NEURON SURVIVAL, AXON GROWTH AND THE TRANSCRIPTION FACTOR SRY-BOX CONTAINING GENE 11 (SOX11)

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Developmental survival, axon growth and differentiation of sensory neurons are mediated through the actions of specific sets of transcriptional signaling complexes (Anderson, 1999). A newly recognized family of transcription factors that appear to have important roles in sensory neuron biology is the Sox family of high-mobility group (HMG) domain proteins. In a screen of transcriptional activity in transgenic animals that overexpress either NGF or GDNF in the skin (NGF-OE and GDNF-OE mice), the transcription factor Sox11 was significantly increased in developing neurons of the trigeminal ganglia. This increase suggests Sox11 expression is trophic factor sensitive and that it may contribute to the transcriptional control of genes involved in the increased survival and axonal projections that has been documented in these transgenic animals (Albers et al., 1994; Zwick et al., 2002). Sox11 was also increased in neurons of adult dorsal root ganglia (DRG) following sciatic nerve cut. The rise in Sox11 in response to enhanced trophic factor level and axotomy has led us to hypothesize that Sox11 is an essential transcriptional regulator in both embryonic and adult systems that is trophic factor responsive.

To further investigate the role of Sox11 and begin to identify transcriptional targets, the level of Sox11 was assayed in the Neuro2A stem cell line (Klebe and Ruddle, 1969), primary dorsal root ganglion (DRG) neurons and *in vivo* after nerve injury. Upon retinoic acid (RA)-induced differentiation of Neuro-2A cells and upon culturing DRG neurons, Sox11 mRNA

increased, suggesting Sox11 was important for expression of genes involved in Neuro2A and primary DRG differentiation and survival. To test this, the level of Sox11 expression was knocked down in Neuro2A cells and cultured DRG neurons by transfection of siRNAs against Sox11. Knockdown of Sox11 in these cells caused cell death and inhibited axon growth. RNAi knockdown of Sox11 *in vivo* after a saphenous nerve crush injury also inhibited axon regeneration. These data suggest that the developmentally regulated transcription factor Sox11 is induced in adult neurons after injury to promote neurite growth and axon regeneration and inhibit apoptosis by regulating genes associated with each of these distinct biological pathways.

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PREFACE

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1.0 INTRODUCTION

1.1 NEUROTROPHINS REGULATE SENSORY NEURON DEVELOPMENT

Neurotrophic growth factor proteins expressed in peripheral target tissues are essential for the survival of specific types of sensory neurons that innervate these tissues (Oppenheim, 1991; Thoenen, 1987). Two neurotrophic factors, nerve growth factor (NGF) and glial-cell line derived neurotrophic factor (GDNF) support the development of two distinct types of cutaneous nociceptor or pain transmitting neurons. NGF produced in the skin binds and signals through the neuronally-expressed receptor tyrosine kinase (trk) A, whereas GDNF binds and signals through a complex containing the receptor tyrosine kinase Ret and a specific glycosyl-phosphatidyl inositol (GPI)-anchored member of the GDNF family receptors (GFRs) (Averill et al., 1995; Verge et al. 1995; Perl, 1996; Pachnis et al. 1993; Molliver et al. 1997; Bennett et al., 1998). NGF and GDNF-dependent nociceptors are thought to underlie transmission of different types of pain. NGF responsive, trkA positive neurons that express the neuropeptides substance P and calcitonin-gene related peptide (CGRP) are thought to mediate inflammatory pain signaling (McMahon 1996), whereas the peptide poor, isolectin B4-binding, GDNF responsive population of nociceptors (Molliver et al. 1997), are thought to underlie neuropathic pain (Boucher et al. 2000).

It is well accepted that sensory neuron survival is dependent on the level of target-derived growth factors. In addition, studies that increase or decrease growth factor signaling have suggested that they affect differentiation as well. Mendell and coworkers (Lewin et al. 1992) found that the level of NGF altered the balance of c-fiber and D-hair innervation in the skin of the rat. Treatment with anti-NGF decreased the number of c-fibers innervating the skin while simultaneously increasing the number of D-hairs. The conclusion from these studies was that NGF was required for the maturation of c-fiber innervation. In another study, Stucky et al (Stucky et al. 1999) found that the heat sensitivity of c-fibers was increased four fold in mice overexpressing NGF, whereas Aδ high threshold mechanoreceptors were twice as sensitive to mechanical stimulation. Mice lacking BDNF (a member of the neurotrophin family) exhibit a loss of mechanical sensitivity in slowly adapting myelinated fibers without affecting survival of these neurons (Carroll et al. 1998). Finally, mice overexpressing NT-3 in skin exhibit non-overlapping dermatomes, indicating significant changes in peripheral projections (Ritter et al. 2001). Taken together, these studies suggest that growth factors can play an important role in both survival *and* phenotypic differentiation of sensory neurons.

During early development (E12), virtually all sensory neurons express trkA and up to 80% continue to express trkA until late in development (ca. E18). At E13, ret and GFRα1 begin to be expressed in sensory neurons and increase throughout embryonic development and postnatally (Baloh et al. 2000). The expression of these receptors extends into the postnatal period when sensory neurons are differentiating. Most importantly for nociceptors, these proteins are expressed at a time when a developmental switch from NGF to GDNF responsiveness occurs (Mu et al. 1993; Pachnis et al. 1993; Widenfalk et al. 1997; Jackman and

Fitzgerald 2000). While CGRP and Ret expression increases into late prenatal and early postnatal stages, trkA expression declines around P0 (Mu et al. 1993; Bennett DL 1996; Molliver et al. 1997). IB4 is not expressed embryonically, appears at birth and increases gradually over the first weeks of development (Bennett DL 1996; Molliver et al. 1997; Guo et al. 2001).

The dynamic changes in neurotrophic growth factor and receptor expression that occur during development of NGF- and GDNF-dependent neurons suggests a high level of temporal and spatial regulation. To examine this regulation and begin to identify genes that might regulate development of various sensory neuron populations, a transgenic mouse model system in which 2.3Kb of the human K14 keratin promoter and enhancer drives expression of various neurotrophic factors in the skin was utilized. Overexpresser animals (OE mice) were used to establish the temporal expression patterns of phenotypic markers of NGF responsive and GDNF responsive nociceptive neurons to determine if a developmental shift in the expression of trkA, CGRP, Ret and IB4 occurs in animals in which excess trophic factors are present. The goal was to determine if trophic factor levels regulated the onset of neuronal phenotypic properties as defined by expression of peptides and receptors. Various embryonic and postnatal stages of development were compared to adult patterns. It was found that overexpression of trophic factors in the periphery did not accelerate expression of phenotypic markers but did result in a change in the distribution of these markers in each transgenic line (Fig.1). No effect was seen at E16.5 or P2 (not shown) although a differential distribution of markers for the subtypes of neurons in the transgenic animals was detected by P8. Mice that overexpress GDNF (GDNF-OEs) had an increase in the percentage of ret-positive cells and a decrease in the percentage of cells immunopositive for trkA. Conversely, NGF-OEs had an increase in the percent of cells

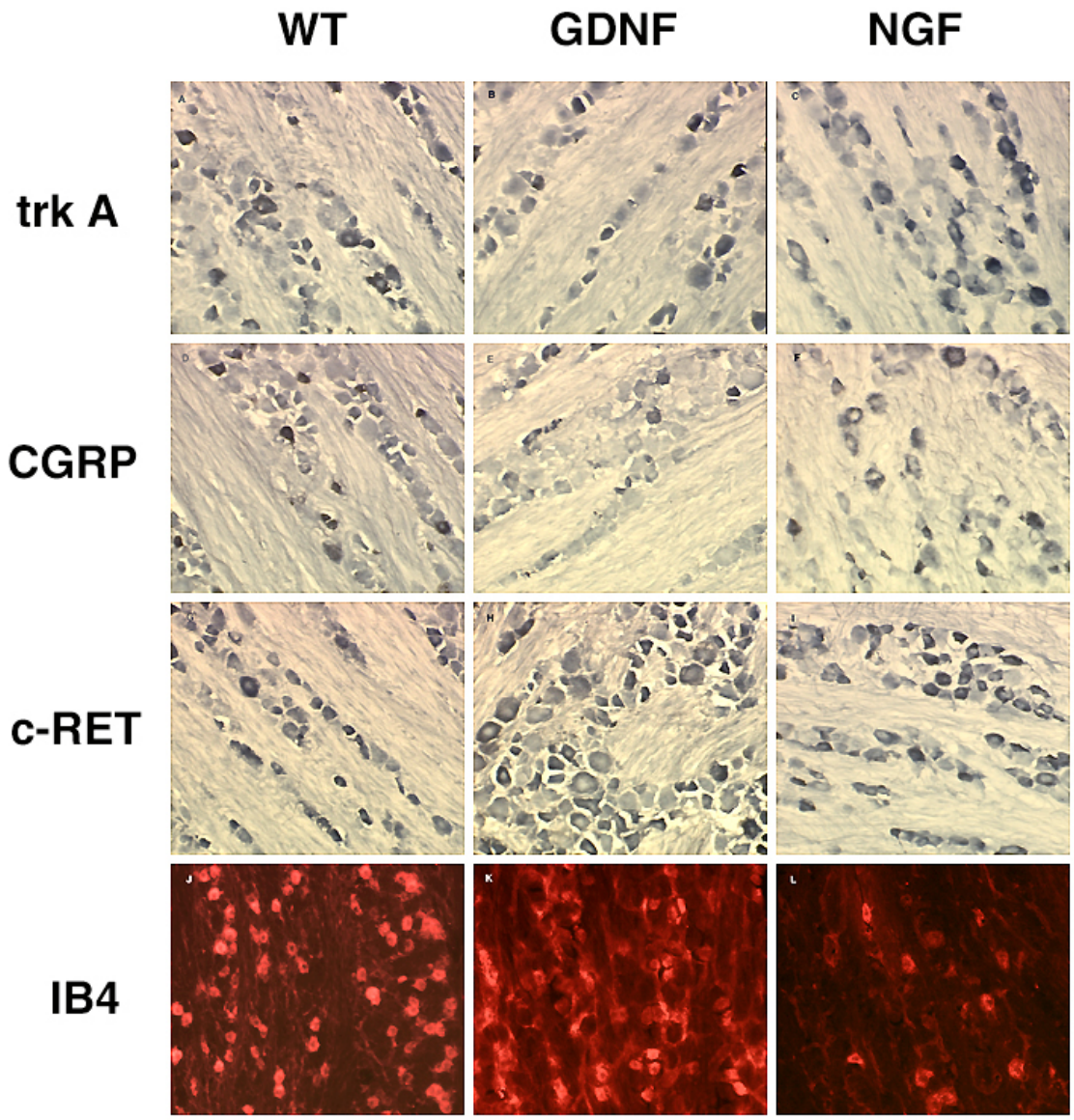
WT
GDNF
NGF
trk A
CGRP
c-RET
IB4

Fig. 1: Light micrographs of trigeminal ganglia (TG) immunoreactive for trkA, CGRP or c-Ret or stained for IB4 at postnatal day 8 in wild type, GDNF-OEs or NGF-OEs. Unlike P2 animals, P8 TGs show an increase in trkA and CGRP positive neurons in the NGF-OE animals and an increase in the number of c-Ret positive cells in both NGF-OE and GDNF-OE animals. Preliminary cell counts show a decrease in the number of IB4 neurons at P8 when overproducing NGF while GDNF overproduction results in a decrease in the number of trkA neurons.

immunopositive for CGRP and trkA and a subsequent decrease in the percent of cells that bind IB4. These preliminary findings suggested that phenotypic switching between NGF-dependence and GDNF-dependence occurs postnatally (by P8).

Having established that enhanced levels of skin-derived trophic factors did not accelerate differentiation but rather enhanced the size of the responsive population, experiments were designed to identify *how* these changes were occurring via neurotrophic regulation, ie. what were the downstream mediators of neuronal development? A large scale genomic screen using Affymetrix gene chips was used to identify transcripts specific to neuron subpopulations. Affymetrix-based analysis of RNA expression using RNA from trigeminal ganglia of embryonic day 16.5 and adult sensory ganglia from GDNF- and NGF-OE mice exhibited several changes in gene expression (Table 1). Classes of genes that showed change that were of particular interest were phosphatases, γ-amino-butyric acid (GABA) receptors and transcription factors. It is feasible to hypothesize that changes in these types of genes would underlie at least some of the anatomical, electrophysiological and behavioral changes characterized in the GDNF and NGF-OEs.

Phosphatase expression analysis. Neuronal phosphatases have been associated with control of neuronal differentiation, axon pathfinding and survival (Stroker and Dutta, 1998). These proteins have been linked to regulation of cytoskeletal function (Sanchez et al., 2000) and neurotrophin mediated cell survival and neurite growth (Tisi et al., 2000). Affymetrix analysis showed transcript levels of two phosphatases, the dual specificity phosphatase 6 (DUSP6) and

Table 1: Microarray analysis of trigeminal ganglia from animals that overexpress either nerve growth factor or glial cell line-derived neurotrophic factor in the skin at embryonic day 16.5 and adults.

Gene	Wild Type	GDNF-OE	Fold change E16.5
diaphorase 4 (NADH/NADPH)	103.654	68.613	-1.511
gamma-aminobutyric acid (GABA-A) receptor, subunit rho 2	181.481	129.576	-1.401
cyclin-dependent kinase 2	114.684	82.814	-1.385
phospholipase A2 group VII (platelet-activating factor acetylhydr	484.9	668.68	1.379
follistatin-like	1288.351	1778.548	1.38
kinesin family member C2	103.629	143.169	1.382
Rho GDP dissociation inhibitor (GDI) gamma	667.518	923.712	1.384
potassium voltage-gated channel, shaker-related subfamily, mei	405.786	568.183	1.4
transforming growth factor beta regulated gene 1	412.141	585.355	1.42
synaptosomal-associated protein, 25 kDa, binding protein	143.348	205.236	1.432
calcium channel, voltage-dependent, alpha2/delta subunit 1	136.158	195.822	1.438
profilin 1	635.851	914.269	1.438
ankyrin 3, epithelial	482.374	694.075	1.439
eukaryotic translation elongation factor 1 delta (guanine nucleoti	357.697	522.938	1.462
gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 2	308.027	450.819	1.464
ribonuclease, RNase A family 4	203.396	299.067	1.47
synaptotagmin 1	101.931	150.236	1.474
ret proto-oncogene	379.675	561.379	1.479
brain derived neurotrophic factor	677.622	1005.034	1.483
synuclein, gamma	1926.744	2864.011	1.486
mitogen activated protein kinase 1	132.369	197.203	1.49
endothelin receptor type B	512.993	783.58	1.527
SRY-box containing gene 11	142.705	243.18	1.704
SRY-box containing gene 11	419.074	766.237	1.828

Gene	Wild Type	GDNF-OE	Fold change Adult
phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (	335.62	209.28	-1.6
cholecystokinin A receptor	342.81	238.17	-1.44
insulin-like growth factor binding protein 5	2329.65	1666.63	-1.4
glutamate receptor, ionotropic, NMDA1 (zeta 1)	237.76	172.16	-1.38
gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 1	525.53	397.71	-1.32
ret proto-oncogene	628.79	865.38	1.38
dual specificity phosphatase 6	304.9	439.74	1.44
glial cell line derived neurotrophic factor family receptor alpha 1	133.96	195.38	1.46
potassium voltage-gated channel, shaker-related subfamily, beta	437.07	659.63	1.51
brain derived neurotrophic factor	591.1	895.48	1.51
protein tyrosine phosphatase, receptor type, O	250.32	386.23	1.54
lymphocyte antigen 86	392.06	1472.43	3.76

Gene	Wild Type	NGF-OE	Fold change Adult
cadherin 3	364.27	206.16	-1.77
gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 1	525.53	311.41	-1.69
kinesin heavy chain member 1B	209.86	130.29	-1.61
calbindin 2	1012.26	654.31	-1.55
SRY-box containing gene 17	375.82	254.57	-1.48
potassium large conductance pH-sensitive, alpha member 3	403.81	273.03	-1.48
cholecystokinin A receptor	342.81	237.31	-1.44
glutamate receptor, ionotropic, kainate 1	596.73	417.54	-1.43
integrin-associated protein	1485.37	1041.54	-1.43
potassium voltage-gated channel, shaker-related, member 3	310.67	219.54	-1.42
eukaryotic translation initiation factor 4, gamma 2	643.09	453.6	-1.42
phosphatidylinositol 3-kinase, regulatory subunit	335.62	239.87	-1.4
regulator of G-protein signaling 4	302.72	429.21	1.42
sodium channel, voltage-gated, type X, alpha	1917.24	2720.86	1.42
potassium voltage-gated channel, shaker-related, member 4	80.04	118.66	1.48
brain derived neurotrophic factor	591.1	894.05	1.51
lymphocyte antigen 86	392.06	833.9	2.13

protein tyrosine phosphatase receptor type O (PTPro), were increased in adult sensory ganglia of NGF- and GDNF-OE transgenic mice. Reverse transcriptase PCR (RT-PCR) analysis of RNA isolated from sensory ganglia confirmed this increase in expression. GDNF overproduction resulted in a greater increase in DUSP6 than NGF overproduction (1.8-fold vs. 1.62-fold, respectively) while NGF-OEs caused a larger increase in PTPro in TG compared to GDNF-OEs (1.64-fold vs 1.44-fold respectively) when compared to adult wild type animals (n=1). PTPRo has been associated with axon outgrowth and differentiation of sensory neurons (Beltran et al. 2003). It is expressed by trkA and trkC neurons of developing DRGs and may enhance motor neuron outgrowth (Stepanek et al. 2005). The role of DUSP6 is less clear but is thought to have the ability to dephosphorylate extracellular signal related kinase (ERK) 2, one of the major mitogen activated protein kinases (MAPKs) (Castelli et a., 2004; Furukawa et al., 2006). It may have a role in axial patterning through regulation of fibroblast growth factor 8 (FGF 8) signaling (Echevarria et al. 2005; Tsang et al. 2006), but also has been linked to the development of psychiatric disorders that include schizophrenia and bipolar disorder (Lee et al. 2006). The increased expression of these phosphatases in sensory neurons exposed to enhanced levels of growth factors may reflect a means by which control can be exerted over the activation of growth factor signaling pathways in neurons.

GABA receptor expression. GABA receptor activation is known to be critical for the development of sensory neuron function (Vinay et al., 1999). RT-PCR quantification of GABA A receptor subunit $\alpha 3$ and $\gamma 2$ in postnatal animals overexpressing GDNF indicated a large down-regulation of the GABA A $\alpha 3$ subunit in GDNF-OE trigeminal ganglia at P8 that returned to wildtype levels by P16. The $\gamma 2$ subunit of the GABA A receptor was unchanged in GDNF-OE

ganglia at P8 but upregulated by P16. No change in the α1 or α2 subunits at either stage was detected when comparing GDNF-OEs to wild type animals (n=1; not shown). Few studies have ascribed a function for the various GABA receptor subunits during the development of DRG neurons but individual subunit expression may play a role in synaptogenesis or cell type electrophysiological properties (Mitchell and Redburn, 1996) resulting in different sensory neuron phenotypes.

Although changes in phosphatase and GABA receptors (and other genes listed in Table 1) may underlie various aspects of the development and maturation of sensory neurons responsive to GDNF and NGF, we chose to focus analysis on transcription factors, specifically, the SRY-Box containing gene 11 or Sox 11, which was found to be elevated in ganglia of the NGF- and GDNF-OE animals.

