model. However, while much research has been undertaken to examine different forms to measure nerve repair, functional recovery remains the gold standard of successful reinnervation. Therefore, to determine whether the lack of C/EBPδ genuinely has a negative effect on nerve regeneration after sciatic nerve injury, several functional analyses were performed to test the sensor and motor recovery following a sciatic nerve crush injury.

Recovery of sensory function, as a measure of the rate and extent of regeneration of sensory axons in the injured sciatic nerve, was assessed in 8 wildtype and 8 C/EBPδ -/- mice by monitoring of the withdrawal reflex in response to stimulation of the hindpaw with Von Frey hairs from day 4 following nerve injury to day 22. Von Frey filaments 1g (Fig. 29A) and 1.66g (Fig. 29B) were used.

A 100% response was detected in the uninjured contralateral side tested in all the animals before and after injury with 1g and 1.66g filaments (data not shown). No response was observed with any of the filaments up to day 9 in the tested animals. By day 14 no response was detected with the 1g filament (Fig. 29A). However, 14 days following nerve injury, flexion withdrawal responses were observed with the heavier 1.66g filament in 87.5% of the wildtype and only 12.5% of C/EBPδ -/- animals (Fig. 29B). By day 18 a response was seen with the lighter 1g filament in 100% of the wildtype and in around 60% of C/EBPδ -/- animals (Fig. 29A). At the same time all the animals in the wildtype and knock-out group responded to the heavier 1.66 filament (Fig. 29B). By day 22 the

withdrawal response frequency with the 1g filament in wildtype was 100% whereas in C/EBPδ -/- animals was 87% (Fig 29A).

These results show that even though C/EBPδ is not essential, it is important for sensory regeneration. This is illustrated by C/EBPδ -/- animals showing a delayed sensory regeneration in the sciatic nerve after a crush injury.

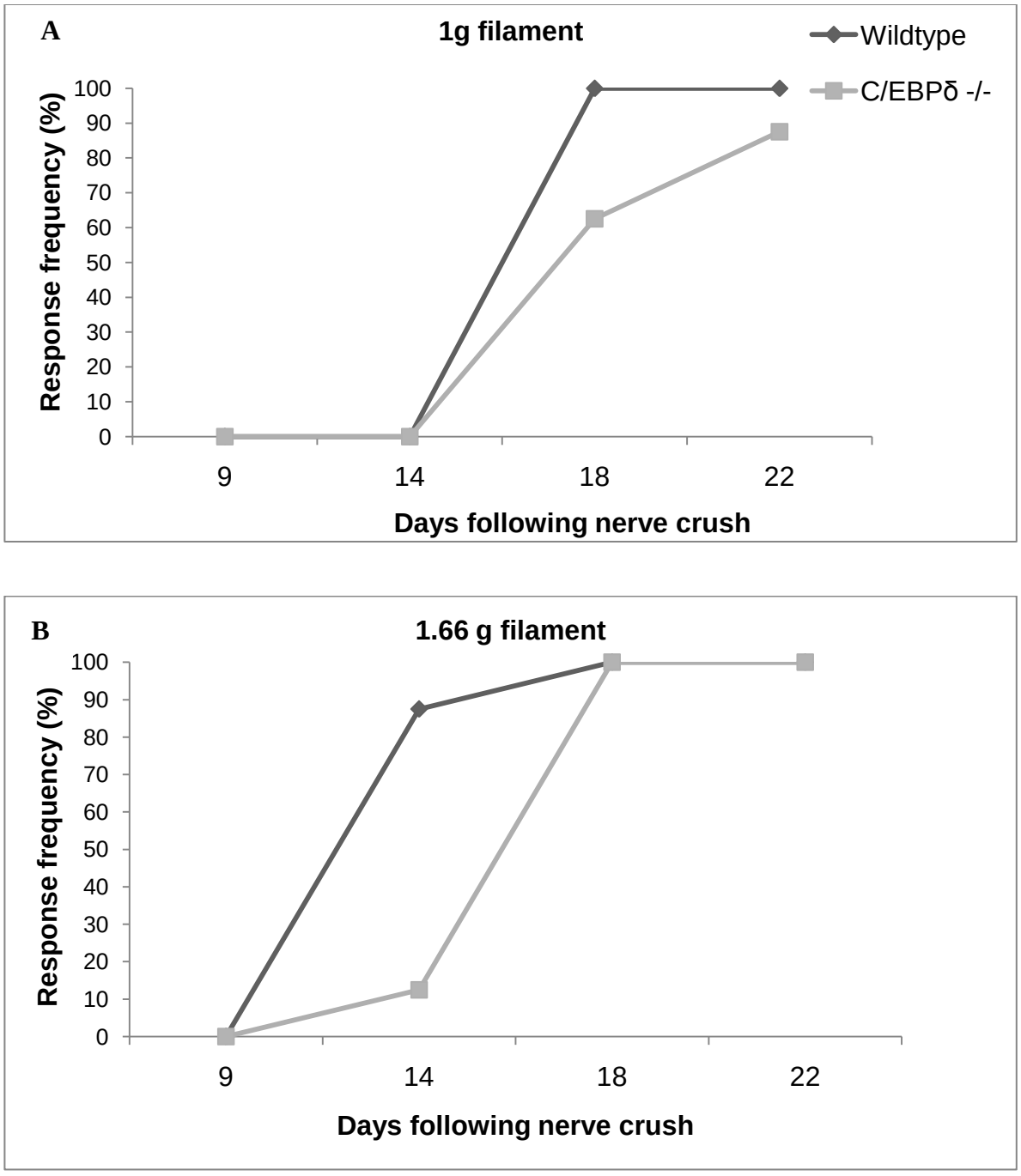


Fig. 29 Mechanical sensitivity test of mice after sciatic nerve crush injury: Responses to repeated mechanical stimuli with two different Von Frey filaments used 1g (A) and 1.66g (B). Each point represents the mean in percentage of the response frequency for wildtype and C/EBPδ -/- animals between 9 and 22 days following sciatic nerve crush. (n = 8)

5.5.2 Grid test

Within a total of 16 mice subjected to unilateral sciatic nerve crush further behavioural testing was performed to evaluate recovery of hind-limb strength in 8 wildtype and 8 C/EBPδ-/- animals. For that purpose we used the grid test which assesses the ability to accurately place the hindpaws during spontaneous exploration of an elevated and inverted grid by analysing the frequency with which the mice failed to accurately grasp the rungs in 10 attempts. The animals were tested from 4 to 28 days following nerve crush.

Initially by day 4 and 8 all the animals in the wildtype and knock-out group failed to grasp the rungs in 10 out 10 attempts (Fig. 30). By day 12 the C/EBPδ -/- mice failed twice as many times as the wildtype group, 8 vs. 4 times out 10 attempts respectively (Fig. 30). This difference in grasping performance, between wildtype and C/EBPδ -/- animals, persisted throughout the testing period. However, the knock-out mice made significantly more footslips than wildtype only at day 12, 16 and 20 following injury, $P<0.05$, $P<0.001$ and $P<0.05$ respectively. Interestingly, at the end of the testing period by day 28, the animals of both groups show an improvement in the grasping ability indicating increased nerve regeneration. However, the average number of footslips was 0.6 fails for wildtype and 1.3 fails for C/EBPδ -/- mice. Even though this might seem like the knock-out group misses the grid twice as many times as the wildtype group, the difference is negligible and not significant.

These data corroborates our previous immunohistochemistry findings indicating that C/EBPδ -/- animals subjected to a crush injury of the sciatic nerve, have delayed nerve regeneration.

