Transcriptional Regulation & Downstream Effectors of Peripheral Nerve Regeneration

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DECLARATION

I, Smriti Patodia, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Unlike the central nervous system, robust axon regeneration after peripheral nerve injury is driven by transcriptional activation of a regeneration programme. Transcription factors form a vital link in the chain of regeneration, converting injury-induced stress signals into increased expression of a wide range of downstream effector molecules like neuropeptides, neurotrophic factors, adhesion molecules and cytoskeletal adaptor proteins. To gain insight into the *in vivo* function of some of these regeneration associated molecules, we examined the effects of global or cell-type specific, and single and combined deletions of transcription factors - STAT3, c-Jun and C/EBPδ, and effector molecules - CAP23 and integrin β1, on axonal regeneration after facial nerve axotomy. The facial nerve axotomy model is a well-established experimental paradigm, providing insights into molecular signals that determine axonal regeneration, target re-innervation and neuronal cell death.

Neuronal STAT3 deletion (STAT3ΔS) caused profound and persistent defects in regeneration and functional recovery after mild (crush) and severe (cut) nerve injuries. Axotomised STAT3-deficient motoneurons appeared shrunk by 50-60% in size but displayed no cell death. There was also a severe reduction in microglial activation and recruitment of lymphocytes, in the expression of regeneration-associated molecules CD44, α7β1 integrin, in the nuclear translocation of ATF3, and in perineuronal sprouting of the CGRP+ and galanin+ facial motoneurons. Deletion of STAT3 in Schwann cells produced no apparent deficiencies in regeneration.

As with neuronal STAT3 deletion, neuronal deletion of c-Jun (c-JunΔS) (Raivich et al, 2004; Ruff et al, 2012) abolishes most of the cell body response, neuronal cell death, and re-innervation after axotomy. Deletion of c-Jun in peripheral nerve Schwann cells (c-JunΔP0) had a very different effect. Neuronal cell death was increased by 2-3 fold, even though most of the cell body response was not affected. Axonal regeneration was reduced, but most of the defect in target re-innervation and functional recovery appeared to be due to excessive neuronal cell death.

Mutants lacking both neuronal c-jun and STAT3 did not show more exacerbated regeneration defects than the single deletions, suggesting that deletion of either

transcription factor – c-Jun or STAT3 – will produce a saturating effect on the regeneration-deficient phenotype. Expression of c-jun itself was not affected in the neuronal STAT3 null mice, and vice versa, confirming the above hypothesis.

Double deletion of c-jun in both neurons and in Schwann cells, blocked neuronal cell death seen in Schwann cell c-Jun deficient mutants, and completely obliterated target re-innervation after facial axotomy, suppressing even the mild 20% regeneration seen in the neuronal c-jun null mutants. Since both neuronal and Schwann cell c-Jun are phosphorylated at its N-terminus following nerve injury, we also explored the effects of c-Jun phosphorylation. Global replacement of all 4 N-terminal c-Jun phosphorylation sites (Ser63&73, Thr91&93) with alanines (c-jun4A) produced a significant increase (1.8x) in neuronal cell death, an approximate 40% reduction in target re-innervation and delayed functional recovery. This phenotype was more alike that observed with Schwann cell rather than neuronal c-Jun deletion.

Global C/EBPδ deletion reduced axotomy induced neuronal cell death, and had moderate effects on microglial activation and axonal sprouting. Combined deletion of C/EBPδ and STAT3 did not exacerbate the defect in regeneration seen with STAT3 deletion alone, but seemed to speed up functional recovery.

Deletion of neuronal CAP23, a downstream effector gene, led to impaired target re-innervation, reduced galanin+ perineuronal sprouting and early microglial function. Neuron-specific deletion of beta 1 integrin, another effector molecule, resulted in a 2.5-fold increase in neuronal cell death, a commensurate 60% reduction in target re-innervation and transiently delayed functional recovery. This phenotype was similar to c-junΔP0 and c-jun4A mutants.

In summary, there appears to be two neuronal sub-populations which are controlled by different aspects of the regenerative programme A) one dependent on 'intrinsic' activation of transcriptional master switches like c-jun and STAT3 in neurons and B) the other on 'extrinsic' post-traumatic trophic signalling elicited by the Jun-expressing and N-terminal phosphorylation-dependent Schwann cells. c-Jun phosphorylation and neuronal β1 integrin appear to be critical co-factors in the signalling response elicited by the Schwann cells.

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APRF – acute phase response factor
ATM - ataxia telangiectasia mutated
AUC – area under curve
ATF2/3 - activating transcription factor 2/3
BDNF - brain-derived neurotrophic factor
CAP23 – cytoskeletal associated protein 23
C/EBP - CCAAT/enhancer binding protein
CGRP – calcitonin gene-related peptide
CREB – cAMP response element binding protein
CNS – central nervous system
CNTF - ciliary neurotrophic factor
DINE – damage induced neuronal endopeptidase
DRG – dorsal root ganglion
ECM – extra-cellular matrix
EDTA – ethylene diamine tetra-acetic acid
EGF - epidermal growth factor
EPO – erythropoietin
ERK – extra-cellular signal-related kinase
FG – fluoro-gold
FGF – fibroblast growth factor
FNA – facial nerve axotomy
GAP43 – growth associated protein 43
GAS - gamma activated sequence
G-CSF - granulocyte-colony stimulating factor
GDC – granular disintegration of cytoskeleton
GDNF- glial cell-derived neurotrophic factor
GFAP – glial fibrillary acidic protein
GSK3 – glycogen synthase kinase 3
ICAM 1- inter cellular adhesion molecule -1
IEG – immediate early gene
IFNγ – interferon gamma
IGF-1/2 - insulin-like growth factor-1/2
IHC – immuno-histo-chemistry
IL-1/6 - interleukin-1/6
ISRE - interferon stimulated regulating element
Jab1 - Jun activation-domain binding protein 1
JAK – Janus kinase
JIP – JNK interacting protein
JNK- jun N-terminal kinase
JNP – jun N-terminal phosphorylation
KLF – Kruppel-like factors
KO- knock-out

LIF - leukaemia inhibitory factor
LPS – lipo-polysaccharide
MAG – myelin associated glycoprotein
MAP(K) – mitogen activated protein (kinase)
M-CSF – macrophage colony stimulating factor
MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR – MiniRuby
NFAT – nuclear factor of activated T-cells
NFκB – nuclear factor kappa-light-chain enhancer of activated B cells
NF-L – neurofilament light chain
NGF - nerve growth factor
NLS- nuclear localisation signal
NT-3 - neurotrophin-3
NT- 4/5 - neurotrophin-4/5
NT-6 - neurotrophin-6
NT-7 - neurotrophin-7
OLV – optical luminosity value
PACAP – pituitary adenylate cyclase activating peptide
PDGF - platelet-derived growth factor
PIP2 - phosphoinositol-4,5-diphosphate
PNI – peripheral nerve injury
PNR – peripheral nerve regeneration
PNS – peripheral nervous system
PTEN – phosphatase and tensin homolog
RAG – regeneration associated gene
SAR – survival adjusted re-innervation
SC – schwann cell
SCG – superior cervical ganglion
SEM – standard error of mean
SH2 – sequence homology 2
SIE- sis-inducible element
SOCS3 - suppressor of cytokine signalling 3
Sox11 – SRY-box containing gene 11
SPRR1A – small proline rich protein 1A
SRF – serum response factor
STAT- signal transducer and activator of transcription
TDP43 – TAR-DNA binding protein 43
TF – transcription factor
TGF- transforming growth factor
TNF – tumour necrosis factor
VIP - vasoactive intestinal peptide
WD- wallerian degeneration
WHM – whisker hair movement

CHAPTER 1 – Introduction

Injury to peripheral nerves triggers a regenerative programme associated with neurite outgrowth, re-innervation of the denervated target and recovery of function. This includes a series of molecular, cellular and ultrastructural responses. The rapid arrival of signals from the injured axon results in a remarkable shift of the injured neuron from a transmitting to a growth promoting phenotype. This is accomplished by the upregulation of a vast array of regeneration-associated genes (RAGs) including the rapid induction of transcription factors and enhanced synthesis of adhesion molecules, growth associated proteins, cytoskeletal elements, cytokines, neuropeptides and other molecules involved in regeneration. The molecular changes are accompanied by pronounced morphological changes in the surviving neurons – the cell body undergoes a 'chromatolytic' reaction characterised by swelling of the neuronal body, increase in cellular metabolism and protein synthesis, and a regional dispersion of Nissl bodies in the neuronal cytoplasm (Lieberman, 1971); and there is a rapid appearance of growth cones at the proximal tip of the lesioned axons; The distal nerve stump undergoes 'Wallerian degeneration'(Waller, 1850), leading to phagocytosis of axonal and myelin debris initially by Schwann cells and later also by invading macrophages (Stoll et al., 1989;Bignami and Ralston, III, 1969).

In addition to the neuronal response, neighbouring non-neuronal glial cells – in the case of brain and spinal cord motoneurons, the astrocytes and microglia - become activated, with microglial expression of immune cell recognition molecules (ICAM1, αMβ2 and αXβ2 integrins, B7 system etc.) and major histocompatibility complex (Bohatschek et al., 2004). There is also a rapid microglia-associated recruitment of lymphocytes within a day after injury which may assist with immune surveillance first of the injured neurons, and later on of the dead neurons (Kalla et al., 2001;Raivich et al., 1998). In the adult mouse facial motor nucleus model which has been studied in extensive detail, neuronal cell death is a delayed phenomenon, peaking at 2 weeks following nerve transection; the immuno-glial activation is characterized by two phases – a fast but moderate response starting within 24h after neuronal injury, and a late but much stronger response to neuronal cell death, peaking at 14 days after axotomy. Neuronal transcription factor deletions blocking cell death will suppress most of the late glioimmune response (Raivich et al., 2004). However, these deletions also inhibit some of the early microglial activation – within 24h and long

before the appearance of cell death, suggesting that these transcription factors may also interfere with the synthesis of injury signals from lesioned but not dying neurons.

In contrast with the PNS, the CNS neurons normally fail to regenerate after injury, possibly due to a combination of diminished intrinsic capacity for regeneration and heightened susceptibility to inhibitory factors of their extracellular environment (Sun et al., 2011;Maier and Schwab, 2006). Identifying the crucial elements responsible for successful regeneration in injured peripheral nerves, and unravelling their underlying signalling pathways and molecular components, is therefore, quintessential in improving regenerative outcomes after peripheral and central nerve injuries. Systematic gene expression profiling using cDNA microarrays has led to a dramatic increase in the number of identified genes regulated in the injured and regenerating neurons (Boeshore et al., 2004;Bosse et al., 2001;Bosse et al., 2006;Kubo et al., 2002;Kury et al., 2004;Michaelevski et al., 2010). Interestingly, roughly half of the regeneration-associated genes are part of both developmental and lesion-induced programmes, suggesting that regeneration partially recapitulates development. However, this proportion of shared developmental and lesion-induced transcripts is substantially lower – only 33% - for genes encoding signal transducers or factors involved in processes such as cell death, immune response, transport and transcriptional regulation. Most of the latter show injury-specific gene expression (Bosse et al., 2006).

Transcription factors are DNA-binding proteins that can activate or repress target gene expression. Their effects are amplified by their ability to bind to multiple promoter regions, causing a large number of target genes to be switched on or off by a single transcription factor. This is especially interesting because deletion of an individual transcription factor can give rise to a vast number of regeneration defects. This introductory chapter aims to summarise the key events ensuing axonal injury in the PNS - the structural, biochemical and molecular changes taking place in the neuronal cell body and the axons, and the contribution of non-neuronal cellular responses. Particular attention has been focused on regeneration associated transcription factors and their downstream signalling targets involved in mounting a successful regenerative response.

Structural changes after peripheral nerve injury

Morphological changes in the neuronal perikaryon

The neuronal cell body response to axotomy (also known as chromatolysis/retrograde reaction) is characterised by the dispersion of parallely arranged rough endoplasmic reticulum cisternae or Nissl bodies into a random, non-linear appearance. The neuronal cell body swells up, the nucleus is displaced to the cellular periphery and the synaptic terminals are retracted. This phenomenon was first described by Frank Nissl in 1894, and it provides a visible evidence of the transformation of the neuronal function from synaptic transmission to growth and repair. The neuronal cell surface gets covered by microglial cells (Raivich and Makwana, 2007). In successfully regenerating axons, the cell bodies remain swollen throughout the regrowth period and are associated with a massive upheaval of metabolism and protein synthesis. In neurons that fail to regenerate, for example those in the CNS, the neuronal cell bodies begin to atrophy both in cell volume and in the number of dendrites, and may persist in the atrophied state for long periods of time (Lieberman, 1971), highlighting the significance of the cell body reaction in successful regeneration

Axonal changes distal to the lesion site & Schwann cell reaction

Following peripheral nerve injury, the portion of the nerve distal to the injury site undergoes a process known as Wallerian degeneration (WD). WD is a set of molecular and cellular events by which degenerating axons and myelin are cleared after injury, and was first described by Augustus Waller in 1850 (Waller, 1850). Nerve injury is quickly followed by the appearance of axolemmal blebs (swelling of the axonal membrane) between 8 and 24 h after injury, and the granular disintegration of the axonal cytoskeleton (GDC). GDC is the disassembly of microtubules, neurofilaments, and other cytoskeleton components resulting in the fragmentation of the axon and this process is usually complete within 3 – 10 days in the PNS (Bignami et al., 1981;Bignami and Ralston, III, 1969;Bignami et al., 1981;Bignami et al., 1981). The myelin sheath also swells, eventually becoming fragmented and breaking down into fatty material that is deposited around the axon (Lassmann et al., 1978;Lassmann et al., 1978).
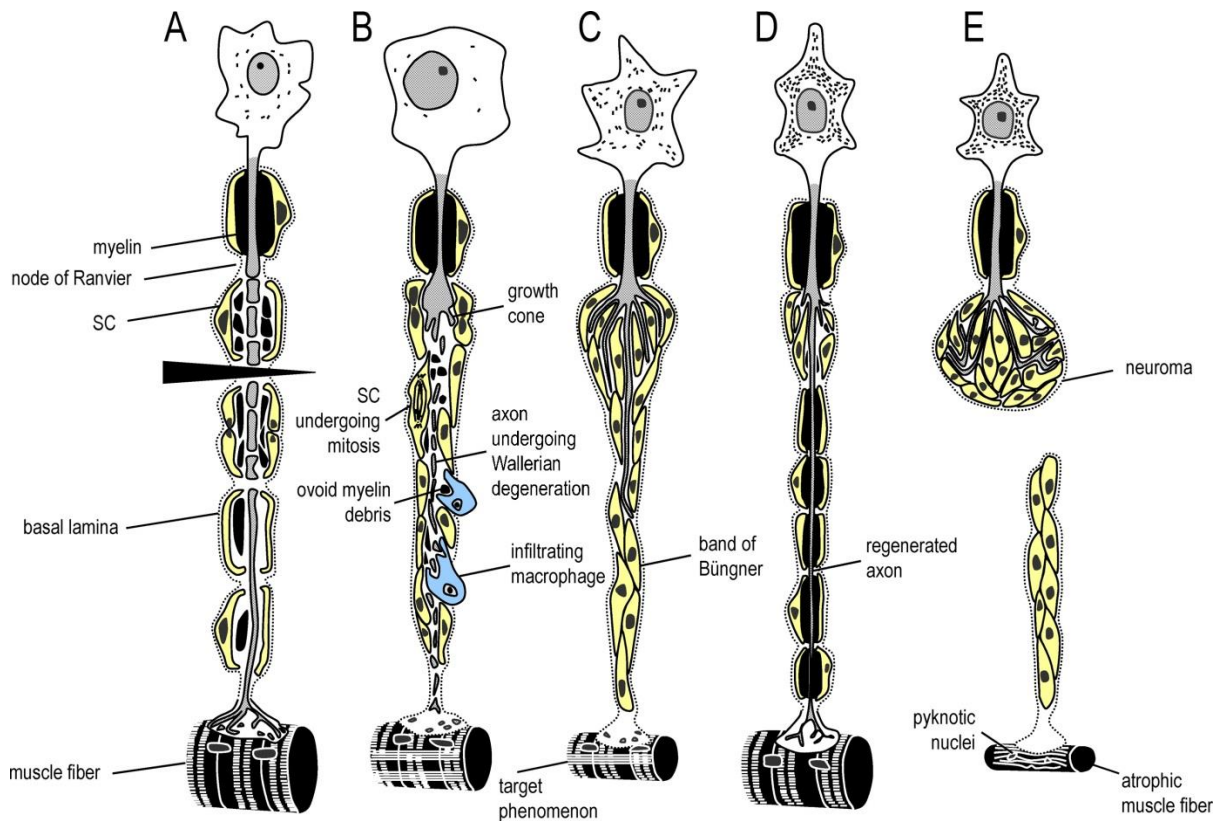
Figure 1.1. Schematic representation of the degenerative and regenerative events associated with peripheral nerve injury. **A.** During the early phase (first few days) after axonal injury (arrowhead), local degenerative events are accompanied by both retrograde and anterograde degeneration of axon and myelin. **B.** During the intermediate phase (a few days to weeks), the anterograde pattern of Wallerian degeneration proceeds to completion with infiltrating macrophages contributing to the removal of tissue debris and SC undergoing mitosis. The axotomised neuronal cell body undergoes reactive, chromatolytic changes and the severed proximal end of the axon develops regenerative axonal sprouts. **C.** Of the numerous axonal sprouts that successfully traverse the injury site (during the first few weeks to months), some re-enter appropriate endoneurial tubes and continue to extend through the distal nerve stump, supported by SC in the bands of Büngner. The target organ/tissue (in this case skeletal muscle) undergoes disuse atrophy. **D.** Successful axon regeneration through the bands of Büngner and the re-establishment of neurotransmission at the neuromuscular junction results in the retraction or dying-back of unsuccessful axon sprouts, the reversal of muscle fibre atrophy, of neuronal cell body chromatolysis and the establishment of maturing SC–axon interactions (including reduced internodal spacing). **E.** Failure of regenerating axonal sprouts to cross the injury site (possibly due to the formation of a physical scarring barrier or the loss of a large segment of nerve) results in neuroma formation. The permanently denervated muscle fibres demonstrate severe atrophy, loss of their characteristic striations and pyknotic nuclei (Reproduced from 'Repairing injured peripheral nerves: Bridging the gap') (Deumens et al., 2010).

Schwann cells (SCs) are crucial for early clearing of neuronal debris and providing directional guidance cues to the regenerating axons. They undergo nuclear and cytoplasmic hypertrophy, and begin to phagocytose axonal and myelin ellipsoids with thick vimentin-rich processes (Liu et al., 1995). 1-3 days after injury, they are joined by macrophages, infiltrating via the disrupted blood brain barrier to complete the destruction

and phagocytosis of all debris (Perry et al., 1987). In all, the process of clearing takes about two weeks. The rapid clearing of neuronal debris is a hallmark of successful regeneration in the peripheral nerves, not seen after CNS injury. The SCs become mitotic and proliferate rapidly, aligning themselves into columns called Bands of Büngner, along the remaining connective tissue basement membrane to provide a path for axon regrowth (Gordon and Gordon, 2010). SCs are also able to attract injured neurons and aid axon elongation by secreting neurotrophic factors such as NGF, BDNF, NT-4 and GDNF and other proteins like F-spondin, collagen, laminin and fibronectin (Bhatheja and Field, 2006;Gordon and Gordon, 2010;Raivich and Makwana, 2007).

Axonal changes proximal to the lesion site – Growth Cone Formation

While the distal stump undergoes degeneration, the proximal stump begins regeneration. Calcium influx after nerve injury signals the resealing of the proximal severed end by a vesicle mediated process. The axon forms a bulbous swelling at the tip due, partly, to the arrival of new axoplasm including cytoskeletal components like neurofilaments and microtubules (Meller, 1987), eventually giving rise to a club-like structure called a 'growth cone' as described by Ramon y Cajal in 1890 (Garcia-Marin et al., 2009). Growth cone formation is associated with accumulation of F-actin at the leading edge, and changes in intracellular calcium and sodium ion levels (Dent and Gertler, 2003).

The growth cone plays a critical role in accurate neurite outgrowth for target re-innervation, by interaction with surrounding non-neuronal cells and the extracellular matrix. Numerous filamentous projections made from actin and tubulin protrude from the injured tip, extending in to the extracellular milieu where they guide growth by searching for pro-regenerative guidance cues (Spira et al., 2003). Regenerating axon tips are progressively re-myelinated as they grow within the SC-lined endoneurial tubes.

Molecular changes after peripheral nerve injury

Early axonal injury sensors

Axonal injury generates three major signalling cues to the injured neurons which triggers a complex, regenerative programme in the neuronal cell body. Firstly, axonal injury interferes with the retrograde flow of trophic signals, disinhibiting the normally suppressed

regenerative process, within 12-24 hours following injury (Raivich et al., 1991b). Secondly, it exposes the tip of the injured axon to the intracellular content of neighbouring axons and SCs containing growth factors like LIF, CNTF, FGF and NT3 (Sendtner et al., 1997) and later to the inflamed neural tissue environment (Lindholm et al., 1987). The above two processes can result in de novo activated molecules carrying a nuclear localisation signal (NLS) which link to importins and are retrogradely transported to the cell body by dynein motors (Hanz et al., 2003;Curtis et al., 1994;Curtis et al., 1993). Recently, it has emerged that injury-induced transcription factors can also be axonally translated and retrogradely transported to the nucleus upon nerve injury (Ben-Yaakov et al., 2012). Thirdly, it causes a disruption of the tight ionic concentration gradient between the axon and the ECM by rapid influx of extracellular ions such as calcium and sodium through the transiently open plasmalemma before it is resealed. This results in depolarisation and transmission of successive injury-mediated action potentials. These molecular and electrical signals cause rapid elevation of calcium and cAMP, which in turn activate multiple signalling pathways (Berdan et al., 1993).

Early signalling in neuronal perikaryon

A crucial aspect of early neuronal signalling is the activation of a diverse array of intracellular signalling cascades. Mitogen activated protein (MAP) kinases pathways represent an important family of retrogradely transported signalling molecules through which extracellular signals are converted in to an intracellular response. Subfamily members of this group include ERK, p38 and JNK, which respond to various cytokines, growth factors and signals of cellular stress by phosphorylation of several substrates including transcription factors, cytoskeletal components and nuclear receptors (Lindwall and Kanje, 2005). MAPK subfamily members are activated themselves by phosphorylation by mitogen-associated protein kinase kinases (MEK) via extensive and convoluted ras /raf pathways (Chang et al., 2003). Recent studies have implicated the involvement of abl and AKT protein kinases and mTOR in generating a specific injury-induced response (Michaelevski et al., 2010). PTEN inactivation is known to activate different downstream pathways, such as Akt and mTOR signalling, and inhibit other signalling molecules, such as GSK-3 and PIP3 (Liu et al., 2010). Downstream effects of the signalling cascades are varied and include activation of specific nuclear transcription factors.

Transcriptional changes

The arrival of injury signals is rapidly followed by the phosphorylation and nuclear localisation of a host of transcription factors. A brief summary of signalling from early sensors to mostly enzymatic cytoplasmic mediators to transcription factors and synthesis of effector molecules is shown in Figure 1.2.

Data from phospho-proteomic and microarray studies have identified nearly 400 redundant axonal signalling networks connected to 39 transcription factors (26 transcription factor families), implicated in the sensory neuron response to axonal injury (Michaelevski et al., 2010). These include c-jun, jun D, ATF3 (Activating transcription factor 3) , CREB (cAMP response element binding protein), STAT3 (Signal transducer and activator of transcription 3), C/EBPs (CCAAT/enhancer binding proteins), p53, Oct-6, NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells), NFATs (Nuclear factor of activated T-cells), KLFs (Kruppel-like factors), Sox11, SnoN, ELK-3, P311, and E47 among others (Magoulas B., 2010;Mason et al., 2003;Moore and Goldberg, 2011;Nadeau et al., 2005;Raivich et al., 2004;Raivich, 2011;Schwaiger et al., 2000;Jankowski et al., 2009;Ruff et al., 2012a;Di Giovanni et al., 2006;Moore et al., 2011). Transcription factors provide a vital link between injury- induced signals and downstream protein expression via gene regulation. Once in the nucleus, they bind to selective DNA promoter regions to increase or repress transcription of specific target genes. Nerve injury can also result in reduced activation of transcription factors such as islet-1, Fra-2, ATF2 and TDP43 (Doyle and Hunt, 1997;Herdegen et al., 1997a;Hol et al., 1999;Moisse et al., 2009;Sato et al., 2009), which probably contributes to the change in gene expression of the injured neuron from a fully differentiated to a growing phenotype (Raivich, 2011). The activation of transcriptional programme is critical for expression of many
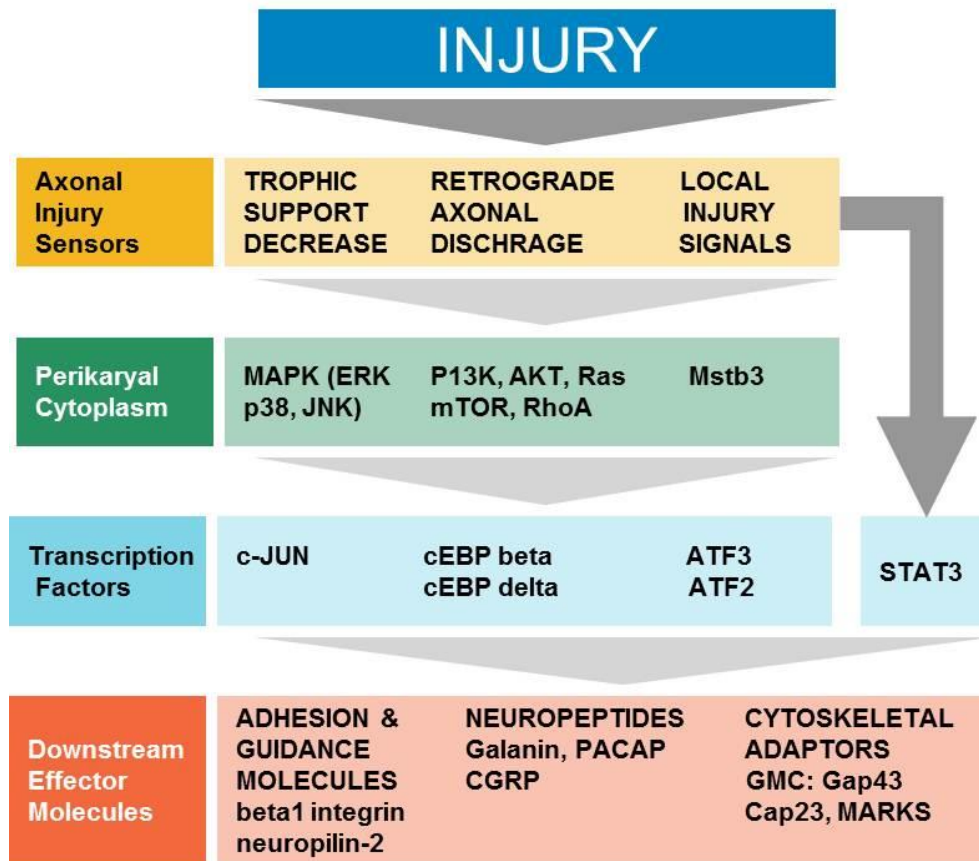
Figure 1.2. Cellular signalling in successful regeneration, from early sensors of injury, to cytoplasmic signals, transcription and downstream effectors (Modified (Raivich, 2011).

target genes implicated in successful regeneration, and blocking of transcription at an early time point after injury frequently changes the regenerative response of injured neurons (Smith and Skene, 1997). Recent advances in cre/loxP technology permitting cell-type and/or time specific genetic knockouts (Sauer, 1998;Akira, 2000) have begun to provide insight into the powerful roles of these transcription factors in orchestrating complex axon growth and regenerative responses. Neuronal deletion of transcription factors frequently causes reduced regenerative ability. An overview of the observed phenotypes is given in Table 1.1, and the individual transcription factors discussed in more detail in the following sections.

Transcription factor G KO or CTS KO	Injury model	KO Phenotype	References
Neuronal c-Jun (CTS)	Facial n.	• Strongly reduced target re-innervation • Delayed functional recovery • Decreased RAG expression and neuronal sprouting • Strongly reduced glial activation and leucocyte recruitment • Enhanced motoneuron survival • Cellular atrophy	Raivich et al., 2004, Makwana et al., 2010, Ruff et al., 2012
Schwann cell c-Jun (CTS)	Facial n. Sciatic n.	• Severely impaired target re-innervation and functional recovery • Drastically reduced motoneuron survival • Delayed myelin breakdown • Reduced trophic factor expression	Fontana et al., 2012 Arthur-Farraj et al., 2012
Jun 2A (G)	Facial n.	• Cellular atrophy	Ruff et al., 2012
Neuronal STAT3 (CTS)	Saphenous n. Facial n. Sciatic n.	• Decreased sprouting • Impaired axonal growth • Phase-specific regulator of growth 'initiation' • Reduced motoneuron survival	Bareyre et al., 2011, Schweizer et al., 2002, Ben-Yaakov et al., 2012
ATF3 (G)	Sciatic n.	• Enhanced speed of regeneration, and increased expression of RAGs in mice constitutively expressing ATF3 in DRGs	Seijffers et al., 2007
CREB (G)		• Impaired axonal/neurite growth • Increased apoptosis of sensory neurons	Lonze et al., 2002, Lonze and Ginty, 2002, Redmond et al., 2002
C/EBP β (G)	Facial n.	• Reduced expression of microtubule Tα1 α tubulin and growth cone protein GAP-43	Nadeau et al., 2005
C/EBP δ (G)	Sciatic n.	• Impaired axonal growth and reduced response to conditioning lesion • Delayed functional recovery • Decreased RAG expression	Lopez de & Magoulas, 2013
SRF (G)	Facial n.	• Impaired neurite outgrowth, growth cone shape and axon guidance • Increased motoneuron death	Stern et al., 2012
Sox 11 (CTS)	Saphenous n.	• Reduced regeneration after nerve crush	Jankowski et al., 2009
p53 (G)	Facial n.	• Decreased neurite outgrowth • Reduced target re-innervation • Decreased motoneuron death	Di Giovanni et al., 2006, Tedeschi et al., 2009, Qin et al., 2009

Table 1.1. Transcription factor deletions and peripheral nerve regeneration: Effects of global (G) and cell type specific (cts) knockouts (KO)

STAT3

Signal Transducer and Activator of Transcription – 3 (STAT3) belongs to the STAT family of seven transcription factors - STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 - that mediate a wide variety of biological functions in the CNS & PNS such as cell

growth, regulation, inflammation and embryological development (Dziennis and Alkayed, 2008).
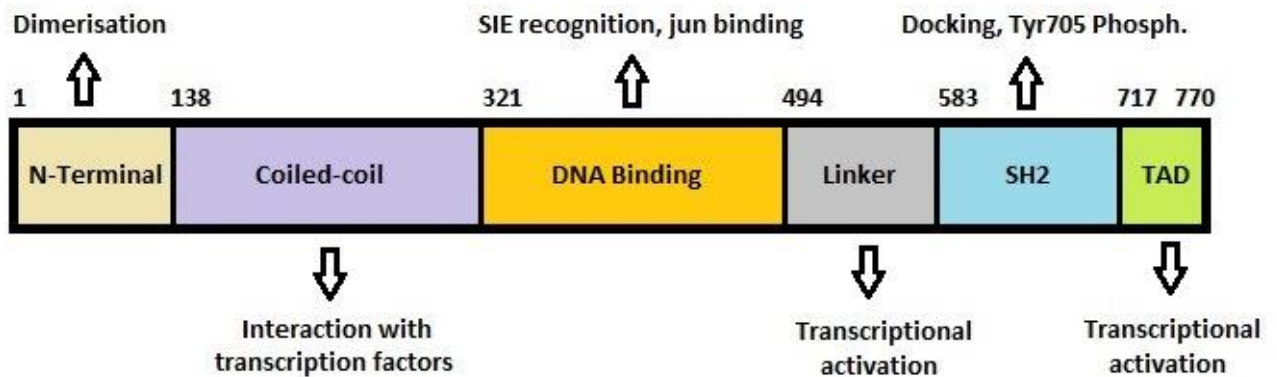
Figure 1.3. Schematic diagram showing the functional domains of STAT3

The STATs are comprised of six regular domains - an amino terminal (for STAT3 dimerisation after activation), a coiled-coil domain (for interaction with other transcription factors and regulatory proteins), a DNA binding domain made up of β-sheets (for recognition of consensus binding regions on gene promoters), an α-helical linker domain (for transcriptional activation and protein-protein interaction), a classical SH2 (sequence – homology) docking domain, and a transcriptional activation domain (Lim and Cao, 2006;Bromberg and Darnell, 2000). There are 2 highly conserved, phosphorylatable amino acids that are frequently required for STAT3 activation – a tyrosine 705 in the SH2 domain and a serine 727 in the 78-amino acid region of the transactivation domain (Bromberg and Darnell, 2000;Dziennis and Alkayed, 2008). Figure 1.3 shows a schematic diagram of the functional domains of STAT3.

STAT3 was identified as an acute phase response factor (Akira et al., 1994) and is expressed cytoplasmically in both neuronal and glial cells during development and in the adult (Gautron et al., 2006;De-Fraja et al., 1998). In the adult brain, STATs are normally quiescent, but are activated in specific subsets of neurons following injury (Schwaiger et al., 2000;Xia et al., 2002) in response to multiple signalling pathways induced by several cytokines and growth factors including IL-6, LIF, CNTF, G-CSF, G-CSFR, EPO, EGF, IGF-1, NGF withdrawal, BDNF, free radicals, excitatory neurotransmitters and other inflammatory mediators that are released after injury (reviewed in (Dziennis and Alkayed, 2008). Propagation of many of these signals (IL-6, LIF, and CNTF) requires a combination of various ligand binding units and a common signal-transducing unit gp130 (Taga and

Kishimoto, 1995), through which they activate the JAK/STAT, Ras/MAPK, ERK, PI3K and other pathways (Akira, 2000;Heinrich et al., 2003). Activation of the gp130 receptor has been implicated in neuronal response to injury and regenerative axonal growth (Habecker et al., 2009).

Injury induced ligand binding to cell-surface receptors activates the associated receptor tyrosine kinase Janus kinases (JAKs), which phosphorylate tyrosine residues on the cytoplasmic portion of the receptor complex that acts as a binding site for STAT proteins. STATs dock onto the phospho-tyrosines via their SH2 domain, and are also phosphorylated by JAK on a tyrosine residue (e.g. Y705 in STAT3). Upon activation, they either hetero- or homodimerise and translocate to the nucleus via importins (Cimica et al., 2011), where they bind to consensus sequences (SIE/GAS/ISRE sequences) to induce gene transcription. Other mechanisms of STAT activation include phosphorylation by non-receptor tyrosine kinases such as Src, direct or indirect phosphorylation by receptors with intrinsic tyrosine kinase activity (i.e. EGF, PDGF, and FGF receptors), and activation by G-protein coupled receptors (Ram and Iyengar, 2001). STAT3 can be deactivated by dephosphorylation or targeted degradation, and recycled back to the cytosol (reviewed by (Dziennis and Alkayed, 2008;Moore and Goldberg, 2011;Lim and Cao, 2006). Further, Suppressor of Cytokine Signalling 3 (SOCS3) or Protein Inhibitors of Activated STATS (PIAS) can also inhibit STAT3 activation (Krebs and Hilton, 2001).

Global STAT3 knockout animals are embryonic lethal, dying prior to gastrulation between E6.5 and 7.5 (Takeda et al., 1997). Cell-type specific STAT3 deletion has greatly improved our knowledge of its function in different tissues (Akira, 2000;Sauer, 1998). STAT3 deletion in T-cells is associated with impaired T-cell proliferation, in macrophages with high susceptibility to endotoxin shock, in keratinocytes with compromised wound healing process, and in mammary glands with delay of involution (Akira, 2000). Nestin-promoter mediated neural-specific disruption of STAT3 resulted in mice which were hyperphagic, obese, diabetic, and infertile (Gao et al., 2004). In the last decade, there has been growing evidence of a role of STAT3 in axonal regeneration. In a recent broad screen for transcription factors involved in regeneration, STAT3 was identified in two independent studies using different approaches (Michaelevski et al., 2010;Smith et al., 2011).

In vitro, STAT3 induces neurite growth in motor and sensory neurons and this growth can be inhibited by the application of SOCS3 or the JAK inhibitor AG490 (Miao et al., 2006;Liu and Snider, 2001). Interestingly, blockade of the ERK pathway had no such effect. STAT3 induces GAP-43, a protein found in growth cones, and GAP-43 expression is inhibited with AG-490, suggesting a role for the JAK/STAT3 pathway in GAP-43 expression and neurite outgrowth and regeneration (Wu and Bradshaw, 1996). Similarly, Qiu et al. blocked regeneration of DRG neurons into the dorsal columns after a conditioning lesion using the same drug in vivo (Qiu et al., 2005).

In 1999, Haas and colleagues observed STAT3 activation at 24h and 5d after rat facial nerve axotomy (Haas et al., 1999). Following rat facial and hypoglossal nerve axotomy, a transient but significant increase in STAT3 mRNA, along with its phosphorylation and nuclear translocation within 3 hours in neurons and 1 day in astrocytes was observed by Schwaiger and his colleagues in 2000. In contrast, these changes were not observed in non-regenerating neurons of Clarke's nucleus, or in the CNS dorsal columns, although both forms of injury upregulated c-jun and GAP43 expression (Schwaiger et al., 2000;Qiu et al., 2005). Transient increases in STAT3 signalling were also found in sprouting neurites and astrocytes following entorhinal cortex lesions in addition to a rise in gp130-receptor cytokines, suggesting a dual role of STAT3 in both astrocytic regulation and axonal sprouting (Xia et al., 2002). In 2004, Nancy Lee and her colleagues showed that axonal STAT3, activated at the injury site, acts as both a retrograde injury signal and as a transcription factor which promotes the survival and regeneration of both sensory and motor neurons. Levels of activated STAT3 increased in the nuclei of regenerating adult cranial motor neurons, sciatic motor neurons and in sensory neuron nuclei after injury. Sciatic nerve lesion led to a very rapid activation of STAT3 in axons at the lesion site within 15 minutes, and by 24 hours STAT3 labelling was detected in the DRG and in the spinal cord (Lee et al., 2004). These levels of STAT3 decreased when regeneration was completed, emphasizing its role in the regenerative process. A conditional knockout of STAT3 in DRG neurons greatly reduced nerve outgrowth from the saphenous nerve after transection (Bareyre et al., 2011).

Local activation and retrograde transport of transcription factors, including STAT3, have been now described in several recent studies. In cell bodies of facial motor neurons, STAT activation following nerve injury can be delayed by three days following treatment

with a blocker of axonal transport, colchicine (Kirsch et al., 2003). Shin et al, have suggested a role of dual leucine zipper kinase (DLK) in the retrograde transport of p-STAT3 to the cell body after axonal injury, but not in the phosphorylation of STAT3 at the site of injury. The upregulation of p-STAT3 in DRGs after axonal injury is abolished in DLK KO mice (Shin et al., 2012). In another study, Ben-Yaakov describes local activation and translation of axonal STAT3 upon injury, followed by retrograde transport to the cell body in association with dynein and importin a5 to modulate survival of peripheral sensory neurons after injury (Ben-Yaakov et al., 2012). Deletion of CNTF, which is abundantly present in myelinating Schwann cells but not in or around the cell bodies of axotomised motoneurons (Dobrea et al., 1992;Rende et al., 1992) caused a delay in the appearance of phosphorylated STAT3 and its nuclear translocation in neuronal cell bodies (Kirsch et al., 2003). This delay points to a signalling cascade beginning with local release of CNTF by damaged myelinating Schwann cells, its local action on adjacent axons, intra-axonal phosphorylation of STAT3 and its retrograde transport to the cell bodies of injured neurons and finally to the nucleus.

Overexpression of active STAT3 also has beneficial effects on neurite outgrowth. Cerebellar granular cells electroporated with a constitutively active form of STAT3, resulted in small increase in neurite outgrowth in neurons cultured on a permissive substrate (Smith et al., 2011). Similarly, when DRG neurons were infected by viral gene transfer with a constitutively active form of STAT3, a large increase in sprouting was found in the spinal cord after a dorsal column injury (Bareyre et al., 2011). However, repetitive in vivo imaging of individual fluorescently labelled axons showed that STAT3 selectively regulates initiation but not later perpetuation of axonal growth, thus acting as a phase-specific regulator of axonal outgrowth in both PNS and CNS (Bareyre et al., 2011). Strong pro-regenerative effects have also been observed when upstream negative regulation of STAT3 by SOCS3 was modified (Sun et al., 2011). Inactivation of floxed neuronal SOCS3, an upstream inhibitor of STAT3, using a viral vector carrying cre recombinase elicited pronounced axonal regeneration in the central, crushed optic nerve model (Sun et al., 2011). This effect could be blocked by concurrent deletion of floxed STAT3. Furthermore, simultaneous deletion of both SOCS and PTEN (negative regulator of mTOR) causes sustained synergistic enhancement of robust axon regeneration, and increased neuronal survival after optic nerve crush (Sun et al., 2011). In a similar vein, deletion of gp130, the common neurokine receptor subunit that transduces the effects of IL6, LIF, CNTF etc., in

the sympathetic neurons of the superior cervical ganglion abolished upregulation, phosphorylation and nuclear translocation of STAT3 after postganglionic axotomy, while also blocking most of the injury response (Habecker et al., 2009;Sachs et al., 2010). Interestingly, injections of cAMP into the DRG in vivo lead to an increase in IL-6 and LIF mRNAs, and in the phosphorylation and nuclear localization of STAT3 (Wu et al., 2007). Some of the identified direct downstream targets of STAT3 include small proline rich protein 1a (SPRR1A), WNT5A and IRF1 which are known to enhance neurite outgrowth (Coqueret and Gascan, 2000;Fujio et al., 2004;Smith et al., 2011). Altogether, these studies show a general trend of early and persistent expression of phosphorylated STAT3 during the regenerative process.

Finally, a number of reports have also implicated STAT3 as an intracellular survival-promoting factor (Qiu et al., 2005;Schwaiger et al., 2000;Schweizer et al., 2002;Smith et al., 2011;Liu and Snider, 2001;Alonzi et al., 2001). In vitro studies have shown that STAT3 expression directly induces bcl-2 and bcl-xL gene expression, which are critical in promoting neuronal survival after injury (Gonzalezgarcia et al., 1995;Dziennis and Alkayed, 2008). Mice lacking STAT3 in facial and spinal motoneurons (controlled by cre expression under NF-L promoter) showed a significant reduction in motoneuron survival after facial nerve lesion, which closely correlated with the extent of motoneuron loss seen in LIF/CNTF KO mice after nerve lesion (Sendtner et al., 1996;Schweizer et al., 2002). Moreover, upregulation of Reg-2 and Bcl-xl expression was reduced in axotomised motoneurons in STAT3 KO mice, suggesting a role of STAT3 in anti-apoptotic signalling. Interestingly, in the study by Schweizer et al., local application of CNTF strongly reduced the apparent motoneuron cell death in the absence of neuronal STAT3 (Schweizer et al., 2002), but which other signalling pathways were involved is unclear. Recently, retrogradely transported axonal STAT3 was shown to primarily have anti-apoptotic effects in sensory neuron cell bodies after nerve injury (Ben-Yaakov et al., 2012).

c-Jun

The AP-1 transcription complex is a well-characterised regulator of neural development and consists of homo- or hetero-dimeric complexes between members of the Jun, c-Fos and ATF/CREB families. A primary component of the AP-1 complex, c-Jun, is produced as an immediate early gene (IEG) following nerve injury and persists at high levels in injured

neurons during the entire peripheral regenerative process (Herdegen et al., 1991;Lindwall and Kanje, 2005;Mason et al., 2003;Raivich et al., 2004;Ruff et al., 2012a). Schwann cells also strongly upregulate c-Jun following axotomy, where it functions as a negative regulator of myelination by cross-inhibiting pro-myelin transcription factor Krox20, and switches on local inflammation (Arthur-Farraj et al., 2007;Latouche et al., 2009;Wilton et al., 2009;Parkinson et al., 2008). Mice with conditional inactivation of c-Jun in Schwann cells show strikingly delayed myelin loss after sciatic nerve injury (Parkinson et al., 2008). Numerous studies, using targeted gene deletions or pharmacological inhibition, have examined the activation, function and cellular basis of c-Jun and suggest a tripartite role of c-Jun action in neural degeneration, inflammation and repair (Raivich, 2008).

Nestin-cre mediated deletion of c-Jun prevented the upregulation of axotomy-associated molecules (CD44, α7β1 integrin, galanin). It also decreased perineuronal sprouting (Makwana et al., 2010) and successful reconnection to peripheral targets by 4-5 fold, and strongly reduced functional recovery (Raivich et al., 2004). This massive reduction in target re-innervation was also observed more than 3 months after facial nerve cut, suggesting that it was a persistent defect, rather than just a delay in the speed of regeneration. Incidentally, functional recovery did not appear to require re-innervation of the target by the same number of neurons. Compared with the Jun-competent littermates, functional recovery showed a longer but nevertheless transient delay, suggesting that long term, target re-innervation by a fraction of neurons could produce extensive functional recovery (Raivich et al., 2004). Thus, although functional recovery is the clinically relevant parameter, a sole reliance on this parameter could overestimate the extent of re-innervation. Non-neuronal responses like leucocyte recruitment and microglial activation were also severely compromised in the mutants. Interestingly, the facial motoneurons in the mutants showed enhanced post-axotomy survival, but were severely shrunken (Raivich et al., 2004), supporting the previously reported functional dichotomy of c-Jun in promoting post-traumatic neuronal cell death as well as axonal regeneration (Herdegen et al., 1997b;Herdegen and Waetzig, 2001).

Since the cre recombinase in these animals was driven by a nestin-promoter expressed in early neuroepithelial cells giving rise to neurons, astrocytes or oligodendrocytes, as well as Schwann cells it is raised the question whether the defects in neuronal regeneration observed were due to the absence of c-Jun in neurons or other nestin+ progenitor-derived

cells. This issue was addressed by using transgenic mice having neuron-specific (synapsin promoter controlled cre-recombination) or Schwann cell-specific (myelin protein zero (P0) promoter controlled cre-recombination) c-Jun deletions.

Neuron-specific c-Jun deletion mirrored the regeneration defect seen with nestin-cre mediated deletion of c-Jun, i.e., strongly reduced speed of axonal regeneration, significantly delayed functional recovery, strongly reduced target re-innervation, enhanced motoneuron survival but cellular atrophy and diminished astrocyte and microglial activation and T-cell influx. A number of different downstream effector targets of this transcription factor were almost completely abolished (Ruff et al., 2012a). Neural c-jun deletion also interfered with central axonal sprouting of the injured motoneurons (Makwana et al., 2010).

The absence of c-Jun in Schwann cells also caused severely impaired axonal regeneration - strongly reduced functional recovery and increased failure of target re-innervation (Arthur-Farraj et al., 2012;Fontana et al., 2012). However, in contrast with neuronal c-jun deletion, it strongly increased cell death of facial motor neurons and sensory DRG neurons after facial and sciatic nerve injuries (Arthur-Farraj et al., 2012;Fontana et al., 2012). Comparison of the distal stumps of WT and c-Jun mutants revealed significant differences in expression of genes implicated in regeneration and trophic support such as BDNF, GDNF, Artemin, Shh, and GAP-43 that failed to upregulate after injury, together with genes that failed to downregulate normally after injury such as the myelin genes Mpz, Mbp, and Cdh1 (Arthur-Farraj et al., 2012). Local application of neurotrophic factors, artemin and GDNF, substantially improved this defect in regeneration (Fontana et al., 2012). GDNF and Artemin bind to their cognate GDNF family α coreceptors (GFRα) and signal via a receptor tyrosine kinase (trk) encoded by the Ret proto-oncogene (also c-Ret) (Durbec et al., 1996;Jing et al., 1996;Treanor et al., 1996). Genetic inactivation of the Ret receptor specifically in neurons resulted in regeneration defects without affecting motoneuron survival, suggesting a paracrine signalling mechanism for communication between Schwann cell neurotrophin production and neuronal regeneration. Altogether, these results propose a critical role of both Schwann cell and neuronal c-Jun in the axonal injury response, which led to the next challenge of understanding the mechanisms underlying c-Jun activation and signalling following axonal injury.

Activation of c-Jun mediated transcription is affected by interactions at 3 major sites: N-terminal phosphorylation at serines 63&73 and threonines 91&93 by the Jun N-terminal Kinases (JNKs), dephosphorylation of Thr239 and the ensuing ubiquitination and degradation, and C-terminal lysine acetylation near aa 257-276 (Morton et al., 2003;Vries et al., 2001). The JNKs are rapidly activated following peripheral nerve injury, and are retrogradely transported to the cell body along with the upstream kinases MEKK1 and p-MKK4, as well as the JNK Interacting Protein (JIP), a scaffold protein that stabilises the interaction between JNK and its upstream kinases (Lindwall and Kanje, 2005;Raivich, 2008). Neuronal injury increases JNK-mediated c-Jun phosphorylation (Herdegen et al., 1998), and application of a JNK inhibitor to explants of injured DRGs reduces c-Jun phosphorylation, ATF3 expression, and neurite outgrowth, without affecting survival (Lindwall et al., 2004).

Removal of Ser63&73 phosphoacceptor sites in the junAA mutant interfered with kainic acid excitotoxicity in hippocampal neurons (Behrens et al., 1999) at levels similar to JNK3 KO (Brecht et al., 2005), identifying c-Jun as an essential substrate of JNK signalling during kainate-induced neuronal apoptosis. While global c-Jun deletion is embryonically lethal, junAA mice showed normal development (albeit slightly smaller than littermate wild-type mice) and were fertile (Behrens et al., 1999). Following optic nerve transection, there was a partial but significant reduction of the apoptosis of retinal ganglion cells in junAA mice, and Fas L has been suggested to be an important target gene regulated by N-terminal Jun phosphorylation during apoptosis (Yoshida et al., 2002). junAA and JNK3 null mutants also showed better neuronal survival after targeted dopaminergic cell death (Brecht et al., 2005;Crocker et al., 2006). Trophic factor deprivation or DNA damage-induced death was significantly delayed in JunAA neurons which correlated with delayed expression of pro-apoptotic genes (Besirli et al., 2005). In contrast, the JNK3 KO or junAA mutations did not interfere with neuronal cell death after facial axotomy (Brecht et al., 2005;Ruff et al., 2012a;Ruff et al., 2012b). This difference could be attributed to the fact that facial axotomy affects peripheral neurons, unlike previous studies which discuss effects of phosphorylation deficient c-Jun on apoptosis of CNS neurons.

Preliminary studies from our group after facial axotomy revealed that JunAA mice do show some neuronal shrinkage, but have no effect on speed of axonal regeneration for fastest axons, functional recovery or target re-innervation. JunAA mice also had a moderate

reduction in post-axotomy CD44 levels and slightly increased astrogliosis (Ruff et al., 2012b). Complete JNK ablation decreased caspase-dependent neuronal cell death in both neuronal and non-neuronal cells (Weston and Davis, 2007). Deletion of JNK1 or JNK3 single genes showed somewhat delayed functional recovery after facial nerve axotomy but the effects were quite moderate; and deletion of JNK2 had no effect on regeneration. Deletion of JNK3 also interfered with T-cell influx, and reduced CD44 levels (Ruff et al., 2012b). The mild effects of junAA and global JNK deletions, and the fact that JNKs can act via c-Jun independent cytoplasmic (stathmin, MAP1b, MAP2) and nuclear (e.g. ATF2, ELK1, p53 etc.) targets (Barnat et al., 2010;Bjorkblom et al., 2005;Bogoyevitch and Kobe, 2006;Westerlund et al., 2011), points to a possible parting of ways: i.e. the observed JNK null effects on regeneration are c-Jun independent, and the strong Jun-dependent effects on regeneration and survival do not require N-terminal phosphorylation. It is possible that complete removal of all 4 JNK dependent N-terminal phosphorylation sites (Thr91&93 as well as Ser63&73) will produce a more severe regeneration phenotype, a hypothesis which will be further investigated in this thesis.

ATF3

Another important regeneration associated transcription factor is Activating Transcription Factor-3 (ATF3), a member of the ATF/CREB family of basic leucine zipper domain (bZIP) transcription factors. It can form homo-dimers or hetero-dimers with other bZIP transcription factors like members of the AP-1 family (ATF2, JunB, JunD, c-Jun, c-fos) and C/EBPs (Hai and Curran, 1991;Hai and Hartman, 2001). ATF3 is normally expressed at low levels, but is rapidly activated in sensory, sympathetic and motor neurons by stress stimuli after peripheral nerve injury (Raivich and Behrens, 2006;Tsujino et al., 2000), optic nerve injury (Takeda et al., 2000), and NGF depletion (Mayumi-Matsuda et al., 1999) but not after central injury (Tsujino et al., 2000). ATF3 immunoreactivity in these regenerating peripheral neurons is concentrated in the nucleus, but not the nucleolus, and cytoplasmic immunoreactivity is sometimes detectable (Hunt et al., 2012). ATF3 expression is also induced in the disconnected Schwann cells and endoneurial fibroblasts distal to the peripheral injury site, though not on central, denervated glia (Hunt et al., 2004). The specific role of injury induced glial ATF3, is currently not clear. ATF3 increases the speed of axonal mitochondrial transport in cultured demyelinated DRG neurons, probably to maintain axonal homeostasis (Kiryu-Seo and Kiyama, 2011). Several pathways have been implicated in activating ATF3 expression such as neurotrophin deprivation (Sachs et al.,

2007), as well as JNK/MAPK and p53-dependent mechanisms (Moore and Goldberg, 2011). ATF3 is axonally transported from the periphery (Lindwall and Kanje, 2005), possibly acting as a retrograde signal like STAT3.

Delivery of ATF3 to adult DRG neurons, neonatal SCG neurons and PC12 cells, enhanced both the number of neurons extending neurites and neurite length (Nakagomi et al., 2003;Pearson et al., 2003;Seijffers et al., 2006). As after conditioning peripheral injury, the pattern of growth consisted of long neurites rather than branched arborized ones (Seijffers et al., 2006). In transgenic mice constitutively expressing ATF3 in adult DRGs, the rate of peripheral nerve regeneration (measured by the nerve pinch test two days after sciatic nerve crush) was enhanced to an extent comparable to that produced by a preconditioning lesion (Seijffers et al., 2007). The expression of some growth-associated genes, such as Hsp27, SPRR1A, CAP23 and c-Jun was increased in the injured neurons, but not of others like the alpha 7 integrin subunit (α7), GAP-43, and STAT3. Unlike peripheral nerve-conditioning lesions, ATF3 overexpression also did not overcome the inhibition produced by CNS myelin in culture, suggesting the mechanisms responsible for axonal regeneration are separate from those involved in overcoming glial-associated inhibition (Seijffers et al., 2007). Thus, although ATF3 appears to contribute to nerve regeneration by increasing the intrinsic growth state of injured neurons and promoting cell survival, its up-regulation is not enough to fully recapitulate the peripheral nerve regeneration program (Seijffers et al., 2007).

ATF3 can bind directly to the Hsp27 promoter and activate its expression in PC12 cells (Nakagomi et al., 2003). In addition to its role as an injury-induced neuronal survival factor, Hsp27 enhances neurite outgrowth in cultured adult DRG neurons (Williams et al., 2006), possibly as a result of interactions with the cytoskeleton. The growth-associated gene SPRR1A promotes axonal outgrowth by interacting with actin structures (Bonilla et al., 2002) and ATF3 may regulate its expression either by binding directly to its promoter or by affecting other transcription factors. ATF3 also appears to have a survival role, preventing JNK-mediated neuronal death (Nakagomi et al., 2003).

ATF3 can form heterodimers with c-Jun, leading to enhanced transcriptional activation of various RAGs and increased neurite outgrowth in neuronal cell lines (Hai and Hartman, 2001;Hai and Curran, 1991). There has been a number of conflicting reports regarding the

correlation between increased ATF3 expression and upregulation of c-Jun in surviving neurons. In 2003, Pearson et al., showed that in the absence of c-Jun, ATF3 failed to enhance neurite outgrowth in PC12 cells, suggesting that a coincident presence of c-Jun and ATF3 may act synergistically (possibly via a physical interaction) to promote nerve regeneration (Lindwall et al., 2004;Pearson et al., 2003). In contrast, in 2006, Seijffers et al., showed enhanced neurite outgrowth in adult DRG neurons by ATF-3 without any increase in endogenous c-Jun levels (Seijffers et al., 2006). The scope of c-Jun and ATF3 expression in DRG neurons after sciatic nerve transection also differs: ATF3 is induced in all injured neurons, while phosphorylated c-Jun is induced only in a small subset of mostly small and medium sized DRG neurons (Tsujino et al., 2000;Lindwall et al., 2004). Sciatic nerve injury induces prolonged activation of JNKs in the DRGs (Kenney and Kocsis, 1998). Inhibiting JNK in this model led to decreased activation of c-Jun and ATF3 expression, and to decreased axonal growth (Lindwall et al., 2004). Recent results from our group show that deletion of neuronal c-Jun or STAT3 interferes with the nuclear transfer of ATF3 in the axotomised facial motor neurons (Patodia and Raivich, unpublished). This effect may be specific for motoneurons – in sympathetic neurons, cell-specific deletion of gp130 interferes with the appearance of phosphorylated nuclear STAT3, but does not affect the nuclear translocation of ATF3 (Habecker et al., 2009). Thus, the extent to which the growth-promoting action of ATF3 is autonomous of c-Jun and STAT3 and dependent on additional injury signals needs to be explored.

CREB

The cAMP response element binding protein (CREB) is a member of the ATF/CREB family of bZIP transcription factors (Hai and Hartman, 2001), and mediates cAMP signalling in the nervous system by forming homo- or hetero-dimers with other bZIP factors (Hannila and Filbin, 2008). The brains of CREB null mice show abnormal development of the corpus callosum and anterior commissure (Rudolph et al., 1998). CREB can be phosphorylated and activated by many kinases, including PKA, PKC, CAMKII, CAMKIV, AKT, MAPKAP K2, and members of RSK and MSK families (Mayr and Montminy, 2001), and it is negatively regulated by phosphatases such as PP1 and PP2A (Sun et al., 1994). Important targets of CREB include arginase I and BDNF (Deng et al., 2009;Mayr and Montminy, 2001)

In vitro cultured DRG and SCG neurons from CREB null mice show shorter neurites than controls. In vivo, these mutant mice exhibit impaired axonal growth and projections. Their

sensory neurons undergo excess apoptosis and degeneration during the period of NGF dependency in the absence of CREB (Lonze and Ginty, 2002;Lonze et al., 2002). In addition, overexpression of a dominant negative CREB leads to decreased dendritic outgrowth in cortical neurons and loss of cAMP or neurotrophin associated neurite outgrowth on an inhibitory substrate (Redmond et al., 2002). Injection of the adenovirus containing constitutively active CREB into DRG neurons enhances nerve regeneration (Gao et al., 2004). This suggests an important role for CREB-mediated gene expression for both survival and axonal growth in PNS neurons. Recently, it has been suggested that axonal translation of CREB mRNAs may precede its phosphorylation, retrograde nuclear translocation and transcriptional activation of pro-survival genes (Cox et al., 2008).

C/EBP β, δ

The C/EBP (CCAAT/enhancer binding protein) family of transcription factors is comprised of six proteins (C/EBPα, β, γ, δ, ε, and ζ), each having unique properties regulating cellular proliferation and differentiation, particularly in hepatocytes, adipocytes and haematopoietic cells (Ramji and Foka, 2002). C/EBPs also play pivotal, non-redundant roles in the development and plasticity of the nervous system, including cell fate determination, apoptosis, synthesis and response to trophic factors and to brain injury and ischemia (Kfoury and Kapatos, 2009). All members have a highly homologous basic leucine-zipper (bZIP) domain which is required for dimerisation, and a DNA binding domain. The C/EBPs can interact with other bZIP and non-bZIP transcription factors (Huang et al., 2007).

C/EBP was shown to be up-regulated following nerve injury in the CNS of adult Lymnaea stagnalis, a freshwater pond snail, capable of spontaneous regeneration following neuronal injury. Knockdown of C/EBP prevented extension of the distal, proximal and intact neurites, in vitro, and postponed recovery of locomotory activity following nerve crush in vivo (Aleksic and Feng, 2012). C/EBPβ is upregulated and phosphorylated across different classes of injured neurons, including the invertebrate Aplysia model. In this model, phosphorylation of C/EBPβ is mediated by the RISK1, a mitogen associated protein kinase homologue related to the ERK family (Sung et al., 2001). In the mouse facial nerve model, C/EBPβ is upregulated in mouse facial motor neurons in an injury dependent manner, and is important for the expression of regeneration-associated microtubule Tα1 α tubulin and growth cone protein GAP-43 (Nadeau et al., 2005).

C/EBP-δ (also known as CELF, CRP3, NF/IL-6) was first characterized as an acute phase inflammatory response gene (Alam et al., 1992). It is widely expressed in spinal cord motor neurons and DRG neurons (Sterneck et al., 1998). Expression of C/EBP-δ is typically low to undetectable in most cell types and tissues, but it is rapidly induced in sensory and sympathetic neurons (Boeshore et al., 2004;Lopez de and Magoulas, 2013) by a variety of extracellular stimuli, (e.g. growth hormones, insulin, IFNγ, IL-1, IL-6, LPS, TNFα, noradrenaline and glutamate), phosphorylated and translocated to the nucleus as an active transcription factor where is induces further IL-6 expression (Ramji and Foka, 2002). The most favourable complex responsible for the transcription of the IL-6 gene requires the interaction between p65 and C/EBPδ, and c-Jun occurring on the NFκB site (Faggioli et al., 2004). A recent study showed that the anti-inflammatory and neuroprotective effects of chrysin on reactive microglial cell induced neurotoxicity was partly mediated by inhibition of C/EBPδ expression at both protein and mRNA level. C/EBPδ deficient microglial cultures produce less NO and TNFα in response to LPS/IFNγ, re-affirming its role in microglial activation and neuroinflammation (Gresa-Arribas et al., 2010). Deletion of both C/EBPβ and C/EBPδ in mouse embryonic fibroblasts caused impaired production of IL-6 and TNFα in response to LPS or IL- 1, suggesting complementary roles of C/EBPβ and C/EBPδ in induction of proinflammatory cytokines (Ramji and Foka, 2002). While C/EBP-δ-deficient mice display no overt phenotype, are fertile and achieve normal life spans, they have a selectively enhanced contextual fear response (Sterneck et al., 1998). A recent study implicates C/EBPδ in axonal regeneration of DRG neurons after sciatic nerve crush injury (Lopez de and Magoulas, 2013). Lack of C/EBPδ resulted in severe impairment of the early phase of regeneration of the sciatic nerve – significantly smaller neurites, delayed motor and sensory function recovery, and failure to induce expression of regeneration-associated genes like SPRR1A (but not of others like GAP-43 and galanin). In addition, following conditioning of the sciatic nerve, dissociated cultured DRG neurons from C/EBPδ KO mice failed to exhibit an enhanced axonal outgrowth (Lopez de and Magoulas, 2013). Nuclear factor IL-3 regulated (NFIL3) represses C/EBP target genes in neuronal outgrowth. Targeting such intrinsic repressors of neuronal regeneration might promote regeneration in the damaged nervous system (MacGillavry et al., 2011).

In summary, a number of intriguing findings implicate a role of C/EBPs in injury response in neurons, i.e., they are mediators of neuro-inflammatory responses in the brain; are

induced in injured neurons of invertebrates (Korneev et al., 1997;Aleksic and Feng, 2012) as well as vertebrates (Nadeau et al., 2005;Lopez de and Magoulas, 2013); have regeneration associated genes like Tα1 α tubulin, GAP-43 and SPRR1A as direct transcriptional targets (Nadeau et al., 2005;Lopez de and Magoulas, 2013); are upregulated by IL-6, an important post-injury molecule (Ramji et al., 1993); are responsive to regenerative cAMP signals in neurons (McCauslin et al., 2006;Yukawa et al., 1998); and are involved in axonal outgrowth in vitro (MacGillavry et al., 2011) and in vivo (Lopez de and Magoulas, 2013). A number of alternative signalling pathways might also converge onto C/EBP family members following axonal injury, e.g., the JAK- STAT pathway has been shown to directly regulate C/EBP-β (Jiang and Zarnegar, 1997).

Sox11

SRY-box containing gene 11 (Sox11), a member of the Sox transcription factor family can partner with Brn1 or -2 to regulate transcription (Kuhlbrodt et al., 1998;Tanaka et al., 2004) and drive expression of neurite growth-associated genes like β-III tubulin, MAP2 (Bergsland et al., 2006), actin-related protein complex 3 (ARPC3)(Jankowski et al., 2006), and SPRR1A (Jing et al., 2012;Bonilla et al., 2002). Sox11 expression in sensory and sympathetic ganglia rises steadily after nerve injury and during axonal regeneration; returning to baseline levels at the end of regeneration (Jankowski et al., 2009;Jankowski et al., 2006). In Neuro2a cells, Sox11 levels dramatically increased with the number of cells extending neurites. Cultured adult DRG neurons treated with Sox11 siRNAs exhibit a significant decrease in regeneration following axotomy as indicated by reduced neurite length and branching index (Jankowski et al., 2006). Conversely, viral-mediated Sox11 overexpression caused increased neurite elongation and branching of cultured mouse DRG neurons (Jing et al., 2012). Similarly, Sox11 overexpression in crushed saphenous nerves accelerated target re-innervation and recovery of sensory function, and increased myelin thickness in vivo (Jing et al., 2012). Injection of Sox11 siRNAs into mouse saphenous nerve caused a transient knockdown of Sox11 mRNA that transiently inhibited in vivo regeneration of both myelinated and unmyelinated axons after nerve crush. Nearly all neurons in the ganglia of crushed nerves that were Sox11 immunopositive showed co-labelling for ATF3 (Jankowski et al., 2009) and treatment with Sox11 siRNAs in vitro and in vivo caused a transcriptional and translational level reduction in ATF3 expression. Interestingly, ATF3 ablation using ATF3 siRNAs did not affect Sox11 levels. Furthermore, expression of SPRR1A, a transcriptional target of Sox11, coincides with the expression of

ATF3 and GAP43 (Starkey et al., 2009). These results suggest that Sox11 may act upstream of ATF3 (Jankowski et al., 2009) and influence nerve regeneration by regulating gene expression of downstream effectors like SPRR1A (Jing et al., 2012). A recent study also implicates a role of Sox11 in regulating BDNF expression in DRGs following sciatic nerve injury (Salerno et al., 2012).

p53

p53 is a member of a family of tumour suppressors together with p63 and p73. Like c-jun, p53 is known to mediate both pro- and anti-apoptotic roles in the nervous system (Jacobs et al., 2006;Culmsee and Mattson, 2005). Over-expression of a dominant negative form of p53 in primary cortical neurons leads to growth cone collapse and decrease in neurite outgrowth, possibly due to a combination of reduced expression of its growth-cone associated target genes Coronin1b, Rab13, and GAP-43 (Di Giovanni et al., 2006;Tedeschi et al., 2009), as well as its local, non-transcriptional activity at the growth cone (Qin et al., 2009). On the other hand, overexpression of wild-type p53 leads to an increase in growth cone size (Qin et al., 2009). Other axonal guidance molecules and their receptors like netrins, semaphorins and ephrins are also regulated by p53 (Arakawa, 2005). In vivo experiments using p53 knockout mice showed a significant decrease in the number of fibres re-innervating the target muscles at 28 days after facial axotomy in the mutant mice when compared with control animals (Di Giovanni et al., 2006). Deletion of p53 also rescued the motor neuron death after hypoglossal nerve injury by preventing expression of the pro-apoptotic gene Noxa (Kiryu-Seo et al., 2005). Taken together, these studies suggest a role for p53 in modulating neurite growth and regeneration.

SRF

Serum Response Factor (SRF) mediated transcriptional gene expression is important for actin cytoskeletal dynamics, developmental neuronal migration, outgrowth and path-finding of neurites, as well as synaptic targeting (Knoll and Nordheim, 2009). Following nerve fibre injury in vitro, SRF improves re-growth of severed neurites and survival of neurons by supressing active caspase 3 expression (Stern et al., 2012). SRF-deficient mice have impaired cell migration, neurite outgrowth, branching, growth cone shape and axon guidance. Administration of constitutively-active SRFVP16, a fusion protein of SRF and the viral VP16 transactivation domain, enhances neuronal motility and increases motoneuron survival by three-fold in vivo. Further, SRF-VP16 and increased microglia and

T cell activation around transected motoneurons in vivo. SRF transcriptional targets after facial axotomy include IEGs like Egr1, Egr2 and actin cytoskeletal genes such as actin isoforms (Actc1, Acta2), calponin (Cnn1), and actinin (Actn3) (Stern et al., 2012). Finally SRF also shares distinct and overlapping functions with transcription factor CREB (Knoll and Nordheim, 2009).

Other transcription factors with potential roles in PNR

A few other transcription factors have been shown to be consistently upregulated following nerve injury and appear to have growth supportive roles in vitro, but there are no studies as yet to substantiate their significance, if any, in peripheral nerve regeneration in vivo.

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is upregulated in DRGs and spinal cord after peripheral nerve crush (Ma and Bisby, 1998;Pollock et al., 2005). It can respond to a stimulus in neurites and retrogradely travel to the nucleus to affect gene transcription. NF-κB signalling can either enhance of inhibit neurite growth depending on the phosphorylation status of its RelA/p65 subunit. Phosphorylation of p65 by IKKβ results in reduced neurite growth, and in neurons without this IKKβ activity, there is increased neurite growth (Gutierrez et al., 2008). Identified neuronal targets of NF-κB include genes for Bcl-2, MnSOD, glutamate receptor subunits, BDNF, and calcium regulating proteins (reviewed by (Mattson, 2005)). In addition, NF-κB has been shown to regulate expression of cell-adhesion molecules like NCAM, tenascin C and β1 integrin in other systems (reviewed by (Moore and Goldberg, 2011). NFκB is also the downstream modulator of neurite growth for fas apoptosis inhibitory molecule (FAIM) signalling. Blocking NFκB activation prevents the increase of neurite growth seen with FAIM overexpression (Sole et al., 2004).

ELK - The ETS-like (ELK) subfamily of transcription factors comprises of ELK1, ELK3, and ELK4 factors (Buchwalter et al., 2004;Ducret et al., 2000). Seven days after peripheral axotomy, a 2.3 fold upregulation of ELK3 mRNA, but not of ELK1 or ELK4 mRNA was detected in adult mouse DRGs (Kerr et al., 2010). The expression of neuropeptide galanin mRNA is upregulated around 80-fold in DRGs following axotomy, and an 18 basepair sequence within its promoter/enhancer region containing overlapping putative ETS, STAT and Smad binding sites, has been shown to be critical for this response (Bacon et al., 2007), making ELK3 a potentially important candidate regulating peripheral nerve regeneration.

Oct-6 - Transcription factors Oct-6 (SCIP/Tst-1), Krox-20 (Egr-2) and Sox 10 are major regulators of Schwann cell differentiation and myelination (Jaegle and Meijer, 1998;Topilko et al., 1994;Bermingham et al., 1996). Oct-6 is present in the cytoplasm of Schwann cells associated with myelinated fibres of intact nerves. Axonal injury induces nuclear Oct-6 expression to promote transcription of genes, possibly those encoding myelin proteins (Scherer et al., 1994;Kawasaki et al., 2003). Oct-6 expression is down-regulated in fully regenerated nerves and in nerves showing chronic axonal loss (Kawasaki et al., 2003).

Cross-talk between transcription factors

Transcription factors form an intricate network in which cross-talk between different pathways leads to an integrated and input-balanced signalling outcome (Moore and Goldberg, 2011;Morrison and Davis, 2003). Some axonal regeneration still occurs in the absence of master regulators of regeneration like c-Jun, highlighting the existence of alternative pathways, and of compensatory and complementary/synergistic mechanisms, which may be shared during axonal outgrowth.

JNK signalling is interconnected with various other cellular signalling pathways, such as NFkB and the JAK/STAT system (Haeusgen et al., 2009;Waetzig et al., 2006), resulting in STAT3 phosphorylation (Levy and Lee, 2002). Besides JNK, c-Jun can also be activated by ERK1/2 (Leppa et al., 1998;Morton et al., 2003;Schwarz et al., 2002). On the other hand, STAT3 can stimulate transcription of several AP-1 members including JunB (Coffer et al., 1995) and c-fos (Higashi et al., 2004;Yang et al., 2003) which have STAT3 responsive elements in their regulatory sequence. Besides the JAK/STAT pathway, gp130 can trigger other signalling streams including the MAPK/ERK pathway (Ernst and Jenkins, 2004).

The interactions between AP-1 and STAT3 are multiple and complex, resulting in mutual modulation of their transcriptional activities. STAT3 and AP-1 factors can bind to independent but closely spaced DNA binding sites in the regulatory sequences of a number of genes, such as α-2 macroglobulin (Schaefer et al., 1995), VIP (Symes et al., 1997), bcl-6 (Arguni et al., 2006), and matrix metallo-proteinases, and synergistically

induce maximal enhancer function (Zhang et al., 1999). AP-1 complexes can also bind directly to STAT3, enhancing STAT3's transcriptional activity (Leu et al., 2001;Schaefer et al., 1995). Mapping of the STAT3-c-jun interactive regions by GST pull-down assays suggested that the STAT3-interactive region lies within its coiled-coiled domain, and in a portion of the DNA binding domain (Figure 1.3). The c-Jun interactive region was within its C-terminal residues 105 to 334. Point mutations within these regions blocked their interaction (Zhang et al., 1999). SOCS3 was discovered for its ability to inhibit tyrosine phosphorylation of STAT3 through binding to gp130 and JAKs (Krebs and Hilton, 2001). It has subsequently been shown that endogenous SOCS3 can also inhibit AP-1 activity by blocking JNK phosphorylation (Miao et al., 2008). Therefore SOCS3 could be an important molecule regulating axonal regeneration by controlling changes in JNK and JAK/STAT signalling pathways after injury.

Additional transcription factors encompassing a compensatory/complementary role include the C/EBP family members C/EBPβ and C/EBPδ. The promoter region of a c-Jun co-activator, an oncogene Jab1, contains binding sequences for C/EBP, GATA, as well as a STAT3 consensus sequence overlapping the C/EBP site. Both, mutation of the C/EBP binding site and inhibition of STAT3, significantly reduced Jab1-promoter activity (Shackleford et al., 2011). C/EBPβ is essential for appropriate induction and maintenance of GAP43 mRNA following axonal injury (Nadeau et al., 2005). While there is no evidence of a direct binding site for C/EBP on the GAP43 gene, it contains a functionally-important AP-1 site for c-jun and c-fos (Weber and Skene, 1998). C/EBPβ can bind to and regulate both c-fos and c-jun (Nadeau et al., 2005), suggesting a coordinated induction of GAP43 by C/EBPβ, c-fos, and c-Jun in injured neurons. It has already been shown that co-expression of c-Fos with c-Jun induces a greater number of cells to produce neurites than c-Jun alone (Leppa et al., 1998). In contrast with C/EBP β, C/EBPδ causes injury induced expression of SPRR1A but not of galanin or GAP43 (Lopez de and Magoulas, 2013). ATF3 has been observed to have a similar regulatory effect as C/EBPδ, as it enhances the intrinsic growth state of DRG neurons by affecting the expression of SPRR1A but not GAP-43 (Seijffers et al., 2007). Similarly, Sox11 has been found to promote nerve regeneration via SPRR1A expression (Jing et al., 2012). On the other hand c-jun is required for the activation of both GAP43 and galanin in response to nerve injury (Raivich et al., 2004). Moreover, C/EBP is activated by CREB in other neuronal systems (Alberini, 2009), and CREB-associated transcription factors interact with C/EBP in axonal outgrowth

in vitro (MacGillavry et al., 2011). It can therefore be speculated that the C/EBPδ pathway interacts with CREB/ATF3 and Sox11 signalling networks but not the AP1 pathway in injury-induced regeneration (Lopez de and Magoulas, 2013).

We have already discussed a number of different studies investigating the complex association between ATF3 and c-jun expression after injury. Besides interacting with leucine zipper transcription factors, ATF3 can also interact with other transcription factors upregulated after peripheral injury, such as STAT3. This could be via co-activators or by synergistic binding to adjacent DNA binding sites. For example, SPRR1A, a target gene for ATF3, is a gp130 pathway protein and its promoter region contains functional AP-1 binding sites (Sark et al., 1998;Pradervand et al., 2004).

Damage-induced neuronal endopeptidase (DINE) is expressed after motor, sensory and sympathetic nerve injuries, brain and spinal cord trauma, and cerebral ischemia (Kiryu-Seo et al., 2000;Boeshore et al., 2004), and is involved in terminal nerve branching (Nagata et al., 2010). DINE mRNA induction is always accompanied by the induction of ATF3, in various injury models (Nakagomi et al., 2003;Ohba et al., 2004). Transcription of DINE is increased 60-fold by Sp1 mediated recruitment of STAT3, c-jun and ATF3 to the DINE promoter in response to LIF up-regulation and NGF withdrawal following injury (Kiryu-Seo et al., 2008).

NFκB can also interact with Jun, ATF, CREB and Fos transcription factors. STAT3 can induce the alternative NFκB pathway, and bind to DNA in a complex with p52, to induce transcription. p53 can also interact with p52 to regulate expression of its target genes (reviewed by (Perkins, 2007).
Various mathematical models of interacting signalling pathways are being developed to predict regenerative outcomes after injury (Moya et al., 2011).Transgenic animal models with deletion of multiple signalling streams will be very useful in identification of functional complementary counterparts.

Downstream targets – effectors of the regenerative programme

The upstream injury sensors, signalling molecules and transcription factors rapidly condition the injured nerve and within 1 – 4 days after injury, the neuronal perikaryon produces a plethora of RNA, protein and glycolipid components which play a vital role in executing axonal regeneration (Raivich, 2011). RAGs is an umbrella term including the vast number of genes that are differentially regulated during nerve regeneration and may be involved in cell-cell signalling, axonal growth and sprouting, and activation of non-neuronal, cellular milieu (Skene and Willard, 1981;Boeshore et al., 2004). Absence of RAGs in CNS neurons is postulated to be one of the causes for their failure in regeneration of axons. An overview of expression and function of downstream effector molecules in peripheral nerve regeneration, and their transcriptional regulation is shown in Table 1.2. This thesis focuses on the roles and regulatory mechanisms of such identified downstream effectors in successful peripheral nerve regeneration, specifically axonal adhesion molecules, chemo-attractant signalling molecules, cell surface-cytoskeletal adaptors and neuropeptides, and also of neurotrophins produced in the injured nerve.

Downstream effectors (DE)	Transcription Factors Driving DE	Expression after Axotomy	Effects of DE deletion on nerve regeneration	References
Adhesion molecules				
α7 β1 integrin	c-jun (N)	↑	- Global α7 deletion strongly reduces regenerative speed after nerve crush	Werner et al., 2000 Raivich et al., 2004
CD44	c-jun (N)	↑	- Reduced neurite outgrowth of transplanted central noradrenergic neurons - Errors in retinal axonal growth in development - *But: so far no effects data on regeneration*	Nagy et al., 1998 Lin and Chan., 2003 Raivich et al., 2004 Ruff et al., 2012
DINE	ATF3, c-jun, STAT3	↑	- Reduced sprouting at developing NMJ - *But: so far no effects data on regeneration*	Kiryo-Seo et al., 2008 Nagata et al., 2010
Neuropeptides				
Galanin	c-jun (N)	↑	- Reduced rate of regeneration - Modulation of pain transmission after injury	Holmes et al., 2000 Raivich et al., 2004 Holmes et al., 2005 Ruff et al., 2012
CGRP	c-jun (N)	↑	- Reduced number of axons crossing from proximal to distal stump	Raivich et al., 2004 Toth et al., 2009 Ruff et al., 2012
Guidance molecules				
Ephrin-B	Sox-2 (SC)	↑	- Disordered axonal outgrowth from proximal to distal stump	Parrinello et al., 2010
Cytoskeletal adaptors				
CAP23	c-jun (N)	↑	- Reduced neurite outgrowth & target re-innervation	Anderson et al., 2006; Verhaagen, Mattson and Raivich, unpublished
GAP43	C/EBPβ (N)	↑	- Abnormal developmental path-finding - *But: so far no effects data on regeneration*	Strittmatter et al., 1995 Nadeau et al., 2005
Neurotrophins				
BDNF	c-jun (SC)	↑	- *So far no effects data on regeneration*	Meyer et al., 1992
GDNF	c-jun (SC)	↑	- Increased neuronal cell death - Reduced functional recovery & speed of regeneration	Fontana et al., 2012
Artemin	c-jun (SC)	↑	- Increased neuronal cell death - Reduced functional recovery & speed of regeneration	Fontana et al., 2012
LIF	c-jun (SC)	↑	- *So far no effects data on regeneration*	Curtis et al., 1994
NGF	c-fos (SC)	↑	- *So far no effects data on regeneration*	Hengerer et al., 1990

Table 1.2. An overview of the transcriptional regulation of expression and function of downstream effector molecules in successful peripheral nerve regeneration (N = neuronal, SC = Schwann cell, NMJ = neuromuscular junction)

Adhesion molecules

Adhesion molecules enable the interaction of the cell surface of the axonal growth cones with the adjacent bands of Büngner consisting of denervated Schwann cells, and the inner lining of the neural tube basal membrane, both structures acting as a scaffold for the growing axon (Grumet et al., 1991). Signalling between adhesion molecules on axonal and Schwann cell surfaces is frequently bi-directional (Quintes et al., 2010;Fricker et al., 2011), but to simplify it for the purpose of axonal regeneration it is helpful to concentrate on axonal molecules that act as receptors for guidance cues from Schwann cells and the associated extracellular matrix.

Regenerating neurons up-regulate a variety of adhesion molecules. These include integrins (e.g., α7β1) which serve as receptors for the matrix and cell surface molecules such as laminin, fibronectin and paxillin (Kloss et al., 1999). There is increased expression of CD44, a receptor for hyaluronic acid (Jones et al., 2000) and galectin-1, a lectin receptor for the galactoside side chains (Horie and Kadoya, 2000). Regenerating axons express cell multimodal surface molecules involved in homophilic binding such as ninjurin (nerve injury-induced protein) (Araki and Milbrandt, 2000), gicerin/CD146, a heterophilic receptor for the neurite outgrowth factor (NOF) (Hiroi et al., 2003), and adhesion molecule FLTR3 which can form complexes with receptors for fibroblast growth factor (FGF) and potentiate FGF signalling (Bottcher et al., 2004). On the functional side, galectin-1 stimulates the migration of Schwann cells from the proximal and distal stumps, assisting in the formation of cellular bridges permitting axonal growth into the distal part of the injured nerve (Horie and Kadoya, 2000). Administration of exogenous galectin-1 promotes, and its removal by antibody neutralisation or through gene deletion, decreases the speed of neurite outgrowth (Horie and Kadoya, 2000;McGraw et al., 2004). Removal of β2-microglobulin causes enhanced synaptic stripping, and produces a moderate, 20% reduction in the ability of regenerating motor axons to cross the proximal to distal gap after nerve cut (Oliveira et al., 2004).

Regenerating axons also contain an assortment of degradative enzymes including urokinase and plasminogen activator, metalloproteinases (MMP) 2, 3 and 9 (Demestre et al., 2004;Shubayev and Myers, 2004) and damage induced neuronal endopeptidase

(DINE) (Kiryu-Seo et al., 2000). Activation of MMP2 and MMP9 is partially dependent on the presence of tPA and/or UPA systems (Siconolfi and Seeds, 2003) and deletion of tPA and/or UPA interferes with functional recovery after peripheral nerve injury (Siconolfi and Seeds, 2001). As with chondroitinase, administration of exogenous MMP2 has been shown to degrade chondroitin sulfate proteoglycans and improve recovery and anatomic re-innervation following nerve transection. Furthermore, in the presence of saturating concentrations of MMP2, co-administration of chondroitinase ABC does not produce an additional improvement, suggesting that both enzymes attack the same target (Zuo et al., 1998). Interestingly, although regenerating sympathetic neurons show increase in MMP2 protein activity, there is no comparative increase in MMP2 mRNA (Leone et al., 2005). However, the axotomised dorsal root ganglion neurons show injury-caused down regulation of TIMP2, an inhibitor of MMP2 (Huang et al., 2011), and there is a similar downward trend in facial motor nucleus (Raivich, Mason, Verhaagen, unpublished), suggesting a regeneration-induced disinhibition of MMP2.

So far, three of the injury-upregulated adhesion molecules – DINE (Kiryu-Seo et al., 2008), CD44 (Jones et al., 2000) and the α7β1 integrin (Werner et al., 2000a) – have been identified as downstream targets of the regeneration-associated transcription factors. In vitro, DINE expression in neuronal cell cultures is strongly upregulated by STAT3, in combination with an ATF3/c-Jun fusion hybrid (Kiryu-Seo et al., 2008). Global deletion of DINE following insertion of loxP sites into its gene is associated with a massive reduction in terminal sprouting of motor axons at the neuromuscular junction (Nagata et al., 2010). Unfortunately, these floxed DINE animals inadvertently suffer from perinatal lethality, precluding direct examination of DINE effect in the adult using the Cre/lox system.

Deletion of neuronal c-Jun interferes with the upregulation of CD44, and the α7 and β1 integrin subunits, which possibly contributes to the very poor regenerative response seen in these animals (Raivich et al., 2004). CD44 expression is strongly upregulated in a wide variety of neural injuries (Jones et al., 2000). Antibody inhibition of CD44 reduces neurite outgrowth of transplanted central noradrenergic neurons (Nagy et al., 1998) as well as creating multiple errors in retinal axonal growth trajectory through the optic chiasm (Lin and Chan, 2003). Further, GFRα-1 receptor for GDNF, a direct c-jun target, can form complexes with integrin β1 after administration of GDNF (Cao et al., 2008), and GDNF can also increase the integrin levels (Chao et al., 2003;Funahashi et al., 2003).

Overall, the integrins are large family of heterodimeric, transmembrane glycoproteins, composed of specifically paired α and β subunits (Kloss et al., 1999;Vogelezang et al., 2001a;Werner et al., 2000b;Gardiner et al., 2005). Facial axotomy leads to a strong, cell-type-specific, and time-point dependent increase in the immunoreactivity for several different integrin subunits in the affected motor nucleus, on neurons, microglia, lymphocytes, and the vascular endothelia (Kloss et al., 1999). Figure 1.5 below shows the time course of β-1 expression after facial nerve axotomy (Kloss et al., 1999).
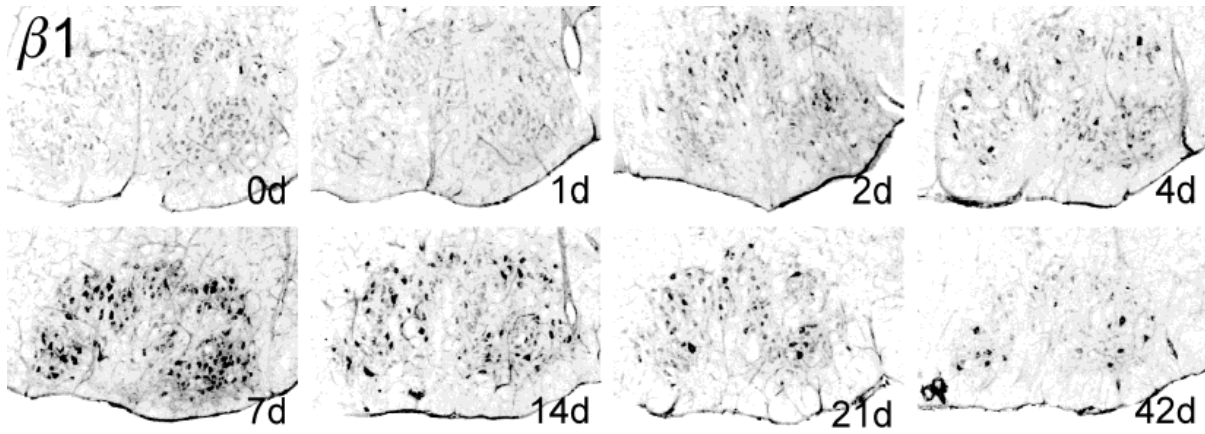
Figure 1.4. β1-integrin immunohistochemistry in normal (0d) and axotomised facial motor nuclei (1d–42d). β1 expression is increased within 2 days after axotomy; Peak expression of this integrin subunit is seen between 7-14 days and, expression is sustained until day 42 (Kloss et al., 1999).