1.2 SOX GENES ARE IMPORTANT REGULATORS OF DEVELOPMENT

SRY-box containing gene 11 (Sox11) is a member of the group C high mobility group (HMG) transcription factor family (Azuma et al., 1999; Hargrave et al. 1997). There are approximately 20 members of the Sox gene family which have been divided into 7 subgroups based on sequence homology within groups. Sox proteins, as members of the HMG superfamily of DNA-binding proteins, may either activate or repress transcription of target genes. HMG proteins are characterized by an 80 amino acid DNA binding domain (the HMG box) that mediates binding to AT rich DNA sequences. HMG box containing transcription factors are thought to interact with other components of the transcriptional machinery and the combination of these factors is thought to be cell type specific (Dailey and Basilico, 2001). The HMG domain is highly conserved among Sox factors, all of which recognize a similar binding motif (WWCAAWG, where W is either an A or T). However, a given cell type can co-express a number of Sox genes, suggesting interactions with other protein partners is required to provide specificity in directing a gene expression pattern for a particular cell type (Kamachi et al, 2000; Wilson and Koopman, 2002).

The expression of many Sox genes has been shown to be regulated both spatially and temporally during development (Gubbay et al., 1990; Wright et al., 1993; Wilson and Koopman, 2002) and all appear to influence various aspects of embryonic development. For example, Sox8 and Sox17 are linked to development of muscles tissues (Schmidt et al., 2003) and the endoderm

(Kanai-Azuma et al., 2002), respectively. During pancreatic development (Lioubinski et al., 2003) and formation of the nervous system (Hargrave et al., 1997) several Sox genes are expressed that include Sox11. Mutations in Sox genes have also been associated with human developmental disorders such as campmelic dysplasia (Sox9; Foster et al., 1994; Wagner et al., 2002) and Waardenburg/Hirschsprung syndrome (Sox10; Herbarth et al., 1994; Kuhlbrodt et al., 1998; Pingault et al., 1998). High levels of Sox11 have also been found to be associated with human gliomas (Wiegle et al., 2001).

Sox11 mRNA is expressed in developing mouse embryos as early as embryonic day 8.5 (E8.5) (Hargrave et al., 1997). By northern hybridization, Sox11 expression was found to gradually increase up to day E13.5 and then decline beginning at E16.5. *In situ* hybridization showed Sox11 expression localized to developing sensory ganglia including the trigeminal ganglion (TG) and dorsal root ganglia (DRGs) as well as the spinal cord (SC; Hargrave et al., 1997). The localization and timing of upregulation suggests that Sox11 has a role in regulating transcription of genes important for neuronal survival and differentiation. Our preliminary data supports this notion in several ways. First, we detected a 2-4-fold increase in Sox11 expression in E16.5 trigeminal ganglia of NGF-OE and GDNF-OE transgenic animals **(Fig.2)**. This increase in expression correlates with the enhancement in neuron survival and axonal projections exhibited by NGF-OE and GDNF-OE mice (Albers et al., 1994; 1996; Figueiredo et al. 1999; Zwick et al., 2002). Anti-Sox11 antibody labeling of E14.5 trigeminal ganglia showed that the expression of Sox11 was primarily restricted to neurons **(Fig. 2b)**.

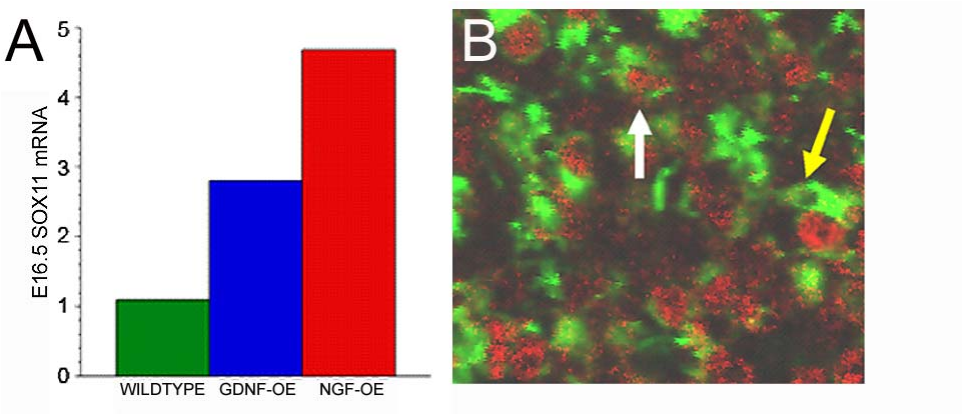

Fig. 2: **A**. Reverse transcriptase (RT)-PCR verification of gene chip data in pooled trigeminal ganglia isolated from E16.5 mice. Relative to WT mice, GDNF-OE mice had a 2.8-fold increase while NGF-OE mice had a 4.3-fold increase. **B.** Confocal micrograph showing Sox11 localization in neurons of E14.5 trigeminal ganglia. The white arrow indicates a neurofilament positive (green, cytoplasm) and Sox11 (red, nucleus) positive neuron. Most cells showed colocalization of Sox11 and NF150, though some appeared Sox11 negative (yellow arrow). Neuronal expression was also evident at E16.5 (not shown).

The increased expression of Sox11 in developing neurons of OE transgenic mice led us to hypothesize that Sox11 is a downstream regulator of survival and differentiation of sensory neurons in a neurotrophic factor dependent manner. To test this possibility the studies of this dissertation examined if the level of Sox11 modulated expression of genes involved in cell survival pathways or affected axon growth in models of differentiation and following neuron injury. Because changes in caspase activation and axon growth were used to assess Sox11 induced changes, a brief description of these processes follows.

1.3 CASPASE MEDIATED PROGRAMMED CELL DEATH

Studies using BrdU and TUNEL labeling showed that the increased survival of neurons in NGF-overexpressing mice was due to a trophic factor–mediated block in apoptotic cell death in developing neurons rather than stimulation of neuronal precursor replication (Figueiredo et al. 1999). Apoptotic programmed cell death is widespread during neurogenesis when approximately 50% of born neurons die. Apoptosis is generally thought to establish the appropriate number of neurons and innervation density of targets (Oppenheim, 1991). Programmed cell death can involve many biological pathways that collectively induce DNA fragmentation, chromatin condensation and membrane disruption and typically involve activation of caspase proteases (Hoshi et al., 1998; Nijhawan et al., 2000; Sakurai et al., 2003). Caspase-mediated apoptosis is regulated by both extrinsic and intrinsic mechanisms. Activation of death receptors such as tumor necrosis factor (TNF) receptors, their co-receptors Traf 1-4 and Fas receptors (Boldin et al., 1995) activate *initiator* caspases such as caspase 8, a major regulator of the apoptosis *effector* caspases such as caspase3 (Boldin et al., 1996; Nijhawan et al., 2000; Muzio et al., 1996; Srinivasula et al., 1996). Caspase 3 (and other effectors) activates many other proteins in the cytoplasm and nucleus involved in DNA fragmentation, actin destabilization, cell shrinkage and formation of apoptotic bodies (Thornberry and Lazebnik, 1998).

All caspases are activated by proteolysis with high specificity for cleavage after an aspartate residue. Once activated, their initial function appears to be associated with cytoplasmic

inactivation of proteins that normally protect the cell from death and activation of proteins that disrupt the cell's cytoskeleton. For example, Bcl-2 proteins which are pro-survival mitochondrial membrane associated proteins that inhibit pro-apoptosis molecules like Bid, Bad and Bax are cleaved by caspases (Adams and Cory, 1998; Xue and Horvitz, 1998). Effector caspases then begin to disrupt molecules that stabilize the cytoskeleton of the cell including those of the nuclear lamina (Thronberry and Lazebnik, 1998), gelosin (Kothakota et al., 1997), focal adhesion kinase (Wen et al, 1997) and p21 activated kinase 2 (Rudel and Bokoch, 1997). Once the cell structure is compromised, caspases can then translocate to the nucleus (Kamada et al., 2004; Ramuz et al., 2002) to activate DNA fragmentation factors and inhibit DNA repair enzymes (Thornberry and Lazebnik, 1998).

Caspases themselves are also regulated by inhibitory enzymes, mainly the inhibitors of apoptosis (IAP) family such as xIAP. IAPs control apoptosis, possibly through direct inhibition of effector caspases (Deveraux et al., 1997). Effector enzymes are thought to be a part of a positive feedback loop in that these caspases can activate other effectors of the cascade. IAPs inhibit the effector activation and thus appear to function as negative regulators of this feedback. It has also been hypothesized that the IAPs also control the cellular localization and concentration of caspases furthering their role in apoptosis regulation (Thornberry and Lazebnik, 1998). More work on how IAPs and other inhibitors regulate caspase function will be necessary to fully understand how caspases regulate apoptosis; however, current knowledge does show that caspases are major regulators of programmed cell death and are an important feature of normal neuronal development. Determining how these pathways are activated by Sox11 was investigated in these studies.

1.4 AXON OUTGROWTH AND NERVE REGENERATION

A major goal in neuroscience is to understand the cellular processes that underlie functional regeneration of neurons in both the peripheral and central nervous system. Regeneration in the PNS is known to occur in many cases following injury whereas regeneration in the CNS does not. Differences between regenerating peripheral and central axons appear to lie in the presence of inhibitory enzymes in the CNS (Kury et al., 2001; Qiu et al., 2001). In addition, transcriptional mechanisms thought to allow recovery in the PNS are not induced in the CNS (Kenny and Koscis, 1997). For example, activating transcription factor 3 (ATF3) is induced in DRG neurons after sciatic nerve axotomy but not after L4 rhizotomy (Shortland et al., 2006).

After injury to a nerve, a period of "dying back" from the site of injury followed by the initiation of anterograde degeneration (Wallerian Degeneration) occurs during the first several days (Baba et al., 1982; Mi et al., 2005; Sebille, 1982; Thomas, 1982). Wallerian Degeneration (WD) is a normal phenomenon that occurs upon injury that serves to degenerate the injured axons and in so doing, clear a path for axon to regenerate. Features associated with WD include inflammatory cell infiltration, glial cell proliferation and ubiquitination.

Injury to a nerve almost always induces inflammation. Within two days, T cells and macrophages infiltrate and clear myelin debris at the injury site (Bruck et al., 1997; Jander, 2001; Perry et al., 1987; Stoll et al., 1989; Stoll and Janner, 1999) and through the distal stump which

is the main source of inhibitory enzymes that block regeneration (Qiu et al., 2000). Upregulation of inflammatory cytokines like IL-1β, IL-6 and IL-10 occurs within 24h of injury and helps to facilitate the inflammatory response (Bolin et al., 1995; Bourde et al., 1996; Gillen et al., 1999). Proteins such as TNF-α also appear to be major factors in inducing inflammation (Liefner et al., 2000). In the CNS however, the inflammatory response appears to be localized to the injury site only, and may be a major reason why long range axon regeneration does not readily occur (George and Griffin, 1994; Perry et al., 1987).

Nerve injury disrupts normal Schwann cell (SwC) association with axons which leads to proliferation and expression of pro-regeneration genes such as N-myc downstream regulated gene 1, which is thought to facilitate SwC to axon signaling (Kury et al., 2001). Once signaling is re-established between neurons and SwCs, other factors such as ninjurin 1 are upregulated and promote growth cone outgrowth (Araki and Milbrandt, 1996). Neurotrophins and their receptors are also induced after nerve injury and their expression by SwCs is thought to promote neuron survival and neurite extension (Paratcha et al., 2001). Injuries to the nerve also cause rapid influx of calcium (Ca^{++}), which promotes ubiquitination leading to targeted protein degradation of neurofilaments and microtubules (Ehlers, 2004). This degeneration of the axon is necessary to promote clearing, macrophage phagocytosis of debris and SwC proliferation.

Several genes that are upregulated following nerve injury may have a role in peripheral nerve regeneration in adult mice. Small proline rich protein 1a, Sprr1a, has been shown to promote neurite outgrowth of primary DRG neurons and enhance peripheral nerve regeneration (Bonilla et al., 2002). Actin related protein complex subunit 3, Arpc3, has not been shown to be

associated with neurite outgrowth specifically, but it is known that Arpc3 is present at the site of active actin filament assembly (Welch et al., 1997) and necessary for actin filament branching (Volkmann et al., 2001) which occurs in growth cones. ATF3 expression is not normally detected in adult DRG neurons, but is significantly upregulated following peripheral nerve axotomy (Campbell et al., 2003; Isacsson et al., 2005; Lindwall et al., 2004; Tsujino et al., 2000) and in cultured DRG neurons (Seijfers et al., 2006). ATF3 is also known to be associated with neurite sprouting (Pearson et al., 2003). Other developmentally regulated genes have also been shown to have a role in adult mice after peripheral nerve injury such as c-jun (Lindwall et al., 2004; Pearson et al., 2003) and extracellular signal related protein kinase (ERK; Chierzi et al. 2005; Doya et al., 2005; Obata et al. 2004). These proteins may have a role in cytoskeletal reorganization in regenerating axons.

2.0 SIGNIFICANCE

The long-range goal of the following studies was to determine how the Sox11 transcription factor affects expression of genes involved in neuronal survival and differentiation and whether this transcription factor has a role in peripheral nerve regeneration. Neurotrophins have a potent effect on neuronal development, and our analysis suggested that one of the possible downstream targets modulated by neurotrophic factor signaling is Sox11. Our data indicate that Sox11 expression is essential for the survival and differentiation of neurons that are either chemically stimulated to differentiate or put under stress of axotomy and culture. Experiments using the neuroblastoma cell line, Neuro2A, show that differentiation of these cells requires expression of Sox11. If Sox11 expression is reduced at the time these cells undergo differentiation, they enter an apoptotic death pathway and do not project neurites. Thus, Sox11 potentially regulates genes associated with neuronal survival and differentiation.

Sox11 mRNA was also found to be elevated in developing sensory neurons in response to increased levels of trophic factors. Its putative role in regulating differentiation of Neuro2A cells suggests it has a central role in regulating genes involved in neuron differentiation. That Sox11 expression is elevated in response to trophic factors also suggests it may be activated in response to injury, which is a known stimulus of trophic factor mediated gene expression in the adult. We therefore also performed experiments to determine the role of Sox11 in adult sensory neurons *in*

vitro and following injury *in vivo*. Based on evidence that shows Sox11 is upregulated in adult primary DRG cultures and in neurons after axotomy, it is possible that Sox11 is part of a transcriptional program utilized during development that is revisited in the adult PNS in response to injury (Aubert et al., 1995). The data outlined herein provides the first information on the role of Sox11 in neuronal survival and nerve regeneration and identifies possible targets of its regulatory action. These studies form the foundation of future studies that will allow us to perhaps understand on a transcriptional level, why peripheral neurons regenerate without difficulty and CNS neurons do not.

3.0 SOX11 TRANSCRIPTION FACTOR IS REQUIRED FOR SURVIVAL AND DIFFERENTIATION OF NEUROBLASTOMA CELLS

3.1 INTRODUCTION

Members of the SRY box-containing (Sox) family of transcription factors are emerging as important transcriptional regulators whose activity underlies the development and differentiation of multiple organ systems. Twenty Sox genes have been identified in the mouse and human genomes and all contain a DNA-binding high mobility group (HMG) domain and protein specific domains implicated in activation and repression of gene transcription (Kamachi et al., 2000; Schepers et al., 2002). Several Sox genes are highly expressed in the developing central and peripheral nervous system and appear to regulate differentiation and cell fate in cell type specific manners. In mouse, Sox10 is expressed in neural crest precursors and has a role in establishing glial cell populations (Paratore et al., 2001). Sox 1, 2 and 3 are expressed in spinal neurons of developing chick embryos and downregulation of these factors is requisite for expression of the neuronal markers NeuroM, NeuN and Tuj1, suggesting an important role in regulating commitment to the neuronal phenotype (Bylund et al., 2003). In *in vitro* models, Sox6, which is also expressed in developing brain, was found to be essential for differentiation and neurite outgrowth of the embryonic carcinoma (EC) cell line P19 following retinoic acid (RA) stimulation (Hamada-Kanazawa et al., 2004). Sox6 was also critical for survival of RA-stimulated P19 cells as evidenced by the increased apoptotic death that ensued upon inhibition of

Sox6 expression. In human EC cells similarly induced to differentiate into a neuronal phenotype with RA, Sox3 expression was up-regulated as Sox2 expression declined, suggesting regulatory roles for Sox2 and Sox3 in EC differentiation (Stevanovic, 2003).

In the peripheral nervous system, the Sox11 transcription factor has been of interest because of its high level of expression in developing trigeminal and dorsal root ganglia (DRG) (Uwanogho et al., 1995; Hargrave et al., 1997) and potential role in the development of gliomas (Weigle et al., 2005). In sensory neurons the high level of embryonic Sox11 expression is markedly reduced at birth (Hargrave et al., 1997; Tanabe et al., 2003; Jankowski et al., 2004), suggesting tight control of its transcriptional activity. Towards identification of the targets and mechanism of Sox11 action, mice that lack a functional Sox11 gene were recently isolated (Sock et al., 2004). Newborn $Sox11^{-/-}$ mice exhibited widespread developmental abnormalities in the heart, lung, stomach, pancreas and skeletal systems and died soon after birth due to the severe defects in organ development. How knockout of Sox11 affected sensory neuron survival is still unclear, although neonatal $Sox^{-/-}$ mice did respond to tactile stimuli suggesting that at least some sensory neurons were present.

We and others have found that Sox11 transcription is markedly elevated in adult sensory neurons following nerve cut injury (Tanabe et al., 2003; Jankowski et al., 2004). Sox11 therefore appears to have a role in developing neuron growth and survival as well as recovery of adult neurons following injury. To better understand the role of Sox11 and the genes it may modulate, we examined its function in regulating differentiation and survival in the mouse neuroblastoma cell line Neuro2a model system. Neuro2a cells can be induced to differentiate into a neuronal phenotype by exposure to retinoic acid (RA), a potent modulator of neurogenesis, neuronal specification and axonal outgrowth (Shea et al., 1985; Maden, 2001). Based on the high level of

Sox11 expression in developing sensory neurons, we hypothesized that Sox11 would have a role in regulating RA-induced differentiation in the Neuro2a model as well. Using siRNA-mediated knockdown, we asked if Sox11 was essential for survival and neurite extension following RA-induced differentiation. Results indicate that Sox11 is important for RA-induced differentiation, neurite outgrowth and transcriptional regulation of genes associated with cell survival.

3.2 MATERIALS AND METHODS

Cell culture. The mouse neuroblastoma cell line Neuro2a (ATCC clone number CCL-131, Manassas, VA) (Olmsted et al., 1970) was maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (MEMS) and 1% penicillin/streptomycin in an incubator set at 37^{o}C and 5% CO_2. Cells used were passaged no more than three times. For all experiments, cells were plated into 12- or 24-well plates at a concentration of 10,000 and 5,000 cells/ well, respectively, or plated into 2- or 4-well chamber slides. Cells were grown in MEMS to 50% confluence (18-24h) and then treated with 20µM retinoic acid (RA, Sigma, St. Louis, MO), siRNAs or both depending on the experiment. The time of RA addition was considered the 0h timepoint. Cells were incubated according to experimental design and at appropriate times RNA was isolated or cultures were fixed for immunocytochemistry. Experiments to determine the effect of siRNA treatment at various times after RA addition were done by setting up cultures as described above and then adding siRNA at various times (0h, 6h, 12h or 24h) after RA addition. Transfected cultures were harvested for RNA or morphological analysis at 24h after siRNA addition.

siRNA treatment. Two hours prior to siRNA transfection fresh medium was added to cultures. Cells were then treated with either 20µm RA alone or in combination with 10nM Sox11 siRNAs or 10nM non-targeting siRNAs (Dharmacon, Lafayette, CO). For transfection, TRANSIT-TKO transfection reagent (Mirus Corporation) was used. 2-4µl of the tranfection reagent was added to 50 or 100µl of serum-free MEM, respectively. The solution was mixed, incubated at room temperature (RT) for 5-20 min. and the appropriate volume of 1µm siRNAs added to obtain a

final concentration of 10nM siRNA per culture well. Solutions were further mixed by pipetting, incubated at RT for 5-20 min and then added to the cultures.