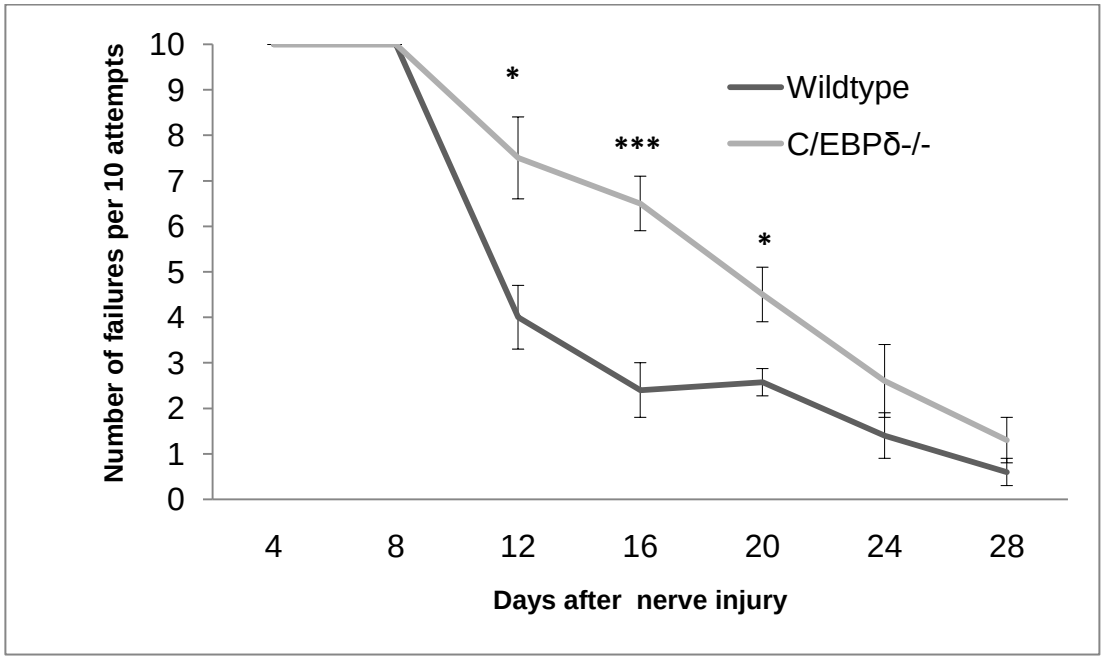


Fig. 30 Grid test in mice after sciatic nerve crush: Mean number of gripping failures for every 10 attempts following sciatic nerve crush recorded in C/EBPδ -/- vs. wildtype mice. Fewer significant gripping failures are achieved by days 12, 16 and 20 after injury in the wildtype group. (M ± S.E.M, n= 8 animals, *=P<0.05, P<0.001***, by student's t-test).

5.5.3 The sciatic static index

In order to assess the degree of functional loss and subsequent recovery after sciatic nerve crush in C/EBPδ -/- compared to wildtype mice, the sciatic static index (SSI) was used. The SSI measures functional loss on a scale from 0 to -100, where 0 corresponds to normal function and a value of −100 corresponds to complete loss of function. The SSI, which is a modified version of the sciatic functional index (SFI), uses the footprints, acquired when the animal is on a static position, and minimizes bias related to gait's velocity. Also, the SSI improves the acquisition of footprints, and is more repeatable and accurate then the SFI. The SSI was carried out by placing the animals in an acrylic cage with a digital camera placed underneath.

Pictures were taken when the animals were static and the images were evaluated to obtain two footprint parameters, the toe spread (TS) which is the distance between the first and fifth toes and the paw length (PL) which is the distance between the tip of the third toe and the most posterior part of the foot in contact with the ground. The TS is defined as the most lateral edges of the proper toes, and the PL as the most anterior edge of the third toe and the most posterior portion in contact with the ground, where the limits of the paw became less clear, pale and discolored.

Once the TS and the PL are measured the SSI can be calculated by using the following formula SFI = 101.3×TSF−54.03×PLF−9.5. The factors for each parameter (TS, and PL) were calculated with the formula: injured−uninjured/uninjured values (e.g. TSF (toe spread factor) = TS ipsilateral−TS contralateral/TS contralateral). The animals were evaluated before injury, shown as day 0 in (Fig. 31) and also on day 4,8,12,16,20,24 and 28 after nerve crush.

Generally the SSI shows a higher loss of function for C/EBPδ -/- mice on each time point after injury except for day 28. By day 8 both groups showed a peak in functional loss with a SSI score of -62±2 for wildtype and -70±3 for C/EBPδ -/- mice. Even though the difference in the SSI score was noticeable by day 12 between wildtype (SSI: – 30±6) and C/EBPδ -/- (SSI: -41±5), this difference was not significant p = 0.09. Statistical significant differences in the regain of function displayed between wildtype and knock-out animals were observed following injury on day 16 (-13±4 vs. -31±5) and day 20 (-5±4 vs. -29±5) (Fig. 31). On day 24 the SSI score for the wildtype group was -6±3 whereas the SSI for C/EBPδ -/- mice was -16±3, p=0.08. By the final evaluation day 28, the wildtype and C/EBPδ -/- groups displayed fully functional recovery demonstrated by resuming background levels (-0.8±3 vs. -1.3±3).

These results validates previous findings showing that animals lacking the C/EBPδ gene recover much slower after sciatic nerve crush than wildtype animals.

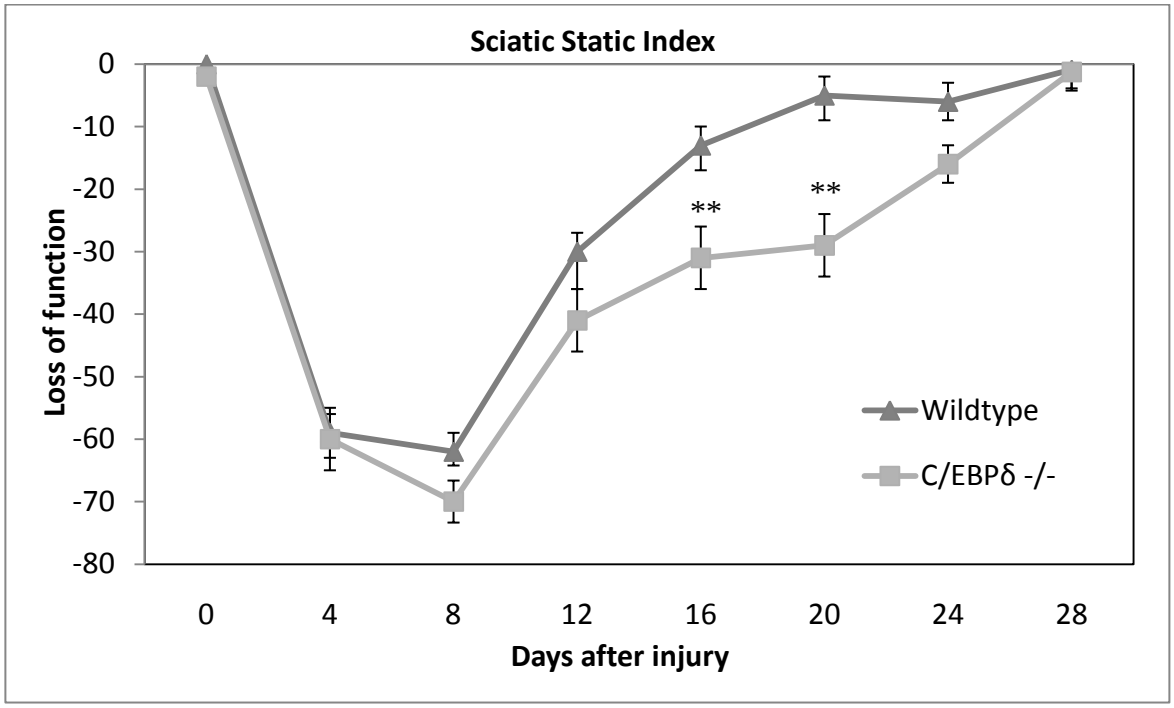


Fig. 31 Sciatic static index analysis shows a delayed recovery of function in C/EBPδ -/- mice: Following sciatic nerve crush, the sciatic static index was calculated by measuring the toe spread (TF) and paw length (PL). Note the differences of the foot posture and the particular footprint parameters between uninjured right and injured left hind limb. Images from wildtype and C/EBPδ -/- animals were taken before injury (0 day) and every 4 days after sciatic nerve crush injury. The knockout group of mice exhibit an initial deficit in recovery of loss function shown by a delayed return to baseline compared with the wildtype group. (Mean ± S.E.M, n= 8, **=P<0.01, by student's t-test).

5.6 Lack of C/EBPδ and the expression of the regeneration associated genes

To identify genes acting downstream of C/EBPδ and to establish whether the expression of genes known to be upregulated following sciatic nerve injury is affected in C/EBPδ -/- mice, the relative expression of GAP-43, galanin and SPRR1A mRNA were analyzed by RT-PCR in L4-L5 DRG neurons. GAP-43, galanin and SPRR1A have previously been shown to be upregulated following axotomy of sensory neurons in lumbar DRG (Benowitz and Routtenberg, 1997; Shadiack et al., 1998; Bonilla et al., 2002).

The expression of GAP-43, galanin and SPRR1A was analyzed 3 days after nerve crush and compared to wildtype mice (Fig. 32). Additionally protein expression was assessed by immunohistochemistry for GAP-43 (Fig. 33) and SPRR1A (Fig. 34) in L4-L5 DRG neurons 3 days after crush injury.