In the case of the α7β1 integrin, global deletion of the α7 subunit causes a strong, approximate 40% reduction in the speed of adult motor axon regeneration after facial nerve axotomy, with a commensurate delay in the re-innervation of its target muscles in the whisker pad (Werner et al., 2000b). Interestingly, these α7-deficient mice show greater up-regulation of the β1 integrin following injury (Figure 1.4), which corresponds with a significant increase in central axonal sprouting (Werner et al., 1998) and may imply compensatory mechanisms involving other, closely related α subunits. Deletion of α7 integrin subunit also abolished the ex vivo conditioning effect: explanting axotomised wild-type sensory ganglia in vitro leads to brisk neurite outgrowth on laminin; the outgrowth is weaker and delayed when using previously uninjured ganglia (Ekstrom et al., 2003). In agreement with these observations, over-expression of α7 subunit in injured neurons also increases axon regeneration (Condic, 2001).
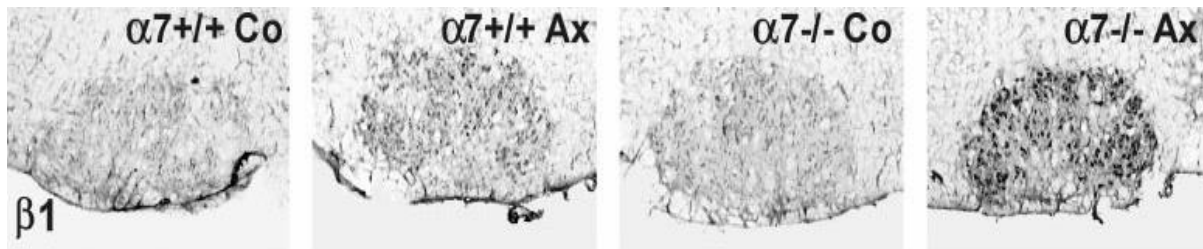
Figure 1.5. β1 integrin immuno-reactivity in normal (Co) and regenerating (Ax) facial motor nuclei of control (α7+/+) and α7 deficient (α7-/-) mice. α7 deficiency causes stronger increase in the neuronal β1 expression 3 d after facial nerve transection, compared to control mice (Werner et al., 2000a).

The absence of all γ1-dependent laminin isoforms, the primary ligands for the α7 β1 integrin, led to similar but more marked and lasting effects. Deletion of the gene for the γ1 laminin chain in SCs, caused a large decrease in the number of axons crossing into the distal portion of the crushed sciatic nerve for up to 30 days (Chen and Strickland, 2003), in contrast to the more transient defect in α7- deficient animals (Werner et al., 2000b).

In addition to cell adhesion migration and axonal outgrowth, integrin signalling is important for non-neuronal and neuronal survival (Lemons and Condic, 2008;Previtali et al., 2001;Tucker and Mearow, 2008). While some α subunits can only dimerise with single β subunits - α1, α2, α3, α5, α7 and α8 with β1, αL, αD, αM and αX with β2, and αIIb with β3; others like α4 (β1,7), α6 (β1,4), α9 (β1,8) and αV (β1,3,5,6,8) can partner with multiple β subunits (Previtali et al., 2001). Table 1.3 lists the 12 members of the β1 integrin family. The β1 subunit connects to actin cytoskeleton in each of this heterodimers, while the α subunit determines ligand specificity. Many of the β1-pairing α subunits (α1, α4, α7, α9, etc.) have been shown to promote axonal regeneration in vitro and following forced expression in vivo (Vogelezang et al., 2001b;Toyota et al., 1990), raising the expectation that complete removal of β1 would produce a severe regeneration-deficient phenotype.

β1 Family	Ligands	Cell type expression
α1β1	Collagens/Sema7A VEGF/TGFβ/PDGF/MCP-1/IL-8 Laminin	Endothelial cells/fibroblasts/ Immature Schwann cells
α2β1	Collagens/E-cadherin/Endorepellin VEGF	Endothelial cells/fibroblasts
α3β1	Laminins/CSPG4	Epithelial cells
α4β1	Fibronectin/VCAM1	Endothelial cells, neurons
α5β1	Fibronectin/Endostatin	Fibroblasts, neurons
α6β1	Laminin	Epithelial cells, glia, neurons and Schwann cells
α7β1	Laminin	Muscle cells and neurons
α8β1	Fibronectin/Vitronectin/Nephronectin	Kidney and ureter bud epithelium
α9β1	Fibronectin/VCAM1/Cytotactin/ Osteopontin/Tenascin-C/VEGF	Endothelial cells
α10β1	Collagens	Chondrocytes
α11β1	Collagens PDGF	Fibroblasts
αVβ1	Fibronectin/Vitronectin	T-cells

Table 1.3 - β1 family of integrins (Allodi et al., 2012)

Neuropeptides

Axon transection frequently causes increased expression of a variety of neuropeptides. There is a pronounced increase of calcitonin gene related peptide (CGRP), galanin, and to a lesser extent pituitary adenylate cyclase activating peptide (PACAP) in subpopulations of cranial and spinal motoneurons (Moore, 1989;Raivich, 2005;Tamas et al., 2012). Sensory DRG neurons show upregulation of galanin, vasculointestinal peptide (VIP), PACAP and neuropeptide Y (NPY) (Xu et al., 1990;Noguchi et al., 1993); sympathetic neurons increase in VIP, galanin, PACAP, cholecystokinin (CCK) and substance P (Zigmond, 2011) . In contrast, expression of substance P or CGRP in the axotomised sensory DRG, or NPY in the sympathetic neurons is reduced (Dumoulin et al., 1992;Habecker et al., 2009). This is in line with the down-regulation of enzymes and transporter systems for adrenergic and cholinergic neurotransmitters in the sympathetic

and motor neurons (Zigmond and Sun, 1997;Kalla et al., 2001). Recent studies point to decreased expression of the Hand2 transcription factor causing the post-axotomy down-regulation for components of the adrenergic system (Pellegrino et al., 2011); it is possible, similar pathways are involved in the down-regulation of some neuropeptides in the injured neurons.

In vitro studies have shown that different neuropeptides like substance P, somatostatin, vasopressin, VIP, calcitonin or alpha-MSH exert a moderate but significant neurite-growth promoting effect on CNS and PNS neurons (Brinton and Gruener, 1987;Narumi and Fujita, 1978;Raivich, 2011). VIP in the SCG and galanin in the DRG may promote neuronal survival after injury (Klimaschewski et al., 1995;Holmberg et al., 2005). Transgenic and pharmacological studies have confirmed a similar role *in vivo* for peptides like galanin, CGRP and PACAP (Raivich, 2011;Moore, 1989). Several of these upregulated neuropeptides have also been recently identified as in vivo downstream targets of regeneration-associated transcription factors. Facial axotomy leads to the appearance of galanin and CGRP positive sprouts peaking at day 14 within the facial nucleus (Makwana et al., 2010). Deletion of neuronal c-Jun interferes with the upregulation of CGRP and galanin in the facial motoneurons (Raivich et al., 2004;Ruff et al., 2012b). Sciatic nerve injury results in gradual and prolonged 3 fold increase in specific CGRP binding to the distal portion of crushed sciatic nerve (Raivich et al., 1992). Local siRNA mediated inhibition of intra-axonal CGRP synthesis strongly reduced the number of axons growing in the conduit between proximal and distal nerve stumps (Toth et al., 2009). Similar inhibitory effects were also observed by blocking the CGRP receptor expressed on neighbouring Schwann cells, via siRNA against the CGRP Receptor Activity Modifying Protein-1 (RAMP1), suggesting that the locally expressed peptide is used to recruit Schwann cell cooperation for neurite outgrowth (Raivich et al., 1992;Toth et al., 2009).

Deletion of galanin or its type 2 receptor expressed in neurons results in a 35% reduction in the rate of peripheral nerve regeneration after sciatic nerve crush (Holmes et al., 2000), modulates pain transmission (Holmes et al., 2005), and reduces conditioning lesion response in DRG neurons (Sachs et al., 2007). In sympathetic neurons, targeted deletion of the core neurokine receptor gp130 that transduces signals for IL6, LIF, CNTF and other neurokines, showed drastically lowered post-axotomy expression for galanin, VIP, and PACAP but did not affect the up-regulation of cholecystokinin (Habecker et al., 2009).

Deletion of gp130 also abolished the nuclear appearance of phosphorylated STAT3 (Habecker et al., 2009), suggesting that this transcription factor may also be involved in the normally occurring post-axotomy upregulation of galanin and associated neuropeptides.

In the case of PACAP, homozygous deletion of the gene reduces initial neurite outgrowth in the first 24h after injury, with a moderate retarding effect (1-2 days) on the re-innervation of peripheral target, and an increased neuro-inflammatory response (Armstrong et al., 2008). Reverse experiments, using local application of PACAP and galanin, showed that either peptide will strongly augment the peripheral branching of regenerating axons, increasing the number of neurons regenerating into each of the identified rami of the facial nerve by up to five-fold (Suarez et al., 2006). However, these pro-sprouting effects were actually associated with impaired outcome, underscoring the importance of misrouting as a major impediment to resumption of coordinated functional activity.

Guidance Signalling

Guidance signals – through cell surface contact or diffusible cues mediating attraction or repulsion – play an important role by binding to receptors on growth cone surfaces, triggering secondary signals and steering axon extension in the correct direction. Overall, there are many different families of guidance molecules, including semaphorins and their receptors – neuropilins and plexins (Tessier-Lavigne, 1998;Huber et al., 2003), ephrins (Tessier-Lavigne, 2000;Parrinello et al., 2010;Koeberle and Bahr, 2004), slits (Koeberle and Bahr, 2004), or inhibitory myelin components like MAG (myelin-associated glycoprotein) which can activate Rho via p75 neurotrophin receptor (p75NTR) (Yamashita et al., 2002). Moreover, lipid rafts present at the growth cone amplify guidance signals (Fujitani et al., 2005;Guirland et al., 2004).

Deletion of the neuropilin-2 reduces the density of the light neurofilament-positive axons in the distal nerve and interferes with functional recovery (Lindholm et al., 2004;Bannerman et al., 2008). Axotomy induces expression of Bex1, an intracellular adaptor molecule which interacts with p75NTR to reduce neurite outgrowth inhibition by myelin inhibitors (Lindholm et al., 2004). Global deletion of Bex1 reduces the number of regenerating axons

crossing the sciatic nerve crush site by more than 50% and impedes functional recovery (Khazaei et al., 2010). Interestingly, deletion of p75 does not affect neuronal survival or the speed of axonal regeneration in the facial nerve model, although p75 null mice show enhanced neuro-inflammatory response compared to their wild-type littermates (Gschwendtner et al., 2003).

In most cases, the in vivo transcriptional regulation for these molecules in neurons has not been clearly delineated. However, some of the Schwann cell function in creating axonal guidance scaffolds is under the control of post-traumatically expressed transcription factor Sox-2, induced by fibroblast ephrin-B signalling. Acting on the Schwann cell EphB2-receptor, the fibroblast ligand induces Sox2, and in a Sox2-dependent manner N-cadherin required for sorting Schwann cells into cell cords that link proximal and distal nerve stump (Parrinello et al., 2010). Interference with this process through EphB2-deletion or blocking antibodies produces a significantly more disordered axonal outgrowth in the gap region between the proximal and distal part of the nerve.

Growth cone and cytoskeletal adaptors

Appearing at the tip of growing axons, the growth cones are specialized, quasi-autonomous structures that are responsible for growth, path-finding and recognition of targets after nerve injury. To aid their navigatory function, they are heavily adorned by adhesion molecules and receptors for chemo-attractive and repulsive signals, discussed previously. Inside the growth cone, cytoskeletal adaptors play a crucial role in mediating connections between the cell surface and cytoskeletal actin-microtubule core of the growing axons (Baas and Ahmad, 2001;Ellezam et al., 2002;Madura et al., 2004). Successful axonal regeneration is accompanied by the appearance of numerous, functionally diverse families of molecules that regulate surface cytoskeletal interaction (Raivich, 2011).

One such family is the GMC family of 'integral' membrane proteins - GAP43/neuromodulin, Myristoylated Alanine-Rich C Kinase Substrate (MARCKS), and cytoskeleton-associated protein 23 (CAP23) (Skene and Willard, 1981;Frey et al., 2000;Bomze et al., 2001). GAP-43 and CAP-23 are among the most abundant proteins in axonal growth cones (Bomze et al., 2001;Goslin and Banker, 1990). Although these GMC molecules do not share structural homologies, they share a number of functional

properties (Frey et al., 2000;Laux et al., 2000). They co-distribute with phosphoinositol-4, 5-diphosphate (PIP2), at the semi-crystalline, plasmalemmal raft regions and modify raft-recruitment of signalling molecules such as src (Laux et al., 2000), bind to acidic phospholipids like PIP2, calcium/calmodulin, protein kinase C and actin filaments via their unique effector domains in a mutually exclusive manner, alter the actin cytoskeleton polymerisation, organization and disassembly, and translate receptor-mediated calcium fluxes into signals guiding growth cone activity (Laux et al., 2000;Henley and Poo, 2004;Kulbatski et al., 2004).

CAP23 is widely expressed during development, maintained in selected brain structures in adult, at particularly high levels in the cortex, hippocampus and DRG neurons, and re-induced by nerve injury and during nerve regeneration. CAP23-deficient mice are born sterile, have pronounced abnormalities at the neuromuscular junction and exhibit high postnatal mortality. This is probably due to the involvement of CAP23 in the maintenance of normal dendritic arborisation and of synapses (Kashihara et al., 2000). Their brains have enlarged ventricles, and pronounced axonal and synaptic ultrastructural abnormalities in the hippocampus and neocortex (Frey, D., L. Xu, and P. Caroni, unpublished observations). The absence of CAP23 leads to deficits in the stability of growth cone actin structures - thinner neurites with a characteristic winding pattern of outgrowth, unusually high densities of microtubules and pronouncedly bulbous growth cones - suggesting that in the absence of CAP23, growth cone actin barriers that restrict microtubule invasion (Forscher and Smith, 1988) are impaired. CAP23 may play a critical role to form an actin-based cortical cytoskeleton at the transition zone between growth cone and neurite (Frey et al., 2000). Neurite elongation normally starts in the tip of the surviving axon, but additional sprouts can develop more proximally at the Nodes of Ranvier.

In vitro, depletion of GAP43 does not impair NGF-elicited neurite outgrowth but leads to poorer adhesion, unstable lamellar extensions devoid of local F-actin, reduced branching and enhanced sensitivity to inhibitory stimuli (Caroni, 1997). In vivo, global deletion of GAP43 interferes with normal developmental path-finding (Strittmatter et al., 1995), and that of CAP23 with inactivity induced sprouting at the neuromuscular synapse (Frey et al., 2000). CAP23 knockouts show an almost complete absence of stimulus-induced nerve sprouting which can be rescued by transgenic overexpression of CAP23 or GAP43,

implying that GAP43 can functionally substitute for CAP23 in vivo (Frey et al., 2000). Overexpression of GAP43 or CAP23 induces excessive neuro-muscular sprouting (Caroni, 1997). Combined overexpression of both GAP43 and CAP23 in the growth cone of adult DRG neurons elicits a transition from local arborisation to long axon extensions *in vitro*, and strongly enhances neurite extension (60-fold) from peripheral sensory neurons into the injured adult mouse spinal cord in vivo. Little regeneration is observed if only one of the two components is overexpressed (Bomze et al., 2001).

GAP43, but not CAP23, is upregulated in cortico-spinal neurons, following proximal intracortical axotomy, but not after distal spinal axotomy (Mason et al., 2003). After peripheral nerve axotomy, the transcriptional upregulation of the mRNAs encoding GAP43 and CAP23 is biphasic, and it is down-regulated after target reconnection (Mason et al., 2002). In the case of GAP43, the early phase (24h after axotomy) is c/EBPβ independent but followed by a c/EBPβ-dependent later phase, with an almost complete disappearance of increased GAP43 mRNA in c/EBPβ null mutants at day 3 (Nadeau et al., 2005). In the case of CAP23, upregulation in the first 24h is unaffected, but the in the 2nd phase (4-14 days), CAP23 expression is reduced in the absence of neuronal c-Jun (Verhaagen, Mattson and Raivich, unpublished observations). Additionally, both GAP-43 and CAP-23 are expressed by Schwann cells in nerve grafts, suggesting that they play a role in the remodelling, and particularly in the elongation, of Schwann cell processes as part of the Bands of Bungner (Mason et al., 2002).

Since long term postnatal survival of GAP43 null mice is rare (Strittmatter et al., 1995), studies in the adult have been limited to the CAP23 deletion (Caroni, 1997;Frey et al., 2000). Recent data using neuron-specific deletion of the floxed CAP23 gene using adeno-associated virus carrying Cre-recombinase, resulted in an approximately 50% reduction in the whisker pad re-innervation (Anderson et al., 2006), the main target of regenerating facial nerve fibres (Werner et al., 2000b), underscoring the importance of this class of adaptor molecules in adult regeneration.

Microtubule disassembly molecules (e.g. SCG10, stathmin, CRMP2 and RB3), a microtubule-targeting group of axonal adaptor molecules are also up-regulated after nerve injury (Iwata et al., 2002;Mori and Morii, 2002). A third family of proteins, the Rho GTPase family (RhoA, Rac, Cdc42 and TC10) members, act as molecular switches regulating cytoskeletal structure, dynamics, and cell adhesion (Etienne-Manneville and Hall, 2002).

Trophic molecules, like NGF and laminin, regulate neurite outgrowth by acting co-ordinately on the same GTPase (Rankin et al., 2008).

Neurotrophins, growth factors, and cytokines

Peripheral nerve injury causes a massive increase in the synthesis and/or availability of a variety of neurotrophic and growth-promoting factors which assist in neuronal survival, immune response and axonal outgrowth following injury (Raivich and Makwana, 2007). These include neurotrophins-3 (Terenghi, 1999), 4/5 (NT3, NT4/5) (English et al., 2005), nerve growth factor (NGF) (Heumann et al., 1987), brain derived neurotrophic factor (BDNF) (Meyer et al., 1992), glial cell-derived neurotrophic factor (GDNF) (Naveilhan et al., 1997), insulin-like growth factors-1/2 (IGF1, IGF2) (Kanje et al., 1989;Glazner et al., 1993), basic fibroblast growth factor (FGF) (Jungnickel et al., 2004), vascular endothelial growth factor (VEGF) (Islamov et al., 2004), leukaemia inhibitory factor (LIF) (Curtis et al., 1994;Haas et al., 1999;Zigmond and Sun, 1997), ciliary neurotrophic factor (CNTF) (Kirsch et al., 2003), interleukin-1 (IL1) (Lindholm et al., 1987), interleukin-6 (IL6) (Hirota et al., 1996), and transforming growth factor-beta 1 (TGFb1) (Lindholm et al., 1992). Following nerve injury, neurotrophin receptors are upregulated on the distal portion of the nerve, in denervated Schwann cells and in growth cones of regenerating axons (Raivich and Kreutzberg, 1994).

The trophic factors IGF1, IGF2, and BDNF, and the TGF β superfamily member GDNF have been implicated in regulating speed of the repair process by stimulating growth of injured axons across the gap between the proximal and distal stumps, and guiding axon extension along the bands of Bungner (Taniuchi et al., 1986;Vogelin et al., 2006;Zhang et al., 2000). Both BDNF and GDNF are induced in the distal stump of the injured axons, and their exogenous application promotes axonal regeneration of chronically injured neurons, thereby completely reversing the harmful effects associated with late nerve repair (Boyd and Gordon, 2003a;Boyd and Gordon, 2003b). Inhibition of endogenous BDNF (Streppel et al., 2002) and GDNF (Sun and Oppenheim, 2003) also decreases the post axotomy collateral sprouting in the peripheral nerve. Overexpression of GDNF in motoneurons has shown to also have positive effects on neuronal survival after axotomy (Zhao et al., 2004). NGF deprivation by neutralizing the endogenous activity with specific antibodies induces axotomy-like changes even in the intact, sensitive sensory and sympathetic neurons

(Shadiack et al., 2001). Factors like IGF-1, LIF, CNTF, BDNF, NGF and NT3 prevent axotomy-induced motoneuron cell death (Moran and Graeber, 2004).

Transgenic deletion of IL6, a multi-functional neurokine rapidly induced after neural injury, causes a mild (15%) reduction in the morphometrically-determined speed of axonal regeneration in the crushed facial motor nerve, and improves central axonal sprouting (Galiano et al., 2001). IL-6 null mice achieve only 50% of sciatic nerve function 30 days after sciatic nerve crush; and induction of regeneration associated gene GAP-43 is also blocked in these mice (Zhong et al., 1999). Combined overexpression of IL6 and its receptor results in improved nerve regeneration (Hirota et al., 1996). A recent study highlights the role of IL6 in promoting regeneration and functional recovery after SCI by reactivating intrinsic growth program of neurons through increased expression of growth associated genes GAP-43, SPRR1A and Arginase I and enhancing synapse formation in vivo (Yang et al., 2012). SPRR1A is induced by gp130 cytokines (Pradervand et al., 2004) and associates with actin in growth cones (Bonilla et al., 2002). Previous studies have reported that conditioning lesion or injection of CNTF into DRGs increase SPRR1A expression (Wu et al., 2007). Post-lesion regulation of CNTF/LIF receptor components indicate a direct and sequential action of CNTF/LIF signalling in survival and regeneration of axotomised facial motoneurons (Haas et al., 1999).

The neuropoeitic cytokines IL6, LIF and CNTF share the signalling receptor gp130 and may mediate their effects by activating transcription factor STAT3. Indeed, absence of the Schwann cell CNTF in the CNTF null mice appears to delay in the appearance of phosphorylated STAT3 and its nuclear translocation in neuronal cell bodies (Kirsch et al., 2003). In LIF KO and in IL-6 KO mice, neurite outgrowth following a conditioning lesion is significantly reduced by 40% and 65% respectively (Cafferty et al., 2004;Cafferty et al., 2001). Exogenous application of IGF1 and IGF2 enhances, and their antibody mediated inhibition reduces the pinch test-determined speed of axonal regeneration (Kanje et al., 1989;Glazner et al., 1993); IGF1 is also partly responsible for the peripheral conditioning (Kanje et al., 1991). Peripheral nerve grafts from mice lacking NT4/5 display decreased growth by regenerating axons of wild-type animals (English et al., 2005).

Studies into transcriptional regulation of induced neurotrophin and growth factor expression in peripheral nerve have concentrated particularly on the role of c-Fos and c-Jun. Sciatic nerve lesions cause a rapid, local increase in c-fos and c-jun mRNA followed

within hours by an increase in NGF mRNA (Hengerer et al., 1990), with endoneurial fibroblasts forming a primary site of NGF synthesis (Lindholm et al., 1987;Heumann et al., 1987). That these two sets of changes – upregulation of c-Fos, and later expression of NGF – are probably linked has been shown by an elegant set of cell culture experiments. Here, heavy metal induced overexpression of c-Fos in fibroblasts from transgenic mice with metallothionein promoter driven Fos resulted in a rapid upregulation of c-Jun and NGF, with c-Fos/Jun heterodimer binding to the AP1 site in in the first intron of the NGF gene critical for NGF mRNA transcription (Hengerer et al., 1990).

Recent studies have shown that Schwann cell-specific deletion of floxed c-Jun using p0 promoter driven Cre, produces a massive increase in facial motoneuron cell death, with a commensurate reduction in peripheral target re-innervation, as well as in functional recovery after facial axotomy (Fontana et al., 2012). Follow-on analysis of neurotrophin and growth factor expression in the injured sciatic nerves of p0:jun mice, revealed a strong deficit in many of the normally upregulated trophic factors, including GDNF, Artemin, BDNF and LIF. Interestingly, administration of recombinant GDNF and Artemin to these p0:jun mice, substantially reduced the post-traumatic neuronal cell death and improved functional recovery, underscoring the importance of Schwann cell c-Jun in providing trophic support for the injured and regenerating neurons (Fontana et al., 2012).

Cell death signals

Nerve axotomy elicits a complex neuronal response that leaves injured neurons poised precariously between death and regeneration. Even in successfully regenerating peripheral nerves, axotomy can induce up to 60% cell death (Kalla et al., 2001;Raivich et al., 2002;Raivich et al., 2004;Raivich et al., 1999a), indicating a normal physiological role of cell death signals in the regenerative effort. Axotomy induced neuronal death can be apoptotic, necrotic or a combination of both (Pettmann and Henderson, 1998). A number of cell surface molecules like fas and TNFR 1 & 2 have been implicated in cell death. They have a cytoplasmic death domain (except TNFR2) and mediate neuronal death by exerting pro-apoptotic signals through FADD (Fas associated death domain) (Ugolini et al., 2003). Downstream cytoplasmic death signals include bax and caspase family members, especially caspase 3 (Chan et al., 2003;Sun and Oppenheim, 2003). Bax is a member of the Bcl-2 protein family, which competes with the survival associated factor

bcl-2 and bcl-x, downstream targets of STAT3, to promote apoptosis (Liu et al., 2011;Nielsen et al., 1999;Stephanou et al., 2000). In ischaemic brain injury in adult rats, bax and caspases appear to act downstream of c-jun phosphorylation via the JNK pathway, with a reduction in neuronal apoptosis by JNK inhibition (Guan et al., 2006;Putcha et al., 2003).

Different types of neurons can widely differ in their response to axotomy, depending on the intrinsic properties of the neuron as well as the regenerative environment of the axon (Goldberg and Barres, 2000). In neurons of the dorsal root ganglia (DRG), which extend branches to both the PNS and CNS, only a PNS lesion but not a CNS lesion induces axonal growth and neuronal survival (Lieberman, 1971). Neuronal survival following axotomy also depends on the distance between the injury site and the cell body (Dai et al., 2000). Axons die back to the proximal collateral branch point which may sustain the nerve fibre. If the injury is too close to the cell body, it normally results in cell death (Fry and Cowan, 1972). Target re-innervation and motoneuronal survival are greater when the epineurium stays intact, i.e., following nerve crush compared with complete transection. Also, the developmental maturity of the animal at the time of facial nerve injury strongly influences neuronal survival - the same peripheral nerve lesion affects neonatal motoneurons much more severely resulting in almost complete cell death as compared to their adult counterparts which show only limited cell death (Moran and Graeber, 2004).

Non-neuronal cellular changes

Accompanying the neuronal response to peripheral injury, non-neuronal cells are also activated in a graded response and cause acute inflammatory changes at the lesion site, in the distal part of the nerve and around the cell body of injured axons. Inflammatory cytokines increase in expression with increasing severity of injury. Initial expression of MCSF, IL-6 and TGFβ1 is followed by later expression of other cytokines like IL-1β, TNFα and IFN-γ (Raivich et al., 1999a). Inflammatory responses to injury serve two functions – clearing of debris and restoring normal function, and providing protection against infectious pathogens (Raivich et al., 1999a). However, they exert only moderate to minimal influence on speed of axonal regeneration (Galiano et al., 2001;Kalla et al., 2001).

Microglial activation

Resting microglia perform an immune surveillance function in the uninjured nervous system. Following nerve insult, they are rapidly activated and act as the first line of defence (Raivich, 2005). Microglial activation proceeds through several stages which are accompanied by changes in cytokine production (Raivich et al., 1999a;Raivich et al., 1999b). The stage of alert (Stage 1) is the first stage of activation and occurs within 24 hours after injury (Raivich et al., 1999a). The microglia proliferate extensively and become hypertrophic, up-regulating molecules with immune function like integrins αM and β2, and leucocyte adhesion molecule, ICAM-1, responsible for lymphocyte recruitment and interaction with the injured neurons (Kloss et al., 1999;Raivich et al., 1999a;Werner et al., 1998). In the homing stage (Stage 2), microglia proliferate and move towards the neuronal cell body and adhere to the damaged structures (Raivich et al., 1999a). Molecules induced in Stage 1 decline, whilst others like α5, α6, and β1 are upregulated around 4 days post-injury (Kloss et al., 1999;Werner et al., 1998). They actively engage in the process of 'synaptic stripping' whereby they displace synaptic inputs from the cell body of the regenerating neuron, which are subsequently wrapped by astrocytic cell processes (Graeber et al., 1993). In the presence of neuronal cell death, microglia become phagocytic (Stage 3a), with 3-20 microglia clustering together to form nodules which degrade the neuronal debris (Raivich et al., 1999a). Phagocytosis also causes activation of adjacent microglia, a process known as bystander activation (Stage 3b). Along with a massive induction of the previously expressed molecular markers, a number of new molecules including cell adhesion molecules for binding and internalising injury-induced debris and for antigen presentation, as well as proteases and pro-inflammatory cytokines are induced by co-stimulatory molecules ICAM-1, B7.2 and αXβ2-integrin (Kloss et al., 1999;Raivich et al., 1999a;Werner et al., 1998;Bohatschek et al., 2004).

Astrocytic response

Concomitant with the proliferation of microglial cells, local astrocytes which normally express very low levels of the GFAP, become reactive and undergo hypertrophy (Raivich et al., 1999a). GFAP is synthesised de novo as early as 24 h after axotomy (Graeber and Kreutzberg, 1986). Following the increase in GFAP synthesis, the reactive astrocytes reorganize their cytoskeleton under the control of specific cytokines like IL-6 (Klein et al., 1997), transforming from protoplasmic to fibrillary astrocytes. This dramatic increase in

GFAP after injury is significantly reduced in IL-6 null mice (Klein et al., 1997). Around 14 days after axotomy, astroglia replace the microglia on the neuronal surface, forming insulating lamellae around the recovering neurons (Kreutzberg, 1993;Graeber and Kreutzberg, 1988). Successful regeneration leads to a retraction of the astrocytic processes and gradual repopulation of the neuronal surface with synaptic terminals (Raivich et al., 1999a).

T-cell recruitment

Lymphocyte recruitment, as with microglial and astrocyte activation, occurs in response to neuronal injury and varies according to the severity of the injury (direct or indirect trauma) (Raivich et al., 1999a). 2-4 days after facial axotomy (indirect trauma), low-level entry of leucocytes (grade 1) is seen, restricted to the facial motor nucleus of the injured neuron (Raivich et al., 1998). Extensive post-traumatic cell death leads to a strong recruitment of CD3, CD11a, and CD44 immunoreactive T-cell (grade 2) at day 14, which aggregate around the phagocytic microglia (Raivich et al., 1998). Pro-inflammatory cytokines expressed by microglia and macrophages, including IL-1, IL-6, IFNγ and TNFα appear to have an important role in leucocyte recruitment (Raivich et al., 1998;Raivich et al., 1999a). More direct trauma like lesions to the brain and spinal cord elicit the entry of both granulocytes and macrophages (grade 3) in addition to the leukocytes and T-cells (Raivich et al., 1999a). These grade 3 leucocytes have a number of cytotoxic effects and result in considerable secondary injury contributing to axonal degeneration and tissue loss (Popovich et al., 1999).

Rate of regeneration, target re-innervation and functional recovery

Following crush injury to the peripheral nerve, axonal regeneration is typically successful even though axons need to regrow and remyelinate over long distances. Regeneration of crushed axons typically proceeds at a rate of 1-2mm per day in humans, and is much faster in rodents (Gutmann, 1942). Regeneration following nerve transection is much more complicated and a number of different theories postulate the crossing over of the proximal fibres to the distal stump and their extension towards their re-innervation targets (Zhang and Yannas, 2005). According to the popular and widely accepted 'basement membrane microtube theory' SCs proliferate and migrate to form linear Bands of Bungner surrounded by basement membrane for axon elongation and myelination. The 'neurotrophic theory'

proposes that elongation of axons and supporting cells is controlled by diffusion of soluble growth-promoting factors from the distal stump. According to 'contact guidance theory', this extension is guided by contact with insoluble substrates which provide tracks for the elongating axons to attach to. As per the 'pressure cuff theory', regeneration across a long gap (>1 cm) is mechanically blocked by circumferential contractile forces exerted by fibroblast, resulting in neuroma formation (Lundborg, 2003). High regenerative activity suppress the pressure cuff theory while facilitating the rest (Zhang and Yannas, 2005). The rate of regeneration can be accelerated by a prior, conditioning lesion (McQuarrie et al., 1977). Electrical stimulation of the proximal stump also reduces the time taken for functional recovery, but does so by reducing the delay before regeneration begins in some motor axons (Al-Majed et al., 2000;Brushart et al., 2002;Gordon et al., 2008). GDNF microspheres implanted at the coaptation site increase fibre diameter, myelin thickness, regenerating axon counts, and contractile muscle force after delayed nerve repair, thereby partially reversing the deleterious effects of chronic nerve injury (Wood et al., 2013;Wood et al., 2012), as mentioned earlier.

In addition to the robust outgrowth of crushed peripheral axons, they have also been shown to accurately reconnect to their original peripheral targets (Nguyen et al., 2002;Fournier and Strittmatter, 2002). In contrast, regeneration after nerve transection is not generally selective for appropriate targets (Miledi and Stefani, 1969), and collateral branches sprouting from the severed axons can randomly grow along different nerve vesicles (polyneural innervation) (Angelov et al., 2005). This can result in somewhat misguided and inaccurate target re-innervation (Gordon and Gordon, 2010;Valero-Cabre and Navarro, 2002) which can lead to synkinesis/pareses, abnormally associated movements, and pathologically altered reflexes (Angelov et al., 2005). Successful peripheral regeneration and complete functional recovery depends on the correct alignment of cut axons and sufficient plasticity of the nervous system for axons to innervate appropriate targets (Navarro et al., 2007).

Hurdles in CNS Regeneration

There are major differences between injury induced responses of the CNS tissue compared with PNS nerves. The numerous factors inhibiting neuronal regeneration in the CNS are complex and overlapping and include (but are not limited to) slower WD, extensive glial cell death and differential axonal arrangement of nerves. WD is rapid and

robust in the PNS but slow and incomplete in the CNS (George and Griffin, 1994). Macrophages are rapidly recruited to sites of WD in peripheral but not central nervous tissue (Perry et al., 1987). Transection of dorsal roots at L1-4, which projects axons into the PNS and CNS, showed that while the peripheral nerve roots were completely clear of myelin debris at 30 days post lesion, myelin and neurofilamentous accumulations were still visible in the spinal cord 90 days after injury (George and Griffin, 1994). The delayed WD is not due to slower axonal degeneration but due to a failure of axonal and myelin debris removal (Vargas and Barres, 2007). Myelin debris contains several inhibitors of regeneration (He and Koprivica, 2004) and their prolonged presence in the CNS creates an inhibitory extracellular environment contributing to the failure of CNS axons to regenerate.

The glial cells in the CNS also behave very differently from SCs following injury. SC phagocytose myelin and axonal debris, proliferate extensively in injured nerves and repopulate lesions sites rapidly to provide guidance pathways for axonal regrowth. Oligodendrocytes lack phagocytic properties (Bignami and Ralston, III, 1969), and in the days following injury, there is extensive glial cell death, resulting in a lesion site largely devoid of astrocytes and oligodendroglia.

Unlike peripheral nerve, axons in the CNS are not structurally separated by perineurial or neurilemmal sheaths, but are organised into tracts of related function and myelinated together without distinct physical barriers separating them. Hence, following injury, there are no biomechanical guidance cues in the CNS that can be compared with the bands of Büngner in peripheral nerves.

Facial Axotomy model

The facial axotomy model is well-established system for studying axonal response and neuronal regeneration (Moran and Graeber, 2004). This model was popularised by Kreutzberg and his colleagues in the 1980s and has since become the prototypic experimental paradigm, allowing a systematic and detailed investigation of the reaction of neuronal and non-neuronal cells and their microenvironment to various types of challenges.
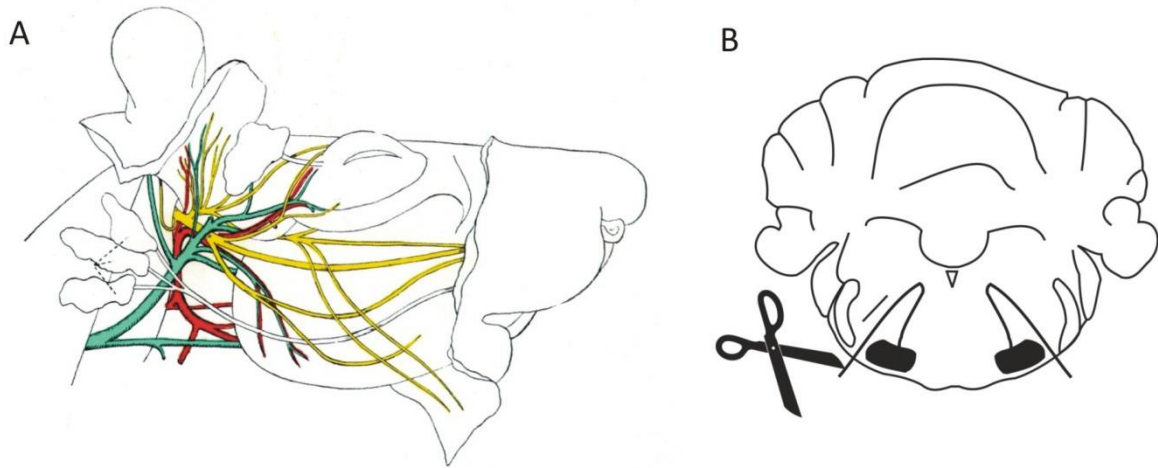
Figure 1.6. Facial Axotomy Model. Schematic drawing showing A: mouse facial nerve (yellow) B: transverse section showing location of facial nucleus in the brainstem (Moran and Graeber, 2004).

The facial nucleus is located in the rostral pons of the brainstem and exists as a semi-circular collection of approximately 2000 motoneurons, which project their axons within the facial nerve (VII cranial nerve) to muscles involved in ear, eyelid, lip and whisker hair movement in mice (Figure 1.6A) (Ashwell, 1982). This model has a number of obvious benefits. Firstly, the facial nerve is bilateral, and unilateral transection of the nerve as it leaves the CNS at the stylomastoid foramen, results in an intra-animal control on the contralateral side (Figure 1.6.B). Researchers benefit from the analytical strength of a paired experimental system with the normal control nucleus conveniently located on the other side of the brain stem. As the nucleus spans a rostral-caudal length of around 1 mm, multiple sections can be taken from each experimental animal providing further virtue to the model. Secondly, the surgical procedure is straightforward and of mild severity compared to other nerve injury models. Nerve crush injury allows re-innervation to take place within 3 weeks after injury, a reasonable time-frame for analysing end-point of regeneration. Nerve transection is a more severe insult. Thirdly, the injury does not involve disruption of the blood-brain-barrier or direct CNS trauma (Moran and Graeber, 2004).

Aims of my project

As outlined, nerve injury is accompanied by increased gene expression for a wide range of regeneration associated molecules. Transcription factors often act as the driving forces of regeneration, regulating the expression of a complex web of target genes simultaneously.

This thesis aims to investigate the likely role of three transcription factors – STAT3, c-jun, and C/EBPδ, and two downstream effector proteins – CAP23, and β1 integrin, in promoting axonal regeneration after peripheral nerve injury *in vivo*. All five molecules are known to be strongly upregulated following injury and during regeneration. Cell-type specific deletions of the individual genes were used to characterise the unambiguous roles of these molecules in nerve regeneration (except for C/EBPδ which was a global deletion). The work was undertaken in the facial axotomy injury paradigm - a reliable and highly reproducible model of peripheral nerve regeneration. It enabled assessment of the effects on the speed and extent of axonal regeneration and sprouting, recovery of motor function, neuronal and non-neuronal cellular response and neuronal survival, resulting from conditional alteration of these proteins.

The first part of the thesis focuses on transcription factors STAT3, c-jun and C/EBPδ.

STAT3 is slowly but assertively emerging as one of the important mediators of injury-induced regeneration, not just in the PNS but also in the CNS. Studies involving STAT3 gene deletions in sensory neurons or in retinal ganglion cells, or its increased expression via viral gene transfer, or by blocking the action of its inhibitors, have shown a promising role for this transcription factor in axonal regeneration and neuronal survival. However, the results from these studies are often conflicting – while some attribute a sustained positive role to STAT3, others claim its involvement is only transient in 'jump-starting' the regenerative response after cut but not crush injuries. Here, neuronal STAT3 KO mice were examined for their short-term and long-term effects on facial nerve injuries of varying severity. Particular attention was paid to measure the various indices of regeneration – early speed of regenerating growth cones, functional and anatomical re-innervation, axotomised motoneuron morphology and survival rates, early and late molecular changes within the injured neurons and the response of the surrounding glial cells. Schwann cell specific deletion of STAT3 was also examined for its role in regeneration.

Previous deletion studies of the transcription factor c-Jun have been particularly instructive, confirming its significance as both a neuronal and Schwann cell-derived factor in regeneration. Neuron-specific deletions of c-Jun have tremendous effects on peripheral regeneration and result in shrunken neurons, but promote neuronal survival. Conversely, Schwann cell-specific deletions lead to increased neuronal death additionally to impaired regeneration. These findings prompted the need to investigate the effects of combined

neuron- and Schwann cell-specific deletions on peripheral regeneration and motoneuron survival. Double knock-out mice with neuron- and Schwann cell-specific deletions using the cre-lox system under the synapsin and myelin protein zero promoters, respectively, were used to study the axonal response and neuronal regeneration in the facial axotomy model. Also examined was the role of N-terminal phosphorylation in c-Jun's regenerative function in response to facial motor nerve transection.

Similarly, mice with global C/EBPδ gene deletion were analysed for effects on regeneration after facial nerve injury. Additionally, to identify functional complementary counterparts, double knockout mice with combined deletions of two transcription factors (for example, c-Jun and STAT3, or C/EBPδ and STAT3) were generated and analysed for greater synergistic reduction in regeneration compared with single deletions.

The second part of the thesis focuses on effector proteins CAP23 and integrin β1 which are direct downstream targets of transcription factors, and form the basic building blocks of nerve repair. Preliminary studies using CAP23 floxed mice and injection of AAV-cre virus have shown a marked reduction in long-term ability to re-innervate the peripheral target in the facial axotomy model. β1 integrin is the only counterpart of α7 integrin, and global deletion of α7 decreases speed of axonal regeneration. Cell-type specific conditional mutants lacking the genes encoding these proteins in neurons were generated, and the effects of these mutants on axonal regeneration analysed as before. Comprehensive investigation of the changes resulting from these specific gene deletions, will allow us to obtain insight into downstream mechanisms controlled by the different transcription factors, and define hierarchies in the regenerative signalling pathways.

Scope of this study

Injury to peripheral nerves can occur due to several causes ranging from physical injury and accidental trauma to pathological conditions and degenerative diseases. Achieving successful regeneration and synchronised functional recovery following neural damage is one of the biggest challenges faced by neuroscientists and clinicians. This applies to both peripheral and central forms of injury. Central axons are normally unable to regenerate across glial scars, whether injury is due to physical trauma (e.g. spinal cord injury), ischemia, stroke and cerebral palsy, or inflammation-associated axonal damage as in

multiple sclerosis, and the modest functional recovery is almost completely due to post-traumatic (regenerative) collateral sprouting of the surviving axonal fibres. Even though neurons in the PNS are capable of regeneration following peripheral nerve trauma, the speed and specificity in the human patient is often sub-optimal. Peripheral nerve injury often results in incomplete functional recovery, and the appearance of involuntary aberrant movements (synkinesis), that may be due to incorrect regenerative sprouting. Understanding the molecular mechanisms underlying nerve regeneration via various regeneration associated molecules and their signalling components will improve the success of central axonal regeneration, as well enhance the speed and specificity of peripheral regeneration.

Chapter 2 – Materials & Methods

Animals

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, and with the approval of the Home Office. All animals were housed in UCL Biological Services Units in the Cruciform and Kathleen Lonsdale buildings, under standard conditions of a 12 hour light-dark cycle, constant temperature (21-23°C) and humidity (60%±5), and access to pelleted food and water ad libitum. Several lines of genetically engineered mice were used in this study. These were sourced from a number of different laboratories and are summarised in Table 2.1.

Genetic modified mice	Background	Source
STAT3 floxed	SVJ129	Shizuo Akira (Osaka U, Japan)
c-jun floxed	C57BL/6	Axel Behrens (Cancer Research, UK)
c-jun 4A	C57BL/6	Axel Behrens (Cancer Research, UK)
CEBP global KO	C57BL/6	Babis Magoulas (Queen Mary, U London, UK)
CAP23 floxed	CD1	Fred Sablitzky (U Nottingham, UK)
β1 integrin floxed	C57BL/6	Reinhard Fässler (Max Planck U, Germany)
Syn:cre	C57BL/6	Axel Behrens (Cancer Research, UK)
P0:cre	C57BL/6	Axel Behrens (Cancer Research, UK)

Table 2.1. Summary of genetic modified animals

Cell type specific single and combined deletions

Neuronal deletion – Knock-in mice carrying floxed allele of the gene/s of interest, say (Y) were crossed with syn::cre transgenic animals expressing cre recombinase under the control of the neuron-specific synapsin promoter (Zhu et al., 2001) twice, to generate mutant mice (Yf/f syn:cre +), in which both alleles of Y gene were inactivated in neurons. Sibling animals lacking the cre transgene, with functional, unrecombined homozygous floxed gene Y (Y f/f syn:cre-), served as controls. For example, Figure 2.1 describes the generation of mutant mice with cell type-specific deletion of STAT3 gene. To prevent germline Y deletion due to testicular synapsin promoter expression (Rempe et al., 2006), mostly female cre+ mice (Yf/f syn:cre) and cre- Yf/f males were used for breeding. Experimental cohorts consisting of homozygous mutants (syn:cre Yf/f) and litter-mate controls (Yf/f) were generated with normal Mendelian frequency (Sauer, 1998).
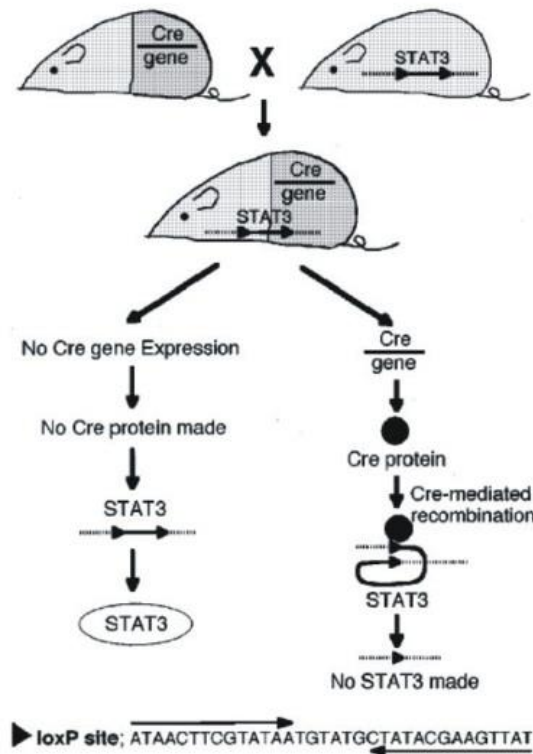
Figure 2.1. Generation of cell-type specific STAT3 deletion by Cre/loxP recombination. Floxed-STAT3 mice, in which two 34 bp loxP sites were introduced 5' and 3' of the STAT3 gene (Akira, 2000), were mated with transgenic mice expressing Cre protein under the control of cell type-specific promoter. The cre enzyme recognizes the loxP sequence motifs and excises the intervening STAT3 gene to generate cell type-specific STAT3 deletion; littermates without cre served as controls.

Schwann cell deletion – Knock-in mice carrying floxed allele of the gene of interest, say (Y) were crossed with P0::cre transgenic animals expressing cre recombinase under the control of the Schwann cell specific Myelin Protein 0 (P0) promoter (Feltri et al., 1999) twice. The loxP sites flanking the Y gene are recognised by cre recombinase, which excises the intervening Y gene in Schwann cells to generate homozygous deletion of gene Y specifically in Schwann cells. Experimental cohorts consisting of homozygous mutants (P0:cre Yf/f) and litter-mate controls lacking the cre transgene (Yf/f) were generated with normal Mendelian frequency.

Combined neuronal and Schwann cell deletion – Transgenic mice having homozygous neuronal deletion of gene Y (syn:cre Yf/f) were crossed with transgenic mice having homozygous Schwann cell deletion of the same gene Y (P0:cre Yf/f). The resulting litters had homozygous mice with combined neuronal and Schwann cell deletions (syn:cre P0:cre Yf/f), only neuronal deletion (syn:cre Yf/f), only Schwann cell deletion (P0:cre Yf/f)

or functional, unrecombined controls (Yf/f), which formed the four experimental groups in our studies.

Jun4A

Jun 4A mutant animals have substitution mutations where the N-terminal serines 63 and 73 (ser63&73) and threonines 91 and 93 (thr91&93) are replaced by alanines. This prevents JNKs from binding to the N-terminal phosphorylation sites, thereby abolishing the effects mediated by JNK phosphorylation of c-jun (Behrens, CRUK). Mice having wild-type phosphorytable c-jun were used as controls.

C/EBPδ

C/EBPδ knockout mice have global homozygous deletion of the C/EBPδ gene (CEBPδ -/-), and were previously generated in the C57BL/6 strain (Sterneck et al., 1998). Briefly, the replacement-type targeting vector was constructed by using 129/Sv mouse genomic DNA and the pGKneobpA and pGK-thymidine kinase cassettes. The coding region of the C/EBPδ gene was replaced by a neomycin-resistance gene, which was also used for their genotyping. Electroporation and selection were performed by using the CJ7 embryonic stem cell line. Two independent ES cell clones with the predicted rearrangements (nos. 433 and 466) were injected into C57BL/6 blastocysts to generate chimeras, which were mated to C57BL/6 females (Sterneck et al., 1998). Experimental cohorts consisted of homozygous null mutants (CEBPδ -/-) and wild-type controls (CEBPδ +/+).

Genotyping

Mice were ear-notched for identification, and less than 0.5 cm of their tail-tip biopsied for genotyping purposes. DNA was extracted from the tails of mice by adding 20µl of 20µg/ml proteinase K (Promega, Madison WI) and 750µl extraction buffer (5% 1M Tris, 20% 0.5M EDTA, 1% SDS, 3.3% 3M NaCl) to each tail and incubating at 55°C overnight in a rotating water-bath. Samples were briefly vortexed and placed on ice, before being centrifuged at 16000g for 10 minutes. 600µl isopropanol was added to the supernatant and the solution mixed by inversion until threads of DNA were visible, centrifuged for a further 10 minutes following which the pellets were washed with 70% ethanol, air dried for 20 minutes, re-suspended in 50µl of Tris-ETDA buffer (TE) and incubated for one hour at 65°C. The extracted DNA was stored at 4°C.

Target gene	Forward primer 5' - 3'	Reverse primer 5' – 3'	Annealing temp (^{0}C)
Generic cre	Mac1 sense ATTACCGGTCGATGCAACGAGT	Mac1 antisense CAGGTATCTCTGACCAGAGTCA	60°C
	Lck cre fwd CGGTCGATGCAACGAGTGATGAGG	Lck cre rev CCAGAGACGGAAATCCATCGCTCG	60°C
Synapsin cre	Syn M3 AGCTTCAGCACCGCGGACAGT	Nestin cre rev TCGTTGCATCGACCGGTAATG	60°C
P0 cre	P0 fwd CCACCACCTCTCCATTGCAC	P0 rev GCTGGCCCAAATGTTCGTGG	60°C
STAT3 WT & flox	STAT3-A CCTGAAGACCAAGTTCATCTGTGTGAC	STAT3-B CACACAAGCCATCAAACTCTGGTCTCC	60°C
c-Jun WT & flox	Lox5 CTCATACCAGTTCGCACAGGCGGC	Lox6 CCGCTAGCACTCACGTTGGTAGGC	60°C
C/EBP δ	C/EBP del fwd ACGACTCCTGCCATGTACG	C/EBP del rev GCTTTGTGGTTGCTGTTGAA	60°C
C/EBP Neo	C/EBP neo fwd AACAAGATGGATTGCACGCAG	C/EBP neo rev GAAGGCGATGCGCTGCGA	60°C
Cap23 WT	P2 AGCAAAGCGTGGCCGTCAAA	P4 CCAAAGCAAGTGAGGTCTGGGTAA	65°C
Cap23 Neo	Pneo ATTCAGGGCACCGGACAGGTCGGTCTTGACAA	P4 CCAAAGCAAGTGAGGTCTGGGTAA	65°C
β1 WT & flox	T56mod GGGTTGCCCTTCCCTCTAG	L1 GTGAAGTAGGTGAAAGGTAAC	60°C

Table 2.2. PCR reaction primers, sequences and annealing temperatures

DNA amplification was carried out in 25 µl PCR reactions (2.5µl 10X buffer, 5µl Q solution, 0.25µl 25 mM dNTP, 0.125 µl of each primer (1nM/µl), 0.2µl Taq polymerase (Qiagen) and 1µl of DNA (diluted 1/20 with DEPC water)). The thermocycling conditions for each PCR were optimised on the T-Gradient PCR machine (Biometra, Germany). Table 2.2 shows the list of primers used in this study.

2% agarose-TAE gels were used for resolving DNA samples. 2 g agarose was added to 100 ml 1x TAE buffer (0.4M Tris, 0.2M sodium acetate, 20mM EDTA, pH), boiled and ethidium bromide was added to a final concentration of 0.2µg/ml. PCR samples were mixed with loading buffer (1x TAE, 50% glycerol, 0.025% bromophenol blue) immediately prior to well loading. Samples were run against 5µl of a 100 basepair DNA ladder (NEB, UK) for 40 mins at a constant voltage of 100V and visualised with a UV transilluminator (MultiImage Light Cabinet, AIC, UK).

Surgeries

Facial Nerve Axotomy

Mutant and control mice of 2-5 months of age were anesthetised with 5% isoflurane (Merial, UK) and the depth of anaesthesia determined by using the paw withdrawal reflex. Oxygen was administered at 1.5L/min. The anaesthesia was subsequently maintained at 3% isoflurane. An incision was made below the right ear at the level of the mastoid process. Superficial muscles were bluntly dissected to expose the facial nerve. For facial nerve cut (1, 14, 30, and 90 days survival) the main branch of the facial nerve, including the retro-auricular branch, was transected at the stylomastoid foramen. For facial nerve crush (4, 10, and 30 days survival), the main branch of the facial nerve was crushed approximately 1mm distal from its exit from the stylomastoid foramen using fine forceps which were held in place for 10 seconds before letting go. The skin was closed with wound clips (AutoClip, USA) and the animal was allowed to recover in a heated chamber (32°C). Successful facial motor nerve transection was confirmed by the absence of motor function in the ipsilateral whisker hair. The animals were sacrificed after 1-90 days with an overdose of Euthatal (Merial, UK).
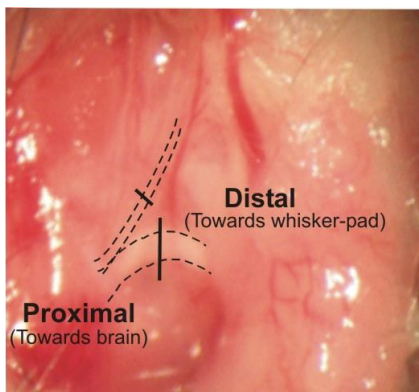
Figure 2.2. Right facial nerve as exposed during facial axotomy. The dotted lines indicate the upper and lower branches of the seventh cranial nerve as it emerges through the stylomastoid foramen. The two solid lines indicate the approximate site of cut/crush injuries.

Retrograde tracing of the facial nerve

Mice were anaesthetised with 10µl/g body weight of 2.5% 2,2,2-tribromoethanol (Avertin) (Sigma-Aldrich, Germany). A midline incision was made between the two whisker pads and a cavity created under both whisker pads. Anatomical re-innervation of the whisker pad was assessed by placing a 2.5x2.5 mm square piece of gel foam (Johnson and Johnson, UK) soaked in 10µl of 4% Fluoro-Gold (FG) (Fluorochrome, Denver, USA) in the

cavity under both whisker pads, 28 days after unilateral facial nerve lesion. Pads were left for 30 minutes, and then removed, and the skin sutured using 6-O prolene sutures (Johnson and Johnson, UK). In addition, some experiments used a double labelling system where 2 µl of 10% MiniRuby (MR) (Invitrogen, USA) was injected subcutaneously into the periorbital muscle in both upper and lower eyelids of each eye. 10µl of 10% buprenorphine (Vetergesic) (VetDispense, UK) was injected subcutaneously for analgesia. The animals recovered in a heated chamber (32°C), and allowed to survive for 48 hours to enable retrograde transport of the fluorescent dyes to the facial motor nuclei.

Viral transfection of facial motor neurons

Lentiviral injections into facial nucleus - Animals were anaesthetised using isoflurane anaesthetic (5% to induce, 2-3% to maintain), an incision was made along the top of the skull with a scalpel and connective tissue was scraped away with the scalpel until the bregma was exposed. Stereotactic co-ordinates for the middle of the facial motor nucleus was previously determined to be 5.3 mm caudal and 1.25 mm lateral to Bregma, and at a 4.7 mm depth from the surface of the brain. Measurements were taken twice before holes were drilled at the appropriate sites using a dental drill. All injections were made using a 10µL Hamilton Syringe (Hamilton Company, Switzerland) with a pulled glass tip attached with superglue. 2 µl of a lentivirus expressing STAT3 and GFP (obtained from Dr. Xuenong Bo, Queen Mary, Univ of London) was injected unilaterally into each animal using a Micro4 MicroSyringe Pump Controller (World Precision Instruments, Sarasota, USA) micropipettor. These animals underwent facial nerve axotomy 4 days post- viral injection, and FG retrograde tracer application 28 days post-axotomy, before being transcardially perfused two days later. Efficiency of viral infection was assessed by eGFP immunohistochemistry.

Retrograde AAV and AV injection into facial nerve – Animals were anaesthetised using isoflurane anaesthetic (5% to induce, 2-3% to maintain) and an incision was made below the right ear to expose the right facial nerve. 2-3 µl of AAV2 virus (from Dr. Joost Verhaagen, Netherlands Institute for Neuroscience), or Ad-Cre-GFP virus (from Vector Biolab – Adenovirus-Type 5 (dE1/E3) expressing CMV driven cre and GFP; Titer 1x 10^10 PFU/ml) was injected into the main branch of the facial nerve. In order to successfully inject the virus into the nerve, the nerve had to be partially snipped to expose cut axon tips; hence all animals underwent partial unilateral facial axotomy. Animals were sacrificed at

10 or 30 days after viral transfection, perfused and brains extracted and sectioned at the level of the facial nucleus. Efficiency of viral infection was assessed by eGFP immunohistochemistry.

Perfusion Fixation

Mice were terminally anaesthetised with intraperitoneal injection of Euthatal (200 mg/ml sodium pentobarbital) or avertin (2.5%) at 1, 14, 30 or 90 days after facial axotomy. Sufficient depth of anaesthesia was determined using the paw withdrawal reflex. A midline incision was made below the xiphoid process and the peritoneal cavity exposed. The rib cage was lifted, and the exposed diaphragm was incised at its junction with the anterior abdominal wall. The incision was continued circumferentially until adequate exposure of the pleural cavity was achieved. Whilst holding the xiphiod process, a deep cut was made along both sides of the rib cage and the anterior section of the ribcage lifted upwards so as to allow access to the mediastinum. A small incision was made at the base of the apex of the heart and a blunt-ended needle, attached to a perfusion pump (Gilson, USA), was inserted into the left ventricle. The inferior vena cava was cut permitting exsanguination. Perfusion was done trans-cardially via the left ventricle at a rate of 0.02L/min for 5 minutes with phosphate buffer containing 0.9% saline (PBS: 10 mM Na_2HPO_4, 0.85% NaCl, pH 7.4), followed by cold (4°C) 4% paraformaldehyde in 10mM phosphate buffered saline solution (PFA/PBS, pH 7.4) at the same flow rate (0.02L/min) for 10 minutes. The brains were extracted and post-fixed on a rotator at 4°C for 2 hours with 1% PFA/PBS (for immunohistochemistry) or overnight with 4% PFA/PBS (for Nissl), followed by cryo-protection in 30% sucrose in PB overnight and then frozen on dry ice for further use (Moller et al., 1996). For extracting facial nerve four days after facial nerve crush, the animals were perfused with 5 minutes of PBS at 0.02L/min followed by 10 minutes with 4% PFA/PBS at 0.02 L/min and 45 minutes with 1% PFA/PBS at 0.01L/min. Brains were extracted and placed in 30% sucrose overnight as before. The facial nerves were extracted and frozen immediately in flat chambers containing O.C.T (Tissue Tek, Netherlands) on dry ice.

Cryostat sectioning

Facial Motor Nucleus

Frozen hindbrain was fixed on a cryostat chuck using an O.C.T. compound (Tissue Tek, Netherlands). The chuck, housed in the cryostat (Leica CM 1900, Germany) was set to a temperature of -15°C while the ambient chamber temperature was set to -20°C. In the brainstem, 40 transverse sections of 20 μm thickness were collected, taken through the level of the facial motor nucleus, to incorporate both ipsilateral and contralateral facial nuclei in their entirety. For immunohistochemistry, 20μm sections were taken, unless where otherwise stated. The sections were collected on 0.5% gelatin-coated slides (Merck, UK), refrozen on dry ice and stored at -80°C.

Facial Nerve

Frozen nerve was fixed on a cryostat chuck using an O.C.T. compound (Tissue Tek, Netherlands). The chuck, housed in the cryostat (Leica, Germany) was set to a temperature of -15°C while the ambient chamber temperature was set to -20°C. 15-20 mm long pieces of facial nerves were sectioned longitudinally at 10μm.The sections were collected on 0.5% gelatin-coated slides (Merck, UK), refrozen on dry ice and stored at -80°C

Light microscopic immunohistochemistry

Facial nucleus and facial nerve sections were retrieved from -80°C, rehydrated in distilled water and spread onto the slide using fine brushes. The sections were dried for 10-15 minutes, circled with a PAP pen (DAKO, Cambridgeshire), and then fixed for 5 minutes in 4% formaldehyde, followed by wash in 0.1M phosphate buffer (PB). Antigen retrieval was done by immersing the slides for 2 min each in 50% acetone/H2O, 100% acetone, 50% acetone/H2O, followed by washing twice in PB, and once in PB/0.1% bovine serum albumin (PB/BSA) (BSA; Sigma, UK). The sections were then blocked for 30 minutes in PB/5% goat or other serum, to match the source of the final, secondary or tertiary antibody (Vector, UK). These pre-incubated sections were incubated overnight at 4°C with the primary antibody at dilutions determined previously through titrations. The sections are then incubated for 1 hour at room temperature with appropriate biotinylated secondary antibody. Source and dilution of all antibodies used are listed in Table 2.3.

Antigen	Antibody	Dilution	Source
Primary			
αM (CD11b)	5C6, RtM	1:5000	Serotec, UK
ATF3 (C-19)	RbP	1:400	Santacruz Biotechnology
αX	HmP	1:400	Chemicon, UK
β1	RtM	1:3000	Chemicon, UK
β 7.2	RtM	1:1600	Pharmigen, UK
CD3	RtM	1:400	Pharmigen, UK
CD44	MAB 2137 RtM	1:400	Chemicon, UK
CGRP	RbP	FMNu 1:5000 FNe 5000	Bachem, UK
c-JUN (H-79)	RbP (FITC-cj)	1:200	Santacruz Biotechnolgy
eGFP	RbP	1:500	Millipore, CA
Galanin	RbP	FMNu 1:2000 FNe1:5000	Bachem, UK
GFAP	RbP	1:6000	Vector, UK
p-STAT3	RbM	1:1000	Abcam, UK
Secondary			
Rabbit Ig	Biot. α-Rb Ig, GtP	1:100	Vector, UK
Rat Ig	Biot. α-Rt Ig, GtP	1:100	Vector, UK
Hamster Ig	Biot. α-Hm Ig, GtP	1:100	Vector, UK

Table 2.3 Summary of antibodies used. Abbreviations. FMNu – facial motor nucleus, FNe- facial nerve **Antigens:** ATF3- Activating Transcription Factor 3, CGRP - Calcitonin Gene-Related Peptide, GFAP – Glial Fibrillary Acidic Protein, p-STAT3 – phospho- Signal Transducer and Activator of Transcription, Ig – Immunoglobulin, **Antibodies:** RtM - rat monoclonal, RbP - rabbit polyclonal, HmP – hamster polyclonal, RbM – rabbit monoclonal, FITC-cj – fluorescein isothiocyanate conjugated, GtP – goat polyclonal.

All incubations with primary and secondary antibodies were carried out in PB/BSA, and finished by washing in 3 changes of PB/BSA. Exposure to the biotinylated secondary antibody was followed by 1 hour incubation with the Avidin-Biotinylated horse radish peroxidase Complex (1:100) (ABC, Vector, Wiesbaden, Germany) and visualized with diaminobenzidine (DAB; Sigma, UK) and hydrogen peroxide (H_2O_2). The reaction mixture consisted of 0.5 g/l DAB, 1:3000 dilution of 30% H2O2 in 10mM PB, pH 7.4, and the sections were allowed to react for 3 - 5 minutes at room temperature. The αX, B 7.2 and CD3-immunoreactivity was enhanced with addition of Co/Ni to the DAB mixture. The DAB reaction was monitored under a light microscope and stopped by washing the sections in 10mM PB followed by distilled water. Sections were subsequently dehydrated by immersion in increasing concentration of ethanols and isopropanol, and then immersed thrice in xylene before being mounted on a glass cover slip with Depex (BDH, UK).

Quantification of light-microscopic Immunohistochemistry

A Sony 3CCD colour video camera (AVT-Horn, Aachen, Germany) was used to obtain 8-bit digital images of the antibody stained sections. Images of both control and axotomised nuclei and for the glass were captured at x10 magnification, using Optimas 6.2 software (Bothwell, WA). Captured images were run against an algorithm to obtain mean and standard deviation values for optical luminosity. Standard deviation was subtracted from the mean for each image and the resulting values for axotomised and control sides were each subtracted from that of the mean optical luminosity of the empty glass slide, to obtain specific optical luminosity values (OLV) for each facial nucleus, on both the control and axotomised sides.

Cresyl violet (Nissl) staining

Tissue sections (20µm) of the facial motor nucleus were rehydrated in ddH2O, spread, and allowed to dry completely. They were fixed in 4% formaldehyde (BDH, UK) overnight followed by 70% ethanol overnight. 1% cresyl violet or Nissl solution was made by mixing 4g cresyl violet powder (BDH, UK) in 40mL 100% Ethanol, shaking and inverting for 15 minutes, then adding this mixture to 360mL warm ddH2O and filtering using Whatman size 4 filter paper. The slides were immersed in this 1% Nissl solution for 6-8 minutes. Excess cresyl violet was removed by washing in water and in increasing concentrations of ethanol (70%, 90%, 95%). This was followed by a de-staining step in 95% ethanol containing 10 drops of glacial acetic acid; the reaction being monitored under a light microscope. The de-staining was stopped by transferring the sections to 100% ethanol, isopropanol, and the slides immersed in xylene thrice, prior to being cover slipped using Depex (BDH, UK).
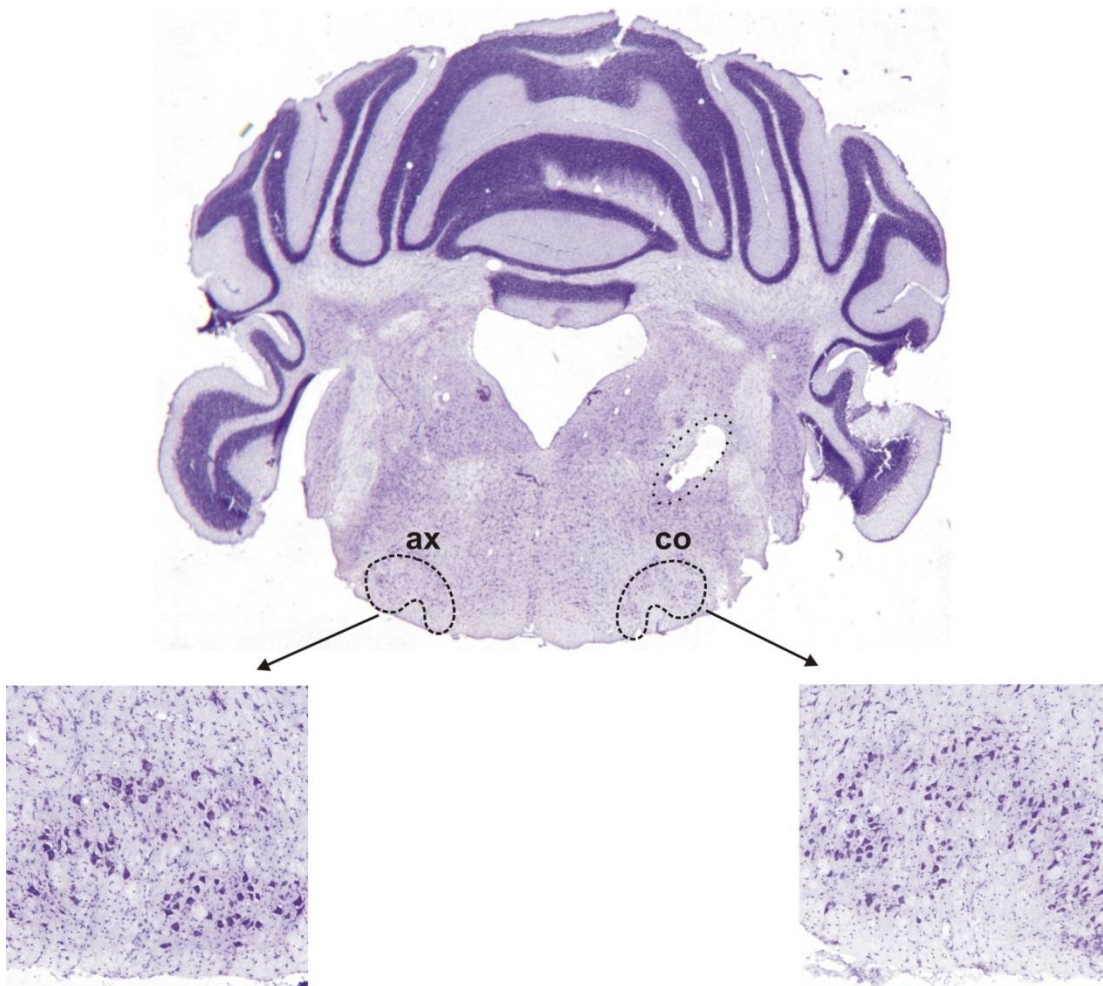
Figure 2.3. A 20µm Nissl-stained coronal brainstem section, showing the unaxotomised/control (co) and axotomised (ax) facial nuclei. The two nuclei are distinguished from each other by a puncture hole made in control side (shown in dotted lines, above the control nucleus). The insets show the two facial nuclei under higher (10X) magnification. Note the chromatolytic neurons on the axotomised side.

Neuronal cell counts

Motoneuron cell counts were determined in the axotomised and unaxotomised facial nuclei 30 and 90 days after a facial nerve cut, and 10 and 30 days after facial nerve crush. Using Nissl-stained 20 µm sections (example Figure 2.3), all the neuronal profiles throughout the entire nucleus were counted and corrected for cell size using the Abercrombie Correction coefficient (Abercrombie, 1946), according to the formula:

N = n x [D/(D + d)],

Where N = the corrected neuronal number; n= the counted number of neurons; D = the section thickness (20um) and d = the mean neuronal diameter, calculated from the cell size. Neuronal cell size was determined from digital images of the stained sections obtained using a Sony 3 CCD video camera and Optimas 6.2 software. The mean diameter (d) was calculated from the mean area (Area), assuming a near spherical form, with the formula: $d = \sqrt{4 \times Area/\pi}$

Speed of facial axonal regeneration

Four days after facial nerve crush, the facial nerve was fixed in-situ by a long perfusion followed by immediate dissection and freezing on dry ice, as described earlier. Nerves were cut longitudinally at 10µm thickness, and the regenerating axons were visualized by immunostaining for galanin or CGRP immunopositive growth cones. Every fifth section was used per antibody, and the distance between the most distal galanin or CGRP+ growth cone and the crush site measured using a Leica microscope with an attached vernier device (Figure 2.4). The distance travelled by the fastest growing axon was calculated using the following formula:

$$d = \sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2}$$

Where d = distance between crush site and fastest growth cone; X_1, Y_1 = co-ordinates of the injury site; X_2, Y_2 = co-ordinates of the most distal immuno-labelled growth cone.
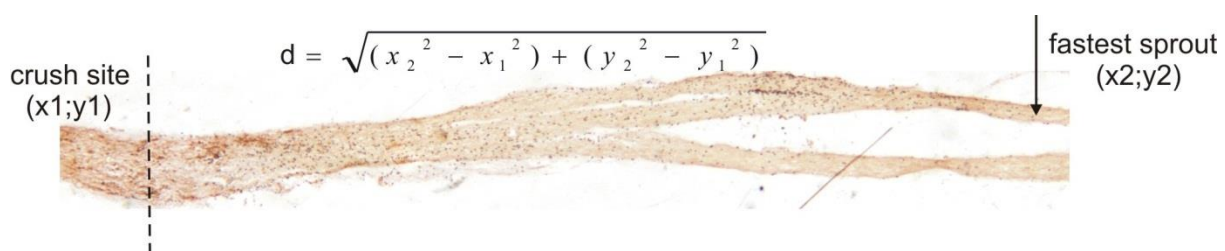
Figure 2.4. Speed of facial nerve regeneration as determined by the distance travelled by fastest axon sprout from the crush side. A longitudinal 10µm CGRP-stained facial nerve section, showing the crush site (identified by swelling of the nerve and concentration of granulocytes), and the furthest CGRP+ sprout. The distance travelled by the fastest growing axon is calculated using the formula shown above, where (X1, Y1) are co-ordinates of the injury site and (X2, Y2), the co-ordinates of the most distal immuno-labelled growth cone.

In cases where the nerve was interrupted or crooked, individual line lengths were determined using the formula above and added or subtracted as appropriate to determine

total length. The average distance for each animal was calculated from four or five tissue sections (Werner et al., 2000).

Assessment of functional recovery

Functional recovery was assessed by scoring the post-traumatic whisker hair motor performance after facial nerve injury. The movement was scored by a blinded observer on every alternate day, starting from seven days after facial axotomy till the end of 28 days (or 8 days for short-term studies and 88 days for long-term studies) when retrograde labelling with Fluorogold is carried out. The whisker hair movement (WHM) is scored between 0 - 3 with 0.5-step intervals; the lack of movement in the ipsilateral vibrissae was assigned a score of 0, the presence of asymmetric, fibrillation-related movement of the vibrissae received a score of 0.5, 1 was given for mild vibrissae sweeping, 2 for moderately good sweep, and 3 for completely normal movement as on the unoperated side. For each individual animal, WHM recovery index was calculated using the linearly interpolating area under the curve (AUC) function on the time course of assessment.

Quantification of re-innervation

Anatomical re-innervation of the whisker pad was determined by retrograde labelling. Quantification of retrograde labelling of facial motoneurons with FG or MR or both was done on every fifth section of the facial nucleus under a fluorescent microscope (Nikon Eclipse E600). FG positive cells were counted in the facial motor nucleus at 450-490 nm wavelength (UV light) using both 64x and 16x filters to enable counting of only strongly FG positive neurons. MR positive cells in each facial nucleus were counted at 510-560 nm wavelength (red light) and the number of double labelled cells (FG & MR positive) were also determined. The overall ratio of labelled neurons in the operated/unoperated side was calculated for each animal. Survival adjusted re-innervation (SAR) was calculated by dividing re-innervation ratio (FG ax/co%) by the percentage of surviving motoneurons (Nissl counts ax/co%).

Statistical testing

The mean ± standard error of mean (SEM) was recorded for all data. When only two groups were present, statistical analysis for axonal regeneration distance, functional recovery analysis, neuronal cell counts, and immunohistochemical staining intensities between mutant mice and control groups, and between the uninjured and injured sides, was performed using a standard two-tailed, unpaired Student's t-test. When more than two groups were present, statistical analysis was done using one-way ANOVA followed by post-hoc Tukey test. When multiple comparisons of data were made, for example, in the behavioural experiments where animals were repeatedly assessed over time, repeated test ANOVA was done. For all statistical analyses, $p < 5\%$ was considered to represent a significant difference between groups.

Chapter 3 – STAT3 Results

Neuronal STAT3, but not Schwann cell STAT3, is essential for successful nerve regeneration after facial nerve cut and crush injuries

STAT3, a member of the Signal Transducer and Activator of Transcription family of transcription factors, is rapidly activated following nerve injury and retrogradely transported into the nucleus (Lee et al., 2004;Ben-Yaakov et al., 2012) where it induces transcription of downstream effector genes. Rapid and prolonged STAT3 phosphorylation following injury is a reliable event following peripheral nerve injury, and has been shown in both sensory and motor neurons (Haas et al., 1999;Schweizer et al., 2002;Bareyre et al., 2011). Upstream of STAT3 activation, various neurotrophic factors such as CNTF, LIF and IL6, have been shown to mediate their neuro-protective effects predominantly via JAK/STAT kinase pathways (Stahl et al., 1995). Schweizer and colleagues have previously reported a role of STAT3 in promoting cell survival after facial axotomy (Schweizer et al., 2002). Sun et al., demonstrated a role for STAT3 in regeneration of retinal ganglion cells after optic nerve crush (Sun et al., 2011). Another study by Bareyre et al, has shown an important but transient role of STAT3 in triggering regeneration of superior cervical ganglion cells after saphenous nerve axotomy (Bareyre et al., 2011). Since global deletions of STAT3 are embryonic lethal, neuron-selective or Schwann-cell selective deletions of STAT3 have been used here to investigate its role in cell survival, target re-innervation, functional recovery, and speed of axonal regeneration following axotomy of the facial motor nerve. By analysing these indices of regeneration at shorter (10 and 30 days after FNA) and longer periods (90 days after FNA), and after mild (crush) and severe (cut) injuries, we have attempted to paint an unequivocal picture of the role of STAT3 in peripheral nerve regeneration. This chapter also looks at the expression of various neuronal regeneration-associated genes by the axotomised cell bodies, as well the expression of non-neuronal molecules assisting in the regenerative effort, to identify direct functional downstream targets of STAT3.

Generation of mutant mice lacking neuronal STAT3

Neuron specific STAT3 gene deletion was achieved by crossing mice with floxed STAT3 alleles (STAT3F/F) with animals expressing cre recombinase under the control of the synapsin promoter (syn::cre), which targets the STAT3ΔS recombination specifically to neurons (Chapter 2 – Figure 2.1) (Kugler et al., 2001;Zhu et al., 2001). The neuronal deletion of STAT3 was confirmed by PCR and immunohistochemistry. While both the STAT3ΔS mutants and the STAT3F/F controls showed a single 300 bp band for the two flowed STAT3 alleles, only the STAT3ΔS mutants gave a positive band for 'cre' (Figure 3.1). Further, homozygous STAT3ΔS recombination was associated with disappearance of STAT3 immunoreactivity in the injured facial motor nucleus (Figure 3.2B).
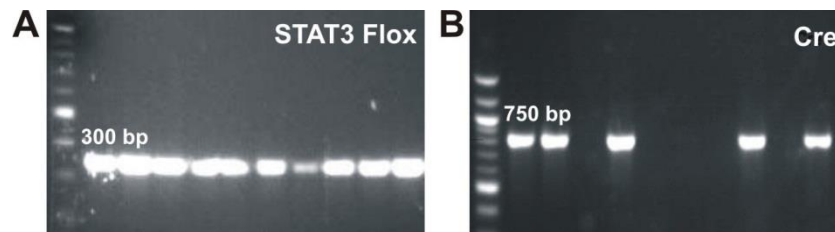
Figure 3.1 - Generation of STAT3ΔS and STAT3 F/F mice. Neuron-specific STAT3 deletion was achieved using synapsin::cre deletion of floxed STAT3 (STAT3ΔS). Agarose gel electrophoresis of products from PCR reactions to detect STAT3 floxed/wild-type allele at 300 bp (A) and cre recombinase allele at 750 bp (B). While both STAT3ΔS mutants and STAT3 F/F controls expressed homozygous floxed STAT3 alleles (A), only the STAT3ΔS mutants showed a band for cre (B).

STAT3 is induced following facial nerve axotomy

Following nerve axotomy, there is an upregulation and translocation of p-STAT3 from the cytoplasm into the nucleus of the injured motoneurons. A time-course of p-STAT3 expression shows that p-STAT3 expression is induced immediately following injury, and activation is sustained for up to 7 days. No expression is seen after 7 days and in the control, uninjured side (Figure 3.2A). Upregulation of activated STAT3 and its nuclear translocation following facial axotomy was completely abolished in the STAT3ΔS mutants (Figure 3.2B).
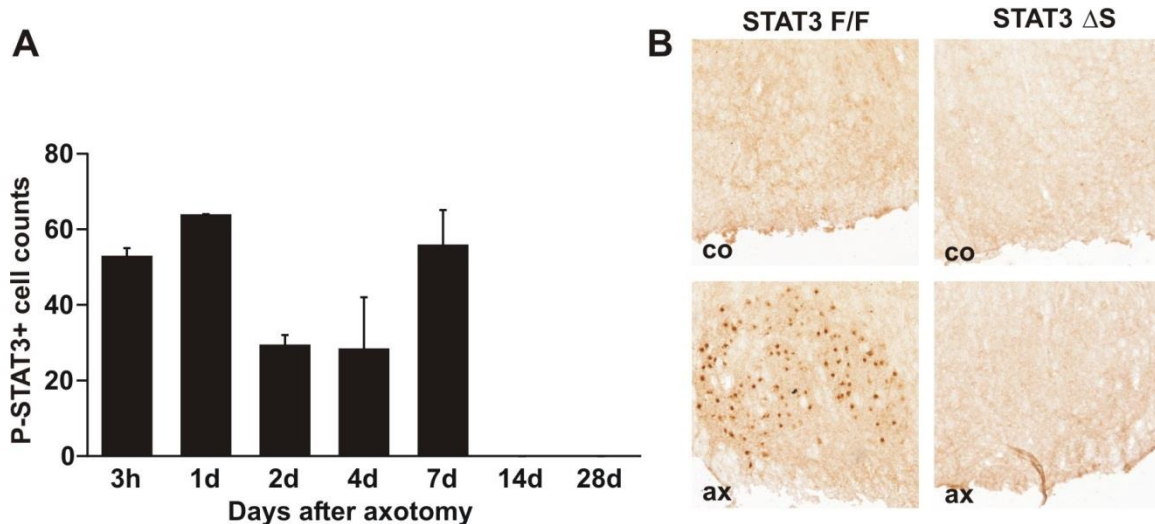
Figure 3.2. A. Time-course of phospho-STAT3 expression following axotomy. p-STAT3 expression is induced immediately following injury, and activation is sustained for up to 7 days. No expression is seen after 7 days and in the control, uninjured side. **B**. Synapsin::cre deletion of floxed STAT3 (STAT3ΔS) removes p-STAT3 immunoreactivity following facial nerve cut. Phospho-STAT3 immunoreactivity in the facial nucleus in STAT3 F/F (left panel) and STAT3ΔS mice (right panel) on the uninjured (co) and injured side (ax), 4 days after facial nerve cut shows that STAT3 is activated and translocated to the nucleus after facial axotomy in STAT3 F/F mice but not in STAT3ΔS mice.

Deletion of neuronal STAT3 interferes with axonal regeneration

Effects on axonal regeneration after facial nerve axotomy at the stylomastoid foramen were assessed by measuring the extent of functional recovery, re-innervation of peripheral target, and speed of axonal elongation in the early phase of nerve regeneration. Regeneration and functional recovery was dramatically reduced in the STAT3ΔS mutants compared to their littermate controls.

Functional recovery

Functional recovery after facial nerve cut was assessed by scoring post-traumatic whisker hair movement from 0 (no movement) to 3 (strong, normal movement). As shown in Figure 3.3 A, STAT3F/F animals commenced recovery of whisker vibrissae at 13 days and improved steadily over the next 2.5 weeks to day 28, reaching a maximum score of 2.4± 0.1. Recovery in homozygous STAT3ΔS mutants was significantly delayed; first noticed at day 15, it then improved with a considerably flatter trajectory, reaching a maximum score of 0.8± 0.1 by day 28. These differences were mirrored by changes in the regeneration index, calculated as Area Under the Curve (AUC) for the functional recovery over days 0-

28 (Figure 3.3B); with an index of 0.97±0.02 for the STAT3 F/F (n=5) and 0.32±0.07 for the STAT3ΔS mutants (n=5, p<0.01%).

To investigate whether the effect of STAT3 deletion led to a final severe defect in recovery, a second experiment was performed; this time the STAT3ΔS and STAT3 F/F animals were allowed to survive and their whisker hair movement assessed for a period of 90 days after facial axotomy. Overall the STAT3ΔS mutants still showed a significantly slower recovery compared to the STAT3 F/F controls till day 54 (Figure 3.3C). Thereafter, recovery of the mutants accelerated, and by day 61, the STAT3ΔS mutants had achieved levels of whisker function similar to that of their littermate controls. The STAT3ΔS mutants then followed closely parallel trajectories of functional recovery till day 90, albeit being slightly lower than the controls, but insignificantly so. The regenerative index for the mutants was still lower than the controls, but this difference was no longer significant (Figure 3.3D). This suggests that while STAT3 is not absolutely essential for functional recovery, its absence significantly delays the regenerative process.