Cell counting, morphology and differentiation measures. Cells grown on chamber slides were fixed 5 min with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) and coverslipped with glycerol for light microscopic analysis on an Olympus BH-2 miscroscope equipped with a differential interference contrast filter. Differentiated cells were quantified using criteria similar to those described in Munch et al (2003). Cells with at least two neurites extending from the soma or one neurite longer than the diameter of the soma were considered differentiated. Cells undergoing apoptosis were identified by shrunken cell membranes, reduced size or disrupted nuclei (Hoshi et al., 1998; Sakurai et al., 2003). Cells are reported as a percent of total cells counted within gridlines of 4 randomly selected squares each 600 μm^2 using a photoetched gridded coverslip (Electron Microscopy Sciences, Hatfield, Pa) at 10x magnification (n=3 per condition). The percent of cleaved caspase 3(casp3*)-labeled cells was calculated by counting the number of casp3* positive cells in 4 randomly selected fields equal to the size of the photoetched grids used for morphological quantification. For each condition, n equaled 3. Statistical significance between conditions was determined by p-values ≤ 0.05.

Cell immunolabeling. After fixation and rinsing, cells were blocked in 0.25% triton X-100 with 5% normal goat serum in PBS for 30 min. and incubated in either goat anti-Sox11 (1:100; Santa Cruz Biotech, Santa Cruz, CA) or rabbit anti-casp3* (1:100; Cell Signaling Technologies, Danvers, MA) overnight at room temperature. Cells were washed in PBS and incubated 2h in 1:500 dilutions of FITC-conjugated donkey anti-rabbit antibody or CY2-conjugated donkey anti-goat (Jackson, West Grove, Pa). Slides were rinsed in PBS, coverslipped in glycerol and images captured using a Leica fluorescence microscope.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Culture medium from triplicate cultures was removed and plates were washed 3 times with PBS. Cells were lysed with RLT buffer (Qiagen) with beta-mercaptoethanol and RNA isolated using the Qiagen RNeasy protocol for animal cells. After RNA isolation, samples were treated with DNase I (Invitrogen) for 15 min at RT and 1 µg of DNased RNA reverse transcribed using Superscript II Reverse Transcriptase according to the manufacturer's protocol (Invitrogen). Samples were stored at -80^{o}C until used in PCR reactions. For realtime PCR, samples of cDNA were added to a SYBR Green MasterMix (Applied Biosystems), run in triplicate on an Applied Biosystems Imager. Ct values (threshold cycles) were normalized to GAPDH by subtracting the target gene from the GAPDH control. A ΔΔCt value was then calculated by subtracting the normalized experimental Ct from the normalized control condition with fold change reported as $2^{\Delta\Delta Ct}$.

SuperArray filter hybridization. Focused DNA microarray filters (GEArray Q Series Mouse Apoptosis Gene Array; MM-002) were used to identify genes involved in cell survival and death that may be regulated by Sox11. Parallel cultures were treated with 20µM RA in combination with either 10nM Sox11 siRNAs or 10nM non-targeting siRNAs. Total RNA (3µg) was isolated at either 12h or 24h post siRNA addition using the Qiagen RNeasy Mini-Kit protocol. RNA was reverse transcribed into ^{32}P-dCTP-labeled probes according to the manufacturer's protocol and annealed with primers at 70^{o}C for 3 min followed by incubation at 37^{o}C for 10 min. A cocktail containing RNase inhibitors and reverse transcriptase was added to the annealing reactions at equal volume and incubated at 37^{o}C for 25 min. RNA was hydrolyzed at 85^{o}C for five min, reactions were placed on ice, and then linear polymerase reactions containing primers specific to the apoptosis superarray were performed using ^{32}P-dCTP spiked reactions according to PCR

parameters provided. Probes were denatured at 94^{o}C for 2 min prior to hybridization. Filters were prehybridized 2 h at 60^{o}C in GEAHyb (Superarray) solution containing 100µg/ml sheared salmon sperm DNA (GEAPrehyb). Denatured probe in 0.75mL of GEAprehyb solution was incubated with filters overnight at 60^{o}C with gentle agitation (5-10 rpm). Filters were washed twice in 2X SSC, 1% SDS at 60^{o}C for 15 min with agitation, twice in 0.1X SSC, 0.5% SDS at 60^{o}C for 15 min. and then covered with plastic wrap and exposed to a phosphoimaging screen for an empirically determined amount of time. Phosphoimaging screens were read on a BioRad phosphoimager and values obtained by subtracting filter background intensity and normalizing to GAPDH controls on the filter.

Statistics: Statistical analysis was determined using Student's t-test with p values < 0.05 as statistical significance.

3.3 RESULTS

3.3.1 SOX11 IS EXPRESSED IN NEURO2A CELLS.

Neuro2a cells provide an excellent model system to study the role of Sox11 in transcriptional regulation of neuronal differentiation. They are easily propagated, they can be stimulated to differentiate by addition of 20μM retinoic acid (RA) and upon differentiation stop dividing and extend neurites (Shea et al., 1985; Sajithlal et al., 2002; Munch et al., 2003; Noguchi et al., 2003). Under normal culture conditions without RA, Neuro2a cells appear as amoeboid neuroblasts that begin to extend neurites after 1-3d in culture. To assess if Sox11 might have a role in regulating genes important for this transition to the differentiated state, we assayed Sox11 mRNA levels at times prior to and coinciding with the initiation of neurite growth (6h, 1d, 2d and 3d post-plating). Reverse transcriptase-PCR (RT-PCR) assays showed that replicating cells with minimal neurite outgrowth had a relatively low level of Sox11 mRNA (Fig. 3A, also see Fig. 6A). At 1d and 2d post-plating, a steady increase in Sox11 mRNA was found that correlated with an increasing number of neurite containing cells (Fig. 3B). By 3d in culture, Sox11 had increased 280% relative to the 6h timepoint level. Thus, a steady increase in Sox11 paralleled the increase in the number of cells exhibiting neurites and undergoing differentiation.

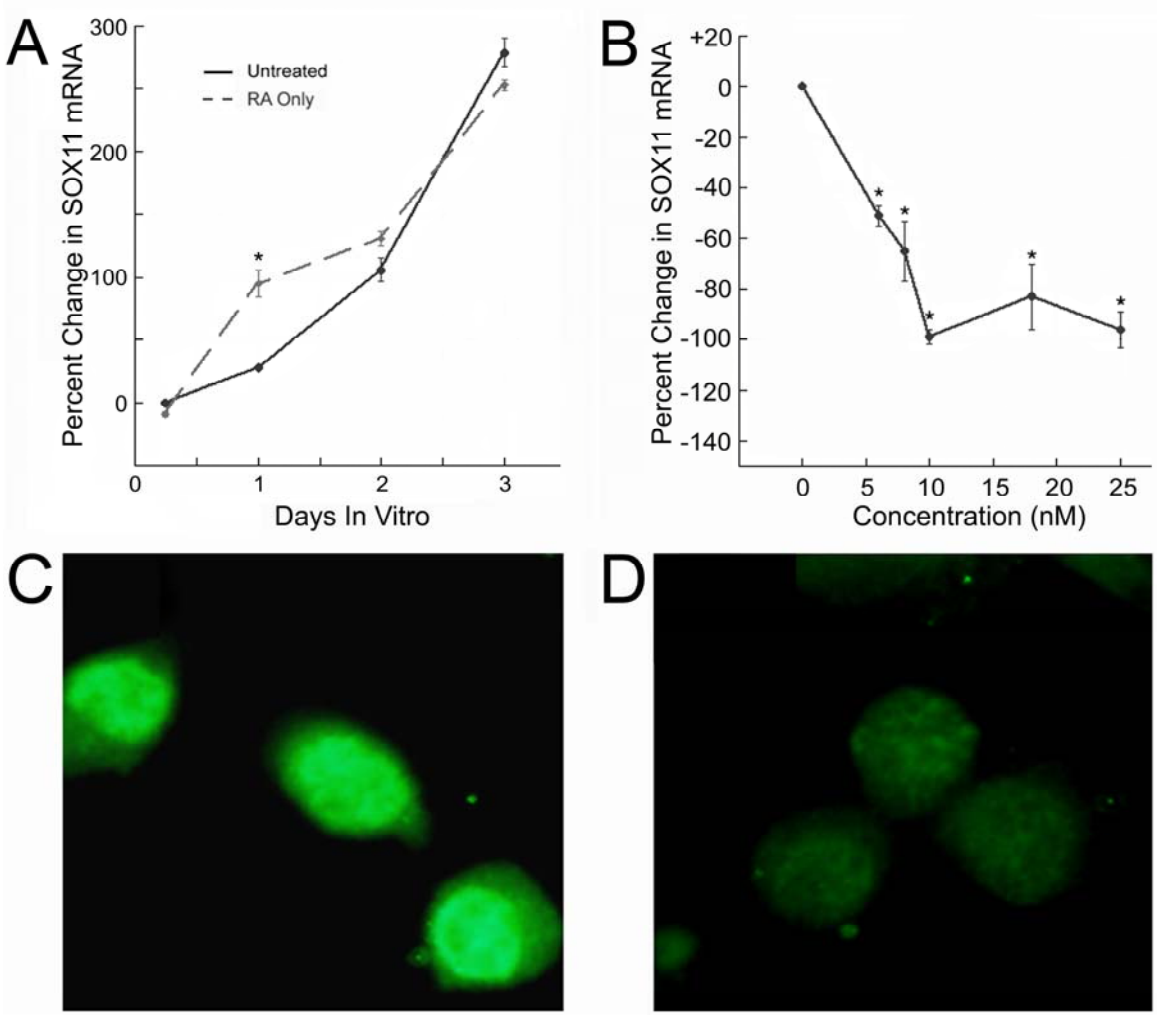
A
Percent Change in SOX11 mRNA
300
200
100
0
Untreated
RA Only
*
0
1
2
3
Days In Vitro
B
Percent Change in SOX11 mRNA
+20
0
-20
-40
-60
-80
-100
-120
-140
*
*
*
*
0
5
10
15
20
25
Concentration (nM)
C
D

Fig. 3: A. Fold change in Sox11 mRNA in Neuro2a cells grown with or without 20μM retinoic acid (RA). Sox11 expression increases gradually in cultures without RA (solid line). Addition of RA caused early stimulation of Sox11 expression with a significant rise seen at 24 h after cell plating (dashed line). Sox11 abundance was equivalent in the two culture conditions at 72 h. N= 3 at each point; p-value <0.001. B. Treatment with Sox11 siRNAs reduces Sox11 mRNA level in Neuro2a cells. Assay was done 24h after addition of a Smart pool siRNAs made against Sox11. Reduction in Sox11 occurs in a dose dependent manner. Asterisk indicates significant change, p< 0.001. Levels are normalized to transfection reagent alone. C. Untreated Neuro2a cells show nuclear immunoreactivity for Sox11 protein. D. Sox11 siRNA treated Neuro2a cells lack nuclear staining for Sox11. Images are of cells 24h after siRNA treatment.

It has previously been shown that Neuro2a cells undergo accelerated differentiation when grown in medium containing 20μM retinoic acid (MEMS-RA) (Shea et al., 1985). To determine if Sox11 has a role in this RA-induced differentiation, cells were treated with RA and the level of Sox11 mRNA measured (Fig. 3A). Neuro2a cells grown in MEMS-RA showed a 100% increase in Sox11 mRNA at 1d, in comparison with the 30% increase in cells grown without RA (Fig. 3A). Sox11 expression continued to increase in RA medium and was increased 250% at 3d, a value not significantly different from that measured in cultures grown without RA. The transient increase in Sox11 mRNA in 1 d-old RA-treated cultures versus untreated cultures suggested Sox11 has a role in Neuro2a differentiation, particularly during early stages.

3.3.2 siRNA-mediated knockdown of Sox11 occurs on translational and transcriptional levels.

To test the role of Sox11 in Neuro2a differentiation, we reduced the cellular level of Sox11 by transfecting cells with a SmartPool of small interfering RNAs (siRNAs) targeted to Sox11. To insure a high transfection efficiency occurred in these cultures, cotransfections of Sox11 siRNAs and CY3-conjugated nontargeting siRNAs were done. Immunofluorescent analysis of transfected cultures showed nearly all (93%) cells were transfected (not shown). We then confirmed Sox11 knockdown on the transcriptional level using RT-PCR assays. Knockdown occurred in a dose dependent manner (Fig. 3B) with 10nM siRNAs producing a maximal 98% knockdown in Sox11 mRNA. Several other non-targeting control experiments and RT-PCR analysis of other genes verified the specificity of the Sox11 knockdown (Fig. 4). Translational level knockdown of Sox11 was also confirmed using Sox11 antibody

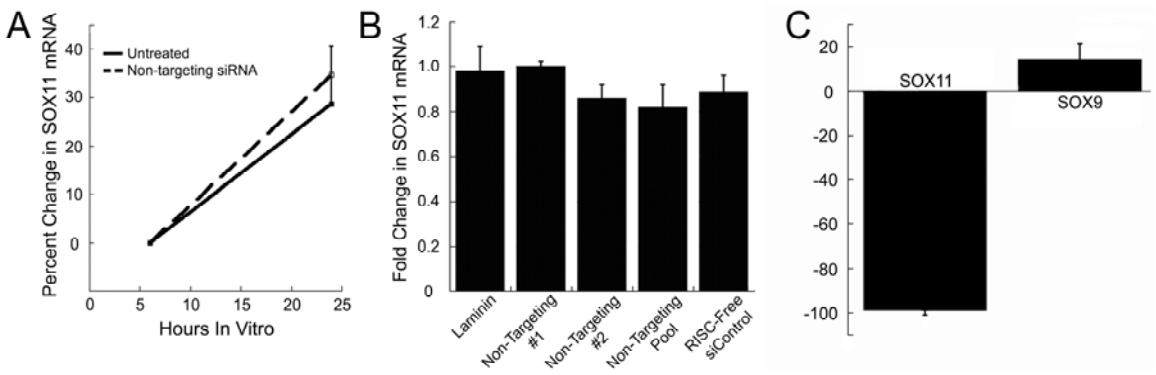

Fig. 4: Control reactions for siRNA knockdown of Sox11 in Neuro2A cells. A. Untreated and non-targeting siRNA transfected cells both have similarly increased levels of Sox11 within 24h. B. Transfection of several other control siRNAs directed at non-Sox11 targets did not change Sox11 mRNA levels and did not affect Neuro2A survival. C. Sox11 targeting siRNAs specifically knock down the expression of Sox11 and not other transcription factors of similar sequence homology like Sox9.

immunolabeling of transfected cells. In untransfected cultures or ones transfected with non-targeting siRNAs, Sox11-positive cells were easily detected (Fig. 3C). In contrast, Sox11-positive cells were clearly reduced in cultures transfected with Sox11 siRNA (Fig. 3D).

3.3.3 Increased expression of Sox11 is required for survival and neurite growth following RA-stimulation.

To determine if Sox11 was required for RA-induced differentiation and neurite outgrowth, Neuro2a cells were treated with a combination of RA and Sox11 siRNAs. As shown in Fig. 3B, cultures grown 24h with RA had a two-fold increase in Sox11 mRNA. With co-addition of Sox11 siRNAs however, RA-treated cultures showed a 59% decrease in Sox11 mRNA by 12h that was maintained out to 24h post RA/siRNA treatment (Fig. 5). Knockdown of Sox11 in RA-treated cultures caused morphological changes as well. Compared to RA-only (Fig. 6B) and siRNA-only (Fig. 6C) cells, cells treated with RA plus Sox11 siRNAs had fewer neurites, many were shrunken and some appeared vacuolated and dying (Fig. 6D). To quantify these morphological changes, we determined the percent of differentiating cells with neurites, the percent of cells exhibiting features of apoptotic cell death (shrunken cell membranes, reduced size, disrupted nuclei) and the percent of activated caspase 3 (casp3*) immunolabeled cells (Fig. 6, 7). In cultures grown for 24h in RA-only, less than 5% of the population had morphological features of apoptotic cells or were casp3*-positive (Fig. 7). Differentiating cells with neurite extensions were prominent and comprised 22% of the population. In contrast, only 8% of cells in RA plus Sox11 siRNA treated cultures showed a differentiated morphology, which was

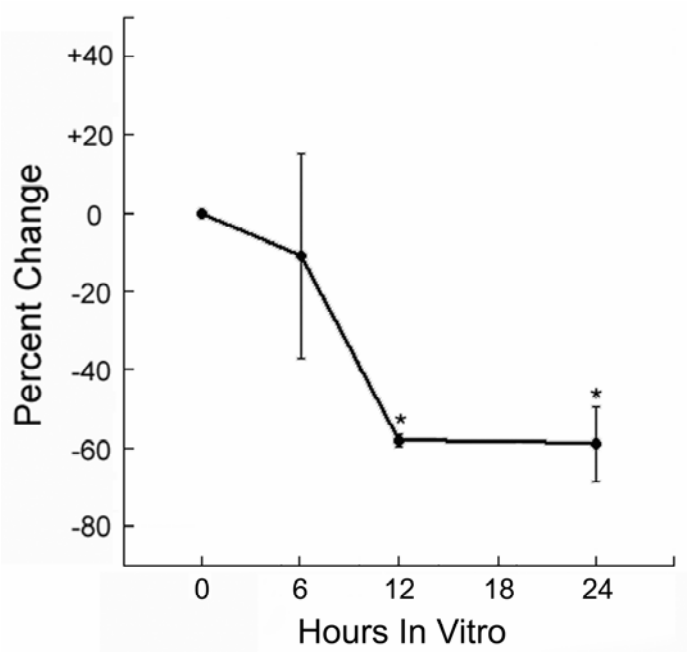

Fig 5: Percent change in Sox11 expression in Neuro2a cells cotreated with RA and Sox11 siRNAs. Transfection of 10nM Sox11 siRNAs blocked the RA-induced increase in Sox11 mRNA within 12h of addition. Knockdown was maintained for up to 24h at which time a 59% decrease was measured. N= 3 for each timepoint. Asterisk indicates $p < 0.001$ relative to transfection reagent only control.