Following nerve crush injury the mRNA expression levels for GAP-43, galanin and SPRR1A were, as expected, upregulated in the wildtype DRG neurons from the injured site in comaparison to the uninjured site (Fig 32A-C). Relative GAP-43 mRNA expression in injured DRG neurons in wildtype and C/EBPδ -/- mice did not show any statistical difference (Fig 32A). A comparable pattern was obeserved in the mRNA expression of galanin which did not show any statistical difference between wildtype and C/EBPδ -/- mice in DRG neurons 3 days after nerve crush (Fig 33). In contrast, the difference between ipsilateral DRG neurons from wildtype and C/EBPδ -/- animals in the SPRR1A realative mRNA expression was statisticly significant (Fig 34). SPRR1A mRNA expression was downregulated approximately 4-fold in knock-out L4-L5 DRG neurons in comparison to wildtypes (Fig. 35). Interestingly, SPRR1A mRNA expression showed no significant difference in C/EBPδ -/- null mice before and after nerve crush ($p= 0,38$).

Similar results following crush injury were observed by immunostaining of L4-L5 DRG neurons with GAP-43 and SPRR1A antibodies. GAP-43 immunostaining was hardly detected in uninjured DRG neurons in wildtype (Fig. 33A) and C/EBPδ null mice (Fig. 33 C). GAP-43 intensity was greatly increased 3 days following nerve crush in injured DRG neurosn from wildtype (Fig. 33B) and C/EBPδ null mice (Fig. 33D). However, there was no difference in the percentage of GAP-43 positive DRG neurons stained after nerve crush between wildtype (48%±5) and C/EBPδ -/- (44%±2) mice (Fig. 33E).

SPRR1A immunostaining was not detected at all in uninjured DRG neurons from wildtype (Fig. 34A) and C/EBPδ null mice (Fig. 34C). Conversely, SPRR1A staining was detected in injured DRG neurons from wildtype and C/EBPδ -/- animals (Fig 34B and D). Although detected, the SPRR1A immunoreactivity observed in DRG neurons from C/EBPδ null mice was significantly lower than in wildtype animals. The percentage of DRG neurons stained with SPRR1A 3 days after nerve injury in wildtype animals was 67%±8 whereas in knock-out it was 9%±2, this was statisticly significant P= 0.002 (Fig 34E).

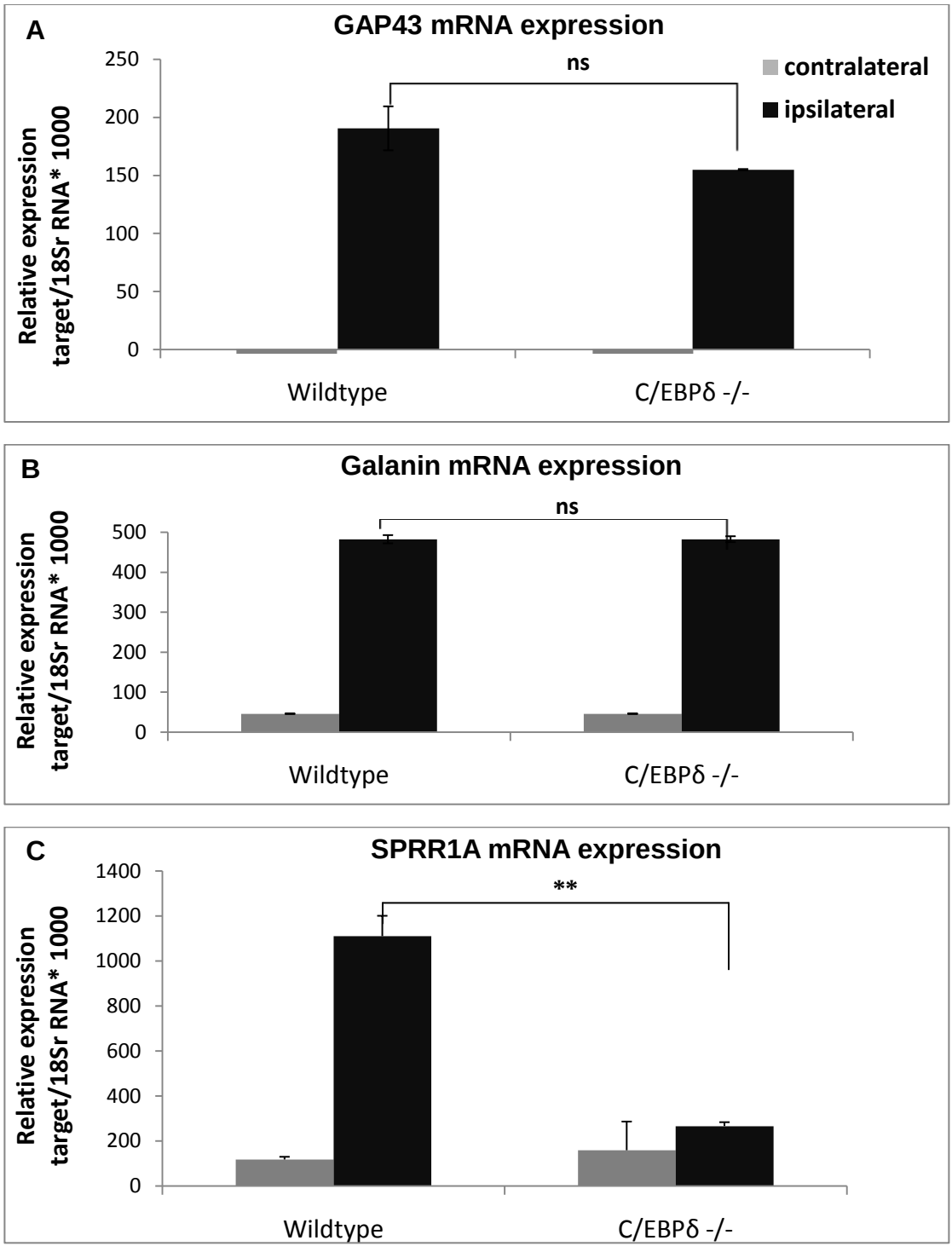


Fig. 32 Relative mRNA expression of regeneration associated genes 3 days after nerve crush: Analysis of relative mRNA expression in L4-L5 DRG neurons of wildtype vs. C/EBPδ -/- mice, contralateral and ipsilateral to nerve crush. (A) GAP-43, (B) Galanin and (C) SPRR1A genes. Target mRNA expression was normalized with 18S rRNA and multiplied by a factor of 1000. (Mean ± S.E.M, n= 4 animals, ns= non significant, **=P<0.01 by student's t-test).