Regeneration following nerve transection is a complicated process and requires the formation of a cellular bridge to allow crossing over of the proximal fibres to the distal stump (Zhang and Yannas, 2005). In contrast, anatomical and functional regeneration is often better after crush injuries since the basal lamina scaffolds for the regenerating growth cones remain intact after nerve crush. To investigate whether the delay in achievement of function after facial nerve cut was a result of the STAT3 deficient axons being unable to form a cellular bridge between the proximal and distal stumps, facial nerve crush was performed, and functional recovery assessed as before by scoring post-traumatic whisker hair movement from 0 (no movement) to 3 (strong, normal movement). While the STAT3 F/F animals started recovering motor function within 9 days after injury and improved steadily, already reaching a maximum score of 3.0 by day 19, recovery in homozygous STAT3ΔS mutants was significantly lower (Figure 3.3E). It was first noticed around day 15, and showed an extremely sluggish improvement to a final score of 1.3± 0.2 by day 28 (figure 3.3.E). In terms of the overall Area under the Curve, STAT3ΔS mutants (0.85±0.06) had a 58% lower regenerative index (p<0.01%) compared with the STAT3F/F mice (2.04±0.03) (Figure 3.3.F).
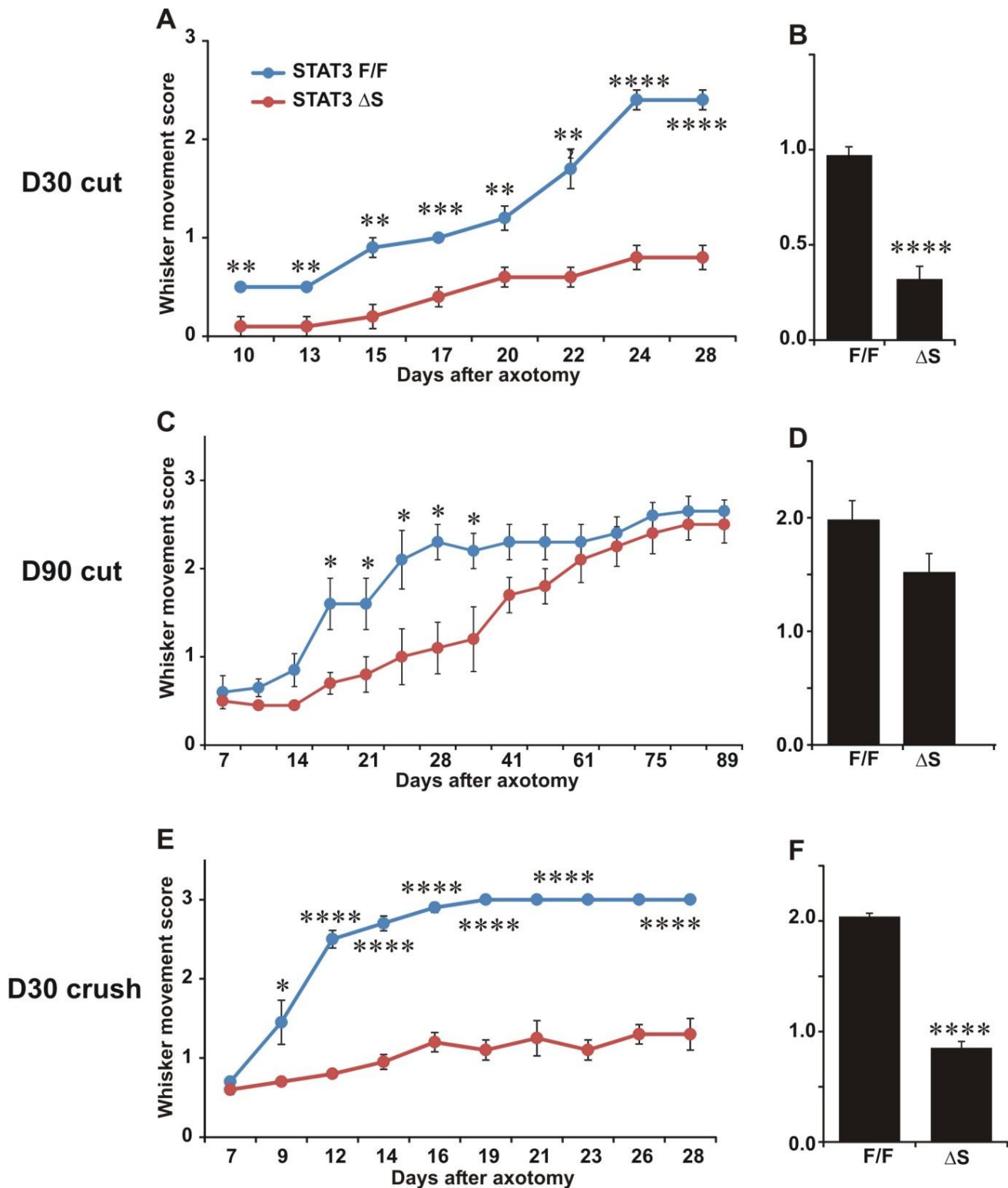
Figure 3.3 – STAT3 is required for successful functional recovery after facial nerve cut and crush injuries. Time course (A,C, E) for functional recovery of whisker hair movement on a scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) and the recovery index (B,D,F) calculated as Area Under The Curve (AUC) show a pronounced reduction and delay in functional recovery in STAT3ΔS mice (n=5) compared to STAT3 F/F mice (n=5) at 30 days post-nerve cut and crush injuries, and a delay in recovery by day 90 (n= 5 for both groups).*p< 5%, ** p<1%, *** p< 0.1%, **** p<0.01% in Repeated Test ANOVA and in unpaired Student's T-Test (for AUC), bars and error bars show the mean and SEM, respectively.

Re-innervation of peripheral target

Regrowth of the axotomised axons and reconnection of the facial nerve to the whisker pad allows functional recovery and is therefore a crucial goal of the regenerative response. To determine whether the observed defect in functional recovery in the STAT3ΔS mutants was due to lack of axonal re-innervation or due to reduced function of successfully re-innervated axons, the re-innervation pattern was analysed in mutant and control groups after cut and crush injuries.

To investigate the crucial time-point when the first regenerating fibres re-innervate the target muscle in the whisker pad after axotomy, 15 wild-type mice underwent facial nerve crush and then retrograde tracing with fluorescent tracers Fluorogold (FG) & MiniRuby (MR) at days 8, 18 and 28 after axotomy (n=5 for each time point). A gel foam insert soaked with FG was implanted for 30 minutes under both whisker pads; MR was injected into the eyelids (Figure 3.4.A). They were sacrificed at days 10, 20 and 30 respectively, and the number of FG and MR positive motoneurons in the contra- and ipsi-lateral facial nucleus quantified. The % (ax/co) of FG+, MR+ and double labelled neurons is shown in Figure 3.4.B. Quantification of MR+ neurons revealed an interesting pattern - the percentage of injured motoneurons positively labelled for MR reduced with increasing time after axotomy, suggesting that MR might be taken up more readily by freshly axotomised neurons, rather than by axons elongating in a later stage. Hence, the number of MR+ may not be an accurate indicator of all reconnected axons and therefore MR was no longer used in these experiments.

The number of FG positive neurons showed a more linear increase - 10 days after axotomy, the wild-type animals showed about 55% re-innervation (%FG ax/co). By day 20, they achieved almost 90% re-innervation which did not improve upon significantly by the end of day 30. Therefore, day 10 and day 30 were chosen as a time-points to investigate the arrival of the first wave of re-innervating axons, and the completion of target re-innervation respectively.
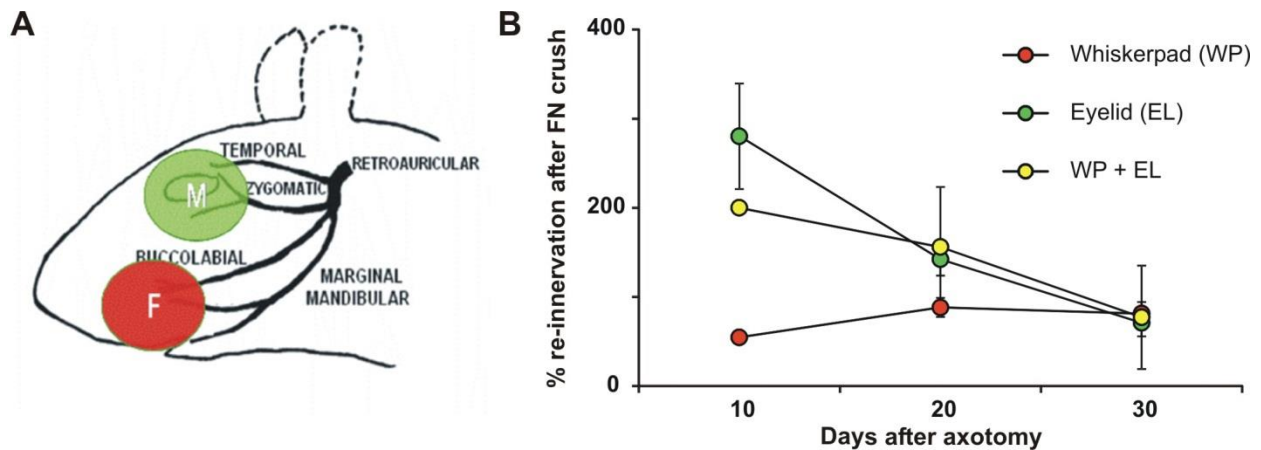
Figure 3.4 – Retrograde labelling with fluorescent tracers – Fluorogold (FG) and Miniruby (MR)   **A.** Schematic diagram showing the application of fluorescent retrograde tracers, MR (in green) to eyelid (EL) and FG (in red) to whisker pad (WP) of control mice at different time-points after crush and cut injuries. These tracers are subsequently quantified in the facial nucleus after 48 hours of retrograde transport. **B.** Time-course of re-innervation after crush injury, showing percentage re-innervation of whisker-pad (WP) and eyelid (EL) by FG and MR respectively at 10, 20 and 30 days after facial nerve crush.

Analysis of re-innervation in the STAT3F/F and STAT3ΔS animals, 10 days after facial nerve crush, showed that STAT3F/F animals had 257±60 FG+ motoneurons on the uninjured side and 181±60 on the injured side. Homozygous STAT3ΔS mutants also showed a similar number (276±22) on the control side, but there was a drastic reduction on the axotomised side, with just 58±14 FG+ motoneurons (Figure 3.5.E). In relative terms (as a percentage of contralateral side), STAT3F/F animals showed retrograde labelling of 72±23%, and STAT3ΔS mutants an almost 3-fold decrease with 21±5% (Figure 3.5.F; $p<5\%$). By 30 days after crush injury, while the control group had achieved 78±6% re-innervation, the mutant group still showed only 24±3% FG+ neurons (% ax/co), a very mild increase since the d10 quantification (Figure 3.5. G, H; $p<0.01\%$).

Nerve crush injury, allows re-innervation to take place as early as 2 weeks after the injury (as shown above). In addition to the robust outgrowth of crushed peripheral axons, they have also been shown to accurately reconnect to their original peripheral targets (Nguyen et al., 2002;Fournier and Strittmatter, 2002). In contrast, complete nerve transection represents a more severe challenge, and the ensuing regeneration is generally not selective for appropriate targets (Miledi and Stefani, 1969), resulting in misguided and inaccurate target re-innervation (Gordon and Gordon, 2010;Valero-Cabre and Navarro, 2002). To investigate an exacerbated regeneration defect after transection injury, FG was applied unilaterally into the right whisker pad 28 days after facial nerve cut, followed by

perfusion (after 48 hours), and cryo-sectioning at the level of the facial motor nuclei. Counting every 5th 20um section, STAT3 F/F animals showed retrograde labelling of 62±7% neurons, and the STAT3ΔS mutants an almost 4-fold decrease with 17±4% (Figure 3.5.A,B; p<0.01%), exceeding the defect in functional recovery. As expected, both the control and mutant groups showed greater reduction re-innervation after facial nerve cut (62% and 17% respectively) than after facial nerve crush (78% and 24% respectively). However, the ratio of re-innervation in the mutants compared to controls remained unchanged between the two injury severities, suggesting that the re-innervation defect in the STAT3ΔS mutants is probably due to a lack of intrinsic regenerative ability arising from neuronal STAT3 deletion, rather than a problem in overcoming the cellular bridge between the proximal and distal stumps.

Finally, in order to investigate whether this reduced re-innervation effect continued throughout the delayed regenerative response in the STAT3ΔS mutants, quantification of the retrograde tracer was done in mutant and control animals 90 days following axotomy. Again, both STAT3ΔS and STAT3 F/F animals showed similar numbers of FG+ motoneurons on the uninjured side. However, while STAT3 F/F animals showed retrograde labelling of 87±3% (ax/co %) motoneurons, the STAT3ΔS mutants still showed only approximately half the level of re-innervation with 45± 2% FG+ cells (ax/co %) (Figure 3.5 C,D; p<0.01%). Altogether, these results suggest that neuronal STAT3 is indispensable for target re-innervation after both crush and cut injuries, but not for functional recovery, following axotomy.
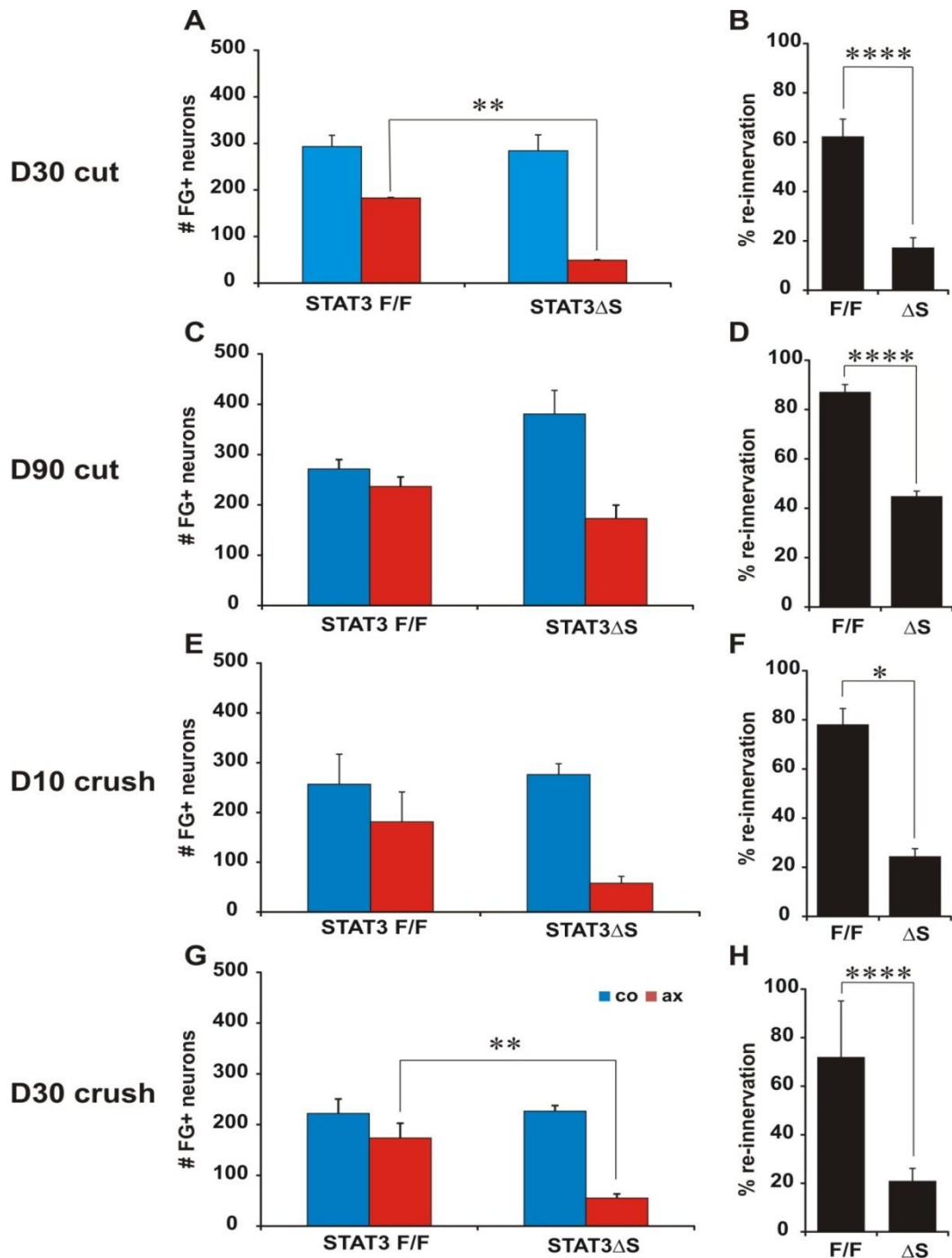
Figure 3.5 - STAT3ΔS mice show impaired peripheral target (whisker pad) re-innervation after facial nerve crush and cut. Whisker pad re-innervation was determined by counting retrogradely labelled Fluorogold-fluorescing facial motoneurons (FG+) at 30, and 90 days after facial nerve cut (A,C) and 10, and 30 days after nerve crush (E,G) in STAT3 F/F controls and STAT3ΔS mutants. Axotomised (ax)/control (co) side ratio – percentage re-innervation – for the FG+ motoneurons are displayed alongside (B, D, F, H). **A, B.** STAT3ΔS mutants (n=7) show almost 75% reduction in whisker pad re-innervation compared to STAT3 F/F controls (n=7) at 30 days after facial nerve cut. **C, D.** Even 90 days after facial nerve cut, neuronal deletion of STAT3 is associated with a 50% decrease in whisker-pad re-innervation. **E, F, G, H.** There is also a sharp 70 % decrease in the number of labelled neurons on the axotomised side in mutant STAT3ΔS compared with control animals at 10 and 30 days after nerve crush. *p< 5%, ** p<1%, *** p< 0.1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Speed of axonal elongation
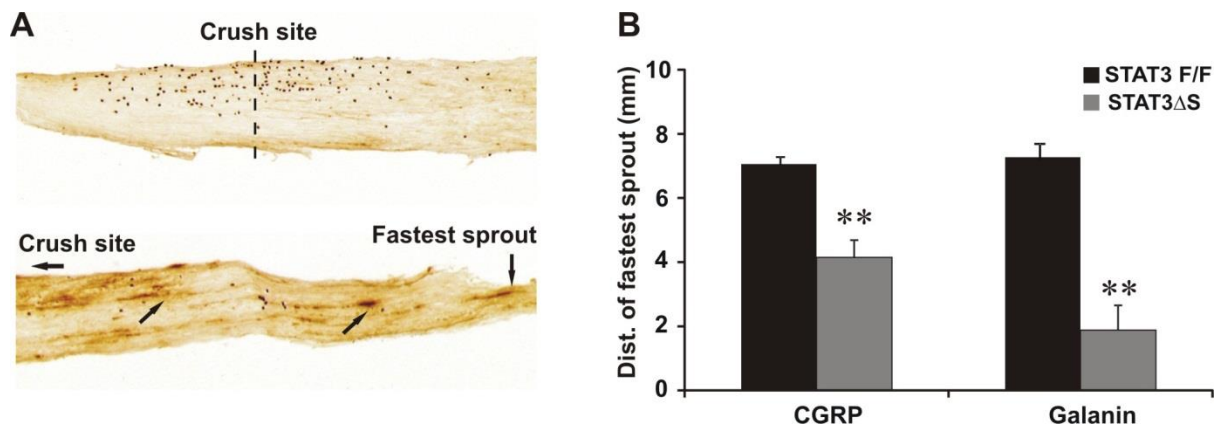
Figure 3.6 – Speed of regeneration and neurite elongation depends on the presence of neuronal STAT3. **A.** The crush site (shown with a dashed line) is characterised by swelling and accumulation of granulocytes, as well as a break in the myelin sheaths observed under UV light. Speed of regeneration was assessed by measuring the distance (in mm) of longitudinal outgrowth of fastest CGRP- and Galanin-immunoreactive axons from the crush site, 4 days after facial nerve injury. Black arrows are pointing to CGRP+ sprouts. **B.** STAT3ΔS deletion (n=4) caused a 40% decrease in outgrowth of CGRP+ neurites and 80% decrease of galanin+ neurites. STAT3 F/F mice showed very similar outgrowth distances for the CGRP- and Galanin-immunoreactive axons. **p<1% in unpaired Students's T-test. Bars and error bars show mean and SEM respectively.

Speed of early outgrowth of nerve fibres was examined using the facial nerve crush paradigm, four days after injury. Unlike nerve 'cut', where regeneration depends on the formation of a cellular bridge between the proximal and distal nerve stumps, in crush injury, the basal lamina scaffolds remain intact, providing a channelled pathway for the regenerating axons. The growth front of the regenerating motor neurites was quantified in longitudinally cut, fixed, 10um thick facial nerve sections using immunoreactivity for the CGRP and galanin neuropeptides (Figure 3.6A). As shown in Figure 3.6 B, in the STAT3 F/F control animals axonal growth front advanced to 7.1±0.3mm for CGRP- and to 7.3±0.5mm for the galanin-positive axons at the end of 4 days. In STAT3ΔS mutants, this axonal elongation was reduced by more than 40% for CGRP positive growth cones, with the fastest neurites reaching a distance of 4.2±0.5 mm from the crush site (p<1%). Galanin positive growth cones regenerated to a maximum distance of 1.9±0.8 mm, a reduction of nearly 80% compared to their control counterparts (p<1%). However, galanin immunoreactivity was significantly reduced in the mutants, making it somewhat unreliable in assessing speed of elongation.

Neuronal STAT3 deletion abolishes neuronal cell loss after axotomy but affected motoneurons show atrophic phenotype

Following axotomy, about 20-40% of lesioned motor neurons die within 30 days after injury, possibly due to lack of trophic support from the de-innervated target (Ferri et al., 1998;Raivich et al., 2002). Figure 3.7A shows the effects of neuron-specific deletion of STAT3 on the Nissl stained sections of control and axotomised facial motor nuclei. All neuronal profiles were counted through the whole facial motor nucleus on the operated and controls sides and the number corrected using the Abercrombie correction coefficient (Abercrombie, 1946).

After axotomy, STAT3F/F control animals showed a pronounced loss of neurons, an effect lost in the STAT3ΔS mutants. Counting motoneuron profiles on the uninjured and injured side throughout the facial motor nucleus revealed a 27±4% loss in STAT3F/F controls, compared with -9±7% in the STAT3ΔS mutants at 30 days after nerve cut (Figure 3.7 B; p<0.1%). However, most of the surviving axotomised STAT3ΔS motoneurons appeared shrunken by almost 50%, showing a dramatic reduction in cellular diameter from 19.5±0.9 to 11.2+/-0.8um (Figure 3.7C; p<0.01%). The opposite effect was observed in their control counterparts which underwent chromatolytic enlargement by 15% from 19.2±0.5 um for the uninjured to 22.1+/-0.8um for injured motoneurons (Figure 3.7C). A similar trend for neuronal survival was seen at day 90 and the surviving neurons continued to be in an atrophic state (Figure 3.7 B,C).

Investigation of motoneuron survival after crush injury also revealed a similar pattern as before. At day 10 post axotomy, STAT3F/F controls showed 18±2% motoneuron loss compared with -6±3% in the STAT3ΔS mutants (Figure 3.7B; p<0.1%). After 30 days, STAT3F/F controls showed 18±5% cell death compared with 8±2% in the STAT3ΔS mutants (Figure 3.7B). At both time-points, axotomised neurons in the STAT3ΔS mutants appeared shrunken by approximately 20%, which was less than the shrinkage seen after nerve transection (Figure 3.7C).
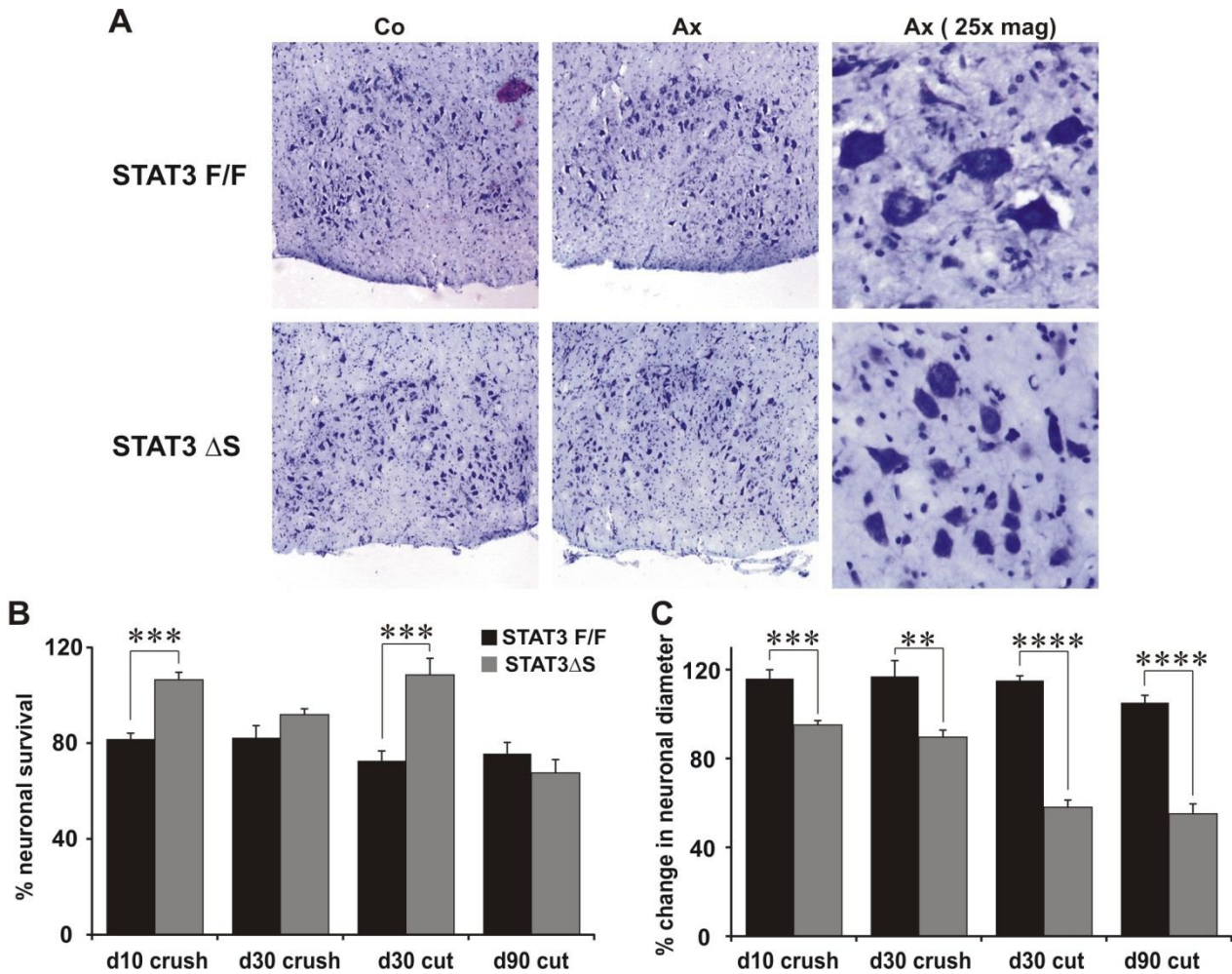
Figure 3.7 – Deletion of neuronal STAT3 prevents motoneuron death following axotomy but induces neuronal atrophy. **A**. Nissl-stained coronal sections of facial motoneurons on the contralateral (co) and axotomised (ax) side, and at higher 25 X magnification of ax side in STAT3 F/F (n =7) and STAT3ΔS (n = 7) mice, 30 days after unilateral facial nerve cut. **B**. Total motoneuron count, corrected for neuronal size, showed a significant increase in neuronal survival after axotomy in the absence of STAT3 after d10 crush and d30 cut. **C.** Percentage change (ax/co%) in neuronal diameter measurements (in μm) showed a mild chromatolytic increase in neuronal size in STAT3 F/F controls, but a significant shrinkage of STAT3ΔS neurons after axotomy. ** p<1%, *** p< 0.1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Survival adjusted re-innervation

To investigate whether alterations in motoneuron survival contributed to the observed reduction in re-innervation and functional recovery, survival adjusted re-innervation (SAR) was calculated by dividing re-innervation (FG %ax/co) by cell survival (Nissl %ax/co). Figure 3.8 shows a comparison of the survival adjusted regenerative abilities between STAT3 F/F controls and STAT3ΔS mutants at 10 and 30 days after crush, and 30 and 90 days after cut. At day 10 after crush, while the controls showed 89% SAR, the mutants

showed a 4-fold reduction with 20% SAR (p<5%). At day 30 after crush, while the controls had 95% SAR, the mutants showed 26% SAR (p<0.01%). The effect was even more dramatic at 30 days after nerve cut, with the control group demonstrating 85% SAR, and the mutant group only 16% SAR (p<0.01%). By day 90 after crush, even though the mutant group showed an increase in their SAR to 68%, it was still significantly lower than their control counterparts who exhibited 116% SAR (p<1%). This data suggests an inherent defect in the ability of the STAT3ΔS mutants to regenerate which is independent of their effects on cell survival.
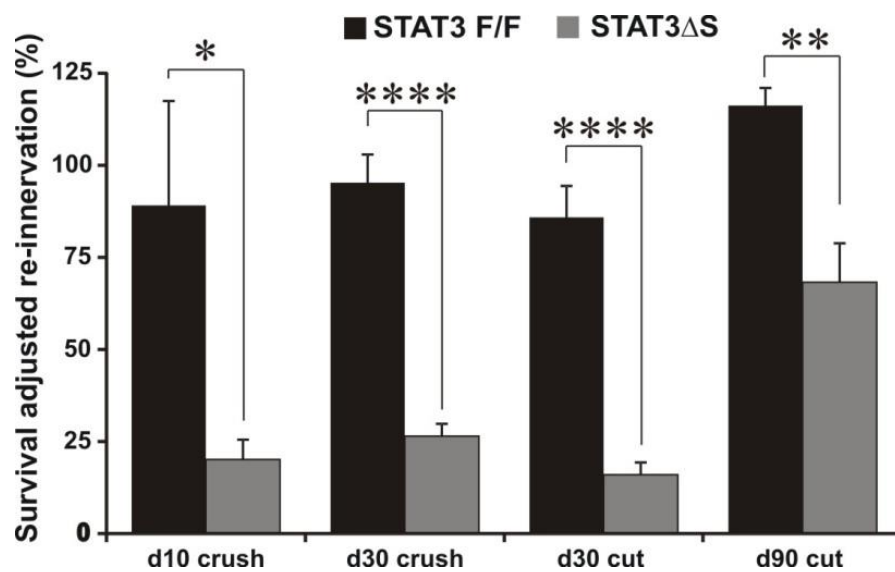
Figure 3.8 – Deletion of neuronal STAT3 causes a massive reduction in survival adjusted re-innervation after nerve cut and crush injuries. Nearly 80% reduction in SAR was seen at d10 after crush, almost 75% reduction at d30 after crush, 82% reduction at d30 after cut, and 40% reduction at d90 after cut. * p<5%, ** p<1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Neuronal molecular response to injury requires neuronal STAT3

Following neuronal injury, numerous morphological changes occur in the neuronal perikaryon, cumulatively known as 'chromatolysis', enabling the injured neuron to switch from a 'transmitting' mode to a 'growth' mode (Lieberman, 1971). The ensuing regeneration programme comprises of changes in transcription factors, adhesion molecules, neuropeptides and growth factor signalling, and an increase in nutrient transport, metabolism and cytoskeletal protein synthesis (Makwana and Raivich, 2005).

Nerve injury induces the activation and nuclear translocation of transcription factors like c-jun and ATF3 (Lindwall and Kanje, 2005;Raivich et al., 2004;Werner et al., 2000b). Neuronal deletion of STAT3 completely blocks the nuclear translocation of ATF3 (Figure 3.9 (a) E-H, (b) D-F; p<0.1%). On the other hand, the induction of c-jun and its translocation to the nucleus was unaffected, quite interestingly so, by the lack of STAT3 (Figure 3.9 (a) A-D, (b) A-C).

Increased levels of neuropeptides, CGRP and galanin, in the facial nucleus have previously been associated with an enhanced sprouting and regenerative response (Arvidsson et al., 1990;Kerr et al., 2001;Streit et al., 1989). Axotomy of the facial nerve causes an increase in the immunoreactivity for CGRP and Galanin of STAT3 F/F at day 14 (Figure 3.9 (a) I-J, M-N). This effect is almost abolished in the STAT3ΔS mutants (Figure 3.9 (a) K-L, O-P). Quantitation of CGRP/Galanin positive sprouts in the facial nucleus show a significant decrease of both neuropeptides (Galanin+ sprouts are reduced from 112±27 to 13±3; CGRP+ sprouts from 62±17 to 3±0.9) in the mutants compared with controls (Figure 3.9 (b) J, K; p<1%).

Facial nerve axotomy in the STAT3 F/F control animals also causes an increase in the immunoreactivity for the adhesion molecule CD44 (Figure 3.9 (a) Q-R). This axotomy induced change is almost completely abolished in STAT3ΔS mice (Figure 3.9 (a) S-T). Direct quantification of immunoreactivity (IR) by luminosity revealed a significant decrease by 90% (p<0.01%) in the amount of CD44-IR on the axotomised side in the STAT3ΔS mutants compared with STAT3 F/F controls. This effect was seen consistently at all three time-points – day 1, day 4 and day 14 after axotomy (Figure 3.9 (b) G-I).

Similarly, upregulation of β1 integrin has been associated with peripheral nerve injury and regeneration (Kloss et al., 1999;Werner et al., 2000b). A significant difference was observed between mutant and control groups, the mutants having 76% less β1 immuno-reactivity compared to the controls (p<5%) (Figure 3.9 (a) U-X, (b) L).
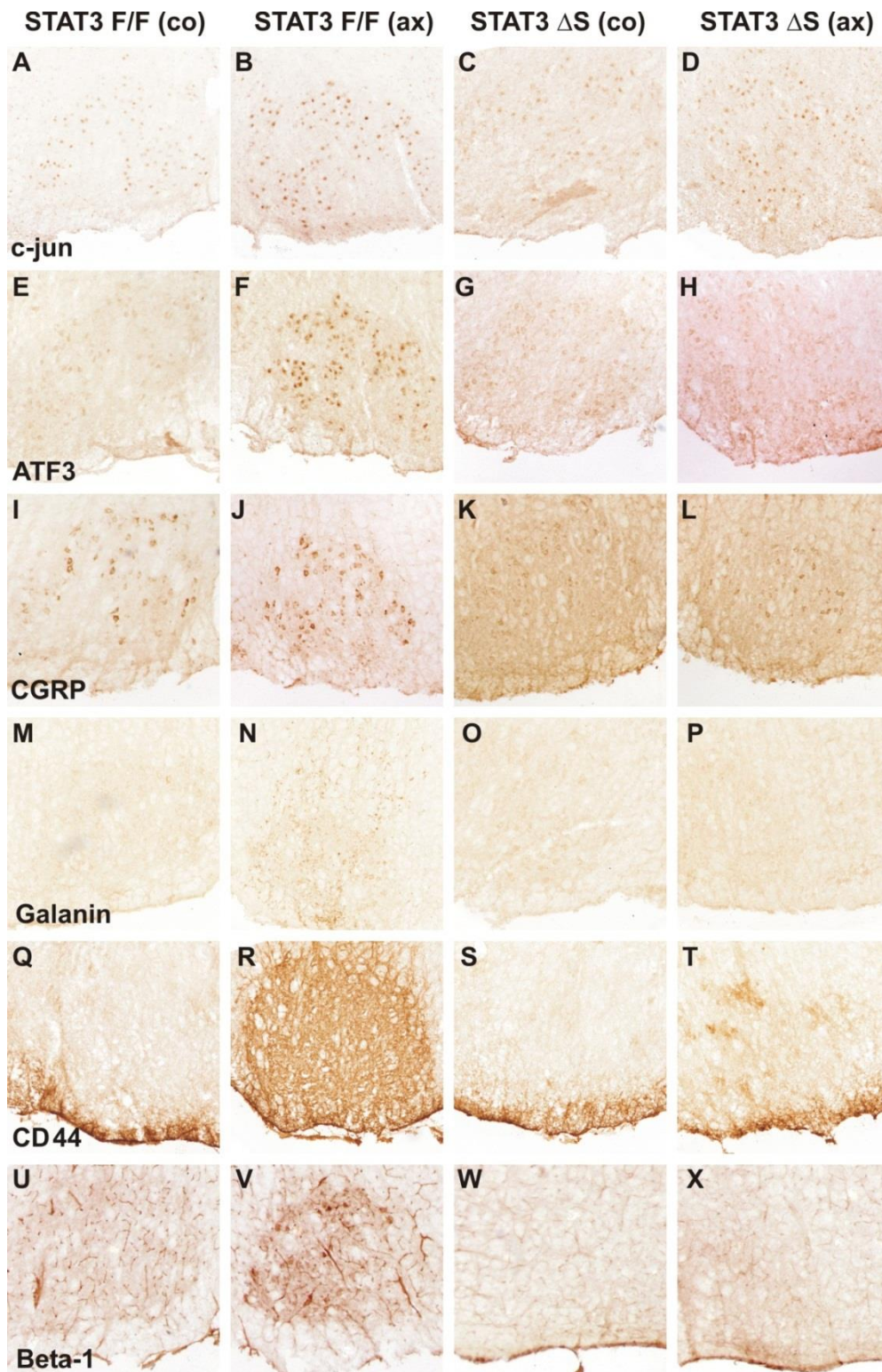
Figure 3.9 (a) – Neuronal axotomy response depends on the presence of STAT3. **A-X:** Facial nucleus axotomy response 14 days after nerve cut in the STAT3 F/F controls (A,B,E,F,I,J,M,N,Q,R, U,V) and the STAT3ΔS mutant (C,D,G,H,K,L,O,P,S,T, W, X) mice on the contralateral side (co) (1st and 3rd column), and on the axotomised side (ax) (2nd and 4th column). After axotomy, STAT3 F/F mice show a prominent increase in transcription factors c-jun (A-B) and ATF3 (E-F), neuropeptides CGRP (I-J) and Galanin (M-N) and adhesion molecules CD44 (Q-R) and beta1-integrin subunit (U-V); most of these changes are significantly reduced or abolished in the STAT3ΔS mutants, except for the activation of transcription factor c-jun, where STAT3 deletion has only a mild effect on its injury induced up-regulation (D).
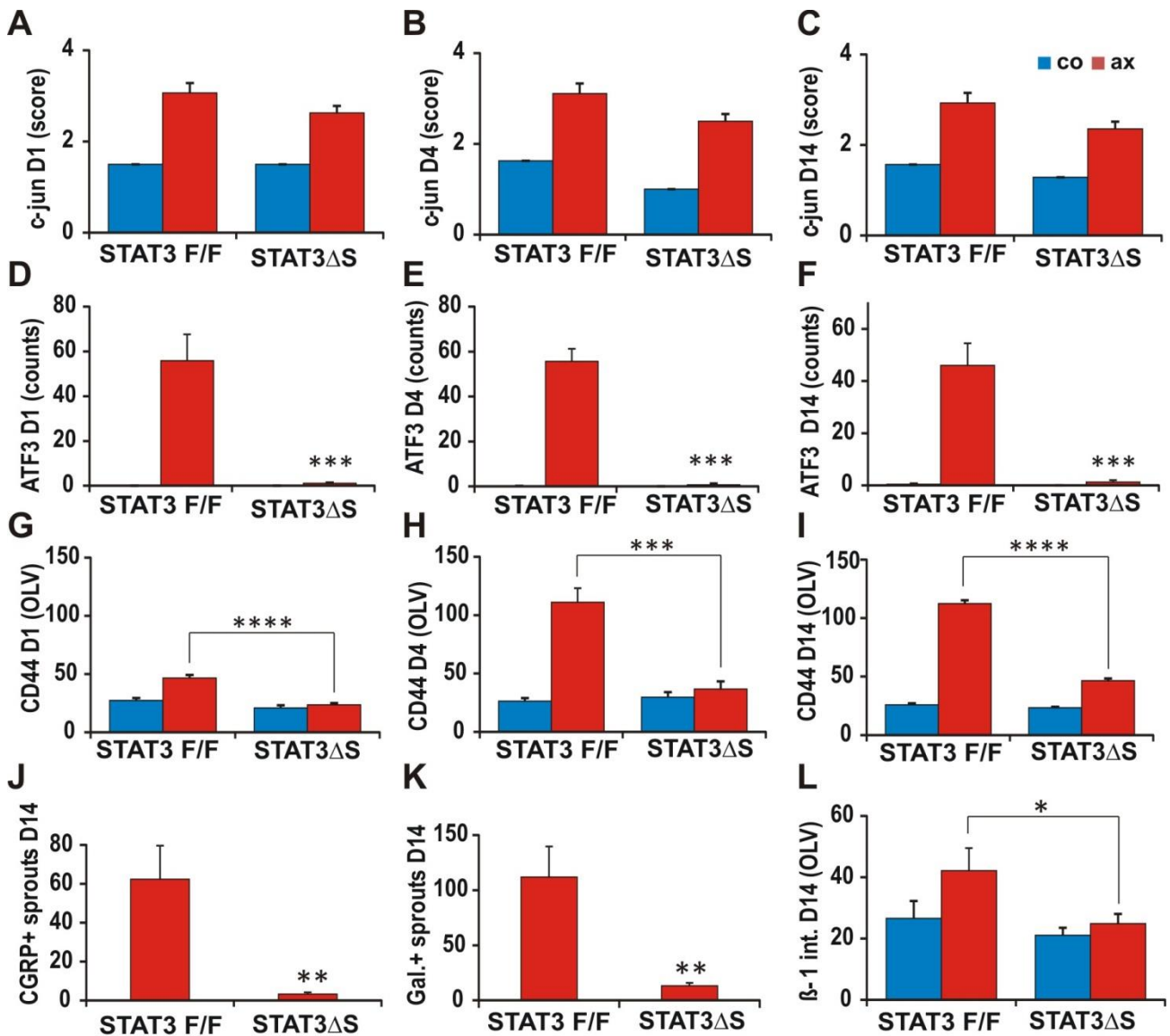
Figure 3.9 (b) – Neuronal axotomy response depends on the presence of STAT3. **A-L.** Quantification of overall facial motor nucleus immunoreactivity by manual counts or OLV units at 1 (d1), 4 (d4) and at 14 days (d 14) after nerve cut for c-jun (A-C), ATF3 (D-F), CD44 (G-I), CGRP (J), Galanin (K), and β-1 integrin (L) in STAT3 F/F (n=5) and STAT3ΔS mice (n=5). The contralateral (co) and axotomised (ax) sides are indicated by blue and red bars respectively. All biochemical markers, except for c-jun, showed a significantly smaller increase in the STAT3ΔS mutants compared to STAT3 F/F controls. *p<5%, ** p<1%, ***p<0.1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Non-neuronal responses like glial activation & immune recruitment require neuronal STAT3

Nerve transection not only triggers changes in the neuron but also results in activation of neighbouring non-neuronal cells, causing inflammatory changes at the level of the affected motor nucleus, associated with activation of microglia and astrocytes, as well as leukocyte recruitment (Graeber et al., 1989;Kalla et al., 2001;Raivich et al., 1991a;Raivich et al., 1996;Schwaiger et al., 1998;Raivich et al., 1998). These changes become particularly pronounced at day 14, which is a time-point corresponding with the peak in neuronal cell death, microglial phagocytosis and the influx of CD3+ T-cells (Raivich et al., 1998).

Microglia, undergo a number of changes in response to neuronal injury, from resting microglia (Stage 0) to state of alert (Stage 1) to homing and adhesion (Stage 2) and finally phagocytosis in the presence of neuronal cell death (Stage 3a) and bystander activation of surrounding non-phagocytic microglia (Stage 3b) (Raivich et al., 1999a). Each of these changes can be characterised immuno-histochemically by using antibodies against specific activation markers. Neuronal STAT3 deletion caused a substantial reduction in αM staining which serves as an early and mid-phase activation marker that normally increases after injury, as seen in the STAT3 F/F controls (Figure 3.10 (a) A-B). Quantification of the immune-labelling by optical luminosity values showed a significant, approximately 80% reduction in injury-induced αM integrin expression at day 1 and day 4 (Figure 3.10 (a) C-D (b) A,B; p<1%), and an almost 90% decrease at day 14 (Figure 3.10 (b) C; p<0.1%) in the STAT3ΔS animals. The late phagocytosis-associated microglial markers, αX and B7.2, were examined 14 days after facial axotomy (Figure 3.10 (a) E-H, I-L). Quantification of αX and B7.2 immunoreactive nodules by manual counts, showed a reduction of both markers in STAT3ΔS mice, with a complete abolition of αX immunoreactive nodules and reduction in B7.2 immunoreactivity by 92% compared with littermate controls (Figure 3.10 (b) D,E; p< 1%).

Injury to the facial nerve also causes local astrocytes to become reactive, undergo hypertrophy and rapidly increase GFAP synthesis (Raivich et al., 1999a) . Therefore, astrocytic activation after injury was measured by GFAP immunoreactivity. Astrocyte activation was unaffected by deletion of STAT3 at day 1, 4 or 14 (Figure 3.10 (a) M-P, (b) G-I).
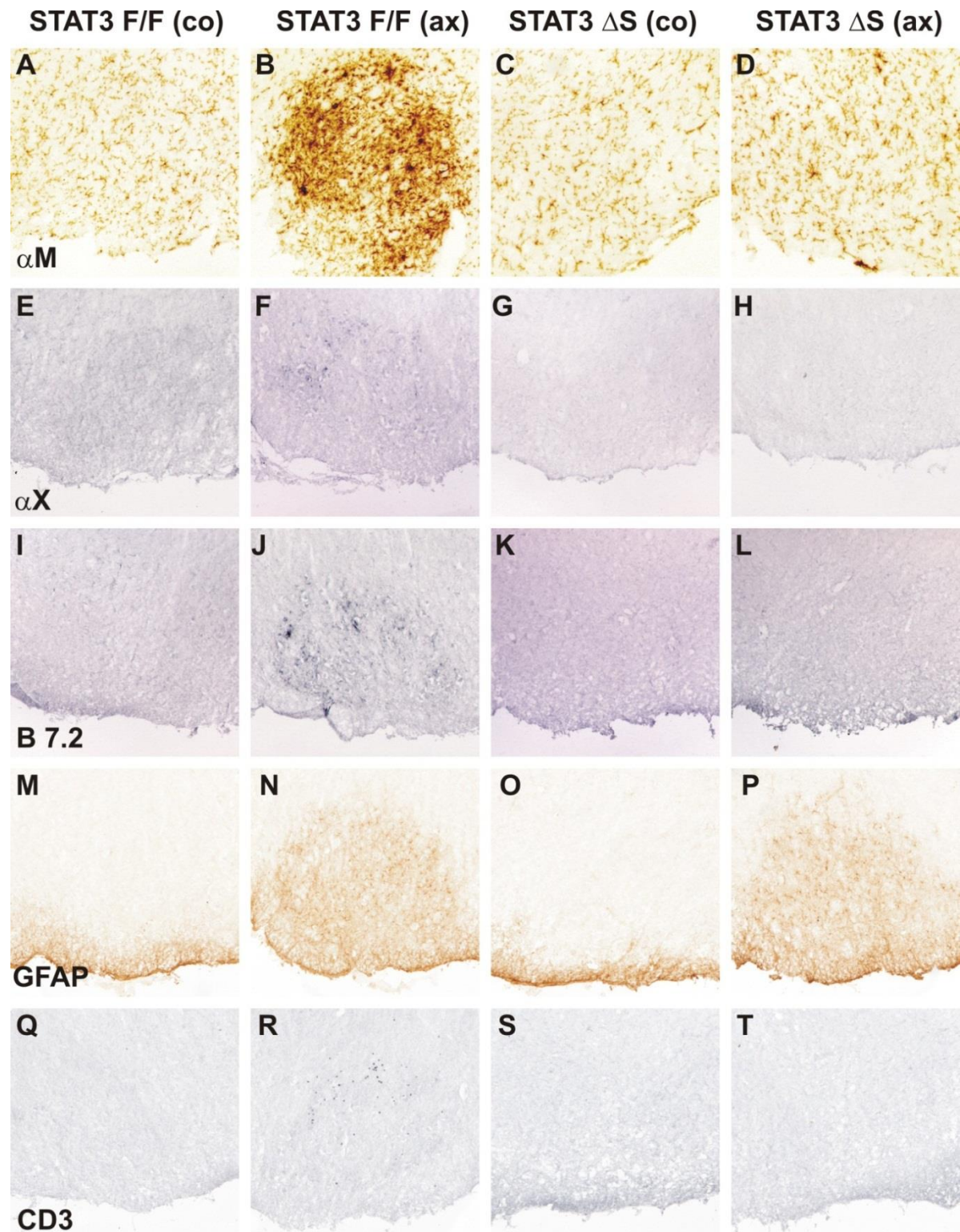
Figure 3.10 (a) - Neuronal STAT3 is required for the non-neuronal neuroinflammatory response after facial axotomy. **A-T:** Microglial (A-L) and astrocyte (M-P) activation, and T-cell influx (Q-T) 14 days after nerve cut in the STAT3 F/F controls (A,B,E,F,I,J,M,N,Q,R) and the STAT3ΔS mutant (C,D,G,H,K,L,O,P,S,T) mice on the contralateral side (co) (1st and 3rd column), and on the axotomised side (ax) (2nd and 4th column). Early and late microglial activation was detected through immunoreactivity for αM, αX and B7.2 integrin subunits, astrocyte activation with GFAP, and T-cells with CD3. After axotomy, STAT3 F/F mice show a prominent increase all 3 components of the neuroinflammatory response. In STAT3ΔS mutants, while the microglial (D, H, L) and T-cell response (T) was completely abolished, the astroglial response (P) remained unaltered.
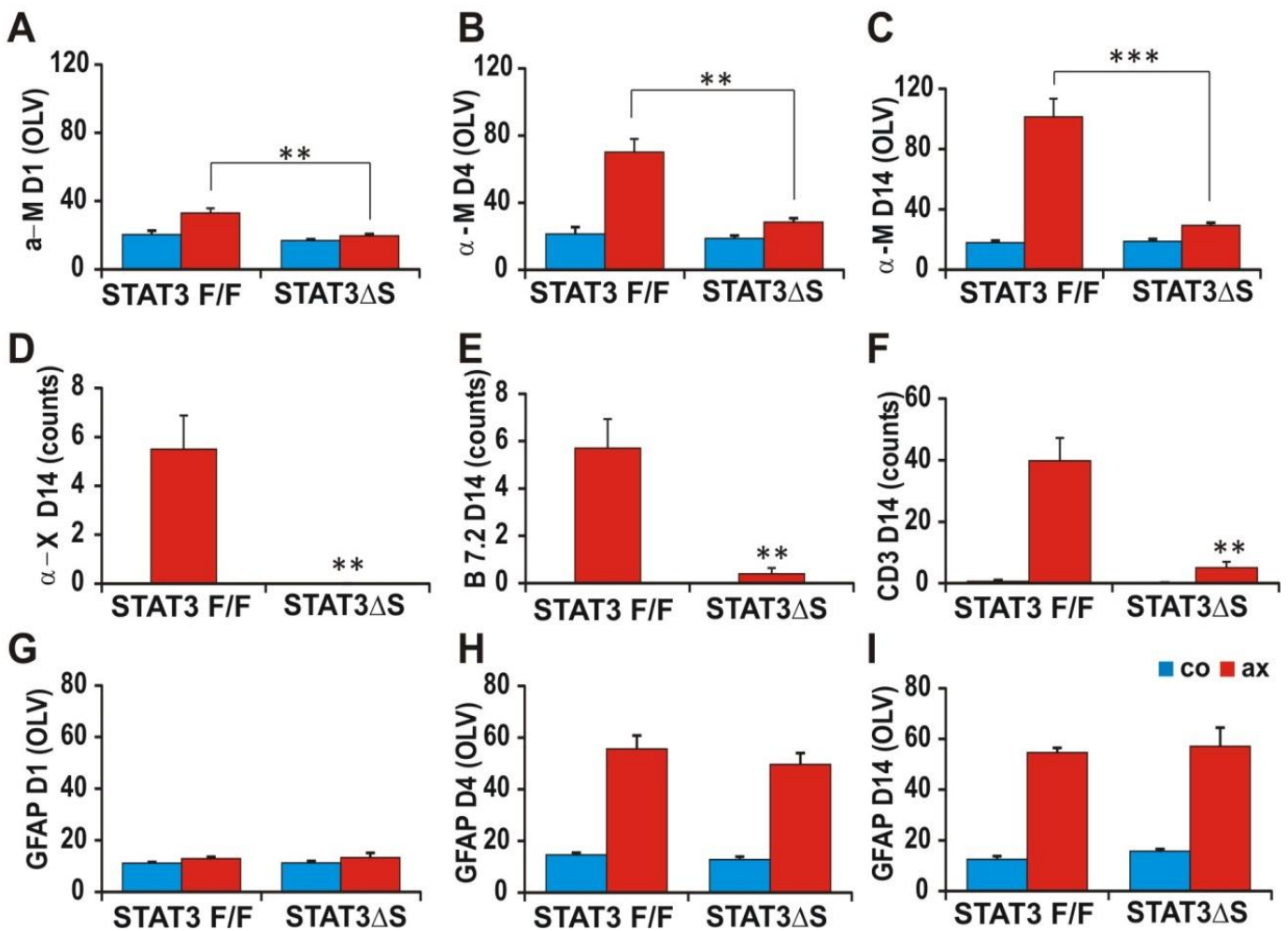
Figure 3.10 (b) Neuronal STAT3 is also required for the non-neuronal neuroinflammatory response after facial axotomy. **A-I.** Quantification of facial nucleus microglial and astrocyte activation at 1 (d1), 4 (d4) and at 14 days (d 14) and CD3+ T-cell influx at 14 days (d14) after nerve cut. STAT3ΔS mice show a significant reduction on the axotomised side for microglial components at all three time-points (A-E) and for T-cell influx (F) at d14. However, no effect of STAT3 deletion was seen on astroglial activation at all time-points analysed (G, I). The contralateral (co) and axotomised (ax) sides are indicated by blue and red bars respectively. *p<5%, ** p<1%, ***p<0.1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Finally, facial axotomy also leads to two different phases of T-lymphocyte entry into the injured facial nucleus. The first is a small increase in T-cell number, 1-4 days after injury; the second is a much stronger 100-fold increase with a peak at day 14 (Raivich et al., 1999a). Infiltrating T-cells were detected using immunoreactivity for CD3 at day 14 (Figure 3.10 (a) Q-T). As shown in Figure 3.10 (b) F, neuronal STAT3 deletion resulted in an 87% reduction in the number of CD3 positive cells (5±2) compared with WT littermates (40±7.5), (p<1%).

Virus-mediated STAT3 transfection of facial motoneurons

All the above experiments utilised a STAT3 KO model to establish its crucial role in regulating all aspects of the facial nerve regenerative response, from biochemical and molecular response of the neuronal soma to the reconnection of growing axons to their target muscles, and the achievement of motor function. The neuronal STAT3 deficient mice showed extremely poor regeneration even at the end of 90 days after injury, and even after milder crush injuries which normally facilitate quick extension of axons along existing tracks to the correct targets. This prompted the next question – can the administration of constitutively active STAT3 to these mutants help in recovering the normal, fast regenerative phenotype seen in the controls. To this end, we chose two different approaches of administering STAT3 to the regenerating motoneurons.

The first approach involved the use of a lentivirus expressing STAT3 and GFP, and direct injection into the facial nucleus. Trial injections with rhodamine were done to reproducibly establish the co-ordinates of the facial nucleus as 5.3 mm caudal and 1.25 mm lateral to Bregma, and at a 4.7 mm depth from the surface of the brain. 2μl of LV-STAT3-GFP was stereo-tactically injected into right facial nuclei of STAT3ΔS mice. These animals underwent facial nerve axotomy 4 days post- viral injection, and FG retrograde tracer application 28 days post-axotomy, before being transcardially perfused two days later. Efficiency of viral infection was assessed by eGFP immunohistochemistry. The transverse spread of the virus was approximately 0.5-0.6 mm. eGFP staining was present along the injection tract, in the reticular neurons above the facial nucleus, but more importantly also in the astrocytes within the facial nucleus. However, none of the facial motoneurons showed positive eGFP staining, suggesting that the virus may spare transfection of the facial motor neurons.

The second approach was to inject adenovirus or adeno-associated virus into the facial nerve and allow it to be retrogradely transported to the facial motor nucleus. 2-3 μl of AAV2 virus, or Ad-Cre-GFP virus expressing cre and GFP was injected into the main brain of the facial nerve. In order to successfully inject the virus into the nerve, the nerve had to be partially snipped to expose cut axon tips; hence all animals underwent partial unilateral facial axotomy. Animals were sacrificed at 10 or 30 days after viral transfection, perfused, and brains extracted and sectioned at the level of the facial nucleus.
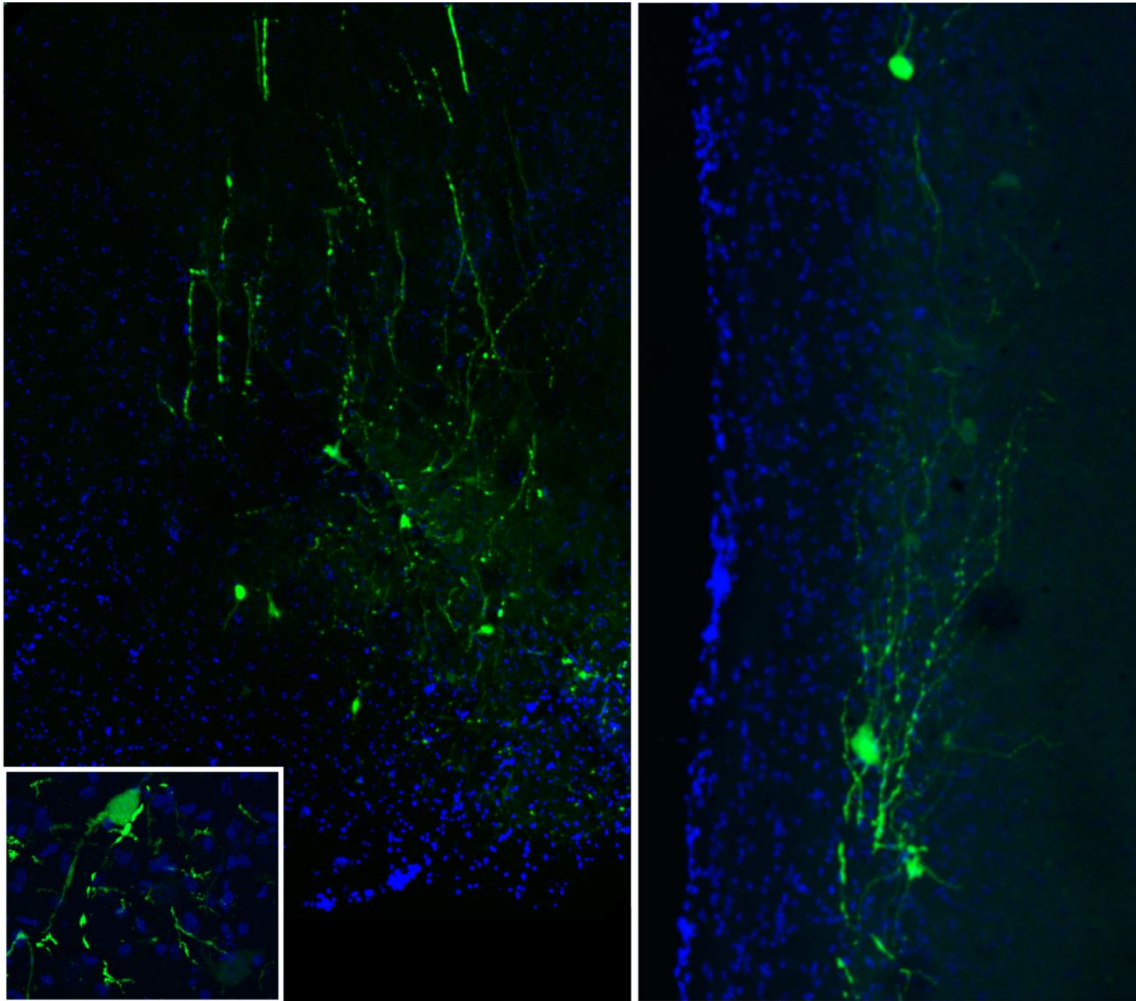
Figure 3.11. Adenoviral transfection of facial and spinal motoneurons. Transfection of facial motor neurons (left, and inset at high magnification), and spinal motor neurons (right) following retrograde injection of Adenovirus-GFP-Cre into facial and sciatic nerve respectively, followed by ten days survival.

While the AAV injected only transfected a few axons, AV injection showed beautiful transfection of many facial motoneurons when stained with an eGFP antibody (Figure 3.11). However, by 30 days, the immuno-staining disappeared, suggesting transient transgene expression after adenovirus injection. These optimisation experiments have paved the way for future experiments analysing the effects of overexpression of constitutively active STAT3, or of point-mutated STAT3 (especially at its phosphorylation sites Tyr705 and Ser727) in STAT3ΔS mice, and thereby identify the mechanism/s through which neuronal STAT3 exerts its multitude of effects in peripheral nerve regeneration.

Schwann cell STAT3 is not required for axonal regeneration or neuronal survival after facial axotomy

In contrast with its neuronal counterpart, conditional mutants with Schwann cell STAT3 deletion did not produce any defects in functional recovery, re-innervation of peripheral target, and motoneuron survival after facial axotomy.

To assess the level of functional recovery post injury, the post-traumatic whisker hair motor performance was scored on a scale of 0 (no movement) to 3 (normal movement as on uninjured side) every alternate day from day 7 after surgery till day 28 (Figure 3.12 A). The STAT3ΔP0 animals showed an almost identical recovery pattern to the STAT3 F/F animals, and by day 28 post axotomy, both the mutant and control animals had achieved similar levels of motor function (2.3±0.1 and 2.4±0.1 respectively).

It has been reported earlier that the whisker hair recovery occurs in a non-linear fashion, with only 20-30% of re-innervating neurons being capable of accounting for a majority of the functional movement (Raivich et al., 2004). This suggests that whisker hair function might not be an accurate indicator of re-innervation levels between groups. Therefore, to analyse differences in re-innervation of the whisker pad, fluorescent tracer FG was injected into the whisker pad 28 days post axotomy, and the mice allowed to survive for 48 hours to enable retrograde transport of the tracer. The level of re-innervation at day 30 following facial axotomy was assessed by counting the number of FG positive motoneurons in the injured facial nucleus and comparing them with the uninjured, control side. Again, there was no difference between the two groups - the overall ratio of labelled FG neurons on the ipsi- vs. the contra-lateral side in the STAT3ΔP0 mutants (n=6) was 56±5% as compared to 52±4% in the STAT3 F/F controls (n=5) (Figure 3.12 B).

Finally, the level of cell survival following facial axotomy was assessed by counting all motoneurons through the whole facial nucleus on the control and injured side using Nissl stained sections, and the number corrected for neuronal sizes by Abercrombie correction (Abercrombie, 1946). The number of motoneurons in the STAT3 F/F controls (co side: 1760±97; ax side: 1288 ± 88; n=5) and the STAT3ΔP0 mutants (co side: 1631 ± 115; ax side: 1324 ± 175; n=6) were similar to each other and in line with previously published data on the facial nucleus (Galiano et al., 2001;Raivich et al., 2004;Sendtner et al., 1997). In terms of percentages, facial axotomy led to a loss of 27% of motoneurons in the

STAT3ΔP0 mice compared to 19% loss in the STAT3 F/F mice (Figure 3.12 C). However, this difference was not statistically significant. In contrast with the neuronal counterpart, STAT3 deletion in Schwann cells did not lead to atrophic morphology of surviving axotomised motoneurons (Figure 3.12 D).
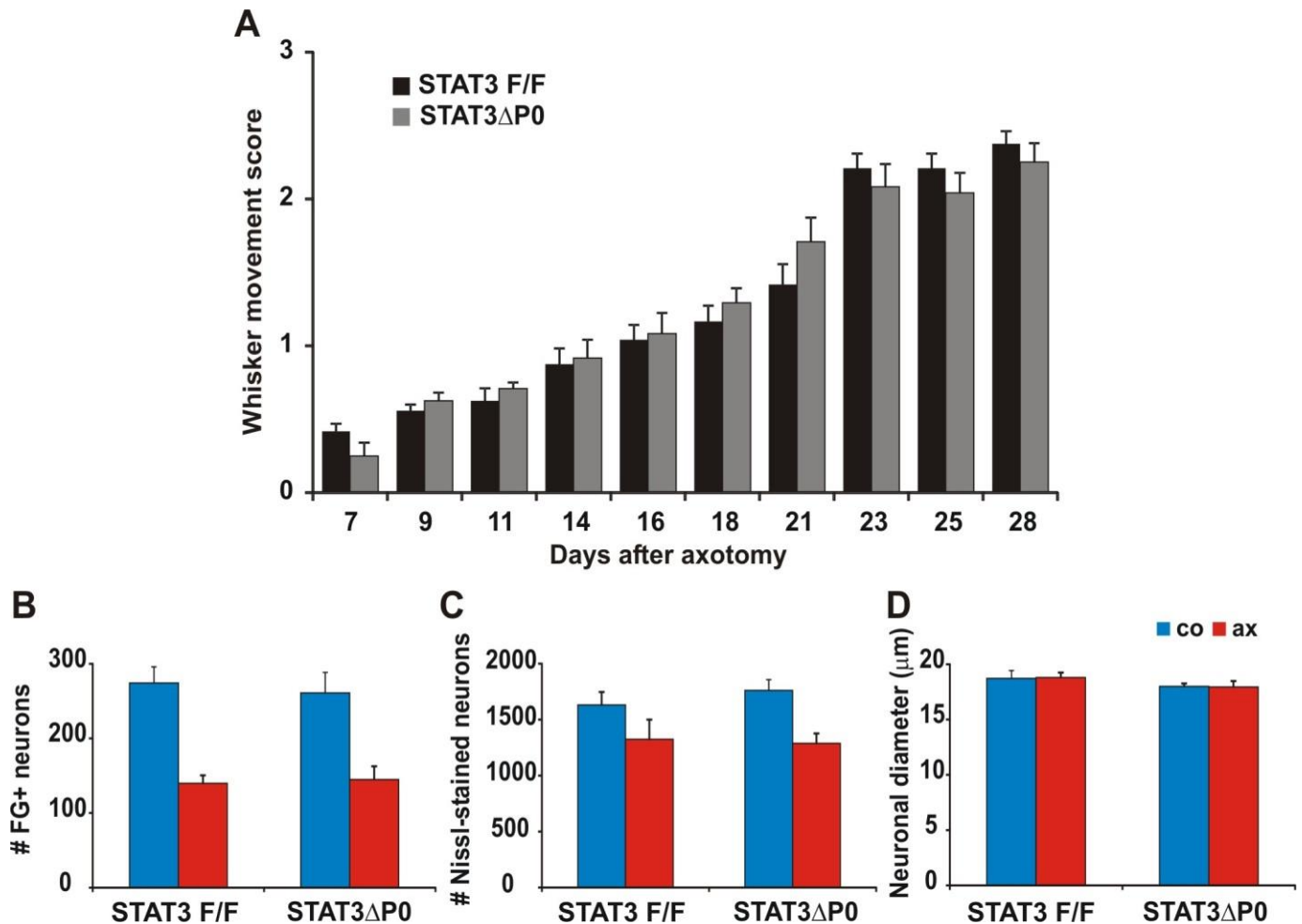
Figure 3.12. Schwann cell STAT3 is not required for axonal regeneration. **A.** Whisker hair movement, measured over a period of 28 days after facial nerve cut showed no difference between the STAT3 F/F control and STAT3ΔP0 mutant groups. **B.** Target re-innervation of the whisker pad, assessed by counting the number of Fluorogold positive motoneurons showed no change in the axotomy response between groups 30 d after facial nerve cut. **C.** Motoneuron cell count of Nissl-stained facial nuclei shows no change in survival 30 d after nerve cut in the absence of Schwann cell STAT3 when comparing control and mutant animals. **D.** No change was seen in neuronal sizes between the two groups either. Bars and error bars show the mean and SEM respectively.

Chapter 3 – STAT3 discussion

Increased synthesis and activity of transcription factors like c-Jun, ATF3, and STAT-3 are among the most consistent changes in injured neurons and have been suggested to play an important role in the neural response to injury and the initiation of the neuronal repair program. In the current study, we used neuron-specific inactivation of STAT3 to demonstrate that the STAT3 transcription factor is an important regulator of the neuronal response to axonal disconnection. Schwann cell STAT3, on the other hand, does not play a role in peripheral nerve regeneration.

Neuronal STAT3 controls functional recovery & speed of axonal elongation after PNI

Synchronised and rapid functional recovery is arguably the overall aim of any regenerative response. To functionally evaluate the role of STAT3 in axon growth, we assayed post-traumatic whisker performance. In the absence of neuronal STAT3, there is a significant delay in functional recovery after peripheral nerve injury. At 30 days after facial nerve cut, while control mice regain nearly 75% of normal whisker function, STAT3 knock-out mice show only 40% of normal whisker movement. It is only about 35 days after injury that they gain some regenerative speed, a lag of 21 days compared to the controls. Even after milder crush injury, the STAT3ΔS mice showed nearly 60% less motor recovery than their control counterparts at 30 days after injury. This suggests that impaired regeneration of STAT3-deficient axons is not due to an inability to cross-over from the proximal to distal stump but possibly due to a delay in growth induction or reduced speed of regenerating axons. Deletion of STAT3 in Schwann cells, on the other hand, produced no defect in functional recovery.

In order to investigate the cause of this prolonged lag phase in STAT3ΔS mice, we looked at the maximum distance of the regenerating neurites from facial nerve crush sites, four days after axotomy. In the controls, the neurites regenerated at a speed of 1-2 mm per day as reported previously (Gutmann, 1942). But the STAT3ΔS mutants showed a 60% reduction in this early elongation speed, as measured by the distance travelled by the fastest CGRP+ growth cone, providing substantial evidence for the delay in their functional recovery. The fact that STAT3 deficient neurons still mount a growth response, albeit with

prolonged delay, achieving levels of functional recovery similar to the controls by day 90, highlight the existence of collateral sprouting mechanisms and/or additional compensatory pathways which result in functional recovery in the absence of STAT3. That being said, speed of recovery is a crucial criterion in determining the overall efficacy of a regenerative programme after injury. Long delays in regeneration can make an acute injury, chronic, and create a whole new plethora of impediments. Tessa Gordon and colleagues have demonstrated in a series of experiments that prolonged disconnection from target reduces the ability of injured motoneurons to regenerate (Furey et al., 2007;Gordon et al., 2011). Considerable research is currently being focused on methods to improve speed of regeneration and STAT3's role in accelerating axonal elongation and achieving faster functional recovery, makes it a desirable candidate for future translational studies.

Neuronal deletion of STAT3 results in impaired target re-innervation

Compared to the impaired functional recovery outcome in STAT3ΔS mice, the defect in re-innervation outcome was even more striking and persistent. STAT3ΔS mice showed a 3-fold reduction in target re-innervation at 10 and 30 days after nerve crush, a 4-fold reduction in re-innervation at day 30 after nerve cut, and only half of normal wild-type re-innervation even at day 90 following cut injury. When adjusted for cell survival, the difference between the groups became even more dramatic, with the mutants (16% SAR) showing more than 5-fold reduction in re-innervation after nerve cut compared to controls (85% SAR). The fact that the functional recovery in the mutants exceeded target re-innervation at day 90 highlights an interesting feature of the regenerative response that full target re-innervation is not necessary for functional recovery. A similar phenomenon has been shown earlier in case of c-jun nestin:cre mice (Raivich et al., 2004). The assessment of whisker hair movement appears to be non-linear, providing an accurate indication of functional reconnections by the first 20-30% neurons, but there-after losing its predictive value, as more neurons reconnect to the target (Raivich et al., 2004). Also, both the groups showed greater re-innervation after facial nerve crush than cut, but the ratio of re-innervation in the mutants compared to controls remained unchanged between the two injury severities, again highlighting the idea that the re-innervation defect in the STAT3ΔS mutants is probably due to an intrinsic regenerative disability arising from neuronal STAT3 deletion, rather than a problem in overcoming the cellular bridge between the proximal and

distal stumps. Once again, no re-innervation defect was seen in STAT3ΔP0 mice, which lack STAT3 in Schwann cells.

A recent study using mice with conditional STAT3 deletion in DRG neurons, has demonstrated a phase-specific role of STAT3 in regulating induction of the neuronal growth programme, but not subsequent perpetuation of axonal growth. Deletion of STAT3 impairs initial regeneration of peripheral DRG axons after a saphenous nerve cut, but by 28 days after lesion, similar proportions of STAT3-competent and STAT3-deficient axons re-approach their termination zone. Once regeneration has been initiated, STAT3-deficient axons grow with the same speed as STAT3-competent axons (Bareyre et al., 2011). They have postulated that axons require STAT3 when growing independently of Schwann cells; but once they come into contact with the Schwann cell tube, they shift into an elongation mode and no longer require STAT3. Thus crush lesions that do not interrupt Schwann cell guidance, do not induce STAT3 expression in the corresponding DRG neurons (Bareyre et al., 2011). Another study, involving STAT3 deletion in adult DRG neurons, followed by sciatic nerve crush, has shown no major differences in neurite outgrowth from STAT3 deficient adult sensory neurons, compared with neurons expressing constitutively active STAT3 (Ben-Yaakov et al., 2012). These results are in stark contrast with our current data which shows that STAT3 is indispensable for full target re-innervation of motoneurons after both cut and crush injuries. Without STAT3, there is a persistent defect in regeneration (measured up to 90 days) and peripheral targets remain terminally denervated; the delayed increase in function could possibly be an effect of collateral sprouting, or due to the existence of alternate pathways that can promote neuronal regeneration in the absence of STAT3, albeit at a much lower efficiency. It is possible that motor (as seen after facial nerve injury) and sensory (as seen after sciatic and saphenous nerve injuries) fibres respond differently after nerve lesions (Lago et al., 2007). Several cell adhesion molecules and growth factors like BDNF are known to be differentially expressed in motor and sensory nerves and may play a role in differential regulation of regeneration of these nerves (Allodi et al., 2012).

STAT3 is required for both cell death & for the trophic state of the surviving neurons

Absence of STAT3 in axotomised facial motoneurons prevented neuronal cell death following facial axotomy. Quantification of total number of motoneurons in the entire facial nucleus revealed a significant decrease in motoneuron death in the axotomised nuclei of STAT3ΔS mice at 30 days after facial nerve transection, in contrast with the STAT3F/F controls, which showed approximately 30% cell death. However, most of the surviving STAT3ΔS motoneurons had a severely atrophic morphology, and remained shrunken by 50% even at 90 days after axotomy. Even after crush lesion, cell death was completely abolished in the mutants, and the axotomised neurons were atrophic by a 20% reduction in cell diameter compared to the controls. Facial motoneurons which fail to reconnect with their target normally die because of the lack of trophic support (Sendtner et al., 1996;Ferri et al., 1998). It appears that in the absence of STAT3 these disconnected neurons are prevented from dying, and are instead held between cell survival and cell death in a shrunken, and most likely a non-functional state. These atrophic neurons also fail to upregulate various RAGs, as seen by immunohistochemistry at days 4 and 14 after axotomy. This phenomenon has been reported earlier in our laboratory in case of neural c-jun knockouts (Raivich et al., 2004;Ruff et al., 2012), suggesting that STAT3, like c-jun, may be acting as a 'double-edged sword' promoting injury-induced neuronal cell death as well as regeneration (Herdegen et al., 1997b). In a study published by Schweizer and colleagues in 2002, it was reported that conditional STAT3 deletion in motoneurons under a neurofilament promoter driven cre, results in the death of 45% of the axotomised motoneurons. However, the cell numbers in this study were not adjusted for their size variability by Abercrombie correction. Moreover, there was no indication that the shrunken neurons were included in their total motoneuron counts, suggesting that the level of cell death seen by Schweizer et al., could be misleading (Schweizer et al., 2002). STAT3 has been consistently referred to as a 'pro-survival' or anti-apoptotic factor, correlating with neuronal survival either directly (Alonzi et al., 2001;Liu and Snider, 2001;Qiu et al., 2005;Schwaiger et al., 2000;Schweizer et al., 2002;Smith et al., 2011), or indirectly via inactivation of its regulator SOCS3 (Miao et al., 2006;Smith et al., 2009). STAT3 has also been reported to promote survival of adult motor neurons (Schweizer et al., 2002), adult sensory neurons (Ben-Yaakov et al., 2012) and of retinal ganglion neurons (Zhang et al., 2008) after injury, and co-localises with anti-apoptotic proteins like bcl-2, bcl-XL and Reg-

2 (Dziennis and Alkayed, 2008). Our current results show that the attributed title of 'survival factor' for STAT3 may be deceptive, and more experiments will be required to ascertain whether like c-jun, STAT3 has the ability to act as a cell death signal. In 2001, Wen et al., observed that after cerebral ischemia, p-STAT3 immunopositive neurons were also TUNEL positive, suggesting that increased expression of p-STAT3 could play a crucial role in ischemia-induced neuron death (Wen et al., 2001). Moreover, several neurotrophic factors which activate STAT3, including CNTF, (Haas et al., 1999), can also mediate their survival effects via other pathways like the Ras pathway which has been shown to support motoneuron survival (Weng et al., 1996), suggesting a dispensable role for STAT3 in neurotrophin-mediated survival of motoneurons.

Further, STAT3ΔS mutant mice showed a marked and progressively widening defect in microglial activation with increasing time following axotomy (-78% at day 1, -80% at day 4, -87% at day14). The smaller reduction in microglial activation in mutant mice during the early phase of the axonal response, and a more drastic difference of microglial activation at a later stage coinciding with the peak of cell death at day 14 (Raivich et al., 1999a;Raivich et al., 1998), confirms that the defects in microglial activation are caused by the reduced death of facial motoneurons in STAT3ΔS mice. T-cell recruitment from the periphery to the damaged brain is triggered by dying cells and quantification of CD3 immunopositive cells at day 14 also showed a significant reduction by 87% in the mutants. Such a nearly total obliteration in the expression of microglial and lymphocytic markers reaffirms the observation that mice lacking neuronal STAT3 do not undergo motoneuron cell death after facial axotomy. It also highlights the importance of STAT-3 dependent signals from neurons in eliciting the non-neuronal responses to injury.

STAT3 is required for the molecular & biochemical response of the cell body to injury

Removal of neuronal STAT3 abolished basal and injury-induced p-STAT3 immunoreactivity, as well as most of the molecular response following facial axotomy. Unpublished data from our laboratory has shown that STAT3 is an early marker of neuronal injury, being up-regulated within 3 hours and reaching a peak at 7 days after injury. Because of its early activation, it plays a potentially vital role in triggering a cascade of biochemical and molecular events essential for efficient regeneration. Up-regulation of RAGs result in the transformation of a transmitting neuron to a regenerative phenotype

that supports axonal elongation and sprouting. Previous studies using genetic deletion and antibody inhibition techniques have shown that a concoction of various transcription factors, neuropeptides, cell adhesion molecules, cytoskeletal elements and growth factors is required for successful regeneration. For example, deletion of neuronal c-jun has been shown to acutely impair peripheral regeneration (Raivich et al., 2004;Ruff et al., 2012). Overexpression of ATF3 speeds up the initiation of axonal outgrowth (Seijffers et al., 2006), whilst deletion of the α7 integrin gene retards axonal regeneration by 35-40% (Werner et al., 2000). Local inhibition of neuropeptide CGRP (Dumoulin et al., 1992;Raivich et al., 1992) and adhesion molecule CD44 (Nagy et al., 1998) and deletion of galanin (Holmes et al., 2000) reduced neurite outgrowth *in vivo*. The current results show that axotomy induced upregulation of all of the above molecules, except for c-jun, is almost completely abolished in the STAT3ΔS mice, suggesting their role in mediating STAT3-dependent axonal growth. In the absence of neuronal STAT3, injury-induced nuclear translocation of ATF3 was completely blocked, but the effect on c-jun activation was relatively mild. The effects of c-jun and STAT3 deletions on various RAGs appear to mimic each other, without affecting the expression of either transcription factor. It is likely that there are molecules which co-ordinate the expression of these two transcription factors in regulating regeneration. For example transcription of damage-induced neuronal endopeptidase (DINE) was shown to be increased 60-fold by Sp1 mediated recruitment of STAT3, c-jun and ATF3 to the DINE promoter following injury (Kiryu-Seo et al., 2008).

It is probable that there are additional, unidentified, transcriptional targets of STAT3 that are necessary for some aspects of the axonal response. Genetic profiling of STAT3F/F and STAT3ΔS mice before and after axotomy, will help in the identification of novel molecules involved in STAT3-dependent neuronal regeneration. Since STAT3 appears to be a key regulator of the axonal reaction, pharmacological augmentation of its activity could represent a promising therapeutic strategy to facilitate axonal regeneration in patients with nerve trauma.

Viral transfection of facial motoneurons

Overexpression or viral vector–mediated knockdown of genes will allow a large range of investigations into gene function during regeneration after peripheral nerve injury.

Rescuing the regeneration deficient phenotype seen in the STAT3ΔS mutants, by administering constitutively active STAT3 or any of its downstream targets, will greatly help our understanding of the exact mechanisms of STAT3 function in regeneration. Stereotactic injection of lentivirus expressing STAT3 and GFP into the facial nucleus resulted in transfection tropism towards astrocytes, but not the motoneurons within the facial nucleus. So, as the next step, adeno-associated virus (AAV) or adenovirus (AV) was injected into the facial nerve and allowed to be retrogradely transported to the facial motor nucleus.

AAV is being increasingly recognised as a useful gene therapy vector, due to its low immunogenicity and toxicity, and has been known to retrogradely transduce motor and sensory neurons (Mason et al., 2010;Hollis et al., 2010). However, in the current study, AAV injection transfected mostly axons, and very few motoneurons. On the other hand, AV injection showed transfection of several facial motoneurons within 10 days. But by 30 days, the immuno-staining disappeared, suggesting transient transgene expression after adenovirus injection. This is consistent with a previous study showing intraneural adenoviral injection into sciatic nerve causes a peak expression of lacZ between 10-14 days after injection, after which it decreases, suggesting that adenoviral expression in nerves of adult mice is limited by host cellular immune response to the virus (Jani et al., 1999). Also, the CMV promoter is known to typically provide rapid and ubiquitous expression; however it is also prone to silencing over time in some tissues, specifically in the brain (Gray et al., 2011), explaining the disappearance of neuronal transfection by day 30 in the facial motoneurons.

ATF3 is rapidly, and consistently activated in sensory, sympathetic and motor neurons by stress stimuli after peripheral nerve injury (Raivich and Behrens, 2006;Tsujino et al., 2000), making it one of the most common and reliable injury markers. Several studies have also highlighted a synergistic action of ATF3 and c-jun, and of ATF3, c-Jun and STAT3. The absence of ATF3 in the STAT3 mutants might be one of the main reasons for the poor regeneration. AV-mediated administration of ATF3 to the STAT3 mutants might help improve regeneration. These optimisation experiments have also paved the way for future experiments analysing the effects of overexpression of constitutively active STAT3, or of point-mutated STAT3 (especially at its phosphorylation sites Tyr705 and Ser727) in STAT3ΔS mice, and thereby identify the mechanism/s through which neuronal STAT3 exerts its multitude of effects in peripheral nerve regeneration.

Chapter 4 – c-Jun Results

Neuronal and Schwann cell c-Jun together contribute to successful nerve regeneration, and N-terminal phosphorylation is not the main regulator of their regenerative function

Upon nerve transection, the expression of many growth and survival-associated transcription factors is dramatically up-regulated. Increased expression of c-jun, an important component of the AP-1 transcription complex, is one of the most immediate, consistent and well-characterised changes taking place in the neuronal soma following axonal transection. In 2004, Raivich et al. reported that mice with a neuro-epithelial stem cell marker Nestin-specific c-Jun deletion (c-JunΔN), exhibit several defects in the neuronal regenerative response. They showed increased cell survival but of atrophic neurons, reduced target-muscle re-innervation, perineuronal sprouting, lymphocyte recruitment and microglial activation after facial nerve axotomy (Raivich et al., 2004). Since neuroepithelial stem cells differentiate into several distinct CNS populations such as neurons, oligodendrocytes, astrocytes and peripheral Schwann cells (Raivich et al., 2004;Heffron et al., 2009), it remained unknown which of these cell types was the key regulator of the phenotype exhibited by c-JunΔN mice. In 2012, another publication from the Raivich laboratory revealed that neuronal c-Jun deletion by neuron-specific synapsin promoter (c-JunΔS) echoed all the regeneration deficits seen in the c-JunΔN mice (Ruff et al., 2012b), suggesting that neuronal c-Jun is the essential regulator of the cellular response to axonal injury and also the resulting neuronal survival and regeneration.

Neuronal c-Jun and STAT3 are not functionally complementary counterparts in facial nerve regeneration

Immuno-histo-chemical and genome wide mRNA microarrays studies (in collaboration with Joost Verhaagen, Netherlands Neuroscience Institute) have confirmed strong overall reduction for regeneration-associated molecules like α7β1 integrin, ATF3, CGRP, Galanin and CAP23 in c-JunΔN and c-JunΔS mice. However, some transcription factors and cytokines such as STAT3 and IL6 were completely unaffected by the absence of c-Jun. Previously reports have shown that STAT3 promotes neurite outgrowth in vitro (Miao et al., 2006;Liu and Snider, 2001;Smith et al., 2011), and neuronal survival and functional repair *in vivo* (Schweizer et al., 2002;Bareyre et al., 2011). Our current study using neuronal

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STAT3 deficient mice (STAT3ΔS) has shown an important role of neuronal STAT3 in achieving successful functional and anatomical regeneration after nerve injury. Briefly, removal of STAT3 gene in facial motoneurons abolished most of the molecular responses following facial axotomy, reduced the speed of axonal regeneration following crush, and prevented most cut axons from reconnecting to their target, preventing functional recovery. Despite blocking cell death, this was associated with a large number of shrunken neurons. STAT3ΔS mutants also had diminished microglial activation and T-cell influx (Chapter 3). Interestingly, STAT3 expression was unaffected by lack of neuronal c-Jun in the c-JunΔS mice and vice-versa (Chapter 3) (Ruff et al., 2012), supporting the hypothesis that neuronal STAT3 and c-Jun might be performing functionally complementary roles in peripheral nerve regeneration and are responsible for the maintenance of a residual neuronal regenerative program in mice lacking either transcription factor. In order to investigate this hypothesis, double mutant mice with combined deletion of both neuronal c-Jun and STAT3 (STAT3+c-JunΔS) were generated by lox-P cre recombination under the control of neuron-specific synapsin promoter. The possibility of c-Jun and STAT3 belonging to complementary signalling pathways in regeneration was investigated by exploring whether the effects of combining both deletions, produced a stronger, synergistic effect on regeneration, than a simple deletion of either STAT3 or c-Jun.

Combined deletion of c-jun & STAT3 interferes with axonal regeneration, but not more than the effects seen with single deletion of either transcription factor

The right facial nerve was cut at the stylomastoid foramen, and the animals allowed to survive for 30 days to allow scoring of post-traumatic whisker motor function, and analysis of target re-innervation and motoneuron survival. All assessments were done similarly as on mice with single gene deletions, to assess synergistic effects and interactions between the two transcription factors.

Functional recovery was assessed by scoring ipsilateral whisker hair movement every other day on a scale of 0 (no movement) to 3 (normal movement). The control mice commenced recovery of whisker vibrissae around day 10 and improved steadily over the next 18 days to reach a final score of 1.8±0.1 at day 28. Recovery in the STAT3+c-JunΔS mutants, also started around day 10, however, the pattern of recovery was much slower than the control mice, achieving a maximum average score of 1.3±0.1 by day 28 (Figure 4.1A). These differences were mirrored by changes in the regenerative index, calculated

as area under the curve for functional recovery over the 28 day period. The controls had an index of 1.04±0.06 (n=5) and the double mutants, a significantly lower index of 0.71±0.03 (n=5; p < 1%, Figure 4.1B). Interestingly, previous studies using single gene deletions have shown a lower recovery index for neuronal STAT3 deletion (AUC = 0.32±0.07) (Chapter 3 Results) and neuronal c-jun deletion (AUC = 0.44± 0.02) alone (Ruff et al., 2012). However, this could simply be variability between the animals, or human observer variability, since the scoring was done at different times and by different people. Overall, these results do not show a stronger delay in functional recovery in the double mutants, compared to the single mutants.