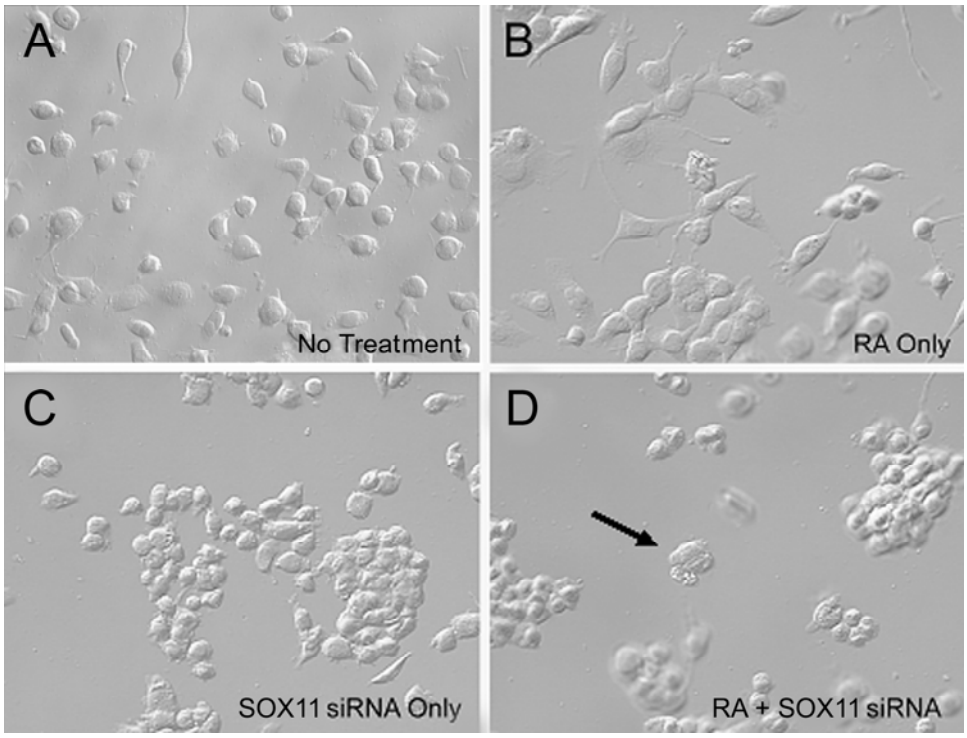

Fig 6: Light micrographs of Neuro2a cells treated with RA and/or Sox11 siRNAs. A. Most Neuro2a cells grown in MEMS media for 24h show typical amoeboid morphology with few neurite projections. B. Cultures treated with RA for 24h contain more cells with extension of neuronal processes and evidence of differentiation. C. Few cells in Neuro2a cultures treated for 24h with siRNA against Sox11 show neurite projections and appear aggregated. D. Neuro2a cultures treated with RA and Sox11 siRNAs are clustered and many appear apoptotic (arrow). All images were taken at 24h post-treatment.

statistically unchanged relative to the percent of differentiated cells in untreated (no RA) cultures. In addition, cultures treated with RA plus Sox11 siRNAs had a significant increase in the percent of cells displaying apoptotic morphologies (18%) and casp3*-immunoreactivity (21%) (Fig. 6D, 7). Cultures treated with Sox11 siRNAs but not RA showed no difference compared to untreated cultures in the percent of differentiated cells, a small increase in the percent of apoptotic cells but no change in casp3*-positive cells (Fig. 7). Thus, a low degree of toxicity may result from RNAi treatment alone. To verify that the siRNA effects on cell death were specific to Sox11, we quantified the extent of differentiation and apoptosis in control siRNA transfected (siControl) and RA plus siControl treated cultures. Addition of control siRNAs did not alter the level of cell death or differentiation in either condition relative to untreated or RA treated cultures (Fig. 7). Taken together, this analysis shows that knockdown of Sox11 expression in RA-treated cultures inhibits RA-induced differentiation and neurite growth and significantly increases cell death.

3.3.4 The ability to knockdown Sox11 in RA-treated cells is dependent on the time of transfection.

To examine if the level of Sox11 is important for later stages of RA-induced differentiation, we attempted knockdown of Sox11 at various times after RA treatment. Cell cultures were treated with 20μm RA and Sox11 siRNAs were then added at 0h (as shown above), 6h, 12h or 24h post RA addition (Fig. 8). The level of Sox11 mRNA (Fig. 8E) and changes in morphology and casp3* (Fig. 8A-D, F) were then assessed. Unexpectedly, we found the level of Sox11 mRNA was only reduced in cultures in which siRNA transfection was done at the time of RA addition

(0h, Fig. 8E). No reduction of Sox11 mRNA was found in cultures transfected at 6h, 12h or 24h after RA addition. The morphology of cells transfected with Sox11 siRNAs 24h after RA addition was consistent with a lack of Sox11 knockdown, i.e., many cells had neurite extensions (Fig. 8B). Cells in parallel cultures transfected at the time of RA addition displayed few neurites and many had apoptotic morphologies (Fig. 8A), as previously observed (Fig. 6). Consistent with the prominent neurite growth in cultures transfected at 24h after RA addition, few if any casp3*-labeled cells were visible (Fig. 8D) which again was in contrast to cultures transfected at the time of RA addition (Fig. 8C).

Changes in morphology and apoptosis were then quantified across treatment groups in which siRNA was added at either 0h, 12h or 24h after RA (Fig. 8F). All measures were done at 24h post siRNA addition. Cells treated concurrently with RA and siRNAs (0h timepoint) showed a percent of differentiated and casp3*-positive cells similar to previous assays (Fig. 7). However, in cultures in which Sox11 siRNAs were added at 6h (not shown) or 12h (Fig. 8F) after RA treatment, the percent of differentiated or casp3*-positive cells was unchanged from RA-only treated cells. For 12h cultures, 27% of cells showed a differentiated phenotype in both RA-only and RA plus siRNA groups and a very low percent of casp3*-positive cells. Similar results were found for cells treated with RA and transfected 24h later with siRNAs to Sox11 (Fig 8F). Co-treated cultures had 38% differentiated cells, which is similar to the 42% percent of cells in the RA-only group. Thus, siRNA knockdown of Sox11 and its resulting effect on Neuro2a differentiation and apoptosis was only possible if it was done at the time RA was added to cultures.

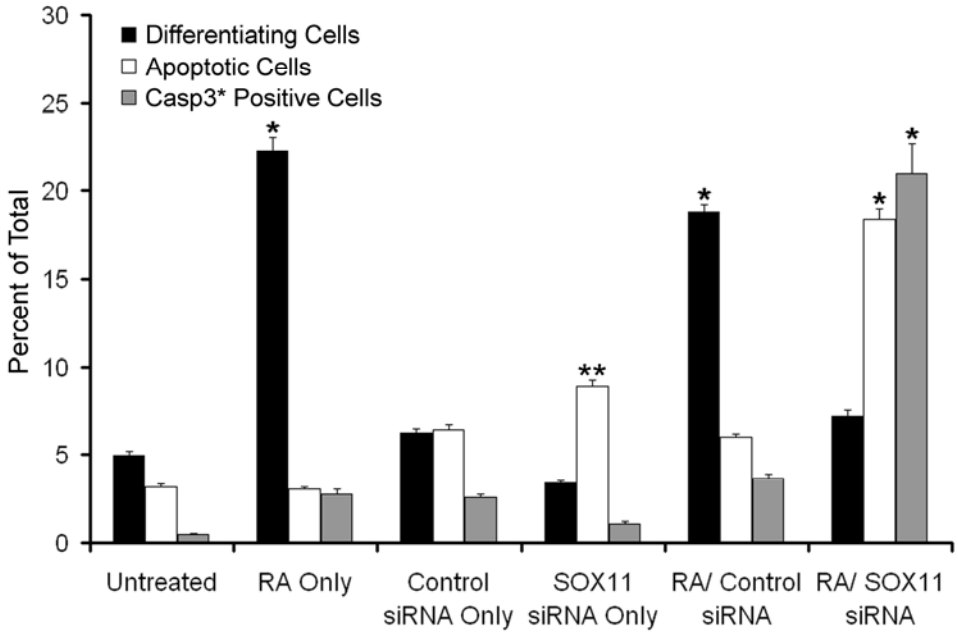
Differentiating Cells
Apoptotic Cells
Casp3* Positive Cells
Percent of Total
30
25
20
15
10
5
0
*
**
Untreated
RA Only
Control siRNA Only
SOX11 siRNA Only
RA/ Control siRNA
RA/ SOX11 siRNA

Fig. 7: Combined treatment of RA and Sox11 siRNA enhances Neuro2a cell death. Chart shows quantification of differentiation (neurite growth) and apoptosis in cultures of Neuro2a cells 24h after treatment with RA alone, control (nontargeting) siRNA only, Sox11 siRNA only, RA plus control siRNA and RA plus Sox11 siRNA. Untreated cultures have few differentiating cells with neurites (black bar), few apoptotic cells (white bar) and virtually no cells immunopositive for activated caspase 3 (grey bar). RA-only treated cultures had a significant increase in the percent of differentiating cells whereas cultures with siRNA alone showed a slight rise in cells that were morphologically apoptotic. Neurite growth and apoptosis was not significantly different in cultures treated with control siRNAs. Co-treatment with RA and control siRNA caused an increase in neurite containing cell, as expected. Combination of RA and Sox11 siRNA reduced neuritre growth and caused a significant increase in the number of apoptotic cells and caspase3 immunoreactivity. The number of cells analyzed for each group was as follows: untreated, 1,674 cells; RA only, 1,275; Control siRNA only, 1,857; Sox siRNA only, 981; RA plus control siRNA, 1,398; RA plus Sox11 siRNA, 1,292. Values on chart are a percent of total cells analyzed. Asterisks indicate $p < 0.001$ relative to untreated controls.

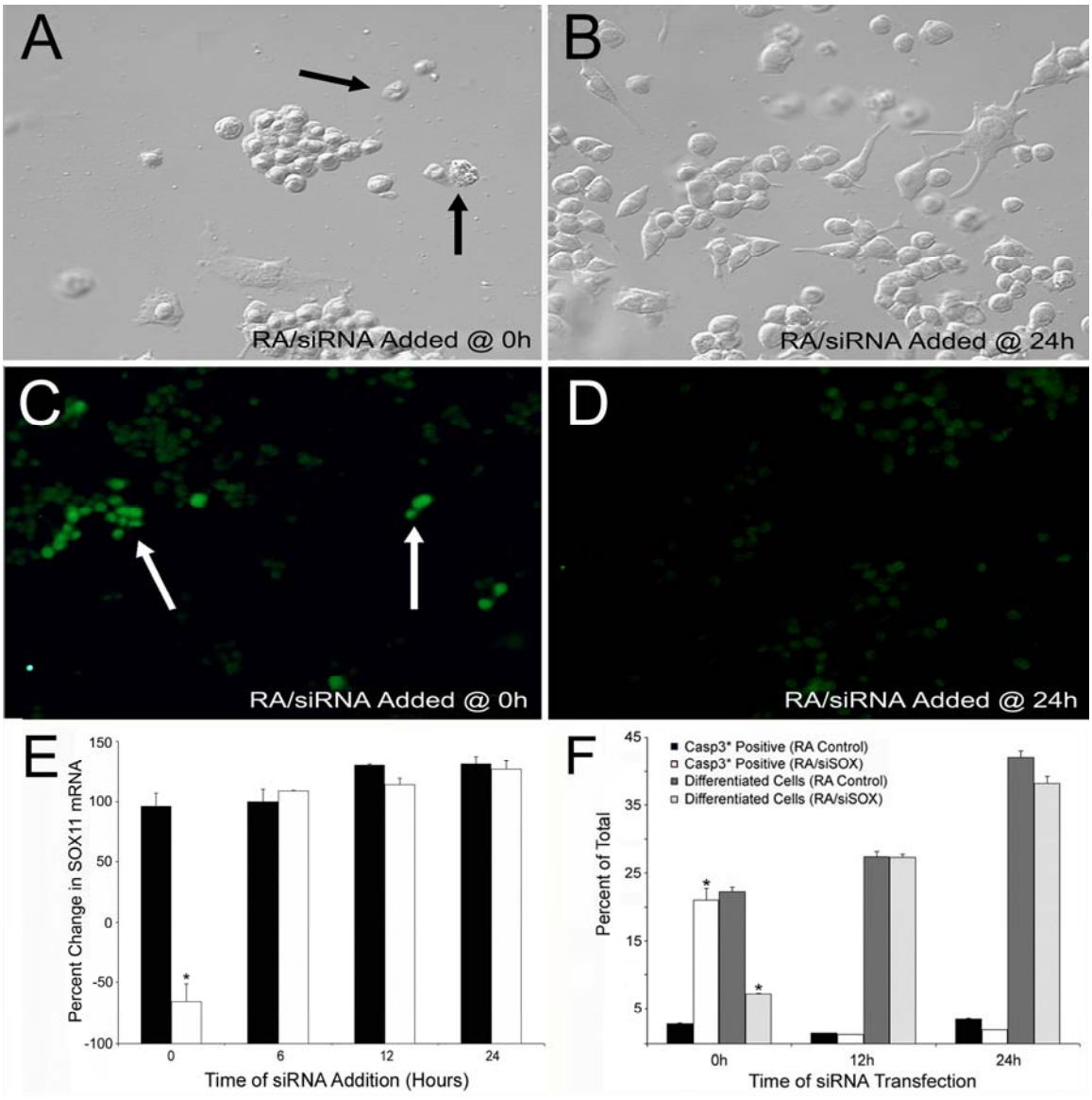
A
RA/siRNA Added @ 0h
B
RA/siRNA Added @ 24h
C
RA/siRNA Added @ 0h
D
RA/siRNA Added @ 24h
E
Percent Change in SOX11 mRNA
150
100
50
0
-50
-100
0
6
12
24
Time of siRNA Addition (Hours)
F
Casp3* Positive (RA Control)
Casp3* Positive (RA/siSOX)
Differentiated Cells (RA Control)
Differentiated Cells (RA/siSOX)
Percent of Total
45
35
25
15
5
0h
12h
24h
Time of siRNA Transfection

Fig. 8: Sox11 expression is critical at the early phase of the transition of Neuro2A cells to a differentiated state. Differential interference contrast (DIC) images of Neuro2a cultures treated with Sox11 siRNAs at the time of retinoic acid (RA) treatment (0h) (A) and 24h after addition of RA (B). Note prevalence of cells with neurites in B but not A. C. Activated caspase 3(casp3*)-labeling (arrows) appears in many cells co-treated with RA and Sox11 siRNAs at the 0h timepoint. D. Cells grown in RA for 24h and then treated with Sox11 siRNAs do not exhibit casp3* labeling. E. The reduction in Sox11 mRNA occurs only when Sox11 siRNAs are added simultaneously with RA. F. Plot of differentiating, apoptotic and casp3*-positive cells in cultures treated with RA and Sox siRNAs at various times after RA stimulation. Cultures in which RA and Sox11 siRNAs are added together (0h) have more casp3* positive cells (white box) and fewer differentiating cells (tan box) compared to RA-alone treated cells. Sox11 siRNA transfected at 12h or 24h after RA addition does not increase the number of casp3*-positive cells or decrease the number of differentiating cells normally seen following RA addition. All samples are n =3. The number of cells analyzed in each group was: At 0h, RA-only, 2351 cells; RA/siRNA, 2419 cells. At 12h, RA-only, 2185 cells; RA/siRNA, 2061 cells. At 24h, RA-only, 2552 cells; RA/siRNA, 3744 cells. Asterisks indicate $p < 0.001$ relative to RA-only control values.

3.3.5 Knockdown of Sox11 alters expression of genes associated with apoptosis.

To identify how knockdown of Sox11 contributes to RA-induced Neuro2a cell death, we assayed gene levels following knockdown using tailored gene arrays spotted with cDNAs encoding genes linked to cell death pathways (Fig. 9). RNA isolated from Neuro2a cultures cotreated with RA or with a combination of RA and Sox11 siRNAs was analyzed at 12h or 24h after transfection. Analysis of gene arrays processed in parallel showed several genes were differentially expressed following Sox11 knockdown (Table 2). For several genes, the change in transcript abundance was different at 12h versus 24h post-transfection suggesting a dynamic change in gene expression at these times.

It is difficult to know whether these changes in gene abundance are directly related to the reduction in Sox11 or whether they represent secondary effects of other transcriptional events. To address this issue and further validate some of the changes indicated by the gene arrays, four genes that contain 1-4 Sox factor HMG binding consensus domains (5'-(A/T)(A/T)CAA(A/T)G-3') within 1-2 Kb of their start codons, were further analyzed using real time RT-PCR assays (Fig. 9A,B). The genes chosen and results of this analysis are: 1) TRAF (TNF receptor-associated factor) family member- associated NFkB activator (TANK) was decreased 31% at 24h in the RA-Sox11 siRNA treated cultures, 2) Bcl2 interacting protein1, NIP3 (BNIP3), was increased 35% at 24h. 3) B lymphoid kinase (Blk) was statistically unchanged, though a trend toward increased expression was found and 4) B-cell lymphoma 2-like 10 (Bcl10), whose abundance was unchanged by Sox11 inhibition and was used to verify

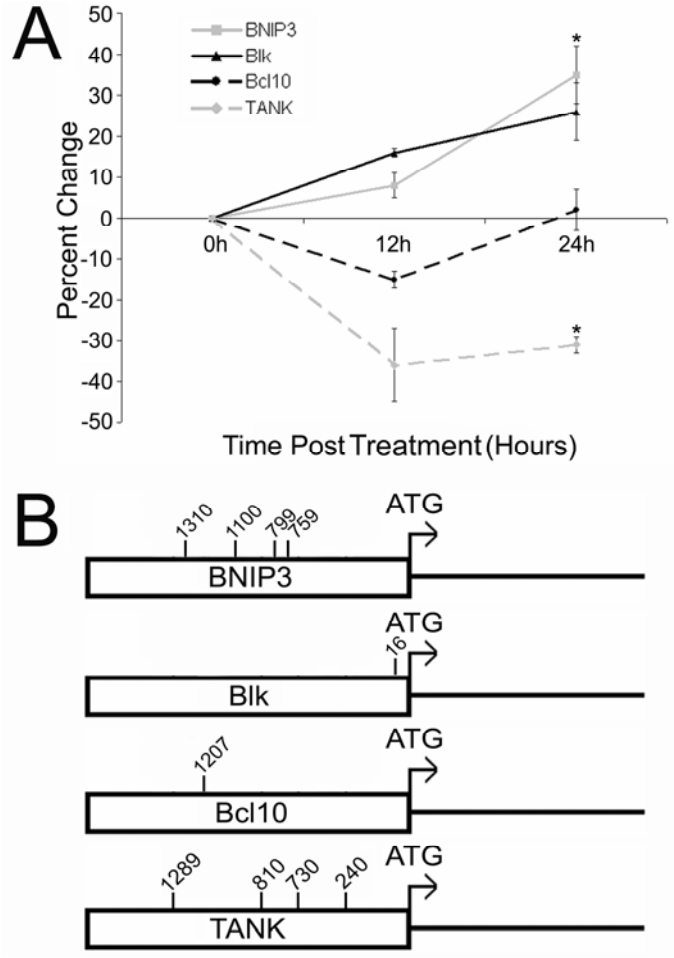

Fig. 9: Knockdown of Sox11 mRNA alters expression of genes involved in cell death pathways. RNA isolated from Neuro2a cells treated with RA or RA plus Sox11 siRNAs for either 12h or 24h were analyzed using gene array filters. Plot shows RT-PCR analysis for 4 genes: Traf member and activator of NFkB (TANK), Bcl2-like 10 (Bcl10), B-lymphoid kinase (Blk) and Bcl2 interacting protein 1 NIP3 (BNIP3). All except Bcl10 were changed following knockdown of Sox11. All contain Sox transcription factor binding sites in their promoter regions. N=3 for each timepoint. Asterisk indicates $p<0.05$.

the accuracy of the gene array. The RT-PCR analysis of gene expression (Fig. 9), which was done in triplicate, was most consistent with the 24h timepoint array data listed in Table 1. This may reflect a more dynamic state in gene expression at the 12h timepoint. Together, these data suggest TANK and BNIP3 are targets of Sox11 transcriptional regulation and may have importance in mediating the RA-induced program of differentiation.

Table 2: Change in mRNA level of apoptotic associated genes in RA-treated Neuro2a cells at 12h and 24h post Sox11 siRNA-mediated knockdown.