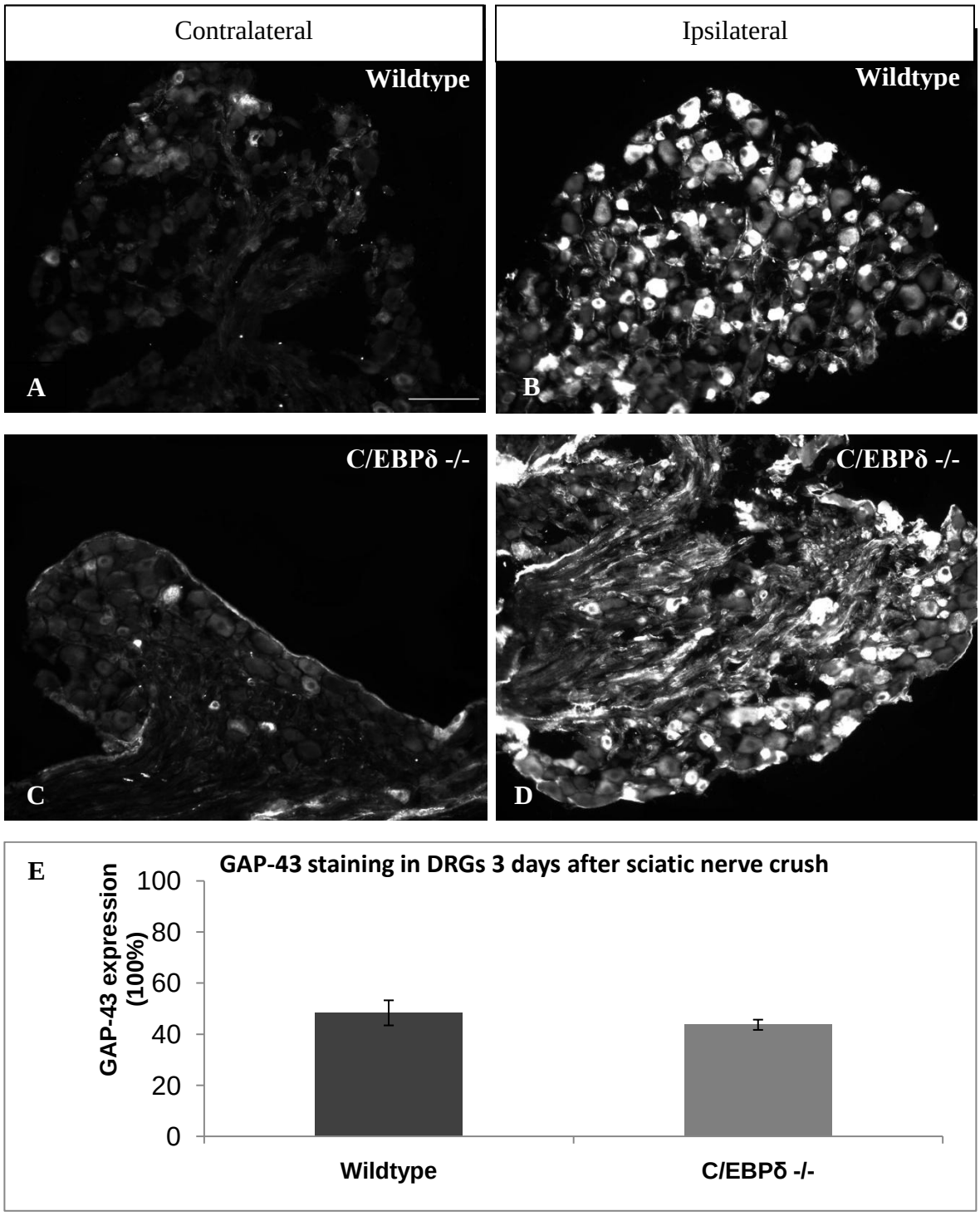


Fig. 33 GAP-43 expression in DRG neurons 3 days following sciatic nerve injury: Immunoreactivity for GAP-43 was analyzed in L4-L5 DRG neurons removed 3 days after sciatic nerve crush. Scale bar = 100 µm. GAP-43 staining detected in lumbar DRG neurons contralateral to nerve injury was barely detectable in wildtype mice (A) and in C/EBPδ -/- mice(C). GAP-43 immunoreactivity is seen in a high proportion of cells in injured DRG neurons of wildtype mice (B) and C/EBPδ -/- mice (D). (E) Percentage of GAP-43 positive DRG neurons from wildtype vs. C/EBPδ-/- mice (M ± S.E.M., n= 4 animals).

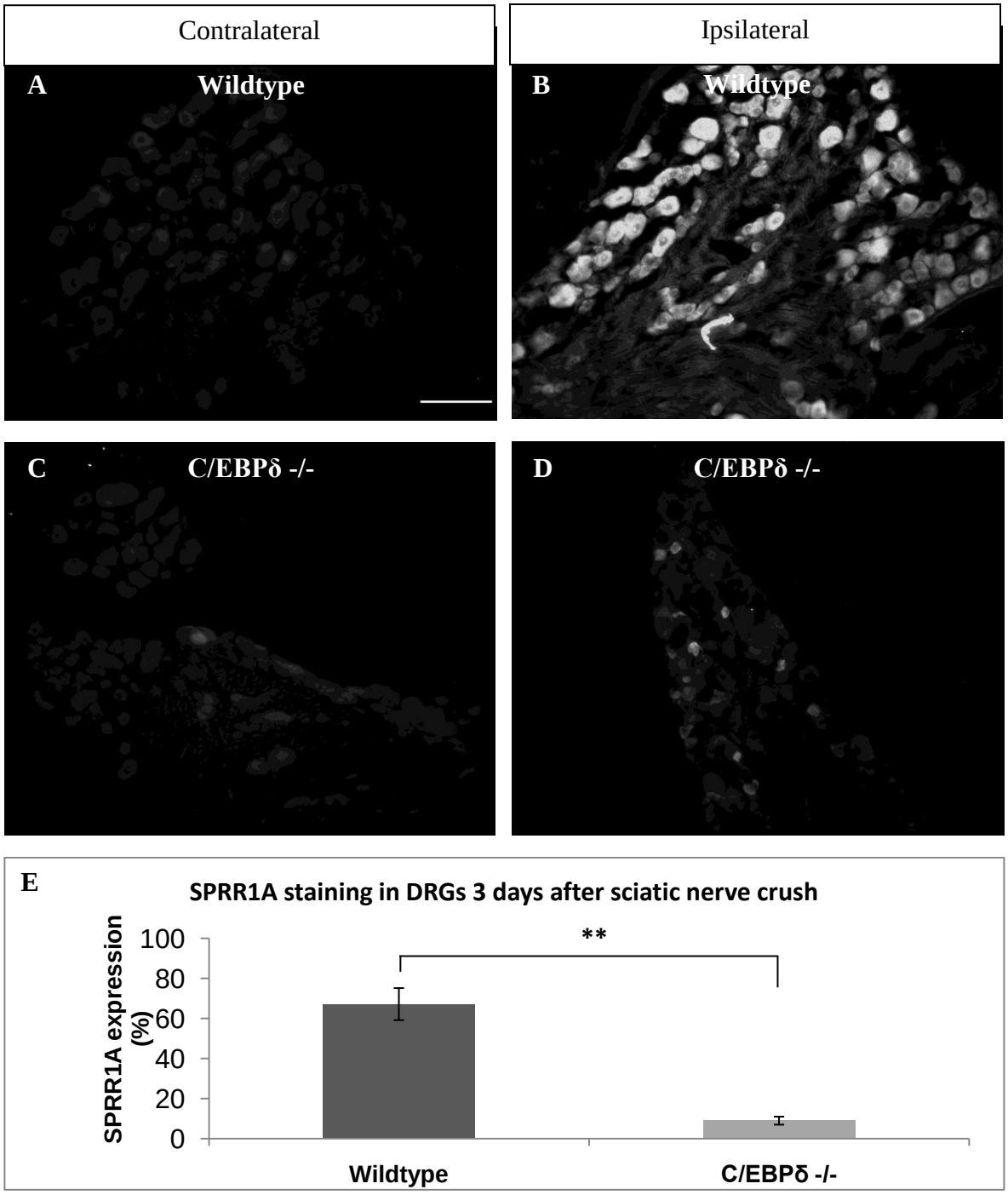


Fig. 34 SPRR1A expression in DRG neurons 3 days following sciatic nerve injury: Immunoreactivity for SPRR1A was analyzed in L4-L5 DRG neurons removed 3 days after sciatic nerve crush. Scale bar = 100 µm. (A) No positive SPRR1A staining is detected in lumbar DRG neurons contralateral to nerve injury in wildtype mice. (B) SPRR1A immunofluorescence is seen in a high proportion of cells in the ipsilateral DRG neurons. (C) No positive immunofluorescence for SPRR1A is observed in DRG contralateral to nerve injury in C/EBPδ-/- mice (D) DRG neurons ipsilateral to nerve injury in C/EBPδ-/- mice show a low proportion of cells expressing SPRR1A. (E) Percentage of SPRR1A positive DRG neurons from wildtype vs. C/EBPδ-/- mice (M ± S.E.M., n= 4 animals, **=P<0.01 by student's t-test).

5.7 Discussion

Evaluation of sciatic nerve function under normal conditions in C/EBPδ null mice

To examine the role of C/EBPδ *in vivo* following the promising results from the *in vitro* experiments, we decided to use C/EBPδ null mice generated by Esta Sterneck's group at the National Institute of Health in the USA (Sterneck et al., 1998). Previously it has been shown that the disruption of other members of the C/EBP family of transcription factors interferes with normal embryonic and postnatal development. For instance, C/EBPα disruption results in perinatal death caused by impaired energy homeostasis (Wang et al., 1995) as well as deficiencies of the hematopoietic system (Zhang et al., 1997). Deletion of C/EBPβ results in female infertility (Sterneck et al., 1997) and the health of C/EBPβ $^{-/-}$ mice deteriorates within weeks to months after birth. Consequently, in order to discern the specific effects of C/EBPδ in the sciatic nerve before addressing its involvement in nerve repair, I looked at transverse sections of uninjured sciatic nerves of wildtype and C/EBPδ -/- mice and compared them by morphometric analysis.

No morphological changes and no differences in the number of axons in the sciatic nerve were observed. Subsequently, in order to determine whether the lack of C/EBPδ has a behavioural effect, a grip strength test was performed in wildtype and knock-out animals. The lack of C/EBPδ in mice does not affect the neuromuscular function since both groups applied the same amount of force to the mesh pull bar at the moment the grasp was released. Consistently, Sterneck and colleagues demonstrated that prenatal and postnatal development of C/EBPδ null mice was normal and the animals were healthy. C/EBPδ -/- mice only displayed significantly more contextual-conditioned fear after a 24-hr retention

interval. However, performance in a battery of behavioural tests indicated that basic neurological functions are normal.