To determine the cause of this defect, 28 days after nerve cut the same experimental cohorts were assessed for target muscle re-innervation. Whisker pads were labelled bilaterally with the fluorescent tracer Fluoro-Gold (FG), followed by 48 hours of retrograde transport. Motoneurons that successfully reconnected with their targets were identified by the presence of the retrograde tracer in the cell bodies, and counted on every fifth section throughout the facial nucleus. Control animals showed retrograde labelling of 69±7% neurons on the axotomised side compared with the uninjured side, which was in agreement with the extent of re-innervation in previous controls from single gene deletion experiments (Chapter 3 Results; (Ruff et al., 2012)). c-jun deletion resulted in a dramatic 8-fold reduction of retrograde labelling to 3.8% ± 1.1% (ax/co%; p<0.1%; (Ruff et al., 2012)) STAT3 deletion alone reduced the percentage of re-innervating neurons by 4-fold to 15±4% (ax/co%; p<0.01%; Chapter 3). Deletion of both neuronal STAT3 and c-jun did not exacerbate this defect in re-innervation. The STAT3+c-JunΔS double mutants showed a 2.5 fold reduction in re-innervation to 26±4% (Figure 4.1C; p<0.1%). In terms of actual numbers, while the unaxotomised facial nucleus had 190±13 FG+ neurons, the axotomised side had 47±5 FG neurons. Although the re-innervation ratio was higher in the double mutants compared to the single mutants, the difference was statistically insignificant, again highlighting variability between experiments done at different times.
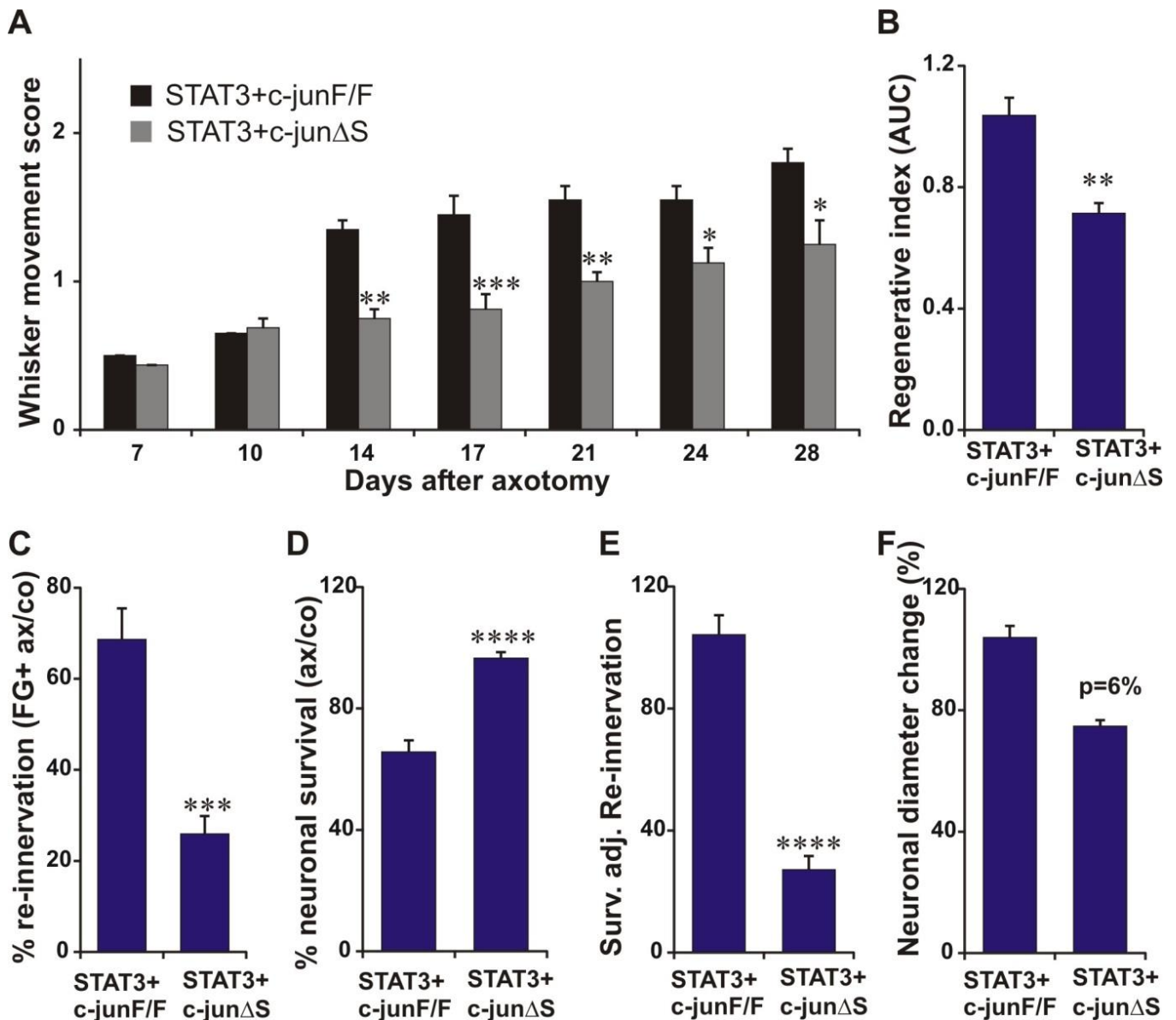
Figure 4.1. Deletion of neuronal STAT3 and c-jun interferes with axonal regeneration, functional recovery, target re-innervation and neuronal cell death. **A,B.** Functional recovery of whisker hair movement after facial nerve cut, also expressed as regenerative index (calculated as Area Under Curve) WHM was scored on a scale of 0 (no movement) to 3 (strong, normal movement); see Materials and methods for details. STAT3+c-JunΔS double mutants show a significantly poorer overall recovery. **C.** Quantification of retrograde labelling of facial motoneurons with Fluorogold 28 days after facial nerve cut. The overall ratio of labelled neurons in the operated/unoperated side is shown. Peripheral target re-innervation of the whisker pad is reduced in the absence of STAT3 and c-jun in neurons. **D.** Changes in neuronal cell number in the axotomised and contralateral facial motor nucleus, 30 days after facial nerve cut. Quantification of Nissl-stained motoneurons show a strong increase in survival 30 d after nerve cut in the absence of neuronal STAT3 and c-jun. **E.** STAT3+c-JunΔS double mutants show significantly reduced survival adjusted re-innervation. **F.** Axotomised neurons in the STAT3+c-JunΔS double mutants are atrophic. Error bars indicate mean +/-standard error of mean (SEM), n=5 animals per group, *p<5%, **p<1%, ***p<0.1%, ****p<0.01% in Repeated Test Anova (for whisker movement score) and in unpaired Student's t-test.

To assess the effects of deleting neuronal STAT3 and c-jun on cell survival after axotomy, motoneuron number on the uninjured and injured sides at 30 days after injury were

compared. It revealed a loss of 34±4% neurons in the control group, injured side. Motoneuron survival was significantly increased in STAT3 and c-jun single and double mutants (Figure 4.1D; p<0.01%). c-Jun single mutants had 99±4% motoneuron survival (Ruff et al., 2012), STAT3 single mutants 109±7% (Chapter 3 – STAT3 Results), and the STAT3+c-JunΔS double mutants 97±2% (p < 0.01%). The opposite effect was observed on cell size – while the controls showed a slight chromatolytic increase in neuronal diameter on the axotomised side, the STAT3+c-JunΔS double mutants showed a 25% shrinkage from 18.4 ± 0.4 µm for the uninjured to 13.7 ± 0.6 µm for injured motoneurons (Figure 4.1F). These results again conform to the previous observations of increased motoneuron survival and cell shrinkage in the STAT3 and c-jun single mutants.

Finally, to explore whether alterations in motoneuron survival contributed to the observed reduction in re-innervation and functional recovery, we looked at survival-adjusted re-innervation. While the controls had an S.A.R. of 104±6%, the STAT3+c-JunΔS double mutants had a S.A.R. of only 27±5%, an almost 4-fold reduction in regeneration capability (Figure 4.1E; p<0.01%). A similar phenomenon was seen in the single gene deletion mutants compared to their control counterparts. In summary, the regenerative abilities of the double mutants seem to be at par with those of the single mutants at 30 days after facial nerve transection. Due to the absence of exacerbated deficiency or complete abolition of nerve regeneration in the double mutants, it seems unlikely that the two transcription factors belong to complementary pathways. Rather, they may be acting in concert to unlock a common signalling mechanism in regeneration, and the absence of either factor is sufficient to block that stream of regeneration.

Neuronal c-Jun and Schwann cell c-jun are functionally complementary counterparts in facial nerve regeneration

In our search to identify master regulator/s of regeneration which exhaustively regulate all aspects of the regenerative response, we explored the possibility of complementary function of transcription factors c-Jun and STAT3. However, what emerged was that the two factors do not have a synergistic or incremental effect on regeneration. In fact, their closely related response in terms of regulating re-innervation, functional recovery, neuronal survival, and molecule changes in the neuronal cell bodies and non-neuronal

cells suggest that they might be acting through the same pathway. This led us to investigate the contribution of non-neuronal cells via alternate mechanisms.

c-Jun in Schwann cells is a global regulator of Wallerian degeneration, governing major aspects of the injury response, including expression of trophic factors, adhesion molecules, the formation of regeneration tracks and myelin clearance. A key function of c- Jun is the activation of a repair program in Schwann cells and the creation of a cell specialised to support regeneration (Arthur-Farraj et al., 2012). Two recent, closely-related studies have highlighted an important role of Schwann cell c-jun in cell survival following peripheral nerve regeneration (Arthur-Farraj et al., 2012;Fontana et al., 2012). The first study using a Schwann cell c-jun deleted mutant expressing cre recombinase under the control of myelin protein P0 promoter showed that after facial nerve injury, the absence of c-Jun caused impaired axonal regeneration and severely increased neuronal cell death. c-Jun deficiency resulted in decreased expression of several neurotrophic factors, including GDNF and Artemin, both of which encode ligands for the Ret receptor tyrosine kinase, and act on neurons via paracrine signalling (Fontana et al., 2012). The second study, using a sciatic nerve injury model, also showed that the absence of c-Jun results in the formation of a dysfunctional repair cell, striking failure of functional recovery, and neuronal death. These observations prompted the question whether transcriptional control of modulatory factors present in, and produced by glial cells in the peripheral system play a role in axonal regeneration and compensate for the loss of functional and anatomical regeneration in the absence of neuronal STAT3 or c-jun. In order to investigate this hypothesis, facial nerve regeneration was assessed in double mutant mice with deletion of both neuronal and Schwann cell c-jun signalling streams.

Combined deletion of neuronal and Schwann cell c-jun completely abolishes target re-innervation after facial nerve regeneration

To investigate the significance of combined c-Jun deletion in neurons and SCs, transgenic mice with neuronal deletion of c-jun (c-JunΔS) were crossed with mice having Schwann cell c-Jun deletion (c-JunΔP0). The resulting litters had mice with combined homozygous neuronal and Schwann cell deletions (c-JunΔSΔP), only neuronal deletion (c-JunΔS), only Schwann cell deletion (c-JunΔP0) or functional, unrecombined controls (c-JunWT), which formed the four experimental groups in these studies.
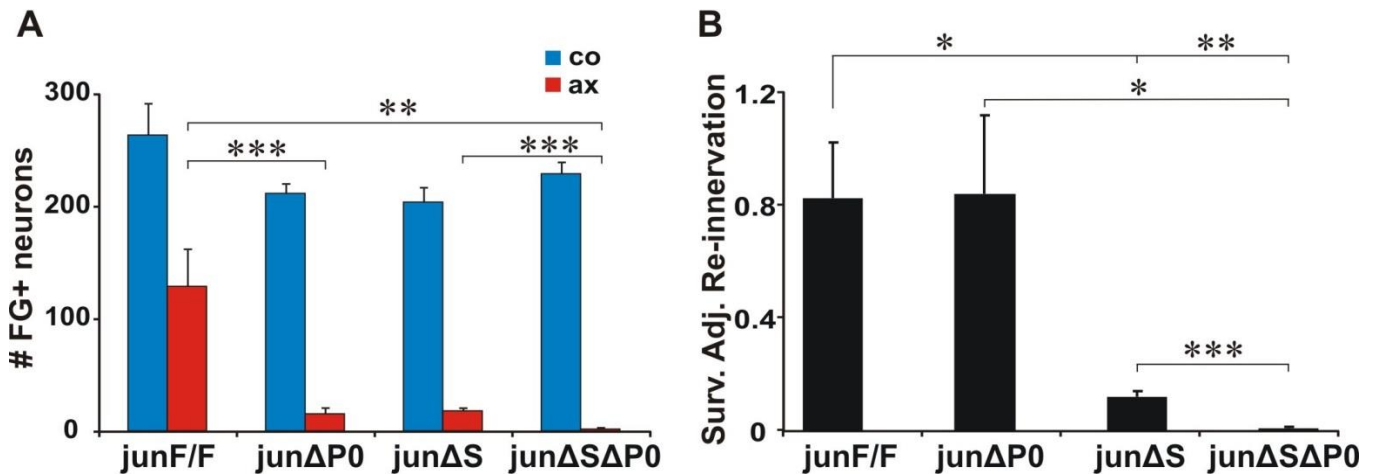
Figure 4.2. Combined deletion of c-jun in neurons and Schwann cells exacerbates the defect in target re-innervation after facial axotomy. **A.** Whisker pad re-innervation was determined by counting retrogradely labelled Fluorogold-fluorescing facial motoneurons (FG+) at 30 days after facial nerve cut. While junF/F animals (n=4) showed 57% reduction in re-innervation, junΔP0 mice (n=8) showed 93% reduction, junΔS mice 91% reduction (n=3) and junΔSΔP0 mice 99% reduction (n=5). **B.** Survival adjusted re-innervation (SAR) was calculated by dividing the ratio of re-innervation by the survival ratios for each group. Note the sharp reduction in SAR in junΔS animals, and complete abolition of SAR in the junΔSΔP0 double mutants. *p< 5%, ** p<1%, *** p< 0.1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM, respectively.

The effects of combined neuron- and SC-specific c-Jun inactivation on axonal regeneration were assessed by the extent of peripheral target re-innervation, and motoneuron survival, using the same cohorts of control and mutant mice. 28 days after nerve cut, the whisker pads were labelled on both sides with a fluorescent tracer Fluoro-Gold (FG), followed by 48 hours of retrograde transport. Motoneurons that successfully reconnected with their targets were identified by the presence of the retrograde tracer in their somas, and counted on every fifth section throughout the facial nucleus. Although control c-JunWT animals showed retrograde labelling of 43 ± 15% on the axotomised side compared with the uninjured side, c-JunΔS animals showed an almost five fold decrease with 9 ± 2%, c-JunΔP0 animals showed more than six-fold decrease with just 7 ± 2% (p<0.1%), and the c-JunΔSΔP0 mice a striking forty-fold decrease with only 1 ± 0% neurons labelled with the fluorescent tracer (Figure 4.2A; p<1%). Statistically speaking, the extent of re-innervation in the double mutant c-JunΔSΔP0 mice was significantly lower than that seen with deletion of c-Jun in neurons (c-JunΔS) or Schwann cells (c-JunΔP0) alone. There was no difference in the number of labelled motoneurons on the unaxotomised side of all four groups of animals.

We next explored whether alterations in motoneuron survival contributed to the observed reduction in re-innervation. Comparison of motoneuron number on the uninjured and injured sides 30 days after injury revealed a loss of 42 ± 5% neurons in the c-JunWT control group, injured side. However, motoneuron death was strikingly increased in c-JunΔP0 animals, with a loss of nearly 90±1% neurons on the injured side, and more than double the loss seen in the controls (p<0.01%). Such striking cell loss in mutants with Schwann cell c-jun deletion has been reported earlier (Arthur-Farraj et al., 2012;Fontana et al., 2012). In complete contrast to this phenotype, motoneuron survival was dramatically increased in the c-JunΔS mutants, with a loss of only 10±1% neurons; a four-fold reduction in cell death compared to the controls (p <1%). Again, this phenomenon has been described by earlier studies from our laboratory using neural and neuronal c-jun KOs (Ruff et al., 2012;Raivich et al., 2004). Interestingly, combination of the two gene deletions, i.e., neuronal and Schwann cell c-jun, in the c-JunΔSΔP0 mice mirrored the increased cell survival phenotype of the c-JunΔS mutant, with a loss of only 5±2% neurons; a significantly higher rate of cell survival than the controls (p<0.1%) and the c-JunΔP0 animals (p<0.001%) (Figure 4.3(a), (b) A,B). The neuronal c-jun deletion appeared to completely block motoneuron loss due to the absence of Schwann cell c-jun in the c-JunΔSΔP0 double mutants.

The four experimental cohorts differed not just in terms of their motoneuron numbers, but also in terms of their morphology (Figure 4.3(a) – 40X mag). While the surviving cell bodies in the c-JunWT and c-JunΔP0 mice appeared chromatolytic, most of the axotomised neurons in the c-JunΔS animals appeared shrunken by 30% of their original cell diameter (p<1%), with the exception of a few chromatolytic, and possibly functional neurons. The axotomised neurons in the c-JunΔSΔP0 double mutants had an even more severe atrophic morphology, with a 40% reduction in cell diameter (p<0.01%), and complete absence of any normal-sized or chromatolytic neurons (Figure 4.3(b)C,D).

The massive variation in cell survival between the groups posed another question – were the observed changes in re-innervation merely the effect of increased cell death in certain groups? To answer this question, survival adjusted re-innervation (SAR) was calculated by dividing the ratio of re-innervation by the survival ratios for each group.
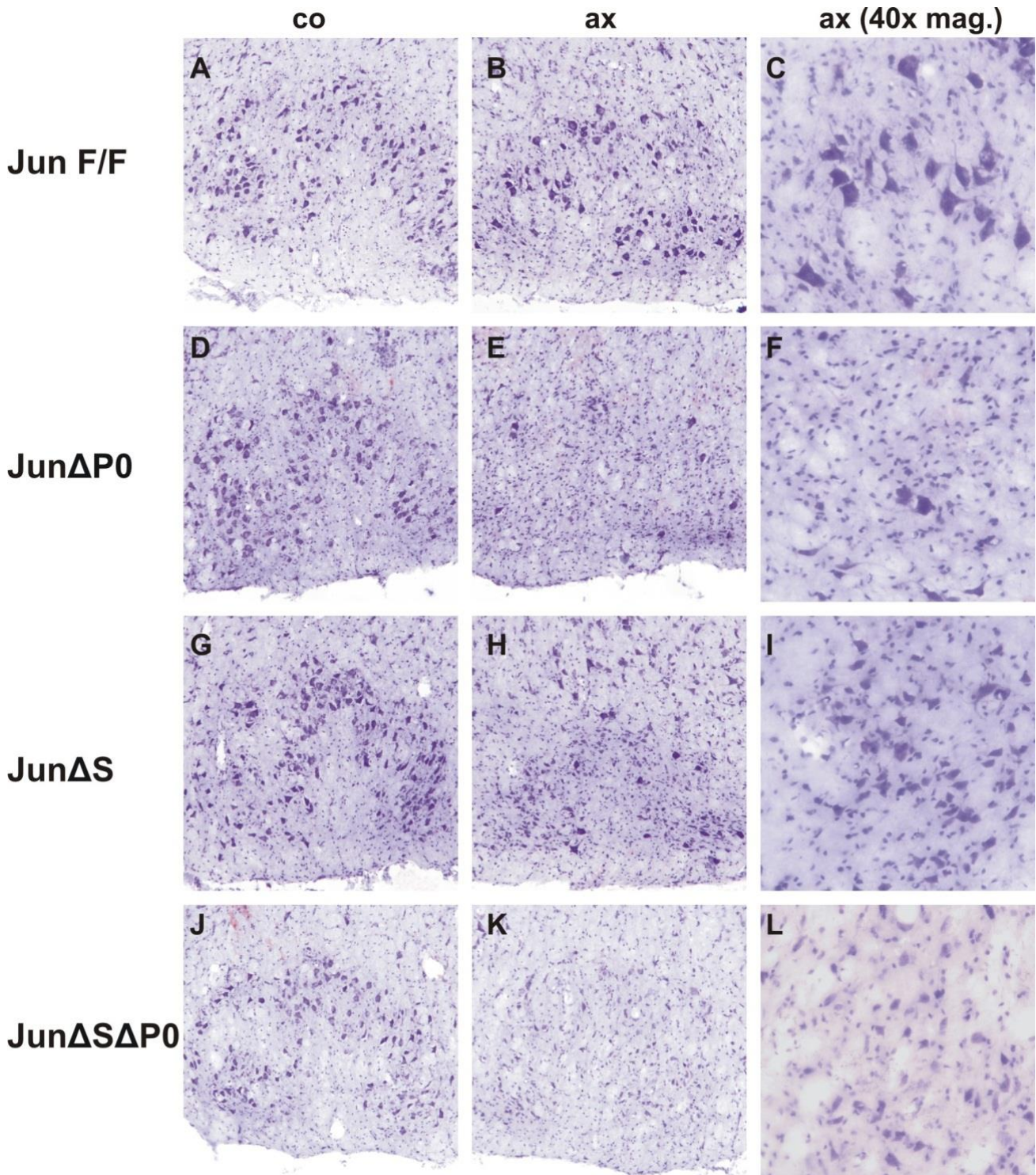
Figure 4.3 (a) Deletion of c-jun in neurons and SCs affects neuronal survival and morphology after facial axotomy. Nissl-stained coronal sections of facial motoneurons on the contralateral (co – A,D,G,J) and axotomised (ax – B,E,H,K) side, and at higher 40 X magnification of ax side (C,F,I,L) in control (junF/F) and mutant (junΔP0, junΔS, and junΔSΔP0) animals, 30 days after unilateral facial nerve cut. Note the strong decrease in cell survival in junΔP0 mice, and the increase in cell survival in junΔS and junΔSΔP0 mice, but of severely atrophic neurons.
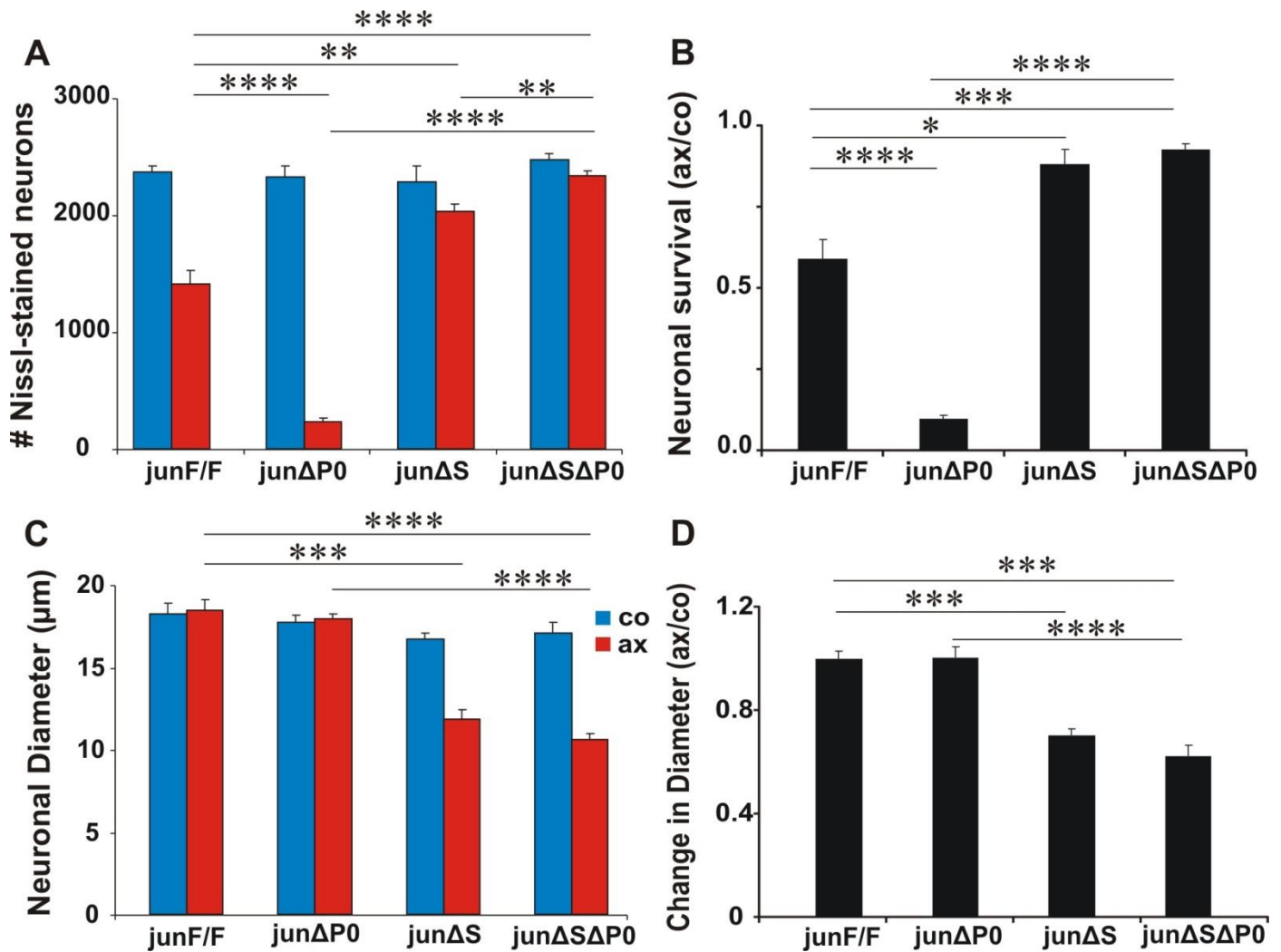
Figure 4.3 (b) Deletion of c-jun in neurons and SCs affects neuronal survival and neuronal morphology after facial axotomy. **A, B.** Total motoneuron count and percentage survival, corrected for neuronal size, showed a significant reduction in neuronal survival after axotomy in the absence of SC c-jun, but significant increase in neuronal survival in the absence of neuronal c-jun or combined deletion of neuronal and SC c-jun. **C, D.** Neuronal diameter measurements (in µm) and percentage change in diameter (ax/co%) showed a mild chromatolytic increase in neuronal size in junF/F controls and junΔP0 mutants, but a significant shrinkage of junΔS and junΔSΔP0 neurons after axotomy. ** $p<1\%$, *** $p< 0.1\%$, **** $p<0.01\%$ in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

The controls had an SAR value of 69±16%, followed closely by the c-JunΔP0 animals with 70±23% (Figure 4.2B). This suggests that the re-innervation defect seen in the Schwann cell c-Jun null mutants is entirely due to the increased neuronal cell death after axotomy. In contrast, the c-JunΔS animals had an SAR value of 10±2%, significantly lower than the controls ($p<1\%$); and the c-JunΔSΔP0 double mutants a nearly complete abolition of survival-adjusted re-innervation with an SAR value of 1±0%, significantly lower than controls ($p<0.01\%$), and neuronal c-Jun deletion alone (Figure 4.2B; $p<0.1\%$).

N-terminal phosphorylation of c-jun is involved in neuronal survival and re-innervation after FNA

The above experiments have clearly delineated an important role for neuronal and SC c-jun in peripheral nerve regeneration. In spite of the pronounced effects of c-jun deletion on axonal regeneration, the exact molecular mechanisms underlying its course of action remain largely unknown. In addition to the presence of c-jun in different regulatory cell types, its phosphorylation state can influence dimer stability and can affect its biological role in regeneration, both in vivo and in vitro (as described previously in Chapter 1 Introduction). c-jun can be activated by N-terminal phosphorylation at serines 63 & 73 and threonines 91 & 93 by Jun N-Terminal Kinases 1, 2 and 3 (JNK1-3) (Raivich, 2008). Other means of c-jun modulation include dephosphorylation of Thr239 which attracts FBW7 ubiquitin ligase (FBW7L) and targets the phosphorylated protein for ubiquitination and subsequent degradation, and C-terminal lysine acetylation near aa257-276 (Vries et al., 2001), which results in activated c-Jun via the ERK pathway (Raivich, 2008). Mutants with global deletion of JNK1, 2 or 3, and alanine replacement of the serine 63&73 sites have shown a mild effect of c-jun phosphorylation on peripheral regeneration (Ruff et al., 2012). It is possible that complete removal of all 4 JNK dependent N-terminal phosphor-acceptor sites (Thr91&93 as well as Ser63&73) will produce a more severe regeneration phenotype. This study uses homozygous c-Jun4A and wild type (wt) mice, and the facial axotomy model, to elucidate the effects of total ablation of c-jun N-terminal phosphorylation on facial nerve regeneration. Based on data characterising survival, regeneration and cellular response of Jun4A mutants following facial nerve injury, it is evident that N-Terminal phosphorylation of c-jun is not the mechanism by which neuronal c-jun triggers majority of its effects in regeneration.

Abolition of c-jun N-terminal phosphorylation has moderate effects on functional recovery and anatomical re-innervation after facial nerve axotomy

Axonal regeneration in c-JunWT controls and c-Jun4A mutants was evaluated by analysing recovery of whisker hair function and target re-innervation after facial nerve axotomy.

Recovery of motor function was assessed by scoring the ipsilateral whisker hair movement on a scale of 0 – 3 (0= no movement, 1= mild movement, 2= moderate movement, and 3 = normal movement as on unoperated side), starting from 7 days after

axotomy, and measured at 2-3 day intervals till day 28. Functional recovery in c-jun 4A mutants was on average slightly lower than that in the c-jun WT controls (Figure 4.4A). The overall recovery of function, as measured by AUC, showed a moderate 18% reduction in the c-Jun4A mutants (0.65±0.05) compared to the c-JunWT controls (0.53±0.5), almost reaching statistical significance with p-value of 7% (Figure 4.4B). Due to the variability between the animals, adding animal numbers to the two groups might push this result towards significance.

A fluorescent tracer, Fluorogold (FG), was used to analyse target re-innervation at 30 days after axotomy. A gel-foam soaked in 10µl FG was applied bilaterally for 30 minutes to the whisker-pads of c-jun4A and c-junWT mice at 28 days after facial axotomy. The mice were allowed to survive for 48 hours to enable retrograde transport of the tracer to the reconnected facial motoneurons in the hindbrain, after which they were perfused, and their brains cryo-sectioned for microscopic analysis. Counting of the FG+ motoneurons revealed a significant, approximately 50% reduction in re-innervation in the c-jun4A mutants (63±8% in c-JunWT to 37±10% in c-Jun4A mice) (Figure 4.4C; p<5%).
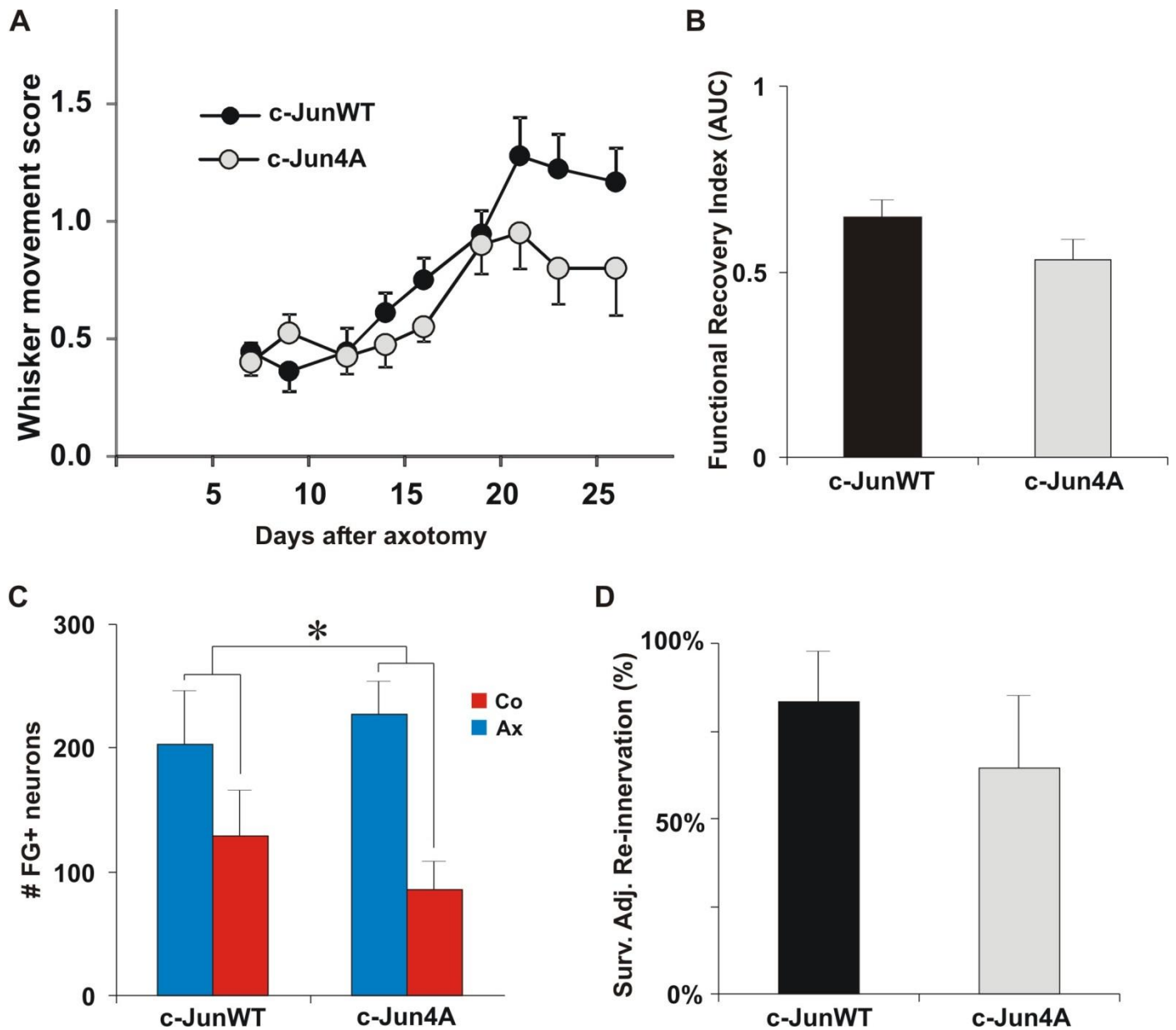
Figure 4.4. c-jun N-terminal phosphorylation and axonal regeneration. **A.** Time-course of functional recovery of whisker hair movement on a scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) shows a trend towards reduced functional recovery in c-jun 4A (n=10) compared to c-jun WT (n=9) at 30 days post-injury. **B.** Regenerative index of functional recovery calculated as Area Under the Curve for the two groups. **C.** Re-innervation of the target whisker-pad muscle at day 30 after unilateral facial nerve axotomy, measured by counting the number of motoneurons positively labelled by retrogradely transported fluorescent dye Fluorogold (FG). There was a significant reduction in re-innervation in the c-Jun4A mice compared to the c-JunWT. **D.** The survival adjusted re-innervations showed no significant difference between groups. Data points and error bars in these graphs show the mean and SEM, respectively, *p<5% in Repeated Test Anova (for whisker movement score) and in unpaired Student's T-test.

Abolition of c-jun N-terminal phosphorylation causes increased cell death and shrinkage following facial nerve axotomy

After axotomy, c-junWT control animals showed 25% neuronal loss, from 1633±118 neurons on the uninjured side to 1208±64 neurons on the injured side. Such level of cell death after axotomy is expected and has been reported in several studies previously. However, the c-jun4A mutants showed significantly higher motoneuron death – an almost 45% reduction in cell survival from 1819 ± 91 on the unaxotomised side to 1041±131 neurons on the axotomised side (Figure 4.5 A, B). Also, most of the surviving axotomised c-Jun4A motoneurons appeared shrunken by 20%, showing a striking reduction in cellular diameter from 18.2±0.2 to 14.7±0.5μm (Figure 4.5C; $p<0.01\%$). The opposite effect was observed in their control counterparts which underwent chromatolytic enlargement by 10% from 17.5±0.4 μm for the uninjured to 19.2±0.3 μm for injured motoneurons (Figure 4.5C; $p<5\%$).
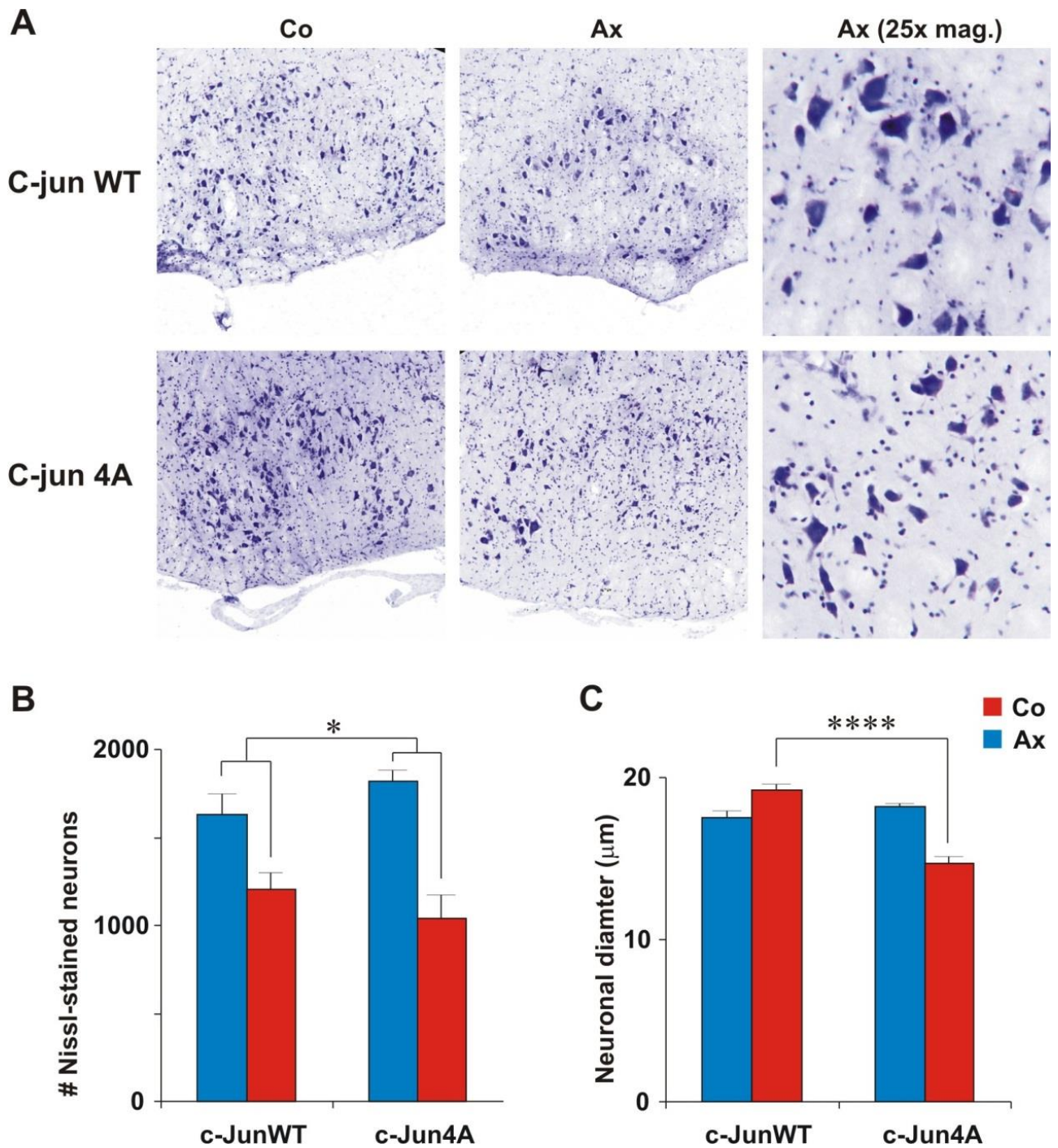
Figure 4.5. N-terminal phosphorylation of c-jun is required for neuronal survival after facial axotomy. **A.** Nissl-stained coronal sections of facial motoneurons on the contralateral (co) and axotomised (ax) side, and at higher 25 X magnification of ax side, in control (c-junWT) and mutant (c-jun4A) mice, 30 days after unilateral facial nerve cut. **B.** Total motoneuron count, corrected for neuronal size, showed a significant reduction in neuronal survival after axotomy in the c-jun4A mice. **C.** Neuronal diameter measurements (in μm) showed a significant shrinkage of c-jun4A neurons after axotomy. *p<5%, ** p<1%, ***p<0.1%, **** p<0.01% in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Because the c-jun4A mutants showed 50% reduction in re-innervation, and 45% reduction in cell death, the next question was to investigate if the increased cell loss led to the reduced target reconnection in these animals. To this end, percentage re-innervation (FG+ ax/co%), was divided by percentage survival (Nissl counts ax/co%), to get an index of survival adjusted re-innervation. While the c-junWT showed 83±15% re-innervation after adjusting for cell survival, the c-jun4A mutants showed a tendency towards lower SAR of 64±21% (Figure 4.4D). However, this difference was statistically insignificant, suggesting that the majority of the regeneration deficits in the mutants are due to excessive motoneuron loss.

The expression of neuronal regeneration associated proteins is unaffected in c-Jun4A mice

Following peripheral nerve transection, the chromatolytic response involves the upregulation of regeneration associated proteins which aid in neurite outgrowth and elongation. The neuropeptide galanin and the cell adhesion molecule CD44 are important mediators of a successful regenerative response and have previously been shown to rely on c-Jun upregulation following axotomy (Raivich et al., 2004). To determine whether the post axotomy effect of c-Jun relies on phosphorylation of the molecule at ser63&73 and thr91&93, the axotomy induced expression of CD44 and galanin in c-Jun4A and c-Jun Wt mice was compared at day 4 and day 14 after facial nerve axotomy (Figure 4.6. A-H). C-Jun4A mice showed no significant difference in CD44 expression or in the expression of galanin+ sprouts at day 4 (Figure 4.6 I, K) or day 14 (Figure 4.6 J, L) following axotomy, indicating that c-Jun dependent upregulation of neuronal injury markers is autonomous of JNK phosphorylation of c-jun following injury. This was in contrast to the moderate reduction (-24%) in CD44 immunoreactivity seen in JunAA mice at day 4 after axotomy.
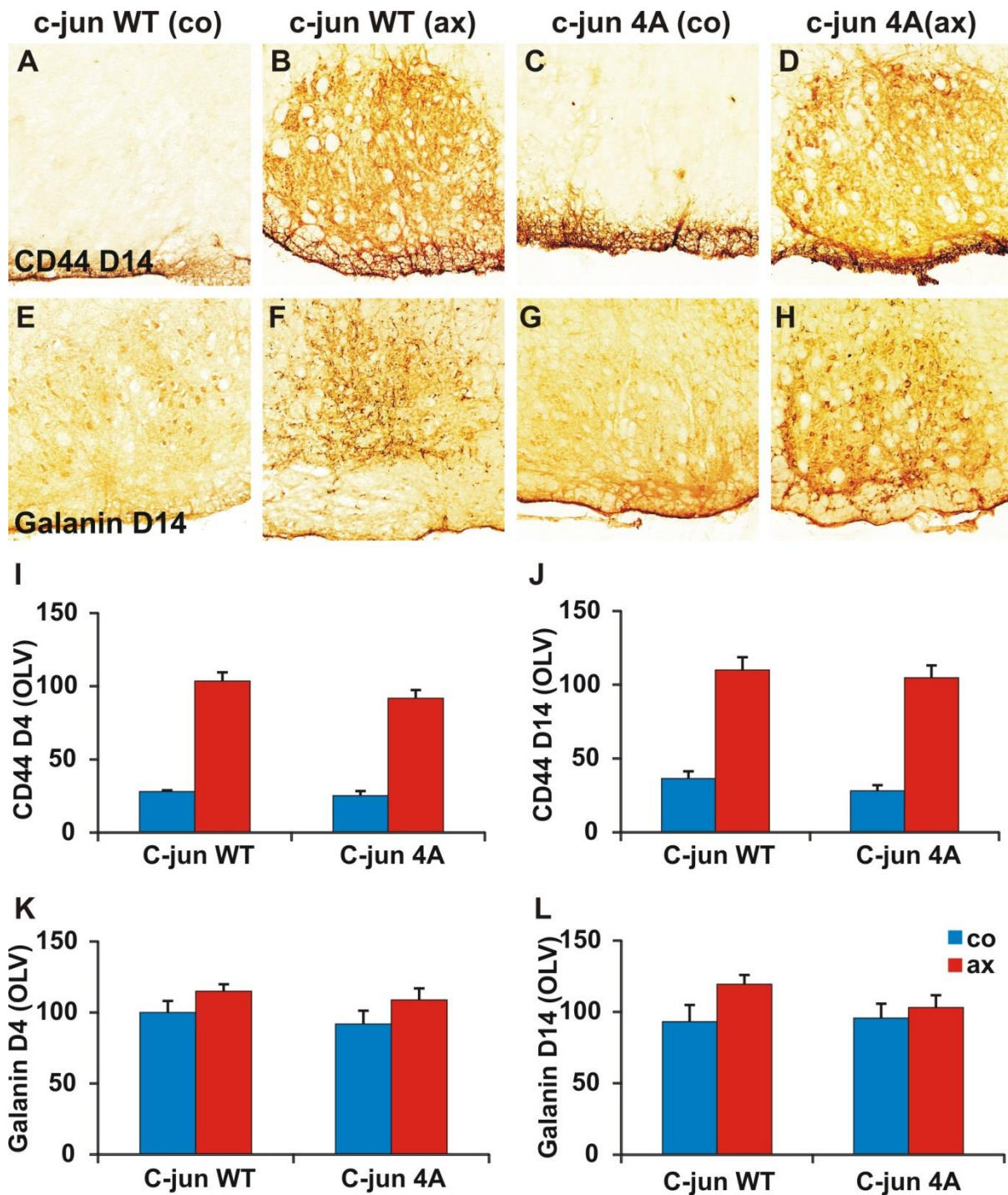
Figure 4.6. Neuronal axotomy response is unaffected in c-jun 4A mice. **A - H.** CD44 and Galanin immunoreactivity on the contralateral (co) and axotomised (ax) facial nucleus at day 14 (d14) following axotomy in c-jun WT (n=5) and c-jun 4A (n=5) animals. **I - L.** Quantification of CD44 and Galanin expression at d4 and at d14 following axotomy. Injured (co) and uninjured (ax) sides are represented by blue and red bars respectively. The abolition of N-terminal phosphoryable c-jun sites, does not impair induction of adhesion molecule, CD44 (I, J) or the expression of galanin positive sprouts (K, L) at either time point analysed. Bars and error bars show mean and SEM respectively.

c-Jun N-terminal phosphorylation is not essential for the activation and recruitment of non-neuronal cells following axotomy

Activation of non-neuronal cells around the cell body of injured axons is an integral part of the regenerative response following nerve injury (Raivich et al., 1998;Raivich et al., 1999a). Neuronal c-jun deletion results in a dramatic reduction in microglial activation and leucocyte recruitment 14 days following axotomy, with an accompanying small but significant reduction in astrocytic activation (Raivich et al., 2004). In contrast, c-Jun4A mice showed no significant difference in early microglial activation as assessed by the expression of the αM integrin subunit at day 4, and only a mild (-20%) but statistically insignificant reduction in late microglial activation at day 14 following axotomy (Figure 4.7 I, J). Both c-JunWt and c-Jun4a mice showed appearance of clustered microglial nodules (Figure 4.7. B, D). Furthermore, astrocytic activation as measured by GFAP immunoreactivity showed no significant difference in c-Jun4A animals compared to c-JunWt controls (Figure 4.6 E-H, K-L).
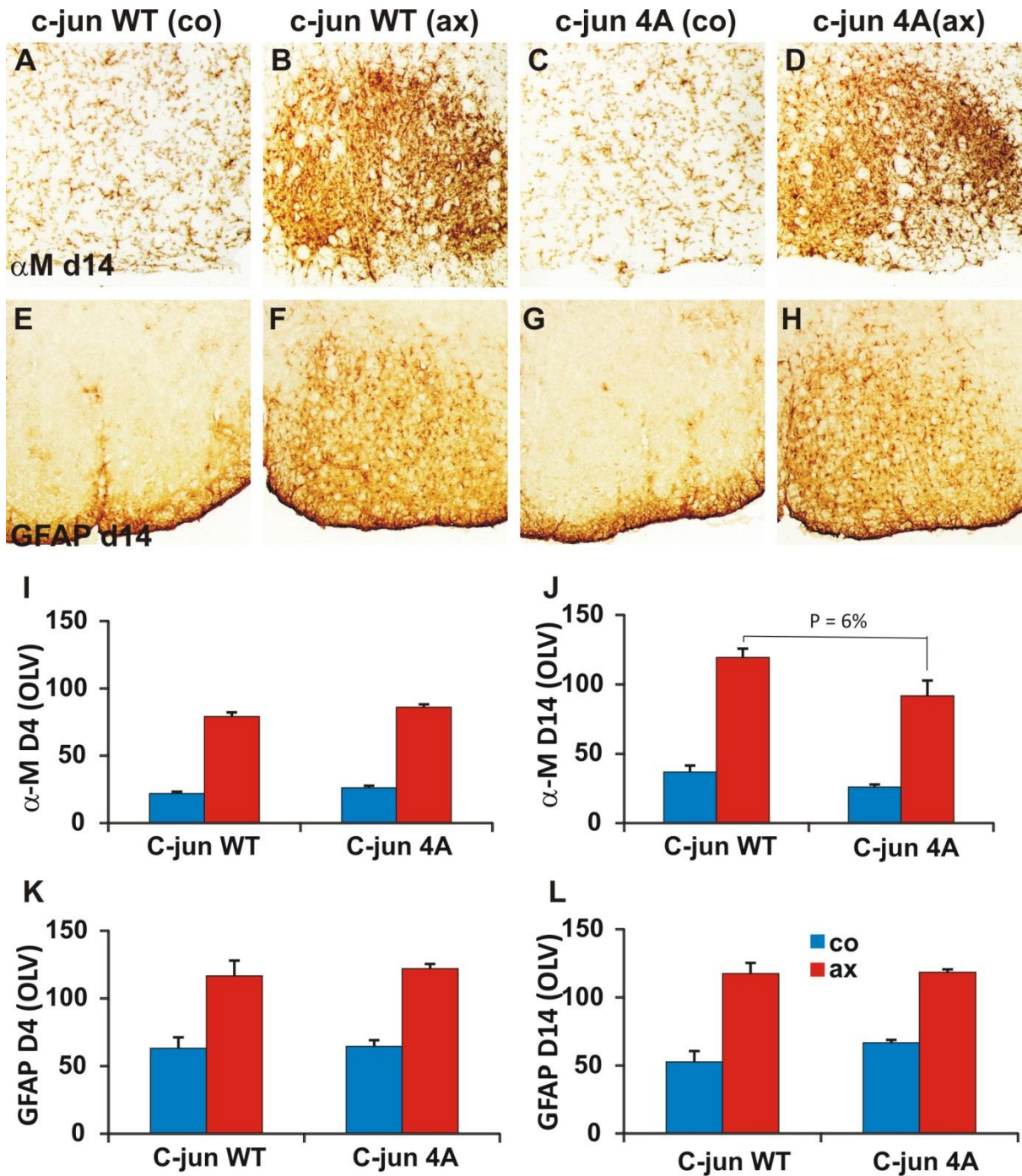
Figure 4.7. Non-neuronal response following axotomy is unaffected in c-jun 4A mice. **A-H.** αM and GFAP immunoreactivity on the contralateral (co) and axotomised (ax) facial nucleus at 14 days following facial nerve axotomy in c-jun WT (n=5) and c-jun 4A (n=5) animals. **I-L.** Quantification of αM and GFAP expression at d4 and at d14 following axotomy. Injured (co) and uninjured (ax) sides are represented by blue and red bars respectively. The abolition of N-terminal phosphoryable c-jun sites, does not impair early (I, K) or late (J, L) microglial (I, J) and astroglial (K, L) activation after injury. Bars and error bars show mean and SEM respectively.

Overall, the c-jun4A mutation results in lesser motoneuron survival, post-traumatic neuronal shrinkage, less recovery and less re-innervation than their control counterparts. Interestingly, the molecular response in the facial nucleus is more or less normal in the mutants, same as in junAA animals, and JNK1-3KOs.

Chapter 4 – c-Jun Discussion

Previous scientific evidence has highlighted an important role for c-jun in injury-induced nerve repair in adult mice. While deletion of neuronal or Schwann cell c-jun has far-reaching consequences on the success of peripheral nerve regeneration - delaying functional recovery and causing a strong, persistent defect in target re-innervation, there is still some residual regeneration in the absence of the transcription factor. Similarly, deletion of neuronal STAT3 greatly impedes target re-innervation, functional recovery, speed of regeneration, and the biochemical and molecular response from injured neurons and neighbouring glial cells. However, a small fraction of the neurons continue to regenerate with an apparently 'normal' rate of regeneration, accounting for delayed yet substantial functional recovery in these animals. This data suggests the likelihood of co-existence of 'two different neuronal populations' – one that is completely devoid of regeneration in the absence of transcription factors, and the other that is transcriptionally independent in its regenerative ability. To explore this idea, regeneration was analysed in double mutants lacking neuronal c-jun (which functions in transcriptional control of downstream effectors of regeneration), and also lacking Schwann cell c-jun (which is important in mediating trophic signalling). Another possibility is that there are multiple transcription factors acting simultaneously, and their concerted action is required for the success of regeneration. In the absence of one, others might be increased via a compensatory mechanism and account for the residual regeneration in the knock-out mutants. In order to investigate this hypothesis, double mutant mice with combined deletion of both neuronal c-Jun and STAT3 were generated, and complete obliteration of regeneration in the absence of these factors was analysed. Finally, we also investigated c-jun N-terminal phosphorylation as a mechanism for c-jun activation and function in injury-induced peripheral nerve regeneration.

Neuronal c-Jun & STAT3 are not functionally complementary counterparts in facial nerve regeneration

The possibility of c-Jun and STAT3 belonging to complementary signalling pathways in regeneration was investigated by exploring whether combining both deletions, produced a stronger, synergistic effect on regeneration, than a simple deletion of either STAT3 or c-Jun. What emerged was that combined deletion of c-jun and STAT3 interfered with functional recovery and re-innervation of whisker-pad, at the same levels as that seen with single deletion of either transcription factor, making it unlikely for them to be complementary counterparts. Motoneuron loss was abolished in the combined mutants, and the surviving neurons were atrophic, again the same effect being seen in the single deletion mutants. A comparison of the early and late neuronal and non-neuronal markers affected by the absence of either STAT3 or c-jun (Chapter 3- STAT3 results) (Ruff et al., 2012), also showed a striking similarity, not only in terms of the markers affected but also in the degree of deficiency (Table 4.1).

Regeneration markers	% change in STAT3ΔS	% change in c-junΔS	% change in STAT3+c-JunΔS
D28 Functional recovery	↓↓	↓↓	↓↓
D28 whisker pad re-innervation	↓↓↓	↓↓↓	↓↓↓
D30 neuronal survival	↓↓	↓↓	↓↓
D4 regeneration speed (CGRP)	↓↓	↓↓	-
D4 regeneration speed (Galanin)	↓↓	↓↓	-
D4 nuclear ATF3	↓↓↓	↓↓↓	-
D4 c-jun score	No change	Abolished	-
D4 nuclear STAT3	Abolished	No change	-
D14 integrin β1(OLV)	↓↓↓	↓↓↓	-
D14 CGRP+ sprouts	↓↓↓	↓↓↓	-
D14 Galanin+ sprouts	↓↓↓	↓↓↓	-
D14 CD44 (OLV)	↓↓↓	↓↓↓	-
D14 α-M integrin (OLV)	↓↓↓	↓↓↓	-
D14 α-X count	↓↓↓	↓↓↓	-
D14 B 7.2 count	↓↓↓	↓↓↓	-
D14 CD3+ T-cell count	↓↓↓	↓↓↓	-
D14 GFAP (OLV)	No change	↓	-

Table 4.1. Comparison of regenerative defects in STAT3ΔS and c-junΔS mice

It is unlikely that either transcription factor is upstream or downstream of each other since normal c-jun expression in seen in STAT3ΔS mice and vice-versa. Rather, it seems that the two transcription factors might be co-regulating various aspects of the regeneration machinery by forming a 'double-keyed lock', whereby absence of any one key (transcription factor) is sufficient to prevent unbolting of the 'lock' (regeneration). It is even possible that there are other transcription factors in addition to c-jun and STAT3, and regeneration might be controlled by a 'multiple-key lock'. A previous study by Kiryu-Seo et al, has already shown that co-ordinated action of 3 transcription factors – ATF3, c-Jun, and STAT3 – is required for the maximal transcription of regeneration associated gene DINE (Kiryu-Seo et al., 2008). As mentioned earlier, the interactions between STAT3 and c-jun could be multiple, overlapping and complex – ranging from direct physical interaction (Zhang et al., 1999;Schaefer et al., 1995) to co-ordinated retrograde transport of p-STAT3 and JIP3/JNK3 by DLK upon axon injury (Shin et al., 2012), and concurrent recruitment by common STAT3 and c-jun binding proteins to gene promoter regions (Kiryu-Seo et al., 2008). Interestingly, in a model of MPTP-mediated Parkinson's disease, c-jun and STAT3 deletions had completely contrasting results on neuronal survival. While neuronal deletion of c-Jun reduced dopaminergic neuron loss in the substantia nigra by 41%, absence of neuronal STAT3 had no effect on death or survival of dopaminergic neurons (Patodia and Raivich, unpublished data). This result adds further complexity to the relationship between c-jun and STAT3, suggesting that they can have convergent or divergent actions depending on the model of injury. Genetic profiling of their target genes by microarrays will help in the identification of molecular recognition patterns for both regeneration signals.

In summary, due to the absence of exacerbated deficiency or complete abolition of nerve regeneration in the double mutants, it seems unlikely that the two transcription factors belong to complementary pathways. Rather, they may be acting in concert to unlock a common signalling mechanism in regeneration, and the absence of either factor is sufficient to block that stream of regeneration. Further investigation will shed light on the prospect of multiple players being involved, and identification of their individual and co-operative roles in peripheral nerve regeneration.

Neuronal c-Jun & Schwann cell c-jun together regulate nerve regeneration after facial axotomy

Analysis of injury-induced regeneration in the absence of neuronal c-jun and STAT3 showed that in spite of substantial shortcomings in all aspects of the usually robust peripheral nerve response, a small fraction of the neurons continued to regenerate unaffectedly, producing small amounts of functional recovery and re-innervation, albeit with a slight delay. This led to the idea that trophic support from Schwann cells and other non-neuronal cells might be sufficient to induce elongation of some axonal fibres even in the absence of neuronal transcription factor mediated recovery. The success of regeneration after peripheral nerve injury has long been attributed to a combination of intrinsic and extrinsic factors. The 'intrinsic' mechanism involves changes in gene transcription, a part of a set of changes referred to as the "cell body response". Equally important is the presence of a permissive environment with axon guidance cues which constitutes the 'extrinsic' component. In CNS neurons, diminished intrinsic capacity for regeneration together with heightened presence of inhibitory factors of their extracellular environment results in poor regeneration (Buchli et al., 2007;Bolsover et al., 2008). Schwann cells are vital contributors to the extrinsic response in peripheral nerve regeneration, regulating Wallerian degeneration, and releasing neurotrophic factors which mediate their effects in a paracrine manner via tyrosine kinase (Trk) receptors on growing axonal tips (Raivich, 2011). Recent studies have shown a crucial role of Schwann cell c-jun in inhibiting myelin gene activation by Krox-20 or cAMP (Parkinson et al., 2008), and forming a de-differentiated Schwann cell state, essential for the generation of a growth supporting environment in injured nerves, and promoting regeneration after injury (Arthur-Farraj et al., 2012). In the absence of c-Jun in Schwann cells, the expression of neurotrophin genes Artn, BDNF-1, GDNF, LIF, and NGF is severely down-regulated, causing severe defects in motoneuron survival, and axonal growth, and resulting in very poor functional recovery (Arthur-Farraj et al., 2012;Fontana et al., 2012). Current data from experiments using animals lacking both neuronal and Schwann-cell c-jun, show that facial nerve regeneration is completely obliterated in the absence of c-jun in the two cell-types. Comparison of motoneuron number on the uninjured and injured sides 30 days after injury revealed that motoneuron survival was strikingly decreased in c-JunΔP0 animals, with loss of nearly 90% neurons, more than double the loss seen in the controls. Such striking cell loss is consistent with earlier reports (Arthur-Farraj et al., 2012;Fontana

et al., 2012). Facial motor neurons require neurotrophic factors for their survival and rapid death of neurons occurs when peripheral support is completely lost (Yetiser and Kahraman, 2008). In complete contrast, motoneuron survival was dramatically increased in the c-JunΔS mutants, a four-fold reduction in cell death compared to the controls, a phenomenon described in earlier studies from our lab (Raivich et al., 2004;Ruff et al., 2012). Interestingly, combination of the neuronal and Schwann cell c-jun deletions reflected the increased cell survival phenotype of the c-JunΔS mutant; neuronal c-jun deletion being sufficient to completely block motoneuron loss due to absence of Schwann cell c-jun in the double mutants. However, all surviving neurons were severely atrophic. Target re-innervation was also strongly compromised with c-JunΔS animals showing an almost five fold decrease, c-JunΔP0 animals more than six-fold decrease, and the c-JunΔSΔP0 mice a striking forty-fold decrease. When adjusted appropriately for cell survival, the double mutants demonstrated a nearly complete abolition of re-innervation with a survival adjusted re-innervation value of 1%.

This study illuminates the yet unexplained conundrum of c-jun having a dual role in cell-death as well as regeneration. Schwann cell c-jun is a mediator of trophic factor signalling in peripheral regeneration, supporting neuronal survival. On the other hand, neuronal c-jun is essential for the increased expression of growth associated molecules, perineuronal sprouting and re-innervation, but also death of severely injured motoneurons. Thus, while the effects of neuronal and Schwann cell c-jun on regeneration is additive, their effects on neuronal survival are antagonistic. Transcriptional control of modulatory factors produced by glial cells can compensate, to a small extent, for the loss of functional and anatomical regeneration in the absence of neuronal c-jun. However, deletion of both neuronal and Schwann cell c-jun results in loss of the cell-body response together with extremely poor neurotrophic environment, affecting the ability of axons to extend and re-innervate, ultimately leading to a complete suppression of peripheral nerve regeneration.

c-Jun N-terminal phosphorylation affects neuronal survival and morphology, and axonal re-innervation after axotomy

Results from previous and current experiments have highlighted a vital role for c-jun in peripheral nerve regeneration (Arthur-Farraj et al., 2012;Fontana et al., 2012;Raivich et al., 2004;Ruff et al., 2012). In the absence of neuronal c-jun, speed of axonal regeneration is

strongly reduced, preventing most axons from making target reconnections and resulting in loss of function (Raivich et al., 2004;Ruff et al., 2012). Deletion of Schwann cell c-jun exacerbates cell death after injury, preventing target re-innervation and leading to loss of muscular function (Arthur-Farraj et al., 2012;Fontana et al., 2012). N-terminal phosphorylation of c-jun by JNKs, represented an attractive mechanism for c-jun's post-axotomy response, since JNKs are known to be rapidly upregulated and retrogradely transported to the cell body, and increase c-jun phosphorylation and stability after injury (Brecht et al., 2005;Herdegen and Leah, 1998;Lindwall and Kanje, 2005). Although N-terminal phosphorylation of c-Jun plays an important role in modifying transcriptional activity and elicits strong effects in stroke, ischemia and excitotoxicity, its effects on regeneration are less clear. Jun4A mice where all 4 JNK dependent N-terminal phosphorylation sites - Thr91&93 as well as Ser63&73 – are removed, were used to assess the effect of c-jun N-terminal phosphorylation in peripheral nerve regeneration.

Usually around 20-40% of lesioned motoneurons are lost after facial nerve axotomy (Raivich et al., 2002). c-JunAA mutants show neuronal shrinkage but not increased cell death or survival (Ruff et al., 2012b). The c-Jun4A mice showed increase in cell death after facial axotomy, suggesting that c-jun4A is a more severe phenotype than c-junAA. Also, the surviving neurons had significantly shrunken neuronal cell bodies, similar to the c-JunAA mice. Interestingly, late microglial activation at day 14 showed a mild (-20%), but statistically insignificant reduction in the c-jun4A mutants compared to controls. It has been implied that injury at the distal axon initiates the early response in the cell body by survival promoting components of JNK signalling that are retrogradely transported along the axon (Lindwall and Kanje, 2005). The data in this present study suggests that c-Jun N-terminal phosphorylation may be required for normal transportation of survival factors to the cell body, which could be important for normal neuronal cell sizes to be regained after axotomy. Increased cell death in the c-jun4A mutants contradicts previously published reports that c-jun N-terminal phosphorylation is required for kainic acid excitotoxicity (Behrens et al., 1999) and for cell death following intrastriatal injections of 6-hydroxydopamine (Brecht et al., 2005). Concomitant phosphorylation of Thr91&93 by JNK and of Thr95 by ATM kinase has been shown to enhance cell death after DNA damage (Vinciguerr et al., 2008). Furthermore, Nateri et al., in 2004 demonstrated that complete abrogation of all four JNP sites, as in the case of our c-jun 4A mutants, blocks FBW7 mediated degradation (Nateri et al., 2004). Conditional deletion of c-jun in Schwann cells,

also showed increased cell death and reduced re-innervation after axotomy (Fontana et al, 2012; Chapter 4-results), suggesting that the effects N-terminal phosphorylation might be important for c-jun action in Schwann cells. In cell culture, enhanced phosphorylation activation of c-Jun with anisomycin, a JNK activator, causes increased production of neurotrophins (Fontana et al., 2012), a function attributed to injury-induced c-jun response in Schwann cells (Arthur-Farraj et al., 2012;Fontana et al., 2012). However, c-jun mediated inhibition of myelin genes in Schwann cells does not appear to depend upon the classical N-terminal phosphorylation of c-jun by JNKs (Parkinson et al., 2008).

Deletion of c-Jun, results in a 74% and 92% decrease in the expression of galanin and CD44 respectively, and microglial activation is impaired by 88% (Raivich et al., 2004). The jun4A mutation, however, did not interfere with the expression of injury-associated neuronal (CD44 and galanin) and non-neuronal markers (microglial, astroglial, and leucocyte), similar to the junAA, and JNK1-3 KOs (Ruff et al., 2012). On the other hand, as in the JNK1&3 null mutants, the c-jun4A strain reproduced the moderate reduction in functional recovery and a 50% lower number of re-innervating motoneurons at 28 days following facial nerve cut. This effect on regeneration, though significant, is considerably smaller than that observed for neuronal c-Jun deletion and is not accompanied by any of the molecular changes in the facial nucleus seen with deletion of c-Jun, suggesting that the regeneration effect of neuronal c-Jun is mostly independent of its N-terminal phosphorylation. However, the similar pattern of increased cell death, and reduced re-innervation in the c-Jun4A and c-JunΔP0 mutants suggest that JNK mediated N-terminal phosphorylation might be important for SC c-jun arbitrated trophic signalling and neuronal survival following facial axotomy.

Taken together, these results suggest that c-Jun N-terminal phosphorylation is not the vital mechanism through which neuronal c-Jun is activated and exerts its functions in nerve regeneration. c-junAA mutants and global deletion of individual JNKs, have very mild effects on regeneration (Ruff et al., 2012) compared to complete c-jun ablation in neurons. c-Jun4A mutants appear to be a combination of the c-junAA and JNK1-3 phenotypes – less re-innervation, functional recovery and neuronal survival, post-traumatic neuronal shrinkage, and very little change in neuronal molecular response - but it does not quite reproduce the changes seen with neural c-jun deletion. Rather its role in mediating excessive neuronal cell death and a moderate reduction in re-innervation after axotomy,

suggests the involvement of c-Jun N-terminal phosphorylation in the post-traumatic response of c-jun in SCs.

In addition to the N-terminal phosphorylation sites, c-jun has additional phosphorylation (Thr239) and acetylation sites (Lys268, 271 and 273) in its hinge and basic regions which are downstream of the glycogen synthase kinase-3 (GSK3) and p300 pathways, the pathways being activated via PI3K or ERK 1 & 2 (Raivich, 2008;Vries et al., 2001;Wang et al., 2006). Therefore, understanding how neuronal c-jun mediates its regenerative effects *in vivo* may warrant a closer investigation of these alternate activation pathways through selective amino-acid replacements, similar to the exchange of the N-terminal serines and threonines. Given the striking role of c-jun in regeneration, unraveling its molecular pathways, will enhance its capacity for use as a clinical target to improve regeneration following peripheral, and potentially also central injury.

Chapter 5 - C/EBPδ Results

C/EBPδ is involved in neuronal survival, microglial activation, and central sprouting following facial nerve axotomy

The transcriptional factor C/EBP delta belongs to the CCAAT/enhancer binding protein transcription factor family of basic leucine zipper binding proteins which show almost ubiquitous expression throughout the body, and respond to signals of injury and inflammation (Ramji and Foka, 2002;Sterneck et al., 1998). Like other C/EBPs, C/EBPδ has previously been shown to be activated by various cytokines, and is also implicated to play a role in axonal regeneration – controlling the expression of downstream regeneration-associated gene SPRR1A, and inducing axonal regeneration of DRG neurons after sciatic nerve crush (Lopez de and Magoulas, 2013). Here, by using mice with global C/EBPδ gene deletion and the facial axotomy model, we have shown that C/EBP acts as a moderate regulator of microglial activation, neuronal sprouting, and cell survival after facial nerve transection.

C/EBPδ is required for efficient neuronal sprouting response, but not for CD44 up-regulation following nerve injury

Upregulation of growth associated proteins by injured neurons is considered to contribute significantly to the success of the final regenerative outcome following injury (Raivich and Makwana, 2007). Numerous chromatolytic changes take place in the cell body of injured axons in order to support growth cone formation and efficient elongation of the axon.

In order to investigate the involvement of C/EBPδ in the neuronal response following facial nerve axotomy, 20μm brainstem sections containing the injured and uninjured facial nuclei were immuno-histochemically stained for neuropeptide galanin (Figure 5.1 A-D) and cell adhesion molecule CD44. Galanin expression is increased in central axonal sprouts within the facial nucleus in the hindbrain, as well as in the regenerating nerves. Counts of galanin positive sprouts in the facial nucleus revealed a 44% decrease in galanin expression (from 84±4 galanin+ sprouts in C/EBPδ+/+ mice to 47±5 in C/EBPδ-/- mice) in the axotomised facial nucleus at day 14 following axotomy (Figure 5.1.E).
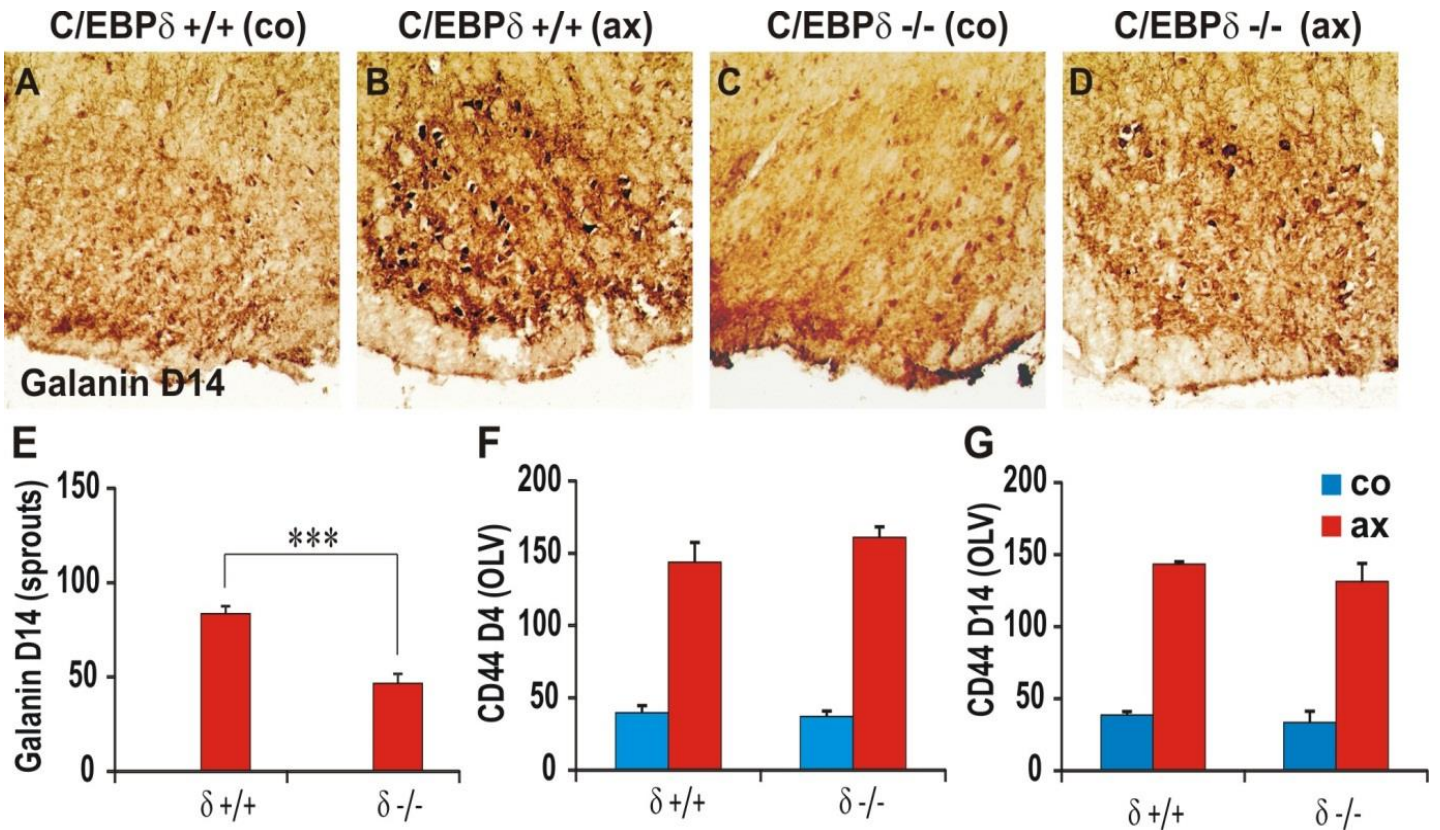
Figure 5.1. CEBPδ is required for neuronal sprouting following axotomy. **A-D.** Galanin immunohistochemistry showing facial nerve axotomy response in the C/EBPδ+/+ controls (A, B) and the C/EBPδ-/- mutants (C, D) on the contralateral side (co) and on the axotomised side (ax). After axotomy, CEBP δ+/+ mice show a prominent increase in neuropeptide galanin (A-B). Deletion of CEBPδ significantly reduces the galanin-associated sprouting response (D). **E-G.** Quantification of overall facial motor nucleus immunoreactivity by manual counts or OLV units at 4 (d4) and 14 days (d 14) after nerve cut for galanin (E) and CD44 (F,G) in CEBPδ+/+ (n=5) and CEBPδ-/- mice (n=5). The contralateral (co) and axotomised (ax) sides are indicated by blue and red bars respectively. Galanin showed a significantly smaller increase in the mutants compared to controls. CEBPδ was not required for induction of CD44 following injury. ***p< 0.1% in unpaired Student's T-Test, bars and error bars represent mean and SEM respectively.

CD44, a cell surface glycoprotein, plays an important role in cell adhesion during neurite outgrowth and in the glial and leucocyte response following nerve injury (Jones et al., 1997;Jones et al., 2000). It is a hyaluronic acid receptor, and its expression is strongly upregulated within the facial motor nucleus after axotomy. Quantification of CD44 by luminosity revealed no difference in CD44 immunoreactivity between wild type and mutant animals at 4 or 14 days after axotomy (Figure 5.1 F, G). These results indicate that endogenous C/EBPδ is required for the induction of galanin positive sprouts following axotomy, but not for the expression of the cell adhesion molecule CD44.

C/EBPδ is required for microglial but not for astroglial or leucocyte response following injury

In addition to the neuronal response, the expression of non-neuronal hematopoietic and neuro-epithelial cells which participate in inflammation, repair and lesion point stability is an essential part of a successful regenerative response (Raivich et al., 1998). This non-neuronal cellular response was assessed by measuring levels of activation of microglia and astroglia, and amount of leucocyte recruitment to the injured facial nucleus. The uninjured nuclei of both C/EBPδ+/+ and C/EBPδ-/- mice had similar levels of resting microglia and protoplasmic astrocytes, and no leucocytes. After axotomy, CEBPδ-/- mice showed a significant decrease in both early (d1, d4) and late (d14) microglial activation as assessed by αM immuno-staining in the facial nucleus (Figure 5.2 A-L). 38% decrease in the expression of the αM integrin microglial subunit was seen at day 1 (Figure 5.2 D, M), reducing to a 24% decrease at day 4 (Figure 5.2 H, N; p<5%) and a 16% decrease at day 14 (Figure 5.2 L, O; p<5%). T-lymphocyte recruitment as measured by counts of CD3+ cells in the axotomised facial nucleus showed no significant differences between C/EBPδ$^{-/-}$ and C/EBPδ+/+ animals (Figure 5.2Q). Similarly, astrocytic activation as measured by GFAP immunoreactivity was unimpaired in the C/EPBδ-/- animals (Figure 5.2P).
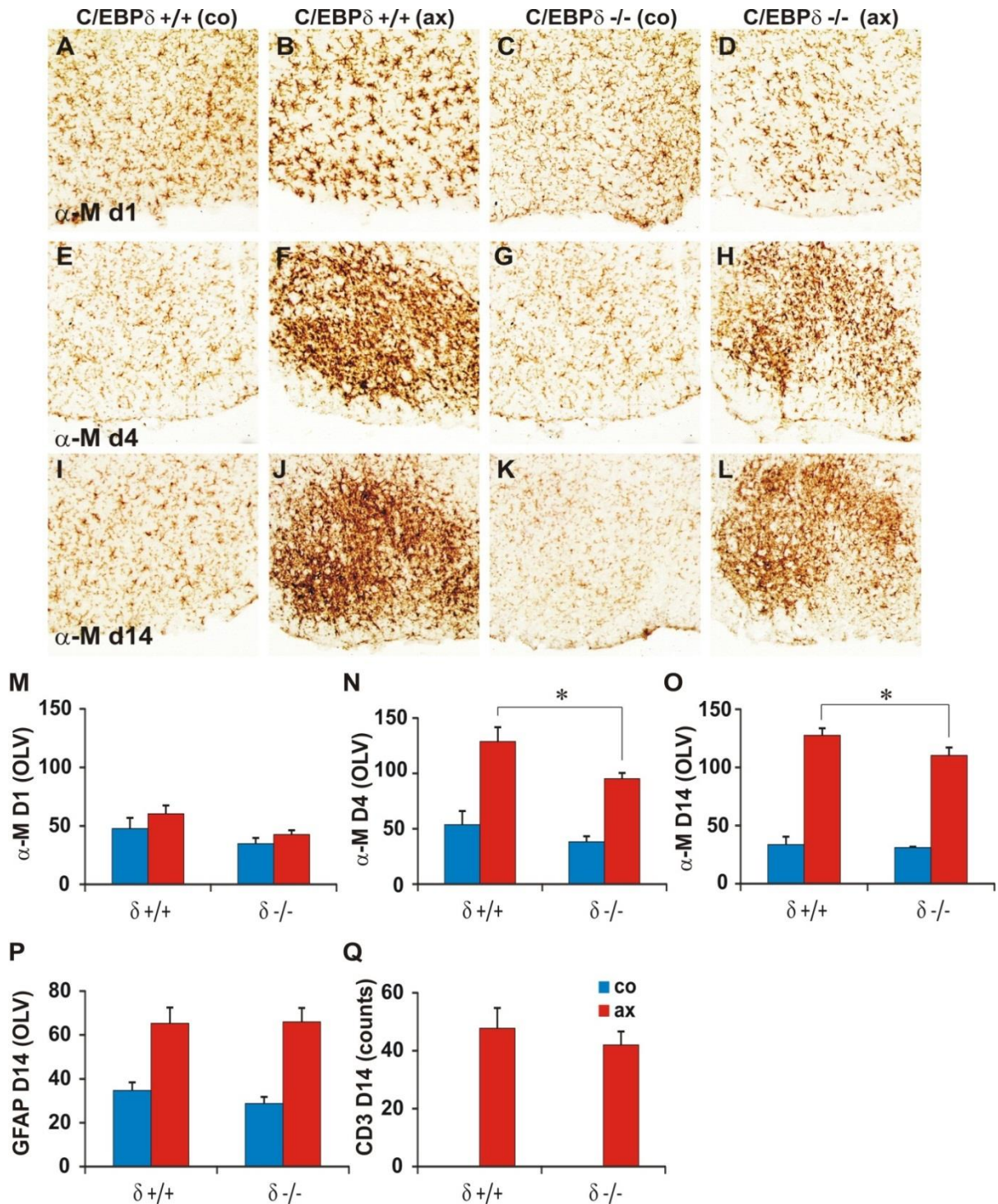
Figure 5.2. CEBPδ is involved in microglial activation following axotomy. **A - L.** Facial nerve axotomy response after nerve cut in the CEBPδ+/+ controls (A,B,E,F,I,J) and the CEBPδ-/- mutants (C,D,G,H,K,L) on the contralateral side (co) (1st & 3rd column), and on the axotomised side (ax) (2nd & 4th column). After FNA, CEBPδ+/+ mice show a prominent increase microglial marker αM integrin (A-B, E-F, I-J) and GFAP (IHC not shown). Deletion of CEBPδ significantly reduces microglial activation at all three time-points analysed (C-D, G-H, K-L). **M - Q.** Quantification of overall facial motor nucleus immunoreactivity by manual counts or OLV units at 1 (d1), 4 (d4) and at 14 days (d 14) after nerve cut for αM integrin (M-O), GFAP (P) and CD+ T-cells (Q) in CEBPδ+/+ (n=5) and CEBPδ-/- mice (n=5). The (co) and (ax) sides are indicated by blue and red bars respectively. αM showed a significantly smaller increase in the mutants compared to controls. CEBPδ was not required for astrocytic activation or T-cell recruitment following injury. *p< 5% in unpaired Student's T-Test, bars and error bars represent mean and SEM respectively.

Combined deletion of global C/EBPδ and neuronal STAT3 does not exacerbate the regeneration defects caused by STAT3 deletion alone

As both STAT3 and C/EBPδ play a role in peripheral regeneration, the likelihood of them being functionally complementary counterparts in peripheral nerve regeneration was investigated. Double KO mice having global C/EBPδ deletion, and neuronal STAT3 deletion were generated. The right facial nerve was cut at the stylomastoid foramen, thereby axotomising the facial motoneurons. The animals were allowed to survive for 30 days. All assessments were done similarly and simultaneously on mice with single gene deletions, and on control mice, to assess synergistic effects and interactions between the two transcription factors.

Functional recovery was assessed by scoring post-traumatic whisker hair movement every other day on a scale of 0 (no movement) to 3 (normal movement). Both the controls, and the mice with single deletion of C/EBPδ, showed recovery by day 11 and improved steadily over the next two week to a score of around 2.4 by day 28; in fact C/EBPδ deletion, rather than impeding motor function recovery after facial nerve cut, resulted in significantly faster recovery compared to controls between day 11 to day 21. Thereafter, the effect disappears, possibly because recovery of controls by that time is already very good. Recovery in the STAT3ΔS mutants was significantly poorer (as observed earlier in Chapter 3 –STAT3 Results), and reached a maximum score of 0.8 ± 0.1 at day28. Deletion of C/EBPδ in addition to neuronal STAT3 deletion resulted in significantly better functional recovery than STAT3 deletion alone, achieving a score of 1.6 ± 0.1 by day 28 (Figure 5.3A). These varied trends in functional recovery are reflected by the Recovery Index (RI) for the four groups (Figure 5.3B).

The same experimental cohorts were also assessed for target muscle re-innervation and motoneuron survival. Whisker pads were labelled bilaterally with the fluorescent tracer Fluoro-Gold (FG), followed by 48 h retrograde transport. Motoneurons that successfully reconnected with their targets were identified by the presence of the retrograde tracer in their somas, and counted on every fifth section throughout the facial nucleus. Control animals showed retrograde labelling of $70\pm7\%$ on the axotomised side compared with the uninjured side. C/EBPδ deletion alone produced no change in re-innervation ability with animals showing $81 \pm 3\%$ labelling. In contrast, neuronal STAT3 deletion drastically reduced the percentage of re-innervating neurons to $15\pm4\%$ (Figure 5.3D).
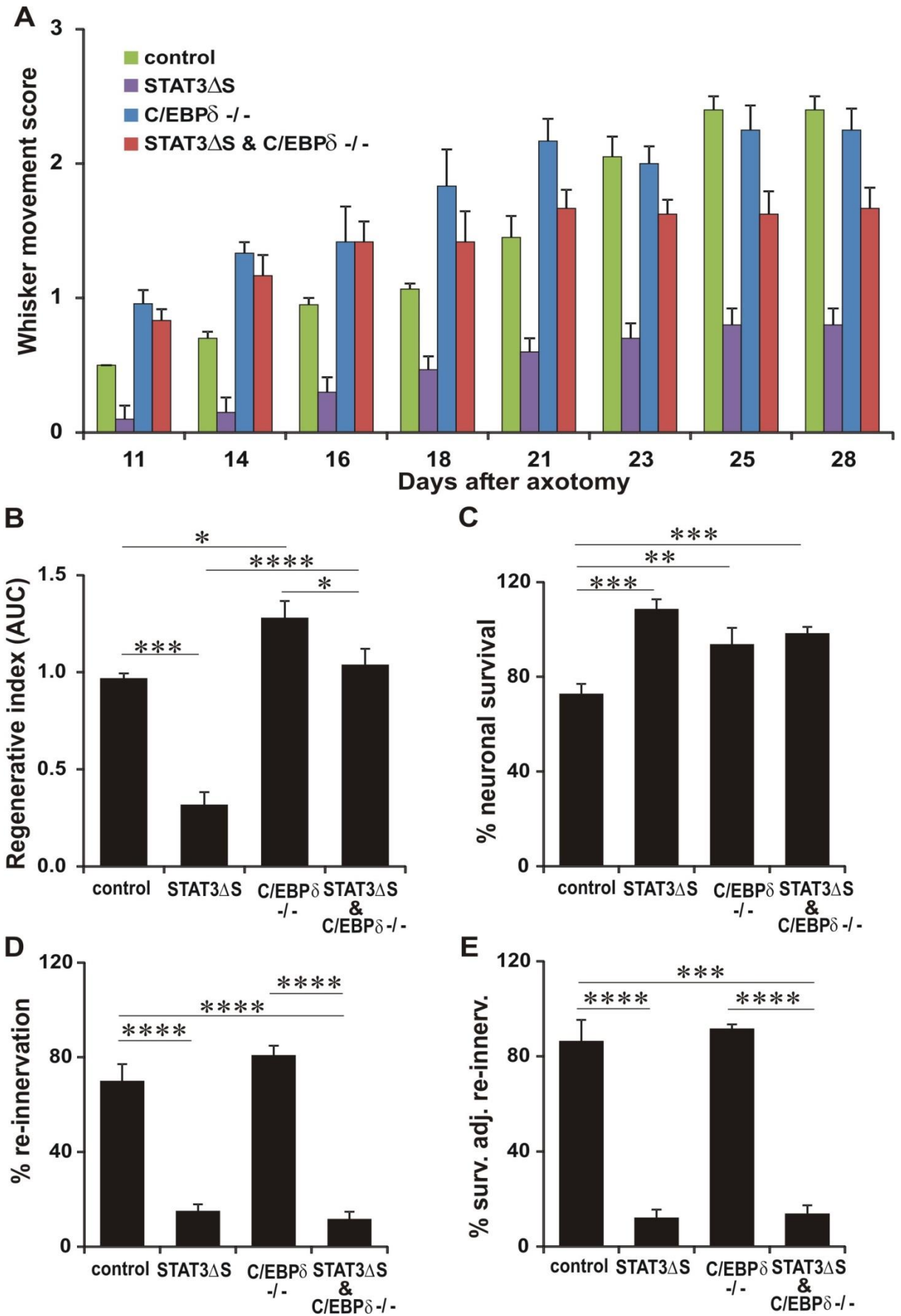
Figure 5.3. Deletion of C/EBPδ improves functional recovery and neuronal survival following axotomy, and combined deletion of C/EBPδ with neuronal STAT3 deletion does not exacerbate the regeneration defect seen with deletion of neuronal STAT3 alone. **A**. Time course for functional recovery of whisker hair movement on a scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) at 30 days post-injury. **B.** Overall regenerative index of functional recovery (calculated as Area Under the Curve). **C.** Percentage survival of facial motoneurons 30 days after axotomy showed increased neuronal survival in absence of either C/EBPδ or neuronal STAT3 or both. **D.** Quantification of Fluorogold retrograde labels following FNA shows reduced re-innervation in the absence of neuronal STAT3 with or without simultaneous global deletion of C/EBPδ. **E.** Survival adjusted re-innervation also shows reduced re-innervation in the absence of neuronal STAT3 with or without simultaneous global deletion of C/EBPδ. $*p< 5\%$, $**p<1\%$, $***p<0.1\%$, $****p<0.01\%$ in unpaired t-test, bars and error bars show the mean and SEM, respectively.

However, the combination of global C/EBPδ, and neuronal STAT3 deletions did not make the phenotype more or less severe than what was observed with neuronal STAT3 deletion, with the animals achieving 12±3% re-innervation by day 30 (Figure 5.3B). Survival adjusted re-innervation showed the same trend (Figure 5.3E).

To explore whether alterations in motoneuron survival contributed to the observed reduced re-innervation and functional recovery, motoneuron number on the uninjured and injured sides at 30 days after injury were compared. It revealed a loss of 27 ± 4% neurons in the control group, injured side. However, motoneuron death was significantly decreased in C/EBPδ and STAT3 single and double mutants. C/EBP single mutants had 94±4% motoneuron survival, STAT3 single mutants 109±7%, and the double mutants 98±3% (Figure 5.3C; $p<0.1\%$). The extent of microglia recruitment correlates with the severity of neuronal cell death (Raivich and Banati, 2004). In agreement with reduced neuronal death in C/EBP and STAT3 single deletions, there was a reduction in the levels of microglial activation in these mutants, as mentioned earlier in this and previous chapters. Therefore, C/EBPδ deletion, while producing a significant effect of its own on neuronal survival, is subsumed in double null mice, suggesting that the C/EBPδ phenotype might be a composite part of the overall neuronal STAT3 mediated response.