Gene	Accession Number	Direction of Change 12h	Direction of Change 24h
Casp8ap2	NM_011997	Increased	Increased
Bnip3	NM_009760	Increased	Increased
Blk	NM_007549	Decreased	Increased
Tank	NM_011529	Increased	Decreased
Tnfrsf21	NM_178589	Increased	Decreased
Fas	NM_007987	Decreased	Decreased
Casp2	NM_007610	Increased	Decreased
Casp3	NM_009810	Increased	Decreased
Casp9	NM_015733	Unchanged	Decreased
Bcl2a1d	NM_007536	Increased	Decreased
Bok	NM_016778	Increased	Decreased
Biklk	NM_007546	Decreased	Decreased
Birc6	NM_007566	Decreased	Decreased
Mcl1	NM_008562	Decreased	Decreased
Bcl2l10	NM_013479	Unchanged	Unchanged

Table 2 Key: Casp8ap2, Casp8 associated protein 2; Bnip3, Bcl2/adenovirus E1B 19kDa-interacting protein 1, NIP3; Blk, B lymphoid kinase; Tank, TRAF family member-associated NfkB activator; Tnfrsf21, TNF receptor superfamily, member 21; Fas, TNF receptor superfamily member; Casp2, Caspase 2; Casp3, Caspase 3; Casp9, Caspase 9; Bcl2a1d, B cell leukemia/lymphoma 2 related protein A1d; Bok, Bcl2 related ovarian killer protein; Biklk, Bcl2-interacting killer-like; Birc6, Bacloviral IAP repeat-containing 6; Mcl1, Myeloid cell leukemia sequence 1; Bcl2l10, Bcl2-like 10.

3.4 DISCUSSION

We show in this study that the transcription factor Sox11 is normally expressed in the Neuro2A neuroblastoma cell line and that its expression is significantly increased as these cells differentiate into neuronal morphologies. Addition of retinoic acid stimulated expression of Sox11 at early phases of Neuro2A cell differentiation and reduction of Sox11 at this time caused a significant increase in apoptotic cells and blocked the normal RA-induced differentiation and neurite outgrowth typically expressed by Neuro2a cells. These changes only occurred if Sox11 knockdown coincided with the time of RA addition, suggesting Sox11 has a crucial role in regulating early transcriptional events associated with the RA-induced commitment of Neuro2a cells to differentiation and survival pathways. Genes potentially involved in regulating cell survival that contain Sox11 binding sites in proximal upstream regulatory regions were also identified as possible targets of Sox11 activity.

In cells grown without RA, Sox11 knockdown had little to no affect on either the number of differentiated or casp3*-positive cells present at 24h after siRNA treatment. In contrast, RA-stimulated cells in which Sox11 was knocked down showed a significant decrease in differentiation (neurite growth) and increase in apoptotic morphology and casp3* immunoreactivity. That Sox11 knockdown in RA-treated cultures increased apoptosis suggests an essential role for Sox11 in regulating early transcriptional events induced by RA that promote entry into differentiation and survival pathways. A likely role for Sox11 is in regulation of genes involved in the RA response, which is transcriptionally mediated through RA binding to the nuclear retinoid receptors, RAR and RXR (Chambon, 1996; Mark et al., 2005). These ligand-activated transcription factors act in conjunction with other transcription factors to stimulate and

inhibit gene expression. Sox11 therefore appears to be required for regulation of genes that facilitate the RA-induced cell differentiation and survival in neuroblastoma cells.

The reduced number of differentiated cells and increased number of apoptotic cells that were found when RA and Sox11 siRNAs were added together (at 0hs) were not seen if siRNAs were added at 6h, 12h or 24h following RA stimulation. The level of Sox11 mRNA was unchanged as well. Why such a short window for effective Sox11 knockdown occurred in Neuro2a cultures is unclear. One possibility is that the increasing level of Sox11 mRNA that was induced by RA was too great to be effectively knocked-down by siRNA addition. Another possibility is that RA-induced differentiation altered the ability of Sox11 siRNAs to be processed efficiently in Neuro2A cells. The entry and processing of transfected siRNAs in RNA-induced silencing complexes (RISCs) may have changed following RA stimulation and as a result, inhibition of siRNA processing occurred. Precedence for inhibition of RISC activity is well documented in neuronal cells of *Caenorhabditis elegans*, which are refractory to RNA interference and resistant to siRNA modulation (Timmons, 2004). A similar mechanism of inhibition may underlie resistance to Sox11 RNAi modulation in Neuro2A cells that are RA-induced to express a neuronal phenotype.

To begin to identify target genes of Sox11 involved in Neuro2a survival and differentiation, tailored filter gene arrays containing cDNAs linked to apoptosis and survival were used. RNA samples from cultures treated with RA alone were compared to RNA isolated from cultures treated with both RA and siRNAs against Sox11. Comparison of probe binding intensities showed that expression of several genes changed in response to Sox11 knockdown. RT-PCR validation of some candidate Sox11 modulated genes, defined by having at least one Sox11 binding site within 2 Kb 5’ of the start codon, showed results consistent with the array

analysis. Genes of interest were TANK, BNIP3, Blk and Bcl10, all of which have the potential to influence entry into apoptosis. TANK, which was decreased following Sox11 knockdown, may have pro-survival activity and appears to regulate apoptosis through its synergistic activation of NFkB with TNF receptor-associated factor 2 (TRAF2). This activation occurs via the CD40 and tumor necrosis factor receptor 1 surface membrane receptors (Cheng and Baltimore, 1996). BNIP3, a pro-apoptotic gene regulated through nitric oxide signaling (Yook et al., 2004), was increased following Sox11 knockdown. Transfection of BNIP3 into MCF-7 and Rat-1 cells induced a rapid onset of apoptosis (Chen et al., 1999) whereas siRNA knockdown of BNIP-3 blocked cell death (Manka et al., 2005). TANK and BNIP-3 expression were both significantly changed after knockdown of Sox11 in RA-treated Neuro2a cells suggesting that Sox11 normally acts to transcriptionally regulate these genes in early phases of RA-induced differentiation. Consistent with this possibility is that the 5' regulatory regions of TANK and BNIP3 contain 4 Sox binding domains, compared with only one domain in the Blk and Bcl10 genes (Fig. 7B). Blk, a pro-apoptotic, mitochondrial membrane associated protein that interacts with the anti-apoptotic protein Bcl2 (Hegde et al., 1998), was increased on the gene array but further RT-PCR validation showed only a trend toward increased expression. Bcl10 was also unchanged on both gene array and RT-PCR analysis.

The requirement for Sox11 in Neuro2a differentiation and survival following RA stimulation parallels findings from other studies of Sox factors in cell lines induced to differentiate using RA stimulation. As previously mentioned, Sox6 was important for differentiation and neurite outgrowth in the RA-stimulated EC cell line P19 (Hamada-Kanazawa et al., 2004). Similarly, human EC cells induced to differentiate with RA showed regulation of Sox2 and Sox3 expression (Stevanovic, 2003). How each of the different Sox factors regulates

gene expression and entry into differentiation programs following RA-stimulation is yet to be defined. These studies do indicate however, that the Sox gene family has key roles in regulating genes underlying differentiation and cell survival.

4.0 DEVELOPMENTALLY REGULATED TRANSCRIPTION FACTOR SRY-BOX CONTAINING GENE 11 (SOX11) IS NECESSARY FOR SURVIVAL AND AXON OUTGROWTH OF PRIMARY DORSAL ROOT GANGLION NEURONS

4.1 INTRODUCTION

SRY-box containing gene 11 (Sox11) is known to be associated with neuronal maturation (Hargrave et al., 1997) and prevention of cell death during differentiation (Jankowski et al., 2006). Sox11 is upregulated during the stage of development that is associated with initial neurotrophin responsiveness and axon outgrowth of primary sensory neurons (Hargrave et al., 1997; Mirnics and Koerber, 1995; Oppenheim, 1991; Uwanogho, 1995) suggesting that it may play a role in how neurotrophins regulate sensory neuron survival and branching in the target of innervation. The upregulation of Sox11 occurs prior to the stage of rapid cell death in the dorsal root ganglion (Figueiredo et al. 2001; Pinon et al., 1996) and inhibition of Sox11 during differentiation of Neuro2A cells induces apoptosis (Jankowski et al. 2006). Sox11 was thus hypothesized to play a dual role during neuronal maturation; one which promotes differentiation and one which prevents programmed cell death *during* differentiation.

Several genes have been implicated in the injury response in adult mice after nerve damage. For example, activated transcription factor 3 (ATF3) is not normally present in adult DRG neurons; however, after nerve axotomy, ATF3 is upregulated and readily detectable in the

injured neurons (Lindwall et al., 2004; Tsujino et al., 2000) and after dissociation and culturing the cells (Seijfers et al., 2006). Changes in ATF3 expression were hypothesized to play a role in axon regeneration. After injury, not only do neurons need to induce axon regeneration machinery, but they must also prevent the induction of apoptotic pathways that may accompany cellular stress (Perrelet et al., 2002; Kutulska et al., 2005). Axotomy does not typically induce the expression of pro- or anti-apoptotic genes (Burnand et al. 2004), but survival of DRG neurons from injury has been linked to the activation of the NFkB pathway (Ferryhough et al., 2005) and JNK signaling (Keramaris et al. 2005). Sox11 appears to regulate multiple genes associated with apoptosis (Jankowski and Albers, 2006) and has also been linked to nerve injury (Jankowski et al. 2004; 2005). In this portion of study, we examine the role of Sox11 in axon growth and neuron survival in *adult* DRG.

Using primary DRG neurons as an *in vitro* model of axotomy (Jankowski et al., 2005; Seijfers et al., 2006), we found that Sox11 is significantly upregulated, similar to its upregulation following axotomy of the sciatic nerve (Jankowski et al., 2004; Chapter 1 and 5). Previous studies have show that this *in vitro* system displays similar transcriptional and growth features as axotomy (Seijfers et al., 2006; Smith and Skene, 1997). Over time in culture, sensory neurons extend axons and develop intricate neuronal networks. Penetratin-1 conjugated siRNAs (Davidson et al., 2001) directed against Sox11 blocked this outgrowth and caused some the DRG neurons to undergo apoptosis as evidenced by immunoreactivity to activated caspase3. The knockdown of Sox11 expression using siRNA transfection of primary DRG neurons also induced an increase in genes known to be associated with apoptosis as well as a decrease in genes known to regulate neurite growth and nerve regeneration.

4.2 MATERIALS AND METHODS

Primary neuron culture: Male C57/Bl6 mice approximately 2-3 months of age were anesthetized with 2.5% avertin and intracardially perfused with 35mL of Hank's balanced salt solution (HBSS; Gibco). DRGs were dissected away and placed into ice cold HBSS. DRGs were then incubated in 3mL of filter sterilized HBSS containing 1mg l-cysteine, 60U papain (Worthington) and 3μL of $NaHCO_3$ at 37^oC for 10 minutes. Solution was removed and 3mL of filter sterilized HBSS containing 12mg collagenase II (Worthington) was added to the DRGs and incubated for an additional 10 minutes at 37^oC. Collagenase solution was removed and DRGs were washed in 1-2 mL of F12 complete growth media (F12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin; Gibco). Media was removed and replaced with 1mL F12 complete media containing 50ng/mL of nerve growth factor (NGF; Harlan). DRGs were triturated 10-20 times with fire polished glass Pasteur pipettes. Cells were plated into six wells of a 12-well culture dish or onto sterile glass coverslips in a 2-well chamber slide coated with 1mg/mL poly-d-lysine/ 20mg/mL laminin in HBSS (Sigma). Cells were allowed to incubate for one to two hours at 37^oC/ 5%CO_2. Cells were then flooded with 1mL of F12 complete growth media containing NGF (50ng/mL) and incubated at 37^oC/ 5% CO_2 for the appropriate times.

Penetratin-1/ siRNA linkage: The use of Penetratin-1 linked siRNAs was used to enhance the transfection efficiency of adult primary neurons *in vitro* since adult neurons are not readily transfected and many transfection reagents can induce cell death (Davidson et al., 2001). Non-targeting and Sox11 targeting siRNAs were obtained from Dharmacon (Minnesota) containing a 5' thiol modification on the sense strands. The non-targeting siRNA duplex also was synthesized with a 5' CY3 molecule conjugated to the antisense strand. Activated Penetratin-1

(Q-Biogene) was reconstituted in sterile water and siRNAs were reconstituted in 1X siRNA buffer (Dharmacon). Equimolar concentrations of activated penetratin-1 were added to each of the reconstituted siRNAs and incubated at 65^{o}C for fifteen minutes followed by incubation at 37^{o}C for one hour. Stocks were brought up to 400nM NaCl to enhance solubility of penetratin linked siRNAs (Pen-siRNAs) and kept at -80^{o}C until used.

Penetratin-siRNA transfection of primary DRG neurons: Pen-siRNAs were heated to 65^{o}C for 15 minutes prior to DRG neuron transfections. When DRG neurons were flooded with F12 complete media containing NGF (see above), 500μL was removed and either penetratin-1 linked control (non-targeting; PenCON) and/ or penetratin-1 linked Sox11 (targeting; PenSox) siRNAs were added to the media that was removed. Pen-siRNAs were mixed by gentle pipetting and added back to the cultures dropwise as a 2X solution for a final concentration of 80nM siRNAs per well. Cells were then allowed to re-incubate at 37^{o}C/ 5% CO_2 for the appropriate times.

RNA Isolations: RNA isolation was performed using Qiagen RNeasy mini kit for animal cells or animal tissues. Briefly, for primary DRG neuron RNA isolation, media was completely removed from culture wells and cells were washed with 0.1M PBS. 400-500μL of buffer RLT containing 10μL/1mL of β-mercaptoethanol (β-ME) was added directly to the culture dish to lyse the cells. Cells were homogenized with the pipette tip and homogenate was added to an RNase-free tube. One volume of 70% ethanol in RNase-free water was added to the samples, mixed by pipetting and added to an RNeasy-Spin column. Samples were centrifuged at 10,500 rpm for 15 seconds then underwent several other centrifugations using buffers RW1 or RPE (Qiagen). RNA was

eluted from the column in 30-35 μL of RNase-free water. RNA concentrations were determined by obtaining A260 readings on a GeneQuant spectrometer.

RT-PCR/ Realtime PCR: Purified RNA was treated with DNase I (Invitrogen) for 15 minutes at room temperature and 1 μg of DNased RNA reverse transcribed in the following manner: 1 μL of random primers were annealed to the DNased RNA by incubating at 68^{o}C for 10 minutes followed by cooling on ice for 5 minutes. A mixture of 5X First Strand Synthesis buffer, 0.1M dithiothreitol, 25mM dNTPs, RNase inhibitor and sterile water was then added to make a 20 μl total volume. Samples were mixed, incubated at 37^{o}C for 2 min, 1 μL of Superscript II Reverse Transcriptase (Invitrogen) was added to each sample and they were incubated at 42^{o}C for 50 min. Samples were then incubated at 70^{o}C for 15 min, cooled on ice for 5 min. and then stored at -80^{o}C until used in PCR reactions. 200 ng of DNA was used in ^{32}P-dCTP spiked PCR reactions and products run on an 8% polyacrylamide gel at 400V, blotted onto Whatman 3MM paper, dried and exposed to a phosphorimager screen read using a BioRad phosphoimager. For realtime PCR, 20ng samples of cDNA were added to a SYBR Green MasterMix (Applied Biosystems) and run in triplicate on an Applied Biosystems Imager. Values were normalized to GAPDH or neuronal specific enolase (NSE). Changes in expression are reported as a ΔΔCt value that is calculated by subtracting the gene expression by the GAPDH/ NSE control for each sample and compared among samples. Fold change is described as $2^{\Delta\Delta Ct}$ (Applied Biosystems).

Immunocytochemistry: For cultured neurons, media was removed from cells at the designated times and rinsed in phosphate buffered saline (PBS). Cells were then fixed in 4% paraformaldehyde for 5 min and were then rinsed again in PBS. Cells were then blocked in

0.25% triton X-100 with 5% normal goat serum in PBS or 0.25% triton X-100 in PBS for 30 min. Incubation in rabbit cleaved caspase3 (casp3*; 1:100; Cell Signaling Technologies), goat Sox11 (1:8000; Aves Labs), mouse N52 (1:500; Sigma) or goat PGP 9.5 (1:500, Ultraclone) overnight at room was followed by washing in PBS and a 2h secondary antibody labeling (CY3 conjugated donkey anti rabbit; CY2-conjugated goat anti rabbit, goat anti mouse or donkey anti goat; FITC-conjugated donkey anti rabbit antibody; biotinylated donkey anti-goat (1:500 in PBS). Cells processed for immunofluorescence were rinsed in PBS, sections were mounted onto superfrost slides (VWR) and coverslipped in glycerol. For certain conditions, cells were incubated in 4,6-diamino-2-phenyl-indole, dihydrochloride (DAPI; 1:300 in PBS) for five minutes and rinsed again in PBS before being coverslipped. Images were taken on a Leica fluorescence microscope or an Olympus BH-2 microscope equipped with differential interference contrast (DIC) optics. Cells incubated in biotinylated secondary antibodies were rinsed in PBS and incubated in avidin biotin perixidase complex (ABC; 1:500 in PBS) for one hour. Cells were then rinsed again in PBS and underwent reaction in 3,3-diaminobenzidine in sterile water with H_2O_2 (0.03%) as a cofactor for an empirically determined mount of time. Cells were then rinsed in PBS, PB and sterile water. Cells were then dehydrated in an ethanol series and xylenes before being coverslipped in DPX (Fluka Biochemicals) with photoetched gridded coverslips (EMS). Images of cells were taken on a Leica DMRX microscope.

In vitro quantification of cell death and axon outgrowth: Cells grown on chamber slides were processed for either activated caspase3 or protein gene product (PGP) 9.5 immunocytochemical labeling as described above. 10-30 total PGP 9.5 stained cells per condition within gridlines of 2 randomly selected squares on opposite ends of the grid each 600 μm^2 using a photoetched

gridded coverslip (Electron Microscopy Sciences) at 20x magnification (n=3 per condition; 387 total cells) were quantified for neurite outgrowth using NIH Image software. Neurite outgrowth was quantified by measuring the total length of all primary neurites and obtaining an average length and average maximum length per neuron and counting the number of branch points per neurite. Branching index was then calculated as the number of branch points divided by the number of primary neurites on each neuron. Cells immunolabeled for casp3* were reported as a percentage of total cells within 4 randomly selected fields at 20x magnification (n=3 per condition; 2178 total cells) on the fluorescence microscope; a field approximately equilvalent to the fields analyzed using the photoetched coverslips with DIC optics.

4.3 RESULTS

4.3.1 Sox11 is expressed in primary DRG neurons undergoing neurite outgrowth.

Analysis of primary DRG neurons grown *in vitro* shows that Sox11 is upregulated over time as neurons extend neurites (Fig 10). mRNA levels of Sox11 are increased slightly (41%) at 3h and gradually increases 233% by four days *in vitro* relative to the mRNA level detected immediately after dissociation and plating (0h). The increase in Sox11 mRNA coincides with the rate at which primary neurites and higher order branches are formed on DRG neurons (Fig. 11). Neurite growth is readily detected in primary DRG neurons within 24h and length and branching continues to increase the longer neurons are allowed to grow *in vitro* (Fig. 11; Table 3). Because cultured DRG neurons can regenerate axons, and they upregulate Sox11 while doing so, we used DRG cultures as an axotomy model and analyzed the functional consequences of siRNA mediated knockdown of Sox11 on survival and neurite growth. Since adult primary DRG neurons are difficult to transfect, we used siRNAs directed against Sox11 that were conjugated to the lipophillic peptide Penetratin-1. Penetratin-mediated delivery of siRNAs greatly enhances transfection efficiency of primary DRG neurons from 1% to 90% *in vitro* (Fig. 12; Davidson et al., 2001).