Sterneck's group assessed gross neurological functions in the C/EBPδ null mice by examining spontaneous behaviours in battery of tests, specifically, limb-extension reflexes in a moving cage, righting, eye-blink, ear-twitch reflexes, whisker-orienting response, wire-suspension test, vertical pole test, motor coordination and sensorimotor behaviours (Sterneck et al., 1998). The C/EBPδ null mutation did not show any deleterious effect on sensory and motor functions or complex behaviours, concurring with our results where the lack of C/EBPδ did not have a negative functional effect. However, it cannot be ruled out that the potential behavioural phenotypes of the mutants could be caused by developmental defects that are not detected at the gross anatomical level.

Sciatic nerve regeneration assessment in C/EBPδ null mice by immunohistochemistry

To evaluate nerve regeneration in the sciatic nerve of C/EBPδ null mice after nerve crush I examined the expression of the regeneration associated gene GAP-43 and the general neuronal marker PGP9.5. I found that GAP-43 immunofluorescence was highly detectable in the sciatic nerve of wildtype animals 3 days and 2 weeks after nerve injury. Conversely, in the injured sciatic nerve from C/EBPδ null, GAP-43 general intensity of immunofluorescence was much lower 3 days and even 2 weeks after nerve crush. Other studies have shown that GAP-43 is present in the growth cones of developing and regenerating adult PNS (Chong et al., 1994; Meiri et al., 1986). Unfortunately, this could cause problems in differentiating between the axon and Schwann cell labeling, as well as in

discriminating between direct and indirect effects. However, it has been shown that GAP-43 is expressed only by non myelin forming Schwann cells and dedifferentiated Schwann cells responding to the loss of axonal contact (Curtis, 1993). This suggests that the upregulation of GAP-43 observed in wildtype mice following nerve crush may be related to increasing Schwann cell density and that the abnormal GAP-43 expression seen in knock-out animals could be due to a negative effect of the lack of C/EBPδ upon Schwann cells. Interestingly, Kamaraju and colleagues showed that silencing of C/EBPδ mRNA and protein expression by introduction of small interference RNA prevents the induction of myelin protein zero (P0) and myelin basic protein (MBP) mRNAs in a Schwann cell myeloma culture model. Kamaraju also suggested that C/EBPδ may be relevant for the switch from embryonic and adult non-myelinating Schwann cells, to the myelinating phenotype expressing the myelin gene products (Kamaraju et al., 2004). Consequently, the difference in the GAP-43 immunoreactivity between wildtype and knock-out mice after nerve crush might not be entirely due to the presence of Schwann cells since according to Kamaraju *et al*. the lack of C/EBPδ only affects myelinating Schwann cells and according to Curtis GAP-43 is only expressed by non myelinating Schwann cells. It is also possible that other non-neuronal cells like macrophages and satellite cells might be affected in the knockout animals which might impair axonal regeneration.

Therefore to elucidate whether the lack of C/EBPδ specifically affects axonal repair, we used PGP9.5 also another marker for nerve regeneration. PGP9.5 is a structural cytoplasmic protein present in all types of mammalian nerves (Thompson et al., 1983). PGP9.5 immunofluorescence was highly detected in the sciatic nerve from wildtype animals 3 days and 2 weeks following nerve injury. In the knock-out animals PGP 9.5

intensity of immunofluorescence was much lower than wildtype 3 days following nerve crush. However, after 2 weeks, PGP9.5 immunoreactivity was comparable to the levels observed in wildtype animals.

These findings show that the lack of C/EBPδ specifically affects axonal regeneration which is not abolished but delayed in the C/EBPδ -/- mice. Further studies are required to assess whether Schwann cells and other non neuronal cells are affected by the lack of C/EBPδ and to clarify PGP9.5 but not GAP-43 high immunoreactivity in knock-out animals 2 weeks after nerve crush.

Certain considerations need to be taken into account when analysing and discussing the results presented here. Variations in background levels might result due to inadequate sample fixation, hydrophobic interactions between tissue and reagent proteins, defective reagents or antibodies. Therefore, an adequate internal positive control should have been used. In this case only negative controls were performed by omitting primary antibodies.

Functional analysis in C/EBPδ null mice: mechanical-sensory tests

Von Frey sensitivity testing is regularly used to quantify the degree of neuropathic pain in various sciatic nerve injury models, such as the chronic constriction injury, the spinal nerve ligation and the partial sciatic ligation models. In this study we have used a modified Von Frey test to evaluate sensory nerve regeneration in C/EBPδ -/- compared to wildtype animals after nerve crush. However, it is erroneous to say that the Von Frey test is a pure sensory test. Since the animals cannot express sensory perception, this test relies on a motor response to a sensory stimulus. Thus, it becomes difficult to separate sensory and motor function. It should be noted that in this case even if the mice may be able to feel pain after

the applied stimulus, due to decreased axonal connections and decreased muscle strength they are unable to lift the foot in response to the stimulus.

Initially I established that 1g and 1.66g Von Frey hair filaments invoke a response in non-injured hindlimbs. Therefore, in order to compare sensory regeneration I subsequently used these Von Frey filaments in injured animals. As axonal connections are re-established, sensory nerve regeneration increases and a decreased resistance to applied force is displayed.

Our findings indicate that sensory nerve regeneration was delayed in C/EBPδ -/- mice. By day 14 only 1 out of 8 knock-out mice (12.5%) showed a response to the 1.66g filament whereas 87.5% of the wildtype mice responded to the same stimulus. Additionally by day 18 all the wildtype animals and only 5 out 8 (62.5%) in the knock-out group evoked a response to the 1g filament. However, the delayed sensory nerve regeneration seen in C/EBPδ -/- animals was not permanent given that all these animals were responding by day 18 after stroke with the 1.66g filament and almost all of them (87.5%) were responding by day 22 after stroke with the lightest 1g Von Frey filament. These findings are consistent with our previous PGP9.5 immunohistochemistry results which showed delayed regeneration following nerve crush in the sciatic nerve of C/EBPδ -/- animals. In that experiment no PGP9.5 staining was detected by day 3, however, by day 14 the PGP9.5 staining intensity in C/EBPδ -/- was as high, however more fragmented, as the wildtype group.

It is important to mention that although high PGP9.5 intensity staining was observed by day 14 in the knock-out animals, sensory regeneration was not properly observed in C/EBPδ -/- mice until day 18. The reason for this difference is that the sections

stained for PGP9.5 were approximately 3mm long distal from the injury site whereas the distance that the regenerating nerves need to cover in order to re-establish sensory connections is much further; therefore it requires longer time to show any response.

Importantly, functional studies have non-linear correlation with reinnervation. Reinnervation and the response of the stimulated nerve can be due to collateral sprouting of uninjured axons. Therefore, functional studies should be combined with other techniques (e.g. anterograde and/or retrograde labelling) to circumvent this issue.

The sciatic static index

The SSI was used to evaluate the sciatic nerve function after injury and repair in C/EBPδ -/- mice. The SSI is a method that relies on the analysis of footprints to determine functional recovery and it has been adapted from the SFI which is one of the most commonly used forms of functional assessment of hindlimb performance by examining walking tracks. Walking track analysis is a direct measurement of function, whereas electrophysiological and histomorphometrical analyses are indirect methods that may not necessarily correlate with the return of motor and sensory nerve functions (Dellon and Mackinnon, 1989). The SFI introduced by DeMedinaceli L et al., (1982) employed ink for staining the feet and allowed the animal to walk in a proper catwalk across white paper. Others used water on the feet and a special moisture-sensitive paper (Lowdon et al., 1988), or developer and a radiographic film in a darkened catwalk (Bain et al., 1989). A serious improvement was the introduction of the video imaging technique to record the footprint (Lin et al., 1992). It allowed digital analysis of the video images and better repeatability. It significantly diminished the number of useless footprints and decreased printing errors.

The SFI adapted to the mouse model of sciatic nerve lesion relies only on the print length and the toe spread. Pawprints are recorded by moistening the hindpaws of each animal with water in combination with bromophenol blue-impregnated paper to allow clear identification of the footprint parameters which permits various gait cycles to choose (Inserra et al., 1998). However, this method may stress the animals and it is also time consuming. Furthermore, some other factors can bias the study, such as foot contractures, autotomy, excrements on the corridor and variations due to gait velocity (Walker et al., 1994; Bain et al., 1989).