Chapter 5 – C/EBPδ Discussion

C/EBPδ plays a role in neuronal survival and microglial activation following axotomy

Analysis of cell survival at 30 days after facial axotomy showed a significant 22% increase in number of surviving motoneurons in the global C/EBPδ knock-outs compared to the control animals. Increased neuronal survival has been seen earlier in the absence of other transcription factors like STAT3 and c-jun (Chapters 3 & 4). Motoneuron survival was also significantly increased in C/EBPδ and STAT3 double mutants, but no more than in the single mutants.

Activation of microglia following axotomy is a hallmark feature of the regenerative response. Microglia respond to signals of stress and injury by extensive proliferation, eventually becoming phagocytic in instances of increased cell death (Raivich et al., 1999a). In this study, C/EBPδ-/- animals showed a mild to moderate, but statistically significant reduction in microglia expression compared to controls at three separate time points. Interestingly, the reduction in microglial activation diminished with increasing time after injury, going from -38% at day 1, to -26% at day 4 and -14% at day 14. C/EBPδ has been long implicated in mediating neuro-inflammatory responses in the brain (Alam et al., 1992;Ramji and Foka, 2002), supporting the above finding of reduced microglial activation in its absence. Further, maximal reduction in microglial activation was seen at the earliest time-point analysed (day 1), suggesting that C/EBPδ mediated regulation of microglia is associated with the injury-induced inflammatory and immune response. The extent of microglia recruitment correlates with the severity of neuronal cell death (Raivich and Banati, 2004), therefore reduced microglial activation in the absence of C/EBPδ reaffirms a role of this transcription factor in inducing cell death. On the other hand, CD3+ T-lymphocytes which accompany microglia in phagocytosis, showed no complementary decrease in expression in the C/EBPδ-/- mutants at day 14. Further work will be required to assess the mechanisms of C/EBPδ mediated microglial activation and motoneuron death, and to determine whether the impairment in microglial activation following axotomy in the C/EBPδ-/- animals is a consequence of C/EBPδ absence in neuronal or other cells.

C/EBPδ deletion improves functional recovery in neuronal STAT3 null mice

The concerted action of several transcriptional factors is the likely mechanism by which they control the activation and expression of a wide range of effector molecules that orchestrate axonal regeneration. Neuronal deletion of STAT3 severely reduced functional recovery and re-innervation of the peripheral target. A few studies have shown that C/EBPδ is associated with the transcriptional response to neuronal injury and with axonal elongation (Lopez de and Magoulas, 2013;MacGillavry et al., 2011). Severely compromised sensory and motor function after sciatic nerve injury in C/EBPδ null mice has been reported in a recent publication (Lopez de and Magoulas, 2013). As both STAT3 and C/EBPδ play a role in peripheral regeneration, the likelihood of them being functionally complementary counterparts in peripheral nerve regeneration was investigated. By using double knock-out mice, the hypothesis that deletion of both signalling streams will lead to a massive reduction in regeneration compared to single gene deletions was tested. Interestingly, our studies showed that C/EBPδ deletion did not impede motor function recovery after facial axotomy. On the contrary, it significantly improved post-traumatic whisker movement, compared to controls and neuronal STAT3 deletion alone. C/EBPδ deletion, when combined with neuronal STAT3 deletion, even improved recovery in the very slow recovering STAT3 mutants. The mechanism for increased functional recovery in the absence of C/EBPδ is as yet unknown, and may include factors like improved myelination. In terms of anatomical re-innervation, C/EBPδ deletion produced no change in re-innervation ability, on its own or in combination with neuronal STAT3 deletion.

C/EBPδ mediates neurite outgrowth following facial nerve axotomy

The neuronal response involves the upregulation of various growth-associated molecules inside the cell body of injured axons leading to axonal elongation at the site of injury and the formation of intra-brainstem sprouts around the cell body of injured axons. Centrally sprouting neurites express neuropeptide galanin (Makwana et al., 2010) and deletion of galanin results in a 35% reduction in rate of peripheral nerve regeneration after sciatic nerve crush (Holmes et al., 2000). In this study, global deletion of transcription factor, C/EBPδ resulted in a 40% reduction in the number of galanin positive sprouts in the facial

nucleus at 14 days following axotomy, underscoring a role for C/EBPδ in mediating neurite growth after injury.

Interestingly, in 2005, Nadeau and colleagues showed a role of C/EBPβ, another member of the C/EBP transcription factor family, in regulating the expression of microtubule α-tubulin protein and GAP43, a growth cone protein, after injury (Nadeau et al., 2005). According to two recent reports, C/EBPs are also involved in axonal outgrowth *in vitro* (MacGillavry et al., 2011), and necessary for the efficient axonal regeneration of DRG neurons after sciatic nerve crush in vivo (Lopez de and Magoulas, 2013). Lack of C/EPBδ caused a major reduction in the regenerative response of DRG neurons to a conditioning lesion, and affected the expression of regeneration-associated molecule SPRR1A, but not of galanin or GAP-43. This is in contrast with our current experiments which show reduction in galanin levels to nearly half in the C/EBPδ null mutants. As STAT3 has also been shown to regulate the expression of galanin positive sprouts following axotomy (Chapter3 – STAT3 Results), concerted action of the two transcription factors might be responsible for the pro-regenerative effects following axotomy. Taken together, these results indicate an important role for C/EBP family of transcription factors in axonal sprouting and outgrowth following injury, possibly through transcriptional activation of cytoskeletal elements which mediate aspects of actin rearrangement, formation of the growth cone, and expression of neuropeptide galanin.

Chapter 6 – CAP23 Results

Neuronal CAP23 plays a role in axonal regeneration, central sprouting and early glial response after facial axotomy

CAP23 is a cytoskeletal-associated protein present in growth cones, with known functions in actin remodelling and anatomical plasticity. CAP-23 is upregulated by facial motor neurons within three days following facial nerve transection (Anderson et al., 2006). CAP23, along with GAP43, another growth cone adaptor, has been shown to induce long neurite extensions *in vitro*, and after spinal cord injury *in vivo* (Bomze et al., 2001). Most CAP-23 KO mice die before adulthood and are therefore unsuitable for studying the role of CAP23 in successful axonal regeneration in adult animals. By using loxP-cre recombinase method, we have generated mice with neuron-selective deletion of the CAP23 gene in order to investigate the *in vivo* function of neuronal CAP23 in motor axon regeneration of peripheral nerves.

Neuronal CAP23 deletion interferes with target re-innervation after FNA

Effects of CAP23 on axonal regeneration were assessed by measuring the extent of functional recovery and re-innervation of peripheral target 30 days after PNI.

Functional Recovery

To assess the level of functional recovery post injury, the post-traumatic whisker hair motor performance was scored on a scale of 0 (no movement) to 3 (normal movement as on uninjured side) every alternate day from day 7 after surgery till day 28 (Figure 6.1A). The CAP23ΔS animals had similar levels of motor performance compared to the CAP23F/F animals right through the 28 day assessment period, and by day 28 post axotomy, the control and mutant animals both had achieved similar motor performances (1.4±0.2 and 1.6±0.3 respectively). When represented as regeneration index, i.e., the Area Under the Curve for the functional recovery over days 0-28 (Figure 6.1B), the two groups again showed similar functional levels with the CAP23F/F mice (n=11) having an index of 0.9±0.1 and the CAP23ΔS mutants (n=9) an index of 0.8±0.1.
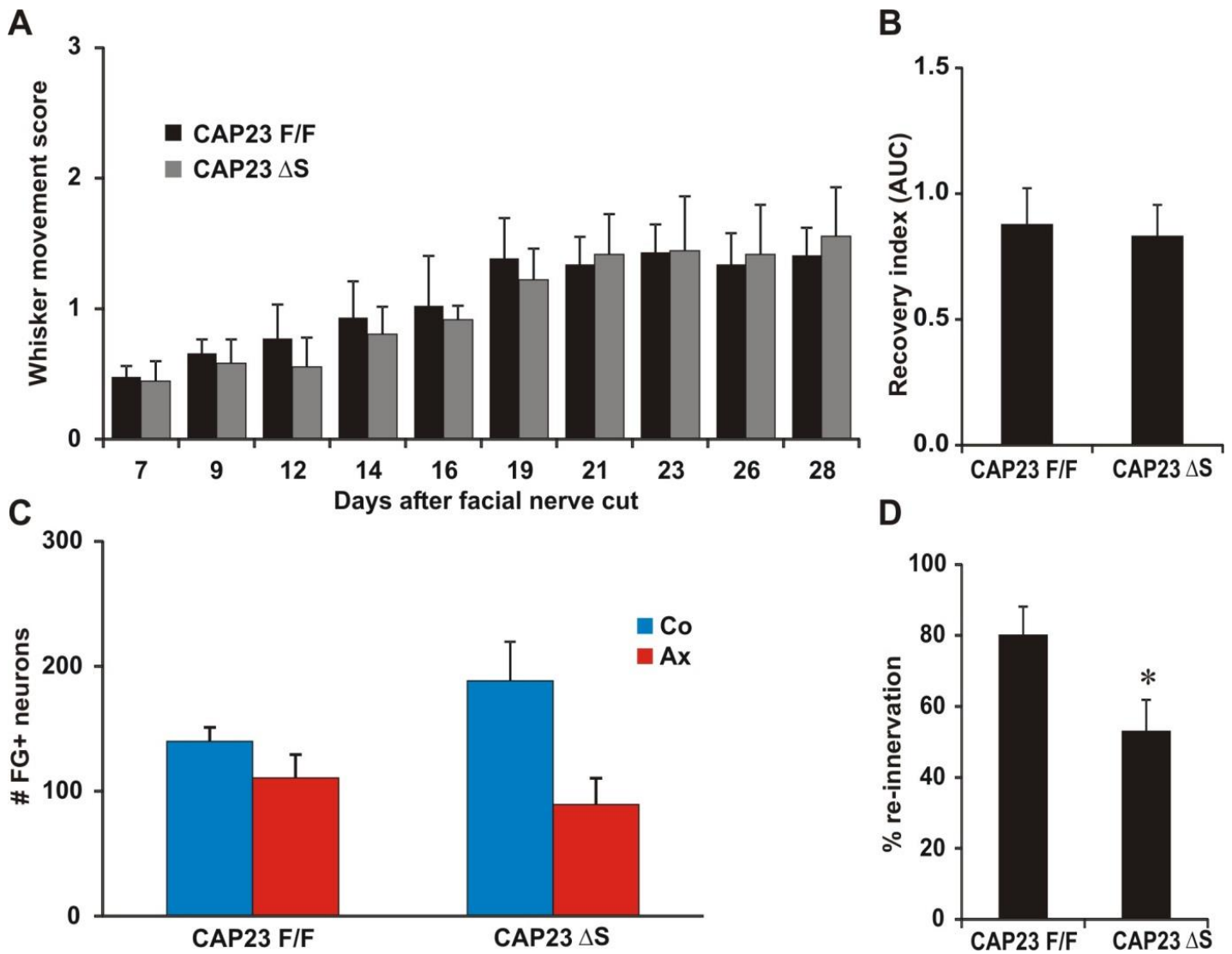
Figure 6.1 – CAP23 regulates re-innervation following nerve axotomy. **A, B** - Time course (A) for functional recovery of whisker hair movement on a scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) and the recovery index (B) calculated as Area Under The Curve (AUC) shows no difference between functional recovery in the CAP23ΔS mutants (n=11) compared to the CAP23 F/F controls (n=9) at 30 days post-injury. **C, D -** Quantification of Fluorogold retrograde labels in the CAP23F/F and CAP23ΔS animals following facial axotomy shows reduced re-innervation in the absence of neuronal CAP23. (C) shows the number of FG labelled motor neurons in the facial nucleus on the contralateral (co) and axotomised (ax) sides at 30 days after facial nerve cut. CAP23ΔS mutants showed about 34% less whisker pad re-innervation compared to CAP23F/F controls. Percentage re-innervation, calculated as a ratio of labelled neurons on the axotomised to the contralateral side (ax/co %) following injury, showed a significant reduction in re-innervation in the absence of CAP23 in the neurons. *p< 5% in unpaired t-test, bars and error bars in these graphs show the mean and SEM, respectively.

Re-innervation of the peripheral target

To determine any differences in re-innervation efficiencies between the CAP23F/F and CAP23ΔS mice, a fluorescent tracer FG was injected into the whisker pads of mice bilaterally, 28 days post axotomy, and the mice allowed to survive for 48 hours to enable retrograde transport of the tracers. The level of re-innervation at day 30 following facial axotomy was assessed by counting the number of FG positive motoneurons in the injured facial nucleus and comparing them with the uninjured, control side. The overall ratio of labelled FG neurons on the ipsi- vs. the contra-lateral side in the CAP23ΔS mutants (n=7) was only 53±9% as compared to 80±8% in the CAP23F/F controls (n=8) (Figure 6.1 C,D). This reduction of re-innervation (-34%) in the mutants is statistically significant, suggesting a role for neuronal CAP23 in peripheral target re-innervation but not for functional recovery.

Neuronal CAP23 does not affect motoneuron survival after PNI

Facial axotomy results in delayed neuronal cell death which peaks at day 14 after injury (Raivich et al., 1998), probably due to lack of trophic support resulting from failed re-innervation (Ferri et al., 1998). The level of cell survival following facial axotomy was assessed by counting all motoneurons through the whole facial nucleus on the control and injured side using Nissl stained sections (Figure 6.2A). The number of motoneurons on the unoperated side of the CAP23F/F controls (3291±135; n=11) and the CAP23ΔS mutants (3411±191; n=8) were similar to each other and in line with previously published data on the facial nucleus (Galiano et al., 2001;Raivich et al., 2004;Sendtner et al., 1997). Compared to the unoperated side, facial axotomy led to a loss of 23±9% of motoneurons in the CAP23ΔS (2536±142 on the axotomised side) animals compared to 33±4% loss in the CAP23F/F animals (2173±130 on the axotomised side). Although, there was no statistical significance, the mutants tended to show a mild increase in cell survival (Figure 6.2.B). No atrophic morphology was seen on either the axotomised or the contralateral side.
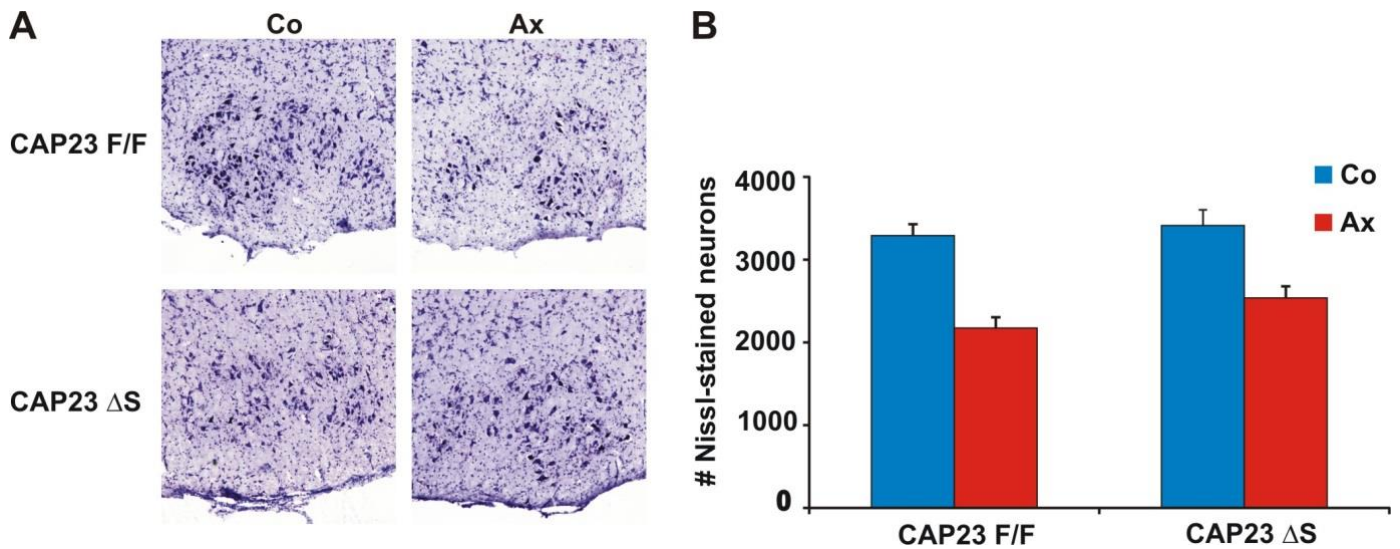
Figure 6.2 – Neuronal CAP23 is not involved in neuronal cell death or survival following peripheral nerve injury. **A.** Micrographs showing cresyl-violet staining of the uninjured (co) and injured (ax) facial nucleus of CAP23F/F and CAP23ΔS mice at day 30 post facial axotomy. **B.** Quantification of motoneuron number in the facial nucleus 30 days after axotomy did not show any difference in the neuronal numbers between the mutants and the controls on the contralateral (blue bars) or the axotomised (red bars) side. Bars and error bars represent mean and SEM respectively.

Neuronal CAP23 is involved in axonal sprouting

Transection of the facial nerve leads to axonal sprouting, which peaks at day 14 post axotomy. These neuronal sprouts express high levels of neuropeptides like galanin and CGRP which can be assessed by measuring the levels of their immunoreactivity in the injured facial nucleus. Quantification of the total galanin levels in the facial nucleus at day 4 and 14 by optical luminosity showed a trend towards reduced galanin levels in the CAP23ΔS mutants compared to the CAP23F/F animals, however the data did not achieve statistical significance (Figure 6.3S). On the other hand, the manual counting of sprouts revealed a dramatic reduction (-46%) in galanin positive sprouts in the CAP23ΔS mutants (44.2±8.8 sprouts on the axotomised side) compared to their littermate controls (82.3±12.7 sprouts on the axotomised side) (Figure 6.3T). These results suggest a mechanism of action for previously reported findings which propose a role of CAP23 in neuronal sprouting (Bomze et al., 2001;Frey et al., 2000).

CAP23 F/F (co) CAP23 F/F (ax) CAP23 ΔS (co) CAP23 ΔS (ax)

A B C D CD44 d4

E F G H CD44 d14

I J K L Galanin d4

M N O P Galanin d14

Q CD44 d4 (OLV)

R CD44 d14 (OLV)

S Galanin d14 (OLV)

T Galanin d14 (sprouts)

Co Ax
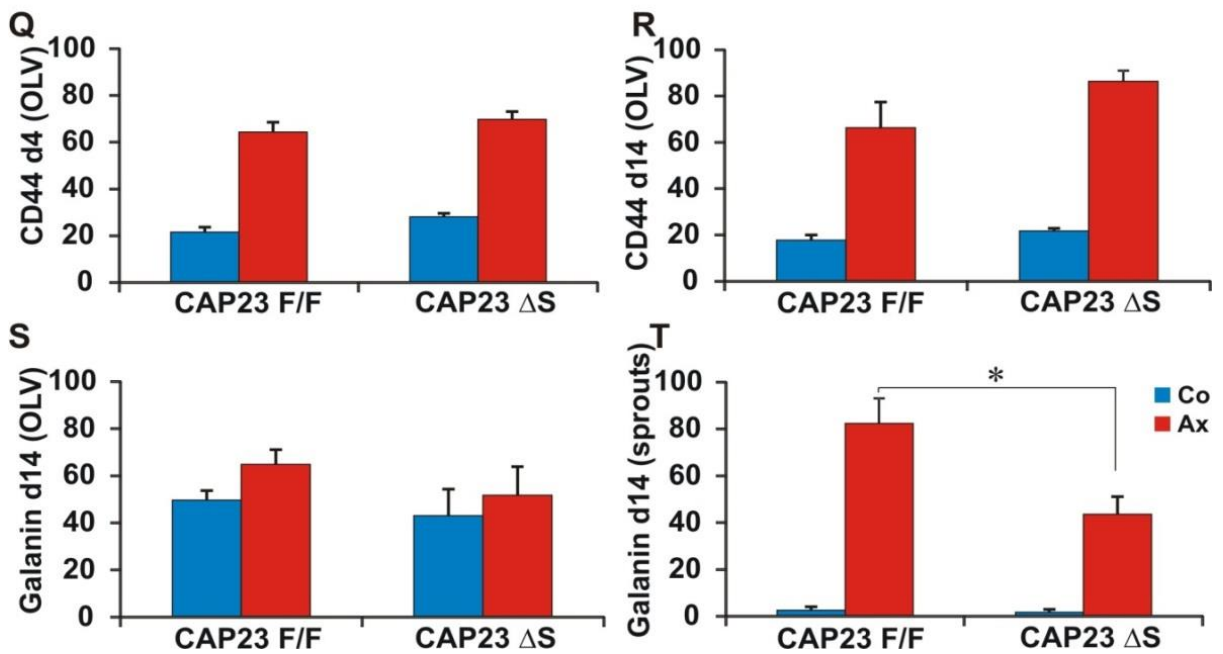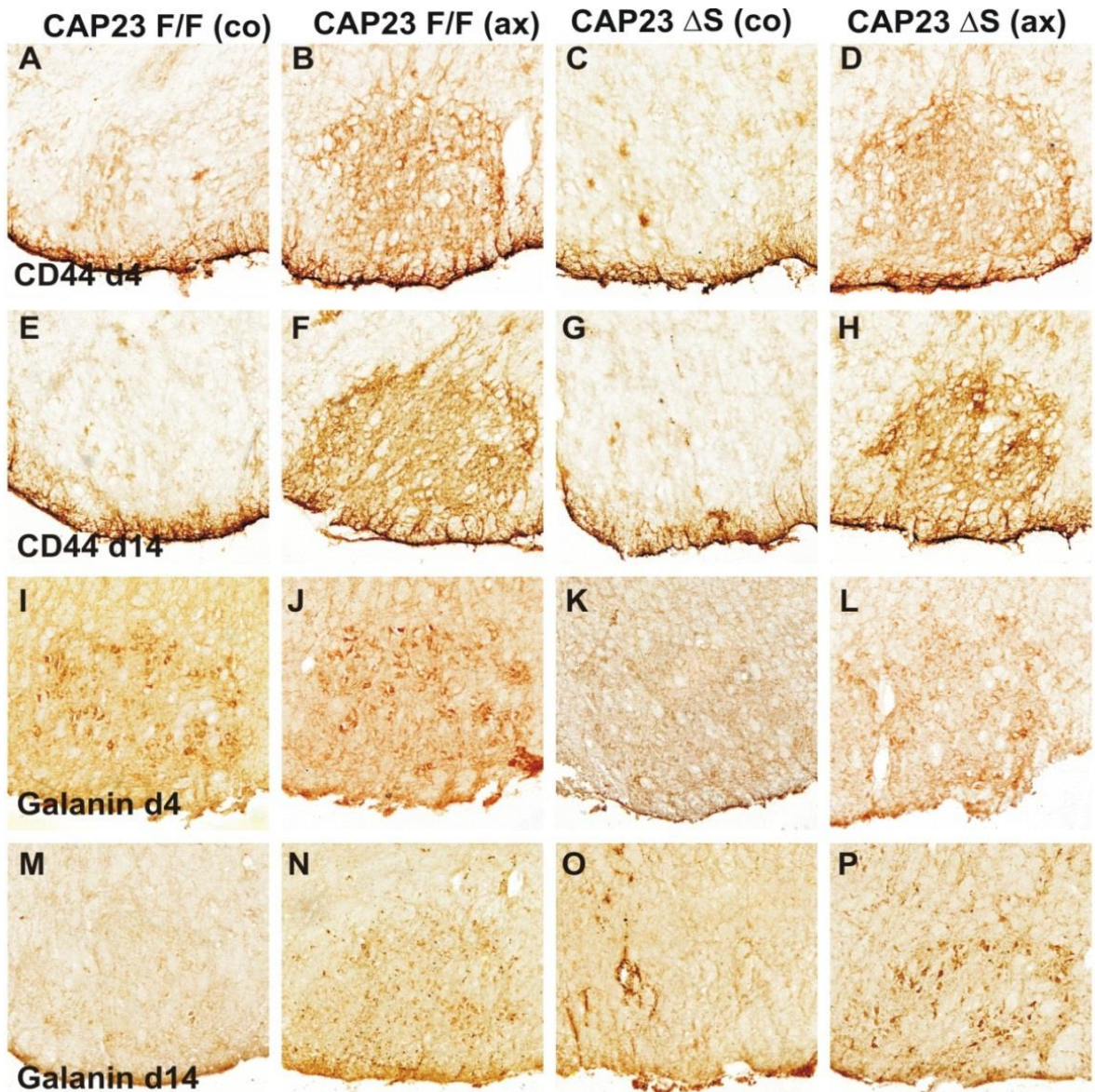
CAP23 F/F CAP23 ΔS

Figure 6.3 – Neuronal CAP23 is required for axonal sprouting but not for other aspects of neuronal injury response. **A - P.** Facial nucleus axotomy response 14 days after nerve cut in the CAP23F/F controls (A,B,E,F,I,J,M,N) and the CAP23ΔS mutant (C,D,G,H,K,L,O,P) mice on the contralateral side (co) (1st and 3rd column), and on the axotomised side (ax) (2nd and 4th column). After axotomy, CAP23F/F mice show a prominent increase in adhesion molecule CD44 (A-B, E-F) and neuropeptide Galanin (I-J, M-N). **Q - T**. Quantification of overall facial motor nucleus immunoreactivity by manual counts or OLV units at 4 (d4) and at 14 days (d 14) after nerve cut for CD44 and Galanin in CAP23F/F (n=5) and CAP23ΔS mice (n=5). The (co) and (ax) sides are indicated by blue and red bars respectively. While CD44 upregulation is not altered in the absence of neuronal CAP23 (C-D, G-H), the number of galanin positive sprouts is significantly reduced in the CAP23 mutants. No galanin-positive growth cones were observed at day 4 in the uninjured or axotomised nucleus of mutants or controls. *p<5%; bars and error bars show mean and SEM respectively.

CD44 is another axonal regeneration marker, up-regulated by the neuronal cell body following facial nerve transection (Jones et al., 1997;Jones et al., 2000). In order to see if this up-regulation was dependent on neuronal CAP23, CD44 immunoreactivity at days 4 and 14 after axotomy was compared between the CAP23ΔS mutants (n=5) and the CAP23F/F controls (n=5) (Figure 6.3. A-H). As demonstrated in Figure 6.3 Q, R, axotomy induced CD44 up-regulation was not affected by CAP23 deletion at either time-point.

Neuronal CAP23 deletion affects early but not late glial response

Nerve injury induces changes in the surrounding non-neuronal cells within the brain. Early microglial activation is characterised by increased immunoreactivity for thrombospondin (TSP), αMβ1 integrin, ICAM and IBA1 within the first 24 hours of injury. After the initial activation, microglia enter a proliferation phase, followed by a phagocytic phase with a peak at d14 post injury, which coincides with the peak of cell death (Raivich et al., 1999a). The level of microglial activation following facial axotomy at d4 and d14 in the CAP23 controls and mutants was assessed by measuring αM immunoreactivity via luminosity (Figure 6.4 A-H). At day 4, the CAP23ΔS animals showed a moderate but significant reduction (-35%; p<5%) in early microglial activation in the axotomised nucleus as compared to the CAP23F/F controls (Figure 6.4D,Q). By day 14, this defect was no longer seen in the mutants, suggesting a delayed onset of microglial activation in the mutants. No statistical significance was found between injury-induced microglial activation at day 14 between the two groups (Figure 6.7R).
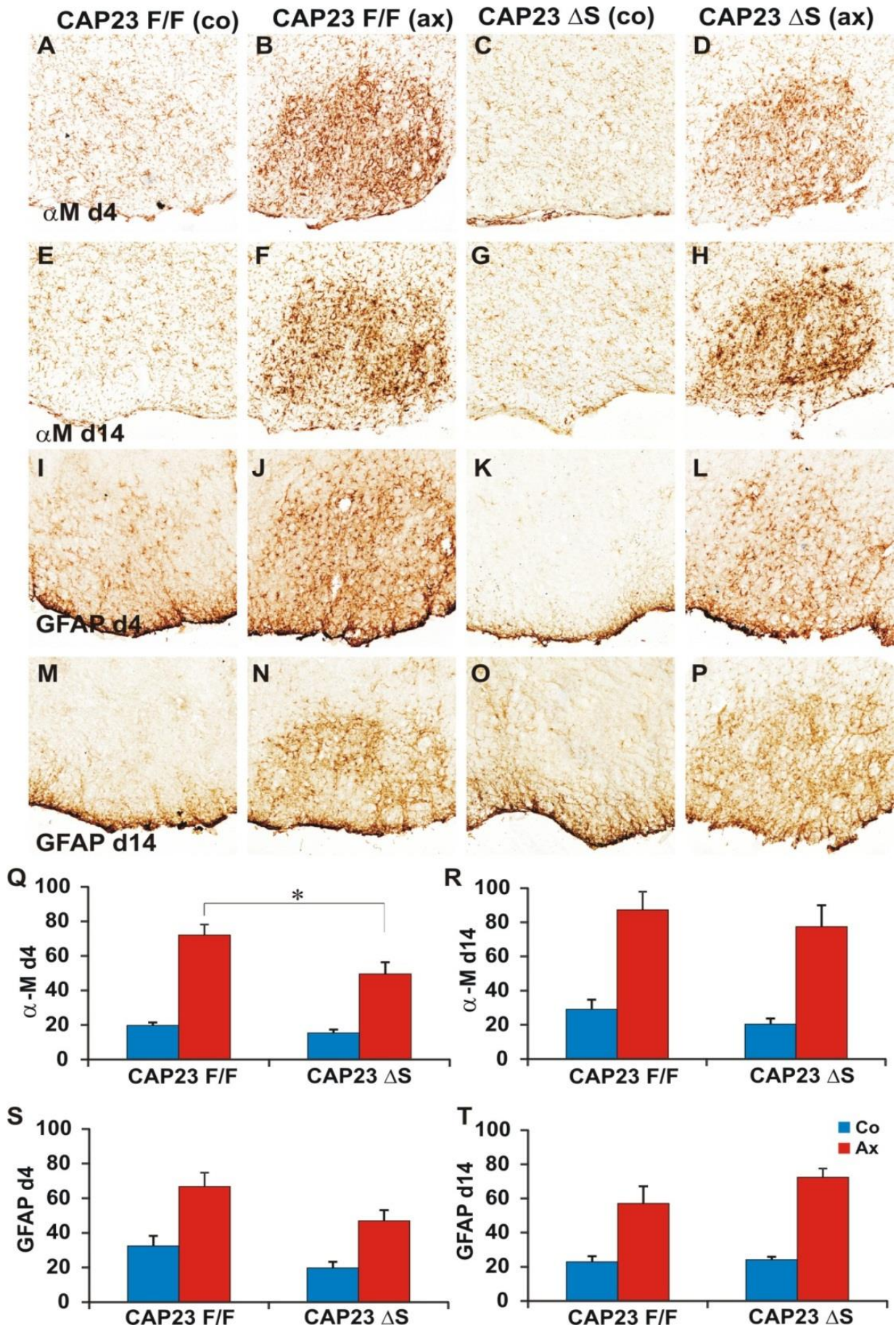
Figure 6.4 – CAP23 deletion affects early but not late glial response following FNA. **A-P.** Microglial (A-H) and astrocyte (I-P) activation after nerve cut in CAP23*F/F* control (A,B,E,F,I,J,M,N) and CAP23ΔS mutant (C,D,G,H,K,L,O,P) mice on the contralateral side (co) (1st & 3rd column), and on the axotomised side (ax) (2nd & 4th column). Glial activation was detected through immunoreactivity for αM and GFAP at days 4 and 14 after axotomy. After axotomy, CAP23F/F mice show a prominent increase in both markers at both time-points. In CAP23ΔS mutants, both astrocytes and microglia showed a decrease in the extent of their early activation but reached normal levels of activation by day 14. **Q - T.** Quantification of microglial and astrocyte activation at 4 (d4) and at 14 days (d 14) after nerve cut show a significant reduction on the (ax) side for microglial components at d4 (Q) but not at d14. Similarly GFAP immunoreactivity showed a trend towards reduced activation (p=8%) in the mutants as compared with controls, at day 4 after facial axotomy.

As with microglia, astrocyte response to injury proceeds through several stages. There is a rapid increase in synthesis of GFAP within 24 hours of axotomy and the appearance of GFAP positive processes at day 2-3 after axotomy (Raivich et al., 1999a). The level of astrocytic activation following facial axotomy at d4 and d14 was assessed by measuring levels of GFAP immunoreactivity via luminosity. As demonstrated in Figure 6.4. S, T, no significant difference was seen between the CAP23ΔS and the CAP23F/F animals at day 4 or 14, however on day 4 there was a trend for mutants to have reduced astrogliosis on the axotomised side (p=8%; Figure 6.4S).

Chapter 6 – CAP23 Discussion

Neuronal CAP23 is required for axonal re-innervation and sprouting

The CAP23ΔS mutants showed reduced re-innervation of the peripheral target by 34% at day 30 after facial axotomy compared to their littermate controls. Interestingly, this defect in axonal regeneration was not reflected by the pattern of whisker hair function recovery in the mutants, suggesting that with moderate reductions in re-innervation, functional recovery can be normal.

Facial axotomy leads to enhanced axonal sprouting, accompanied by an increase in immunoreactivity for neuropeptides like galanin and CGRP in the axotomised facial nucleus. These molecules are also present in growth cones and can enhance growth cone extension by overcoming the inhibitory cues in the extracellular environment. In CAP23ΔS animals, a significant reduction in galanin positive sprouts by nearly 50% was seen at day 14 post injury in the axotomised facial nucleus. No significant changes were

seen in overall luminosity measurement of galanin immunoreactivity in the facial nucleus, as this measurement included cellular galanin, as well as those localized to the growth cones. Cellular galanin peaks at day 4 post injury, but is still present at day 14 (Werner et al., 2000b). In 2000, Frey et al., showed that overexpression of CAP23 in adult neurons of transgenic mice promoted nerve sprouting and CAP23 knockout mice failed to produce stimulus induced nerve sprouting at adult neuromuscular junction (Frey et al., 2000). Experiments with pre- and post-synaptic toxins have shown that in the presence of CAP23, sprouting is stimulated by a mechanism that responds to reduced neurotransmitter release and may be independent of post-synaptic activation (Caroni, 1997). Terminal Schwann cells respond to the change in neurotransmitter release and mediate the growth promoting signal (Son and Thompson, 1995) which, in the presence of CAP23, results in axonal sprouting. At the structural level, CAP23, along with the other GMC proteins, is a major intrinsic determinant of anatomical plasticity in neurons, and appears to support axonal sprouting by remodelling the actin cytoskeleton for the formation of stable growth cones (Laux et al., 2000). In the absence of CAP23, actin structures do not accumulate at the leading edge and motile growth cones cannot form, causing loss of directed outgrowth and axonal sprouting, as demonstrated in the current results. These results point towards a role of CAP23 mediated axonal sprouting in re-innervation after peripheral nerve injury.

Neuronal CAP23 is involved in early microglial activation, but not in regulation of neuronal survival after axotomy

Activation of microglial cells and astrocytes show a graded response after injury. Microglial activation occurs in four stages - passing through initial alert and homing stages to later phagocytic and bystander activation phases. αM expression, therefore, has a biphasic profile, with an early peak during days 1-4 and a late peak at day 14, accompanied by a peak in cell death (Moller et al., 1996). Deletion of CAP23 affects microglial activation in a stage-specific way, causing a significant reduction (-45%) in early microglial activation at day 4, with only a mild (-12%) and statistically insignificant reduction at day 14, in the axotomised facial nucleus. The conundrum of activated microglia being potentially harmful or beneficial to injured neurons has been a long-standing debate (Streit, 1993;Polazzi and Monti, 2010). Their ability to enhance or inhibit neuronal survival and regeneration is the outcome of a delicate balance of their neuroprotective and neurotoxic effects. In the

current study, reduction in early microglial activation (at day 4) in the absence of neuronal CAP23 did not affect neuronal survival at day 30. This suggests a role for CAP23 in regulating early microglial activation in immune surveillance and inflammatory response, rather than in neuronal survival. To test this hypothesis, motoneuron survival was assessed in mutant and control groups 30 days after axotomy. Indeed, no difference was seen in the ratios of unaxotomised to axotomised motoneuron numbers.

Chapter 7 - β-1 integrin Results

β-1 integrin is required for neuronal survival following axotomy, but its effects on cellular injury response are moderate

Peripheral nerve injury is normally accompanied by a strong increase in regeneration-associated molecules that have been suggested to play a key role in successful nerve repair. Integrins are a large family of glycoprotein receptors for extracellular matrix proteins, and consist of different heterodimers composed of an α and a β subunit. Both α7 and β1 integrin subunits are upregulated after axonal injury and have been detected on regenerating axons and growth cones as part of the neuronal response to axonal injury (Kloss et al., 1999). Global deletion of the gene encoding α7 integrin results in problems in neurite outgrowth, and reduced speed of axonal regeneration and re-innervation of peripheral targets after axotomy (Werner et al., 2000). β1 integrin, the only counterpart of α7, plays a critical role for the NT3-mediated outgrowth of sensory neurons in-vitro, and is strongly increased in α7 null mice, probably as a compensatory mechanism (Werner et al., 2000). In neural c-Jun-deficient mice, α7 upregulation is dramatically reduced, and this reduction of α7 may be the one of the reasons for such poor regeneration in these animals (Raivich et al., 2004). Similarly, neuronal deletions of c-jun and STAT3 almost completely abolish injury-induced β1 expression, identifying β1 as an important transcriptional target and downstream effector molecule in regeneration (Ruff et al., 2012) (Chapter 3 – STAT3 results).

By using the standard facial axotomy model and neuron-specific β1 knockout mice, this chapter aims to characterise the role of neuronal β1 in regulating the speed and extent of axonal regeneration and sprouting, motor function recovery, neuronal survival, and the expression of regeneration, inflammation and astrocytic markers. Transgenic mice with neuron-specific deletion of β1-integrin were generated by crossing mice with floxed β1-integrin alleles with mice expressing synapsin promoter-controlled cre recombinase. Littermates without 'cre' served as controls. Altogether, these results suggest a moderate effect of neuronal β1-integrin deletion on the molecular and cellular response to injury, at levels comparable to that of α7 deletion; and a strong reduction in target re-innervation, probably due to its effect of greatly reducing neuronal cell survival.

β1 integrin is required for efficient axonal regeneration

After nerve transection, the injured neurons respond by axonal sprouting and elongation of growth cones towards the target muscle, ultimately resulting in recovery of nerve function. To investigate the efficiency of axonal elongation, the overall movement of whisker hair, a key target of facial innervation, was scored blindly, on a scale of 0 (no movement) to 3 (strong, normal movement). Twenty-eight days after the facial nerve was cut, both control and mutant mice showed strong normal movement on the uninjured side. On the injured side, β1F/F control animals showed an average motor score of 2.38 ± 0.15, but motor performance was slightly lower in β1ΔS mice (2.25 ± 0.10) (Figure 7.1A). In terms of regenerative index (RI), calculated as area under the curve for the 28 day recovery period, the β1F/F had an index of 1.23 ± 0.05, while the mutants had an approximately 10% lower index of 1.09 ± 0.07. However, this overall regenerative index was not significantly different between the groups (Figure 7.1B).

To determine differences in muscle re-innervation, the fluorescent tracer Fluorogold was injected into the whisker pad 28 days after the facial nerve was cut, followed by a 48 hour period of retrograde transport. Fluorogold application on the uninjured side served as intra-animal control, with similar numbers of retrogradely labelled motoneurons per section, 586 ± 30 and 543 ± 53 on the uninjured side in control and mutant mice, respectively. The overall ratio of labelled neurons on the axotomised versus the contralateral side (ax/co ratio) in control animals was $69.4\% \pm 3.7\%$. In the absence of β1 integrin, it was $28.4\% \pm 4.5\%$, nearly 2.5-fold lower (Figure 7.1D; $p < 0.01\%$) than their control litter-mates.

Speed of axonal elongation was determined by analysing early re-innervation after crush injury. Unilateral facial nerve crush was performed on 5 β1 F/F controls and 5 β1ΔS mutant mice, and eight days later FG was bilaterally applied to the whisker pads. The animals were allowed to survive for another two days, before being sacrificed. While both groups showed similar number of neurons on the uninjured side (170 ± 17 in controls and 147 ± 9 in mutants), there was a significant reduction in the number of neurons on the axotomised side for the mutants compared to the controls (33 ± 18 in controls and 0.4 ± 0.4 in mutants).
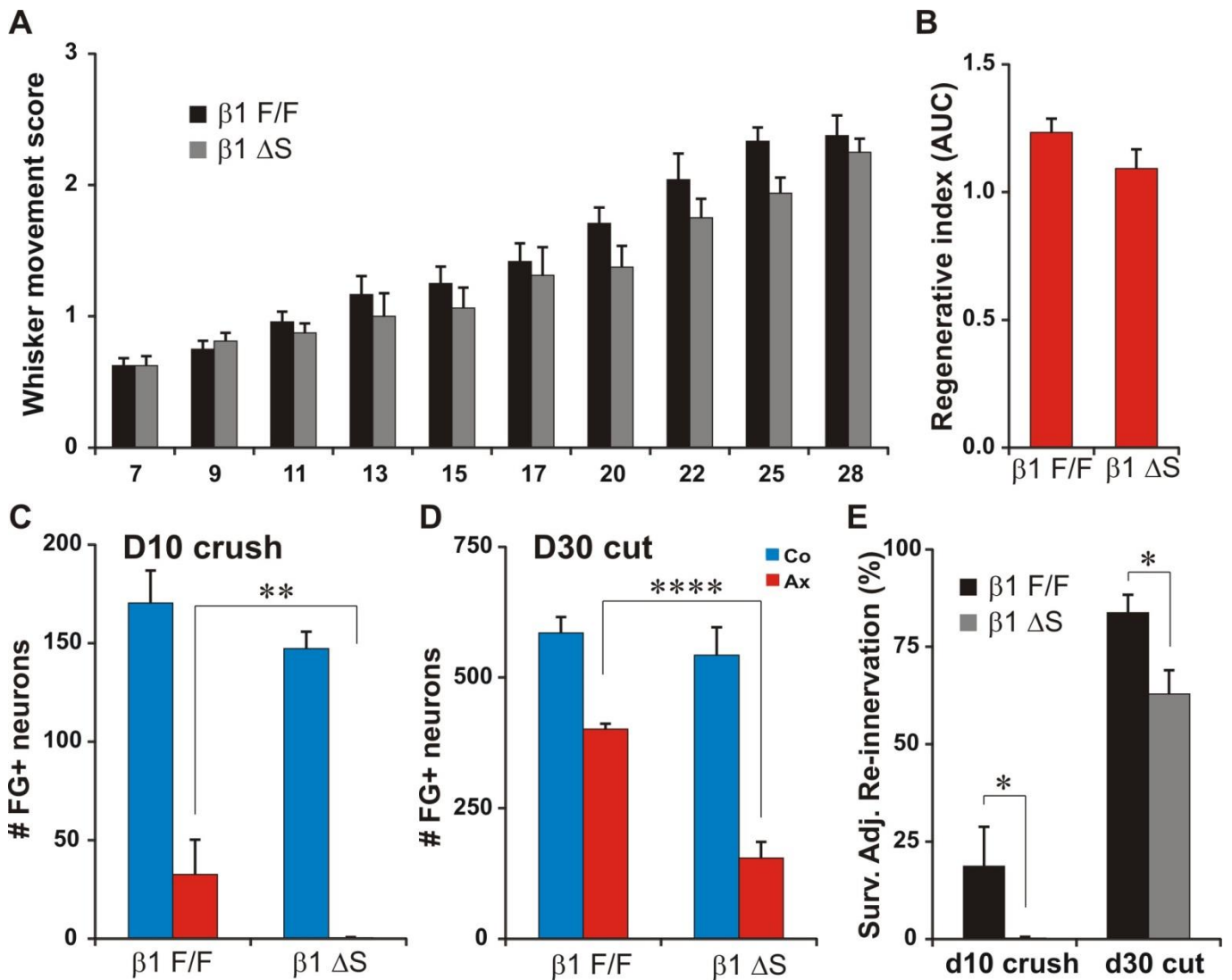
Figure 7.1. Beta-1 integrin and axonal regeneration. **A.** Time-course of functional recovery of whisker hair movement on a scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) shows a trend towards reduced functional recovery in β1ΔS mice (n=6) compared to β1 F/F mice (n=4) at 30 days post-injury. **B.** Regenerative index of functional recovery calculated as Area Under the Curve for the two groups. **C, D.** Re-innervation of the target whisker-pad muscle at day 10 after unilateral facial nerve crush and 30 days after facial nerve cut, measured by counting the number of motoneurons positively labelled by retrogradely transported fluorescent dye Fluorogold (FG). There was a significant reduction in re-innervation in the β1ΔS mice compared to the β1 F/F at both time-points and after both injury severities. **E.** Survival adjusted re-innervation (SAR) gives an index of the regenerative abilities of surviving axotomised neurons. There was a moderate reduction in SAR after d10 crush and after d30 cut in the β1ΔS mice. Data points and error bars in these graphs show the mean and SEM, respectively, *$p<5\%$, **$p<1\%$, ***$p<0.1\%$, ****$p<0.01\%$ in Repeated Test Anova (for whisker movement score) and in unpaired Student's T-test.

Comparison of the ax/co ratios showed 20% ± 10% re-innervation ratio in the controls, but almost no re-innervation in the mutants, with an ax/co value of 0.3%±0.3% ($p<1\%$ for log ax/co; Figure 7.1C).

Absence of β1 integrin increases axotomy-induced cell death

A substantial number of neurons die after nerve transection, probably due to failed target re-innervation and subsequent lack of trophic support (Sendtner et al., 1996). To assess whether neuronal β1 plays a role in axotomy-induced cell death, all motoneurons in the facial motor nucleus were counted using 20 μm Nissl-stained serial brain-stem sections.

Ten days after facial nerve crush, the control animals did not show much loss of neurons, with 1659 ± 63 on the operated and 1489 ± 42 neurons on the unoperated side. However, 13%±4% cell death was seen in the β1ΔS mice (1167 ± 67 motoneurons on the axotomised and 1339 ± 71 on the unoperated side (p < 0.1%)). This was a 22% higher cellular loss compared to the wild-type animals (Figure 7.2 B, D; p<1%).

One month after facial nerve transection, there was much higher neuronal cell death in both groups, compared to that seen with crush injury. But while there was a loss of 17±3% neurons in the injured facial motor nucleus of control mice (1481 ± 61 neurons on the operated side compared to 1786 ± 65 neurons on the unoperated side) (Figure 7.2 A, C, D), in β1ΔS mice, neuronal loss by axotomy was increased more than 3-fold to 56±4% (717 ± 73 motoneurons on the axotomised and 1609 ± 44 on the unoperated side, p < 0.01% (Figure 7.2.A, C, D).

To investigate the increased cell death in the mutants as a causal factor for their reduced re-innervation, Survival Adjusted Re-innervation (SAR) was calculated by dividing the re-innervation ratios by the survival ratios for each group. While the controls showed a SAR of 84%±5% at d30 after nerve transection, the mutants showed a moderate 25% lower SAR of 63%± 6% (Figure 7.1E; p<5%), compared to a drastic 60% reduction in re-innervation seen earlier (when not adjusted for increased cellular loss). This suggests that the involvement of β1 in regulating target re-innervation at d30 is mostly due to its effect of greatly reducing neuronal cell survival. However, at ten days after facial nerve crush, while the controls showed an SAR of 19%±10%, the mutants showed a 98% greater reduction in SAR with a value of only 0.3%± 0.3% (Figure 7.1E; p<5%). The much stronger effect seen at d10 compared to d30 after axotomy, suggests a transient effect of β1 deletion on regeneration. This is similar to the transitory effect seen with global deletion of the gene encoding α7 integrin subunit, where the mutants showed reduced re-innervation at day 9 after crush injury, but the re-innervation defect disappeared by day 21 (Werner et al., 2000a).
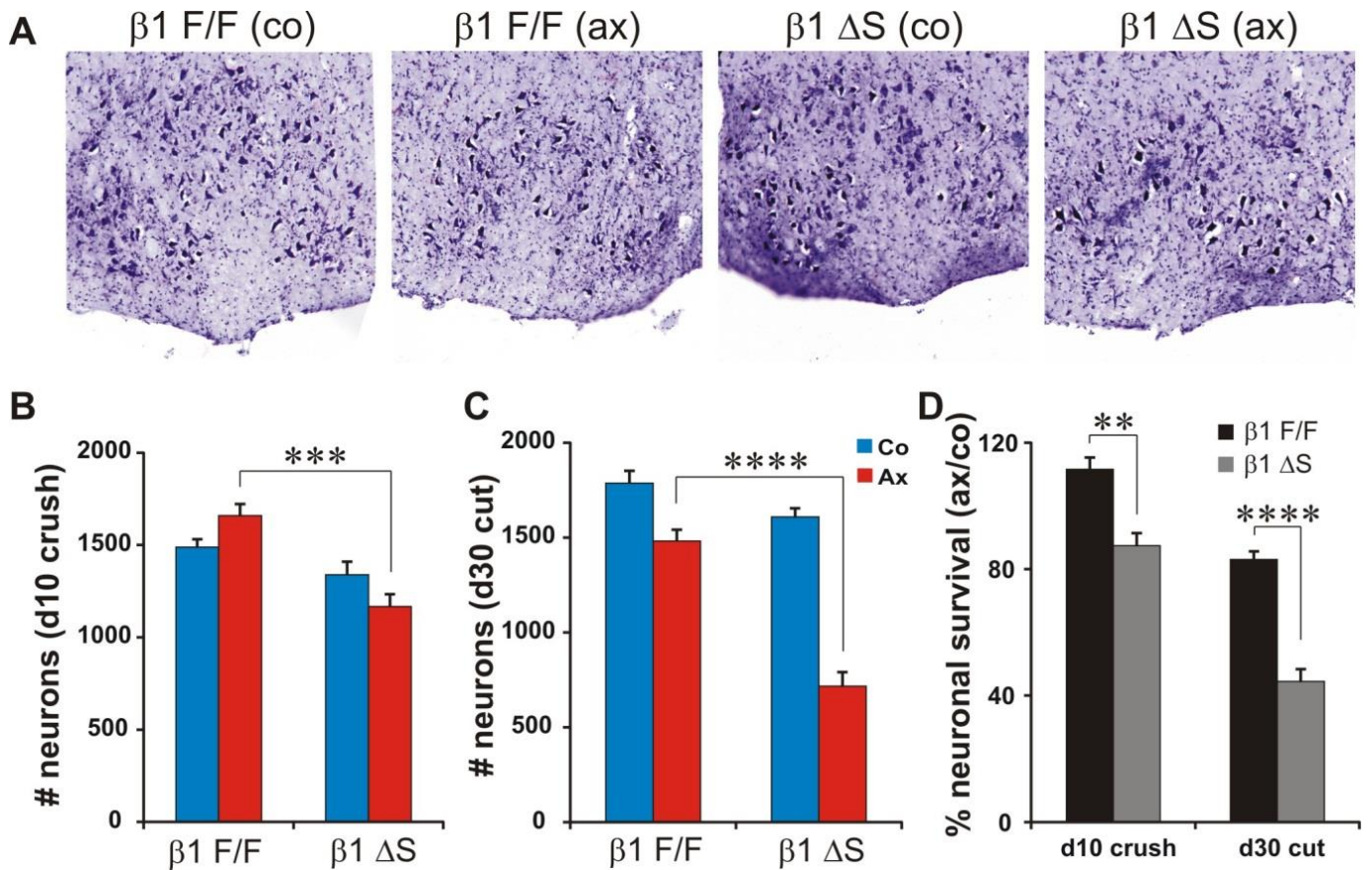
Figure 7.2. Neuronal β1 integrin is required for motoneuron survival after facial axotomy. **A.** Nissl-stained coronal sections of facial motoneurons on the contralateral (co) and axotomised (ax) side in β1 F/F and β1ΔS mice, 30 days after unilateral facial nerve cut. **B.** Total motoneuron count, corrected for neuronal size, showed a significant reduction in neuronal survival 10 days after facial nerve crush in the β1ΔS mice. **C.** Total motoneuron count, corrected for neuronal size, showed a significant reduction in neuronal survival 30 days after facial nerve cut in the β1ΔS mice. **D.** Motoneuron survival expressed as a percentage of the unaxotomised side show in similar trend of significantly reduced neuronal survival in β1ΔS mutant mice after milder crush injury and more severe cut injury. ** $p<1\%$, *** $p< 0.1\%$, **** $p<0.01\%$ in unpaired Student's T-Test, bars and error bars show the mean and SEM respectively.

Measurement of neuronal diameters showed normal chromatolytic morphology of axotomised neurons in both groups, after both cut and crush injuries (data not shown here). Overall, these data highlight a role for β1 integrin in the survival of facial motoneurons following nerve axotomy.

Effect of β1-integrin deletion on neuronal axotomy response

In order to understand the molecular mechanism of β1 function during the chromatolytic response, the expression of regeneration associated molecules was explored in β1ΔS mice. Since previous studies have pointed to an important role of the neuropeptides galanin and CGRP, the neuronal adhesion molecules α7 integrin, and the CD44 hyaluronic acid receptor in neurite elongation (Jones et al., 1997;Jones et al., 2000;Werner et al., 2000b;Wynick, 2001;Lin and Chan, 2003), we determined if their expression was affected in the absence of neuronal β1 integrin at 14 days after axotomy. As demonstrated in Figure 7.3, all four molecules show a strong increase in protein expression on the axotomised sides of both β1F/F control animals and β1ΔS mutant animals. Quantification of the staining intensity was done by manual counting and/or by measuring their optical luminosity values (OLV) using Optimas software. Quantification of CD44 immunoreactivity by luminosity revealed a 20% higher increase in axotomy-induced expression in the mutants compared to the controls (Figure 7.3S; $p < 5\%$). There was no difference in the optical luminosity values for injury-induced expression of α7- integrin, CGRP, and galanin between groups (Figure 7.3 V,Q,T). Besides being present in the neuronal somas, galanin and CGRP are also present at the growing tips of axonal sprouts, and can be quantified manually by counting under a light microscope. While there was no difference between the number of CGRP-positive sprouts in the axotomised facial nuclei of the controls and mutants, the number of galanin-positive sprouts were reduced by nearly 35% in the β1ΔS mutant mice (from 313 ± 53 β1F/F ax side, to 201 ± 21 β1ΔS ax side). However this difference marginally shied away from being statistically significant (Figure 7.3U). Quantification of injury-induced expression of CD44, CGRP and galanin at 4 days after crush, did not show any difference between the two groups (data not shown).

β1 F/F (co)   β1 F/F (ax)   β1 ΔS (co)   β1 ΔS (ax)

A   B   C   D

CGRP

E   F   G   H

Galanin

I   J   K   L

CD44

M   N   O   P

α7

Q   R   S

T   U   V
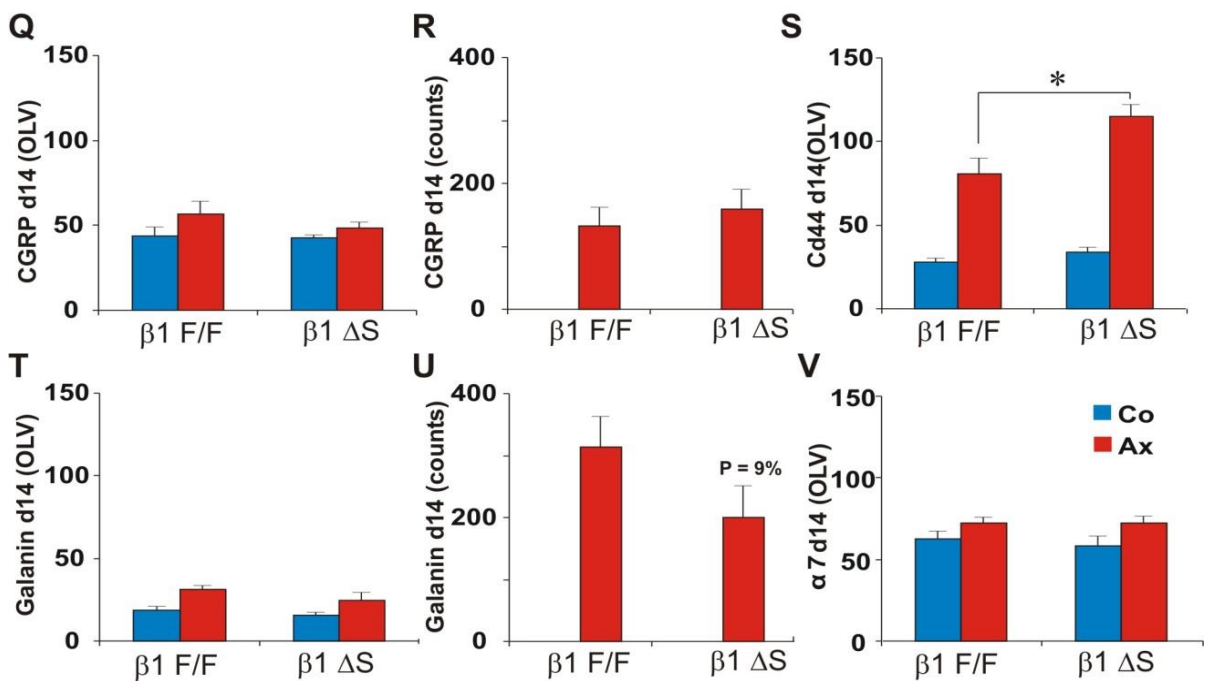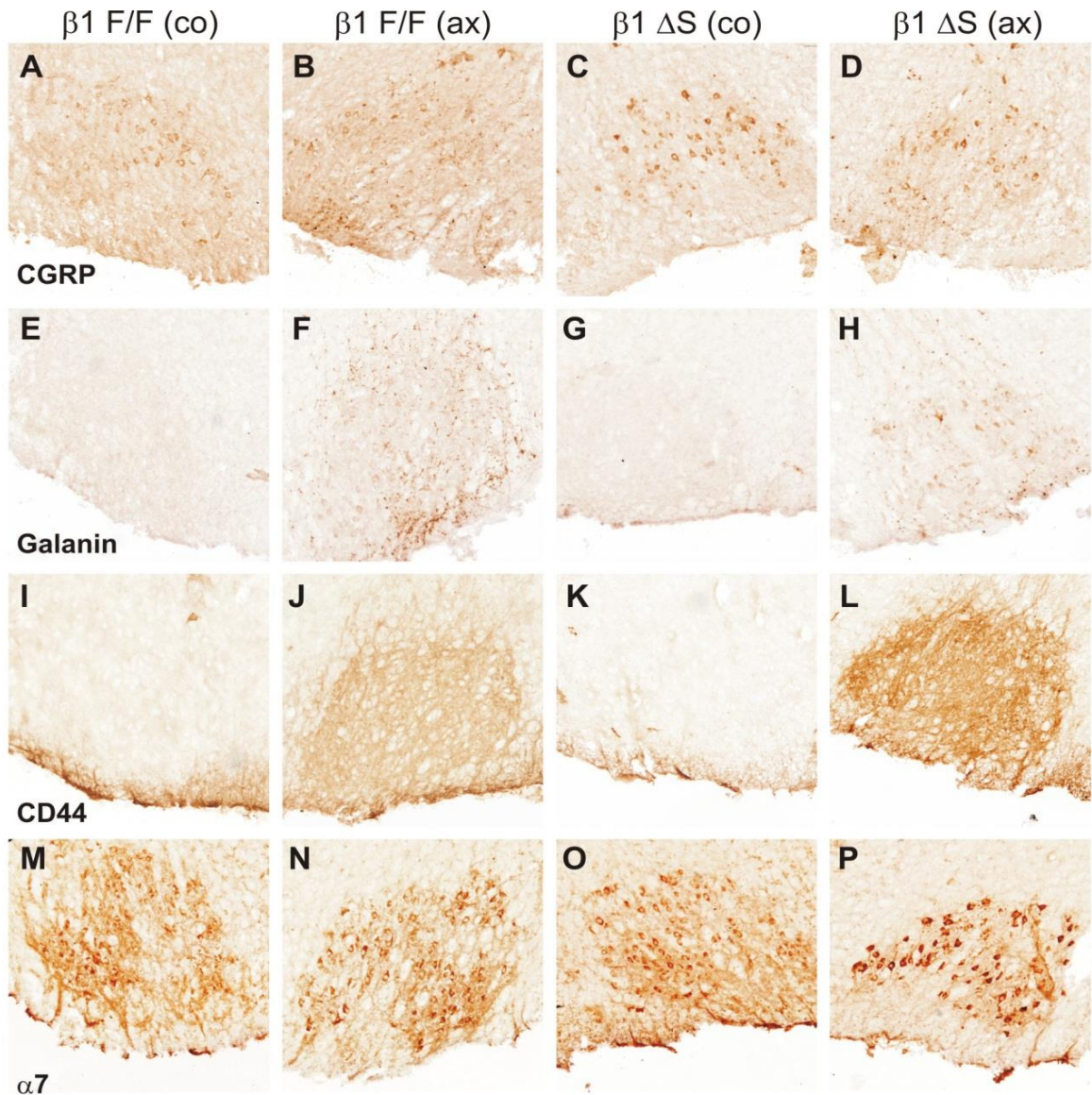
Figure 7.3. Beta-1 integrin and neuronal axotomy response. Facial nucleus axotomy response 14 days after nerve cut in the β1F/F controls (A,B,E,F,I,J,M,N) and the β1ΔS mutant (C,D,G,H,K,L,O,P) mice on the contralateral side (co) (1st and 3rd column), and on the axotomised side (ax) (2nd and 4th column). After axotomy, β1 F/F mice (n=5) show a prominent increase in neuropeptides CGRP (A-B) and Galanin (E-F) and adhesion molecules CD44 (I-J) and α7-integrin subunit (M-N); most of these changes are reproduced in the β1ΔS mutants (n=5), except for a significantly higher expression of CD44 (L, S). Quantification of overall facial motor nucleus immunoreactivity by manual counts or OLV units (Q-V) showed a 20% higher increase in CD44 (S), and a trend towards reduced galanin sprouting (U) in the mutants. The contralateral (co) and axotomised (ax) sides are indicated by blue and red bars respectively. *p<5% in Student's unpaired T-test. Bars and error bars show mean and SEM respectively.

Effect of β1-integrin deletion on non-neuronal axotomy response

Nerve transection not only triggers changes in the neuron itself, but also results in reactive changes in non-neuronal, brain-resident cells of neuro-epithelial (astrocytes) and hematopoietic (microglia and T cells) origin (Raivich et al., 1999a;Raivich et al., 1999b;Schwaiger et al., 1998). Immunohistochemistry was carried out on day 4 and 14 tissue using antibodies that stain for T-cells (CD3), activated microglia (αM), phagocytic microglia (αX), and astrocytes (GFAP) (Raivich and Kreutzberg, 1994).

Early and late microglial activation, as measured by the increase in immunoreactivity for the αM integrin subunit, at days 4 and 14 after axotomy was completely unaffected by the absence of neuronal β1 integrin as seen in β1ΔS mice. The numbers of infiltrating lymphocytes and phagocytic microglia were counted under a light microscope. There was no difference in the number of CD3+ T-cells in the axotomised facial nucleus of β1F/F and β1ΔS mice (Figure 7.4 I-L, S). Interestingly, the number of αX+ phagocytic cells was increased by 3-fold in the β1ΔS mutant animals (from 5.2 ± 0.7 in controls to 15.6 ± 3.4 in mutants (Figure 7.4. E-H, R; p<5%), a response corroborating the increased cell death seen in the mutants after nerve cut and crush injuries. Astroglial activation measured by GFAP immunoreactivity also showed a nearly 40% higher axotomy-induced increase in β1ΔS mice, compared to their control counterpart (Figure 7.4 M-P, T; p<5%).
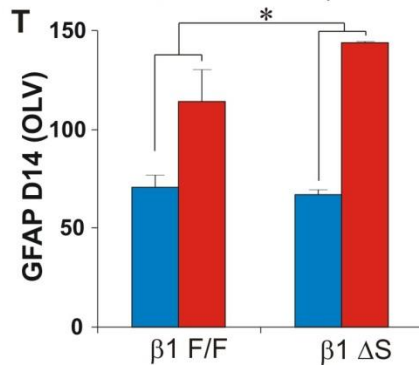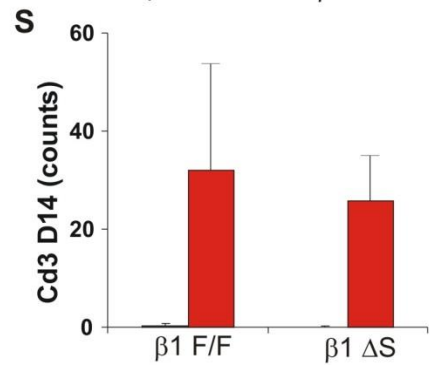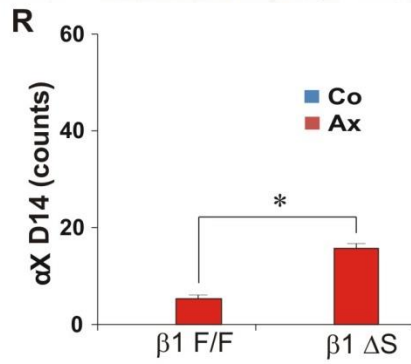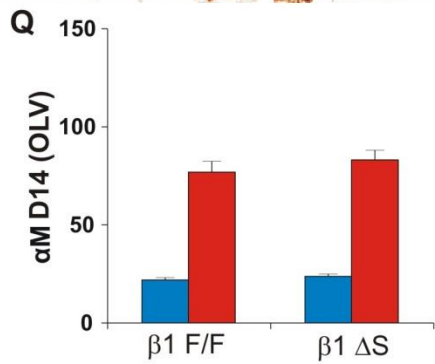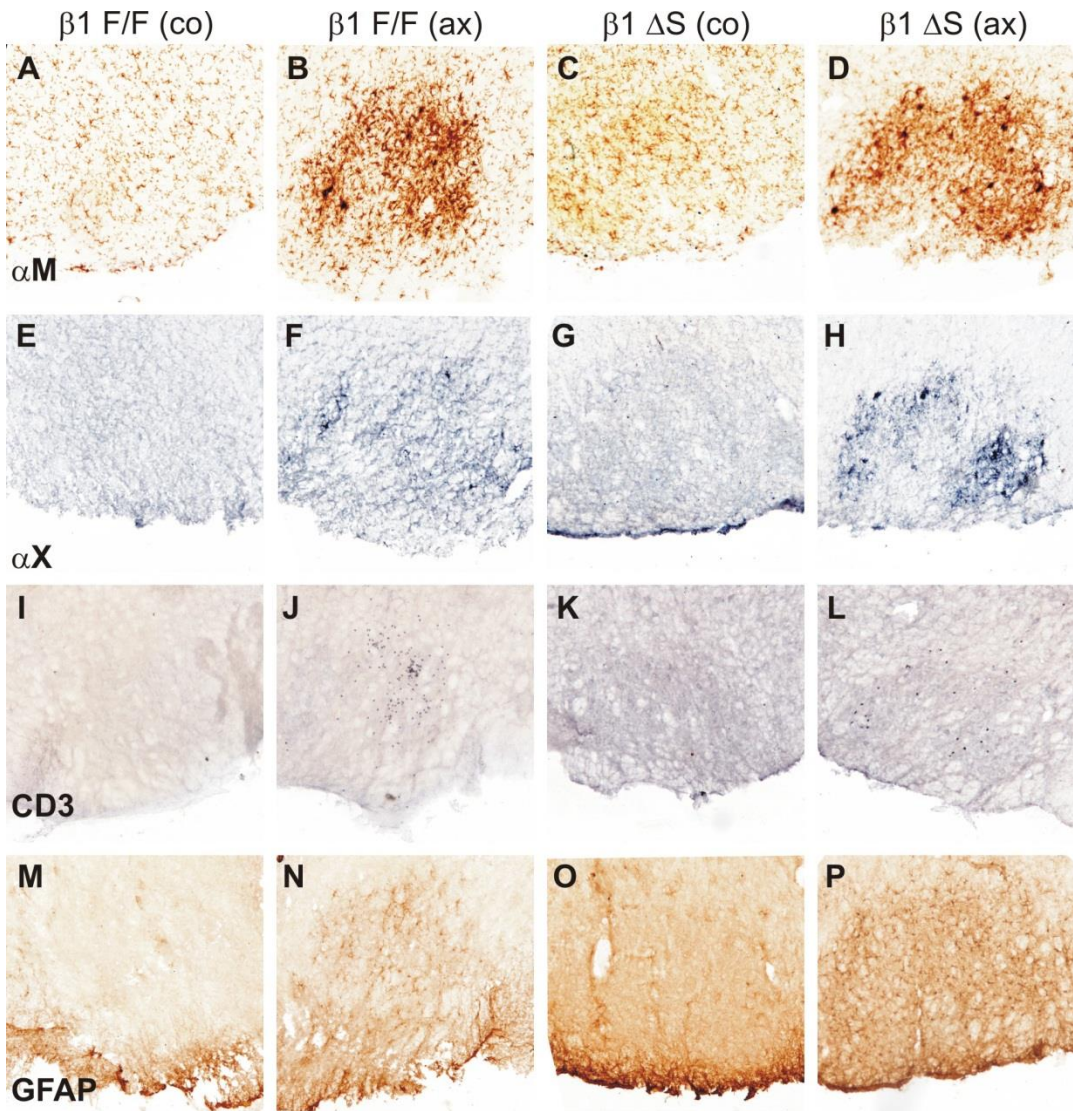
Figure 7.4. Beta-1 integrin and non-neuronal axotomy response. Facial nucleus axotomy response 14 days after nerve cut in the β1F/F controls (A,B,E,F,I,J,M,N) and the β1ΔS mutant (C,D,G,H,K,L,O,P) mice on the contralateral side (co) (1st and 3rd column), and on the axotomised side (ax) (2nd and 4th column). After axotomy, β1 F/F mice (n=5) show a prominent increase in microglial activation (A-B; E-F), T-cell recruitment (I-J) and astrocytic activation (M-N); these changes are reproduced in the β1ΔS mutants (n=5), with significantly higher expression of αX (G, H, R) and GFAP (O, P, T). Quantification of overall facial motor nucleus immunoreactivity by manual counts or OLV units (Q-T) showed a 66% higher increase in αX (R), and a 40% higher expression of GFAP (T) in the mutants. The contralateral (co) and axotomised (ax) sides are indicated by blue and red bars respectively. Bars and error bars show mean and SEM respectively, *p<5% in unpaired Student's T-test.

Chapter 7 - β1 integrin Discussion

Adhesion molecule β1 integrin is upregulated on regenerating axons and growth cones following axonal injury (Kloss et al., 1999). Deletion of neuronal c-Jun or neuronal STAT3 interferes with the upregulation β1 integrin subunit (Ruff et al., 2012) (Chapter 3 – STAT3 results), which possibly contributes to the very poor regenerative response seen in these animals. This implicates β1 as an important transcriptional target and downstream effector molecule in regeneration, warranting further investigation of its in vivo function in peripheral nerve regeneration. By using the standard facial axotomy model and neuron-specific β1 knockout mice, we have shown an important role for this integrin in regulating axonal re-innervation and neuronal survival after facial nerve cut and crush injuries.

Neuronal β1 integrin deletion, like α7 deletion, has a transient effect on axonal regeneration

After nerve transection, the injured neurons respond by axonal sprouting and elongation of growth cones towards the target muscle, ultimately resulting in recovery of nerve function. Both α7 and β1 integrin subunits are present in growth cones at the tip of the elongating axons within a regenerating nerve and participate in maintaining cellular contact between neurons and SCs. Previous studies have shown that following FNA, transgenic deletion of the α7 subunit leads to a reduced rate of axonal outgrowth and delayed re-innervation of the whisker-pad, a peripheral target of the facial nerve. This delay in regeneration is transient with the α7 null mutants catching up with the controls by day 21 after axotomy (Werner et al., 2000). Similar to α7 deletion, in the absence of β1 integrin, survival adjusted re-innervation was reduced by 98% at day 10, but by day 30 the reduction was only 25% compared to control mice that expressed normal functional β1 integrin in their

neurons. This suggests the presence of other axonal molecules which promote regeneration, leading to a compensation of regenerative function in the absence of α7β1 integrin, albeit with a slight delay. On the other hand, motor performance, as assessed by post-traumatic whisker movement, was only slightly lower in β1ΔS mice and the overall regenerative index was not significantly different between the groups. Collateral sprouting could be playing a role (Raivich and Makwana, 2007) as many branches re-innervating the whisker pad could be originating from one neuron. This non-linear correlation between re-innervation and functional recovery has been previously observed, with only 20-30% of nerve re-innervation accounting for substantial functional recovery (Raivich et al., 2004).

Facial axotomy is also followed by increase in the number of CGRP/galanin + central sprouts. The β1ΔS mutants showed a trend towards reduced galanin-positive sprouts within the facial nucleus by nearly 35%. In contrast, the α7 null animals showed enhanced sprouting after axotomy (Makwana et al., 2010). However, this could be due to the particularly strong, apparently compensatory, increase of the β1 subunit after axotomy in the α7 deficient mice (Werner et al., 2000a). These results highlight an important role of β1 integrin, possibly in combination with other α subunits, in regulating axotomy induced sprouting.

Finally, the absence of the α7 or β1 integrin subunits did not have a major impact on the cellular response in the axotomised facial nucleus. The moderate effects of increased microglial and astrocytic activation in the β1 null mutants could be secondary to the increased neuronal cell death seen in these mutants. The absence of α7 did not appear to affect the response to injury by glia or lymphocytes, suggesting that the immune surveillance of the injured CNS is unaffected by the absence of the α7 subunit (Werner et al., 2000). The effect of α7 deletion on neuronal survival and activation of phagocytic microglial nodules is as yet unknown and further investigation will provide more clues. Overall, the lack of major changes in the central neuroglial response to injury suggests a peripheral site of action for α7β1 adhesion molecule in interaction with the extracellular matrix in the injured peripheral nerve, to drive neuronal survival and prompt axonal regeneration after injury.

β1 integrin is required for neuronal survival after FNA

In addition to cell adhesion migration and axonal outgrowth, integrin signalling is important for neuronal survival (Lemons and Condic, 2008;Previtali et al., 2001;Tucker and Mearow,

2008). A substantial number of neurons die after nerve transection (Sendtner et al., 1996). Deletion of the β1 integrin in motoneurons strongly reduced neuronal survival after facial axotomy. The effect was quite pronounced, reducing survival by half from 80% in the controls to just about 40% in the mutant mice at d30 after facial nerve cut. Even after milder crush injury, where the two ends of the nerve stump are not completely disconnected, the mutants still had a 22% higher neuronal loss compared to the controls. Motoneuron numbers in the mutant and control mice without injury were similar, so lack of β1 does not seem to impede developmental survival, the effect is just post-traumatic.

In the current study, it is unclear whether the neurons are dying from lack of retrograde trophic support due to an inability to reconnect with the appropriate targets in the absence of β1 guidance-signalling, or whether the lack of β1 is somehow directly triggering cell death, and hence there is less re-innervation. Deprivation of trophic factors can lead to failure of re-innervation, and eventually cell death (Sendtner et al., 1996). Laminins are present in SCs as well as in the basal membranes, and may provide trophic support to the regenerating axons (Ide, 1996). Laminin-integrin interactions stimulate extension and directional guidance of neurites and growth cones (Woolley et al., 1990;Wallquist et al., 2002). The α7β1 integrin is a specific receptor for laminin-1 (α1β1γ1), laminin-2 (α2β1γ1), and laminin-4 (α2β2γ1) (Previtali et al., 2001). Increased cell death in the absence of neuronal β1-integrin could be due to reduced laminin-based trophic factor signalling.

To investigate the increased cell death in the mutants as a causal factor for their reduced re-innervation, survival adjusted re-innervation was analysed for the two groups. This is important to understand if the reduced re-innervation in the mutants is due to a reduced intrinsic regenerative ability of the β1 deficient neurons or if it is simply due to increased motoneuron death, while the surviving neurons continue to regenerate with the same efficiency as the wild-type neurons. The mutants showed a moderate 25% lower SAR than the controls, compared to a drastic 60% reduction in re-innervation seen earlier (when not adjusted for increased cellular loss), suggesting that the majority of β1 effects on re-innervation is due to its regulation of neuronal cell survival. Overall, this data demonstrates an important role for β1 integrin in survival of facial motoneurons following nerve axotomy. What remains to be seen is which of the various α subunits, that form hetero-dimeric partners with β1 subunit, might be involved in regulating neuronal cell death.

Neuronal β1-integrin deletion increases CD44 expression after FNA

Nerve transection causes pronounced changes distal to the site of axotomy, but also in and around the cell body of the affected neurons. Somas of injured motoneurons increase protein synthesis of adhesion molecules and neuropeptides (Makwana and Raivich, 2005). In order to understand the molecular mechanism of β1 function during the neuronal chromatolytic response, the expression of regeneration associated molecules CD44, CGRP, galanin and α7 integrin was explored in β1ΔS mice. Axotomy-induced expression of CD44 was increased by 20% in the mutants compared to the controls. CD44 expression is strongly upregulated in a wide variety of neural injuries (Jones et al., 2000). Like β1 integrin, it is an adhesion molecule, and is suggested to be supportive of neurite outgrowth. Antibody mediated inhibition of CD44 reduces neurite outgrowth of transplanted central noradrenergic neurons (Nagy et al., 1998) as well as creating multiple errors in retinal axonal growth trajectory through the optic chiasm (Lin and Chan, 2003). Deletion of neuronal c-Jun or neuronal STAT3 interferes with the upregulation of CD44 and β1 integrin (Ruff et al., 2012), (Chapter 3 -Results). Therefore the increase in CD44 expression could be a transcriptionally mediated compensatory mechanism that accounts for the absence of β1 in the mutants. Alternatively, it could be an auxillary effect of the increased cell death in the β1ΔS mutants, since early expression of this molecule at 4 days after nerve crush did not show any difference between the two groups.

The number of centrally sprouting axons expressing the neuropeptide CGRP was unaffected, while galanin-positive central sprouts showed a trend towards a moderate reduction. Surprisingly, no change was seen in the expression of α7 integrin subunit either. β1 is the only dimeric partner for α7 subunit. Global deletion of the α7 subunit caused a 40% decrease in the speed of regeneration of the facial nerve in vivo, and a compensatory 70% increase in β1-subunit expression. Since β1 can dimerise with several different α subunits, many of which have been implicated in axonal regeneration in vitro (Werner et al., 2000b;Vogelezang et al., 2001a;Toyota et al., 1990), it can be supposed that the regeneration defect in the β1ΔS animals might be due to other closely related α subunits.

Deletion of neuronal β1-integrin causes increased astrocytic and phagocytic microglia activation

Nerve transection not only triggers changes in the neuron itself, but also results in reactive changes in non-neuronal cells such as astrocytes, microglia and T cells. Activated microglial cells proliferate and upregulate activation markers such as αMβ2 integrin (Raivich and Kreutzberg, 1994).

Early and late microglial activation, as well as T-cell recruitment was completely unaffected by the absence of neuronal β1 integrin. Neuronal cell death, leads to a further transformation of microglia into phagocytotic cells, which usually form microglial clusters that remove neural debris (Raivich et al., 1999a). The number of αX+ phagocytic cells was increased by 3-fold in the mutant animals, a response corroborating the 3-fold increase in cell death seen in the mutants after nerve cut and crush injuries. Phagocytic microglia show strong immunoreactivity for aM-, aX-, a5-, a6-, b1-, and b2- subunits at 14 days following axotomy and could reflect processes which facilitate endocytosis and degradation of neuronal debris (Raivich et al., 1999a).

Following nerve injury, reactive astrocytes are also activated, upregulating cytoskeletal proteins like GFAP and converting to a fibrillary phenotype (Raivich et al., 1999a;Graeber and Kreutzberg, 1986). Astroglial activation measured by GFAP immunoreactivity also showed a nearly 40% higher axotomy-induced increase in β1ΔS mice, compared to their control counterparts. The main function of reactive fibrillary astrocytes is to create a physical barrier between damaged and healthy cells, by transiently enveloping injured neuronal somas with thin and flat cytoplasmic processes. Severe damage or inflammation can lead to much stronger astrocytic proliferation, eventually forming glial scars which are inhibitory to neurite outgrowth (Raivich et al., 1998). In the current study, the strong increase in astrocyte response might be responsible for reduced regeneration due to scarring. It could also be an ancillary effect of the increased neuronal cell death seen in the β1ΔS mutants.

In summary, the current study suggests that the β1 integrin subunit is an important mediator of axonal regeneration, particularly by reducing excessive injury-induced cell death. Moreover, the lack of major changes in the central neuroglial response to injury indicates a peripheral site of action for this cell adhesion molecule in the interaction with the extracellular matrix in the injured peripheral nerve.

Chapter 8 – Summary of Results & Final Discussion

Unlike the central nervous system, axon regeneration after peripheral nerve injury is usually robust. There are two major determinants of successful nerve regeneration in the PNS - intrinsic growth ability and an extrinsic permissive environment (Raivich and Makwana, 2007). The 'intrinsic' growth ability is driven by transcriptional activation of a regeneration programme, whereby transcription factors convert injury-induced stress signals into increased expression of a wide range of downstream effector molecules like neuropeptides, neurotrophic factors, adhesion molecules and cytoskeletal adaptor proteins. These downstream effector proteins regulate the actin-tubulin machinery of growth-cones, integrate intracellular signalling with stimulatory and inhibitory signals from the local environment, and translate them into axon elongation. The importance of the intrinsic growth state of injured neurons is perfectly exemplified by the preconditioning lesion example, whereby prior lesion to the peripheral branch of sensory DRG neurons, results in increased growth of their central axons after dorsal column injury (Neumann and Woolf, 1999). The 'extrinsic' factors depend on the existence of a local permissive environment, which consists of the molecular network organized by Schwann cells and macrophages at the injury site, and by astrocytes and microglia around the cell bodies (Raivich, 2005). This environment also produces neurotrophic factors, cytokines, neuropeptides, cell adhesion molecules and numerous ECM molecules which act on the neuronal cell bodies and growing axons to support their extension towards the final destination. Even though all these different molecules appear to be tangibly involved, their direct injury-associated transcriptional regulation is still unclear – for example, little is known about the factors involved in down-regulating the sensitivity to inhibitory and chemorepulsive stimuli in peripheral regeneration even though there is evidence for the presence of such regeneration blockers. Nevertheless, recent advances using cell-type specific deletion of transcription factors and their downstream targets is allowing more and more pieces of the puzzle to be placed together, making visible broad lines of identified chain of activities contributing to recovery, and underscoring the role of transcription factors as master-switches of the regeneration programme. However, our knowledge of regeneration associated transcription factors and their targets is still limited. To gain insight into the *in vivo* function of some of these regeneration associated molecules, we examined the effects of global or cell-type specific deletions of transcription factors -

STAT3, c-jun and C/EBPδ, and effector molecules - CAP23 and integrin β1, on axonal regeneration after facial nerve axotomy. The facial nerve axotomy model is a well-established experimental paradigm, providing insights into molecular signals that determine axonal regeneration, target re-innervation and neuronal cell death.

Conditional mice mutants, lacking STAT3 in their neurons, had profound defects in the usual retrograde response, with a severe early and persistent reduction by 75-90% in microglial activation and recruitment of lymphocytes, and 4 days after crush injury in the speed of axonal elongation in the distal stump. At 30 days, axotomised STAT3-deficient motoneurons appeared shrunk by 50-60% in size but displayed no cell death in contrast to results from earlier studies (Schweizer et al., 2002). Functional recovery was dramatically reduced and the mutants showed 80% less target muscle re-innervation. Interestingly, functional recovery tends to catch up after prolonged periods of regeneration, possibly due to rerouting and collateral sprouting, while anatomic re-innervation is still brought down – in the case of STAT3 deletion, the re-innervation of the whisker hair pad, a major target of facial nerve regeneration, 3 months after nerve cut still only received approximately a third of the normal number of axons. Interestingly, the effects of STAT3 deletion are milder in the dorsal root ganglia (Bareyre et al., 2011;Ben-Yaakov et al., 2012) suggesting the presence of complementary or alternative pathways, in the peripheral sensory neurons. As with neuronal c-Jun deletion which interferes with regeneration (Raivich et al., 2004), expression of RAGs like CD44, α7β1-integrin, perineuronal sprouting of the CGRP+ and galanin+ facial motoneurons, as well as the nuclear translocation of ATF3 was also greatly diminished or abolished in the STAT3 mutants. However, c-Jun expression itself was not affected, suggesting that deletion of either transcription factor – c-Jun or STAT3 – will produce a very similar regeneration-deficient phenotype. Mutants lacking both neuronal c-jun and STAT3 did not show more exacerbated regeneration defects than the single deletions, further verifying the above observation, and demonstrating that the combination of the two factors is not complementary in peripheral nerve regeneration.

Several studies have shown a key role for the transcription factor c-Jun in allowing regeneration to occur (Arthur-Farraj et al., 2012;Fontana et al., 2012;Raivich et al., 2004;Ruff et al., 2012b). In the facial nerve axotomy model, deletion of c-Jun throughout brain neuroepithelium-derived cells (Raivich et al., 2004) or just in neurons (Ruff et al., 2012b) abolishes most of the cell body response after axotomy. It also abolishes post-

traumatic neuronal cell death, causes most of the motoneurons to atrophy, and very strongly reduces the ability of their axons to regenerate, reinnervate their peripheral targets and contribute to functional recovery, as also confirmed in this thesis.

Deletion of c-Jun in peripheral nerve Schwann cells (the c-junΔP0 mutants) produced a very different effect (Arthur-Farraj et al., 2012;Fontana et al., 2012). Neuronal cell death is increased by 2-3 fold, even though most of the cell body response is not affected. Axonal regeneration is reduced, but most of the defect in target re-innervation and functional recovery appears to be due to excessive neuronal cell death. Deletion of Schwann cell c-Jun interferes with local production of neurotrophins, including BDNF, LIF, GDNF and Artemin; in the same vein supplementation with exogenous GDNF and Artemin promotes neuronal survival, target re-innervation as well as functional recovery (Fontana et al., 2012). When the two phenotypes were combined by deleting c-jun in both neurons and in Schwann cells, an almost complete abolition of re-innervation was seen in the mutants, significantly lower than the already low levels of re-innervation seen in the single mutants. In terms of axotomy-induced neuronal cell death, the neuronal c-jun deletion phenotype appeared to supersede the deletion in Schwann cells, with the axotomised motoneurons showing massively increased cell survival, and severely atrophic morphology. Thus, the c-JunΔS and c-JunΔP0 mutations appear to have additive effects on regeneration, but their effects on neuronal survival are antagonistic.

Since both neuronal and Schwann cell c-Jun are strongly phosphorylated at the N-terminus following nerve injury, we explored c-Jun phosphorylation as the mechanism behind the striking functions of c-Jun in regeneration. Global replacement of all 4 N-terminal c-Jun phosphorylation sites (Ser63&73, Thr91&93) with alanines (jun4A) did not interfere with the expression of injury-associated neuronal and non-neuronal markers. On the other hand, as in the JNK1&3 null mutants, the jun4A strain reproduced the moderate reduction in functional recovery and a 50% lower number of re-innervating motoneurons at 28 days following facial nerve cut. However, this effect on regeneration is considerably smaller than that observed with neuronal c-Jun deletion, suggesting that the regeneration effect of c-Jun is mostly independent of its N-terminal phosphorylation. The mutation produced a significant increase (1.8x) in neuronal cell death, but mostly of shrunken neurons, a phenomenon also seen in the junAA mutants (Ruff et al., 2012b). Thus the jun4A mutants seem to be a combination of the junAA and global JNK deletions. The

moderate effects of these mutants suggest that c-Jun activation specifically in the neuronal, non-peripheral part of the transcriptional regeneration program is not affected by N-terminal phosphorylation. However, c-jun N-terminal phosphorylation appears to be important for neuronal survival, implicating a role in trophic signalling elicited by the c-Jun in Schwann cells. At present, no information is available with regards to the regeneration-associated c-Jun effects of Thr239 dephosphorylation, or for C-terminal acetylation.