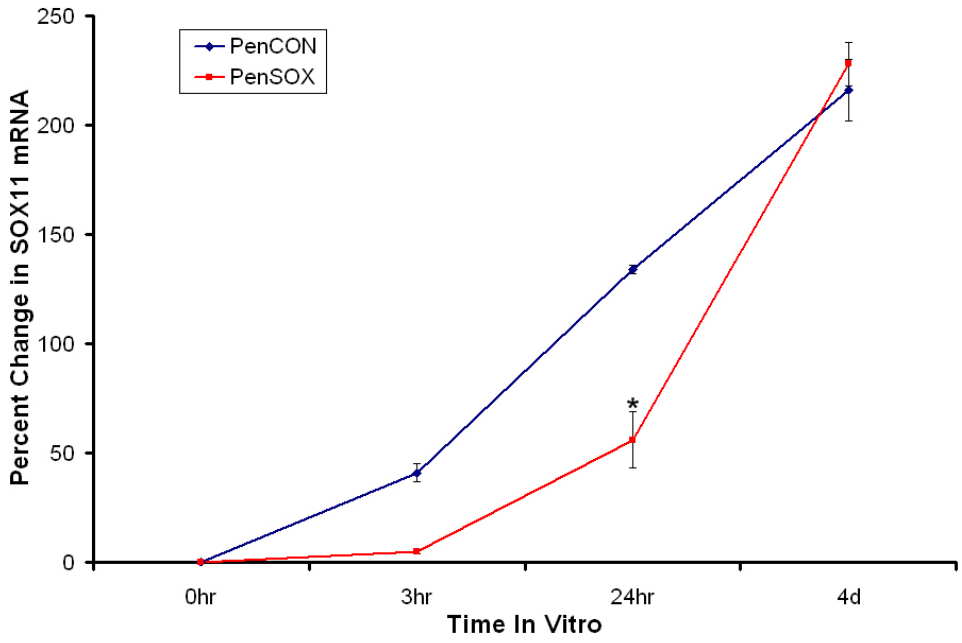

Fig. 10: Percent change in expression of Sox11 mRNA in primary DRG neurons *in vitro* transfected with control (PenCON) or Sox11 (PenSOX) siRNAs. In PenCON transfected cultures, Sox11 mRNA gradually increases over time (blue line). In PenSOX transfected DRG cultures however, Sox11 mRNA is inhibited for at least 24h and does not return to normal levels until four days *in vitro* (red line). * p value < 0.05, relative to controls.

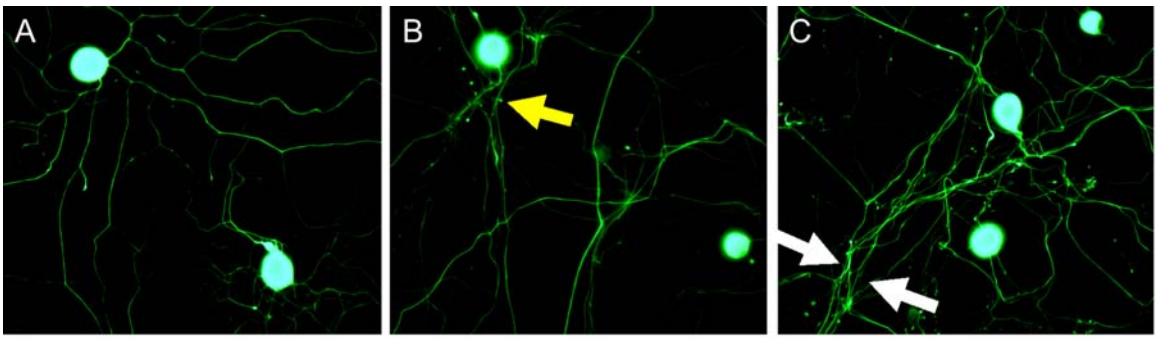

Fig. 11: Light micrographs of primary DRG neurons at 1, 4 and 7 DIV immunoreactive for neurofilament. Panel A shows neurons cultured for 24 hours. Neurite branching is typically in all directions from the cell body. Panel B shows neurons cultured for 4 days. Neurites are beginning to be grouped together (facing arrows) and punctuate labeling suggests synaptic incidence (yellow arrow). Panel C shows primary neurons grown in culture for seven days. Cells are increasingly more interconnected and bundled into groups of neurites (facing arrows).

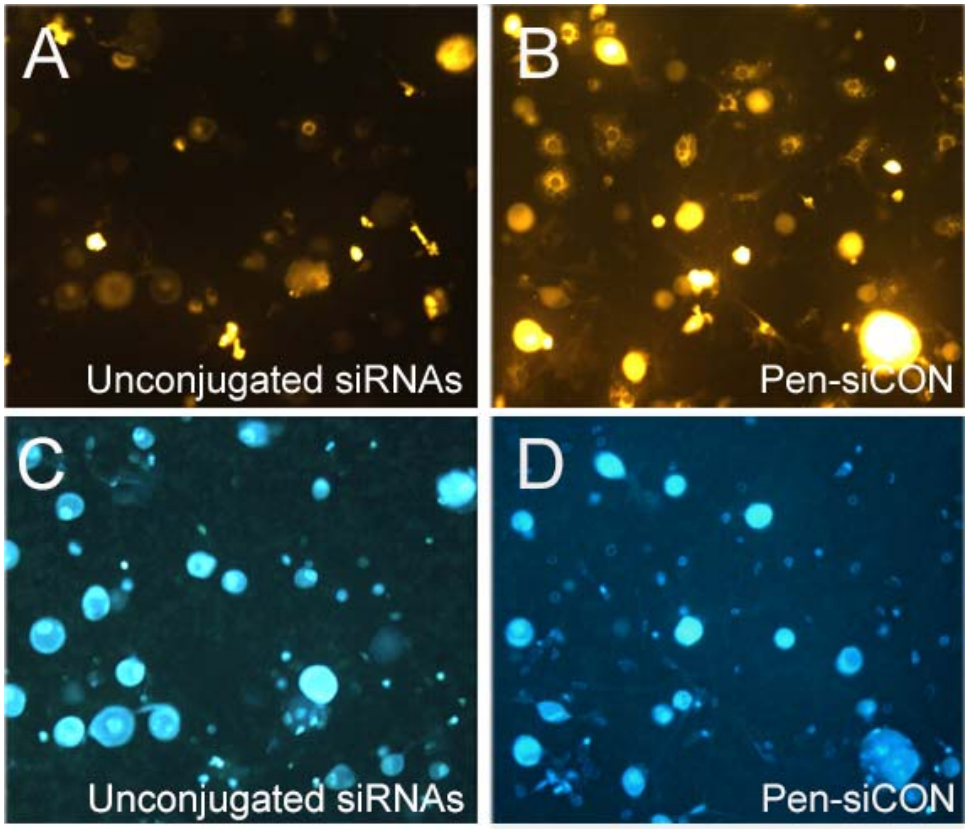

Fig. 12: Light micrographs of primary DRG neurons transfected with siRNAs that are linked to a CY3 tag that were unconjugated (A) or conjugated to Penetratin-1 (B). Transfection of adult primary DRG neurons with siRNAs not conjugated to penetratin-1 results in low transfection efficiency (A), whereas conjugation to penetratin-1 greatly increases the transfection efficiency (B). Panels C and D show labeling of cultures with the nuclear stain DAPI.

Transfection of primary DRG neurons with penetratin linked siRNAs directed against Sox11 (Pen-SOX siRNAs) caused a transient but significant knockdown of Sox11 mRNA. Knockdown of Sox11 mRNA lasted at least 24h and returned to control levels (PenCON siRNAs) by four days (Fig. 10). Immunocytochemical localization of Sox11 shows that PenCON transfected DRG neurons had heavy nuclear and low to moderate cytoplasmic staining for Sox11 protein (not shown) whereas PenSOX transfected DRG neurons showed minimal cytoplasmic staining for Sox11 protein and no nuclear staining (Fig. 13). Initial knockdown in Sox11 protein could be detected as early as 3hr in a small percentage of cells (not shown). The largest knockdown in protein expression was detected at 24h (Fig. 13). After four to seven days *in vitro* (DIV), Sox11 staining returned to normal levels (not shown). To confirm that these findings were specific to neurons and not contaminating glial cells, primary DRG cultures were colabeled with antibodies to Sox11 and NeuN (a neuronal marker) and DAPI, a nuclear dye that stains all nuclei. This analysis showed that only NeuN positive neurons were positive for Sox11 (Fig. 14). Non neuronal cells which were NeuN negative and DAPI positive did not label with anti-Sox11 (Fig. 14). Given the specificity of the Sox11 siRNAs (see above), the effects of knocking down Sox11 in DRG cultures is highly likely to be specific to neurons.

4.3.2 The increased expression of Sox11 is essential for neurite outgrowth.

Since neurite outgrowth correlated to the increase in Sox11 expression in primary DRG neurons, we analyzed the functional consequences of Sox11 knockdown on outgrowth by measuring neurite length and branching in PenSOX transfected DRG neurons. At 24h, PenSOX transfected DRG neurons showed no significant deficit in neurite length; however, these cells

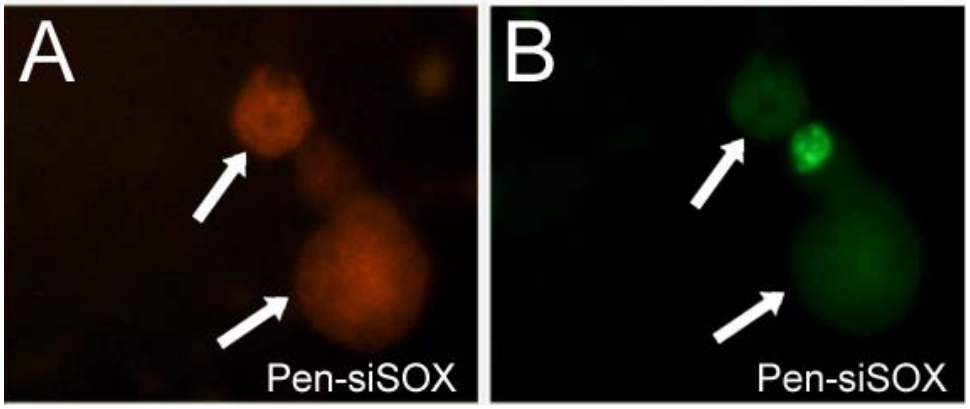

Fig. 13: Light micrographs of primary DRG neurons transfected with both penetratin-1 conjugated, CY3-tagged control siRNAs and Sox11 siRNAs (Pen-siSOX). 24h after transfection, cultures were immunolabeled for Sox11. In transfected cells (Panel A arrows; red), no Sox11 immunolabeling is present (Panel B arrows). Only non-transfected cells show labeling for Sox11 protein (B; green labeled nuclei).

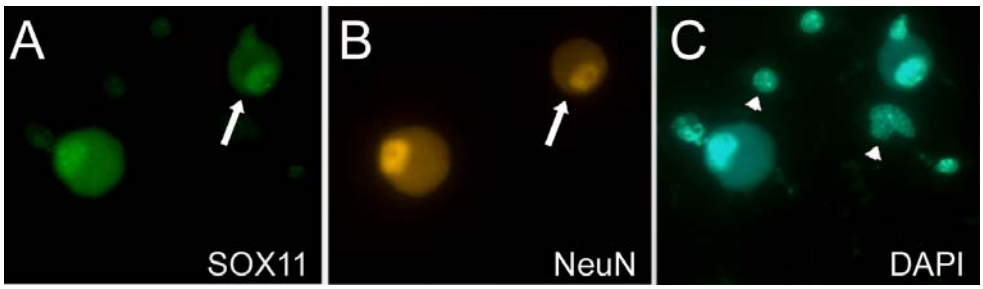

Fig. 14: Light micrographs of primary DRG cultures immunolabeled for Sox11 and NeuN and stained for DAPI. Sox11 positive cells (Panel A; arrow) were found to be neuronal (Panel B; arrow) and not non-neuronal (Panel C; arrowheads).

had a significant decrease in branching compared to PenCON transfected cells (Table 3).

After four DIV, PenSOX transfected cells did exhibit a significant decrease in neurite length compared to control transfected neurons at 4d and a continued deficit in branching (Fig. 15; Table 3). To determine if all or some of the primary neurites were affected by Sox11 knockdown, we analyzed maximum neurite length in PenSOX transfected DRG neurons. The average length of the longest primary neurite was not significantly different between PenCON and PenSOX treated DRG neurons at 24h but in PenSOX treated cultures it was statistically decreased by 4d (Fig. 15; Table 3).

To determine if particular populations of neurons were differentiatlly affected by Sox11 knockdown, we analyzed neurite growth in different cell sizes. Average and maximum neurite length was not affected in any population of sensory neurons after Sox11 knockdown at 24h (Fig. 16a and e); however, there was a statistically significant reduction in branching index in the smallest and largest diameter cells (Fig. 16c). Medium diameter cells were less affected by Sox11 inhibition at 24h. After four days *in vitro*, the smallest and largest cells still retained a significant reduction in average and maximum neurite length; however, some neurons of medium diameter exhibited neurite growth deficits in the absence of Sox11 (Fig. 16b and f). Surprisingly, after four DIV, nearly all neuron size classes in PenSOX treated cultures had deficits in branching with the exception of cells in the 20-24 and 28 to 32 μm diameter bins (Fig. 16d).

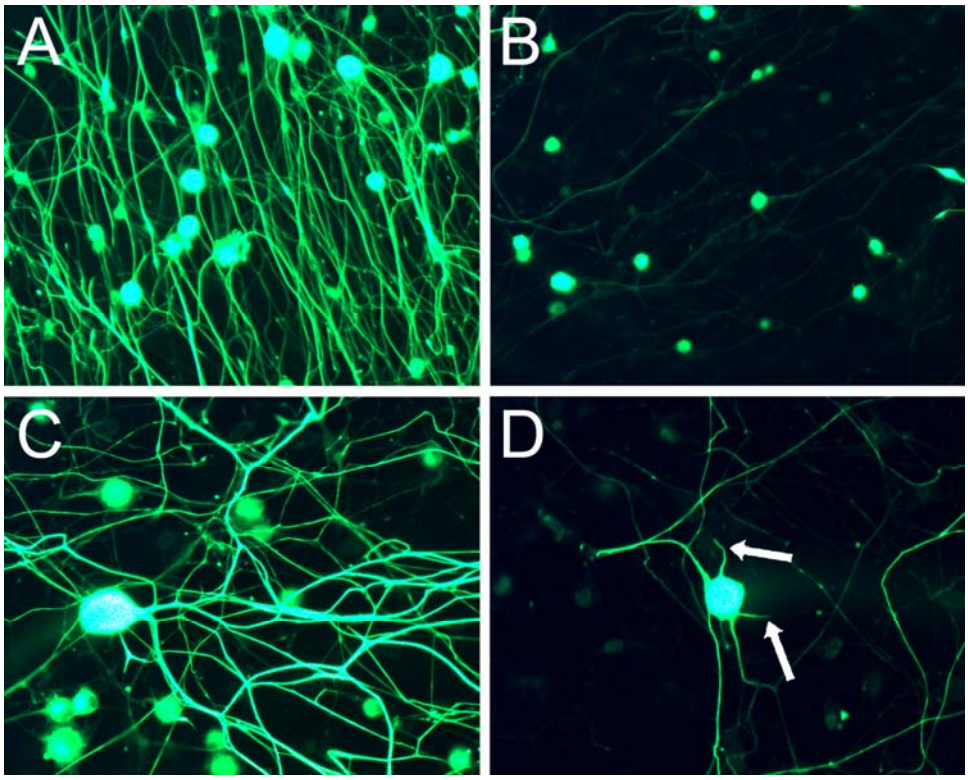

Fig. 15: Light micrographs of primary DRG neurons transfected with control (Pen-siCON) and/or Sox11 targeting (Pen-siSOX) siRNAs immunoreactive for neurofilament 200 (N52). At four days, Pen-siCON transfected neurons display longer neurites and enhanced branching (panels A and C) while Pen-siSOX transfected neurons do not show enhanced neurite lengthening or branching (panels B and D). In fact, many primary neurites are visibly shorter in Pen-siSOX treated cultures than in control cutltures (arrows).

Table 3: Quantification of Neurite Outgrowth and Cleaved Caspase3* Immunoreactivity in Primary DRG Neurons Transfected with either PenCON or PenSOX siRNAs

	PenCON 24h	PenSOX 24h	PenCON 4d	PenSOX 4d
Average Length (μm)	82.12+/- 12.1	73.41+/- 10.7	112.98+/- 7.7**	80.2+/- 8.7*
Branching Index (BI)	1.78+/- 0.18	1.01+/- 0.14*	2.21+/- 0.32#	0.92+/- 0.12*
Average Maximum Length (μm)	122.47+/- 15.1	105.29+/- 16.2	153.48+/- 10.9#	108.62+/- 11.9*
Percent Caspase3* Positive Cells	11% (n=535)	20% (n=633)*	10% (n=507)	20% (n=503)*

* p value < 0.03, relative to time matched controls
** p value < 0.03, relative to 24h controls
# p value <0.07, relative to 24h controls

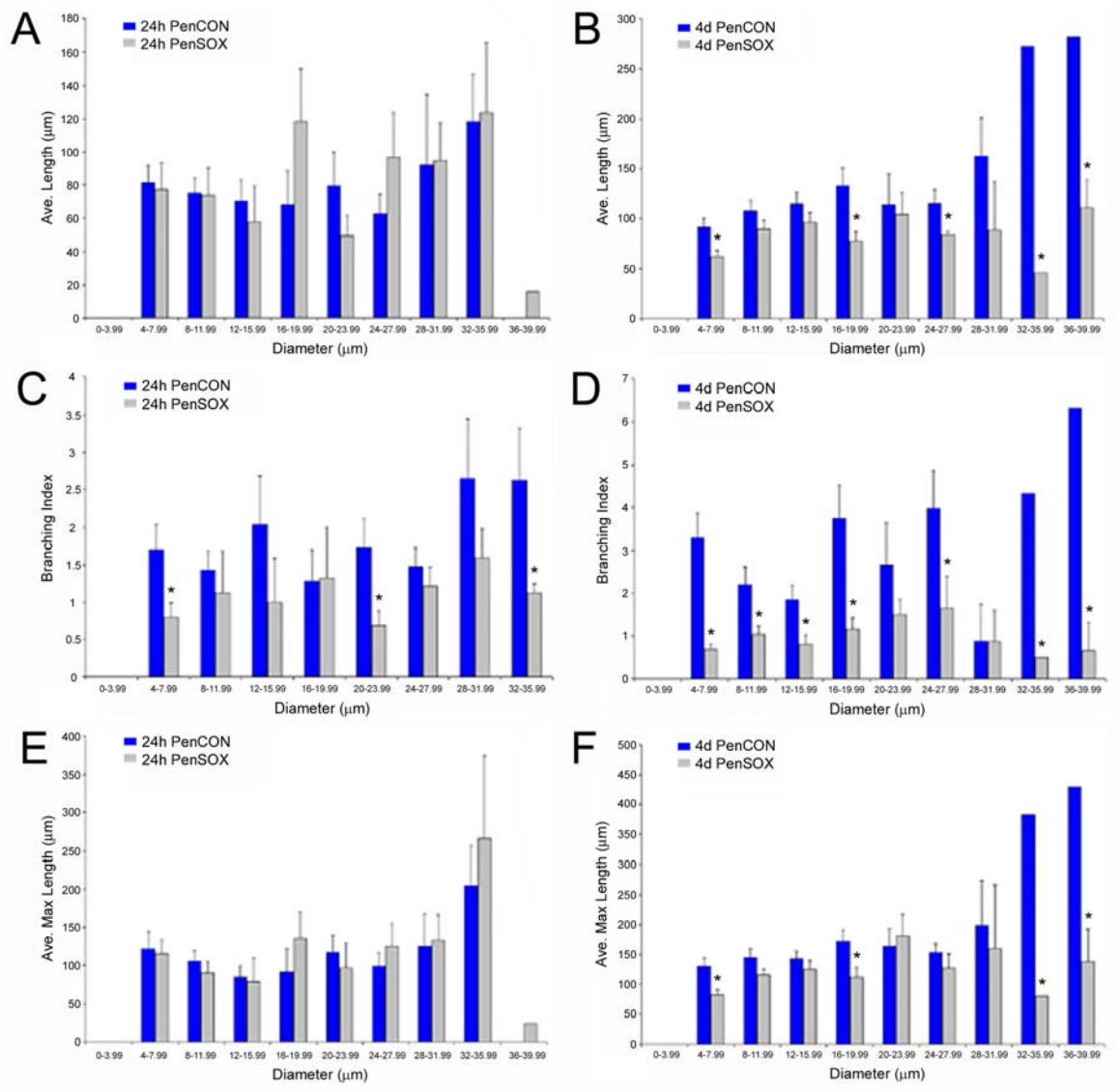

Fig 16: Cell size histogram of neurite outgrowth in primary DRG neurons transfected with control (PenCON) or Sox11 targeting (PenSOX) siRNAs. There were no detectable differences in PenSOX treated cells compared to PenCON at 24h for average length or maximum length; however, a few groups of cells had a significant reduction in branching. At 4d however, several groups of cells had problems with neurite lengths (average and maxium) while almost all groups displayed deficits in branching indices. * p value < 0.05, relative to controls.