The SSI on the other hand introduced by Bervar, (2000), and adapted to the mouse model by Baptista et al. (2007), is more practical to perform and is not so influenced by gait's velocity. The images are simpler to be acquired and analyzed since it only requires an acrylic cage, a digital camera and an image software. Compare to ink, photo methods are more accurate and reproducible. SSI doesn't suffer from poor variability due to difficulties with smeared footprints, contractures and variations on gait velocity. Additionally, due to the low quality of some prints the process to record footprints has sometimes to be repeated which stresses the animals contributing to greater variations on the measurements. The photos used on the SSI analysis eliminate most of these difficulties, since the researcher can wait for the moment where the animals are stopped to acquire the images. There is no bias due to smeared footprints, as the hindpaws are seen as they are, or to gait velocity variations, because the animals are not moving.

To calculate the SSI score, the formula $101.3\times TSF - 54.03\times PLF - 9.5$ was used. The formula developed by Baptista and colleagues, shows the big influence of the toes spread factor (TSF) as a measure of gait dysfunction due to sciatic nerve lesion (Baptista et al.,

2007). The TSF parameter represents the main cause of changes and may be used alone as indicative of the lesion's status. The paw length factor (PLF) represents a small but significant contribution to the formula according to Baptista's group. This parameter expresses the ability of the soleous and gastrocnemious muscles to maintain the heel elevated during walking in rodents. Because these muscles are innervated by the sciatic nerve, when impaired they cannot maintain the normal ankle angle, letting a bigger portion of the paw to be in contact with the floor. Although the PL may be very variable, due to the difficulty to establish its posterior aspect, its significance has also been demonstrated in the ink track method, which generated the SFI formula for the mouse (Inserra et al., 1998).

The highest loss of function for the wildtype and C/EBPδ -/- mice was showed at day 8 after nerve crush (SSI= -62 vs. -70). This is in line with observations of others where the highest loss of function (SSI and SFI= -65) was observed after 1 week following sciatic nerve crush in mice (Baptista et al., 2007). 16 and 20 days after nerve crush a statistical significant difference was noticed in the recovery of function between wildtype (SFI: -16 and -5) and C/EBPδ knock-out animals (SFI: -31 and -29). Previously it has been shown that upon full recovery animals achieved a SFI and SSI score between -10 and -5 (Baptista et al., 2007; Dijkstra et al., 2000; Dinh et al., 2009). According to these results, our findings indicate that wildtype animals recovered normal gait function at the third week after nerve crush whereas C/EBPδ -/- animals achieved full recovery at the fourth week after crush.

The SSI results confirm our previous findings, from the motor-sensory analysis, grid test, morphometrical assessment and immunohistochemistry examination of regeneration markers that indicate an important delay of approximately 30% - 40% in the

functional recovery of C/EBPδ -/- animals following sciatic nerve injury. Similar delay in functional recovery after sciatic nerve crush have been shown by mice lacking the cell cycle inhibitory protein $p21^{Cip1/WAF1}$ (Tomita et al., 2006), mice lacking the genes for the key components of the plasminogen system, tissue plasminogen activator (tPA), urokinase (uPA), and plasminogen knockout mice (Siconolfi and Seeds, 2001). Comparably, deletion of the gene encoding the neuronal cell adhesion molecule alpha7 integrin subunit, causes a severe, almost 40% reduction in the speed of the motor axon regeneration after facial axotomy and a significant delay in the reinnervation of its peripheral target (Werner et al., 2000). Additionally, several reports have shown the involvement of other transcription factors in nerve repair. Transcription factors such as c-Jun, ATF-3 are normally expressed at low levels in adult DRG neurons and rise significantly following peripheral axotomy, modulating adult injury responses (Lindwall et al., 2004; Tsujino et al., 2000; Raivich et al., 2004). Recently, it has also been shown that the transcription factor Sox-11 is required for a successful regeneration following peripheral nerve injury (Jankowski et al., 2009).

Furthermore, a recent report suggesting the importance of C/EBPs in the plasticity of the nervous system, identify 115 C/EBP target genes in PC12 cells that include transcription factors, neurotransmitter receptors, ion channels, protein kinases and synaptic vesicle proteins. C/EBPs binding sites were located primarily within introns, suggesting novel regulatory functions, and were associated with binding sites for other developmentally important transcription factors. Experiments using dominant negatives showed C/EBPβ to repress transcription of a subset of target genes. Target genes in rat brain were subsequently found to preferentially bind C/EBPα, β and δ. (Kfoury and Kapatos, 2009).

These previous results reveal the importance of the C/EBPδ gene in nerve repair after a sciatic nerve crush injury. However, two important details must be noticed: i) even though reinnervation and functional recovery are correlated, this correlation is not linear, since only 20–30% of the final reinnervation is required to detect recovery (Raivich et al., 2004). ii) The lack of C/EBPδ affects nerve regeneration but it is not essential for recovery of normal gait function since knock-out animals ultimately although belatedly regenerate. The C/EBP family is a large group of transcription factors with 6 different members and multiple isoforms that can interact with each other and with the members of other transcription factor families and proteins; therefore it wouldn't be surprising that another member, related transcription factor or protein compensates for the lack of C/EBPδ expression in the knock-out animals. Previously it has been shown that C/EBP family members can compensate for the lack of a specific subtype (Hu et al., 1998; Gery et al., 2005; Chen et al., 2000b) and it has been suggested that C/EBPβ might compensate for the lack of C/EBPδ *in vivo* (Borrelli et al., 2007). Conversely it has also been suggested C/EBPδ as the most plausible candidate to compensate for the lack of C/EBPβ in C/EBPβ-deficient animals upon LPS treatment (Hu et al., 1998). A lack of C/EBPβ expression would be expected to further reduce functional recovery in a regeneration model. Therefore, it should be interesting to study nerve regeneration in mice deficient in both C/EBPβ and C/EBPδ expression. However, it has been shown that approximately 85% of C/EBPβ (-/-) δ (-/-) mice died at early neonatal stage (Tanaka et al., 1997), so a conditional approach should be considered.

Lack of C/EBPδ and the expression of the regeneration associated genes

We have shown that the C/EBPδ is upregulated following nerve crush injury and also that the lack of C/EBPδ delays functional recovery after peripheral injury. Because transcription factors which are upregulated early in the regenerative process could be important for the expression of multiple downstream genes, we investigated the expression of several regeneration associated genes known to be upregulated after sciatic nerve injury in C/EBPδ -/- mice before and after nerve crush.

The genes selected were galanin, GAP-43 and SPRR1A. Previously it has been shown by other groups that galanin (Boeshore et al., 2004; Shadiack et al., 1998), GAP-43, reviewed by (Benowitz and Routtenberg, 1997) and SPRR1A (Nilsson et al., 2005; Bonilla et al., 2002) are all upregulated after sciatic nerve injury.

Here we report that the relative mRNA expression and also the protein expression of galanin and GAP-43 are equally upregulated in L4-L5 DRG neurons from wildtype and C/EBPδ -/- mice following sciatic nerve injury. On the other hand, in C/EBPδ -/- mice the mRNA and protein expression of SPRR1A was significantly reduced compared to wildtype animals. These results suggest that SPRR1A may act downstream of C/EBPδ in a neuronal regeneration cascade. To date, there is no direct connection between C/EBPδ and SPRR1A expression. However, several lines of experimental evidence indirectly suggest a possible association between the roles of SPRR1A as regeneration associated gene and the expression of C/EBPδ. For instance, SPRR1A induction by axotomy is upregulated during successful regeneration and blockade of SPRR1A decreases the outgrowth potential of regenerating adult neurons (Bonilla et al., 2002), two features which are also displayed by C/EBPδ. Furthermore, SPRR2A which is another member of the SPRR family and

normally expressed in the epidermis, is misexpressed in C/EBPβ -/- mice (Grimm et al., 2002). Additionally, the SPRR1A promoter contains functional AP-1 binding sites (Pradervand et al., 2004; Sark et al., 1998) which are recognized by homodimers of Jun or heterodimers of Jun/Fos, ATF/CREB bZIP transcription factors (Hai and Curran, 1991) and it is known that C/EBPs can bind to and regulate two AP-1 transcription factors, c-*fos* and c-*jun*, (Hsu et al., 1994). Furthermore, C/EBPβ has a binding site located on the SPRR1A promoter (Pradervand et al., 2004) which was discovered in a major pathway involved in cardiomyocyte protection that resembles a signalling pathway involved in nerve repair. In this pathway IL-6 cytokines bind to gp130/LIFR receptor which activates STAT3, PI-3K, and MAPK signalling cascade. Then MAPK activates C/EBPβ and AP-1. Thereafter, C/EBPβ binds to SPRR1A promoter inducing SPRR1A gene transcription (Pradervand et al., 2004). Interestingly, the induction of SPRR1A by C/EBPβ could explain the low although present mRNA and protein expression in C/EBPδ null mice before and after sciatic nerve injury. Recently it has been shown that SPRR1A induction is regeneration-specific and distinct from developmental programs of gene expression (Starkey et al., 2009). Therefore, these findings may be important for the future use of C/EBPδ as a regeneration-specific marker in CNS and PNS injury studies in mice. Further experiments are required to determine how C/EBPδ influences peripheral nerve repair and whether this occurs by controlling the expression of other regeneration associated genes following nerve injury. For that purpose microarray analysis could be used.