C/EBPδ has recently emerged as another important regeneration associated transcription factor. Lack of C/EPBδ causes a major reduction in the regenerative response of DRG neurons to a conditioning lesion, and affects the expression of regeneration-associated molecule SPRR1A (Lopez de and Magoulas, 2013). In 2005, Nadeu et al., showed a role of C/EBPβ, another member of the C/EBP transcription factor family, in regulating the expression of microtubule α-tubulin protein and GAP43, a growth cone protein, after injury (Nadeau et al., 2005). The current studies demonstrate the involvement of CEBPδ in early and late microglial activation and in neuronal cell death, but not in the astroglial or leucocyte response following peripheral nerve injury. Global C/EBPδ deletion impaired central axonal sprouting, causing a 40% reduction in the number of galanin positive sprouts in the facial nucleus at 14 days following axotomy. Since STAT3 has also been shown to regulate the expression of galanin positive sprouts, and neuronal survival following axotomy, it appeared that concerted action of the two transcription factors might be responsible for the pro-regenerative effects following axotomy. Indeed, combined deletion of global C/EBP and neuronal STAT3 did not exacerbate the regeneration defects caused by STAT3 deletion alone. Rather deletion of C/EBPδ, improved functional recovery in the neuronal STAT3 null mice.

We next explored the *in vivo* functional and anatomical effects of deleting two downstream effector molecules, CAP23 and integrin β1, on regeneration. Deletion of neuronal CAP23, a cytoskeletal protein and candidate target of regeneration associated transcription factors, led to impaired early microglial function and almost 50% reduction in galanin+ perineuronal sprouting at day 14. This observation fits in with the implicated role of C/EBP family of transcription factors in axonal sprouting and outgrowth following injury, possibly through transcriptional activation of cytoskeletal elements which mediate aspects of actin rearrangement, and formation of the growth cone.

Neuron-specific deletion of beta 1 integrin resulted in approximately 2.5-fold increase in neuronal cell death, a commensurate 60% reduction in target re-innervation and transiently delayed functional recovery. These preliminary results suggest that the β1ΔS phenotype is roughly on par with that observed for the global α7 deletion. This coincides with the fact that α7 is the main neuronal α subunit that appears upregulated following axotomy (Kloss et al., 1999;Vogelezang et al., 2001b;Werner et al., 2000). The neuronal response of the cell body was largely unaffected, and the mutations interfered primarily with the post-traumatic trophic response, resulting in massive amounts of cell death. The β1ΔS mutant mice also showed 69% increase in the number of phagocytic microglia compared to control mice, and significantly higher astrocytic activation, which are probably related to the increased cell death seen in these mutants. The β1ΔS phenotype showed a striking similarity to the jun4A and c-junΔP0 mice, specifically in terms of immensely increased neuronal cell death, reduced axonal regeneration and sprouting, and minimal effects on the cellular response within the facial nucleus.

In summary, this data suggests the likelihood of co-existence of two different and mutually exclusive neuronal populations which are controlled by different aspects of the regenerative programme - a larger population dependent on 'intrinsic' activation of transcriptional master switches like c-jun and STAT3 in neurons, and which is completely devoid of regeneration on the absence of these neuronal transcription factors; and the other, a smaller population dependent on 'extrinsic' post-traumatic trophic signalling elicited by Schwann cells. This smaller population of neurons is independent of the cell body transcription cascade in its regenerative ability. Neuronal β1 integrin appears to be a critical co-factor in trophic signalling elicited by the Jun-expressing and N-terminal phosphorylation-dependent Schwann cells. This notion of the two neuronal populations being exclusive, but collectively exhaustive is further strengthened by the complete obliteration of re-innervation in mice having simultaneous deletion of c-jun in neurons and in Schwann cells. This bifurcation of the neuronal populations also provides an explanation for the bivalent role of c-jun in regeneration - Neuronally expressed c-Jun regulates the cellular response to injury and is essential for cell death, while Schwann cell c-Jun is an important regulator of peripheral nerve growth that is necessary for neuronal survival. Unlike c-jun, the Schwann cell counterpart of STAT3 was not involved in trophic signalling in regeneration.

Since the neuronal transcription factor - dependent regeneration programme provides the bulk of the regenerative response seen after peripheral nerve injury, with only about 20% neurons continuing to regenerate in their absence (Chapter 3- STAT3 Results; Chapter 4- c-jun results), (Raivich et al., 2004;Ruff et al., 2012), it is important to identify the various components of the transcription signalling cascades. As mentioned before, transcription factors trigger the activation of multiple intracellular signalling pathways, causing the expression of a cocktail of regeneration-associated genes and other downstream effector molecules which interact with each other and the extracellular environment to determine the fate of the injured neurons. By using conditional/global deletions and the facial axotomy paradigm, we have identified and described distinct as well as overlapping roles of transcription factors STAT3, c-Jun and C/EBPδ, and effector molecules CAP23 and β1 integrin in achieving a desirable regenerative outcome after peripheral nerve injury. Comparison of the cellular and molecular responses following deletion of c-Jun or STAT3 shows a complete overlap: both deletions interfered with induction of regeneration-associated adhesion molecules α7β1 integrin and CD44, both deletions abolished neuronal cell death but induced atrophy, and both also prevented microglial activation and T-cell recruitment. Interestingly, both transcription factors had little or no effect on reactive astrocyte expression. Neuronal c-Jun and STAT3 double mutants confirmed this overlap between the two transcription factors. The effects of STAT3 and C/EBPδ deletion also showed a partial overlay. The high degree of co-operation among these regeneration associated transcription factors proposes that they may contribute to a larger transcriptional complex that collectively regulates intrinsic growth ability. In fact, these transcription factors may be the multiple keys required to unbolt the lock on regeneration. In the absence of any key, regeneration cannot be triggered. It is unclear how these multiple factors interact with each other to induce transcription of their target genes.

STAT3, c-Jun, and ATF-3 are activated by the LIF-gp130 signalling and JNK signalling pathways for the transactivation of DINE gene (Kato et al., 2002;Kiryu-Seo et al., 2000;Kiryu-Seo and Kiyama, 2004). One of proposed theories is that Sp1, a general transcription factor, functions as a scaffolding protein to recruit c-Jun, ATF3, and STAT3, and binds directly to the GC-rich region located proximally to the transcription start site of the DINE promoter (Kiryu-Seo et al., 2008). It has been reported earlier that gene promoters without the AP1 site or the STAT3 binding site were activated by c-Jun or

STAT3 through an interaction with Sp1 (Chen and Chang, 2000;Kardassis et al., 1999). This Sp1 mediated recruitment of c-Jun/ATF3/STAT3 provides a reasonable and practical explanation for increased expression of numerous effectors simultaneously in injured neurons. It is possible that there are several other players in addition to these three transcription factors for co-ordinated control of intrinsic regenerative ability.

In addition to the concerted action of transcription factors as described above, there may be other transcription factors that are complementary to the c-Jun-STAT3 axis. A large number of regeneration associated transcription factors have been identified by microarray and gene profiling studies (Michaelevski et al., 2010). Candidate genes include p53, SRF, Sox11, NF-κB, ELK, Oct-6 etc., and their known in vitro /in vivo functions in regeneration have been described earlier (Chapter 1- Introduction). Identification of functionally complementary counterparts is important to understand what compensatory mechanisms are in place, and how they act together in order to produce a reliable and robust regenerative effort. However, this is easier said than done. Although a large number of molecules are widely-known markers of injured neurons, their complex interactions with other transcription factors, and their varied roles in different cell types makes the assessment of their role in the injured nervous system difficult. For example, because of the highly contrasting response of c-Jun in neurons and Schwann cells, future therapies will have to be specifically targeted to a particular cell-type to avoid undesirable effects. Further, the role of STAT3 appears to be much more prominent in motoneurons than in sensory or sympathetic neurons. Therefore to truly understand the signals which govern cell survival, axonal regeneration and target re-innervation following axotomy, it is not enough to take these molecules out of their context and study them individually.

It is important to mention here that most experimental approaches have their limitations, and the ones used in these studies are no exception. The use of cre/loxP technology for generating cell-type-specific gene deletions, as used in our studies, has greatly expanded the versatility with which biological questions can be addressed *in vivo*. However, there can be problems associated with the cre/loxP recombination system such as irregular or non-specific pattern of transgene expression due to promoters being 'leaky', variable recombination efficiencies, as well as potential influences of genetic background and breeding strategies of mice. Overlooking these issues can influence accuracy of the genetic targeting strategies, and overestimate phenotypic interpretations. For example, in

our mouse models with neuron-specific gene deletions, mostly female cre+ mice and cre- males were used for breeding to prevent germline gene deletion due to testicular synapsin promoter expression (Rempe et al., 2006). Secondly, a method of manual counting of neurons followed by Abercrombie correction was employed to analyse levels of cell survival after axotomy. The Abercrombie method counts neuronal segments in histological sections and then applies a correction formula to convert the segment count to neuronal number. However, this correction has been widely criticised for assuming the cellular diameter or nuclear height to be constant, and for missing out on neuronal segments (Coggeshall, 1992;Hedreen, 1998). The preferred approach is the 'physical dissector' or stereological approach which identifies 'tops' of neuronal profiles in a known volume, giving the numerical density of the neuronal population (Coggeshall, 1992;West, 2002). However, due to the atrophic nature of surviving motoneurons seen in many of our conditional KO mice models, manual counting of neurons provided a more reliable estimate compared to stereological measurements. Finally, antibodies against specific antigens can cross-react with other proteins, giving false-positives in the tissue. Specificity of antibody mediated stain must therefore be controlled and verified by other methods such as disappearance of staining in knock-outs, Western blotting, and RNA analysis to name a few.

In conclusion, if regeneration were akin to a tree, transcription factors would represent the main trunk of the regenerative response, controlling the arborisation of smaller branches symbolising downstream effector molecules which play niche roles in fine tuning specialised aspects of regeneration. This branching pattern of regeneration results in multiple, overlapping pathways acting in concert to create an intricate system that balances cell death and survival, recruits non-neuronal cells and culminates in target re-innervation and functional recovery. Without the central trunk, the branches are not supported, therefore the tree of regeneration collapses, as seen in the current studies. Discovering complementary/compensatory molecules in regeneration, along with signalling cascades and molecular targets of known players will undoubtedly be a huge step forward in devising clinical therapies following traumatic peripheral and even central nerve injuries.

Reference List

Abercrombie M (1946) Estimation of Nuclear Population from Microtome Sections. Anatomical Record 94:239-247.

Akira S (2000) Roles of STAT3 defined by tissue-specific gene targeting. Oncogene 19:2607-2611.

Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, Kishimoto T (1994) Molecular-Cloning of Aprf, A Novel Ifn-Stimulated Gene Factor-3 P91-Related Transcription Factor Involved in the Gp130-Mediated Signaling Pathway. Cell 77:63-71.

Al-Majed AA, Neumann CM, Brushart TM, Gordon T (2000) Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J Neurosci 20:2602-2608.

Alam T, An MR, Papaconstantinou J (1992) Differential Expression of 3 C/Ebp Isoforms in Multiple Tissues During the Acute Phase Response. Journal of Biological Chemistry 267:5021-5024.

Alberini CM (2009) Transcription Factors in Long-Term Memory and Synaptic Plasticity. Physiological Reviews 89:121-145.

Aleksic M, Feng ZP (2012) Identification of the role of C/EBP in neurite regeneration following microarray analysis of a L. stagnalis CNS injury model. BMC Neurosci 13:2.

Allodi I, Udina E, Navarro X (2012) Specificity of peripheral nerve regeneration: Interactions at the axon level. Progress in Neurobiology 98:16-37.

Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UAK, Muller W, Musiani P, Poli V, Davies AM (2001) Role of STAT3 and PI 3-Kinase/Akt in mediating the survival actions of cytokines on sensory neurons. Molecular and Cellular Neuroscience 18:270-282.

Anderson PN, Zhang JY, Ahmed BY, Sablitsky F, Raivich G, Lieberman AR, Verhaagen J (2006) Deletion of CAP-23 in adult mouse motor neurons delays axonal regeneration following facial nerve transection . Society for Neuroscience Abstracts C46.

Angelov DN, Guntinas-Lichius O, Wewetzer K, Neiss WF, Streppel M (2005) Axonal branching and recovery of coordinated muscle activity after transection of the facial nerve in adult rats. Adv Anat Embryol Cell Biol 180:1-130.

Arakawa H (2005) P53, apoptosis and axon-guidance molecules. Cell Death and Differentiation 12:1057-1065.

Araki T, Milbrandt J (2000) Ninjurin2, a novel homophilic adhesion molecule, is expressed in mature sensory and enteric neurons and promotes neurite outgrowth. J Neurosci 20:187-195.

Arguni E, Arima M, Tsuruoka N, Sakamoto A, Hatano M, Tokuhisa T (2006) JunD/AP-1 and STAT3 are the major enhancer molecules for high Bcl6 expression in germinal center B cells. Int Immunol 18:1079-1089.

Armstrong BD, Abad C, Chhith S, Cheling-Lau G, Hajji OE, Noblita H, Waschek JA (2008) Impaired nerve regeneration and enhanced neuroinflarnmatory response in mice lacking pituitary adenylyl cyclase activating peptide. Neuroscience 151:63-73.

Arthur-Farraj P, Baskaran A, Parkinson D, Turmaine M, Feltri M, Wrabetz L, Behrens A, Mirsky R, Jessen K (2007) Schwann cells without c-Jun show retarded demyelination and dedifferentiation after peripheral nerve injury. Society for Neuroscience Abstracts 480.

Arthur-Farraj PJ, Latouche M, Wilton DK, Quintes S, Chabrol E, Banerjee A, Woodhoo A, Jenkins B, Rahman M, Turmaine M, Wicher GK, Mitter R, Greensmith L, Behrens A, Raivich G, Mirsky R, Jessen KR (2012) c-Jun Reprograms Schwann Cells of Injured Nerves to Generate a Repair Cell Essential for Regeneration. Neuron 75:633-647.

Arvidsson U, Johnson H, Piehl F, Cullheim S, Hokfelt T, Risling M, Terenius L, Ulfhake B (1990) Peripheral-Nerve Section Induces Increased Levels of Calcitonin Gene-Related Peptide (Cgrp)-Like Immunoreactivity in Axotomized Motoneurons. Experimental Brain Research 79:212-216.

Ashwell KW (1982) The Adult-Mouse Facial-Nerve Nucleus - Morphology and Musculotopic Organization. Journal of Anatomy 135:531-538.

Baas PW, Ahmad FJ (2001) Force generation by cytoskeletal motor proteins as a regulator of axonal elongation and retraction. Trends in Cell Biology 11:244-249.

Bacon A, Kerr NCH, Holmes FE, Gaston K, Wynick D (2007) Characterization of an enhancer region of the galanin gene that directs expression to the dorsal root ganglion and confers responsiveness to axotomy. Journal of Neuroscience 27:6573-6580.

Bannerman P, Ara J, Hahn A, Hong L, McCauley E, Friesen K, Pleasure D (2008) Peripheral Nerve Regeneration Is Delayed in Neuropilin 2-Deficient Mice. J Neurosci Res 86:3163-3169.

Bareyre FM, Garzorz N, Lang C, Misgeld T, Buning H, Kerschensteiner M (2011) In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. Proc Natl Acad Sci U S A 108:6282-6287.

Barnat M, Enslen H, Propst F, Davis RJ, Soares S, Nothias F (2010) Distinct Roles of c-Jun N-Terminal Kinase Isoforms in Neurite Initiation and Elongation during Axonal Regeneration. Journal of Neuroscience 30:7804-7816.

Behrens A, Sibilia M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nature Genetics 21:326-329.

Ben-Yaakov K, Dagan SY, Segal-Ruder Y, Shalem O, Vuppalanchi D, Willis DE, Yudin D, Rishal I, Rother F, Bader M, Blesch A, Pilpel Y, Twiss JL, Fainzilber M (2012) Axonal transcription factors signal retrogradely in lesioned peripheral nerve. EMBO J 31:1350-1363.

Berdan RC, Easaw JC, Wang R (1993) Alterations in Membrane-Potential After Axotomy at Different Distances from the Soma of An Identified Neuron and the Effect of Depolarization on Neurite Outgrowth and Calcium-Channel Expression. Journal of Neurophysiology 69:151-164.

Bergsland M, Werme M, Malewicz M, Perlmann T, Muhr J (2006) The establishment of neuronal properties is controlled by Sox4 and Sox11. Genes & Development 20:3475-3486.

Bermingham JR, Scherer SS, OConnell S, Arroyo E, Kalla KA, Powell FL, Rosenfeld MG (1996) Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. Genes & Development 10:1751-1762.

Besirli CG, Wagner EF, Johnson EM (2005) The limited role of NH2-terminal c-Jun phosphorylation in neuronal apoptosis: Identification of the nuclear pore complex as a potential target of the JNK pathway. Journal of Cell Biology 170:401-411.

Bhatheja K, Field J (2006) Schwann cells: Origins and role in axonal maintenance and regeneration. International Journal of Biochemistry & Cell Biology 38:1995-1999.

Bignami A, Dahl D, Nguyen BT, Crosby CJ (1981) The Fate of Axonal Debris in Wallerian Degeneration of Rat Optic and Sciatic-Nerves - Electron-Microscopy and Immunofluorescence Studies with Neurofilament Antisera. Journal of Neuropathology and Experimental Neurology 40:537-550.

Bignami A, Ralston HJ, III (1969) The cellular reaction to Wallerian degeneration in the central nervous system of the cat. Brain Res 13:444-461.

Bjorkblom B, Ostman N, Hongisto V, Komarovski V, Filen JJ, Nyman TA, Kallunki T, Courtney MJ, Coffey ET (2005) Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: Role of microtubule-associated protein 2 as an effector. Journal of Neuroscience 25:6350-6361.

Boeshore KL, Schreiber RC, Vaccariello SA, Sachs HH, Salazar R, Lee J, Ratan RR, Leahy P, Zigmond RE (2004) Novel changes in gene expression following axotomy of a sympathetic ganglion: A microarray analysis. Journal of Neurobiology 59:216-235.

Bogoyevitch MA, Kobe B (2006) Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiology and Molecular Biology Reviews 70:1061-1095.

Bohatschek M, Kloss CUA, Pfeffer K, Bluethmann H, Raivich G (2004) B7.2 on activated and phagocytic microglia in the facial axotomy model: regulation by interleukin-1 receptor type 1, tumor necrosis factor receptors 1 and 2 and endotoxin. Journal of Neuroimmunology 156:132-145.

Bolsover S, Fabes J, Anderson PN (2008) Axonal guidance molecules and the failure of axonal regeneration in the adult mammalian spinal cord. Restor Neurol Neurosci 26:117-130.

Bomze HM, Bulsara KR, Iskandar BJ, Caroni P, Skene JHP (2001) Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nature Neuroscience 4:38-43.

Bonilla IE, Tanabe K, Strittmatter SM (2002) Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. Journal of Neuroscience 22:1303-1315.

Bosse F, Hasenpusch-Theil K, Kury P, Muller HW (2006) Gene expression profiling reveals that peripheral nerve regeneration is a consequence of both novel injury-dependent and reactivated developmental processes. J Neurochem 96:1441-1457.

Bosse F, Kury P, Muller HW (2001) Gene expression profiling and molecular aspects in peripheral nerve regeneration. Restor Neurol Neurosci 19:5-18.

Bottcher RT, Pollet N, Delius H, Niehrs C (2004) The transmembrane protein XFLRT3 forms a complex with FGF receptors and promotes FGF signalling. Nature Cell Biology 6:38-U8.

Boyd JG, Gordon T (2003a) Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. Experimental Neurology 183:610-619.

Boyd JG, Gordon T (2003b) Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Molecular Neurobiology 27:277-323.

Brecht S, Kirchhof R, Chromik A, Willesen M, Nicolaus T, Raivich G, Wessig J, Waetzig V, Goetz M, Claussen M, Pearse D, Kuan CY, Vaudano E, Behrens A, Wagner E, Flavell RA, Davis RJ, Herdegen T (2005) Specific pathophysiological functions of JNK isoforms in the brain. European Journal of Neuroscience 21:363-377.

Brinton RE, Gruener R (1987) Vasopressin Promotes Neurite Growth in Cultured Embryonic Neurons. Synapse 1:329-334.

Bromberg J, Darnell JE (2000) The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19:2468-2473.

Brushart TM, Hoffman PN, Royall RM, Murinson BB, Witzel C, Gordon T (2002) Electrical stimulation promotes motoneuron regeneration without increasing its speed or conditioning the neuron. J Neurosci 22:6631-6638.

Buchli AD, Rouiller E, Mueller R, Dietz V, Schwab ME (2007) Repair of the injured spinal cord - A joint approach of basic and clinical research. Neurodegenerative Diseases 4:51-56.

Buchwalter G, Gross C, Wasylyk B (2004) Ets ternary complex transcription factors. Gene 324:1-14.

Cafferty WBJ, Gardiner NJ, Das P, Qiu J, McMahon SB, Thompson SWN (2004) Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. Journal of Neuroscience 24:4432-4443.

Cafferty WBJ, Gardiner NJ, Gavazzi I, Powell J, McMahon SB, Heath JK, Munson J, Cohen J, Thompson SWN (2001) Leukemia inhibitory factor determines the growth status of injured adult sensory neurons. Journal of Neuroscience 21:7161-7170.

Cao JP, Yu JK, Li C, Sun Y, Yuan HH, Wang HJ, Gao DS (2008) Integrin beta 1 is involved in the signaling of glial cell line-derived neurotrophic factor. Journal of Comparative Neurology 509:203-210.

Caroni P (1997) Intrinsic neuronal determinants that promote axonal sprouting and elongation. Bioessays 19:767-775.

Chan YM, Yick LW, Yip HK, So KF, Oppenheim RW, Wu WT (2003) Inhibition of caspases promotes long-term survival and reinnervation by axotomized spinal motoneurons of denervated muscle in newborn rats. Experimental Neurology 181:190-203.

Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA, McCubrey JA (2003) Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia 17:1263-1293.

Chao CC, Ma YL, Chu KY, Lee EHY (2003) Integrin alpha v and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. Neurobiology of Aging 24:105-116.

Chen BK, Chang WC (2000) Functional interaction between c-Jun and promoter factor Sp1 in epidermal growth factor-induced gene expression of human 12(S)-lipoxygenase. Proc Natl Acad Sci U S A 97:10406-10411.

Chen ZL, Strickland S (2003) Laminin gamma 1 is critical for Schwann cell differentiation, axon myelination, and regeneration in the peripheral nerve. Journal of Cell Biology 163:889-899.

Cimica V, Chen HC, Iyer JK, Reich NC (2011) Dynamics of the STAT3 Transcription Factor: Nuclear Import Dependent on Ran and Importin-beta 1. Plos One 6:e20188.

Coffer P, Lutticken C, Vanpuijenbroek A, Klopdejonge M, Horn F, Kruijer W (1995) Transcriptional Regulation of the Junb Promoter - Analysis of Stat-Mediated Signal-Transduction. Oncogene 10:985-994.

Coggeshall RE (1992) A Consideration of Neural Counting Methods. Trends in Neurosciences 15:9-13

Condic ML (2001) Adult neuronal regeneration induced by transgenic integrin expression. Journal of Neuroscience 21:4782-4788.

Coqueret O, Gascan H (2000) Functional interaction of STAT3 transcription factor with the cell cycle inhibitor p21(WAF1/CIP1/SD11). Journal of Biological Chemistry 275:18794-18800.

Cox LJ, Hengst U, Gurskaya NG, Lukyanov KA, Jaffrey SR (2008) Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. Nature Cell Biology 10:149-159.

Crocker SJ, Hayley SP, Smith PD, Mount MP, Lamba WR, Callaghan SM, Slack RS, Park DS (2006) Regulation of axotomy-induced dopaminergic neuron death and c-Jun

phosphorylation by targeted inhibition of cdc42 or mixed lineage kinase. J Neurochem 96:489-499.

Culmsee C, Mattson MP (2005) p53 in neuronal apoptosis. Biochemical and Biophysical Research Communications 331:761-777.

Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, DiStefano PS (1993) Retrograde Axonal-Transport of Ciliary Neurotrophic Factor Is Increased by Peripheral-Nerve Injury. Nature 365:253-255.

Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, Zhu Y, Lindsay RM, DiStefano PS (1994) Retrograde Axonal-Transport of Lif Is Increased by Peripheral-Nerve Injury - Correlation with Increased Lif Expression in Distal Nerve. Neuron 12:191-204.

Dai CF, Kanoh N, Li KY, Wang ZM (2000) Study on facial motoneuronal death after proximal or distal facial nerve transection. American Journal of Otology 21:115-118.

De-Fraja C, Conti L, Magrassi L, Govoni S, Cattaneo E (1998) Members of the JAK/STAT proteins are expressed and regulated during development in the mammalian forebrain. J Neurosci Res 54:320-330.

Demestre M, Wells GM, Miller KM, Smith KJ, Hughes RAC, Gearing AJ, Gregson NA (2004) Characterisation of matrix metalloproteinases and the effects of a broad-spectrum inhibitor (BB-1101) in peripheral nerve regeneration. Neuroscience 124:767-779.

Deng J, Zhang XL, Wang JW, Teng LL, Ge J, Takeiviori H, Xiong ZQ, Zhou Y (2009) Expression and Regulated Nuclear Transport of Transducers of Regulated Creb 1 in Retinal Ganglion Cells (Retracted Article. See Vol. 169, Pg. 950, 2010). Neuroscience 159:1023-1031.

Dent EW, Gertler FB (2003) Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron 40:209-227.

Deumens R, Bozkurt A, Meek MF, Marcus MAE, Joosten EAJ, Weis J, Brook GA (2010) Repairing injured peripheral nerves: Bridging the gap. Progress in Neurobiology 92:245-276.

Di Giovanni S, Knights CD, Rao M, Yakovlev A, Beers J, Catania J, Avantaggiati ML, Faden AI (2006) The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. Embo Journal 25:4084-4096.

Dobrea GM, Unnerstall JR, Rao MS (1992) The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. Brain Res Dev Brain Res 66:209-219.

Doyle CA, Hunt SP (1997) Reduced nuclear factor kappa B (p65) expression in rat primary sensory neurons after peripheral nerve injury. Neuroreport 8:2937-2942.

Ducret C, Maira SM, Lutz Y, Wasylyk B (2000) The ternary complex factor Net contains two distinct elements that mediate different responses to MAP kinase signalling cascades. Oncogene 19:5063-5072.

Dumoulin FL, Raivich G, Haas CA, Lazar P, Reddington M, Streit WJ, Kreutzberg GW (1992) Calcitonin gene-related peptide and peripheral nerve regeneration. Ann N Y Acad Sci 657.

Durbec P, MarcosGutierrez CV, Kilkenny C, Grigoriou M, Wartiovaara K, Suvanto P, Smith D, Ponder B, Costantini F, Saarma M, Sariola H, Pachnis V (1996) GDNF signalling through the Ret receptor tyrosine kinase. Nature 381:789-793.

Dziennis S, Alkayed NJ (2008) Role of signal transducer and activator of transcription 3 in neuronal survival and regeneration. Rev Neurosci 19:341-361.

Ekstrom PAR, Mayer U, Panjwani A, Pountney D, Pizzey J, Tonge DA (2003) Involvement of alpha 7 beta 1 integrin in the conditioning-lesion effect on sensory axon regeneration. Molecular and Cellular Neuroscience 22:383-395.

Ellezam B, Dubreuil C, Winton M, Loy L, Dergham P, Selles-Navarro I, McKerracher L (2002) Inactivation of intracellular Rho to stimulate axon growth and regeneration. Spinal Cord Trauma: Regeneration, Neural Repair and Functional Recovery 137:371-380.

English AW, Meador W, Carrasco DI (2005) Neurotrophin-4/5 is required for the early growth of regenerating axons in peripheral nerves. European Journal of Neuroscience 21:2624-2634.

Ernst M, Jenkins BJ (2004) Acquiring signalling specificity from the cytokine receptor gp130. Trends in Genetics 20:23-32.

Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. Nature 420:629-635.

Faggioli L, Costanzo C, Donadelli M, Palmieri M (2004) Activation of the interleukin-6 promoter by a dominant negative mutant of c-Jun. Biochimica et Biophysica Acta-Molecular Cell Research 1692:17-24.

Feltri ML, D'Antonio M, Previtali S, Fasolini M, Messing A, Wrabetz L (1999) P0-Cre transgenic mice for inactivation of adhesion molecules in Schwann cells. Charcot-Marie-Tooth Disorders 883:116-123.

Ferri CC, Moore FA, Bisby MA (1998) Effects of facial nerve injury on mouse motoneurons lacking the p75 low-affinity neurotrophin receptor. Journal of Neurobiology 34:1-9.

Fontana X, Hristova M, Da Costa C, Patodia S, Thei L, Makwana M, Spencer-Dene B, Latouche M, Mirsky R, Jessen KR, Klein R, Raivich G, Behrens A (2012) c-Jun in Schwann cells promotes axonal regeneration and motoneuron survival via paracrine signaling. Journal of Cell Biology 198:127-141.

Forscher P, Smith SJ (1988) Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol 107:1505-1516.

Fournier AE, Strittmatter SM (2002) Regenerating nerves follow the road more traveled. Nature Neuroscience 5:821-822.

Frey D, Laux T, Xu L, Schneider C, Caroni P (2000) Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. Journal of Cell Biology 149:1443-1453.

Fricker FR, Lago N, Balarajah S, Tsantoulas C, Tanna S, Zhu N, Fageiry SK, Jenkins M, Garratt AN, Birchmeier C, Bennett DLH (2011) Axonally Derived Neuregulin-1 Is Required for Remyelination and Regeneration after Nerve Injury in Adulthood. Journal of Neuroscience 31:3225-3233.

Fry FJ, Cowan WM (1972) Study of Retrograde Cell Degeneration in Lateral Mammillary Nucleus of Cat, with Special Reference to Role of Axonal Branching in Preservation of Cell. Journal of Comparative Neurology 144:1-6.

Fujio Y, Matsuda T, Oshima Y, Maeda M, Mohri T, Ito T, Takatani T, Hirata M, Nakaoka Y, Kimura R, Kishimoto T, Azuma J (2004) Signals through gp130 upregulate Wnt5a and contribute to cell adhesion in cardiac myocytes. Febs Letters 573:202-206.

Fujitani M, Honda A, Hata K, Yamagishi S, Tohyama M, Yamashita T (2005) Biological activity of neurotrophins is dependent on recruitment of Rac1 to lipid rafts. Biochemical and Biophysical Research Communications 327:150-154.

Funahashi H, Takeyama H, Sawai H, Furuta A, Sato M, Okada Y, Hayakawa T, Tanaka M, Manabe T (2003) Alteration of integrin expression by glial cell line-derived neurotrophic factor (GDNF) in human pancreatic cancer cells. Pancreas 27:190-196.

Furey MJ, Midha R, Xu QG, Belkas J, Gordon T (2007) Prolonged target deprivation reduces the capacity of injured motioneurons to regenerate. Neurosurgery 60:723-732.

Galiano M, Liu ZQ, Kalla R, Bohatschek M, Koppius A, Gschwendtner A, Xu S, Werner A, Kloss CU, Jones LL, Bluethmann H, Raivich G (2001) Interleukin-6 (IL6) and cellular response to facial nerve injury: effects on lymphocyte recruitment, early microglial activation and axonal outgrowth in IL6-deficient mice. Eur J Neurosci 14.

Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY (2004) Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci U S A 101:4661-4666.

Garcia-Marin V, Garcia-Lopez P, Freire M (2009) The Growth Cone as Seen Through Cajal's Original Histological Preparations and Publications. Journal of the History of the Neurosciences 18:197-210.

Gardiner NJ, Fernyhough P, Tomlinson DR, Mayer U, von der Mark H, Streuli CH (2005) alpha 7 integrin mediates neurite outgrowth of distinct populations of adult sensory neurons. Molecular and Cellular Neuroscience 28:229-240.

Gautron L, de Smedt-Peyrusse V, Laye S (2006) Characterization of STAT3-expressing cells in the postnatal rat brain. Brain Research 1098:26-32.

George R, Griffin JW (1994) Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: the dorsal radiculotomy model. Exp Neurol 129:225-236.

Glazner GW, Lupien S, Miller JA, Ishii DN (1993) Insulin-Like Growth Factor-Ii Increases the Rate of Sciatic-Nerve Regeneration in Rats. Neuroscience 54:791-797.

Goldberg JL, Barres BA (2000) The relationship between neuronal survival and regeneration. Annual Review of Neuroscience 23:579-612.

Gonzalezgarcia M, Garcia I, Ding LY, Oshea S, Boise LH, Thompson CB, Nunez G (1995) Bcl-X Is Expressed in Embryonic and Postnatal Neural Tissues and Functions to Prevent Neuronal Cell-Death. Proc Natl Acad Sci U S A 92:4304-4308.

Gordon T, Brushart TM, Chan KM (2008) Augmenting nerve regeneration with electrical stimulation. Neurol Res 30:1012-1022.

Gordon T, Gordon K (2010) Nerve regeneration in the peripheral nervous system versus the central nervous system and the relevance to speech and hearing after nerve injuries. Journal of Communication Disorders 43:274-285.

Gordon TG, Tyreman N, Raji MA (2011) The Basis for Diminished Functional Recovery after Delayed Peripheral Nerve Repair. Journal of Neuroscience 31:5325-5334.

Goslin K, Banker G (1990) Rapid Changes in the Distribution of Gap-43 Correlate with the Expression of Neuronal Polarity During Normal Development and Under Experimental Conditions. Journal of Cell Biology 110:1319-1331.

Graeber MB, Bise K, Mehraein P (1993) Synaptic Stripping in the Human Facial Nucleus. Acta Neuropathologica 86:179-181.

Graeber MB, Kreutzberg GW (1986) Astrocytes increase in glial fibrillary acidic protein during retrograde changes of facial motor neurons. J Neurocytol 15:363-373.

Graeber MB, Kreutzberg GW (1988) Delayed astrocyte reaction following facial nerve axotomy. J Neurocytol 17:209-220.

Graeber MB, Raivich G, Kreutzberg GW (1989) Increase of transferrin receptors and iron uptake in regenerating motor neurons. J Neurosci Res 23.

Gray SJ, Foti SB, Schwartz JW, Bachaboina L, Taylor-Blake B, Coleman J, Ehlers MD, Zylka MJ, Mccown TJ, Samulski RJ (2011) Optimizing Promoters for Recombinant Adeno-Associated Virus-Mediated Gene Expression in the Peripheral and Central Nervous System Using Self-Complementary Vectors. Human Gene Therapy 22:1143-1153.

Gresa-Arribas N, Serratosa J, Saura J, Sola C (2010) Inhibition of CCAAT/enhancer binding protein delta expression by chrysin in microglial cells results in anti-inflammatory and neuroprotective effects. J Neurochem 115:526-536.

Grumet M, Mauro V, Burgoon MP, Edelman GM, Cunningham BA (1991) Structure of A New Nervous-System Glycoprotein, Nr-Cam, and Its Relationship to Subgroups of Neural Cell-Adhesion Molecules. Journal of Cell Biology 113:1399-1412.

Gschwendtner A, Liu Z, Hucho T, Bohatschek M, Kalla R, Dechant G, Raivich G (2003) Regulation, cellular localization, and function of the p75 neurotrophin receptor (p75NTR) during the regeneration of facial motoneurons. Mol Cell Neurosci 24.

Guan QH, Pei DS, Liu XM, Wang XT, Xu TL, Zhang GY (2006) Neuroprotection against ischemic brain injury by SP600125 via suppressing the extrinsic and intrinsic pathways of apoptosis. Brain Research 1092:36-46.

Guirland C, Suzuki S, Kojima M, Lu B, Zheng JQ (2004) Lipid rafts mediate chemotropic guidance of nerve growth cones. Neuron 42:51-62.

Gutierrez H, O'Keeffe GW, Gavalda R, Gallagher D, Davies AM (2008) Nuclear factor kappa B signaling either stimulates or inhibits neurite growth depending on the phosphorylation status of p65/RelA. Journal of Neuroscience 28:8246-8256.

Gutmann E (1942) Factors affecting recovery of motor function after nerve lesions. J Neurol Psychiatry 5:81-95.

Haas CA, Hofmann HD, Kirsch M (1999) Expression of CNTF/LIF-receptor components and activation of STAT3 signaling in axotomized facial motoneurons: evidence for a sequential postlesional function of the cytokines. J Neurobiol 41:559-571.

Habecker BA, Sachs HH, Rohrer H, Zigmond RE (2009) The Dependence on gp130 Cytokines of Axotomy Induced Neuropeptide Expression in Adult Sympathetic Neurons. Developmental Neurobiology 69:392-400.

Haeusgen W, Boehm R, Zhao Y, Herdegen T, Waetzig V (2009) Specific Activities of Individual C-Jun N-Terminal Kinases in the Brain. Neuroscience 161:951-959.

Hai T, Curran T (1991) Cross-Family Dimerization of Transcription Factors Fos Jun and Atf Creb Alters Dna-Binding Specificity. Proc Natl Acad Sci U S A 88:3720-3724.

Hai T, Hartman MG (2001) The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. Gene 273:1-11.

Hannila SS, Filbin MT (2008) The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Experimental Neurology 209:321-332.

Hanz S, Perlson E, Willis D, Zheng JQ, Massarwa R, Huerta JJ, Koltzenburg M, Kohler M, van-Minnen J, Twiss JL, Fainzilber M (2003) Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40:1095-1104.

He Z, Koprivica V (2004) The Nogo signaling pathway for regeneration block. Annu Rev Neurosci 27:341-368.

Hedreen JC (1998) What was wrong with the Abercrombie and empirical cell counting methods? A review. Anatomical Record 250:373-380.

Heffron DS, Landreth GE, Samuels IS, Mandell JW (2009) Brain-Specific Deletion of Extracellular Signal-Regulated Kinase 2 Mitogen-Activated Protein Kinase Leads to Aberrant Cortical Collagen Deposition. American Journal of Pathology 175:2586-2599.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochemical Journal 374:1-20.

Hengerer B, Lindholm D, Heumann R, Ruther U, Wagner EF, Thoenen H (1990) Lesion-Induced Increase in Nerve Growth-Factor Messenger-Rna Is Mediated by C-Fos. Proc Natl Acad Sci U S A 87:3899-3903.

Henley J, Poo M (2004) Guiding neuronal growth cones using Ca2+ signals. Trends in Cell Biology 14:320-330.

Herdegen T, Blume A, Buschmann T, Georgakopoulos E, Winter C, Schmid W, Hsieh TF, Zimmermann M, Gass P (1997a) Expression of activating transcription factor-2, serum response factor and cAMP/Ca response element binding protein in the adult rat brain following generalized seizures, nerve fibre lesion and ultraviolet irradiation. Neuroscience 81:199-212.

Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, Hunter T, Karin M (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. Journal of Neuroscience 18:5124-5135.

Herdegen T, Kummer W, Fiallos CE, Leah J, Bravo R (1991) Expression of C-Jun, Jun-B and Jun-D Proteins in Rat Nervous-System Following Transection of Vagus Nerve and Cervical Sympathetic Trunk. Neuroscience 45:413-422.

Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Rev 28:370-490.

Herdegen T, Skene P, Bahr M (1997) The c-Jun transcription factor - Bipotential mediator of neuronal death, survival and regeneration. Trends in Neurosciences 20:227-231.

Herdegen T, Waetzig V (2001) AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration. Oncogene 20:2424-2437.

Heumann R, Korsching S, Bandtlow C, Thoenen H (1987) Changes of Nerve Growth-Factor Synthesis in Nonneuronal Cells in Response to Sciatic-Nerve Transection. Journal of Cell Biology 104:1623-1631.

Higashi N, Kunimoto H, Kaneko S, Sasaki T, Ishii M, Kojima H, Nakajima K (2004) Cytoplasmic c-Fos induced by the YXXQ-derived STAT3 signal requires the co-operative MEK/ERK signal for its nuclear translocation. Genes Cells 9:233-242.

Hiroi S, Tsukamoto Y, Sasaki F, Miki N, Taira E (2003) Involvement of gicerin, a cell adhesion molecule, in development and regeneration of chick sciatic nerve. Febs Letters 554:311-314.

Hirota H, Kiyama H, Kishimoto T, Taga T (1996) Accelerated nerve regeneration in mice by upregulated expression of interleukin (IL) 6 and IL-6 receptor after trauma. Journal of Experimental Medicine 183:2627-2634.

Hol EM, Schwaiger FW, Werner A, Schmitt A, Raivich G, Kreutzberg GW (1999) Regulation of the LIM-type homeobox gene islet-1 during neuronal regeneration. Neuroscience 88:917-925.

Hollis ER, Jamshidi P, Lorenzana AO, Lee JK, Gray SJ, Samulski RJ, Zheng BH, Tuszynski MH (2010) Transient Demyelination Increases the Efficiency of Retrograde AAV Transduction. Molecular Therapy 18:1496-1500.

Holmberg K, Kuteeva E, Brumovsky P, Kahl U, Karlstrom H, Lucas GA, Rodriguez J, Westerblad H, Hilke S, Theodorsson E, Berge OG, Lendahl U, Bartfai T, Hokfelt T (2005) Generation and phenotypic characterization of a galanin overexpressing mouse. Neuroscience 133:59-77.

Holmes FE, Mahoney S, King VR, Bacon A, Kerr NCH, Pachnis V, Curtis R, Priestley JV, Wynick D (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. Proc Natl Acad Sci U S A 97:11563-11568.

Holmes FE, Mahoney SA, Wynick D (2005) Use of genetically engineered transgenic mice to investigate the role of galanin in the peripheral nervous system after injury. Neuropeptides 39:191-199.

Horie H, Kadoya T (2000) Identification of oxidized galectin-1 as an initial repair regulatory factor after axotomy in peripheral nerves. Neuroscience Research 38:131-137.

Huang AM, Rudelius M, Sharan S, McAllister JM, Raffeld M, Christenson LK, Sterneck E (2007) The Cebpd (C/EBPdelta) gene is induced by luteinizing hormones in ovarian theca and interstitial cells but is not essential for mouse ovary function. PLoS One 2:e1334.

Huang B, Zhao X, Zheng LB, Zhang L, Ni B, Wang YW (2011) Different Expression of Tissue Inhibitor of Metalloproteinase Family Members in Rat Dorsal Root Ganglia and Their Changes After Peripheral Nerve Injury. Neuroscience 193:421-428.

Huber AB, Kolodkin AL, Ginty DD, Cloutier JF (2003) Signaling at the growth cone: Ligand-receptor complexes and the control of axon growth and guidance. Annual Review of Neuroscience 26:509-563.

Hunt D, Hossain-Ibrahim K, Mason MR, Coffin RS, Lieberman AR, Winterbottom J, Anderson PN (2004) ATF3 upregulation in glia during Wallerian degeneration: differential expression in peripheral nerves and CNS white matter. BMC Neurosci 5:9-21.

Hunt D, Raivich G, Anderson PN (2012) Activating transcription factor 3 and the nervous system. Front Mol Neurosci 5:7.

Ide C (1996) Peripheral nerve regeneration. Neuroscience Research 25:101-121.

Islamov R, Chintalgattu V, Katwa L, Murashov A (2004) Endothelin and vascular endothelial growth factor signaling in axotomized spinal motoneurons. Faseb Journal 18:A1066.

Iwata T, Namikawa K, Honma M, Mori N, Yachiku S, Kiyama H (2002) Increased expression of mRNAs for microtubule disassembly molecules during nerve regeneration. Brain Res Mol Brain Res 102:105-109.

Jacobs WB, Kaplan DR, Miller FD (2006) The p53 family in nervous system development and disease. J Neurochem 97:1571-1584.

Jaegle M, Meijer D (1998) Role of Oct-6 in Schwann cell differentiation. Microscopy Research and Technique 41:372-378.

Jani A, Menichella D, Jiang HY, Chbihi T, Acsadi G, Kamholz J, Shy ME (1999) Overcoming cellular immunity to prolong adenoviral-mediated gene expression in sciatic nerve. Charcot-Marie-Tooth Disorders 883:397-414.

Jankowski MP, Cornuet PK, McILwrath S, Koerber HR, Albers KM (2006) SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. Neuroscience 143:501-514.

Jankowski MP, McIlwrath SL, Jing XT, Cornuet PK, Salerno KM, Koerber HR, Albers KM (2009) Sox11 transcription factor modulates peripheral nerve regeneration in adult mice. Brain Research 1256:43-54.

Jiang JG, Zarnegar R (1997) A novel transcriptional regulatory region within the core promoter of the hepatocyte growth factor gene is responsible for its inducibility by cytokines via the C/EBP family of transcription factors. Molecular and Cellular Biology 17:5758-5770.

Jing SQ, Wen DZ, Yu YB, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 85:1113-1124.

Jing X, Wang T, Huang S, Glorioso JC, Albers KM (2012) The transcription factor Sox11 promotes nerve regeneration through activation of the regeneration-associated gene Sprr1a. Exp Neurol 233:221-232.

Jones LL, Kreutzberg GW, Raivich G (1997) Regulation of CD44 in the regenerating mouse facial motor nucleus. Eur J Neurosci 9:1854-1863.

Jones LL, Liu Z, Shen J, Werner A, Kreutzberg GW, Raivich G (2000) Regulation of the cell adhesion molecule CD44 after nerve transection and direct trauma to the mouse brain. J Comp Neurol 426:468-492.

Jungnickel J, Claus P, Gransalke K, Timmer M, Grothe C (2004) Targeted disruption of the FGF-2 gene affects the response to peripheral nerve injury. Molecular and Cellular Neuroscience 25:444-452.

Kalla R, Liu Z, Xu S, Koppius A, Imai Y, Kloss CU, Kohsaka S, Gschwendtner A, Moller JC, Werner A, Raivich G (2001) Microglia and the early phase of immune surveillance in the axotomized facial motor nucleus: impaired microglial activation and lymphocyte recruitment but no effect on neuronal survival or axonal regeneration in macrophage-colony stimulating factor-deficient mice. J Comp Neurol 436:182-201.

Kanje M, Skottner A, Lundborg G, Sjoberg J (1991) Does Insulin-Like Growth Factor-I (Igf-1) Trigger the Cell Body Reaction in the Rat Sciatic-Nerve. Brain Research 563:285-287.

Kanje M, Skottner A, Sjoberg J, Lundborg G (1989) Insulin-Like Growth Factor-I (Igf-I) Stimulates Regeneration of the Rat Sciatic-Nerve. Brain Research 486:396-398.

Kardassis D, Papakosta P, Pardali K, Moustakas A (1999) c-Jun transactivates the promoter of the human p21(WAF1/Cip1) gene by acting as a superactivator of the ubiquitous transcription factor Spl. Journal of Biological Chemistry 274:29572-29581.

Kashihara M, Miyata S, Kumanogoh H, Funatsu N, Matsunaga W, Kiyohara T, Sokawa Y, Maekawa S (2000) Changes in the localization of NAP-22, a calmodulin binding membrane protein, during the development of neuronal polarity. Neurosci Res 37:315-325.

Kato R, Kiryu-Seo S, Kiyama H (2002) Damage-induced neuronal endopeptidase (DINE/ECEL) expression is regulated by leukemia inhibitory factor and deprivation of nerve growth factor in rat sensory ganglia after nerve injury. Journal of Neuroscience 22:9410-9418.

Kawasaki T, Oka N, Tachibana H, Akiguchi I, Shibasaki H (2003) Oct6, a transcription factor controlling myelination, is a marker for active nerve regeneration in peripheral neuropathies. Acta Neuropathologica 105:203-208.

Kenney AM, Kocsis JD (1998) Peripheral axotomy induces long-term c-Jun amino-terminal kinase-1 activation and activator protein-1 binding activity by c-Jun and junD in adult rat dorsal root ganglia in vivo. Journal of Neuroscience 18:1318-1328.

Kerr BJ, Thompson SWN, Wynick D, McMahon SB (2001) Endogenous galanin is required for the full expression of central sensitization following peripheral nerve injury. Neuroreport 12:3331-3334.

Kerr N, Pintzas A, Holmes F, Hobson SA, Pope R, Wallace M, Wasylyk C, Wasylyk B, Wynick D (2010) The expression of ELK transcription factors in adult DRG: Novel isoforms, antisense transcripts and upregulation by nerve damage. Molecular and Cellular Neuroscience 44:165-177.

Kfoury N, Kapatos G (2009) Identification of neuronal target genes for CCAAT/enhancer binding proteins. Mol Cell Neurosci 40:313-327.

Khazaei MR, Halfter H, Karimzadeh F, Koo JH, Margolis FL, Young P (2010) Bex1 is involved in the regeneration of axons after injury. J Neurochem 115:910-920.

Kirsch M, Terheggen U, Hofmann HD (2003) Ciliary neurotrophic factor is an early lesion-induced retrograde signal for axotomized facial motoneurons. Molecular and Cellular Neuroscience 24:130-138.

Kiryu-Seo S, Hirayama T, Kato R, Kiyama H (2005) Noxa is a critical mediator of p53-dependent motor neuron death after nerve injury in adult mouse. Journal of Neuroscience 25:1442-1447.

Kiryu-Seo S, Kato R, Ogawa T, Nakagomi S, Nagata K, Kiyama H (2008) Neuronal injury-inducible gene is synergistically regulated by ATF3, c-Jun, and STAT3 through the interaction with Sp1 in damaged neurons. Journal of Biological Chemistry 283:6988-6996.

Kiryu-Seo S, Kiyama H (2004) Dine (damage induced neuronal endopeptidase). Protein and Peptide Letters 11:451-460.

Kiryu-Seo S, Kiyama H (2011) The nuclear events guiding successful nerve regeneration. Front Mol Neurosci 4:53.

Kiryu-Seo S, Sasaki M, Yokohama H, Nakagomi S, Hirayama T, Aoki S, Wada K, Kiyama H (2000) Damage-induced neuronal endopeptidase (DINE) is a unique metallopeptidase expressed in response to neuronal damage and activates superoxide scavengers. Proc Natl Acad Sci U S A 97:4345-4350.

Klein MA, Moller JC, Jones LL, Bluethmann H, Kreutzberg GW, Raivich G (1997) Impaired neuroglial activation in interleukin-6 deficient mice. Glia 19:227-233.

Klimaschewski L, Unsicker K, Heym C (1995) Vasoactive-Intestinal-Peptide But Not Galanin Promotes Survival of Neonatal Rat Sympathetic Neurons and Neurite Outgrowth of Pc12 Cells. Neurosci Lett 195:133-136.

Kloss CU, Werner A, Klein MA, Shen J, Menuz K, Probst JC, Kreutzberg GW, Raivich G (1999) Integrin family of cell adhesion molecules in the injured brain: regulation and cellular localization in the normal and regenerating mouse facial motor nucleus. J Comp Neurol 411:162-178.

Knoll B, Nordheim A (2009) Functional versatility of transcription factors in the nervous system: the SRF paradigm. Trends Neurosci 32:432-442.

Koeberle PD, Bahr M (2004) Growth and guidance cues for regenerating axons: Where have they gone? Journal of Neurobiology 59:162-180.

Korneev S, Federov A, Collins R, Blackshaw S, Davies J (1997) A subtractive cDNA library from an identified regenerating neuron is enriched in sequences up-regulated during nerve regeneration. Invertebrate Neuroscience 3:185-192.

Krebs DL, Hilton DJ (2001) SOCS proteins: Negative regulators of cytokine signaling. Stem Cells 19:378-387.

Kreutzberg GW (1993) Dynamic Changes in Motoneurons During Regeneration. Restor Neurol Neurosci 5:59-60.

Kubo T, Yamashita T, Yamaguchi A, Hosokawa K, Tohyama M (2002) Analysis of genes induced in peripheral nerve after axotomy using cDNA microarrays. J Neurochem 82:1129-1136.

Kugler S, Meyn L, Holzmuller H, Gerhardt E, Isenmann S, Schulz JB, Bahr M (2001) Neuron-specific expression of therapeutic proteins: Evaluation of different cellular promoters in recombinant adenoviral vectors. Molecular and Cellular Neuroscience 17:78-96.

Kuhlbrodt K, Herbarth B, Sock E, Enderich J, Hermans-Borgmeyer I, Wegner M (1998) Cooperative function of POU proteins and SOX proteins in glial cells. Journal of Biological Chemistry 273:16050-16057.

Kulbatski I, Cook DJ, Tator CH (2004) Calcium entry through L-type calcium channels is essential for neurite regeneration in cultured sympathetic neurons. Journal of Neurotrauma 21:357-374.

Kury P, Abankwa D, Kruse F, Greiner-Petter R, Muller HW (2004) Gene expression profiling reveals multiple novel intrinsic and extrinsic factors associated with axonal regeneration failure. European Journal of Neuroscience 19:32-42.

Lago N, Rodriguez FJ, Guzman MS, Jaramillo J, Navarro X (2007) Effects of motor and sensory nerve transplants on amount and specificity of sciatic nerve regeneration. J Neurosci Res 85:2800-2812.

Lassmann H, Ammerer HP, Jurecka W, Kulnig W (1978) Ultrastructural Sequence of Myelin Degradation .2. Wallerian Degeneration in Rat Femoral Nerve. Acta Neuropathologica 44:103-109.

Latouche M, Wilton D, Arthur-Farraj P, DaCosta C, Mitter R, Turmaine M, Raivich G, Behrens A, Mirsky R, Jessen KR (2009) Schwann Cell C-Jun Regulates Glial Support for Survival of Pns Neurons After Injury. Glia 57:S158.

Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P (2000) GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J Cell Biol 149:1455-1472.

Lee N, Neitzel KL, Devlin BK, MacLennan AJ (2004) STAT3 phosphorylation in injured axons before sensory and motor neuron nuclei: potential role for STAT3 as a retrograde signaling transcription factor. J Comp Neurol 474:535-545.

Lemons ML, Condic ML (2008) Integrin signaling is integral to regeneration. Experimental Neurology 209:343-352.

Leone L, De Stefano ME, Del Signore A, Petrucci TC, Paggi P (2005) Axotomy of sympathetic neurons activates the metalloproteinase-2 enzymatic pathway. Journal of Neuropathology and Experimental Neurology 64:1007-1017.

Leppa S, Saffrich R, Ansorge W, Bohmann D (1998) Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. Embo Journal 17:4404-4413.

Leu JI, Crissey MAS, Leu JP, Ciliberto G, Tau R (2001) Interleukin-6-induced STAT3 and AP-1 amplify hepatocyte nuclear factor 1-mediated transactivation of hepatic genes, an adaptive response to liver injury. Molecular and Cellular Biology 21:414-424.

Levy DE, Lee CK (2002) What does Stat3 do? Journal of Clinical Investigation 109:1143-1148.

Lieberman AR (1971) The Axon Reaction - A Review of the Principal Features of Perikaryal Responses to Axon Injury. Int Rev Neurobiol 14:49-124.

Lim CP, Cao XM (2006) Structure, function, and regulation of STAT proteins. Molecular Biosystems 2:536-550.

Lin L, Chan SO (2003) Perturbation of CD44 function affects chiasmatic routing of retinal axons in brain slice preparations of the mouse retinofugal pathway. European Journal of Neuroscience 17:2299-2312.

Lindholm D, Castren E, Kiefer R, Zafra F, Thoenen H (1992) Transforming Growth Factor-Beta-1 in the Rat-Brain - Increase After Injury and Inhibition of Astrocyte Proliferation. Journal of Cell Biology 117:395-400.

Lindholm D, Heumann R, Meyer M, Thoenen H (1987) Interleukin-1 Regulates Synthesis of Nerve Growth-Factor in Nonneuronal Cells of Rat Sciatic-Nerve. Nature 330:658-659.

Lindholm T, Skold MK, Suneson A, Carlstedt T, Cullheim S, Risling M (2004) Semaphorin and neuropilin expression in motoneurons after intraspinal motoneuron axotomy. Neuroreport 15:649-654.

Lindwall C, Dahlin L, Lundborg G, Kanje M (2004) Inhibition of c-Jun phosphorylation reduces axonal outgrowth of adult rat nodose ganglia and dorsal root ganglia sensory neurons. Molecular and Cellular Neuroscience 27:267-279.

Lindwall C, Kanje M (2005) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. Molecular and Cellular Neuroscience 29:269-282.

Liu HM, Yang LH, Yang YJ (1995) Schwann-Cell Properties .3. C-Fos Expression, Bfgf Production, Phagocytosis and Proliferation During Wallerian Degeneration. Journal of Neuropathology and Experimental Neurology 54:487-496.

Liu K, Lu Y, Lee JK, Samara R, Willenberg R, Sears-Kraxberger I, Tedeschi A, Park KK, Jin D, Cai B, Xu B, Connolly L, Steward O, Zheng B, He Z (2010) PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat Neurosci 13:1075-1081.

Liu RY, Snider WD (2001) Different signaling pathways mediate regenerative versus developmental sensory axon growth. Journal of Neuroscience 21:-RC164.

Liu Z, Qiu YH, Li B, Ma SH, Peng YP (2011) Neuroprotection of Interleukin-6 Against NMDA-Induced Apoptosis and Its Signal-Transduction Mechanisms. Neurotoxicity Research 19:484-495.

Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605-623.

Lonze BE, Riccio A, Cohen S, Ginty DD (2002) Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron 34:371-385.

Lopez de HL, Magoulas C (2013) Lack of the transcription factor C/EBPdelta impairs the intrinsic capacity of peripheral neurons for regeneration. Exp Neurol 239:148-157.

Lundborg G (2003) Nerve injury and repair - a challenge to the plastic brain. Journal of the Peripheral Nervous System 8:209-226.

Ma VY, Bisby MA (1998) Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. Brain Research 797:243-254.

MacGillavry HD, Cornelis J, van der Kallen LR, Sassen MM, Verhaagen J, Smit AB, van Kesteren RE (2011) Genome-wide gene expression and promoter binding analysis identifies NFIL3 as a repressor of C/EBP target genes in neuronal outgrowth. Mol Cell Neurosci 46:460-468.

Madura T, Yamashita T, Kubo T, Tsuji L, Hosokawa K, Tohyama M (2004) Changes in mRNA of Slit-Robo GTPase-activating protein 2 following facial nerve transection. Molecular Brain Research 123:76-80.

Maier IC, Schwab ME (2006) Sprouting, regeneration and circuit formation in the injured spinal cord: factors and activity. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1611-1634.

Makwana M, Raivich G (2005) Molecular mechanisms in successful peripheral regeneration. Febs J 272.

Makwana M, Werner A, Acosta-Saltos A, Gonitel R, Pararajasingham A, Ruff C, Rumajogee P, Cuthill D, Galiano M, Bohatschek M, Wallace AS, Anderson PN, Mayer U, Behrens A, Raivich G (2010) Peripheral facial nerve axotomy in mice causes sprouting of motor axons into perineuronal central white matter: time course and molecular characterization. J Comp Neurol 518:699-721.

Mason MR, Lieberman AR, Anderson PN (2003) Corticospinal neurons up-regulate a range of growth-associated genes following intracortical, but not spinal, axotomy. Eur J Neurosci 18:789-802.

Mason MR, Lieberman AR, Grenningloh G, Anderson PN (2002) Transcriptional upregulation of SCG10 and CAP-23 is correlated with regeneration of the axons of peripheral and central neurons in vivo. Mol Cell Neurosci 20:595-615.

Mason MRJ, Ehlert EME, Eggers R, Pool CW, Hermening S, Huseinovic A, Timmermans E, Blits B, Verhaagen J (2010) Comparison of AAV Serotypes for Gene Delivery to Dorsal Root Ganglion Neurons. Molecular Therapy 18:715-724.

Mattson MP (2005) NF-kappa B in the survival and plasticity of neurons. Neurochemical Research 30:883-893.

Mayr B, Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nature Reviews Molecular Cell Biology 2:599-609.

Mayumi-Matsuda K, Kojima S, Nakayama T, Suzuki H, Sakata T (1999) Scanning gene expression during neuronal cell death evoked by nerve growth factor depletion. Biochimica et Biophysica Acta-Gene Structure and Expression 1489:293-302.

McCauslin CS, Heath V, Colangelo AM, Malik R, Lee S, Mallei A, Mocchetti I, Johnson PF (2006) CAAT/enhancer-binding protein delta and cAMP-response element-binding protein mediate inducible expression of the nerve growth factor gene in the central nervous system. Journal of Biological Chemistry 281:17681-17688.

McGraw J, McPhail LT, Oschipok LW, Horie H, Poirier F, Steeves JD, Ramer MS, Tetzlaff W (2004) Galectin-1 in regenerating motoneurons. European Journal of Neuroscience 20:2872-2880.

McQuarrie IG, Grafstein B, Gershon MD (1977) Axonal Regeneration in Rat Sciatic-Nerve - Effect of A Conditioning Lesion and of Dbcamp. Brain Research 132:443-453.

Meller K (1987) Early Structural-Changes in the Axoplasmic Cytoskeleton After Axotomy Studied by Cryofixation. Cell Tissue Res 250:663-672.

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H (1992) Enhanced Synthesis of Brain-Derived Neurotrophic Factor in the Lesioned Peripheral-Nerve - Different Mechanisms Are Responsible for the Regulation of Bdnf and Ngf Messenger-Rna. Journal of Cell Biology 119:45-54.

Miao T, Wu DS, Zhang Y, Bo XN, Subang MC, Wang P, Richardson PM (2006) Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons. Journal of Neuroscience 26:9512-9519.

Miao TZ, Wu DS, Zhang Y, Bo XN, Xiao F, Zhang XY, Magoulas C, Subang MC, Wang P, Richardson PM (2008) SOCS3 suppresses AP-1 transcriptional activity in neuroblastoma cells through inhibition of c-Jun N-terminal kinase. Molecular and Cellular Neuroscience 37:367-375.

Michaelevski I, Segal-Ruder Y, Rozenbaum M, Medzihradszky KF, Shalem O, Coppola G, Horn-Saban S, Ben-Yaakov K, Dagan SY, Rishal I, Geschwind DH, Pilpel Y, Burlingame AL, Fainzilber M (2010) Signaling to Transcription Networks in the Neuronal Retrograde Injury Response. Science Signaling 3:-ra53.

Miledi R, Stefani E (1969) Non-Selective Re-Innervation of Slow and Fast Muscle Fibres in Rat. Nature 222:569-571.

Moisse K, Volkening K, Leystra-Lantz C, Welch I, Hill T, Strong MJ (2009) Divergent patterns of cytosolic TDP-43 and neuronal progranulin expression following axotomy: Implications for TDP-43 in the physiological response to neuronal injury. Brain Research 1249:202-211.

Moller JC, Klein MA, Haas S, Jones LL, Kreutzberg GW, Raivich G (1996) Regulation of thrombospondin in the regenerating mouse facial motor nucleus. Glia 17:121-132.

Moore DL, Apara A, Goldberg JL (2011) Kruppel-like transcription factors in the nervous system: novel players in neurite outgrowth and axon regeneration. Mol Cell Neurosci 47:233-243.

Moore DL, Goldberg JL (2011) Multiple transcription factor families regulate axon growth and regeneration. Developmental Neurobiology 12:1186-1211.

Moore RY (1989) Cranial Motor Neurons Contain Either Galanin Gene-Related Or Calcitonin Gene-Related Peptide-Like Immunoreactivity. Journal of Comparative Neurology 282:512-522.

Moran LB, Graeber MB (2004) The facial nerve axotomy model. Brain Res Rev 44:154-178.

Mori N, Morii H (2002) SCG10-related neuronal growth-associated proteins in neural development, plasticity, degeneration, and aging. J Neurosci Res 70:264-273.

Morrison DK, Davis RJ (2003) Regulation of map kinase signaling modules by scaffold proteins in mammals. Annual Review of Cell and Developmental Biology 19:91-118.

Morton S, Davis RJ, McLaren A, Cohen P (2003) A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. Embo Journal 22:3876-3886.

Moya C, Huang Z, Cheng P, Jayaraman A, Hahn J (2011) Investigation of IL-6 and IL-10 signalling via mathematical modelling. Iet Systems Biology 5:15-26.

Nadeau S, Hein P, Fernandes KJ, Peterson AC, Miller FD (2005) A transcriptional role for C/EBP beta in the neuronal response to axonal injury. Mol Cell Neurosci 29:525-535.

Nagata K, Kiryu-Seo S, Maeda M, Yoshida K, Morita T, Kiyama H (2010) Damage-Induced Neuronal Endopeptidase Is Critical for Presynaptic Formation of Neuromuscular Junctions. Journal of Neuroscience 30:6954-6962.

Nagy JI, Price ML, Staines WA, Lynn BD, Granholm AC (1998) The hyaluronan receptor RHAMM in noradrenergic fibers contributes to axon growth capacity of locus coeruleus neurons in an intraocular transplant model. Neuroscience 86:241-255.

Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H (2003) Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. Journal of Neuroscience 23:5187-5196.

Narumi S, Fujita T (1978) Stimulatory Effects of Substance-P and Nerve Growth Factor (Ngf) on Neurite Outgrowth in Embryonic Chick Dorsal Root-Ganglia. Neuropharmacology 17:73-76.

Nateri AS, Riera-Sans L, Da Costa C, Behrens A (2004) The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. Science 303:1374-1378.

Navarro X, Vivo M, Valero-Cabre A (2007) Neural plasticity after peripheral nerve injury and regeneration. Progress in Neurobiology 82:163-201.

Naveilhan P, ElShamy VM, Emfors P (1997) Differential regulation of mRNAs for GDNF and its receptors Ret and GDNFR alpha after sciatic nerve lesion in the mouse. European Journal of Neuroscience 9:1450-1460.

Neumann S, Woolf CJ (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23:83-91.

Nguyen QT, Sanes JR, Lichtman JW (2002) Pre-existing pathways promote precise projection patterns. Nature Neuroscience 5:861-867.

Nielsen M, Kaestel CG, Eriksen KW, Woetmann A, Stokkedal T, Kaltoft K, Geisler C, Ropke C, Odum N (1999) Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. Leukemia 13:735-738.

Noguchi K, Deleon M, Nahin RL, Senba E, Ruda MA (1993) Quantification of Axotomy-Induced Alteration of Neuropeptide Messenger-Rnas in Dorsal-Root Ganglion Neurons with Special Reference to Neuropeptide-y Messenger-Rna and the Effects of Neonatal Capsaicin Treatment. J Neurosci Res 35:54-66.

Ohba N, Kiryu-Seo S, Maeda M, Muraoka M, Ishii M, Kiyama H (2004) Expression of damage-induced neuronal endopeptidase (DINE) mRNA in peri-infarct cortical and thalamic neurons following middle cerebral artery occlusion. J Neurochem 91:956-964.

Oliveira AL, Thams S, Lidman O, Piehl F, Hokfelt T, Karre K, Linda H, Cullheim S (2004) A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy. Proc Natl Acad Sci U S A 101:17843-17848.

Parkinson DB, Bhaskaran A, Arthur-Farraj P, Noon LA, Woodhoo A, Lloyd AC, Feltri ML, Wrabetz L, Behrens A, Mirsky R, Jessen KR (2008) c-Jun is a negative regulator of myelination. Journal of Cell Biology 181:625-637.

Parrinello S, Napoli I, Ribeiro S, Digby PW, Fedorova M, Parkinson DB, Doddrell RDS, Nakayama M, Adams RH, Lloyd AC (2010) EphB Signaling Directs Peripheral Nerve Regeneration through Sox2-Dependent Schwann Cell Sorting. Cell 143:145-155.

Pearson AG, Gray CW, Pearson JF, Greenwood JA, During MJ, Dragunow M (2003) ATF3 enhances c-Jun-mediated neurite sprouting. Molecular Brain Research 120:38-45.

Pellegrino MJ, Parrish DC, Zigmond RE, Habecker BA (2011) Cytokines inhibit norepinephrine transporter expression by decreasing Hand2. Molecular and Cellular Neuroscience 46:671-680.

Perkins ND (2007) Integrating cell-signalling pathways with NF-kappa B and IKK function. Nature Reviews Molecular Cell Biology 8:49-62.

Perry VH, Brown MC, Gordon S (1987) The Macrophage Response to Central and Peripheral-Nerve Injury - A Possible Role for Macrophages in Regeneration. Journal of Experimental Medicine 165:1218-1223.

Pettmann B, Henderson CE (1998) Neuronal cell death. Neuron 20:633-647.

Polazzi E, Monti B (2010) Microglia and neuroprotection: From in vitro studies to therapeutic applications. Progress in Neurobiology 92:293-315.

Pollock G, Pennypacker KR, Memet S, Israel A, Saporta S (2005) Activation of NF-kappa B in the mouse spinal cord following sciatic nerve transection. Experimental Brain Research 165:470-477.

Popovich PG, Guan Z, Wei P, Huitinga I, van RN, Stokes BT (1999) Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp Neurol 158:351-365.

Pradervand S, Yasukawa H, Muller OG, Kjekshus H, Nakamura T, St Amand TR, Yajima T, Matsumura K, Duplain H, Iwatate M, Woodard S, Pedrazzini T, Ross J, Firsov D, Rossier BC, Hoshijima M, Chien KR (2004) Small proline-rich protein 1A is a gp130 pathway- and stress-inducible cardioprotective protein. Embo Journal 23:4517-4525.

Previtali SC, Feltri ML, Archelos JJ, Quattrini A, Wrabetz L, Hartung HP (2001) Role of integrins in the peripheral nervous system. Progress in Neurobiology 64:35-49.

Putcha GV, Le SY, Frank S, Besirli CG, Clark K, Chu BY, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM (2003) JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron 38:899-914.

Qin QY, Baudry M, Liao GH, Noniyev A, Galeano J, Bi XN (2009) A Novel Function for p53: Regulation of Growth Cone Motility through Interaction with Rho Kinase. Journal of Neuroscience 29:5183-5192.

Qiu J, Cafferty WBJ, McMahon SB, Thompson SWN (2005) Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. Journal of Neuroscience 25:1645-1653.

Quintes S, Goebbels S, Saher G, Schwab MH, Nave KA (2010) Neuron-glia signaling and the protection of axon function by Schwann cells. Journal of the Peripheral Nervous System 15:10-16.

Raivich G (2005) Like cops on the beat: the active role of resting microglia. Trends in Neurosciences 28:571-573.

Raivich G (2008) c-Jun expression, activation and function in neural cell death, inflammation and repair. J Neurochem 107:898-906.

Raivich G (2011) Transcribing the path to neurological recovery-From early signals through transcription factors to downstream effectors of successful regeneration. Annals of Anatomy 193:248-258.

Raivich G, Banati R (2004) Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. Brain Res Rev 46:261-281.

Raivich G, Behrens A (2006) Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain. Progress in Neurobiology 78:347-363.

Raivich G, Bluethmann H, Kreutzberg GW (1996) Signaling molecules and neuroglial activation in the injured central nervous system. Keio J Med 45:239-247.

Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, Hristova M, Nateri AS, Makwana M, Riera-Sans L, Wolfer DP, Lipp HP, Aguzzi A, Wagner EF, Behrens A (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43:57-67.

Raivich G, Bohatschek M, Kloss CUA, Werner A, Jones LL, Kreutzberg GW (1999a) Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Rev 30:77-105.

Raivich G, Dumoulin FL, Streit WJ, Kreutzberg GW (1992) Calcitonin gene-related peptide (CGRP) in the regenerating rat sciatic nerve. Restor Neurol Neurosci 4:107-115.

Raivich G, Gehrmann J, Kreutzberg GW (1991a) Increase of macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor receptors in the regenerating rat facial nucleus. J Neurosci Res 30:682-686.

Raivich G, Hellweg R, Kreutzberg GW (1991b) NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. Neuron 7:151-164.

Raivich G, Jones LL, Kloss CUA, Werner A, Neumann H, Kreutzberg GW (1998) Immune surveillance in the injured nervous system: T-Lymphocytes invade the axotomized mouse facial motor nucleus and aggregate around sites of neuronal degeneration. Journal of Neuroscience 18:5804-5816.

Raivich G, Jones LL, Werner A, Bluthmann H, Doetschmann T, Kreutzberg GW (1999b) Molecular signals for glial activation: pro- and anti-inflammatory cytokines in the injured brain. Acta Neurochir Suppl 73:21-30.

Raivich G, Kreutzberg GW (1994) Pathophysiology of glial growth factor receptors. Glia 11:129-146.

Raivich G, Liu ZQ, Kloss CUA, Labow M, Bluethmann H, Bohatschek M (2002) Cytotoxic potential of proinflammatory cytokines: combined deletion of TNF receptors TNFR1 and TNFR2 prevents motoneuron cell death after facial axotomy in adult mouse. Experimental Neurology 178:186-193.

Raivich G, Makwana M (2007) The making of successful axonal regeneration: genes, molecules and signal transduction pathways. Brain Res Rev 53:287-311.

Ram PT, Iyengar R (2001) G protein coupled receptor signaling through the Src and Stat3 pathway: role in proliferation and transformation. Oncogene 20:1601-1606.

Ramji DP, Foka P (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365:561-575.

Ramji DP, Vitelli A, Tronche F, Cortese R, Ciliberto G (1993) The 2 C/Ebp Isoforms, Il-6Dbp/Nf-Il6 and C/Ebp-Delta/Nf-Il6-Beta, Are Induced by Il-6 to Promote Acute Phase Gene-Transcription Via Different Mechanisms. Nucleic Acids Research 21:289-294.

Rankin SL, Guy CS, Mearow KM (2008) Neurite outgrowth is enhanced by laminin-mediated down-regulation of the low affinity neurotrophin receptor, p75NTR. J Neurochem 107:799-813.

Redmond L, Kashani AH, Ghosh A (2002) Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron 34:999-1010.

Rempe D, Vangeison G, Hamilton J, Li Y, Jepson M, Federoff HJ (2006) Synapsin I cre transgene expression produces germline recombination in male mice progeny. Genesis 44:44-49.

Rende M, Muir D, Ruoslahti E, Hagg T, Varon S, Manthorpe M (1992) Immunolocalization of ciliary neuronotrophic factor in adult rat sciatic nerve. Glia 5:25-32.

Rudolph D, Tafuri A, Gass P, Hammerling G, Arnold B, Schutz G (1998) Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. Proc Natl Acad Sci U S A 95:4481-4486.

Ruff CA, Staak N, Patodia S, Kaswich M, Rocha-Ferreira E, Da Costa C, Brecht S, Makwana M, Fontana X, Hristova M, Rumajogee P, Galiano M, Bohatschek M, Herdegen T, Behrens A, Raivich G (2012) Neuronal c-Jun is required for successful axonal regeneration, but the effects of phosphorylation of its N-terminus are moderate. J Neurochem 121:607-618.

Sachs HH, Rohrer H, Zigmond RE (2010) The conditioning lesion effect on sympathetic neurite outgrowth is dependent on gp130 cytokines. Experimental Neurology 223:516-522.

Sachs HH, Schreiber RC, Shoemaker SE, Sabe A, Reed E, Zigmond RE (2007) Activating transcription factor 3 induction in sympathetic neurons after axotomy: Response to decreased neurotrophin an ailability. Neuroscience 150:887-897.

Salerno KM, Jing X, Diges CM, Cornuet PK, Glorioso JC, Albers KM (2012) Sox11 modulates brain-derived neurotrophic factor expression in an exon promoter-specific manner. J Neurosci Res 90:1011-1019.

Sark MWJ, Fischer DF, de Meijer E, van de Putte P, Backendorf C (1998) AP-1 and ets transcription factors regulate the expression of the human SPRR1A keratinocyte terminal differentiation marker. Journal of Biological Chemistry 273:24683-24692.

Sato T, Takeuchi S, Saito A, Ding W, Bamba H, Matsuura H, Hisa Y, Tooyama I, Urushitani M (2009) Axonal Ligation Induces Transient Redistribution of Tdp-43 in Brainstem Motor Neurons. Neuroscience 164:1565-1578.

Sauer B (1998) Inducible gene targeting in mice using the Cre/lox system. Methods-A Companion to Methods in Enzymology 14:381-392.

Schaefer TS, Sanders LK, Nathans D (1995) Cooperative Transcriptional Activity of Jun and Stat3-Beta, A Short-Form of Stat3. Proc Natl Acad Sci U S A 92:9097-9101.

Scherer SS, Wang DY, Kuhn R, Lemke G, Wrabetz L, Kamholz J (1994) Axons Regulate Schwann-Cell Expression of the Pou Transcription Factor Scip. Journal of Neuroscience 14:1930-1942.

Schwaiger FW, Hager G, Raivich G, Kreutzberg GW (1998) Cellular activation in neuroregeneration. Prog Brain Res 117.

Schwaiger FW, Hager G, Schmitt AB, Horvat A, Hager G, Streif R, Spitzer C, Gamal S, Breuer S, Brook GA, Nacimiento W, Kreutzberg GW (2000) Peripheral but not central axotomy induces changes in Janus kinases (JAK) and signal transducers and activators of transcription (STAT). Eur J Neurosci 12:1165-1176.

Schwarz CS, Seyfried J, Evert BO, Klockgether T, Wullner U (2002) Bcl-2 up-regulates ha-ras mRNA expression and induces c-Jun phosphorylation at Ser73 via an ERK-dependent pathway in PC 12 cells. Neuroreport 13:2439-2442.

Schweizer U, Gunnersen J, Karch C, Wiese S, Holtmann B, Takeda K, Akira S, Sendtner M (2002) Conditional gene ablation of Stat3 reveals differential signaling requirements for survival of motoneurons during development and after nerve injury in the adult. J Cell Biol 156:287-297.

Seijffers R, Allchorne AJ, Woolf CJ (2006) The transcription factor ATF-3 promotes neurite outgrowth. Molecular and Cellular Neuroscience 32:143-154.

Seijffers R, Mills CD, Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. Journal of Neuroscience 27:7911-7920.

Sendtner M, Gotz R, Holtmann B, Escary JL, Masu Y, Carroll P, Wolf E, Brem G, Brulet P, Thoenen H (1996) Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Current Biology 6:686-694.

Sendtner M, Gotz R, Holtmann B, Thoenen H (1997) Endogenous ciliary neurotrophic factor is a lesion factor for axotomized motoneurons in adult mice. Journal of Neuroscience 17:6999-7006.

Shackleford TJ, Zhang Q, Tian L, Vu TT, Korapati AL, Baumgartner AM, Le XF, Liao WS, Claret FX (2011) Stat3 and CCAAT/enhancer binding protein beta (C/EBP-beta) regulate Jab1/CSN5 expression in mammary carcinoma cells. Breast Cancer Res 13:R65.

Shadiack AM, Sun Y, Zigmond RE (2001) Nerve growth factor antiserum induces axotomy-like changes in neuropeptide expression in intact sympathetic and sensory neurons. Journal of Neuroscience 21:363-371.

Shin JE, Cho Y, Beirowski B, Milbrandt J, Cavalli V, DiAntonio A (2012) Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. Neuron 74:1015-1022.

Shubayev VI, Myers RR (2004) Matrix metalloproteinase-9 promotes nerve growth factor-induced neurite elongation but not new sprout formation in vitro. J Neurosci Res 77:229-239.

Siconolfi LB, Seeds NW (2001) Mice lacking tPA, uPA, or plasminogen genes showed delayed functional recovery after sciatic nerve crush. Journal of Neuroscience 21:4348-4355.

Siconolfi LB, Seeds NW (2003) Mice lacking tissue plasminogen activator and urokinase plasminogen activator genes show attenuated matrix metalloproteases activity after sciatic nerve crush. J Neurosci Res 74:430-434.

Skene JHP, Willard M (1981) Characteristics of Growth-Associated Polypeptides in Regenerating Toad Retinal Ganglion-Cell Axons. Journal of Neuroscience 1:419-426.

Smith DS, Skene JHP (1997) A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. Journal of Neuroscience 17:646-658.

Smith PD, Sun F, Park KK, Cai B, Wang C, Kuwako K, Martinez-Carrasco I, Connolly L, He ZG (2009) SOCS3 Deletion Promotes Optic Nerve Regeneration In Vivo. Neuron 64:617-623.

Smith RP, Lerch-Haner JK, Pardinas JR, Buchser WJ, Bixby JL, Lemmon VP (2011) Transcriptional profiling of intrinsic PNS factors in the postnatal mouse. Mol Cell Neurosci 46:32-44.

Sole C, Dolcet X, Segura MF, Gutierrez H, Diaz-Meco MT, Gozzelino R, Sanchis D, Bayascas JR, Gallego C, Moscat J, Davies AM, Comella JX (2004) The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-kappa B signaling. Journal of Cell Biology 167:479-492.

Son YJ, Thompson WJ (1995) Nerve Sprouting in Muscle Is Induced and Guided by Processes Extended by Schwann-Cells. Neuron 14:133-141.

Spira ME, Oren R, Dormann A, Gitler AD (2003) Critical calpain-dependent ultrastructural alterations underlie the transformation of an axonal segment into a growth cone after axotomy of cultured Aplysia neurons. Journal of Comparative Neurology 457:293-312.

Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE, Yancopoulos GD (1995) Choice of Stats and Other Substrates Specified by Modular Tyrosine-Based Motifs in Cytokine Receptors. Science 267:1349-1353.

Starkey ML, Davies M, Yip PK, Carter LM, Wong DJN, McMahon SB, Bradbury EJ (2009) Expression of the Regeneration-Associated Protein SPRR1A in Primary Sensory Neurons and Spinal Cord of the Adult Mouse Following Peripheral and Central Injury. Journal of Comparative Neurology 513:51-68.

Stephanou A, Brar BK, Knight RA, Latchman DS (2000) Opposing actions of STAT-1 and STAT-3 on the Bcl-2 and Bcl-x promoters. Cell Death and Differentiation 7:329-330.

Stern S, Sinske D, Knoll B (2012) Serum response factor modulates neuron survival during peripheral axon injury. J Neuroinflammation 9:78.

Sterneck E, Paylor R, Jackson-Lewis V, Libbey M, Przedborski S, Tessarollo L, Crawley JN, Johnson PF (1998) Selectively enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein delta. Proc Natl Acad Sci U S A 95:10908-10913.

Stoll G, Griffin JW, Li CY, Trapp BD (1989) Wallerian Degeneration in the Peripheral Nervous-System - Participation of Both Schwann-Cells and Macrophages in Myelin Degradation. J Neurocytol 18:671-683.

Streit WJ (1993) Microglial Neuronal Interactions. Journal of Chemical Neuroanatomy 6:261-266.

Streit WJ, Dumoulin FL, Raivich G, Kreutzberg GW (1989) Calcitonin gene-related peptide increases in rat facial motoneurons after peripheral nerve transection. Neurosci Lett 101:143-148.

Streppel M, Azzolin N, Dohm S, Guntinas-Lichius O, Haas C, Grothe C, Wevers A, Neiss WF, Angelov DN (2002) Focal application of neutralizing antibodies to soluble neurotrophic factors reduces collateral axonal branching after peripheral nerve lesion. European Journal of Neuroscience 15:1327-1342.

Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC (1995) Neuronal Pathfinding Is Abnormal in Mice Lacking the Neuronal Growth Cone Protein Gap-43. Cell 80:445-452.

Suarez V, Guntinas-Lichius O, Streppel M, Ingorokva S, Grosheva M, Neiss WF, Angelov DN, Klimaschewski L (2006) The axotomy-induced neuropeptides galanin and pituitary adenylate cyclase-activating peptide promote axonal sprouting of primary afferent and cranial motor neurones. European Journal of Neuroscience 24:1555-1564.

Sun F, Park KK, Belin S, Wang D, Lu T, Chen G, Zhang K, Yeung C, Feng G, Yankner BA, He Z (2011) Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature 480:372-375.

Sun PQ, Enslen H, Myung PS, Maurer RA (1994) Differential Activation of Creb by Ca2+/Calmodulin-Dependent Protein-Kinases Type-Ii and Type-Iv Involves Phosphorylation of A Site That Negatively Regulates Activity. Genes & Development 8:2527-2539.

Sun W, Oppenheim RW (2003) Response of motoneurons to neonatal sciatic nerve axotomy in Bax-knockout mice. Molecular and Cellular Neuroscience 24:875-886.

Sung YJ, Povelones M, Ambron RT (2001) RISK-1: A novel MAPK homologue in axoplasm that is activated and retrogradely transported after nerve injury. Journal of Neurobiology 47:67-79.

Symes A, Gearan T, Eby J, Fink JS (1997) Integration of Jak-Stat and AP-1 signaling pathways at the vasoactive intestinal peptide cytokine response element regulates ciliary neurotrophic factor-dependent transcription. J Biol Chem 272:9648-9654.

Taga T, Kishimoto T (1995) Signaling Mechanisms Through Cytokine Receptors That Share Signal-Transducing Receptor Components. Current Opinion in Immunology 7:17-23.

Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci U S A 94:3801-3804.

Takeda M, Kato H, Takamiya A, Yoshida A, Kiyama H (2000) Injury-specific expression of activating transcription factor-3 in retinal ganglion cells and its colocalized expression with phosphorylated c-Jun. Investigative Ophthalmology & Visual Science 41:2412-2421.

Tamas A, Reglodi D, Farkas O, Kovesdi E, Pal J, Povlishock JT, Schwarcz A, Czeiter E, Szanto Z, Doczi T, Buki A, Bukovics P (2012) Effect of PACAP in Central and Peripheral Nerve Injuries. Int J Mol Sci 13:8430-8448.

Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing NH, Kondoh H (2004) Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. Molecular and Cellular Biology 24:8834-8846.

Taniuchi M, Clark HB, Johnson EM (1986) Induction of Nerve Growth-Factor Receptor in Schwann-Cells After Axotomy. Proc Natl Acad Sci U S A 83:4094-4098.

Tedeschi A, Nguyen T, Puttagunta R, Gaub P, Di Giovanni S (2009) A p53-CBP/p300 transcription module is required for GAP-43 expression, axon outgrowth, and regeneration. Cell Death and Differentiation 16:543-554.

Terenghi G (1999) Peripheral nerve regeneration and neurotrophic factors. Journal of Anatomy 194:1-14.

Tessier-Lavigne M (1998) Axon guidance by netrins and their receptors. Developmental Biology 198:168.

Tessier-Lavigne M (2000) Wiring the brain: Molecular mechanisms of axon guidance. American Journal of Medical Genetics 96:455-456.

Topilko P, Schneidermaunoury S, Levi G, Baronvanevercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 Controls Myelination in the Peripheral Nervous-System. Nature 371:796-799.

Toth CC, Willis D, Twiss JL, Walsh S, Martinez JA, Liu WQ, Midha R, Zochodne DW (2009) Locally Synthesized Calcitonin Gene-Related Peptide Has a Critical Role in Peripheral Nerve Regeneration. Journal of Neuropathology and Experimental Neurology 68:326-337.

Toyota B, Carbonetto S, David S (1990) A Dual Laminin Collagen Receptor Acts in Peripheral-Nerve Regeneration. Proc Natl Acad Sci U S A 87:1319-1322.

Treanor JJS, Goodman L, deSauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, BujBello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A (1996) Characterization of a multicomponent receptor for GDNF. Nature 382:80-83.

Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Molecular and Cellular Neuroscience 15:170-182.

Tucker BA, Mearow KM (2008) Peripheral Sensory Axon Growth: From Receptor Binding to Cellular Signaling. Canadian Journal of Neurological Sciences 35:551-566.

Ugolini G, Raoul C, Ferri A, Haenggeli C, Yamamoto Y, Salaun D, Henderson CE, Kato AC, Pettmann B, Hueber AO (2003) Fas/tumor necrosis factor receptor death signaling is required for axotomy-induced death of motoneurons in vivo. Journal of Neuroscience 23:8526-8531.

Valero-Cabre A, Navarro X (2002) Functional impact of axonal misdirection after peripheral nerve injuries followed by graft or tube repair. Journal of Neurotrauma 19:1475-1485.

Vargas ME, Barres BA (2007) Why is Wallerian degeneration in the CNS so slow? Annual Review of Neuroscience 30:153-179.

Vinciguerr M, Esposito L, Salzano S, Madeo A, Nagel G, Maggiolini M, Gallo A, Musti AM (2008) Negative charged threonine 95 of c-Jun is essential for c-Jun N-terminal kinase-dependent phosphorylation of threonine 91/93 and stress-induced c-Jun biological activity. International Journal of Biochemistry & Cell Biology 40:307-316.