4.3.3 Primary DRG neurons deprived of Sox11 undergo apoptotic cell death.

Our data thus far suggested that Sox11 is important for neurite growth and nerve regeneration. However, it was also noticed that the morphology of some of the PenSOX transfected cells appeared apoptotic as evidenced by shrunken cell membranes and disrupted nuclei (not shown). We therefore quantified the percentage of PenSOX treated DRG cells that expressed activated caspase 3 (casp3*), a marker of apoptosis. Transfection of DRG cultures with PenCON siRNAs caused 11% (59/535 cells) of neurons to express casp3* immunoreactivity at 24h. In contrast, DRG cultures trasnfected with PenSOX siRNAs showed 20% (127/633 cells) of the cells were immunopositive for casp3*. At fourDIV, the number of PenCON treated cells decreased to 10% (51/507 cells) but the percentage of PenSOX transfected DRG cells immunoreactive for casp3* remained at 20% (101/503 cells; Fig. 17; Table 3). Because Sox11 is found to be exclusively localized to neurons (Fig. 14), inhibition of Sox11 with siRNAs in DRG cultures should only directly affect apoptosis in these cells. Although we cannot rule out an indirect affect of Sox11 inhibition in neurons on glial cell apoptosis in these experiments, closer examination of cultures immunostained for casp3*, NeuN and DAPI showed that activated caspase3 immunoreactive cells in primary DRG cultures were indeed neuronal (Fig. 18). Therefore, we are confident that the quantification of casp3* positive cells in primary DRG cultures treated with siRNAs were an accurate representation of *neuronal* cell death.

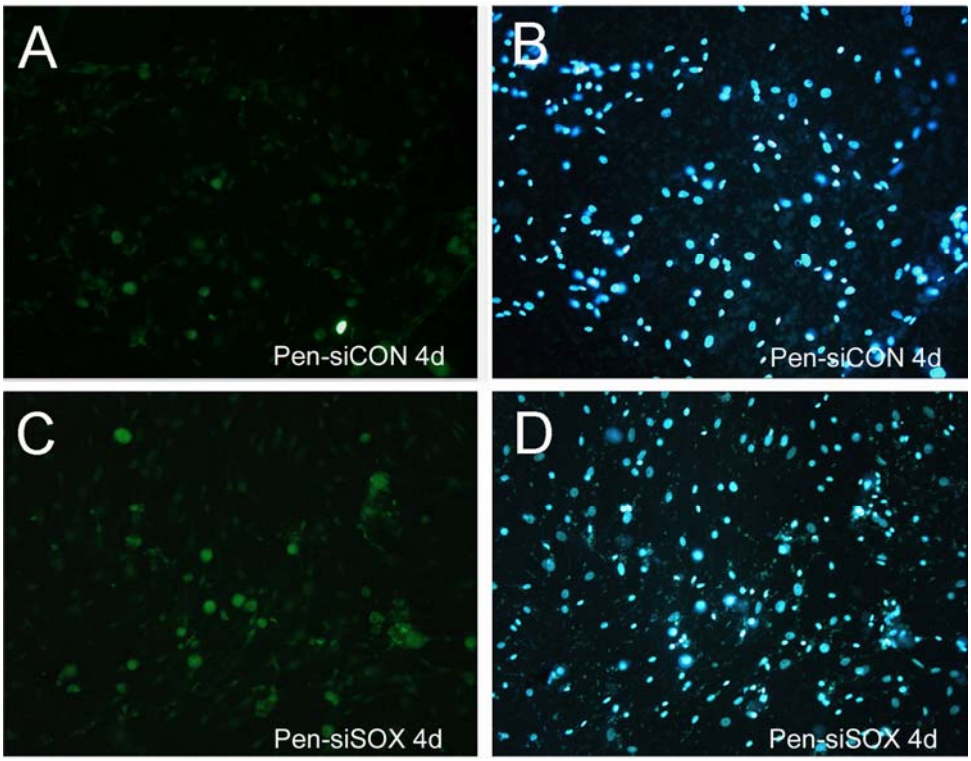

Fig. 17: Immunocytochemical localization of activated caspase3 (casp3*) in primary DRG cells transfected with either control (Pen-siCON) and/or Sox11 targeting (Pen-siSOX) siRNAs and stained for DAPI. At four days *in vitro*, Pen-siCON transfected cells show minimal immunoreactivity for casp3* (panel A) whereas Pen-siSOX transfected cultures show enhanced casp3* immunolabeling (panel C). DAPI staining was performed to show presence of all cells in the fields (panels B and D).

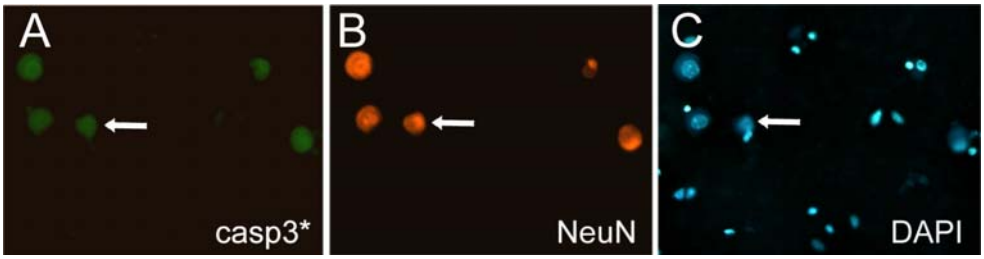

Fig. 18: Light micrographs of primary DRG neurons transfected with Sox11 targeting (PenSOX) siRNAs immunolabeled for activated caspase 3 (casp3*), neuronal specific nuclear protein (NeuN) and nuclear stain, DAPI at 24h *in vitro*. Immunolabeling of PenSOX transfected DRG cultures show that casp3* positive cells (panel A) were neuronal (panel B) and not non-neuronal (panel C; arrows).

4.3.4 Knockdown of Sox11 alters the expression of genes associated with neurite outgrowth and apoptosis.

Because knockdown of Sox11 in primary DRG neurons *in vitro* causes deficits in neurite growth as well as induction of apoptosis in some cells, we sought to determine the mechanism by which Sox11 might be mediating these changes. To determine if known outgrowth associated genes were differentially regulated by Sox11 knockdown, we analyzed the expression of brain derived neurotrophic factor (BDNF), small proline rich protein 1a (Sprr1a), profilin, actin related protein 2/3 complex subunits 3 and 4 (Arpc3 and 4), and activated transcription factor 3 (ATF3) in DRG neurons transfected with PenSOX siRNAs over time. Each of these genes has multiple Sox11 binding sites in the upstream promoter region. BDNF (2 binding sites with in 0.5kb), Arpc4 (4 sites within 2kb) and profilin (2 sites within 2kb) were not significantly altered by Sox11 knockdown at any timepoint analyzed (not shown). However, Sprr1a (3 sites within 2kb) was increased, and Arpc3 (3 sites within 2kb) and ATF3 (2 sites within 2kb) were decreased by Sox11 inhibition at 3h and 24h and returned to control levels by 4d (Table 4).

We then used tailored realtime PCR profiler gene arrays to simultaneously analyze the expression of 88 genes involved in the process of apoptosis in DRG neurons treated with PenSOX siRNAs at 24h We chose to analyze expression at the 24h timepoint since the induction of caps3* immunoreactivity first appeared at this time and lasted for several days thereafter. Twenty-seven apoptosis associated genes were significantly changed as a result of sox11 knockdown. Of these, 5 contained Sox11 binding sites in their upstream promoter regions

suggesting that Sox11 may directly regulate their expression and were therefore analyzed using RT-PCR (Table 4). Traf member and activator of NfkB, or TANK (5 sites within 2kb) was initially decreased by lack of Sox11 but surpassed control levels by four days *in vitro*. Traf1 (3 sites within 2kb) showed a trend toward increased expression as a result of Sox11 inhibition at all time points tested but was not statistically significant. BH3-interacting domain death agonist, or Bid (5 sites within 3kb) was decreased in PenSOX treated cultures at 3hr, but was increased at 24h and 4d although also only showed a trend toward statistical significance (Table 4).

Table 4: Realtime PCR analysis of neurite growth and apoptosis associated genes in primary DRG neurons

Gene Assayed	Percent Change Following SOX11 Knockdown		
	3h	*24h*	*4d*
PenCON- ATF3	156 +/- 7.0	280 +/- 1.0	346 +/- 13.0
PenSOX- ATF3	105 +/- 3.0	220 +/- 2.0**	357 +/- 7.0
PenCON- Arpc3	21 +/- 0.01	123 +/- 0.03	187 +/- 0.1
PenSOX- Arpc3	14 +/- 0.01	28 +/- 0.04*	253 +/- 0.1
PenCON- Sprr1a	674 +/- 0.1	3603 +/- 0.3	5122 +/- 0.8
PenSOX- Sprr1a	916 +/- 0.2	6679 +/- 0.1	4935 +/- 0.4
PenCON- Traf1	4 +/- 1.0	17 +/- 1.1	29 +/- 6.8
PenSOX- Traf1	12 +/- 0.4	20 +/- 5.0	53 +/- 4.5
PenCON- Bid	-11 +/- 7.1	-40 +/- 2.7	16 +/- 1.3
PenSOX- Bid	-40 +/- 4.3	-12 +/- 5.2	42 +/- 0.6
PenCON- TANK	-32 +/- 0.03	69 +/- 0.14	204 +/- 5
PenSOX- TANK	-131 +/- 0.003**	-7 +/- 0.06	430 +/- 2

* p value < 0.05; ** p value < 0.07

4.4 DISCUSSION

The data described herein show that the developmentally regulated Sox11 transcription factor has an important role in survival and neurite growth in primary DRG neurons. Our previous study showed that Sox11 was necessary for Neuro2A survival and differentiation in response to retinoic acid application (Jankowski et al. 2006). A similar mechanism may function in cultured adult primary DRG neurons with reduced levels of Sox11. Inhibition of Sox11 in primary DRG neurons induces some cells to undergo apoptosis and express activated capsase3 immunoreactivity. These neurons also displayed abnormalities in neurite growth and branching suggesting a role for Sox11 in peripheral nerve regeneration.

4.4.1 Sox11 is Involved in Neuronal Survival and Differentiation.

In this study we have shown that Sox11 is an important regulator of neurite growth and branching in primary DRG neurons and neurons with reduced levels of Sox11 are more susceptible to apoptosis. Primary DRG neurons grown *in vitro* enhance their levels of Sox11 mRNA in relation to the extent of neurite growth and branching. Using Penetratin-1 linked siRNAs directed against Sox11, we have shown that primary DRG neurons are readily transfected with high efficiency and display a transient but effective knockdown of Sox11 mRNA and protein. A near complete knockdown of Sox11 in primary DRG neurons does not occur until 24hrs which may explain the lack of a significant deficit in neurite extension at the

24h timepoint. However, at 4 DIV, neurite growth is clearly inhibited in cells transfected with PenSOX siRNAs relative to PenCON transfected neurons. Control DRG neurons continually grow longer neurites *in vitro* whereas PenSOX transfected cells halt their neurite extension suggesting that Sox11 is necessary for continued neurite elongation and outgrowth. In contrast to neurite length analysis, branching indices indicate that higher order branching of primary neurites is affected immediately upon inhibition of Sox11. The BI is decreased in PenSOX transfected neurons with in 24h of transfection compared to PenCON transfected neurons. BI is also halted for several days, similar to what is shown with average neurite length. These features of neurite growth appear to affect all primary neurites extending from the somas since maximum neurite length is affected to the same degree by PenSOX siRNA transfection as average neurite length.

This study also showed that 20% of DRG neurons undergo apoptosis as a result of Sox11 inhibition similar to previous studies (Jankowski et al. 2005; 2006). It is unclear if the population of cells that display outgrowth deficits are the same as those that are casp3* immunopositive. Analysis of cells that were processed for neurite outgrowth measurements suggests that more cells display neurite extention and branching deficits than those that are casp3* positive (not shown). This would suggest that neurite growth is affected in all cells by Sox11 inhibition whereas only a small population of those cells will undergo programmed cell death. It is not clear if the degree of Sox11 inhibition or some other factor contributes to cell death in neurons that lack sufficient levels of Sox11. To address this, future experiments can determine if the degree of Sox11 inhibition in individual cells correlates with expression of apoptotic markers.

4.4.2 Sox11 Regulates the Expression of Genes Associated with Neurite Outgrowth and Apoptosis.

Microarray analysis of cultures treated with Sox11 siRNA showed that knockdown of Sox11 in DRG neurons modulated expression of genes involved in neurite extension and programmed cell death. Genes that appeared to be modulated on arrays were selected for further RT-PCR validation based on whether their proximal regions (2-3Kbp of the transcriptional start site) contained Sox consensus binding domains. The presence of Sox binding sites greatly enhances the probability that these genes are activated/ repressed by Sox11.

Sprr1a is upregulated in the brain after trauma (Marklund et al., 2005) and is known to promote neurite outgrowth of primary DRG neurons and enhance peripheral nerve regeneration (Bonilla et al., 2002). Like many genes in Sox11 siRNA treated DRG cultures, the change in Sprr1a expression was dynamic, with no change early on, upregulation after 24h and return to control levels by four DIV. Sprr1a has 3 Sox consensus sites in its proximal promoter allowing for the possibility that Sox11 contributes to its transcriptional regulation. Little is known about Arpc3 and its role in neurite growth. It is known that Arpc3 is present at the site of active actin filament assembly (Welch et al., 1997) and necessary for actin filament branching (Volkmann et al., 2001). Sox11 significantly regulates the expression of Arpc3 in primary DRG neurons suggesting it may have a role in neurite growth. The transcription factor ATF3 is known to be associated with neurite sprouting (Pearson et al., 2003) and is induced in cells upon injury (Campbell et al., 2003; Isacsson et al., 2005; Lindwall et al., 2003; Tsujino et al., 2000). ATF3

mRNA was highly regulated by the level of Sox11 in primary DRG neurons. ATF3 has two Sox binding sites in its proximal promoter and could therefore be modulated by Sox11 expression. Although ATF3 has Sox binding sites, no ATF3 binding sites are present in the proximal promoter of Sox11 suggesting ATF3 does not regulate Sox11.

Sox11 knockdown also alters expression of genes associated with survival and apoptosis. Traf1 is a mediator of cell death that interacts with tumor necrosis factor receptors (Lee and Lee, 2002) and regulates of c-Jun N-terminal kinase (JNK) and members of the NfkB signaling pathway (Cha et al., 2003). Traf1 has 3 Sox binding sites and Sox11 knockdown enhances Traf1 expression at all time points tested. Thus, Sox11 may have direct control over the transcription of this mediator of apoptosis. TANK was reduced following siRNA inhibition of Sox11 (Jankowski et al., 2006), and appears to be a pro-survival gene that interacts with NfkB signaling pathways (Cheng and Baltimore, 2003). TANK has five Sox binding sites within 2 kb of the transcriptional start site and is another candidate for direct regulation by Sox11. Although these data suggest that Sox11 can regulate the expression of genes associated with neurite growth and apoptosis, reporter assays and chromatin binding studies to show direct interaction at the consensus sequence will be necessary to confirm these interactions.

4.4.3 Conclusions.

The results from these studies parallel previous findings using the neuroblastoma cell line Neuro2A and indicate that Sox11 regulates transcription of genes involved in control of apoptosis and neurite growth in adult sensory neurons *in vitro*. Knockdown of Sox11 in DRG

neurons by transfection of Penetratin-linked siRNAs induced a two-fold increase in the number of apoptotic cells and an arrest in neurite growth that lasted for several days. Potential downstream targets of Sox11 action in relation to apoptosis include the TANK and Traf1 genes, both of which are linked to cell survival. Sox11 also appears to play a role in axon growth, possibly through its regulation of pro-neurite growth genes like ATF3, Sprr1a and Arpc3. Given these outcomes, we continued our studies to determine if Sox11 has a role in adult injury responses through promotion of nerve regeneration *in vivo*.

5.0 SOX11 IS REQUIRED FOR EXPRESION OF ATF3 AND NERVE REGENERATION *IN VIVO*.

5.1 INTRODUCTION

In adult mammals, mechanisms of repair and survival that are stimulated following nerve injury in DRG neurons may in some ways reactivate those stimulated during embryonic development (Bahr, 2000; Silver, 1994). Several genes have been implicated in the injury response in adult mice after nerve damage. Activating transcription factor 3 (ATF3) in particular is not normally present in adult DRG neurons; however, after nerve axotomy, this transcription factor is upregulated and readily detectable in the injured neurons (Tsujino et al., 2000). ATF3 has not been specifically linked to development of sensory neurons, but it has been linked to pro-apoptotic and survival functions depending on the tissue type (Fan et al., 2002; Hai and Hartman, 2001; Hartman et al., 2004). In the nervous system, it appears to be associated with survival and/ or neurite outgrowth (Lindwall and Kanje, 2005; Seijfers et al., 2006). Many developmentally regulated genes have also been shown to play a role in adult mice after peripheral nerve injuries, e.g., c-jun (Lindwall et al., 2004; Pearson et al., 2003) and extracellular signal related protein kinase (ERK; Chierzi et al. 2005; Doya et al., 2005; Obata et al. 2004). Although each of these genes plays an essential role in the injury response, it is unclear whether these genes are necessary for nerve regeneration *in vivo*.

The mechanisms of regenerating peripheral and central axons are different in many aspects. Differences mainly lie in the presence of inhibitory enzymes in the CNS that are either not present or inhibited in the periphery (Kury et al., 2001; Qiu et al., 2001) and a lack of transcriptional mechanisms that are normally induced in the PNS that are not induced in the CNS (Kenny and Koscis, 1997). Once these mechanisms are initiated, several events occur to regenerate peripheral axons. After injury to a nerve, a period of "dying back" from the site of injury and initiation of Wallerian Degeneration occurs during the first several days (Baba et al., 1982; Mi et al., 2005; Sebille, 1982; Thomas, 1982). Wallerian Degeneration (WD) is a response that occurs upon injury to peripheral nerves that serves to degenerate the injured axons and clear a path for the axons to regenerate. Studies have shown that WD involves many processes including macrophage infiltration, glial cell proliferation, and ubiquitination followed by transcription of pro-regeneration genes.

For DRG neurons to undergo successful regeneration, they must induce anti-apoptotic mechanisms to avoid neuron death following injury. Several pro- and anti-apoptotic genes are differentially regulated in the DRGs after axotomy (Costigan et al., 2002) and many are not changed (Burnand et al., 2004). The transcription factor Sox11 has been shown to regulate anti-apoptotic mechanisms in primary DRG cultures and neurite growth (Jankowski et al., 2006). The dual function of this developmentally regulated transcription factor makes it an attractive candidate to be a master regulator of the adult injury response. In the current study, we further investigate the role of Sox11 in DRG axon regeneration and neuron survival in adult mice after nerve injury.