Chapter 6 - Summary and future studies

Studies in the last two decades on the regulation of genes involved in cellular responses, such as cellular differentiation and neuronal repair, have led to the identification of key transcription factors that act as master regulators by interacting with regulatory sequences present in the promoter and enhancer regions of target genes. Transcription factors belonging to the C/EBP family, with many pathophysiological conditions associated with their defective function, fall in this category. Although C/EBPα, β and δ expression has been reported in the brain and systematic analysis on the function and properties of C/EBP proteins has been performed in a range of tissues such as liver and adipose, much less is known about the role of C/EBPs in the nervous system, especially in nerve regeneration after nerve injury. Indirect evidence documented in non-neuronal tissues have shown that C/EBPs can interact with well known genes present in the nerve regeneration signalling cascade such as ArgI, cAMP, CREB, NGF, SPRR1A and STAT-3, suggesting a possible involvement of the C/EBPs in neuronal repair. Consequently, the aim of my PhD project was to study the involvement of C/EBPs, especially C/EBPδ during neuronal repair in the PNS.

A hallmark for the involvement of a transcription factor in neuronal repair would be the variation of its expression and activity after nerve injury, during axonal repair. This change in expression could regulate downstream target genes and account for the dramatic structural changes seen in axonal regrowth. Therefore our first goal was to determine if the expression of the members of the C/EBP family is altered following peripheral sciatic nerve crush.

In this thesis, I have shown that C/EBPβ –γ and –δ mRNA expression were upregulated in L4-L5 DRGs *in vivo* 4 hours after nerve crush. This increase was sustained up to 3days only for C/EBPδ, indicating that although all C/EBP isoforms share substantial sequence identity, they do have different functions and react differently to the same pathological condition in this case axonal injury.

In this thesis, I also showed that in an *in vitro* model that the overall C/EBP transcriptional activity and specifically C/EBPβ and –δ mRNA expression are upregulated during neurite outgrowth induced by the addition of cAMP. Subsequently, in the same *in vitro* model, I showed that the use of a conditional dominant inhibitor of all the C/EBPs and the specific conditional C/EBPδ antisense construct decrease the neurite length. However, I have to admit the fact that the transfection efficiency was not very high and that I couldn't demonstrate the downregulation of the C/EBPs by the conditional constructs. However, I'm confident that the decrease in neurite length is affected by the downregulation of C/EBPs since I showed that axonal elongation was greatly reduced in DRG neuronal cultures from C/EBPδ null mice. These new lines of evidence, besides showing a direct role of C/EBPδ during neurite elongation, also indicate that C/EBPδ is an important factor of the neurons intrinsic growth capacity.

Moreover C/EBPs might be a downstream effector of the second messenger cAMP, which is known to promote axonal outgrowth *in vitro* (Neumann et al., 2002; Qiu et al., 2002) and improve regeneration and functional recovery after spinal cord injury *in vivo* (Pearse et al., 2004). Therefore, C/EBPδ might be an interesting candidate to enhance axonal regeneration following an injury in the CNS.

Another interesting finding in my thesis was the increase of C/EBPδ after treatment of neuronal culture with the histone deacetyltransferase inhibitor, TSA. It has been reported that TSA switches the intrinsic neuronal genetic program from a non permissive to a permissive pattern leading to increased neurite outgrowth (Gaub P, 2008). Therefore future epigenetic studies should investigate in detail the processes of histone acetylation/deacetylation on primary sensory neurones, their impact on neuronal repair and the possible participation of the C/EBPs.

The effect of the lack of C/EBPδ in axon regeneration after peripheral nerve injury had not been studied previously. Here I show that C/EBPδ null mice have equivalent amount of axons in the sciatic nerve compared to their wildtype counterparts. Additionally no difference was observed in the neuromuscular function indicating that the lack of C/EBPδ does not affect the anatomy or function of the sciatic nerve under normal conditions. These results confirm previous findings where no deleterious effects on sensory and motor functions or complex behaviours were observe in C/EBPδ null mice (Sterneck et al., 1998).

More importantly, in my thesis I showed by immunohistochemistry, morphometrical studies and different functional analyses tests a direct detrimental effect of the lack of C/EBPδ after sciatic nerve crush and during axonal regeneration leading to delayed recovery of function. Additionally, in my thesis I showed that after nerve crush the lack of C/EBPδ downregulates the expression of the regeneration associated gene SPRR1A which is normally highly upregulated by axotomized neurons and promotes axonal outgrowth (Nilsson et al., 2005; Bonilla et al., 2002). These results show that C/EBPδ affects the expression of a gene directly involved in nerve repair; it also shows that C/EBPδ

is important during nerve repair yet not indispensable for functional recovery. However, in this thesis it wasn't determined whether non neuronal cells (e.g. Schwann cells, macrophages and satellite cells) are affected in the C/EBPδ -/- mice which could influence the delay in regeneration observed after sciatic nerve injury. Therefore, future studies in C/EBPδ should include immunostaining of non neuronal cells following injury.

In future studies, it would also be valuable to use microarrays and proteomics to disclose gene expression profile at both translational and transcriptional levels in C/EBPδ-/- mice following injury to identify downstream targets and dissect the signalling cascade leading to this delay in nerve repair. Since nerve regeneration in C/EBPδ -/- mice is delayed and not completely impaired, it would be important to determine whether the lack of C/EBPδ becomes eventually redundant due to compensatory mechanism upregulating the expression of other C/EBP members. Therefore, in C/EBPδ -/- mice, the expression of the other C/EBP members should be measured at different time points following nerve injury. Additionally, since C/EBPβ has also been shown to be upregulated after nerve injury and during neurite sprouting, the concomitant lack of C/EBPδ and C/EBPβ should be investigated in a sciatic crush injury model.

The findings of my thesis illustrate that peripheral axotomy provides several insights into axonal regeneration, showing the importance of the transcriptional factor C/EBPδ during neuronal repair *in vivo*. These results offers some hope for successful repair or at least enhanced regeneration after injury in the CNS, therefore, future studies should concentrate on the effect of C/EBPδ overexpression in the CNS after nerve injury. Since it is likely that the *in vivo* CNS regeneration depends on the concerted action of multiple proteins to achieve robust axon growth, C/EBPδ should be overexpressed in combination

with other known genes involved in nerve repair, for instance GAP-43, CAP-23, c-jun, SPRR1A and STAT-3.

Appendix

Generation of a C/EBP luciferase transgenic reporter mouse

Here we report the generation of a C/EBP-luciferase transgenic mouse that can be used as a tool to assess the expression of the C/EBP family of transcription factors *in vivo* under different pathophysiological conditions such as inflammation or neuronal repair.

The use of transgenic reporter mice to investigate regulation of genes in complex biological situations can be helpful to overcome the limitations of other techniques. For example, the effects of environmental factors on tissue culture cells cannot be correlated directly to the in vivo conditions, in part because the cell culture environment is artificial and cannot replicate all processes that occur in an intact animal. Thus it is required to use animal models both to validate in vitro data and to extend the knowledge relevant to the in vivo situation.

The C/EBP-luciferase transgenic model that we generated can be used in combination with molecular imaging, allowing us to visualize and quantify C/EBP activity under biological processes at the cellular level within intact living animals. Furthermore, non-invasive imaging provides the possibility of repetitive assessments in the same animal allowing the animal to serve as its own control, thus reducing significance of differences between individuals.

The production of transgenic C/EBP reporter mice was achieved by the use of pro-nuclear microinjection where the transgene (see Fig. 18D) in multiple copies is injected into one of the nuclei of a fertilized mouse zygote. Because of the randomness of integration

and the variability of the transgene copy number, it was necessary to analyze several transgenic mouse lines to estimate the reliability of the expression.

To image luciferase generated luminescence, the substrate luciferin is delivered intraperitoneally to ensure rapid distribution in the whole mouse, finally reaching the inside of the cells. Luciferin is oxidized to oxyluciferin by those cells that are producing luciferase, leading to the emission of light (Fig. 35). Detection and quantification of the photons generated by the luciferin-luciferase reaction requires that the mice destined for imaging must be placed in a light-tight chamber. To image the C/EBP mediated luciferase activity the mice are injected with 2 mg D-luciferin ($\sim$130 mg/kg). Five minutes later, the mice are placed in the light-tight chamber, where luminescence from these mice is collected for 5 min by using an IVIS-100 system from Xenogen.