Vogelezang MG, Liu ZQ, Relvas JB, Raivich G, Scherer SS, ffrench-Constant C (2001) alpha 4 Integrin is expressed during peripheral nerve regeneration and enhances neurite outgrowth. Journal of Neuroscience 21:6732-6744.

Vogelin E, Baker JM, Gates J, Dixit V, Constantinescu MA, Jones NF (2006) Effects of local continuous release of brain derived neurotrophic factor (BDNF) on peripheral nerve regeneration in a rat model. Experimental Neurology 199:348-353.

Vries RGJ, Prudenziati M, Zwartjes C, Verlaan M, Kalkhoven E, Zantema A (2001) A specific lysine in c-Jun is required for transcriptional repression by E1A and is acetylated by p300. Embo Journal 20:6095-6103.

Waetzig V, Zhao Y, Herdegen T (2006) The bright side of JNKs - Multitalented mediators in neuronal sprouting, brain development and nerve fiber regeneration. Progress in Neurobiology 80:84-97.

Waller AV (1850) 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. Philos Trans R Soc London 140:423-429.

Wallquist W, Patarroyo M, Thams S, Carlstedt T, Stark B, Cullheim S, Hammarberg H (2002) Laminin chains in rat and human peripheral nerve: Distribution and regulation during development and after axonal injury. Journal of Comparative Neurology 454:284-293.

Wang YN, Chen YJ, Chang WC (2006) Activation of extracellular signal-regulated kinase signaling by epidermal growth factor mediates c-jun activation and p300 recruitment in keratin 16 gene expression. Molecular Pharmacology 69:85-98.

Weber JRM, Skene JHP (1998) The activity of a highly promiscuous AP-1 element can be confined to neurons by a tissue-selective repressive element. Journal of Neuroscience 18:5264-5274.

Wen TC, Peng H, Hata R, Desaki J, Sakanaka M (2001) Induction of phosphorylated-Stat3 following focal cerebral ischemia in mice. Neuroscience letters 303:153-156.

Weng GH, Markus MA, Markus A, Winkle A, Borasio GD (1996) P21(ras) supports the survival of chick embryonic motor neurones. Neuroreport 7:1077-1081.

Werner A, Kloss CU, Walter J, Kreutzberg GW, Raivich G (1998) Intercellular adhesion molecule-1 (ICAM-1) in the mouse facial motor nucleus after axonal injury and during regeneration. J Neurocytol 27:219-232.

Werner A, Willem M, Jones LL, Kreutzberg GW, Mayer U, Raivich G (2000) Impaired axonal regeneration in alpha7 integrin-deficient mice. The Journal of neuroscience 20:1822-1830.

West MJ (2002) Design-based stereological methods for counting neurons. Do Seizures Damage the Brain 135:43-51

Westerlund N, Zdrojewska J, Padzik A, Komulainen E, Bjorkblom B, Rannikko E, Tararuk T, Garcia-Frigola C, Sandholm J, Nguyen L, Kallunki T, Courtney MJ, Coffey ET (2011) Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. Nature Neuroscience 14:305-313.

Weston CR, Davis RJ (2007) The JNK signal transduction pathway. Current Opinion in Cell Biology 19:142-149.

Williams KL, Rahimtula M, Mearow KM (2006) Heat shock protein 27 is involved in neurite extension and branching of dorsal root ganglion neurons in vitro. J Neurosci Res 84:716-723.

Wilton D, Arthur-Farraj P, Latouche M, Turmaine M, Behrens A, Mirsky R, Raivich G, Jessen K (2009) Schwann Cell C-Jun Is Essential for Peripheral Nerve Regeneration. Glia 57:S158.

Wood MD, Gordon T, Kemp SW, Liu EH, Kim H, Shoichet MS, Borschel GH (2012) Functional motor recovery is improved due to local placement of GDNF microspheres after delayed nerve repair. Biotechnol Bioeng1-10.

Wood MD, Gordon T, Kim H, Szynkaruk M, Phua P, Lafontaine C, Kemp SW, Shoichet MS, Borschel GH (2013) Fibrin gels containing GDNF microspheres increase axonal regeneration after delayed peripheral nerve repair. Regen Med 8:27-37.

Woolley AL, Hollowell JP, Rich KM (1990) Fibronectin-Laminin Combination Enhances Peripheral-Nerve Regeneration Across Long Gaps. Otolaryngology-Head and Neck Surgery 103:509-518.

Wu DS, Zhang Y, Bo XN, Huang WL, Xiao F, Zhang XY, Miao TZ, Magoulas C, Subang MC, Richardson PM (2007) Actions of neuropoietic cytokines and cyclic AMP in regenerative conditioning of rat primary sensory neurons. Experimental Neurology 204:66-76.

Wu YY, Bradshaw RA (1996) Induction of neurite outgrowth by interleukin-6 is accompanied by activation of Stat3 signaling pathway in a variant PC12 cell (E2) line. Journal of Biological Chemistry 271:13023-13032.

Wynick D (2001) Response: can galanin also be considered as growth-associated protein 3.2? Trends in Neurosciences 24:496.

Xia XG, Hofmann HD, Deller T, Kirsch M (2002) Induction of STAT3 signaling in activated astrocytes and sprouting septal neurons following entorhinal cortex lesion in adult rats. Molecular and Cellular Neuroscience 21:379-392.

Xu XJ, Wiesenfeldhallin Z, Villar MJ, Fahrenkrug J, Hokfelt T (1990) On the Role of Galanin, Substance-P and Other Neuropeptides in Primary Sensory Neurons of the Rat - Studies on Spinal Reflex Excitability and Peripheral Axotomy. European Journal of Neuroscience 2:733-743.

Yamashita T, Higuchi H, Tohyama M (2002) The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. Journal of Cell Biology 157:565-570.

Yang E, Lerner L, Besser D, Darnell JE, Jr. (2003) Independent and cooperative activation of chromosomal c-fos promoter by STAT3. J Biol Chem 278:15794-15799.

Yang P, Wen H, Ou S, Cui J, Fan D (2012) IL-6 promotes regeneration and functional recovery after cortical spinal tract injury by reactivating intrinsic growth program of neurons and enhancing synapse formation. Exp Neurol 236:19-27.

Yetiser S, Kahraman E (2008) An analysis of time-dependent changes of neurotrophic factors (BDNF, CNTF) in traumatic facial nerve injury of a nerve-cut and nerve-crush model in rats. Otology & Neurotology 29:392-396.

Yoshida K, Behrens A, Le-Niculescu H, Wagner EF, Harada T, Imaki J, Ohno S, Karin M (2002) Amino-terminal phosphorylation of c-Jun regulates apoptosis in the retinal ganglion cells by optic nerve transection. Investigative Ophthalmology & Visual Science 43:1631-1635.

Yukawa K, Tanaka T, Tsuji S, Akira S (1998) Expressions of CCAAT/enhancer-binding proteins beta and delta and their activities are intensified by cAMP signaling as well as Ca2+/calmodulin kinases activation in hippocampal neurons. Journal of Biological Chemistry 273:31345-31351.

Zhang C, Li H, Liu MG, Kawasaki A, Fu XY, Barnstable CJ, Zhang SSM (2008) STAT3 activation protects retinal ganglion cell layer neurons in response to stress. Experimental Eye Research 86:991-997.

Zhang M, Yannas IV (2005) Peripheral nerve regeneration. Adv Biochem Eng Biotechnol 94:67-89.

Zhang XK, Wrzeszczynska MH, Horvath CM, Darnell JE (1999) Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. Molecular and Cellular Biology 19:7138-7146.

Zhang ZJ, Soucacos PN, Beris AE, Bo JY, Ioachim E, Johnson EO (2000) Long-term evaluation of rat peripheral nerve repair with end-to-side neurorrhaphy. Journal of Reconstructive Microsurgery 16:303-311.

Zhao ZQ, Alam S, Oppenheim RW, Prevette DM, Evenson A, Parsadanian A (2004) Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. Experimental Neurology 190:356-372.

Zhong J, Dietzel ID, Wahle P, Kopf M, Heumann R (1999) Sensory impairments and delayed regeneration of sensory axons in interleukin-6-deficient mice. Journal of Neuroscience 19:4305-4313.

Zhu Y, Romero MI, Ghosh P, Ye ZY, Charnay P, Rushing EJ, Marth JD, Parada LF (2001) Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. Genes & Development 15:859-876.

Zigmond RE (2011) gp130 cytokines are positive signals triggering changes in gene expression and axon outgrowth in peripheral neurons following injury. Front Mol Neurosci 4:62.

Zigmond RE, Sun Y (1997) Regulation of neuropeptide expression in sympathetic neurons - Paracrine and retrograde influences. Neuropeptides in Development and Aging 814:181-197.

Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D (1998) Degradation of chondroitin sulfate proteoglycan enhances the neurite-promoting potential of spinal cord tissue. Experimental Neurology 154:654-662.

Appendix

Role of transcription factors in peripheral nerve regeneration

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Following axotomy, the activation of multiple intracellular signaling cascades causes the expression of a cocktail of regeneration-associated transcription factors which interact with each other to determine the fate of the injured neurons. The nerve injury response is channeled through manifold and parallel pathways, integrating diverse inputs, and controlling a complex transcriptional output. Transcription factors form a vital link in the chain of regeneration, converting injury-induced stress signals into downstream protein expression via gene regulation. They can regulate the intrinsic ability of axons to grow, by controlling expression of whole cassettes of gene targets. In this review, we have investigated the functional roles of a number of different transcription factors – c-Jun, activating transcription factor 3, cAMP response element binding protein, signal transducer, and activator of transcription-3, CCAAT/enhancer binding proteins β and δ, Oct-6, Sox11, p53, nuclear factor kappa-light-chain-enhancer of activated B cell, and ELK3 – in peripheral nerve regeneration. Studies involving use of conditional mutants, microarrays, promoter region mapping, and different injury paradigms, have enabled us to understand their distinct as well as overlapping roles in achieving anatomical and functional regeneration after peripheral nerve injury.

Keywords: transcription, axotomy, facial nerve, neurite, DRG, regeneration, c-Jun, ATF3, STAT3

INTRODUCTION

Injury to peripheral nerves elicits a sequence of molecular, cellular, and ultrastructural responses which are vital in triggering a successful regenerative program associated with neurite outgrowth, re-innervation of the denervated target and recovery of function. The rapid arrival of signals from the injured axon results in a remarkable shift of the injured neuron from a transmitting to a growth promoting phenotype. This is accomplished by the upregulation of a vast array of regeneration-associated genes (RAGs) including the rapid induction of transcription factors and enhanced synthesis of adhesion molecules, cytoskeletal elements, growth factors, cytokines, neuropeptides, and other molecules involved in regeneration.

The molecular changes are accompanied by pronounced morphological changes in the surviving neurons – the cell body undergoes a "chromatolytic" reaction characterized by swelling of the neuronal body, increase in cellular metabolism and protein synthesis, and a regional dispersion of Nissl bodies in the neuronal cytoplasm (Lieberman, 1971); and there is a rapid appearance of growth cones at the proximal tip of the lesioned axons. The distal nerve stump undergoes "Wallerian degeneration" (Waller, 1850; Dubový, 2011), leading to phagocytosis of axonal and myelin debris by Schwann cells and later also by invading macrophages.

In addition to the neuronal response, neighboring non-neuronal glial cells – in the case of brain and spinal cord motoneurons, the astrocytes, and microglia – become activated, with microglial expression of immune cell recognition molecules (ICAM1, aMb2, and aXb2 integrins, B7 system, etc.) and major histocompatibility complex (Werner et al., 1998; Bohatschek et al.,

2004). There is also a rapid microglia-associated recruitment of lymphocytes within a day after injury which may assist with immune surveillance first in the absence, and later on in the presence of neuronal cell death (Raivich et al., 1998; Kalla et al., 2001). In the adult mouse facial motor nucleus model which has been studied in extensive detail, neuronal cell death is a delayed phenomenon, peaking at 2 weeks following nerve transection; the glioimmune activation is characterized by two phases – a fast but moderate response starting within 24 h after neuronal injury, and a late but much stronger response to neuronal cell death, peaking at 14 days after axotomy. Neuronal transcription factor deletions blocking cell death will suppress most of the late glioimmune response (Raivich et al., 2004; Patodia et al., 2011). However, these deletions also inhibit some of the early microglial activation – within 24 h and long before the appearance of cell death, suggesting that these transcription factors may also interfere with the synthesis of injury signals from lesioned but not dying neurons.

In contrast with the PNS, the CNS neurons normally fail to regenerate after injury, possibly due to a combination of diminished intrinsic capacity for regeneration and a heightened susceptibility to increased inhibitory factors of their extracellular environment (Maier and Schwab, 2006; Sun et al., 2011). Identifying the crucial elements responsible for successful regeneration in injured peripheral nerves, and unraveling their underlying signaling pathways and molecular components will be quintessential in improving regenerative outcomes after peripheral and central nerve injuries.

Systematic gene expression profiling using cDNA microarrays has led to a dramatic increase in the number of identified genes

regulated in the injured and regenerating neurons (Bosse et al., 2001, 2006; Costigan et al., 2002; Kubo et al., 2002; Boeshore et al., 2004; Kury et al., 2004; Michaelevski et al., 2010). Interestingly, roughly half of the RAGs are part of both developmental and lesion-induced programs, suggesting that regeneration partially recapitulates development. However, this proportion of shared developmental and lesion-induced transcripts was substantially lower – only 33% – for genes encoding signal transducers or factors involved in processes such as cell death, immune response, transport, and transcriptional regulation. Most of the latter show injury-specific gene expression (Bosse et al., 2006).

Transcription factors are DNA binding proteins that can activate or repress target gene expression. Their effects are amplified by their ability to bind to multiple promoter regions, causing a large number of genes to be switched on or off by a single transcription factor. In this review, we will be discussing the key molecular events ensuing peripheral nerve injury, focusing on various transcription factors that have been implicated to play a role in neuronal regeneration.

EARLY AXONAL INJURY SENSORS

Axonal injury generates three major signaling cues to the injured neurons:

A. It interferes with the retrograde flow of trophic signals, disinhibiting the normally suppressed regenerative process, within 12–24 h following injury (Raivich et al., 1991).

B. It exposes the tip of the injured axons to the intracellular content of neighboring axons and Schwann cells containing growth factors like LIF, CNTF, FGF, and NT3 (Elde et al., 1991; Sendtner et al., 1997; Kirsch et al., 2003), and later to the inflamed neural tissue environment (Lindholm et al., 1987). The above two processes can result in *de novo* activated molecules carrying a nuclear localization sequence (NLS) which link to importins and are retrogradely transported to the cell body by dynein motors (Schmied et al., 1993; Hanz et al., 2003).

C. It causes a disruption of the tight ionic concentration gradient between the axon and the extracellular matrix by rapid influx of extracellular ions such as calcium and sodium through the transiently open plasmalemma before it is resealed (Yoo et al., 2003). This results in depolarization and transmission of successive injury-mediated action potentials. These molecular and electrical signals cause rapid elevation of calcium and cAMP, which in turn activate multiple downstream pathways (Berdan et al., 1993).

TRANSCRIPTIONAL CHANGES

The arrival of injury signals is rapidly followed by the phosphorylation and nuclear localization of a host of transcription factors. A brief summary of signaling from early sensors to mostly enzymatic cytoplasmic mediators to transcription factors and synthesis of effector molecules is shown in **Figure 1**. Data from phospho-proteomic and microarray studies
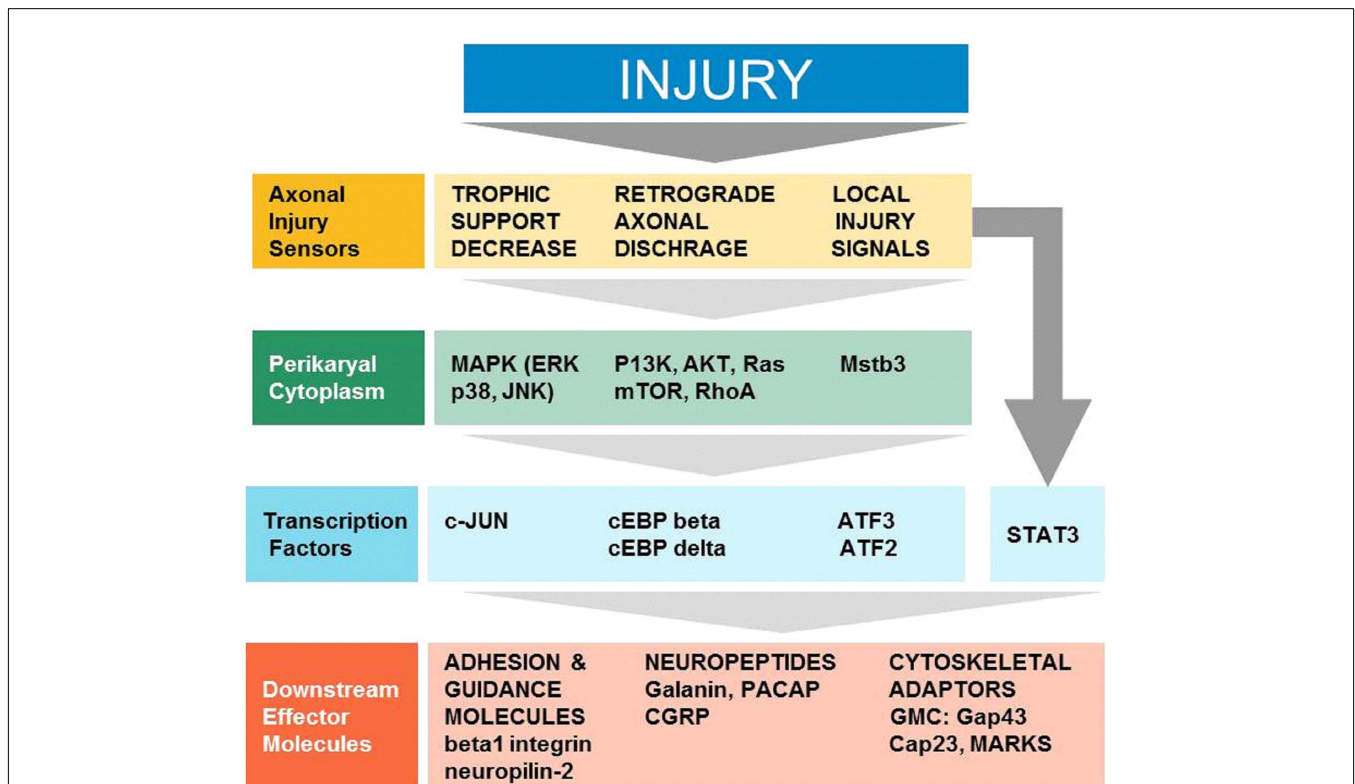

FIGURE 1 | Cellular signaling in successful regeneration, from early sensors of injury, to cytoplasmic signals, transcription, and downstream effectors (**Modified** Raivich, 2011).

have identified nearly 400 redundant axonal signaling networks connected to 39 transcription factors (26 transcription factor families), implicated in the sensory neuron response to axonal injury (Michaelevski et al., 2010). These include c-Jun, Jun D, activating transcription factor 3 (ATF3), cAMP response element binding protein (CREB), signal transducer and activator of transcription (STAT3), CCAAT/enhancer binding proteins (C/EBPs), p53, Oct-6, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), nuclear factor of activated T-cells (NFATs), Kruppel-like factors (KLFs), Sox11, SnoN, ELK3, P311, and E47 among others (Schwaiger et al., 2000; Mason et al., 2003; Raivich et al., 2004; Nadeau et al., 2005; Di Giovanni et al., 2006; Jankowski et al., 2009; Ruff et al., 2009; Magoulas and Lopez-de Heredia, 2010; Moore and Goldberg, 2011; Raivich, 2011). Once in the nucleus, they bind to selective DNA promoter regions to increase or repress transcription of specific target genes. Nerve injury can also result in reduced activation of transcription factors such as islet-1, Fra-2, ATF2, and TDP43 (Doyle and Hunt, 1997; Herdegen et al., 1997a; Hol et al., 1999; Moisse et al., 2009; Sato et al., 2009), which probably contributes to the change in gene expression of the injured neuron from a fully differentiated to a growing phenotype (Raivich, 2011). The activation of transcriptional program is critical for expression of many target genes implicated in successful regeneration, and blocking of transcription at an early time point after injury changes the regenerative response of injured neurons (Smith and Skene, 1997). Recent advances in cre/loxP technology permitting cell-type and/or time specific genetic knockouts (Sauer, 1998; Akira, 2000) have begun to provide insight into the powerful roles of these transcription factors in orchestrating complex axon growth and regenerative responses. An overview of the observed phenotypes is given in **Table 1**.

C-Jun

The AP-1 transcription complex is a well-characterized regulator of neural development and consists of homo- or hetero-dimeric complexes between members of the Jun, c-Fos, and ATF/CREB families. A primary component of the AP-1 complex, c-Jun, is produced as an immediate early gene (IEG) following nerve injury and persists at high levels in injured neurons during the entire peripheral regenerative process (Herdegen et al., 1991; Kenney and Kocsis, 1998; Mason et al., 2003; Raivich et al., 2004; Lindwall and Kanje, 2005; Ruff et al., 2009). Schwann cells also strongly upregulate c-Jun following axotomy, where it functions as a negative regulator of myelination and switches on local inflammation (Arthur-Farraj et al., 2007; Latouche et al., 2009; Wilton et al., 2009). Numerous studies, using targeted gene deletions or pharmacological inhibition, have examined the activation, function, and cellular basis

Table 1 | Transcription factor deletions and peripheral nerve regeneration: effects of global (G) and cell-type specific (cts) knockouts (KO).

Transcription factor G KO or CTS KO	KO phenotype	Reference
Neuronal c-Jun (CTS)	Strongly reduced target re-innervation	Raivich et al. (2004), Makwana et al. (2010), Ruff et al. (2009)
	Delayed functional recovery	
	Decreased RAG expression and neuronal sprouting	
	Strongly reduced glial activation and leukocyte recruitment	
	Enhanced motoneuron survival	
	Cellular atrophy	
Schwann cell c-Jun (CTS)	Severely impaired axonal regeneration	Ruff et al. (2009)
	Drastically reduced motoneuron survival	
Jun 2A (G)	Cellular atrophy	Ruff et al. (2009)
Jun 4A (G)	Moderately reduced target re-innervation and functional recovery	Patodia et al. (unpublished)
	Cellular atrophy	
ATF3 (G)	Enhanced speed of regeneration in mice constitutively expressing ATF3 in DRGs	Seijffers et al. (2007)
CREB (G)	Impaired axonal/neurite growth	Lonze et al. (2002), Lonze and Ginty (2002), Redmond et al. (2002)
	Increased apoptosis of sensory neurons	
Neuronal STAT3 (CTS)	Strongly reduced target re-innervation	Bareyre et al. (2011), Patodia et al. (2011)
	Delayed functional recovery	
	Decreased RAG expression and neuronal sprouting	
	Strongly reduced glial activation and leukocyte recruitment	
	Enhanced motoneuron survival	
	Cellular atrophy	
C/EBPβ (G)	Reduced expression of microtubule Tα1 α tubulin and growth cone protein GAP-43	Nadeau et al. (2005)
C/EBPδ (G)	Impaired axonal growth and reduced response to conditioning lesion	Magoulas and Lopez-de Heredia (2010), Patodia et al. (unpublished)
	Reduced microglial activation	
Sox 11 (CTS)	Reduced regeneration after nerve crush	Jankowski et al. (2009)
p53 (G)	Growth cone collapse	Di Giovanni et al. (2006), Tedeschi et al. (2009), Qin et al. (2009)
	Decreased neurite outgrowth	
	Reduced target re-innervation	

of c-Jun and suggest a tripartite role of c-Jun action in neural degeneration, inflammation, and repair (Raivich, 2008).

Nestin-cre mediated deletion of c-Jun prevented the upregulation of axotomy-associated molecules (CD44, α7β1 integrin, galanin). It also decreased perineuronal sprouting (Makwana et al., 2010) and successful reconnection to peripheral targets by four to fivefold, and strongly reduced functional recovery (Raivich et al., 2004). This massive reduction in target re-innervation was also observed more than 3 months after facial nerve cut, suggesting that it was a persistent defect, rather than just a delay in the speed of regeneration. Incidentally, functional recovery did not appear to require re-innervation of the target by the same number of neurons. Compared with the Jun-competent littermates, functional recovery showed a longer but nevertheless transient delay, suggesting that long term, target re-innervation by a fraction of neurons could produce extensive functional recovery (Raivich et al., 2004). Importantly, though functional recovery is clinically the critical parameter, a sole reliance on this parameter could overestimate the extent of re-innervation. Non-neuronal responses like leukocyte recruitment and microglial activation were also severely compromised in the mutants. Interestingly, the facial motoneurons in the mutants showed enhanced post-axotomy survival, but were severely shrunken (Raivich et al., 2004), supporting the previously reported functional dichotomy of c-Jun in promoting post-traumatic neuronal cell death as well as axonal regeneration (Herdegen et al., 1997b; Herdegen and Waetzig, 2001).

Because this *cre* Recombinase was driven by a nestin-promoter expressed in early neuroepithelial cells giving rise to neurons, astrocytes, or oligodendrocytes, as well as Schwann cells it is raised the question whether the effects observed in neuronal regeneration were due to the absence of c-Jun in neurons or other nestin+ progenitor-derived cells. This issue was addressed by using transgenic mice having neuron-specific (synapsin promoter controlled cre-recombination) or Schwann cell-specific [myelin protein zero (P0) promoter controlled cre-recombination] c-Jun deletions. Neuron-specific c-Jun deletion mirrored the regeneration defect seen with nestin-cre mediated deletion of c-Jun, i.e., strongly reduced speed of axonal regeneration, significantly delayed functional recovery, strongly reduced target re-innervation, enhanced motoneuron survival but cellular atrophy and diminished astrocyte and microglial activation and T-cell influx (Ruff et al., 2009). The absence of c-Jun in Schwann cells also caused severely impaired axonal regeneration. However, it strongly increased neuronal cell death after nerve injury (Ruff et al., 2009). These results suggest a critical role of both Schwann cell and neuronal c-Jun in the axonal injury response, which led to the next challenge of understanding the mechanisms underlying its activation and signaling following axonal injury.

Activation of c-Jun mediated transcription is affected by interactions at three major sites: N-terminal phosphorylation at serines 63 and 73 and threonines 91 and 93 by the Jun N-terminal kinases (JNKs) and the ensuing ubiquitination and degradation, dephosphorylation of Thr239, and C-terminal lysine acetylation near aa 257–276 (Vries et al., 2001; Morton et al., 2003). The JNKs are rapidly activated following peripheral nerve injury, and are retrogradely transported to the cell body along with the upstream

kinases MEKK1 and p-MKK4, as well as the JNK interacting protein (JIP), a scaffold protein that stabilizes the interaction between JNK and its upstream kinases (Lindwall and Kanje, 2005), reviewed in Raivich (2008). Neuronal injury increases JNK-mediated c-Jun phosphorylation (Herdegen et al., 1998; Kenney and Kocsis, 1998), and application of a JNK inhibitor to explants of injured DRGs reduces c-Jun phosphorylation, ATF3 expression, and neurite outgrowth, without affecting survival (Lindwall et al., 2004).

Removal of Ser63 and 73 phosphoacceptor sites in the JunAA mutant interfered with kainic acid excitotoxicity in hippocampal neurons (Behrens et al., 1999) at levels similar to JNK3 KO (Brecht et al., 2005), identifying c-Jun as an essential substrate of JNK signaling during kainate-induced neuronal apoptosis. While global c-Jun deletion is embryonic lethal, JunAA mice showed normal development (albeit slightly smaller than littermate wild-type mice) and were fertile (Behrens et al., 1999). Following optic nerve transection, there was a partial but significant reduction of the apoptosis of retinal ganglion cells in JunAA mice, and Fas L has been suggested to be an important target gene regulated by N-terminal Jun phosphorylation during apoptosis (Yoshida et al., 2002). JunAA and JNK3 null mutants also showed better neuronal survival after targeted dopaminergic cell death (Brecht et al., 2005; Crocker et al., 2006). Trophic factor deprivation or DNA damage-induced death was significantly delayed in JunAA neurons which correlated with delayed expression of pro-apoptotic genes (Besirli et al., 2005). In contrast, the JNK3 KO or JunAA mutations did not interfere with neuronal cell death after facial axotomy (Brecht et al., 2005; Ruff et al., 2009). This difference could be attributed to the fact that facial axotomy affects peripheral neurons, unlike previous studies which discuss effects of phosphorylation deficient c-Jun on apoptosis of CNS neurons.

Preliminary studies from our group after facial axotomy revealed that JunAA mice do show some neuronal shrinkage, but have no effect on speed of axonal regeneration for fastest axons, functional recovery, or target re-innervation (Ruff et al., 2009). Deletion of JNK1 or JNK3 single genes showed somewhat delayed functional recovery after facial nerve axotomy but the effects were quite moderate; and deletion of JNK2 had no effect on regeneration (Ruff et al., 2009). The mild effects of JunAA and global JNK deletions, and the fact that JNKs can act via c-Jun independent cytoplasmic (stathmin, MAP1b, MAP2) and nuclear (e.g., ATF2, ELK1, p53, etc.) targets (Björkblom et al., 2005; Bogoyevitch and Kobe, 2006; Barnat et al., 2010; Westerlund et al., 2011), points to a possible parting of ways: i.e., the observed JNK null effects on regeneration are c-Jun independent, and the strong Jun-dependent effects on regeneration and survival do not require N-terminal phosphorylation. Recent studies using Jun4A mice where all four JNK dependent N-terminal phosphorylation sites – Thr91 and 93 as well as Ser63 and 73 – are removed (Patodia et al., unpublished observations) partially confirm this notion. The Jun4A mutation does not interfere with the expression of injury-associated neuronal and non-neuronal markers. On the other hand, as in the JNK1 and 3 null mutants, the Jun4A strain reproduces the moderate reduction in functional recovery and a 40% lower number of reinnervating motoneurons at 28 days following facial nerve cut. However, this effect on regeneration is considerably smaller than that observed for neuronal c-Jun deletion, suggesting that

the regeneration effect of c-Jun is mostly independent of its N-terminal phosphorylation. At present, no information is available with regards to the regeneration-associated Jun effects of Thr239 dephosphorylation, or for C-terminal acetylation.

ACTIVATING TRANSCRIPTION FACTOR 3

Activating transcription factor 3 is a member of the ATF/CREB family of basic leucine zipper domain (bZIP) transcription factors. It can form homo- or hetero-dimers with other bZIP transcription factors like members of the AP-1 family (ATF2, JunB, JunD, c-Jun, c-fos) and c/EBPs (Hai and Curran, 1991; Hai and Hartman, 2001). ATF3 is normally expressed at low levels, but is rapidly activated in sensory, sympathetic, and motor neurons by stress stimuli after peripheral nerve injury (Tsujino et al., 2000; Raivich and Behrens, 2006; Hyatt Sachs et al., 2007; Zigmond and Vaccariello, 2007), optic nerve injury (Takeda et al., 2000), and NGF depletion (Mayumi-Matsuda et al., 1999) but not after central injury (Tsujino et al., 2000). ATF3 expression is also induced in the disconnected Schwann cells and endoneurial fibroblasts distal to the peripheral injury site, though not on central, denervated glia (Hunt et al., 2004). Several pathways have been implicated in activating ATF3 expression such as neurotrophin deprivation (Hyatt Sachs et al., 2007), as well as JNK/SAPK and p53-dependent mechanisms (Moore and Goldberg, 2011). ATF3 is axonally transported from the periphery (Lindwall and Kanje, 2005), possibly acting as a retrograde signal.

Delivery of ATF3 to adult DRG neurons, neonatal SCG neurons and PC12 cells, enhanced both the number of neurons extending neurites and neurite length (Nakagomi et al., 2003; Pearson et al., 2003; Seijffers et al., 2006). As after conditioning peripheral injury, the pattern of growth consisted of long neurites rather than branched arborized ones (Seijffers et al., 2007). In transgenic mice constitutively expressing ATF3 in adult DRGs, the rate of peripheral nerve regeneration (measured by the nerve pinch test 2 days after sciatic nerve crush) was enhanced to an extent comparable to that produced by a preconditioning lesion (Seijffers et al., 2007). The expression of some growth-associated genes, such as Hsp27, SPRR1A, and c-Jun was increased in the non-injured neurons, but not of others like the alpha 7 integrin subunit (α7), GAP-43, CAP23, and STAT3. Unlike peripheral nerve-conditioning lesions, ATF3 overexpression also did not overcome the inhibition produced by CNS myelin in culture, suggesting the mechanisms responsible for axonal regeneration are separate from those involved in overcoming glial-associated inhibition (Seijffers et al., 2007).

Activating transcription factor 3 can bind directly to the Hsp27 promoter and activate its expression in PC12 cells (Nakagomi et al., 2003). In addition to its role as an injury-induced neuronal survival factor, Hsp27 enhances neurite outgrowth in cultured adult DRG neurons (Williams et al., 2006), possibly as a result of interactions with the cytoskeleton. The growth-associated gene SPRR1A promotes axonal outgrowth by interacting with actin structures (Bonilla et al., 2002) and ATF3 may regulate its expression either by binding directly to its promoter or by affecting other transcription factors. ATF3 also appears to have a survival role, preventing JNK-mediated neuronal death (Nakagomi et al., 2003). Thus, although ATF3 appears to contribute to nerve regeneration by increasing

the intrinsic growth state of injured neurons, its upregulation is not enough to fully recapitulate the peripheral nerve regeneration program (Seijffers et al., 2007).

Activating transcription factor 3 can form hetero-dimers with c-Jun, leading to enhanced transcriptional activation of various RAGs and increased neurite outgrowth in neuronal cell lines (Hai and Curran, 1991; Hai and Hartman, 2001; Pearson et al., 2003). There has been a number of conflicting reports regarding the correlation between increased ATF3 expression and upregulation of c-Jun in surviving neurons. In 2003, Pearson et al., showed that in the absence of c-Jun, ATF3 failed to enhance neurite outgrowth in PC12 cells, suggesting that a coincident presence of c-Jun and ATF3 may act synergistically (possibly via a physical interaction) to promote nerve regeneration (Pearson et al., 2003; Lindwall et al., 2004). In contrast, Seijffers et al. (2006) showed enhanced neurite outgrowth in adult DRG neurons by ATF3 without any increase in endogenous c-Jun levels. The scope of c-Jun and ATF3 expression in DRG neurons after sciatic nerve transection also differs: ATF3 is induced in all injured neurons, phosphorylated c-Jun only in a small subset of mostly small and medium sized DRG neurons (Kenney and Kocsis, 1998; Tsujino et al., 2000; Lindwall et al., 2004). Sciatic nerve injury induces prolonged activation of JNKs in the DRGs (Kenney and Kocsis, 1998). Inhibiting JNK in this model led to decreased activation of c-Jun and ATF3 expression, and to decreased axonal growth (Lindwall et al., 2004). Recent results from our group show that deletion of neuronal c-Jun or STAT3 interferes with the nuclear transfer of ATF3 in the axotomized facial motor neurons (Patodia and Raivich, unpublished). This effect may be specific for motoneurons – in sympathetic neurons, cell-specific deletion of gp130 interferes with the appearance of phosphorylated nuclear STAT3, but does not affect the nuclear translocation of ATF3 (Habecker et al., 2009). Thus, the extent to which the growth promoting action of ATF3 is autonomous of c-Jun and STAT3 and dependent on additional injury signals needs to be explored.

cAMP RESPONSE ELEMENT BINDING PROTEIN

The CREB is a member of the ATF/CREB family of bZIP transcription factors (Hai and Hartman, 2001), and mediates cAMP signaling in the nervous system by forming homo- or hetero-dimers with other bZIP factors (Hannila and Filbin, 2008). The brains of CREB null mice show abnormal development of the corpus callosum and anterior commissure (Rudolph et al., 1998). CREB can be phosphorylated and activated by many kinases, including PKA, PKC, CAMKII, CAMKIV, AKT, MAPKAP K2, and members of RSK and MSK families (Mayr and Montminy, 2001), and it is negatively regulated by phosphatases such as PP1 and PP2A (Sun et al., 1994). Important targets of CREB include arginase I and BDNF (Mayr and Montminy, 2001; Cai et al., 2002; Deng et al., 2009).

In vitro cultured DRG and SCG neurons from CREB null mice show shorter neurites than controls. *In vivo*, these mutant mice exhibit impaired axonal growth and projections. Their sensory neurons undergo excess apoptosis and degeneration during the period of NGF dependency in the absence of CREB (Lonze and Ginty, 2002; Lonze et al., 2002). In addition, overexpression of a dominant negative CREB leads to decreased dendritic outgrowth

in cortical neurons and loss of cAMP or neurotrophin associated neurite outgrowth on an inhibitory substrate (Redmond et al., 2002). This suggests an important role for CREB-mediated gene expression for both survival and axonal growth in PNS neurons. Recently, it has been suggested that axonal translation of CREB mRNAs may precede its phosphorylation, nuclear translocation, and transcriptional activation of pro-survival genes (Cox et al., 2008).

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION-3

Signal transducer and activator of transcription-3 belongs to the STAT family of seven transcription factors – STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 – that mediate a wide variety of biological functions in the CNS and PNS such as cell growth, regulation, inflammation, and embryological development (Dziennis and Alkayed, 2008). The STATs are comprised of six regular domains – an amino terminal (for STAT3 dimerization after activation), coiled-coil domain (for interaction with other transcription factors and regulatory proteins), DNA binding domain made up of β-sheets (for recognition of consensus binding regions on gene promoters), α-helical linker domain (for transcriptional activation and protein–protein interaction), a classical SH2 (sequence – homology) docking domain, and a transcriptional activation domain (Bromberg and Darnell, 2000; Lim and Cao, 2006). There are two highly conserved, phosphorylatable amino acids that are frequently required for STAT3 activation – a tyrosine 705 in the SH2 domain and a serine 727 in the 78-amino acid region of the transactivation domain (Bromberg and Darnell, 2000; Dziennis and Alkayed, 2008).

Injury-induced ligand binding to cell-surface receptors activates the associated receptor tyrosine kinase Janus kinases (JAKs), which phosphorylate tyrosine residues on the cytoplasmic portion of the receptor complex that acts as a binding site for STAT proteins. STATs dock onto the phospho-tyrosines via their SH2 domain, and are also phosphorylated by JAK on a tyrosine residue (e.g., Y705 in STAT3). Upon activation, they either hetero- or homodimerize and translocate to the nucleus via importins (Cimica et al., 2011), where they bind to consensus sequences (SIE/GAS/ISRE sequences) to induce gene transcription. Other mechanisms of STAT activation include phosphorylation by non-receptor tyrosine kinases such as Src, direct, or indirect phosphorylation by receptors with intrinsic tyrosine kinase activity (i.e., EGF, PDGF, and FGF receptors), and activation by G-protein coupled receptors (Ram and Iyengar, 2001). STAT3 can be deactivated by dephosphorylation or targeted degradation, and recycled back to the cytosol (reviewed by Lim and Cao, 2006; Dziennis and Alkayed, 2008; Moore and Goldberg, 2011). Further, Suppressor of Cytokine Signaling 3 (SOCS3) or protein inhibitors of activated STATS (PIAS) can also inhibit STAT3 activation (Krebs and Hilton, 2001).

In the adult brain, STATs are normally quiescent, but are activated following injury in response to multiple signaling pathways induced by several cytokines and growth factors including IL-6, LIF, CNTF, G-CSF, G-CSFR, EPO, EGF, IGF-1, NGF withdrawal, BDNF, free radicals, excitatory neurotransmitters and other inflammatory mediators that are released after injury

(reviewed in Dziennis and Alkayed, 2008). Propagation of many of these signals (IL-6, LIF, and CNTF) requires a combination of various ligand binding units and a common signal-transducing unit gp130 (Taga and Kishimoto, 1995), through which they activate the JAK/STAT, Ras/MAPK, extracellular regulated-signal kinase (ERK), PI3K, and other pathways (Akira, 2000; Heinrich et al., 2003). Activation of the gp130-receptor has been implicated in neuronal response to injury and regenerative axonal growth (Habecker et al., 2009; Hyatt Sachs et al., 2010).

Signal transducer and activator of transcription-3 was identified as an acute phase response factor (Akira et al., 1994) and is expressed cytoplasmically in both neuronal and glial cells during development and in the adult (De-Fraja et al., 1998; Gautron et al., 2006). Nervous system insult leads to STAT3 activation in specific subsets of neurons with the pattern of activation dependent on the nature of the insult (Schwaiger et al., 2000; Xia et al., 2002). Global STAT3 knockout animals are embryonic lethal, dying prior to gastrulation between E6.5 and 7.5 (Takeda et al., 1997). Cell-type specific STAT3 deletion has greatly improved our knowledge of its function in different tissues (Sauer, 1998; Akira, 2000). STAT3 deletion in T-cells is associated with impaired T-cell proliferation, in macrophages with high susceptibility to endotoxin shock, in keratinocytes with compromised wound healing process, and in mammary glands with delay of involution (Akira, 2000). Nestin-promoter mediated neural-specific disruption of STAT3 resulted in mice which were hyperphagic, obese, diabetic, and infertile (Gao et al., 2004).

In the last decade, there has been growing evidence of a role of STAT3 in axonal regeneration. *In vitro*, STAT3 induces neurite growth in motor and sensory neurons and this growth can be inhibited by the application of SOCS3 (Liu and Snider, 2001; Miao et al., 2006). STAT3 induces GAP-43, a protein found in growth cones, and GAP-43 expression is inhibited with the JAK inhibitor AG-490, suggesting a role for the JAK/STAT3 pathway in GAP-43 expression and neurite outgrowth and regeneration (Wu and Bradshaw, 1996).

Haas et al. (1999) observed STAT3 activation at 24 h and 5 days after rat facial nerve axotomy. Following rat facial and hypoglossal nerve axotomy, a transient but significant increase in STAT3 mRNA, along with its phosphorylation and nuclear translocation within 3 h in neurons and 1 day in astrocytes was observed by Schwaiger et al. (2000). In contrast, these changes were not observed in non-regenerating neurons of Clarke's nucleus, although both forms of injury upregulated c-Jun and GAP-43 expression (Schwaiger et al., 2000). Transient increases in STAT3 signaling were also found in sprouting neurites and astrocytes following entorhinal cortex lesions in addition to a rise in gp130-receptor cytokines, suggesting a dual role of STAT3 in both astrocytic regulation and axonal sprouting (Xia et al., 2002). Lee et al. (2004) showed that axonal STAT3, activated at the injury site, acts as both a retrograde injury signal and as a transcription factor which promotes the survival and regeneration of both sensory and motor neurons. Levels of activated STAT3 increased in the nuclei of regenerating adult cranial motor neurons, sciatic motor neurons and in sensory neuron nuclei after injury. Sciatic nerve lesion led to a very rapid activation of STAT3 in axons at the lesion site within 15 min, and by 24 h STAT3 labeling was detected in

the DRG and in the spinal cord (Lee et al., 2004). These levels of STAT3 decreased when regeneration was completed, emphasizing its role in the regenerative process.

Deletion of CNTF, which is abundantly present in myelinating Schwann cells but not in or around the cell bodies of axotomized motoneurons (Dobrea et al., 1992; Rende et al., 1992) caused a delay in the appearance of phosphorylated STAT3 and its nuclear translocation in neuronal cell bodies (Kirsch et al., 2003). This delay points to a signaling cascade beginning with local release of CNTF by damaged myelinating Schwann cells, its local action on adjacent axons, intra-axonal phosphorylation of STAT3 and its retrograde transport to the cell bodies of injured neurons and finally to the nucleus. Recent studies using repetitive *in vivo* imaging of individual fluorescently labeled axons have shown that STAT3 selectively regulates initiation but not later perpetuation of axonal growth, thus acting as a phase-specific regulator of axonal outgrowth in both PNS and CNS (Bareyre et al., 2011). Altogether, these studies show a general trend of early and persistent expression of phosphorylated STAT3 during the regenerative process.

A number of *in vitro* and *in vivo* studies have also implicated the role of STAT3 as an intracellular survival-promoting factor. *In vitro* studies using cell lines have shown that STAT3 expression directly induces *bcl-2* and *bcl-xL* gene expression, which are critical in promoting neuronal survival after injury (Dziennis and Alkayed, 2008). Mice lacking STAT3 in facial and spinal motoneurons (controlled by cre expression under NF-L promoter) showed a significant reduction in motoneuron survival after facial nerve lesion, which closely correlated with the extent of motoneuron loss seen in LIF/CNTF KO mice after nerve lesion (Sendtner et al., 1996; Schweizer et al., 2002). Moreover, upregulation of Reg-2 and Bcl-xl expression was reduced in axotomized motoneurons in STAT3 KO mice, suggesting a role of STAT3 in anti-apoptotic signaling. Interestingly, in the study by Schweizer et al. (2002) local application of CNTF strongly reduced the apparent motoneuron cell death in the absence of neuronal STAT3, but which other signaling pathways were involved is unclear.

Preliminary data from our lab suggests an important role for neuronal STAT3 in axonal regeneration (Patodia et al., 2011). Conditional mice mutants, lacking STAT3 in their neurons, had profound defects in the usual retrograde response, with a severe early and persistent reduction by 75–90% in microglial activation and recruitment of lymphocytes, and 4 days after injury in speed of axonal elongation in the distal stump. At 30 days, axotomized STAT3-deficient motoneurons appeared shrunk by 50–60% in size but displayed no cell death in contrast to results published earlier (Schweizer et al., 2002). Functional recovery was dramatically reduced and the mutants showed 80% less target muscle re-innervation. As with neuronal c-Jun deletion which interferes with regeneration (Raivich et al., 2004), expression of RAGs like CD44, β1 integrin, CGRP, galanin as well as the nuclear translocation of ATF3 was also greatly diminished or abolished in the STAT3 mutants. However, c-Jun expression itself was not affected, suggesting that deletion of either transcription factor – c-Jun or STAT3 – will produce a very similar regeneration-deficient phenotype.

CCAAT/ENHANCER BINDING PROTEIN β, δ

The C/EBP family of transcription factors is comprised of six proteins (C/EBPα, β, γ, δ, ε, and ζ), each having unique properties regulating cellular proliferation and differentiation, particularly in hepatocytes, adipocytes, and hematopoietic cells (Ramji and Foka, 2002). C/EBPs also play pivotal, non-redundant roles in the development and plasticity of the nervous system, including cell fate determination, apoptosis, synthesis and response to trophic factors and responses to brain injury and ischemia (Kfoury and Kapatos, 2009). All members have a DNA binding domain and a highly homologous bZIP domain which is required for dimerization. The C/EBPs can interact with other bZIP and non-bZIP transcription factors (Huang et al., 2007).

CCAAT/enhancer binding protein-δ (also known as CELF, CRP3, and NF/IL-6) expression is typically low to undetectable in most cell-types and tissues, but it is rapidly induced by a variety of extracellular stimuli (e.g., growth hormone, insulin, IFNγ, IL-1, IL-6, LPS, TNFα, noradrenaline, and glutamate), phosphorylated, and translocated to the nucleus as an active transcription factor where is induces further IL-6 expression (Ramji and Foka, 2002). The most favorable complex responsible for the transcription of the IL-6 gene requires the interaction between p65 and C/EBPδ, with c-Jun occurring on the NF-κB site (Faggioli et al., 2004). C/EBPδ is elevated in hypoxia, glaucoma, carcinoma, traumatic brain injury and in the brains of Alzheimer's disease patients (Huang et al., 2007; Yang et al., 2007). A recent study showed that the anti-inflammatory and neuroprotective effects of chrysin on reactive microglial cell induced neurotoxicity was partly mediated by inhibition of C/EBPδ expression at both protein and mRNA levels. C/EBPδ-deficient microglial cultures produce less NO and TNFα in response to LPS/IFNγ, re-affirming its role in microglial activation and neuroinflammation (Gresa-Arribas et al., 2010). C/EBPβδ double knockout in mouse embryonic fibroblasts showed particularly impaired production of IL-6 and TNFα in response to LPS or IL-1, suggesting complementary roles of C/EBPβ and C/EBPδ in induction of proinflammatory cytokines (Ramji and Foka, 2002).

CCAAT/enhancer binding protein β is upregulated and phosphorylated across different classes of injured neurons, including the invertebrate *Aplysia* model. In this model, phosphorylation of *Aplysia* C/EBPβ is mediated by the RISK1, a mitogen associated protein kinase homolog related to the ERK family (Sung et al., 2001). In the mouse facial nerve model, C/EBPβ is important for the expression of regeneration-associated microtubule Tα1 α tubulin and growth cone protein GAP-43, suggesting that C/EBPβ is a critical component of the regenerative response (Nadeau et al., 2005).

In the case of C/EPBδ, which is upregulated in sensory and sympathetic neurons (Boeshore et al., 2004; Magoulas and Lopez-de Heredia, 2010), initial studies using cultured primary sensory neurons from C/EPBδ null mice show impaired axonal growth and a reduced response to the conditioning lesion (Magoulas and Lopez-de Heredia, 2010). Preliminary results from our studies also confirm this finding in the motor, facial nerve injury model as well as indicate a role for C/EBPδ in early and late microglial activation (Patodia et al., unpublished).

In summary, a number of intriguing findings implicate a role of C/EBPs in injury response in neurons, i.e., they are mediators of neuro-inflammatory responses in the brain; are induced in injured neurons of invertebrates (Korneev et al., 1997; Sung et al., 2001) as well as vertebrates (Nadeau et al., 2005); have RAGs like Tα1 α tubulin and GAP-43 as direct transcriptional targets (Nadeau et al., 2005); and are upregulated by IL-6, an important post-injury molecule (Ramji et al., 1993). A number of alternative signaling pathways might also converge onto C/EBP family members following axonal injury, e.g., the JAK–STAT pathway has been shown to directly regulate C/EBPβ (Jiang and Zarnegar, 1997).

Oct-6

Transcription factors Oct-6 (SCIP/Tst-1), Krox-20 (Egr-2), and Sox 10 are major regulators of Schwann cell differentiation and myelination (Topilko et al., 1994; Bermingham et al., 1996; Jaegle and Meijer, 1998). A fragment distal to the Oct-6 gene, containing two DNase I-hypersensitive sites, acts as the Schwann cell-specific enhancer (SCE) and is responsible for the complex signaling between Schwann cells and axons driving myelination, during both normal peripheral nerve development and regeneration (Mandemakers et al., 2000). Oct-6 is present in the cytoplasm of Schwann cells associated with myelinated fibers of intact nerves. Axonal injury induces nuclear Oct-6 expression during the acute degenerative stage, reaching peak expression levels in the subsequent regenerative stage (Scherer et al., 1994; Kawasaki et al., 2003), suggesting that at the time of regeneration, Oct-6 is translocated to the nucleus to promote transcription of genes, possibly those encoding myelin proteins. Oct-6 expression is down-regulated in fully regenerated nerves and in nerves showing chronic axonal loss (Kawasaki et al., 2003). Therefore, Oct-6 may be a marker for dedifferentiation of adult Schwann cells and active nerve regeneration.

Sox11

SRY-box containing gene 11 (Sox11), a member of the Sox transcription factor family, is highly expressed in developing sensory neurons and at low levels in adult neurons (Jankowski et al., 2006; Moore and Goldberg, 2011). There has been limited study of Sox11 targets in axonal growth, but Sox11 can partner with Brn1 or -2 to regulate transcription (Kuhlbrodt et al., 1998; Tanaka et al., 2004) and drive expression of neurite growth-associated genes like β-III tubulin, MAP2 (Bergsland et al., 2006) and actin-related protein complex 3 (Arpc3; Jankowski et al., 2006). Sox11 expression in sensory and sympathetic ganglia is highly sensitive to nerve cut and crush injuries, rising steadily after injury and during subsequent regeneration, and returning to baseline levels at the end of regeneration (Boeshore et al., 2004; Jankowski et al., 2006, 2009). In Neuro2a cells, Sox11 levels dramatically increased with the number of cells extending neurites. In vitro experiments using cultured adult DRG neurons treated with Sox11 siRNAs exhibit a significant decrease in regeneration following axotomy as indicated by reduced neurite length and branching index (Jankowski et al., 2006). This suggests a correlation between Sox11 and neurite growth.

Injection of Sox11 siRNAs into mouse saphenous nerve caused a transient knockdown of Sox11 mRNA, that transiently inhibited

in vivo regeneration after nerve crush. Electron microscopy analysis of Sox11 RNAi-injected nerves showed that regeneration of both myelinated and unmyelinated axons was inhibited. Nearly all neurons in the ganglia of crushed nerves that were Sox11 immunopositive showed co-labeling for ATF3 (Jankowski et al., 2009) and treatment with Sox11 siRNAs in vitro and in vivo caused a transcriptional and translational level reduction in ATF3 expression. Interestingly, ATF3 ablation using ATF3 siRNAs did not affect Sox11 levels, suggesting that Sox11 may act upstream of ATF3 and influence regulation of its gene expression (Jankowski et al., 2009). These anatomical and expression data support an intrinsic role for Sox11 in axonal regeneration that may involve interaction with ATF3.

p53

p53 is a member of a family of tumor suppressors together with p63 and p73. Like c-Jun, p53 is known to mediate both pro- and anti-apoptotic roles in the nervous system (Culmsee and Mattson, 2005; Jacobs et al., 2006). p53 can undergo multiple types of post-translational modifications including acetylation, phosphorylation, and ubiquitination, which can affect its localization and function (Lavin and Gueven, 2006; Tedeschi et al., 2009). In vitro and in vivo experiments have shown that p53 plays a role in promoting neurite growth. Overexpression of a dominant negative form of p53 in primary cortical neurons leads to growth cone collapse and decrease in neurite outgrowth, possibly due to a combination of reduced expression of its growth cone associated target genes Coronin1b, Rab13, and GAP-43 (Di Giovanni et al., 2006; Tedeschi et al., 2009), as well as its local, non-transcriptional activity at the growth cone (Qin et al., 2009). On the other hand, overexpression of wild-type p53 leads to an increase in growth cone size (Qin et al., 2009). Other axonal guidance molecules and their receptors like netrins, semaphorins, and ephrins are also regulated by p53 (Arakawa, 2005). In vivo experiments using p53 knockout mice showed a significant decrease in the number of fibers reinnervating the target muscles at 28 days after facial axotomy in the mutant mice when compared with control animals (Di Giovanni et al., 2006). Taken together, these studies suggest a role for p53 in modulating neurite growth and regeneration. Given its well described role in apoptosis of neurons and non-neuronal cells, it will be interesting to investigate its effects on neuronal survival after peripheral nerve injury.

NUCLEAR FACTOR KAPPA-LIGHT-CHAIN-ENHANCER OF ACTIVATED B CELLS

Nuclear factor kappa-light-chain-enhancer of activated B cells is an inducible transcription factor dimer made up of 5 subunits, bound as homo- or hetero-dimers: RelA/p65, c-Rel, RelB, p50, and p52. Inhibitor of κB (IκB) family of proteins can bind to NF-κB dimer and prevent its nuclear translocation. Dissociation or proteasomal degradation of IκB reveals NF-κB's nuclear localization sequence (NLS), allowing it to move to the nucleus and modulate gene transcription by binding to promoter and enhancer sequences (reviewed by Moore and Goldberg, 2011).

Nuclear factor kappa-light-chain-enhancer of activated B cells is significantly upregulated in DRGs and spinal cord after peripheral nerve crush (Ma and Bisby, 1998; Pollock et al., 2005). It can

respond to a stimulus in neurites and retrogradely travel to the nucleus to affect gene transcription. NF-κB signaling can either enhance of inhibit neurite growth depending on the phosphorylation status of its RelA/p65 subunit. Phosphorylation of p65 by IKKβ results in reduced neurite growth, and in neurons without this IKKβ activity, there is increased neurite growth (Gutierrez et al., 2008). Identified neuronal targets of NF-κB include genes for Bcl-2, MnSOD, glutamate receptor subunits, BDNF, and calcium regulating proteins (reviewed by Mattson, 2005). In addition, NF-κB has been shown to regulate expression of cell-adhesion molecules like NCAM, tenascin C and β1 integrin in other systems (reviewed by Moore and Goldberg, 2011). NF-κB is also the downstream modulator of neurite growth for fas apoptosis inhibitory molecule (FAIM) signaling. Blocking NF-κB activation prevents the increase of neurite growth seen with FAIM overexpression (Sole et al., 2004).

ELK

The ETS-like (ELK) subfamily of transcription factors comprises of ELK1, ELK3, and ELK4 factors which can have different potential protein–protein interactions, and respond differently to MAP kinase signaling pathways (Ducret et al., 2000; Buchwalter et al., 2004). Seven days after peripheral axotomy, a 2.3-fold upregulation of ELK3 mRNA, but not of ELK1 or ELK4 mRNA was detected in adult mouse DRGs (Kerr et al., 2010). The expression of neuropeptide galanin mRNA is upregulated around 80-fold in DRGs following axotomy, and an 18 bp sequence within its promoter/enhancer region containing overlapping putative ETS, STAT, and Smad binding sites, has been shown to be critical for this response (Bacon et al., 2007), making ELK3 a potentially important player in peripheral nerve regeneration. It would be interesting to see if the injury-induced ELK3 isoforms can differently affect neuronal survival and/or regeneration after axonal injury.

CROSS-TALK BETWEEN TRANSCRIPTION FACTORS

Transcription factors form an intricate network in which cross-talk between different pathways leads to an integrated and input-balanced signaling outcome (Morrison and Davis, 2003; Moore and Goldberg, 2011). Some axonal regeneration still occurs in the absence of master regulators of regeneration like c-Jun, highlighting the existence of alternative pathways, and of compensatory and complementary/synergistic mechanisms, which may be shared during axonal outgrowth.

Jun N-terminal kinase signaling is interconnected with various other cellular signaling pathways, such as NFkB and the JAK/STAT system (Waetzig et al., 2006; Haeusgen et al., 2009), resulting in STAT3 phosphorylation (Levy and Lee, 2002). Besides JNK, c-Jun can also be activated by ERK1/2 (Leppa et al., 1998; Schwarz et al., 2002; Morton et al., 2003). On the other hand, STAT3 can stimulate transcription of several AP-1 members including JunB (Coffer et al., 1995) and c-fos (Yang et al., 2003; Higashi et al., 2004) which have STAT3 responsive elements in their regulatory sequence.

The interactions between AP-1 and STAT3 are multiple and complex, resulting in mutual modulation of their transcriptional activities. STAT3 and AP-1 factors can bind to independent but closely spaced DNA binding sites in the regulatory sequences of

a number of genes, such as α-2 macroglobulin (Schaefer et al., 1995), VIP (Symes et al., 1997), bcl-6 (Arguni et al., 2006), and matrix metallo-proteinases and synergistically induce maximal enhancer function (Zhang et al., 1999). AP-1 complexes can also bind directly to STAT3, enhancing STAT3's transcriptional activity (Schaefer et al., 1995; Leu et al., 2001). Mapping of the STAT3–c-Jun interactive regions by GST pull-down assays suggested that the STAT3-interactive region lies within its coiled-coiled domain, and in a portion of the DNA binding domain. The c-Jun interactive region was within its C-terminal residues 105–334. Point mutations within these regions blocked their interaction (Zhang et al., 1999). SOCS3 was discovered for its ability to inhibit tyrosine phosphorylation of STAT3 through binding to gp130 and JAKs (Krebs and Hilton, 2001). It has subsequently been shown that endogenous SOCS3 can also inhibit AP-1 activity by blocking JNK phosphorylation (Miao et al., 2008). Therefore SOCS3 could be an important molecule regulating axonal regeneration by controlling changes in JNK and JAK/STAT signaling pathways after injury.

Additional transcription factors encompassing a compensatory/complementary role include the C/EBP family members C/EBPβ and C/EBPδ. The promoter region of a c-Jun coactivator, an oncogene Jab1, contains binding sequences for C/EBP, GATA, as well as a STAT3 consensus sequence overlapping the C/EBP site. Both, mutation of the C/EBP binding site and inhibition of STAT3, significantly reduced Jab1-promoter activity (Shackleford et al., 2011). C/EBPβ is essential for appropriate induction and maintenance of GAP-43 mRNA following axonal injury (Nadeau et al., 2005). While there is no evidence of a direct binding site for C/EBP on the GAP-43 gene, it contains a functionally important AP-1 site for c-Jun and c-fos (Weber and Skene, 1998). C/EBPβ can bind to and regulate both c-fos and c-Jun (Nadeau et al., 2005), suggesting a coordinated induction of GAP-43 by C/EBPβ, c-fos, and c-Jun in injured neurons. It has already been shown that co-expression of c-Fos with c-Jun induces a greater number of cells to produce neurites than c-Jun alone (Leppa et al., 1998).

We have already discussed a number of different studies investigating the complex association between ATF3 and c-Jun expression after injury. Besides interacting with leucine zipper transcription factors, ATF3 can also interact with other transcription factors upregulated after peripheral injury, such as, STAT3. This could be via co-activators or by synergistic binding to adjacent DNA binding sites. For example, SPRR1A, a target gene for ATF3, is a gp130 pathway protein and its promoter region contains functional AP-1 binding sites (Sark et al., 1998; Pradervand et al., 2004). The levels of SPRR1A expression in DRGs of injured wild-type mice are higher than in DRGs of non-injured transgenic mice expressing constitutively active ATF3, suggesting that other downstream signals in addition to ATF3 are likely to govern SPRR1A expression. C/EBPβ, for example, is induced after peripheral nerve injury (Nadeau et al., 2005) and a functional C/EBPβ binding site is located on the SPRR1A promoter (Pradervand et al., 2004). Recently, transcription of DINE (damage-induced neuronal endopeptidase), an enzyme involved in terminal nerve branching (Nagata et al., 2010), was shown to be increased 60-fold by Sp1 mediated recruitment of STAT3, c-Jun, and ATF3 to the DINE promoter in response to LIF upregulation and NGF withdrawal following injury (Kiryu-Seo et al., 2008).

Nuclear factor kappa-light-chain-enhancer of activated B cell can also interact with Jun, ATF, CREB, and Fos transcription factors. STAT3 can induce the alternative NF-κB pathway, and bind to DNA in a complex with p52, to induce transcription. p53 can also interact with p52 to regulate expression of its target genes (reviewed by Perkins, 2007).

Mathematical models of interacting signaling pathways have been developed to predict regenerative outcomes after injury (Moya et al., 2011).Transgenic animal models with deletion of multiple signaling streams will be very useful in identification of functional complementary counterparts.

DOWNSTREAM TARGETS OF TRANSCRIPTION FACTORS

Transcription factors rapidly condition the injured nerve and within 1–4 days after injury, the neuronal perikaryon produces a plethora of RNA, protein, and glycolipid components which play a vital role in executing axonal regeneration. Downstream targets of the transcription factors include a vast number of RAGs that may be involved in cell–cell signaling, axonal growth and sprouting, and activation of the non-neuronal, cellular milieu. Successful axonal regeneration is accompanied by the appearance of functionally diverse families of molecules that regulate surface cytoskeletal interaction. Examples of regeneration-associated cytoskeletal families include the GMC family of "integral" membrane proteins – GAP-43, MARCKS, and CAP23 (Skene and Willard, 1981; Bomze et al., 2001), microtubule disassembly molecules (e.g., SCG10, stathmin, CRMP2, and RB3; Iwata et al., 2002; Mori and Morii, 2002), and the Rho GTPase family (RhoA, Rac, Cdc42, and TC10) members which act as molecular switches regulating cytoskeletal structure, dynamics, and cell-adhesion (Etienne-Manneville and Hall, 2002). Regenerating neurons also upregulate adhesion molecules like integrins α7 and β1 (Kloss et al., 1999), CD44 (Jones et al., 2000), galectin-1 (Horie and Kadoya, 2000), β2-microglobulin (Oliveira et al., 2004), ninjurin (Araki and Milbrandt, 2000), and gicerin/CD146 (Hiroi et al., 2003). Neurotrophins like BDNF and

GDNF are induced in the distal stump of the injured axons, and their exogenous application promotes axonal regeneration of chronically injured neurons (Boyd and Gordon, 2003a,b). *In vitro* studies have shown that different neuropeptides like substance P, somatostatin, vasopressin, calcitonin, and alpha-MSH exert a moderate but significant neurite growth promoting effect on CNS and PNS neurons (Narumi and Fujita, 1978; Brinton and Gruener, 1987; Raivich, 2011). Transgenic and pharmacological studies have confirmed a similar role *in vivo* for peptides like galanin (Holmes et al., 2000; Wynick et al., 2001), CGRP (Toth et al., 2009), and PACAP (Pituitary Adenylate Cyclase Activating Peptide; Kimura et al., 2003; Suarez et al., 2006; Armstrong et al., 2008).

CONCLUDING REMARKS

Injury to peripheral nerves can occur due to several causes ranging from physical injury and accidental trauma to pathological conditions and degenerative diseases. Achieving successful regeneration and synchronized functional recovery following neural damage is one of the biggest challenges faced by neuroscientists and clinicians. Transcription factors are master switches controlling the expression of multiple gene programs, and orchestrating complex axon growth and regenerative responses. Blocking transcription using RNA polymerase II inhibitors at an early stage after injury inhibits the capacity of dissociated neurons to elongate neurites (Smith and Skene, 1997), highlighting their crucial role in efficient regeneration after nerve injury. In this review, we have attempted to summarize the roles of a number of different transcription factors in peripheral nerve regeneration and their complex web of interactions. An overview of the observed phenotypes following global and cell-type specific deletions is given in **Table 1**. Understanding the molecular mechanisms underlying nerve regeneration via various regeneration-associated molecules and their signaling components will not only help to enhance the speed and specificity of peripheral regeneration but improve the success of central axonal regeneration, as well.

REFERENCES

Akira, S. (2000). Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene* 19, 2607–2611.

Akira, S., Nishio, Y., Inoue, M., Wang, X. J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. (1994). Molecular cloning of Aprf, a novel Ifn-stimulated gene factor-3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 77, 63–71.

Arakawa, H. (2005). P53, apoptosis and axon-guidance molecules. *Cell Death Differ.* 12, 1057–1065.

Araki, T., and Milbrandt, J. (2000). Ninjurin2, a novel homophilic adhesion molecule, is expressed in mature sensory and enteric neurons and promotes neurite outgrowth. *J. Neurosci.* 20, 187–195.

Arguni, E., Arima, M., Tsuruoka, N., Sakamoto, A., Hatano, M., and Tokuhisa, T. (2006). JunD/AP-1 and STAT3 are the major enhancer molecules for high Bcl6 expression in germinal center B cells. *Int. Immunol.* 18, 1079–1089.

Armstrong, B. D., Abad, C., Chhith, S., Cheung-Lau, G., Hajji, O. E., Nobuta, H., and Waschek, J. A. (2008). Impaired nerve regeneration and enhanced neuroinflammatory response in mice lacking pituitary adenylyl cyclase activating peptide. *Neuroscience* 151, 63–73.

Arthur-Farraj, P., Baskaran, A., Parkinson, D., Turmaine, M., Feltri, M., Wrabetz, L., Behrens, A., Mirsky, R., and Jessen, K. (2007). Schwann cells without c-Jun show retarded demyelination and dedifferentiation after peripheral nerve injury. *Abstr. Soc. Neurosci.* 480.

Bacon, A., Kerr, N. C. H., Holmes, F. E., Gaston, K., and Wynick, D. (2007). Characterization of an enhancer region of the galanin gene that directs expression to the dorsal root ganglion and confers responsiveness to axotomy. *J. Neurosci.* 27, 6573–6580.

Bareyre, F. M., Garzorz, N., Lang, C., Misgeld, T., Buning, H., and Kerschensteiner, M. (2011). In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6282–6287.

Barnat, M., Enslen, H., Propst, F., Davis, R. J., Soares, S., and Nothias, F. (2010). Distinct roles of c-Jun N-terminal kinase isoforms in neurite initiation and elongation during axonal regeneration. *J. Neurosci.* 30, 7804–7816.

Behrens, A., Sibilia, M., and Wagner, E. F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.* 21, 326–329.

Berdan, R. C., Easaw, J. C., and Wang, R. (1993). Alterations in membrane potential after axotomy at different distances from the soma of an identified neuron and the effect of depolarization on neurite outgrowth and calcium channel expression. *J. Neurophysiol.* 69, 151–164.

Bergsland, M., Werme, M., Malewicz, M., Perlmann, T., and Muhr, J. (2006). The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev.* 20, 3475–3486.

Bermingham, J. R., Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L., and Rosenfeld, M. G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev.* 10, 1751–1762.

Besirli, C. G., Wagner, E. F., and Johnson, E. M. (2005). The limited role of NH2-terminal c-Jun phosphorylation in neuronal apoptosis: identification of the nuclear pore complex as a potential target of the JNK pathway. *J. Cell Biol.* 170, 401–411.

Björkblom, B., Ostman, N., Hongisto, V., Komarovski, V., Filén, J. J., Nyman, T. A., Kallunki, T., Courtney, M. J., and Coffey, E. T. (2005). Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. *J. Neurosci.* 25, 6350–6361.

Boeshore, K. L., Schreiber, R. C., Vaccariello, S. A., Sachs, H. H., Salazar, R., Lee, J., Ratan, R. R., Leahy, P., and Zigmond, R. E. (2004). Novel changes in gene expression following axotomy of a sympathetic ganglion: a microarray analysis. *J. Neurobiol.* 59, 216–235.

Bogoyevitch, M. A., and Kobe, B. (2006). Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol. Mol. Biol. Rev.* 70, 1061–1095.

Bohatschek, M., Kloss, C. U., Hristova, M., Pfeffer, K., and Raivich, G. (2004). Microglial major histocompatibility complex glycoprotein-1 in the axotomized facial motor nucleus: regulation and role of tumor necrosis factor receptors 1 and 2. *J. Comp. Neurol.* 470, 382–399.

Bomze, H. M., Bulsara, K. R., Iskandar, B. J., Caroni, P., and Skene, J. H. P. (2001). Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat. Neurosci.* 4, 38–43.

Bonilla, I. E., Tanabe, K., and Strittmatter, S. M. (2002). Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. *J. Neurosci.* 22, 1303–1315.

Bosse, F., Hasenpusch-Theil, K., Kury, P., and Muller, H. W. (2006). Gene expression profiling reveals that peripheral nerve regeneration is a consequence of both novel injury-dependent and reactivated developmental processes. *J. Neurochem.* 96, 1441–1457.

Bosse, F., Kury, P., and Muller, H. W. (2001). Gene expression profiling and molecular aspects in peripheral nerve regeneration. *Restor. Neurol. Neurosci.* 19, 5–18.

Boyd, J. G., and Gordon, T. (2003a). Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. *Exp. Neurol.* 183, 610–619.

Boyd, J. G., and Gordon, T. (2003b). Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. *Mol. Neurobiol.* 27, 277–323.

Brecht, S., Kirchhof, R., Chromik, A., Willesen, M., Nicolaus, T., Raivich, G., Wessig, J., Waetzig, V., Goetz, M., Claussen, M., Pearse, D., Kuan, C. Y., Vaudano, E., Behrens, A., Wagner, E., Flavell, R. A., Davis, R. J., and Herdegen, T. (2005). Specific pathophysiological functions of JNK isoforms in the brain. *Eur. J. Neurosci.* 21, 363–377.

Brinton, R. E., and Gruener, R. (1987). Vasopressin promotes neurite growth in cultured embryonic neurons. *Synapse* 1, 329–334.

Bromberg, J., and Darnell, J. E. (2000). The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19, 2468–2473.

Buchwalter, G., Gross, C., and Wasylyk, B. (2004). Ets ternary complex transcription factors. *Gene* 324, 1–14.

Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R. R., and Filbin, M. T. (2002). Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. *Neuron* 35, 711–719.

Cimica, V., Chen, H. C., Iyer, J. K., and Reich, N. C. (2011). Dynamics of the STAT3 transcription factor: nuclear import dependent on Ran and importin-β1. *PLoS ONE* 6, e20188. doi:10.1371/journal.pone.0020188

Coffer, P., Lutticken, C., Vanpuijenbroek, A., Klopdejonge, M., Horn, F., and Kruijer, W. (1995). Transcriptional regulation of the Jun b promoter – analysis of STAT-mediated signal-transduction. *Oncogene* 10, 985–994.

Costigan, M., Befort, K., Karchewski, L., Griffin, R. S., D'Urso, D., Allchorne, A., Sitarski, J., Mannion, J. W., Pratt, R. E., and Woolf, C. J. (2002). Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci.* 3, 16. doi:10.1186/1471-2202-3-16

Cox, L. J., Hengst, U., Gurskaya, N. G., Lukyanov, K. A., and Jaffrey, S. R. (2008). Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. *Nat. Cell Biol.* 10, 149–159.

Crocker, S. J., Hayley, S. P., Smith, P. D., Mount, M. P., Lamba, W. R., Callaghan, S. M., Slack, R. S., and Park, D. S. (2006). Regulation of axotomy-induced dopaminergic neuron death and c-Jun phosphorylation by targeted inhibition of cdc42 or mixed lineage kinase. *J. Neurochem.* 96, 489–499.

Culmsee, C., and Mattson, M. P. (2005). p53 in neuronal apoptosis. *Biochem. Biophys. Res. Commun.* 331, 761–777.

De-Fraja, C., Conti, L., Magrassi, L., Govoni, S., and Cattaneo, E. (1998). Members of the JAK/STAT proteins are expressed and regulated during development in the mammalian forebrain. *J. Neurosci. Res.* 54, 320–330.

Deng, J., Zhang, X. L., Wang, J. W., Teng, L. L., Ge, J., Takeiviori, H., Xiong, Z. Q., and Zhou, Y. (2009). Expression and regulated nuclear transport of transducers of regulated Creb 1 in retinal ganglion cells. *Neuroscience* 159, 1023–1031.

Di Giovanni, S., Knights, C. D., Rao, M., Yakovlev, A., Beers, J., Catania, J., Avantaggiati, M. L., and Faden, A. I. (2006). The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. *EMBO J.* 25, 4084–4096.

Dobrea, G. M., Unnerstall, J. R., and Rao, M. S. (1992). The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. *Brain Res. Dev. Brain Res.* 66, 209–219.

Doyle, C. A., and Hunt, S. P. (1997). Reduced nuclear factor kappa B (p65) expression in rat primary sensory neurons after peripheral nerve injury. *Neuroreport* 8, 2937–2942.

Dubový, P. (2011). Wallerian degeneration and peripheral nerve conditions for both axonal regeneration and neuropathic pain induction. *Ann. Anat.* 193, 267–275.

Ducret, C., Maira, S. M., Lutz, Y., and Wasylyk, B. (2000). The ternary complex factor Net contains two distinct elements that mediate different responses to MAP kinase signalling cascades. *Oncogene* 19, 5063–5072.

Dziennis, S., and Alkayed, N. J. (2008). Role of signal transducer and activator of transcription 3 in neuronal survival and regeneration. *Rev. Neurosci.* 19, 341–361.

Elde, R., Cao, Y. H., Cintra, A., Brelje, T. C., Pelto-Huikko, M., Junttila, T., Fuxe, K., Pettersson, R. F., and Hökfelt, T. (1991). Prominent expression of acidic fibroblast growth factor in motor and sensory neurons. *Neuron* 7, 349–364.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629–635.

Faggioli, L., Costanzo, C., Donadelli, M., and Palmieri, M. (2004). Activation of the interleukin-6 promoter by a dominant negative mutant of c-Jun. *Biochim. Biophys. Acta* 1692, 17–24.

Gao, Q., Wolfgang, M. J., Neschen, S., Morino, K., Horvath, T. L., Shulman, G. I., and Fu, X. Y. (2004). Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4661–4666.

Gautron, L., de Smedt-Peyrusse, V., and Laye, S. (2006). Characterization of STAT3-expressing cells in the postnatal rat brain. *Brain Res.* 1098, 26–32.

Gresa-Arribas, N., Serratosa, J., Saura, J., and Sola, C. (2010). Inhibition of CCAAT/enhancer binding protein delta expression by chrysin in microglial cells results in anti-inflammatory and neuroprotective effects. *J. Neurochem.* 115, 526–536.

Gutierrez, H., O'Keeffe, G. W., Gavalda, R., Gallagher, D., and Davies, A. M. (2008). Nuclear factor kappa B signaling either stimulates or inhibits neurite growth depending on the phosphorylation status of p65/RelA. *J. Neurosci.* 28, 8246–8256.

Haas, C. A., Hofmann, H. D., and Kirsch, M. (1999). Expression of CNTF/LIF-receptor components and activation of STAT3 signaling in axotomized facial motoneurons: evidence for a sequential postlesional function of the cytokines. *J. Neurobiol.* 41, 559–571.

Habecker, B. A., Sachs, H. H., Rohrer, H., and Zigmond, R. E. (2009). The dependence on gp130 cytokines of axotomy induced neuropeptide expression in adult sympathetic neurons. *Dev. Neurobiol.* 69, 392–400.

Haeusgen, W., Boehm, R., Zhao, Y., Herdegen, T., and Waetzig, V. (2009). Specific activities of individual c-Jun N-terminal kinases in the brain. *Neuroscience* 161, 951–959.

Hai, T., and Curran, T. (1991). Cross-family dimerization of transcription factors fos, Jun and atf creb alters DNA-binding specificity. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3720–3724.

Hai, T., and Hartman, M. G. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 273, 1–11.

Hannila, S. S., and Filbin, M. T. (2008). The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. *Exp. Neurol.* 209, 321–332.

Hanz, S., Perlson, E., Willis, D., Zheng, J. Q., Massarwa, R., Huerta, J. J., Koltzenburg, M., Kohler, M., van-Minnen, J., Twiss, J. L., and Fainzilber, M. (2003). Axoplasmic importins enable retrograde injury signaling in lesioned nerve. *Neuron* 40, 1095–1104.

Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Muller-Newen, G., and Schaper, F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J.* 374, 1–20.

Herdegen, T., Blume, A., Buschmann, T., Georgakopoulos, E., Winter, C., Schmid, W., Hsieh, T. F., Zimmermann, M., and Gass, P. (1997a). Expression of activating transcription factor-2, serum response factor and cAMP/Ca response element binding protein in the adult rat brain following generalized seizures, nerve fibre lesion and ultraviolet irradiation. *Neuroscience* 81, 199–212.

Herdegen, T., Skene, P., and Bahr, M. (1997b). The c-Jun transcription factor – bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci.* 20, 227–231.

Herdegen, T., Claret, F. X., Kallunki, T., Martin-Villalba, A., Winter, C., Hunter, T., and Karin, M. (1998). Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. *J. Neurosci.* 18, 5124–5135.

Herdegen, T., Kummer, W., Fiallos, C. E., Leah, J., and Bravo, R. (1991). Expression of C-Jun, Jun-B and Jun-D proteins in rat nervous-system following transection of vagus nerve and cervical sympathetic trunk. *Neuroscience* 45, 413–422.

Herdegen, T., and Waetzig, V. (2001). AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration. *Oncogene* 20, 2424–2437.

Higashi, N., Kunimoto, H., Kaneko, S., Sasaki, T., Ishii, M., Kojima, H., and Nakajima, K. (2004). Cytoplasmic c-Fos induced by the YXXQ-derived STAT3 signal requires the co-operative MEK/ERK signal for its nuclear translocation. *Genes Cells* 9, 233–242.

Hiroi, S., Tsukamoto, Y., Sasaki, F., Miki, N., and Taira, E. (2003). Involvement of gicerin, a cell adhesion molecule, in development and regeneration of chick sciatic nerve. *FEBS Lett.* 554, 311–314.

Hol, E. M., Schwaiger, F. W., Werner, A., Schmitt, A., Raivich, G., and Kreutzberg, G. W. (1999). Regulation of the LIM-type homeobox gene islet-1 during neuronal regeneration. *Neuroscience* 88, 917–925.

Holmes, F. E., Mahoney, S., King, V. R., Bacon, A., Kerr, N. C., Pachnis, V., Curtis, R., Priestley, J. V., and Wynick, D. (2000). Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11563–11568.

Horie, H., and Kadoya, T. (2000). Identification of oxidized galectin-1 as an initial repair regulatory factor after axotomy in peripheral nerves. *Neurosci. Res.* 38, 131–137.

Huang, A. M., Rudelius, M., Sharan, S., McAllister, J. M., Raffeld, M., Christenson, L. K., and Sterneck, E. (2007). The Cebpd (C/EBPdelta) gene is induced by luteinizing hormones in ovarian theca and interstitial cells but is not essential for mouse ovary function. *PLoS ONE* 2, e1334. doi:10.1371/journal.pone.0001334

Hunt, D., Hossain-Ibrahim, K., Mason, M. R., Coffin, R. S., Lieberman, A. R., Winterbottom, J., and Anderson, P. N. (2004). ATF3 upregulation in glia during Wallerian degeneration: differential expression in peripheral nerves and CNS white matter. *BMC Neurosci.* 5, 9. doi:10.1186/1471-2202-5-9

Hyatt Sachs, H., Rohrer, H., and Zigmond, R. E. (2010). The conditioning lesion effect on sympathetic neurite outgrowth is dependent on gp130 cytokines. *Exp. Neurol.* 223, 516–522.

Hyatt Sachs, H., Schreiber, R. C., Shoemaker, S. E., Sabe, A., Reed, E., and Zigmond, R. E. (2007). Activating transcription factor 3 induction in sympathetic neurons after axotomy: response to decreased neurotrophin availability. *Neuroscience* 150, 887–897.

Iwata, T., Namikawa, K., Honma, M., Mori, N., Yachiku, S., and Kiyama, H. (2002). Increased expression of mRNAs for microtubule disassembly molecules during nerve regeneration. *Brain Res. Mol. Brain Res.* 102, 105–109.

Jacobs, W. B., Kaplan, D. R., and Miller, F. D. (2006). The p53 family in nervous system development and disease. *J. Neurochem.* 97, 1571–1584.

Jaegle, M., and Meijer, D. (1998). Role of Oct-6 in Schwann cell differentiation. *Microsc. Res. Tech.* 41, 372–378.

Jankowski, M. P., Cornuet, P. K., McILwrath, S., Koerber, H. R., and Albers, K. M. (2006). SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. *Neuroscience* 143, 501–514.

Jankowski, M. P., McIlwrath, S. L., Jing, X. T., Cornuet, P. K., Salerno, K. M., Koerber, H. R., and Albers, K. M. (2009). Sox11 transcription factor modulates peripheral nerve regeneration in adult mice. *Brain Res.* 1256, 43–54.

Jiang, J. G., and Zarnegar, R. (1997). A novel transcriptional regulatory region within the core promoter of the hepatocyte growth factor gene is responsible for its inducibility by cytokines via the C/EBP family of transcription factors. *Mol. Cell. Biol.* 17, 5758–5770.

Jones, L. L., Liu, Z., Shen, J., Werner, A., Kreutzberg, G. W., and Raivich, G. (2000). Regulation of the cell adhesion molecule CD44 after nerve transection and direct trauma to the mouse brain. *J. Comp. Neurol.* 426, 468–492.

Kalla, R., Liu, Z., Xu, S., Koppius, A., Imai, Y., Kloss, C. U., Kohsaka, S., Gschwendtner, A., Möller, J. C., Werner, A., and Raivich, G. (2001). Microglia and the early phase of immune surveillance in the axotomized facial motor nucleus: impaired microglial activation and lymphocyte recruitment but no effect on neuronal survival or axonal regeneration in macrophage-colony stimulating factor-deficient mice. *J. Comp. Neurol.* 436, 182–201.

Kawasaki, T., Oka, N., Tachibana, H., Akiguchi, I., and Shibasaki, H. (2003). Oct6, a transcription factor controlling myelination, is a marker for active nerve regeneration in peripheral neuropathies. *Acta Neuropathol.* 105, 203–208.

Kenney, A. M., and Kocsis, J. D. (1998). Peripheral axotomy induces long-term c-Jun amino-terminal kinase-1 activation and activator protein-1 binding activity by c-Jun and JunD in adult rat dorsal root ganglia in vivo. *J. Neurosci.* 18, 1318–1328.

Kerr, N., Pintzas, A., Holmes, F., Hobson, S. A., Pope, R., Wallace, M., Wasylyk, C., Wasylyk, B., and Wynick, D. (2010). The expression of ELK transcription factors in adult DRG: novel isoforms, antisense transcripts and upregulation by nerve damage. *Mol. Cell. Neurosci.* 44, 165–177.

Kfoury, N., and Kapatos, G. (2009). Identification of neuronal target genes for CCAAT/enhancer binding proteins. *Mol. Cell. Neurosci.* 40, 313–327.

Kimura, H., Kawatani, M., Ito, E., and Ishikawa, K. (2003). Effects of pituitary adenylate cyclase-activating polypeptide on facial nerve recovery in the guinea pig. *Laryngoscope* 113, 1000–1006.

Kirsch, M., Terheggen, U., and Hofmann, H. D. (2003). Ciliary neurotrophic factor is an early lesion-induced retrograde signal for axotomised facial motoneurons. *Mol. Cell. Neurosci.* 24, 130–138.

Kiryu-Seo, S., Kato, R., Ogawa, T., Nakagomi, S., Nagata, K., and Kiyama, H. (2008). Neuronal injury-inducible gene is synergistically regulated by ATF3, c-Jun, and STAT3 through the interaction with Sp1 in damaged neurons. *J. Biol. Chem.* 283, 6988–6996.

Kloss, C. U., Werner, A., Klein, M. A., Shen, J., Menuz, K., Probst, J. C., Kreutzberg, G. W., and Raivich, G. (1999). Integrin family of cell adhesion molecules in the injured brain: regulation and cellular localization in the normal and regenerating mouse facial motor nucleus. *J. Comp. Neurol.* 411, 162–178.

Korneev, S., Federov, A., Collins, R., Blackshaw, S., and Davies, J. (1997). A subtractive cDNA library from an identified regenerating neuron is enriched in sequences up-regulated during nerve regeneration. *Invert. Neurosci.* 3, 185–192.

Krebs, D. L., and Hilton, D. J. (2001). SOCS proteins: negative regulators of cytokine signalling. *Stem Cells* 19, 378–387.

Kubo, T., Yamashita, T., Yamaguchi, A., Hosokawa, K., and Tohyama, M. (2002). Analysis of genes induced in peripheral nerve after axotomy using cDNA microarrays. *J. Neurochem.* 82, 1129–1136.

Kuhlbrodt, K., Herbarth, B., Sock, E., Enderich, J., Hermans-Borgmeyer, I., and Wegner, M. (1998). Cooperative function of POU proteins and SOX proteins in glial cells. *J. Biol. Chem.* 273, 16050–16057.

Kury, P., Abankwa, D., Kruse, F., Greiner-Petter, R., and Muller, H. W. (2004). Gene expression profiling reveals multiple novel intrinsic and extrinsic factors associated with axonal regeneration failure. *Eur. J. Neurosci.* 19, 32–42.

Latouche, M., Wilton, D., Arthur-Farraj, P., DaCosta, C., Mitter, R., Turmaine, M., Raivich, G., Behrens, A., Mirsky, R., and Jessen, K. R. (2009). Schwann cell c-Jun regulates glial support for survival of PNS neurons after injury. *Glia* 57, S158.

Lavin, M. F., and Gueven, N. (2006). The complexity of p53 stabilization

and activation. *Cell Death Differ.* 13, 941–950.

Lee, N., Neitzel, K. L., Devlin, B. K., and MacLennan, A. J. (2004). STAT3 phosphorylation in injured axons before sensory and motor neuron nuclei: potential role for STAT3 as a retrograde signaling transcription factor. *J. Comp. Neurol.* 474, 535–545.

Leppa, S., Saffrich, R., Ansorge, W., and Bohmann, D. (1998). Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. *EMBO J.* 17, 4404–4413.

Leu, J. I., Crissey, M. A. S., Leu, J. P., Ciliberto, G., and Tau, R. (2001). Interleukin-6-induced STAT3 and AP-1 amplify hepatocyte nuclear factor 1-mediated transactivation of hepatic genes, an adaptive response to liver injury. *Mol. Cell. Biol.* 21, 414–424.

Levy, D. E., and Lee, C. K. (2002). What does STAT3 do? *J. Clin. Invest.* 109, 1143–1148.

Lieberman, A. R. (1971). The axon reaction – a review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* 14, 49–124.

Lim, C. P., and Cao, X. M. (2006). Structure, function, and regulation of STAT proteins. *Mol. Biosyst.* 2, 536–550.