Fig. 33

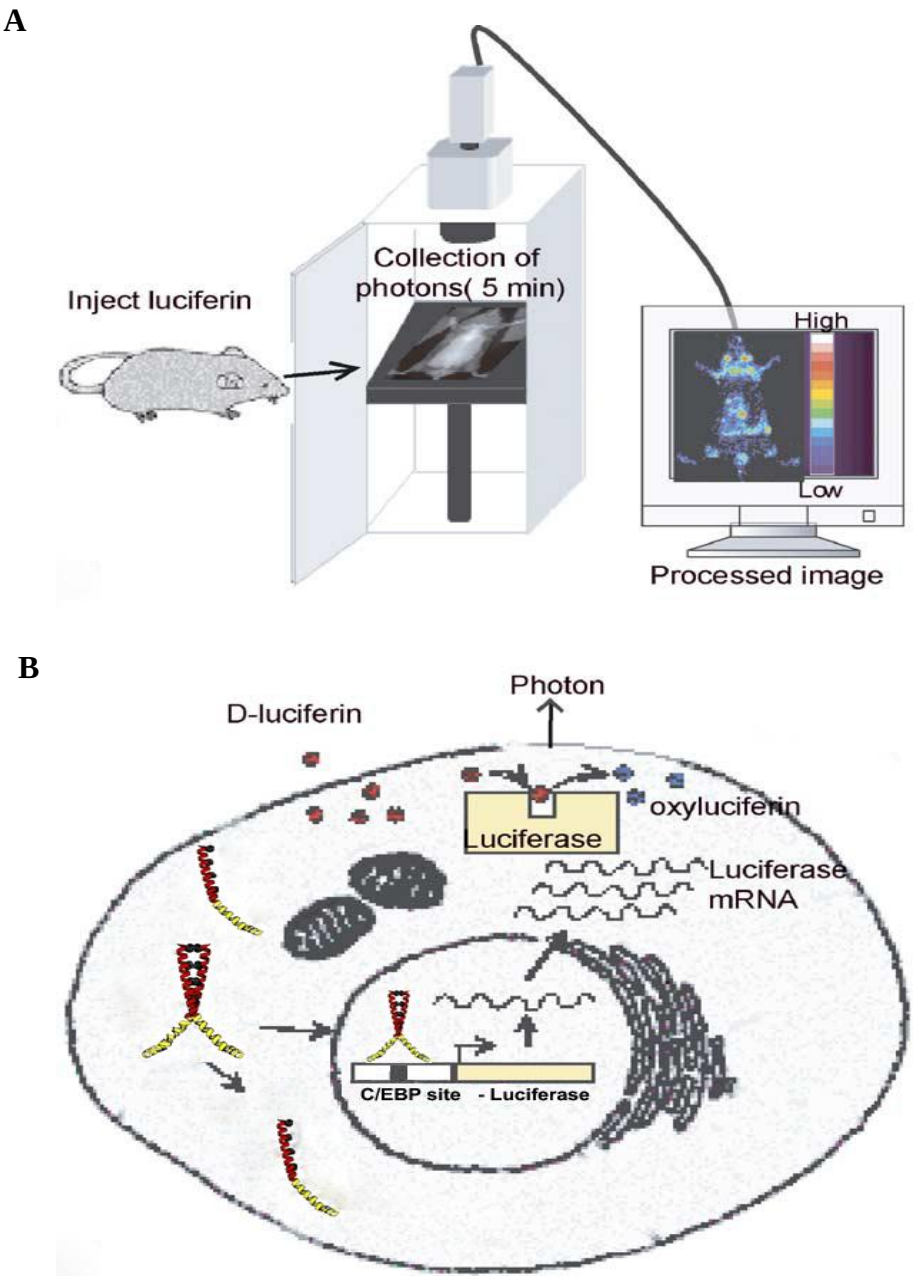


Fig. 35 Schematic overview of non-invasive bioluminescent imaging of transgenic reporter mice: (A) D-Luciferin (2 mg per mouse) is injected intraperitoneally. Shortly afterwards the mice are placed in a light sealed cabinet for a 5 min light collection. The light is amplified and finally processed digitally. (B) After the injection of luciferin, the substrate is taken up by the cells, where the intracellular amount of C/EBP mediated luciferase determines the light output from each cell. Modified from (Carlsen et al., 2004).

Validation of the model

The C/EBP luciferase mice generated in our laboratory were the result of the screening of fifteen different founders by biochemical analyzes of the luciferase activity in various tissues following LPS injection. The mouse line selected reported faithfully the expected increases in C/EBPs activity after stimulation with LPS. However, this upregulation in the C/EBP expression was only observed in the brain (Fig. 36). C/EBP activity was not detected in transgenic animals not injected with LPS *in vivo* (Fig. 37A). The pronounced increase in luciferase activity in the brain, detected biochemically with the luciferase assay, was corroborated by in vivo imaging observations after injection with LPS (Fig. 37B).

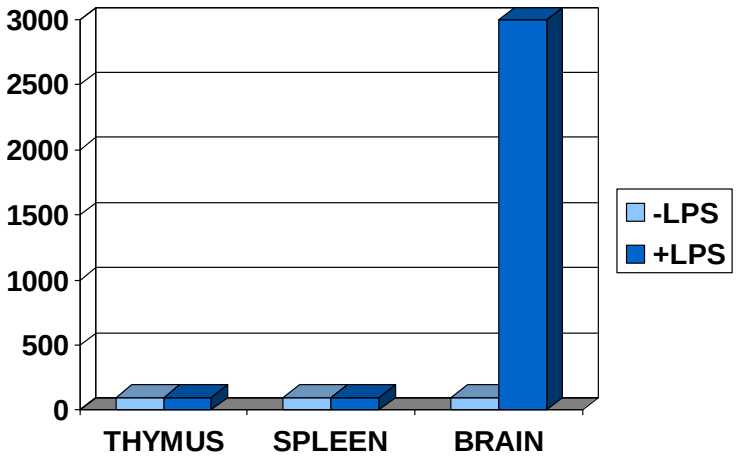


Fig. 36 C/EBP upregulation in the brain of transgenic mice following LPS injection: The luciferase assay did not detect expression of C/EBPs in thymus or liver of transgenic animals injected with LPS. Increased C/EBP expression in the brain of transgenic animals was detected 4 hrs after LPS injection. As control transgenic animals without LPS injection were used.

- LPS **+ LPS**

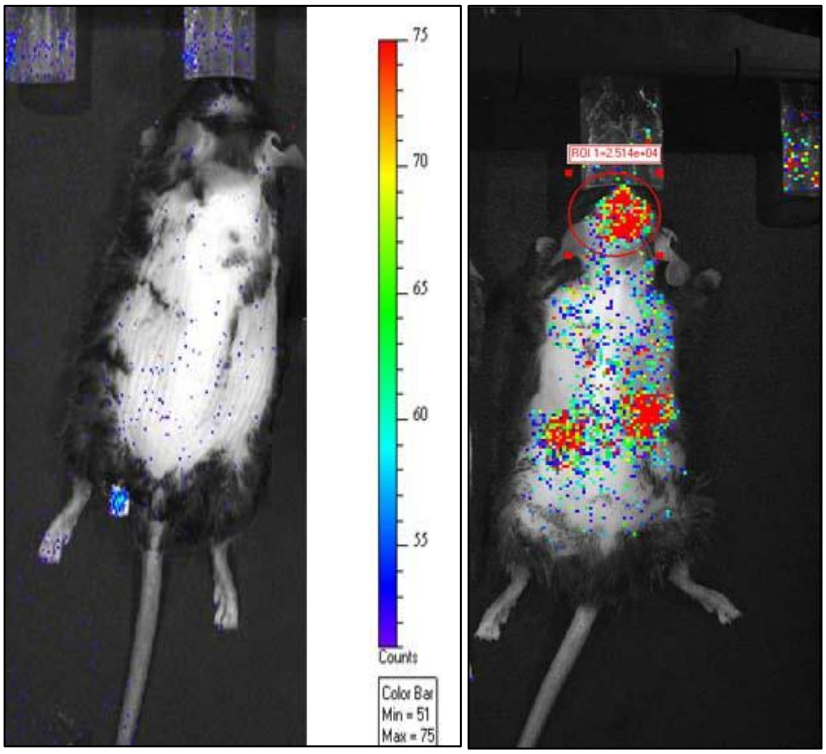


Fig. 37 Molecular imaging of C/EBP activity in transgenic animals: Non-invasive imaging of induced C/EBP activity as seen in mice treated with or without LPS (2 mg/kg). The image was taken 4 hrs following LPS injection.

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