Lindholm, D., Heumann, R., Meyer, M., and Thoenen, H. (1987). Interleukin-1 regulates synthesis of nerve growth-factor in nonneuronal cells of rat sciatic-nerve. *Nature* 330, 658–659.

Lindwall, C., Dahlin, L., Lundborg, G., and Kanje, M. (2004). Inhibition of c-Jun phosphorylation reduces axonal outgrowth of adult rat nodose ganglia and dorsal root ganglia sensory neurons. *Mol. Cell. Neurosci.* 27, 267–279.

Lindwall, C., and Kanje, M. (2005). Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. *Mol. Cell. Neurosci.* 29, 269–282.

Liu, R. Y., and Snider, W. D. (2001). Different signaling pathways mediate regenerative versus developmental sensory axon growth. *J. Neurosci.* 21, RC164.

Lonze, B. E., and Ginty, D. D. (2002). Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 35, 605–623.

Lonze, B. E., Riccio, A., Cohen, S., and Ginty, D. D. (2002). Apoptosis, axonal growth defects, and

degeneration of peripheral neurons in mice lacking CREB. *Neuron* 34, 371–385.

Ma, V. Y., and Bisby, M. A. (1998). Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. *Brain Res.*797, 243–254.

Magoulas, B., and Lopez-de Heredia, L. (2010). Functional analysis of the transcription factor C/EBPdelta in neuronal repair. *FENS Abstr.* 5, 040.12.

Maier, I. C., and Schwab, M. E. (2006). Sprouting, regeneration and circuit formation in the injured spinal cord: factors and activity. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 1611–1634.

Makwana, M., Werner, A., Acosta-Saltos, A., Gonitel, R., Pararajasingham, A., Ruff, C., Rumajogee, P., Cuthill, D., Galiano, M., Bohatschek, M., Wallace, A. S., Anderson, P. N., Mayer, U., Behrens, A., and Raivich, G. (2010). Peripheral facial nerve axotomy in mice causes sprouting of motor axons into perineuronal central white matter: time course and molecular characterization. *J. Comp. Neurol.* 518, 699–721.

Mandemakers, W., Zwart, R., Jaegle, M., Walbeehm, E., Visser, P., Grosveld, F., and Meijer, D. (2000). A distal Schwann cell-specific enhancer mediates axonal regulation of the Oct-6 transcription factor during peripheral nerve development and regeneration. *EMBO J.* 19, 2992–3003.

Mason, M. R., Lieberman, A. R., and Anderson, P. N. (2003). Corticospinal neurons up-regulate a range of growth-associated genes following intracortical, but not spinal, axotomy. *Eur. J. Neurosci.* 18, 789–802.

Mattson, M. P. (2005). NF-kappa B in the survival and plasticity of neurons. *Neurochem. Res.* 30, 883–893.

Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* 2, 599–609.

Mayumi-Matsuda, K., Kojima, S., Nakayama, T., Suzuki, H., and Sakata, T. (1999). Scanning gene expression during neuronal cell death evoked by nerve growth factor depletion. *Biochim. Biophys. Acta* 1489, 293–302.

Miao, T., Wu, D. S., Zhang, Y., Bo, X. N., Subang, M. C., Wang, P., and Richardson, P. M. (2006). Suppressor of cytokine signaling-3 suppresses the ability of activated

signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons. *J. Neurosci.* 26, 9512–9519.

Miao, T. Z., Wu, D. S., Zhang, Y., Bo, X. N., Xiao, F., Zhang, X. Y., Magoulas, C., Subang, M. C., Wang, P., and Richardson, P. M. (2008). SOCS3 suppresses AP-1 transcriptional activity in neuroblastoma cells through inhibition of c-Jun N-terminal kinase. *Mol. Cell. Neurosci.* 37, 367–375.

Michaelevski, I., Segal-Ruder, Y., Rozenbaum, M., Medzihradszky, K. F., Shalem, O., Coppola, G., Horn-Saban, S., Ben-Yaakov, K., Dagan, S. Y., Rishal, I., Geschwind, D. H., Pilpel, Y., Burlingame, A. L., and Fainzilber, M. (2010). Signalling to transcription networks in the neuronal retrograde injury response. *Sci. Signal.* 3, 1–11.

Moisse, K., Volkening, K., Leystra-Lantz, C., Welch, I., Hill, T., and Strong, M. J. (2009). Divergent patterns of cytosolic TDP-43 and neuronal progranulin expression following axotomy: implications for TDP-43 in the physiological response to neuronal injury. *Brain Res.* 1249, 202–211.

Moore, D. L., and Goldberg, J. L. (2011). Multiple transcription factor families regulate axon growth and regeneration. *Dev. Neurobiol.* 71, 1186–1211.

Mori, N., and Morii, H. (2002). SCG10-related neuronal growth-associated proteins in neural development, plasticity, degeneration, and aging. *J. Neurosci. Res.* 70, 264–273.

Morrison, D. K., and Davis, R. J. (2003). Regulation of map kinase signaling modules by scaffold proteins in mammals. *Annu. Rev. Cell Dev. Biol.* 19, 91–118.

Morton, S., Davis, R. J., McLaren, A., and Cohen, P. (2003). A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. *EMBO J.* 22, 3876–3886.

Moya, C., Huang, Z., Cheng, P., Jayaraman, A., and Hahn, J. (2011). Investigation of IL-6 and IL-10 signalling via mathematical modelling. *IET Syst. Biol.* 5, 15–26.

Nadeau, S., Hein, P., Fernandes, K. J., Peterson, A. C., and Miller, F. D. (2005). A transcriptional role for C/EBP beta in the neuronal response to axonal injury. *Mol. Cell. Neurosci.* 29, 525–535.

Nagata, K., Kiryu-Seo, S., Maeda, M., Yoshida, K., Morita, T., and Kiyama, H. (2010). Damage-induced neuronal endopeptidase is critical for presynaptic formation

of neuromuscular junctions. *J. Neurosci.* 30, 6954–6962.

Nakagomi, S., Suzuki, Y., Namikawa, K., Kiryu-Seo, S., and Kiyama, H. (2003). Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. *J. Neurosci.* 23, 5187–5196.

Narumi, S., and Fujita, T. (1978). Stimulatory effects of substance-P and nerve growth factor (NGF) on neurite outgrowth in embryonic chick dorsal root ganglia. *Neuropharmacology* 17, 73–76.

Oliveira, A. L., Thams, S., Lidman, O., Piehl, F., Hokfelt, T., Karre, K., Linda, H., and Cullheim, S. (2004). A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy. *Proc. Natl. Acad. Sci. U.S.A.* 101, 17843–17848.

Patodia, S., Hristova, M., Makwana, M., Rumajogee, P., Akira, S., and Raivich, G. (2011). Transcription factor STAT3 is required for efficient regeneration after peripheral nerve injury. *Abstr. Soc. Neurosci.* 784.01/BB22.

Pearson, A. G., Gray, C. W., Pearson, J. F., Greenwood, J. A., During, M. J., and Dragunow, M. (2003). ATF3 enhances c-Jun-mediated neurite sprouting. *Brain Res. Mol. Brain Res.* 120, 38–45.

Perkins, N. D. (2007). Integrating cell-signalling pathways with NF-kappa B and IKK function. *Nat. Rev. Mol. Cell Biol.* 8, 49–62.

Pollock, G., Pennypacker, K. R., Memet, S., Israel, A., and Saporta, S. (2005). Activation of NF-kappa B in the mouse spinal cord following sciatic nerve transection. *Exp. Brain Res.* 165, 470–477.

Pradervand, S., Yasukawa, H., Muller, O. G., Kjekshus, H., Nakamura, T., St Amand, T. R., Yajima, T., Matsumura, K., Duplain, H., Iwatate, M., Woodard, S., Pedrazzini, T., Ross, J., Firsov, D., Rossier, B. C., Hoshijima, M., and Chien, K. R. (2004). Small proline-rich protein 1A is a gp130 pathway- and stress-inducible cardioprotective protein. *EMBO J.* 23, 4517–4525.

Qin, Q. Y., Baudry, M., Liao, G. H., Noniyev, A., Galeano, J., and Bi, X. N. (2009). A novel function for p53: regulation of growth cone motility through Interaction with rho kinase. *J. Neurosci.* 29, 5183–5192.

Raivich, G. (2008). c-Jun expression, activation and function in neural cell death, inflammation and repair. *J. Neurochem.* 107, 898–906.

Raivich, G. (2011). Transcribing the path to neurological recovery-from early signals through transcription factors to downstream effectors of successful regeneration. *Ann. Anat.* 193, 248–258.

Raivich, G., and Behrens, A. (2006). Role of the AP-1 transcription factor c-Jun in developing, adult and injured brain. *Prog. Neurobiol.* 78, 347–363.

Raivich, G., Bohatschek, M., Da Costa, C., Iwata, O., Galiano, M., Hristova, M., Nateri, A. S., Makwana, M., Riera-Sans, L., Wolfer, D. P., Lipp, H. P., Aguzzi, A., Wagner, E. F., and Behrens, A. (2004). The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* 43, 57–67.

Raivich, G., Hellweg, R., and Kreutzberg, G. W. (1991). NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. *Neuron* 7, 151–164.

Raivich, G., Jones, L. L., Kloss, C. U., Werner, A., Neumann, H., and Kreutzberg, G. W. (1998). Immune surveillance in the injured nervous system: T-lymphocytes invade the axotomized mouse facial motor nucleus and aggregate around sites of neuronal degeneration. *J. Neurosci.* 18, 5804–5816.

Ram, P. T., and Iyengar, R. (2001). G protein coupled receptor signaling through the Src and STAT3 pathway: role in proliferation and transformation. *Oncogene* 20, 1601–1606.

Ramji, D. P., and Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* 365, 561–575.

Ramji, D. P., Vitelli, A., Tronche, F., Cortese, R., and Ciliberto, G. (1993). The 2 c/EBP isoforms, IL-6DBP/NF-IL6 and c/EBP-delta/NF-IL6 beta, are induced by IL-6 to promote acute phase gene-transcription via different mechanisms. *Nucleic Acids Res.* 21, 289–294.

Redmond, L., Kashani, A. H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron* 34, 999–1010.

Rende, M., Muir, D., Ruoslahti, E., Hagg, T., Varon, S., and Manthorpe, M. (1992). Immunolocalization of ciliary neuronotrophic factor in adult rat sciatic nerve. *Glia* 5, 25–32.

Rudolph, D., Tafuri, A., Gass, P., Hammerling, G., Arnold, B., and Schutz, G. (1998). Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response

element binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 95, 4481–4486.

Ruff, C., Da Costa, C., Staak, N., Rumajogee, P., Makwana, M., Acosta-Saltos, A., Kaswich, M., Brecht, S., Hristova, M., Riera-Sans, L., Mirsky, R., Herdegen, T., Jessen, K., Behrens, A., and Raivich, G. (2009). The role of c-Jun and its N-terminal phosphorylation in facial nerve regeneration. *Abstr. Soc. Neurosci.* 510.9/B42.

Sark, M. W. J., Fischer, D. F., de Meijer, E., van de Putte, P., and Backendorf, C. (1998). AP-1 and ets transcription factors regulate the expression of the human SPRR1A keratinocyte terminal differentiation marker. *J. Biol. Chem.* 273, 24683–24692.

Sato, T., Takeuchi, S., Saito, A., Ding, W., Bamba, H., Matsuura, H., Hisa, Y., Tooyama, I., and Urushitani, M. (2009). Axonal ligation induces transient redistribution of TDP-43 in brainstem motor neurons. *Neuroscience* 164, 1565–1578.

Sauer, B. (1998). Inducible gene targeting in mice using the Cre/lox system. *Methods* 14, 381–392.

Schaefer, T. S., Sanders, L. K., and Nathans, D. (1995). Cooperative transcriptional activity of Jun and STAT3-beta, a short-form of STAT3. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9097–9101.

Scherer, S. S., Wang, D. Y., Kuhn, R., Lemke, G., Wrabetz, L., and Kamholz, J. (1994). Axons regulate Schwann-cell expression of the pou transcription factor Scip. *J. Neurosci.* 14, 1930–1942.

Schmied, R., Huang, C. C., Zhang, X. P., Ambron, D. A., and Ambron, R. T. (1993). Endogenous axoplasmic proteins and proteins containing nuclear localization signal sequences use the retrograde axonal transport/nuclear import pathway in *Aplysia* neurons. *J. Neurosci.* 13, 4064–4071.

Schwaiger, F. W., Hager, G., Schmitt, A. B., Horvat, A., Hager, G., Streif, R., Spitzer, C., Gamal, S., Breuer, S., Brook, G. A., Nacimiento, W., and Kreutzberg, G. W. (2000). Peripheral but not central axotomy induces changes in Janus kinases (JAK) and signal transducers and activators of transcription (STAT). *Eur. J. Neurosci.* 12, 1165–1176.

Schwarz, C. S., Seyfried, J., Evert, B. O., Klockgether, T., and Wullner, U. (2002). Bcl-2 up-regulates ha-ras mRNA expression and induces c-Jun phosphorylation at Ser73 via an ERK-dependent pathway in PC 12 cells. *Neuroreport* 13, 2439–2442.

Schweizer, U., Gunnersen, J., Karch, C., Wiese, S., Holtmann, B., Takeda, K.,

Akira, S., and Sendtner, M. (2002). Conditional gene ablation of STAT3 reveals differential signaling requirements for survival of motoneurons during development and after nerve injury in the adult. *J. Cell Biol.* 156, 287–297.

Seijffers, R., Allchorne, A. J., and Woolf, C. J. (2006). The transcription factor ATF-3 promotes neurite outgrowth. *Mol. Cell. Neurosci.* 32, 143–154.

Seijffers, R., Mills, C. D., and Woolf, C. J. (2007). ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J. Neurosci.* 27, 7911–7920.

Sendtner, M., Gotz, R., Holtmann, B., Escary, J. L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brulet, P., and Thoenen, H. (1996). Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. *Curr. Biol.* 6, 686–694.

Sendtner, M., Gotz, R., Holtmann, B., and Thoenen, H. (1997). Endogenous ciliary neurotrophic factor is a lesion factor for axotomized motoneurons in adult mice. *J. Neurosci.* 17, 6999–7006.

Shackleford, T. J., Zhang, Q., Tian, L., Vu, T. T., Korapati, A. L., Baumgartner, A. M., Le, X. F., Liao, W. S., and Claret, F. X. (2011). STAT3 and CCAAT/enhancer binding protein beta (C/EBP-beta) regulate Jab1/CSN5 expression in mammary carcinoma cells. *Breast Cancer Res.* 13, R65.

Skene, J. H. P., and Willard, M. (1981). Characteristics of growth-associated polypeptides in regenerating toad retinal ganglion-cell axons. *J. Neurosci.* 1, 419–426.

Smith, D. S., and Skene, J. H. P. (1997). A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *J. Neurosci.* 17, 646–658.

Sole, C., Dolcet, X., Segura, M. F., Gutierrez, H., Diaz-Meco, M. T., Gozzelino, R., Sanchis, D., Bayascas, J. R., Gallego, C., Moscat, J., Davies, A. M., and Comella, J. X. (2004). The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-kappa B signaling. *J. Cell Biol.* 167, 479–492.

Suarez, V., Guntinas-Lichius, O., Streppel, M., Ingorokva, S., Grosheva, M., Neiss, W. F., Angelov, D. N., and Klimaschewski, L. (2006). The axotomy-induced neuropeptides galanin and pituitary adenylate cyclase-activating peptide promote axonal sprouting of primary afferent

and cranial motor neurones. *Eur. J. Neurosci.* 24, 1555–1564.

Sun, F., Park, K. K., Belin, S., Wang, D., Lu, T., Chen, G., Zhang, K., Yeung, C., Feng, G., Yankner, B. A., and He, Z. (2011). Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. *Nature* 480, 372–375.

Sun, P. Q., Enslen, H., Myung, P. S., and Maurer, R. A. (1994). Differential activation of Creb by Ca2+/calmodulin-dependent protein-kinases type-II and type-IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* 8, 2527–2539.

Sung, Y. J., Povelones, M., and Ambron, R. T. (2001). RISK-1: a novel MAPK homologue in axoplasm that is activated and retrogradely transported after nerve injury. *J. Neurobiol.* 47, 67–79.

Symes, A., Gearan, T., Eby, J., and Fink, J. S. (1997). Integration of Jak-STAT and AP-1 signaling pathways at the vasoactive intestinal peptide cytokine response element regulates ciliary neurotrophic factor-dependent transcription. *J. Biol. Chem.* 272, 9648–9654.

Taga, T., and Kishimoto, T. (1995). Signaling mechanisms through cytokine receptors that share signal-transducing receptor components. *Curr. Opin. Immunol.* 7, 17–23.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Targeted disruption of the mouse STAT3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci. U.S.A.* 94, 3801–3804.

Takeda, M., Kato, H., Takamiya, A., Yoshida, A., and Kiyama, H. (2000). Injury-specific expression of activating transcription factor-3 in retinal ganglion cells and its colocalized expression with phosphorylated c-Jun. *Invest. Ophthalmol. Vis. Sci.* 41, 2412–2421.

Tanaka, S., Kamachi, Y., Tanouchi, A., Hamada, H., Jing, N. H., and Kondoh, H. (2004). Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. *Mol. Cell. Biol.* 24, 8834–8846.

Tedeschi, A., Nguyen, T., Puttagunta, R., Gaub, P., and Di Giovanni, S. (2009). A p53-CBP/p300 transcription module is required for GAP-43 expression, axon outgrowth, and regeneration. *Cell Death Differ.* 16, 543–554.

Topilko, P., Schneidermaunoury, S., Levi, G., Baronvanevercooren, A., Chennoufi, A. B., Seitanidou, T., Babinet, C., and Charnay, P. (1994). Krox-20 controls myelination in the

peripheral nervous system. *Nature* 371, 796–799.

Toth, C. C., Willis, D., Twiss, J. L., Walsh, S., Martinez, J. A., Liu, W. Q., Midha, R., and Zochodne, D. W. (2009). Locally synthesized calcitonin gene-related peptide has a critical role in peripheral nerve regeneration. *J. Neuropathol. Exp. Neurol.* 68, 326–337.

Tsujino, H., Kondo, E., Fukuoka, T., Dai, Y., Tokunaga, A., Miki, K., Yonenobu, K., Ochi, T., and Noguchi, K. (2000). Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: a novel neuronal marker of nerve injury. *Mol. Cell. Neurosci.* 15, 170–182.

Vries, R. G. J., Prudenziati, M., Zwartjes, C., Verlaan, M., Kalkhoven, E., and Zantema, A. (2001). A specific lysine in c-Jun is required for transcriptional repression by E1A and is acetylated by p300. *EMBO J.* 20, 6095–6103.

Waetzig, V., Zhao, Y., and Herdegen, T. (2006). The bright side of JNKs – multitalented mediators in neuronal sprouting, brain development and nerve fiber regeneration. *Prog. Neurobiol.* 80, 84–97.

Waller, A. V. (1850). 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. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 140, 423–429.

Weber, J. R. M., and Skene, J. H. P. (1998). The activity of a

highly promiscuous AP-1 element can be confined to neurons by a tissue-selective repressive element. *J. Neurosci.* 18, 5264–5274.

Werner, A., Kloss, C. U., Walter, J., Kreutzberg, G. W., and Raivich, G. (1998). Intercellular adhesion molecule-1 (ICAM-1) in the mouse facial motor nucleus after axonal injury and during regeneration. *J. Neurocytol.* 27, 219–232.

Westerlund, N., Zdrojewska, J., Padzik, A., Komulainen, E., Björkblom, B., Rannikko, E., Tararuk, T., Garcia-Frigola, C., Sandholm, J., Nguyen, L., Kallunki, T., Courtney, M. J., and Coffey, E. T. (2011). Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. *Nat. Neurosci.* 14, 305–313.

Williams, K. L., Rahimtula, M., and Mearow, K. M. (2006). Heat shock protein 27 is involved in neurite extension and branching of dorsal root ganglion neurons in vitro. *J. Neurosci. Res.* 84, 716–723.

Wilton, D., Arthur-Farraj, P., Latouche, M., Turmaine, M., Behrens, A., Mirsky, R., Raivich, G., and Jessen, K. (2009). Schwann cell C-Jun is essential for peripheral nerve regeneration. *Glia* 57, S158.

Wu, Y. Y., and Bradshaw, R. A. (1996). Induction of neurite outgrowth by interleukin-6 is accompanied by activation of STAT3 signaling pathway in a variant PC12 cell (E2) line. *J. Biol. Chem.* 271, 13023–13032.

Wynick, D., Thompson, S. W., and McMahon, S. B. (2001). The role of galanin as a multi-functional neuropeptide in the nervous system. *Curr. Opin. Pharmacol.* 1, 73–77.

Xia, X. G., Hofmann, H. D., Deller, T., and Kirsch, M. (2002). Induction of STAT3 signaling in activated astrocytes and sprouting septal neurons following entorhinal cortex lesion in adult rats. *Mol. Cell. Neurosci.* 21, 379–392.

Yang, E., Lerner, L., Besser, D., and Darnell, J. E. Jr. (2003). Independent and cooperative activation of chromosomal c-fos promoter by STAT3. *J. Biol. Chem.* 278, 15794–15799.

Yang, Z. Y., Quigley, H. A., Pease, M. E., Yang, Y. Q., Qian, J., Valenta, D., and Zack, D. J. (2007). Changes in gene expression in experimental glaucoma and optic nerve transection: the equilibrium between protective and detrimental mechanisms. *Invest. Ophthalmol. Vis. Sci.* 48, 5539–5548.

Yoo, S., Nguyen, M. P., Fukuda, M., Bittner, G. D., and Fishman, H. M. (2003). Plasmalemmal sealing of transected mammalian neurites is a gradual process mediated by Ca(2+)-regulated proteins. *J. Neurosci. Res.* 74, 541–551.

Yoshida, K., Behrens, A., Le-Niculescu, H., Wagner, E. F., Harada, T., Imaki, J., Ohno, S., and Karin, M. (2002). Amino-terminal phosphorylation of c-Jun regulates apoptosis in the retinal ganglion cells by optic nerve

transection. *Invest. Ophthalmol. Vis. Sci.* 43, 1631–1635.

Zhang, X. K., Wrzeszczynska, M. H., Horvath, C. M., and Darnell, J. E. (1999). Interacting regions in STAT3 and c-Jun that participate in cooperative transcriptional activation. *Mol. Cell. Biol.* 19, 7138–7146.

Zigmond, R. E., and Vaccariello, S. A. (2007). Activating transcription factor 3 immunoreactivity identifies small populations of axotomized neurons in rat cervical sympathetic ganglia after transection of the preganglionic cervical sympathetic trunk. *Brain Res.* 1159, 119–123.

ORIGINAL ARTICLE

Neuronal c-Jun is required for successful axonal regeneration, but the effects of phosphorylation of its N-terminus are moderate

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Abstract

Although neural c-Jun is essential for successful peripheral nerve regeneration, the cellular basis of this effect and the impact of c-Jun activation are incompletely understood. In the current study, we explored the effects of neuron-selective c-Jun deletion, substitution of serine 63 and 73 phosphoacceptor sites with non-phosphorylatable alanine, and deletion of Jun N-terminal kinases 1, 2 and 3 in mouse facial nerve regeneration. Removal of the floxed *c-jun* gene in facial motoneurons using cre recombinase under control of a neuron-specific synapsin promoter (junΔS) abolished basal and injury-induced neuronal c-Jun immunoreactivity, as well as most of the molecular responses following facial axotomy. Absence of neuronal Jun reduced the speed of axonal regeneration following crush, and prevented most cut axons from reconnecting to their target, significantly reducing functional recovery. Despite blocking cell death, this was associated with a large number of shrunken neurons. Finally, junΔS mutants also had diminished astrocyte and microglial activation and T-cell influx, suggesting that these non-neuronal responses depend on the release of Jun-dependent signals from neighboring injured motoneurons. The effects of substituting serine 63 and 73 phosphoacceptor sites (junAA), or of global deletion of individual kinases responsible for N-terminal c-Jun phosphorylation were mild. junAA mutants showed decrease in neuronal cell size, a moderate reduction in post-axotomy CD44 levels and slightly increased astrogliosis. Deletion of Jun N-terminal kinase (JNK)1 or JNK3 showed delayed functional recovery; deletion of JNK3 also interfered with T-cell influx, and reduced CD44 levels. Deletion of JNK2 had no effect. Thus, neuronal c-Jun is needed in regeneration, but JNK phosphorylation of the N-terminus mostly appears to not be required for its function.

Keywords: brain repair, peripheral nerve regeneration, phosphorylation, transcription factor.

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Transection of peripheral nerves or their axons (axotomy) usually results in successful regeneration, by a part of the axon still connected to its cell body growing back to and reinnervating the peripheral target (Lieberman 1971; Kreutzberg 1996). Recent studies using transgenic animal models and viral vectors have demonstrated important regeneration-promoting roles for several newly expressed cell adhesion molecules (Werner *et al.* 2000; Hiroi *et al.* 2003, Makwana *et al.* 2009), neurotrophic and growth factor signals (English *et al.* 2011; Makwana *et al.* 2009) and neuropeptides (Sachs *et al.* 2007). The fact that many of these molecular changes occur at the same time following injury has focused interest on axotomy-induced transcription factors that may orchestrate this regenerative response. Deletion of CCAAT enhancer binding protein (cEBP) beta, cEBP delta, sox11 and signal transducer and activator of

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transcription (STAT)3 has been associated with a reduction in the initiation of axonal outgrowth and/or functional recovery (Nadeau *et al.* 2005; Jankowski *et al.* 2009; Magoulas and Lopez De Heredia 2010; Bareyre *et al.* 2011). The effects were particularly pronounced with brain-wide deletion of c-Jun, which prevented the up-regulation of axotomy-associated molecules (CD44, alpha7-beta1 integrin, galanin), decreased successful reconnection to peripheral targets by 4- to 5-fold, and strongly reduced functional recovery (Raivich *et al.* 2004). Interestingly, cre recombinase-mediated deletion of floxed c-Jun was also associated with enhanced neuronal post-axotomy survival, but of severely shrunken motoneurons. Because this cre recombinase was driven by the nestin-promoter, expressed in early neuroepithelial cells giving rise to neurons, astrocytes or oligodendroglia, as well as in Schwann cell precursors, it is still unclear whether the effects observed in neuronal regeneration were due to its absence in neurons or other nestin+ progenitor-derived cells (Rao 1999; Akiyama *et al.* 2001; Kanu *et al.* 2010).

The activator protein 1 (AP1) transcription complex is a well-characterized regulator of neural development and response to trauma, excitotoxic or hypoxic-ischemic injury and consists of homo- or heterodimeric complexes between members of the Jun, c-Fos and activating transcription factor (ATF)/cyclic adenosine monophosphate (AMP) response element-binding protein (CREB) families (Angel and Karin 1992). Its primary component, c-Jun, is produced as an immediate early gene following nerve injury and persists at high levels during the entire peripheral regenerative process (Herdegen *et al.* 1991; Jenkins and Hunt 1991). With the exception of retinal ganglion cell neurons (Herdegen *et al.* 1993; Koistinaho *et al.* 1993), up-regulation of c-Jun is either transient or absent following transection of central fibers, and may contribute to the reduced ability of centrally injured neurons to regenerate (Anderson *et al.* 1998). Activation of the c-Jun mediated transcription apparatus is affected by interactions at three major sites: (i) N-terminal phosphory-lation at Ser 63 and 73 and Thr 91 and 93 (Smeal *et al.* 1994; Morton *et al.* 2003) and the ensuing ubiquitination and degradation (Nateri *et al.* 2004), (ii) dephosphorylation of Thr239 (Morton *et al.* 2003) and (iii) the C-terminal lysine acetylation near aa257–276 (Vries *et al.* 2001). The best studied is N-terminal phosphorylation through Jun N-termi-nal kinases 1, 2 and 3 (JNK1–3), which can alter AP1 binding activity in the absence of de novo protein synthesis (Dérijard *et al.* 1994; Kallunki *et al.* 1994). These JNK mitogen-activated protein kinases are rapidly activated following peripheral nerve injury and increase the amount of phospho-c-Jun in the process (Waetzig *et al.* 2006). Depending on the type of neural injury, deletion of JNK1, 2 and 3 has been shown to a prodegenerative or protective effect (Pirianov *et al.* 2007; Haeusgen *et al.* 2009). Removal of serine 63 and 73 phosphoacceptor sites in the junAA

mutant also interferes with exitotoxicity (Behrens *et al.* 1999), but relatively little is known about their involvement in axotomy response and neurite outgrowth *in vivo*. In the current study, we therefore examined the function, cellular basis and possible activation of c-Jun in peripheral regener-ation, using mutants with neuron-specific deletion of c-Jun, global deletion of JNK1, 2 or 3, and alanine replacement of the serine 63 and 73 sites.

Materials and methods

Animals, surgical procedures and tissue treatment

Mice carrying floxed *c-jun* allele ($jun^{F/F}$) (Behrens *et al.* 2002) were crossed with syn::cre animals expressing cre recombinase under the control of the synapsin promoter syn::cre (Zhu *et al.* 2001), twice, to obtain homozygous CNS neuron-specific deletion of *jun* ($jun\Delta S$). To prevent germline *c-jun* deletion due to testicular synapsin promoter expression (Hoesche *et al.* 1993; Street *et al.* 2005), only female cre+ mice (syn::cre $jun^{F/F}$) and cre- $jun^{F/F}$ males were used for breeding, generating $jun\Delta S$ mutants and $jun^{F/F}$ littermate controls with normal Mendelian frequency. Homozygous JunAA, with serine 63 and 73 residues replaced with alanine (A) (Behrens *et al.* 1999) and wild-type (wt) mice were derived as offspring of heterozygous (AA/wt) breeder pairs. JNK1, 2 and 3 null mouse strains (Yang *et al.* 1997; Dong *et al.* 1998; Kuan *et al.* 1999) were crossed with C57Bl/6 mice, to obtain F1 heterozygotes, and in the second step, the F2 homozygote and wild-type breeder pairs that were used to generate mutants and their controls as described in detail elsewhere (Brecht *et al.* 2005).

Mutant and wild-type controls 8–10 weeks old were anesthetised with 10 µg/g 2,2,2-tribromoethanol (Sigma, Dorset, UK), the right facial nerve was either crushed (for the day 4 time point) or cut for all other time points at the stylomastoid foramen, and the animals killed after 4–30 days with an overdose of Euthatal. The animals were first perfused as described by Moller *et al.* (1996) with 150 mL phosphate-buffered saline (PBS) followed by 200 mL of 4% paraformaldehyde in PBS, 2 h post-fixed with 1% paraformalde-hyde in PBS, cryoprotected in 30% sucrose in PBS and then frozen on dry ice. The immunohistochemical methods, assessment of axonal regeneration and functional recovery, retrograde tracing and quantification of neuronal survival were performed as previously (Moller *et al.* 1996; Raivich *et al.* 1998, 2002, 2004) and described in detail in the Methods section of the Supplementary material.

Results

Generation of mutant mice

Cell type specific c-jun gene deletion was achieved by crossing mice with a floxed c-jun allele ($jun^{F/F}$) with animals expressing cre recombinase under the control of the synapsin promoter (syn::cre), which targets the $jun\Delta S$ recombination specifically to neurons (Kügler *et al.* 2001; Zhu *et al.* 2001). Homozygous JunAA and wild-type (wt) mice were derived as offspring of heterozygous (AA/wt) breeder pairs. JNK1, 2 and 3 knockout mice and their controls were produced from F2 homozygous breeder pairs derived from heterozygous

mice on C57Bl/6 background. All mutants used in the current study displayed normal brain structure and histology on H&E and NeuN stains on forebrain (cortex, hippocampus, basal ganglia) and hind-brain (cerebellum, metencephalon) sections (not shown). Homozygous *jun*ΔS recombination was associated with disappearance of basal immunoreactivity throughout the brain, for example in hippocampus and dentate gyrus (Fig. 1a and b), or the uninjured facial motor nucleus (Fig. 1c and d). Motoneuron c-Jun immunoreactivity is strongly up-regulated by facial nerve cut (Fig. 1f) and this increase was abolished in the *jun*ΔS mutant (Fig. 1e). Previous study from our groups revealed the disappearance of phospho-serine63 immunoreactivity in the neuronal nuclei of the axotomized facial motor nucleus in junAA mice but also a persistence of phosphoserine 73-like and as well as of total c-Jun immunoreactivity. In the same study, single JNK deletions were not associated with changes in overall phospho-Jun immunoreactivity (Brecht *et al.* 2005). This persistence of total c-Jun immunoreactivity in neuronal nuclei of the axotomized facial motor nucleus in the junAA mice was also reconfirmed in the current study (data not shown).
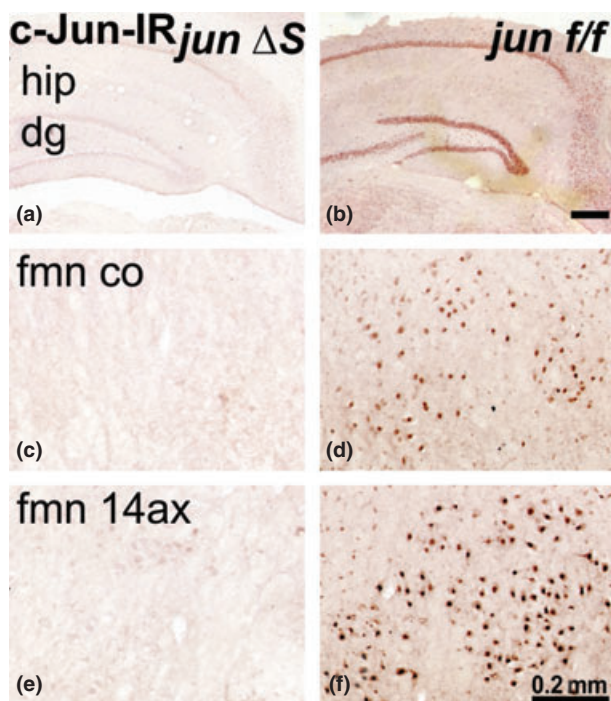

Fig. 1 Synapsin::cre deletion of floxed c-jun (junΔS) removes c-Jun immunoreactivity in normal brain and following facial nerve cut. (a, b) Hippocampus, (c, d) uninjured facial motor nucleus, (e, f) facial nucleus 14 days after facial nerve cut. Micrographs on the left (a, c, e) show the junΔS mutants, those on the right (b, d, f) the control, junF/F animals. Note the strong c-Jun immunoreactivity on the right, in the junF/F hippocampus and hippocampal dentate gyrus (hip and dg), as well as in the large motoneuron nuclei of the facial nucleus (d, f), particularly following nerve injury (f). Scale bar: 0.2 mm.

Deletion of neuronal c-jun interferes with axonal regeneration

Effects on axonal regeneration after facial nerve axotomy at the stylomastoid foramen were assessed by the extent of functional recovery, reinnervation of peripheral target, and speed of axonal elongation in the early phase of nerve regeneration. Functional recovery after facial nerve cut was scored by observed whisker hair movement from 0 (no movement) to 3 (strong, normal movement on uninjured side) by two observers unaware of the animal genotype three times per week for 28 days. As shown in Fig. 2a, $jun^{F/F}$ animals commenced recovery of whisker vibrissae at 12 days and improved steadily over the next 2.5 weeks to day 28. Recovery in homozygous *jun*ΔS mutants was significantly delayed ($p < 0.01$ in unpaired *t*-test, unless indicated, all further tests are also unpaired *t*-test); first noticed at day 19, it then improved but with a less steep trajectory than in the junF/F mice. These differences were mirrored by changes in the regeneration index, calculated as area under the curve for the functional recovery over days 0–28 (Fig. 2b); with an index of 1.00 ± 0.04 for the $jun^{F/F}$ ($n = 5$ animals) and 0.44 ± 0.02 for the *jun*ΔS mutants ($n = 6$ animals, $p < 0.001$).

To determine whether this defect was due to lack of axonal reinnervation or simply reduced function of successfully reinnervated axons, a gelfoam insert soaked with fluorescent tracer Fluorogold (FG) was implanted for 30 min under both whisker pads 28 days after facial nerve cut. The contralateral whisker pad served as an intra-animal, uninjured control. This was followed by 48 h of recovery and retrograde transport to the facial nucleus, and then by perfusion, freezing the brainstem and cutting through the whole brainstem at the level of the facial motor nuclei. The distribution of FG-labelled motoneurons on the the previously uninjured and on the injured side is shown in Fig. 2c,d,g and h. Counting every fifth 20 um section, $jun^{F/F}$ animals ($n = 5$) revealed 168 ± 10 FG+ motoneurons on the uninjured and 57 ± 5 on the injured side. Homozygous *jun*ΔS mutants ($n = 6$) also showed a very similar number (173 ± 9) on the control side, but there was a very drastic reduction on the axotomized side, with just 7 ± 2 FG+ motoneurons (Fig. 1e, $p < 0.001$). In relative terms (as percent of contralateral side) $jun^{F/F}$ animals showed retrograde labelling of $32.9 \pm 4.4\%$, and *jun*ΔS mutants a more than 8-fold decrease with $3.8\% \pm 1.1\%$ (Fig. 1f, $p < 0.001$), exceeding the defect in functional recovery.

As a next step, we explored whether these defects can be attributed to lack of activation of Jun protein function (as opposed to one that is simply absent) by performing these experiments in phosphorylation-deficient homozygous mutants lacking N-terminal phosphoacceptor sites serine-63 and 73 (junAA), or Jun N-terminal Kinases 1, 2 or 3 (JNK1–3), following them for up to 50 days after facial nerve cut. In the case of JunAA (Fig. 3a and b), functional recovery in mutants was on average just barely lower than that in the
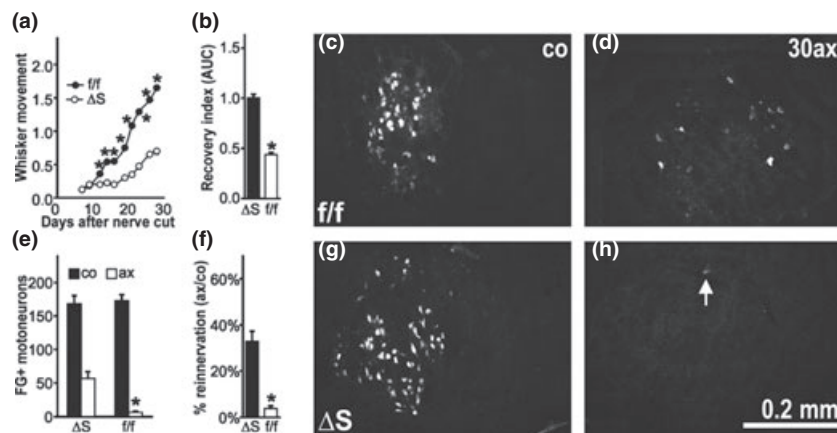
Fig. 2 Neuronal c-Jun is required for successful functional recovery and target reinnervation after facial nerve axotomy. (a, b) Time course for functional recovery of whisker hair movement on the scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) (a) and the recovery index (b) calculated as area under the curve (AUC) show pronounced reduction and delay in recovery junΔS mice ($n = 6$ mice) compared with the junF/F controls ($n = 5$ mice). *$p < 0.05$ in unpaired *t*-test, bars and error bars in these and all following graphs show the mean and SEM, respectively. In panel (a), the significance levels were adjusted for multiple testing using Bonferroni correction. (c–h) Peripheral target (whisker pad) reinnervation using 48 h retrograde transport of FluoroGold to facial motor nucleus 30 days after nerve cut. (c, d, g, h) Micrographs showing FluoroGold labelling on the contralateral control (co; c, g) and axotomized side (ax; d, h). Retrograde labelling on the uninjured side is confined to a discrete facial subnucleus (c, g); after injury, motoneurons reinnervating the whisker pad are found throughout the facial nucleus (f). (e, f) Number of retrogradely labelled, counted motoneurons on the contralateral and axotomized side (e) and the axotomized/control side ratio (f). Neuronal deletion of c-Jun is associated with a more than 8-fold decrease in whiskerpad reinnervation (c, d and arrow in h).

wild-type controls, with the only significant difference ($p < 0.05$) at 12 days following facial nerve cut. Homozygous deletion of JNK2 (Fig. 3i and j) had no significant effect. In contrast, deletion of JNK1 (Fig. 3e and f) and JNK3 (Fig. 3m and n) showed a significant delay in functional recovery at different time points after nerve cut. Similar effects were also observed for the regeneration index, with significant reduction for JNK1 (Fig. 3f, $p < 0.02$) and a similar trend for JNK3 (Fig. 3n, $p = 0.074$).

To see whether these defects were matched by a deficit in target reinnervation, we next determined the extent of 48 h retrograde transport of Fluorogold, 50 days after facial nerve cut. However, as shown for junAA in Fig. 3c and d, for JNK1 in Fig. 3g and h, for JNK2 in Fig. 3k and l and for JNK3 in Fig. 3o and p, all 4 mutants revealed reconnection rates that were very similar to those for their wild-type controls.

As functional recovery following nerve cut depends on the gradual formation of a non-neuronal cellular bridge between the proximal and distal nerve stump and could be affected by JNK deletions, we also examined the extent of early nerve fibre outgrowth following facial nerve crush, where, despite axotomy, the basal lamina scaffolds remain intact. The growth front of the regenerating motor neurites 4 days after facial nerve crush was quantified in longitudinal facial nerve sections of 10 um thickness using immunoreactivity for the calcitonin gene related peptide (CGRP) and galanin neuropeptides expressed in axotomized facial motoneurons (Werner *et al.* 2000). As shown in Fig. 4a, in the *jun^{F/F}* control animals' axonal growth front advanced to 5.8 ± 0.1 mm for

CGRP- and to 5.4 ± 0.2 mm for the galanin-positive axons. In *jun*ΔS mutants, this axonal elongation was reduced by approx. 60% for both types of neuropeptide containing axons ($p < 0.0001$). In contrast to *jun*ΔS, homozygous deletion of JNK1 (Fig. 4c), JNK2 (Fig. 4d) or JNK3 (Fig. 4e) did not affect the extent of axonal elongation, compared with their wild-type controls. Similar lack of effect on early phase of regeneration following nerve crush was also observed for junAA (Fig. 4b).

Neuronal c–Jun deletion abolishes neuronal cell loss after nerve cut but affected motoneurons show atrophic phenotype

Facial axotomy is normally associated with the cell death of 20–40% of lesioned motoneurons within 30 days after nerve cut (Raivich *et al.* 2002) and pan-CNS deletion of c-Jun has been shown to interfere with this process (Raivich *et al.* 2004). Figure S1a–f shows the effects of neuron-specific deletion of jun on the Nissl stained sections of control (Figure S1a and c) and axotomized (Figure S1b and d–f) facial motor nuclei. After facial nerve cut, *jun^{F/F}* control animals showed a pronounced loss of neurons (Figure S1b), an effect lost in the *jun*ΔS mutants. However, many of the axotomized *jun*ΔS motoneurons appeared shrunken and with much paler Nissl staining (Figure S1d, compare also Figure S1e and f). Counting motoneuron profiles on the uninjured and injured side throughout the facial motor nucleus revealed a 38 ± 5% loss in *jun^{F/F}* controls, compared with just 1 ± 4% in the *jun*ΔS mutants (Figure S1i).
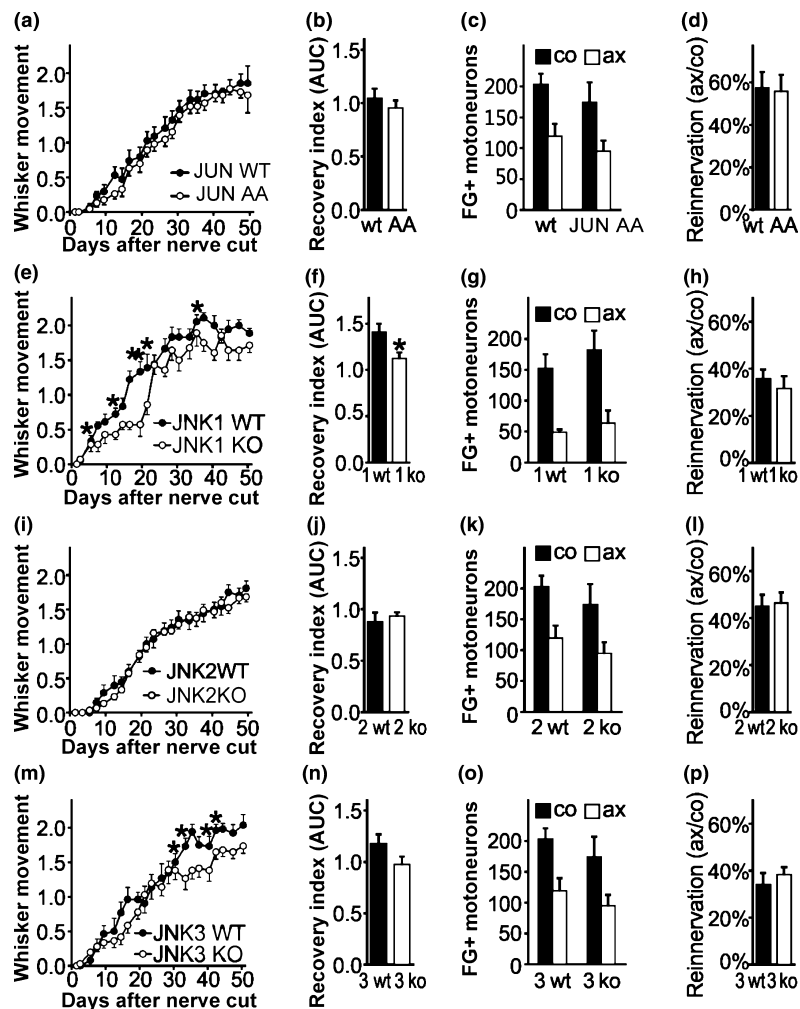
Fig. 3 (a–c, e–g, i–k, m–o) Functional recovery (time course: a, e, i, m; recovery index: b, f, j, n) and whisker pad reinnervation determined by counting retrogradely labelled Fluorogold-fluorescing facial motoneurons (FG+) 30 days after axotomy (c, g, k, o) in JunAA (a–d), JNK1 null (e–h), JNK2 null (i–l) and JNK3 null (m–p) mutants (KO) and their wild-type counterparts (WT). (d, h, l, p) Axotomized (ax)/control (co) side ratio – percent reinnervation – for the FG+ motoneurons. Overall, there was very little effect for junAA (n = 23 mutants and 17 control animals) and JNK2 (n = 30 mutants and 17 controls), but deletion of JNK1 (n = 7 mutants and 9 controls) or JNK3 (n = 18 mutants and 12 controls) was associated with reduced functional recovery (e, f, m).

However, none of the four mutants showed an effect on the anatomical reinnervation of the whisker pad at 30 days after facial nerve cut, detected using Fluorogold retrograde labelling (third and fourth column, n = 7/7, 9/10, 6/5 and 6/9 for the junAA, JNK1, JNK2 and JNK3 null mutants and their controls, respectively. *p < 0.05 in unpaired t-test. In panels (a, e, i and m), the significance levels were adjusted for multiple testing using Bonferroni correction. Filled bars in the third column (c, g, k and o) show FG+ motoneuron number on the control, contralateral side, and empty bars the axotomized side in the same group of animals. In all other graphs, filled bars or circles represent wild-type, and empty bars, mutant animals.

However, opposite effects were observed on cell size (Figure S1m): in $jun^{F/F}$ controls, average neuronal cell diameter showed a trend towards increase on the axotomized side, from 21.5 ± 0.7 um for the uninjured to 23.2 ± 0.8 um for injured motoneurons (p = 0.085), the junΔS mice a decrease from 22.1 ± 0.3 to 15.5 ± 0.8 um (p < 0.005). Compared with controls, the junAA mutants exhibited no effect on neuronal loss (33% ± 8% vs. 35% ± 2% for control and mutant mice, see Figure S1j), but still a significant 14 ± 2% decrease in neuronal size 30 days after facial nerve

cut (p < 0.05, Figure S1n). Deletion of JNK1 or JNK3 was not associated with changes in neuronal loss (Figure S1k and l) or neuronal cell size (Figure S1o and p, respectively).

Neuronal molecular response to injury depends on neuronal c–Jun

In neurons capable of regeneration, axonal injury engenders a switch to a regeneration programme comprising of transcription factor changes, followed by adhesion molecules, neuropeptides and growth factor signalling, and an
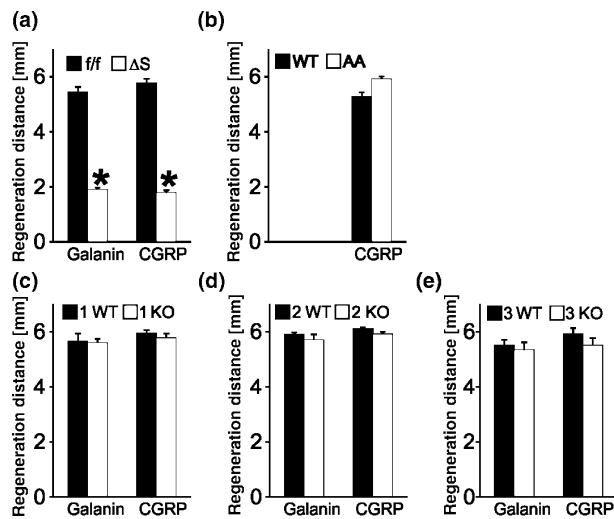
Fig. 4 Facial nerve regeneration relies on the presence of neuronal c-Jun. Longitudinal outgrowth of fastest CGRP- and galanin-immuno-reactive axons in mm below the crush site, 4 days after facial nerve injury. (a–e) Effects of junΔS (a, $n = 6$ control junF/F and 4 junΔS animals), junAA (b, $n = 5$ control and 4 mutant mice) and JNK1 (c, $n = 7$ controls and 4 null mutant mice), JNK2 (d, $n = 2$ controls and 3 null mutant mice) and JNK3 (e, $n = 5$ controls and 5 null mutant mice). Note the very similar outgrowth distances for the CGRP- and galanin-immunoreactive axons. JunΔS deletion caused a 65–70% decrease in neurite outgrowth, all other mutations had no significant effect. *$p < 0.05$ in unpaired *t*-test. Only sections stained for CGRP immunoreactivity were available for junAA and its controls.

increase in nutrient transport, metabolism and cytoskeletal protein synthesis (Makwana and Raivich 2005, Raivich and Makwana 2007). As animals for the day 4 time point were used for speed of regeneration experiment in the crushed facial nerve (Fig. 4), the day 4 facial motor nucleus data are following nerve crush; the day 14 data are following cut.

As shown in left upper part of Fig. 5, axotomy of the facial nerve in the $jun^{F/F}$ control animals causes an increase in the immunoreactivity for the adhesion molecules CD44 (Fig. 5a and b) and the beta1 integrin subunit (Fig. 5e and f), neuropeptides CGRP (Fig. 5i and j) and galanin (Fig. 5m and n) and the ATF3 transcription factor (Fig. 5q and r). These axotomy-induced changes are decreased or even, as in the case of CD44, completely abolished in the $jun\Delta S$ mice (Fig. 5c,d,g,h,k,l,o,p,,s and t). Direct quantification of immunoreactivity in Fig. 6 revealed a significant decrease ($p < 0.05$) in the amount of immunoreactivity on the axotomized side in the $jun\Delta S$ mouse mutants compared with junF/F controls (Fig. 6a,b,g,h,m,n,s,t,y and ad), as well as a smaller (Fig. 6a,b,h,n,s and y) or even no increase (Fig. 6g,m and t) of the immunoreactivity from the control to the axotomized side, on average, $jun\Delta S$ mice showed an up to 80–100% smaller increase 4 days after facial nerve crush, or 14 days after nerve cut. To determine, whether these changes depend on c-Jun phosphorylation, we next examined changes

in CD44, CGRP and ATF3 immunoreactivity in the JNK1, 2 and 3 knockouts and the JunAA mutants at 4 days after nerve crush and 14 days after facial nerve cut. Compared with their controls, JunAA and JNK3 null mutants showed a significant but moderate reduction in CD44 immunoreactivity, at one of the two tested time points – Fig. 6f (−24%), and Fig. 6k (−37%), respectively, for the axotomized to control side difference. The CGRP levels in the case of JNK3 also ATF3 levels were not affected. Deletion of JNK1 (Fig. 6c,i,o,u,z and ae,) or JNK2 (Fig. 6d,j,p,v,aa and af,) had no effect on one of the three tested immunohistochemical neuronal injury-associated markers.

As shown in the Supplementary materials, very similar results – massive inhibition in case of neuronal c-Jun deletion, but at most just moderate effects following deletion of JNK1 or JNK3, or in the junAA mutants were also observed for glial activation and for recruitment of T cells into the facial motor nucleus (Figures S2 and S3).

Discussion

The present data confirm the essential role of neuronal c-Jun for the ability of injured neurons to regenerate following axonal disconnection. Removal of the loxP targeted (floxed) *jun* gene with cre recombinase under the control of a neuron-specific synapsin promoter abolished basal and injury-induced neuronal c-Jun immunoreactivity, as well as most of the molecular response following facial axotomy. This absence of neuronal Jun strongly reduced the speed of axonal regeneration following crush, prevented the vast majority of transected axons after cut from reconnecting to their peripheral target, and impaired functional recovery. Despite blocking neuronal cell death, this was also associated with a large number of shrunken neurons. Finally, junΔS mutants also had diminished astrocyte and microglial activation and T-cell influx, demonstrating that this non-neuronal response depends on the release of Jun-dependent signals from neighboring injured motoneurons.

Compared with complete *jun* gene excision in neurons, the effects of removing serine 63 and 73 phosphoacceptor sites (junAA), or of global deletion of individual kinases responsible for N-terminal c-Jun phosphorylation were mild. The reason global JNK null or junAA mutants were used, because unlike the case with floxed c-Jun, only global mutants were available, and because our primary question was whether the regeneration effects of c-Jun could be reproduced by interfering with its N-terminal phosphorylation. Interestingly, the junAA mutants showed decrease in neuronal cell size, a very moderate reduction in post-axotomy CD44 levels and slightly increased astrogliosis. Deletion of JNK1 or JNK3 showed reduced functional recovery; deletion of JNK3 also interfered with T-cell influx, and somewhat reduced CD44 levels. Deletion of JNK2 had no effect. Thus, neuronal c-Jun is needed in regeneration, but most of this activity may not
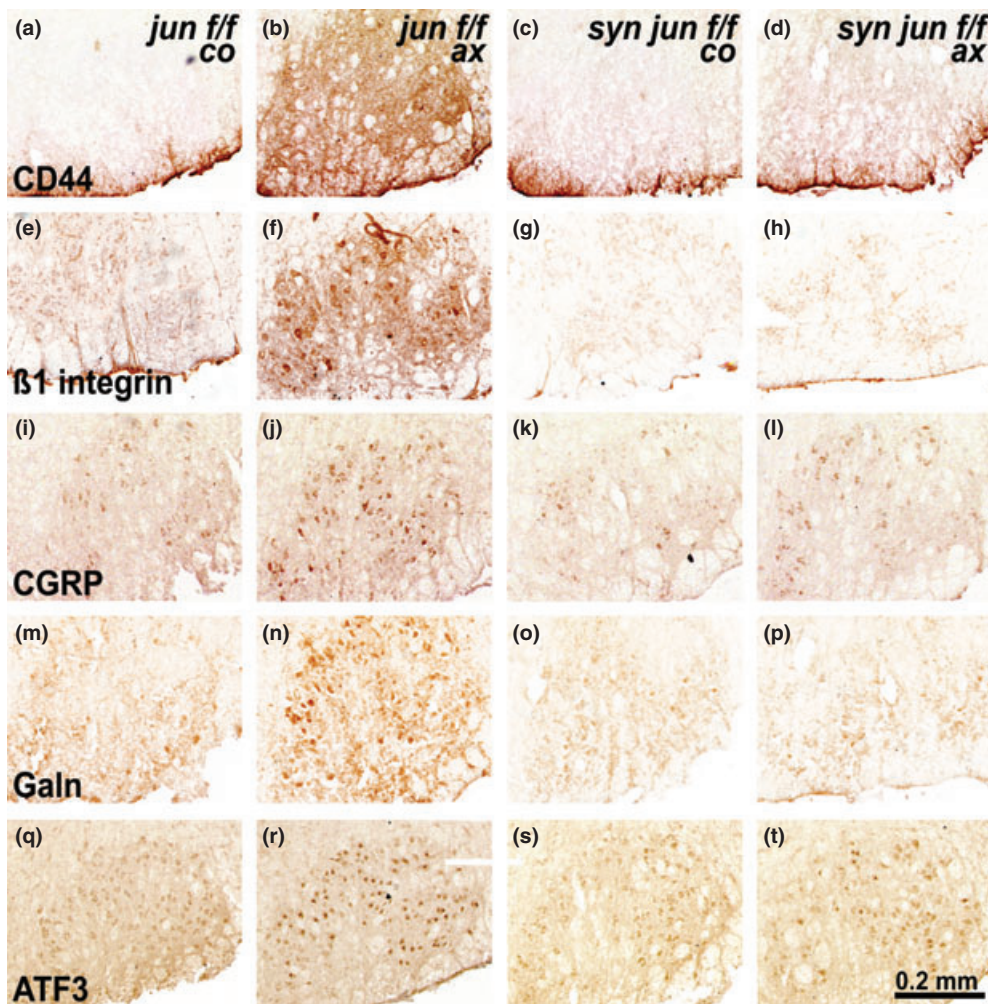
Fig. 5 Biochemical components of neuronal axotomy response depend on the presence of c-Jun. (a–t) Facial nucleus axotomy response 14 days after nerve cut in the junF/F controls (a, b, e, f, i, j, m, n, q, r) and the junΔS mutant (c, d, g, h, k, l, o, p, s, t) mice. Contralateral side (co) -first and third column, Axotomized side (ax) – second and fourth column. After axotomy, junF/F mice show a prominent increase in adhesion molecules CD44 (a–d) and beta1-integrin subunit (e–h), neuropeptides CGRP (i–l) and galanin (m–p) and TF ATF3 (q–t); these changes are reduced or abolished in the junΔS mutants. Scale bar 0.2 mm.

require the presence of individual JNKs or that of the 63 and 73 acceptor sites.

Although cell-type selective excision of target genes is now standard tool in identifying cell-specific function, this can lead to problems if the promoter used to drive cre recombinase is too broad. For example, the nestin promoter used in brain-wide *jun* deletion is expressed in early neuroepithelial cells giving rise to neurons, but also to astrocytes or oligodendroglia. Outside the brain, nestin expression is also found in the islet, liver and muscle stem cells, as well as in Schwann cell precursors (Rao 1999; Akiyama *et al.* 2001; Kanu *et al.* 2010), raising the question whether the regeneration differences observed in that study were also due to *jun* deletion in other cells of neuroepithelial origin. Schwann cells strongly up-regulate c-Jun following axotomy, and removal of Schwann cell c-Jun (Parkinson

et al. 2008) has recently been shown to interfere with axonal regeneration. This problem is resolved in the present study because synapsin promoter expression is highly restricted, limited to the testis and to maturing and mature neurons in the CNS and peripheral ganglia (Hoesche *et al.* 1993; Street *et al.* 2005).

In the currently used facial axotomy model, deletion of neuronal c-Jun produces four sets of effects: it interferes with the expression of numerous target genes involved in molecular changes after facial nerve axotomy, it impairs the flow of activation and recruitment signals to neighboring non-neuronal cells, it abolishes regenerative capacity in most neurons, and it promotes the survival of shrunken and probably still axotomized neuronal cell bodies. Peripheral nerve cut and crush injuries sometimes elicit differing responses at the level of neuronal cell body (Bareyre *et al.*
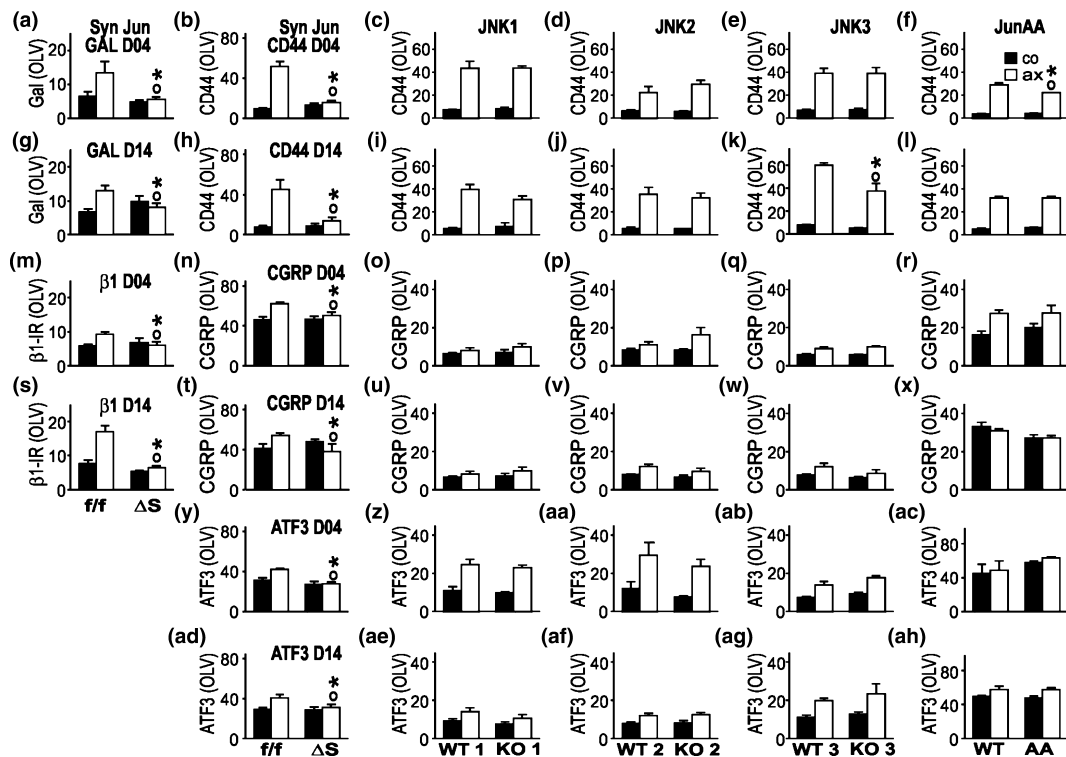
Fig. 6 Effects of neuronal deletion of jun (junΔS, a, b, g, h, m, n, s, t, y, ad, first and second column), the global deletion of JNK1 (c, i, o, u, z, ae, third column), JNK2 (d, j, p, v, aa, af, fourth column), and JNK3 (e, k, q, w, ab, ag, fifth column), and of the junAA mutation (f, l, r, x, ac, ah, sixth column) on changes in neuronal markers following axotomy. Quantification of overall facial motor nucleus immunoreactivity for galanin (a, g) and beta 1 integrin (m, s), CD44 (b–f and h–l), CGRP (n–r and t–x) and ATF3 (y–ah) using OLV units. To facilitate finding the staining and time point on the graph, the row labels (second column, top right of each graph) are the same for the graphs in the third to sixth column). (a–e, m–r, y–ac) 4 days after facial nerve crush, (g–l, s–x, ad–ah) 14 days after facial nerve cut. Filled bars: contralateral (co), empty bars: axotomized (ax) facial nucleus. In each graph, the first two bars represent

the data for control animals (wild-type or junF/F) and the third and fourth bar those for the mutant ones. *$p < 0.05$ (unpaired *t*-test) for the difference on the axotomized side between the mutant and control group. $p < 0.05$ (unpaired *t*-test) for the difference in immunostaining increase due to axotomy (staining intensity on the control side subtracted from that on the axotomized side) between the respective mutant and the wild-type or junF/F control group. $n = 3–7$ animals per group, in each control, and in each mutant group. All five tested markers – galanin, beta1 integrin, CD44, CGRP and ATF3 showed significantly smaller increase or even (g, m, t) the absence of increase, in the junΔS mutants compared with junF/F controls, at both time points – day 4 and day 14. There was no effect for JNK1 or JNK2, and significant but slight decrease for CD44 at day 14 for JNK3 null and at day 4 for junAA.

2011), but in the current case, the molecular responses studied were strongly affected by both types of axotomy, and this response reduced in the absence of c-Jun. Overall, Jun-dependent molecular changes in injured neurons appear pivotal for the latter three sets of functional effects. In the case of axonal regeneration, alpha7beta1 integrin, CD44, galanin and ATF3 have been shown to play an important role in neurite outgrowth. Deletion of the alpha7 integrin gene retards axonal regeneration by 35–40% (Werner *et al.* 2000), removal of the galanin gene delayed sensory functional recovery (Sachs *et al.* 2007); and antibody inhibition of CD44 reduces neurite outgrowth of transplanted central noradrenergic neurons (Nagy *et al.* 1998) as well as creating multiple errors in retinal axonal growth trajectory through the optic chiasm (Lin and Chan 2003). Over-expression of ATF3 also speeds up the initiation of axonal outgrowth, involved in the *in vivo* conditioning response (Seijffers *et al.* 2007). In all

four target genes, enhanced expression after injury depends on the presence of neuronal c-Jun. CD44, α7 and galanin have previously described AP1 consensus binding sequences (Lee *et al.* 1993; Nishida *et al.* 1997; Anouar *et al.* 1999) and ATF3 has been identified as a downstream target of c-Jun (Mei *et al.* 2008). Neuronal p53, cathepsinB and noxA have been implicated in axotomy-induced cell death (Kiryu-Seo *et al.* 2005; Sun *et al.* 2010; Tedeschi and Di Giovanni 2009), and axotomy-induced chemokines including fractal-kine and monocyte chemotactant protein (MCP)1 (Harrison *et al.* 1998; Flügel *et al.* 2001; Schreiber *et al.* 2001) could contribute to non-neuronal recruitment and activation; here the extent of Jun-dependence for the effects of these signals will need to be demonstrated in future studies.

Previous studies revealed that activation of the c-Jun-mediated transcriptional apparatus is affected by interactions at three major sites: (i) N-Terminal phosphorylation at serine

63 and 73 and threonine 91 and 93 (Smeal *et al.* 1994; Morton *et al.* 2003), (ii) dephosphorylation of threonine 239 involved in the ubiquitination and removal of c-Jun (Morton *et al.* 2003) and (iii) C-terminal lysine acetylation near aa257–276 via p300 (Vries *et al.* 2001; also see Raivich 2008 for a review of the signalling pathways involved in the activation of these Jun-domains). In the case of N-terminal phosphorylation, axonal injury is associated with retrograde transport of phosphorylated JNK, the upstream kinases {[[Mitogen-activated protein (MAP)] kinase kinase (MEK or MKK)} kinase (MEKK)1 and phospho - MKK4, as well as the JNK-interacting protein, a scaffold protein that stabilises the interaction between JNK and its upstream kinases (Lindwall and Kanje 2005a,b). Moreover, phosphorylation of Jun will improve dimer stability and thus affect its biological role in the nervous system. Substitution of JNK phosphorylation motifs with those for protein kinase A (PKA) abolishes c-Jun-mediated JNK effects (Smeal *et al.* 1994). Replacement of the serine 63 and 73 phosphoacceptor sites in the junAA mice prevents kainic acid excitotoxity (Behrens *et al.* 1999), to a similar extent as global JNK3 deletion (Yang *et al.* 1997; Brecht *et al.* 2005). Although JNKs have no effect on retinal photoreceptor apoptosis (Grimm *et al.* 2001), junAA and JNK3 null mutants show better neuronal survival after targeted dopaminergic cell death (Brecht *et al.* 2005). Complete JNK ablation – either by deletion or inhibition – has also been shown to decreased caspase-dependent neuronal cell death in both neuronal and non-neuronal cells (Weston and Davis 2007).

Importantly, not all effects of JNK deletion need to be mediated through N-terminal phosphorylation of Jun, and thus would not be observed in the case of junAA – in the current study, this primarily concerns the speed of functional recovery which is normal in junAA, but reduced in JNK1 and 3 null mutations. Interestingly, deletion of JNK3 also strongly reduces cell death of neonatal facial motor neurons after nerve cut, but does not interfere with up-regulation of c-Jun or the phosphorylation of the serine 63 and 73 residues (Keramaris *et al.* 2005). In parentheses, single deletions of JNK2 or 3 significantly decreased cell death in neonatal models (Keramaris *et al.* 2005); as shown in the current study, this effect was not observed following adult axotomy in animals lacking JNK1, 2 or 3 (or the serine 63 and 73 phosphorylation sites), pointing to differences in molecular mechanisms involved in neonatal and adult post-axotomy cell death. So far more than 50 proteins have been shown to be substrates for JNK (Bogoyevitch and Kobe 2006), including various transcription factors in addition to c-Jun (e.g. ATF2, E twenty-six (ETS)-like transcription factor (ELK)1, p53, STAT1/3, etc.), nuclear hormone receptors, apoptosis-regulating bcl2 family members, and signalling and structural molecules involved in cell movement and process outgrowth such as MAP2 (Björkblom *et al.* 2005), MAP1b (Barnat *et al.* 2010) and stathmin (Westerlund *et al.*

2011). Nuclear JNK localization is a requirement for JNK-mediated cell death (Björkblom *et al.* 2008) but effects on neurite outgrowth can be mediated by JNK phosphorylation of cytoplasmic targets (Björkblom *et al.* 2005; Barnat *et al.* 2010; Westerlund *et al.* 2011).

With regards to neurite outgrowth, global *in vitro* inhibition of JNK1/2 and 3 with 10 μM SP600125 strongly reduces and at 200 μM completely blocks outgrowth from the sensory nodose ganglion and dorsal root neurons (Lindwall *et al.* 2004; see also Barnat *et al.* 2010), as well as from sympathetic neurons (Lindwall and Kanje 2005a). *In vitro* studies also showed that deletion of JNK2 or JNK3 interferes with initial dorsal root ganglia (DRG) neurite outgrowth, and deletion of JNK1 and particularly of JNK2 with neurite length. It is possible that combined deletion of all 3 JNKs would have a more pronounced effect, but unfortunately combined deletion of JNK1 and 2 or of all 3 JNKs is embryonic lethal (Kuan *et al.* 1999; Sabapathy *et al.* 1999). Interestingly, functional recovery was not affected in junAA mice (Fig. 3a), suggesting the better performance in the JNK1 and 3 wild-type animals could be due to extra-Jun effects of the JNKs. The regeneration effects shown in the current study in Fig. 3e–p were moderate, with deletions of JNK1 or JNK3 reducing functional recovery, but not interfering with the extent of reinnervation 50 days after facial nerve cut.

In the cerebellar granule cell neurons, inhibition of JNK with 10 uM SP600125, or of the more upstream JNK pathway with mixed lineage kinase inhibitor CEP11004 blocks the ATF3 up-regulation and the ATF3/Phospho-c-Jun mediated cell death following potassium deprivation (Mei *et al.* 2008). However in the current study, deletion of JNK1,2 or 3, or the junAA mutation had no effect on the post-axotomy increase in ATF3 (Fig. 6y–ah), or on neuronal cell death (supplementary Fig. 1). Interestingly, although the junAA mutation did not reduce the cell death it did enhance cell shrinkage. This could suggest that N-terminal phosphorylation brought about through retrograde transport of the kinases (Lindwall and Kanje 2005b) may be important in maintaining the trophic state of surviving neurons. Theoretically, activated JNKs will also lead to the phosphorylation of threonine 91 and 93, which depends on the presence of negatively charged phospho-threonine 95, a target of ataxia teleangiectasia mutated kinase but not of JNK (Vinciguerra *et al.* 2008). Acting in concert, phosphorylation of the three threonines will enhance cell death in response to DNA damage (Vinciguerra *et al.* 2008). Because the three Thr residues are still present in junAA, a similar mechanism may be involved in responsible for reduced functional recovery in the JNK1 or JNK3 null mutants, but not in the junAA mice. Preliminary data using mutant jun4A mice where all 4 JNK-dependent sites were replaced by alanines (S. Patodia, G. Raivich and A. Behrens, unpublished data), do show similar neuronal shrinkage as in junAA, but in addition a moderate but significant decrease in functional recovery (as

with JNK1 or JNK3 deletions) as well as an approximately 40% reduction in whisker pad target reinnervation 30 days after facial nerve cut. Nevertheless, this reinnervation effect is 3–4 times smaller in magnitude than that observed following neuronal deletion of c-Jun (Fig. 2e and f).

It is possible that removal of all 4 phosphoacceptor sites is also needed to reproduce the massive reduction in T-cell influx in the JNK3 null mutants 14 days after facial nerve cut, a peak phase in neuronal cell death, microglial phagocytosis and lymphocyte recruitment (Raivich *et al.* 1998), but this would need to be ascertained in future studies. What the current study does is that it confirms that the pro-regeneration, pro-inflammatory and pro-cell death c-Jun effects observed with nestin-promoter driven expression of Cre recombinase are indeed due to to the neuronal expression of c-Jun. By comparison, the JNK phosphorylation of the N-terminus mostly appears to not be required for these functions.

Acknowledgements

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Supplementary materials and methods.

Figure S1. Deletion of neuronal c-jun induces neuronal shrinkage but prevents cell death.

Figure S2. Neuronal c-Jun is also required for the non-neuronal neuroinflammatory response after facial nerve cut.

Figure S3. Effects of neuronal deletion of c-Jun (junΔS, a, f, k, p, u), global deletion of JNK1 (b, g, l, q, v), JNK2 (c, h, m, r, w), and JNK3 (d, i, n, s, x) and the JunAA mutation (e, j, o, t, y) on microglial and and astrocyte activation and on T-cell recruitment in the facial motor nucleus.

Table S1. Summary of antibody data for the cellular response immunostainings.

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References

Akiyama Y, Honmou O, Kato T, Uede T, Hashi K and Kocsis JD (2001) Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord. *Exp. Neurol.* **167**, 27–39.

Anderson PN, Campbell G, Zhang Y and Lieberman AR (1998) Cellular and molecular correlates of the regeneration of adult mammalian CNS axons into peripheral nerve grafts. *Prog. Brain Res.* **117**, 211–232.

Angel P and Karin M (1992) Specific members of the Jun protein family regulate collagenase expression in response to various extracellular stimuli. *Matrix Suppl.* **1**, 156–164.

Anouar Y, Lee HW and Eiden LE (1999) Both inducible and constitutive activator protein-1-like transcription factors are used for transcriptional activation of the galanin gene by different first and second messenger pathways. *Mol. Pharmacol.* **56**, 162–169.

Bareyre FM, Garzorz N, Lang C, Misgeld T, Büning H and Kerschensteiner M (2011) In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. *Proc. Natl Acad. Sci. USA* **108**, 6282–6287.

Barnat M, Enslen H, Propst F, Davis RJ, Soares S and Nothias F (2010) Distinct roles of c-Jun N-terminal kinase isoforms in neurite initiation and elongation during axonal regeneration. *J. Neurosci.* **30**, 7804–7816.

Behrens A, Sibilia M and Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.* **21**, 326–329.

Behrens A, Sibilia M, David JP, Möhle-Steinlein U, Tronche F, Schütz G and Wagner EF (2002) Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver. *EMBO J.* **21**, 1782–1790.

Björkblom B, Ostman N, Hongisto V, Komarovski V, Filén JJ, Nyman TA, Kallunki T, Courtney MJ and Coffey ET (2005) Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. *J. Neurosci.* **25**, 6350–6361.

Björkblom B, Vainio JC, Hongisto V, Herdegen T, Courtney MJ and Coffey ET (2008) All JNKs can kill, but nuclear localization is critical for neuronal death. *J. Biol. Chem.* **283**, 19704–19713.

Bogoyevitch MA and Kobe B (2006) Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol. Mol. Biol. Rev.* **70**, 1061–1095.

Brecht S, Kirchhof R, Chromik A *et al.* (2005) Specific pathophysiological functions of JNK isoforms in the brain. *Eur. J. Neurosci.* **21**, 363–377.

Dérijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M and Davis RJ (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025–1037.

Dong C, Yang DD, Wysk M, Whitmarsh AJ, Davis RJ and Flavell RA (1998) Defective T cell differentiation in the absence of JNK1. *Science* **282**, 2092–2095.

English AW, Cucoranu D, Mulligan A, Rodriguez JA and Sabatier MJ (2011) Neurotrophin-4/5 is implicated in the enhancement of axon regeneration produced by treadmill training following peripheral nerve injury. *Eur. J. Neurosci.* **33**, 2265–2271.

Flügel A, Hager G, Horvat A, Spitzer C, Singer GM, Graeber MB, Kreutzberg GW and Schwaiger FW (2001) Neuronal MCP-1 expression in response to remote nerve injury. *J. Cereb. Blood Flow Metab.* **21**, 69–76.

Grimm C, Wenzel A, Behrens A, Hafezi F, Wagner EF and Remé CE (2001) AP-1 mediated retinal photoreceptor apoptosis is independent of N-terminal phosphorylation of c-Jun. *Cell Death Differ.* **8**, 859–867.

Haeusgen W, Boehm R, Zhao Y, Herdegen T and Waetzig V (2009) Specific activities of individual c-Jun N-terminal kinases in the brain. *Neuroscience* **161**, 951–959.

Harrison JK, Jiang Y, Chen S *et al.* (1998) Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc. Natl Acad. Sci. USA* **95**, 10896–10901.

Herdegen T, Kummer W, Fiallos CE, Leah J and Bravo R (1991) Expression of c-JUN, JUN B and JUN D proteins in rat nervous system following transection of vagus nerve and cervical sympathetic trunk. *Neuroscience* **45**, 413–422.

Herdegen T, Bastmeyer M, Bähr M, Stuermer C, Bravo R and Zimmermann M (1993) Expression of JUN, KROX, and CREB transcription factors in goldfish and rat retinal ganglion cells following optic nerve lesion is related to axonal sprouting. *J. Neurobiol.* **24**, 528–543.

Hiroi S, Tsukamoto Y, Sasaki F, Miki N and Taira E (2003) Involvement of gicerin, a cell adhesion molecule, in development and regeneration of chick sciatic nerve. *FEBS Lett.* **554**, 311–314.

Hoesche C, Sauerwald A, Veh RW, Krippl B and Kilimann MW (1993) The 5′-flanking region of the rat synapsin I gene directs neuron-specific and developmentally regulated reporter gene expression in transgenic mice. *J. Biol. Chem.* **268**, 26494–26502.

Horie H and Kadoya T (2000) Identification of oxidized galectin-1 as an initial repair regulatory factor after axotomy in peripheral nerves. *Neurosci. Res.* **38**, 131–137.

Jankowski MP, McIlwrath SL, Jing X, Cornuet PK, Salerno KM, Koerber HR and Albers KM (2009) Sox11 transcription factor modulates peripheral nerve regeneration in adult mice. *Brain Res.* **1256**, 43–54.

Jenkins R and Hunt SP (1991) Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. *Neurosci. Lett.* **129**, 107–110.

Kallunki T, Su B, Tsigelny I, Sluss HK, Dérijard B, Moore G, Davis R and Karin M (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.* **8**, 2996–3007.

Kanu N, Penicud K, Hristova M, Wong B, Irvine E, Plattner F, Raivich G and Behrens A (2010) The ATM cofactor ATMIN protects against oxidative stress and accumulation of DNA damage in the aging brain. *J. Biol. Chem.* **285**, 38534–38542.

Keramaris E, Vanderluit JL, Bahadori M, Mousavi K, Davis RJ, Flavell R, Slack RS and Park DS (2005) c-Jun N-terminal kinase 3 deficiency protects neurons from axotomy-induced death in vivo through mechanisms independent of c-Jun phosphorylation. *J. Biol. Chem.* **280**, 1132–1141.

Kiryu-Seo S, Hirayama T, Kato R and Kiyama H (2005) Noxa is a critical mediator of p53-dependent motor neuron death after nerve injury in adult mouse. *J. Neurosci.* **25**, 1442–1447.

Koistinaho J, Hicks KJ and Sagar SM (1993) Long-term induction of c-jun mRNA and Jun protein in rabbit retinal ganglion cells following axotomy or colchicine treatment. *J. Neurosci. Res.* **34**, 250–255.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* **19**, 312–318.

Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P and Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22**, 667–676.

Kügler S, Meyn L, Holzmüller H, Gerhardt E, Isenmann S, Schulz JB and Bähr M (2001) Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinant adenoviral vectors. *Mol. Cell. Neurosci.* **17**, 78–96.

Lee TH, Klampfer L, Shows TB and Vilcek J (1993) Transcriptional regulation of TSG6, a tumor necrosis factor- and interleukin-1-inducible primary response gene coding for a secreted hyaluronan-binding protein. *J. Biol. Chem.* **268**, 6154–6160.

Lieberman AR (1971) The axon reaction: a review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* **14**, 49–124.

Lin L and Chan SO (2003) Perturbation of CD44 function affects chiasmatic routing of retinal axons in brain slice preparations of the mouse retinofugal pathway. *Eur. J. Neurosci.* **17**, 2299–2312.

Lindwall C and Kanje M (2005a) The Janus role of c-Jun: cell death versus survival and regeneration of neonatal sympathetic and sensory neurons. *Exp. Neurol.* **196**, 184–194.

Lindwall C and Kanje M (2005b) Retrograde axonal transport of JNK signaling molecules influence injury induced nuclear changes in p-c-Jun and ATF3 in adult rat sensory neurons. *Mol. Cell. Neurosci.* **29**, 269–282.

Lindwall C, Dahlin L, Lundborg G and Kanje M (2004) Inhibition of c-Jun phosphorylation reduces axonal outgrowth of adult rat nodose ganglia and dorsal root ganglia sensory neurons. *Mol. Cell. Neurosci.* **27**, 267–279.

Magoulas B and Lopez De Heredia L (2010) Functional analysis of the transcription factor C/EBPdelta in neuronal repair. *FENS Abstr. 5, 040* **1**, 2.

Makwana M and Raivich G (2005) Molecular mechanisms in successful peripheral regeneration. *FEBS J.* **272**, 2628–2638.

Makwana M, Werner A, Acosta-Saltos A et al. (2009) Peripheral facial nerve axotomy in mice causes sprouting of motor axons into perineuronal central white matter: Time course and molecular characterization. *J. Comp. Neurol.* **518**, 699–721.

Mei Y, Yuan Z, Song B, Li D, Ma C, Hu C, Ching YP and Li M (2008) Activating transcription factor 3 up-regulated by c-Jun N-terminal kinase/c-Jun contributes to apoptosis induced by potassium deprivation in cerebellar granule neurons. *Neuroscience* **151**, 771–779.

Moller JC, Klein MA, Haas S, Jones LL, Kreutzberg GW and Raivich G (1996) Regulation of thrombospondin in the regenerating mouse facial motor nucleus. *Glia* **17**, 121–132.

Morton S, Davis RJ, McLaren A and Cohen P (2003) A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. *EMBO J.* **22**, 3876–3886.

Nadeau S, Hein P, Fernandes KJL, Peterson AC and Miller FD (2005) A transcriptional role for C/EBP beta in the neuronal response to axonal injury. *Mol. Cell. Neurosci.* **29**, 525–535.

Nagy JI, Price ML, Staines WA, Lynn BD and Granholm AC (1988) The hyaluronan receptor RHAMM in noradrenergic fibers contributes to axon growth capacity of locus coeruleus neurons in an intraocular transplant model. *Neuroscience* **86**, 241–255.

Nateri AS, Riera-Sans L, Da Costa C and Behrens A (2004) The ubiquitin ligase SCFFbw7 antagonizes apoptotic JNK signaling. *Science* **303**, 1374–1378.

Nishida K, Kitazawa R, Mizuno K, Maeda S and Kitazawa S (1997) Identification of regulatory elements of human alpha 6 integrin subunit gene. *Biochem. Biophys. Res. Commun.* **241**, 258–263.

Parkinson DB, Bhaskaran A, Arthur-Farraj P et al. (2008) c-Jun is a negative regulator of myelination. *J. Cell Biol.* **181**, 625–637.

Pirianov G, Brywe KG, Mallard C, Edwards AD, Flavell RA, Hagberg H and Mehmet H (2007) Deletion of the c-Jun N-terminal kinase 3 gene protects neonatal mice against cerebral hypoxic-ischaemic injury. *J. Cereb. Blood Flow Metab.* **27**, 1022–1032.

Raivich G (2008) c-Jun expression, activation and function in neural cell death, inflammation and repair. *J. Neurochem.* **107**, 898–906.

Raivich G and Makwana M (2007) The making of successful axonal regeneration: genes, molecules and signal transduction pathways. *Brain Res. Rev.* **53**, 287–311.

Raivich G, Jones LL, Kloss CU, Werner A, Neumann H and Kreutzberg GW (1998) Immune surveillance in the injured nervous system: T-lymphocytes invade the axotomized mouse facial motor nucleus

and aggregate around sites of neuronal degeneration. *J. Neurosci.* **18**, 5804–5816.

Raivich G, Liu ZQ, Kloss CU, Labow M, Bluethmann H and Bohatschek M (2002) Cytotoxic potential of proinflammatory cytokines: combined deletion of TNF receptors TNFR1 and TNFR2 prevents motoneuron cell death after facial axotomy in adult mouse. *Exp. Neurol.* **178**, 186–193.

Raivich G, Bohatschek M, Da Costa C *et al.* (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* **43**, 57–67.

Rao MS (1999) Multipotent and restricted precursors in the central nervous system. *Anat. Rec.* **257**, 137–148.

Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M and Wagner EF (1999) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech. Dev.* **89**, 115–124.

Sachs HH, Wynick D and Zigmond RE (2007) Galanin plays a role in the conditioning lesion effect in sensory neurons. *NeuroReport* **18**, 1729–1733.

Schreiber RC, Krivacic K, Kirby B, Vaccariello SA, Wei T, Ransohoff RM and Zigmond RE (2001) Monocyte chemoattractant protein (MCP)-1 is rapidly expressed by sympathetic ganglion neurons following axonal injury. *NeuroReport* **12**, 601–606.

Seijffers R, Mills CD and Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J. Neurosci.* **27**, 7911–7920.

Smeal T, Hibi M and Karin M (1994) Altering the specificity of signal transduction cascades: positive regulation of c-Jun transcriptional activity by protein kinase A. *EMBO J.* **13**, 6006–6010.

Street K, Xu G, Hall K, Intano G, McCarrey J, Herbert D, Kilimann MW and Walter CA (2005) Rat snapsin 1 promoter mediated transgene expression in testicular cell types. *DNA Cell Biol.* **24**, 133–140.

Sun L, Wu Z, Baba M, Peters C, Uchiyama Y and Nakanishi H (2010) Cathepsin B-dependent motor neuron death after nerve injury in the adult mouse. *Biochem. Biophys. Res. Commun.* **399**, 391–395.

Tedeschi A and Di Giovanni S (2009) The non-apoptotic role of p53 in neuronal biology: enlightening the dark side of the moon. *EMBO Rep.* **10**, 576–583.

Vinciguerra M, Esposito I, Salzano S, Madeo A, Nagel G, Maggiolini M, Gallo A and Musti AM (2008) Negative charged threonine 95 of c-Jun is essential for c-Jun N-terminal kinase-dependent phosphorylation of threonine 91/93 and stress-induced c-Jun biological activity. *Int. J. Biochem. Cell Biol.* **40**, 307–316.

Vries RG, Prudenziati M, Zwartjes C, Verlaan M, Kalkhoven E and Zantema A (2001) A specific lysine in c-Jun is required for transcriptional repression by E1A and is acetylated by p300. *EMBO J.* **20**, 6095–6103.

Waetzig V, Zhao Y and Herdegen T (2006) The bright side of JNKs-Multitalented mediators in neuronal sprouting, brain development and nerve fiber regeneration. *Prog. Neurobiol.* **80**, 84–97.

Werner A, Willem M, Jones LL, Kreutzberg GW, Mayer U and Raivich G (2000) Impaired axonal regeneration in alpha7 integrin-deficient mice. *J. Neurosci.* **20**, 1822–1830.

Westerlund N, Zdrojewska J, Padzik A *et al.* (2011) Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. *Nat. Neurosci.* **14**, 305–313.

Weston CR and Davis RJ (2007) The JNK signal transduction pathway. *Curr. Opin. Cell Biol.* **19**, 142–149.

Yang DD, Kuan C-Y, Whitmarsh AJ, Rincón M, Zheng TS, Davis RJ, Rakic P and Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389**, 865–870.

Zhu Y, Romero MI, Ghosh P, Ye Z, Charnay P, Rushing EJ, Marth JD and Parada LF (2001) Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev.* **15**, 859–876.