The effects of Sox11 knockdown *in vitro* prompted us to analyze its effects *in vivo* following saphenous nerve axotomy or crush. The saphenous nerve injury model was chosen due to the simplicity of surgery and the fact that the axonal composition of the saphenous nerve is well characterized. The saphenous nerve extends from lumbar DRGs L2 and L3 in the mouse and is a purely cutaneous nerve that is comprised of 80% unmyelinated small and medium diameter fibers and 20% myelinated axons (Baron et al. 1988; Stucky et al. 1999). These axons innervate hairy skin from the mouse knee to regions on the foot extending to the toes (Kitao et al., 2003; Koltzenberg et al., 2000). Saphenous nerve crush was found to induce a similar level of Sox11 mRNA elevation in the L2/L3 DRGs as previously shown after saphenous or sciatic nerve axotomy (Jankowski et al., 2004; 2005). Because nerve crush is an injury that has a more consistent regeneration profile than nerve cut we chose to use this approach. We found that Penetratin-1 linked Sox11 siRNAs injected into the saphenous nerve were retrogradely transported to the cell bodies and induced a knockdown in Sox11 mRNA expression. Targeted siRNA injection into the saphenous nerve prior to axotomy or nerve crush also induced a knockdown in the pro-regeneration gene ATF3 and decreased the extent of regeneration compared to control injected and crushed nerves. These data suggest that Sox11, a developmentally regulated transcription factor associated with neuronal maturation and survival, is an important regulator of events that follow peripheral nerve injury and allow nerve regeneration.

5.2 MATERIALS AND METHODS

Sciatic/ Saphenous nerve axotomy/ crush and siRNA injections: Male Swiss Webster mice (Hilltop) approximately 4-6 weeks of age were anesthetized by a mixture of ketamine and xylazine (90 mg/kg and 10 mg/kg, respectively) for saphenous nerve injury. A small incision was made in the groin area over the saphenous nerve. The nerve was cleaned of connective tissue, transected and wounds closed using 7.0 silk sutures. Sciatic nerve axotomized mice were placed in an enclosed chamber for initial anesthetization by isofluorane and then fitted into a facemask to receive isofluorane throughout the procedure. The animal was closely shaved on the left side, the skin wiped with betadine and an incision made at the lumbar level. A mini-Goldstein retractor (Fine Science Tools, Foster City, CA) was inserted into the incision and opened, providing an area in which to work. Once identified, the sciatic nerve was tightly ligated with 6.0 silk sutures and transected distal to the suture. The muscles were then sutured and the wound was closed with microclips (Roboz, Gaithersburg, MD).

Nerve crush was performed using number 5 forceps (Fine Science Tools, Forester City, CA) held together for approximately 4-5 seconds. Incisions were closed using 2.0 silk sutures. In animals that received siRNA injection, 0.3-0.4μL of 90μM Penetratin-1 linked control (PenCON) or Sox11 targeting (Pen-SOX) siRNAs were pressure injected directly into the saphenous nerve using a glass microelectrode connected to a pico-spritzer. All nerve cuts/

crushes were made distal to the injection site approximately 1-2mm without cleaning the surrounding area and without damage to any blood vessels. Animals were allowed to survive for 2-28 days after nerve injury for immunocytochemical, electron microscopic and/ or PCR analysis.

RNA Isolations: All RNA isolations were performed using Qiagen RNeasy mini kit for animal tissues. For fresh DRGs RNA isolation, animals were anesthetized with ketamine/ xylazine and intracardially perfused with ice cold 0.9% NaCl. Ganglia were removed and placed in RNase-free tubes on dry ice. Then 500μL of β-ME containing RLT buffer was added to the ganglia and homogenized with a polytron generator for 1 minute. Homogenates were centrifuged at 14,000 rpm for three (3) minutes and supernatant was transferred to an RNase-free tube. One volume of 70% ethanol in RNase-free water was added to the samples, mixed by pipetting and added to an RNeasy-Spin column. Samples were centrifuged at 10,500 rpm for 15 seconds then underwent several other 15sec to 2min centrifugations using buffers RW1 or RPE (Qiagen). RNA was eluted from the column in 35 μL of RNase-free water. RNA concentrations were determined by obtaining A260 readings on a GeneQuant spectrometer. RT reactions and realtime PCR were performed as described previously (Chapter Three; Jankowski and Albers, 2006).

Immunocytochemistry: Animals were anesthetized with a lethal dose of ketamine/ xylazine and intracardially perfused with 4% paraformaldehyde. Ganglia were then postfixed in 4% paraformaldehyde for 1 hour and rinsed in PBS. Ganglia were removed and embedded in 10% gelatin in 0.1M PB. Sections were cut at 50μm and collected in 0.1M PBS. Sections were rinsed 3x in 0.1M PBS and then blocked in 0.25% triton X-100 with 5% normal goat serum in PBS for

30 min. Incubation in rabbit cleaved caspase3 (casp3*; 1:100; Cell Signaling Technologies), goat Sox11 (1:100; Santa Cruz), or rabbit ATF3 (1:500, Santa Cruz) overnight at room was followed by washing in PBS and a 2h secondary antibody labeling (CY3 conjugated goat anti rabbit; CY2-conjugated goat anti rabbit or anti goat; FITC-conjugated donkey anti rabbit antibody; 1:500 in PBS). Sections were rinsed in PBS, sections were mounted onto superfrost slides (VWR) and coverslipped in glycerol. Images were taken on an Olympus confocal fluorescence microscope and complied in Adobe Photoshop.

Electron Microscopy. Animals for electron miscroscopic analysis were intracardially perfused with ice cold saline. Saphenous nerves were exposed and fixed with 2% gluteraldehyde/ 4% paraformaldehyde in the animal for 5 minutes prior to dissection. Once dissected, the nerves were post fixed in 2% gluteraldehyde/ 4% paraformaldehyde for two hours. Nerves were then cut into 1mm blocks, post-fixed in 2% osmium tetroxide in 0.1M PB for one hour and dehydrated in an increasing series of ethanols and propylene oxide. Blocks were then embedded in EMBed 812 (Electron Microscopy Sciences). Ultra-thin sections (80-90nm) were cut with an ultramicrotome and collected on formvar/ carbon coated copper slot grids. Grids were counter stained in uranyl acetate and lead citrate and analyzed on a Morgagni electron microscope. Images were captured using AMT digital imaging software and digital camera connected to the microscope. Images taken of the entire nerve at 4400x were compiled into montages using Adobe Photoshop.

In vivo quantification of axon regeneration: Digital images obtained from EM processing were analyzed offline. After montage construction, numbers of non-degenerating axons of normal

appearance were counted in the sections analyzed at seven and 14 days in control and experimental mice and averaged across animals (n=3 per condition). Axons of comparable morphologically to uninjured nerves and those not meeting the criteria for electron-dense degeneration according to Peters et al. (1991) and Mugnaini and Friedrich (1981) were used to establish the criteria for degenerating axons in the saphenous nerve. These criteria do not meet those established for filamentous, flocculent or watery degeneration (Mugnaini and Friedrich, 1981). Briefly, degenerating axons were determined to be electron dense with swollen mitochondria (when present) and associated with surrounding glial cells. Numbers of non-degenerating axons present at 2mm, 4mm and 6mm distal to the site of the nerve crush at various times after injury were recorded and presented as an average difference in the number of regenerating fibers between PenCON and PenSOX injected mice. Data were then segregated into myelinated and unmyelinated groups to distinguish differences in regeneration rate between fiber types. Statistical significance between conditions was determined using Student's t-test with p-value set at $p < 0.05$.

5.3 RESULTS

5.3.1 Sox11 is upregulated in DRGs after saphenous nerve axotomy.

To confirm that Sox11 is upregulated in DRGs of mice with peripheral nerve injury, RNA was isolated from the L2/L3 or L4/L5 DRGs of animals that underwent saphenous or sciatic nerve axotomy, respectively at three, four, five, seven, 14 and/ or 28 days post nerve transection. The saphenous nerve was chosen due to the simplicity of the procedure, ease of access to the nerve and minimal damage that occurs to surrounding tissues due to the surgery. Expression assays on the sciatic nerve were done for comparison. Sox11 mRNA was found to be increased in the L2/L3 DRGs 2.84-fold three days post saphenous nerve axotomy and continued to increase to 3.47-fold at day five. Seven days after saphenous nerve axotomy, Sox11 mRNA was determined to be 3.11-fold higher in the ipsilateral ganglia than the contralateral ganglia and returned to baseline after four weeks (Fig. 19a). Sox11 mRNA was increased 8.73-fold four days after sciatic nerve axotomy in the L4/L5 DRGs. Levels of Sox11 mRNA continued to increase to a 13.1-fold increase at seven days and fell to 10.1-fold at 14 days (Fig. 19. No change in Sox11 mRNA was detected in the spinal cord at any time point after sciatic nerve axotomy (not shown).

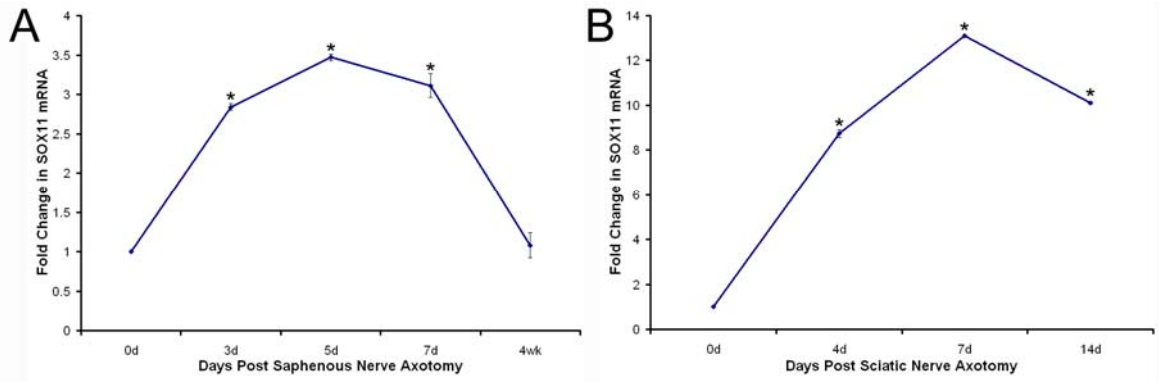

Fig. 19: Fold Change in Sox11 mRNA in L2/L3 DRGS after axotomy of the saphenous and in L4/L5 DRGs after axotomy of the sciatic nerves. **A**: Saphenous nerve axotomy induces an increase in Sox11 mRNA that is increased 2.84-fold within three days, peaks at day five to 3.47-fold and remains elevated for at east seven days at 3.1-fold. After four weeks however, Sox11 mRNA levels returns to baseline. **B**: Axotomy of the sciatic nerve shows a similar pattern of Sox11 expression four days after axotomy. Sox11 levels rise 8.73-fold higher than contralateral ganglia at four days. Sciatic nerve axotomy induces a peak in Sox11 mRNA expression at day seven where levels reach a 13.1-fold increase. After two weeks, Sox11 is still elevated 10.1-fold relative to the contralateral side. * p value < 0.05, relative to contralateral DRGs.

Immunocytochemical analysis of Sox11 expression shows that Sox11 is readily detectable after sciatic nerve injury in the ipsilateral L4 DRG but not the contralateral ganglion (Fig. 20). A low level nuclear and cytoplasmic staining is evident under baseline conditions (Fig 20), but after injury to the sciatic nerve, Sox11 nuclear and cytoplasmic staining is enhanced (Fig. 20). To verify that axotomy induces injury in the DRGs, we performed immunocytochemical labeling for ATF3 after saphenous nerve axotomy in the ipsilateral and contralateral L3 DRGs. Nuclear labeling of ATF3 is easily detected in the injured ganglion three days after saphenous nerve axotomy but not in the uninjured ganglion (Fig. 20 and d).

5.3.2 Injection of Penetratin-1 conjugated siRNAs against Sox11 into the saphenous nerve knocks down mRNA expression.

To determine if Sox11 upregulation after axotomy was necessary for peripheral nerve regeneration and if the effect of Sox11 inhibition on neurite outgrowth *in vitro* correlated to a deficit in axon re-growth *in vivo*, our approach was to knock down the expression of Sox11 in the sensory neurons that contribute to the saphenous nerve. To accomplish this, penetratin-1 linked control (PenCON) or Sox11 targeting (PenSOX) siRNAs were injected into the saphenous nerve. siRNAs were taken up into nerve fibers and retrogradely transported back to the cell bodies in the L2/L3 DRGs (Fig. 21. Retrograde transport of siRNAs was determined to take approximately two days although no RNA knockdown of Sox11 was detected in PenSOX injected mice at this time point relative to PenCON injected animals. PenSOX siRNA induced mRNA knockdown was first detected in the DRGs of mice that

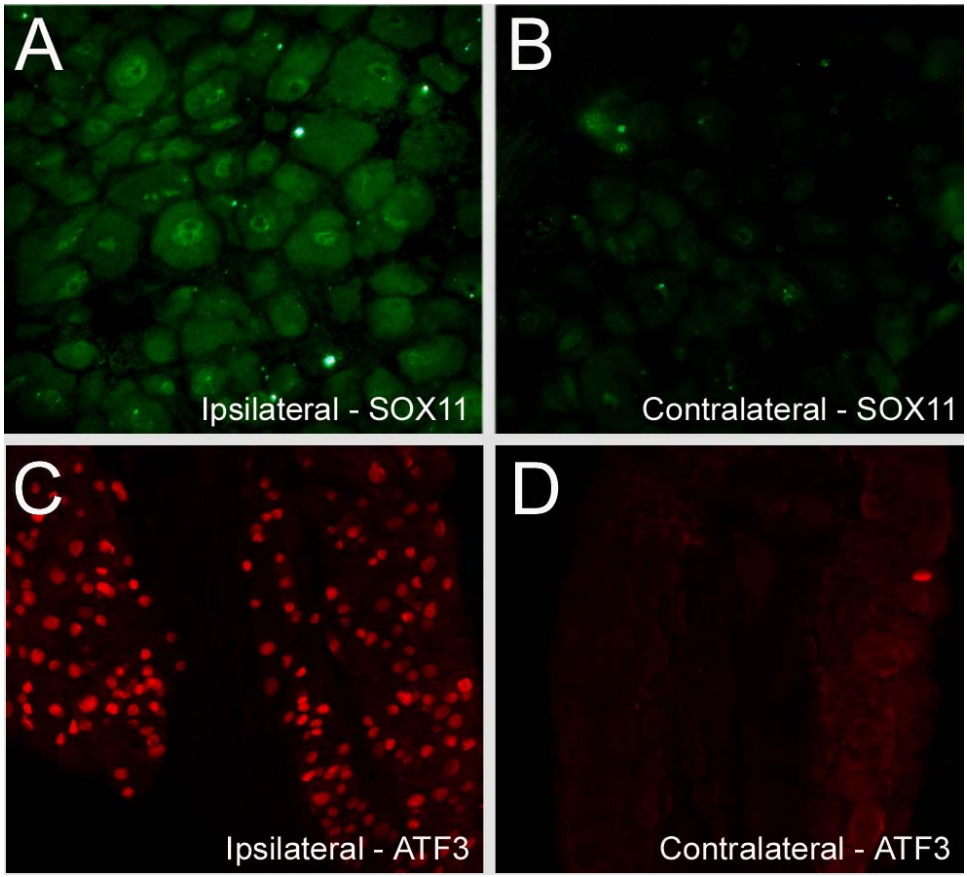

Fig. 20: Light micrographs of L4 DRGs immunoreactive for Sox11 seven days after axotomy of the sciatic nerve and of L3 DRGs immunopositive for ATF3 three days after axotomy of the saphenous nerve. Sox11 is readily detected in nuclei of the majority of sensory neurons, presumably those that were injured by nerve cut on the ipsilateral side (panel A). Some low level staining is detectable on the contralateral side (panel B). Axotomy also induces expression of ATF3 in the ipsilateral L3 DRGs (panel C) three days after axotomy of the saphenous nerve but not in the contralateral DRG (panel D).

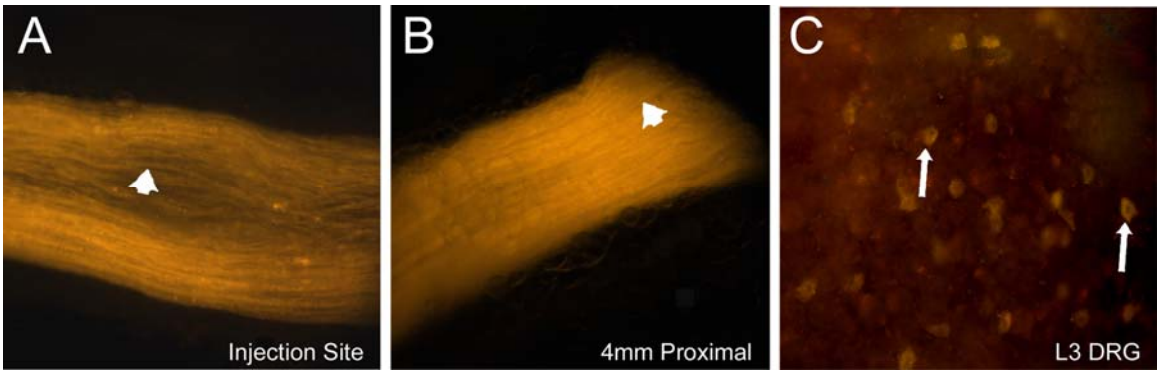

Fig. 21: Light micrographs of saphenous nerve and L3 dorsal root ganglion (DRG) from an animal injected two days previously with CY3-tagged, Penetratin-1 linked control (PenCON) siRNAs. **A**: PenCON siRNAs are taken up by the fibers of the saphenous nerve upon injection (arrowhead). **B**: siRNAs are also readily detectable in axons of the saphenous nerve 4mm proximal to the injection site (arrowhead). **C**: Whole mount DRGs display several somas that also show that the siRNAs are retrogradely transported by evidence of cytoplasmic CY3 fluorescence in the DRG after saphenous nerve injection of PenCON siRNAs (arrows).

underwent saphenous nerve axotomy after three days (Fig. 22).

Animals that were injected with PenCON siRNAs *and* axotomized had 4.63-fold higher levels of sox11 mRNA in the L2/L3 DRGs whereas PenSOX injected animals had only a 3.74-fold increase in sox11 mRNA. The increase in expression was found to be higher in animals that were both injected *and* axotomized versus mice that received nerve cut or injection alone (Figs. 22 and 23; and data not shown). PenSOX injected mice displayed a higher (4.33-fold) level of Sox11 mRNA expression at five days post axotomy than PenCON injected animals; however, both controls and experimental groups returned to a similar level of expression (approx. 3.1-fold) by day seven compared to axotomized animals alone (Fig. 23).

5.3.3 Inhibition of sox11 *in vivo* blocks ATF3 expression and nerve regeneration.

To determine if injection plus axotomy was causing a larger injury than either injury alone, we performed realtime PCR analysis for ATF3 on mice that received saphenous nerve axotomy alone and on mice that were injected with siRNAs and underwent axotomy. Saphenous nerve axotomy induced a 22-fold increase in ATF3 mRNA three days after injury (not shown), whereas axotomy plus injection of siRNAs into the saphenous nerve induced a 26-fold increase in ATF3 (Fig. 24). Surprisingly, injection of PenSOX siRNAs in animals that also experienced nerve cut had a reduction in the level of ATF3 mRNA (19-fold increase) relative to PenCON injected mice.

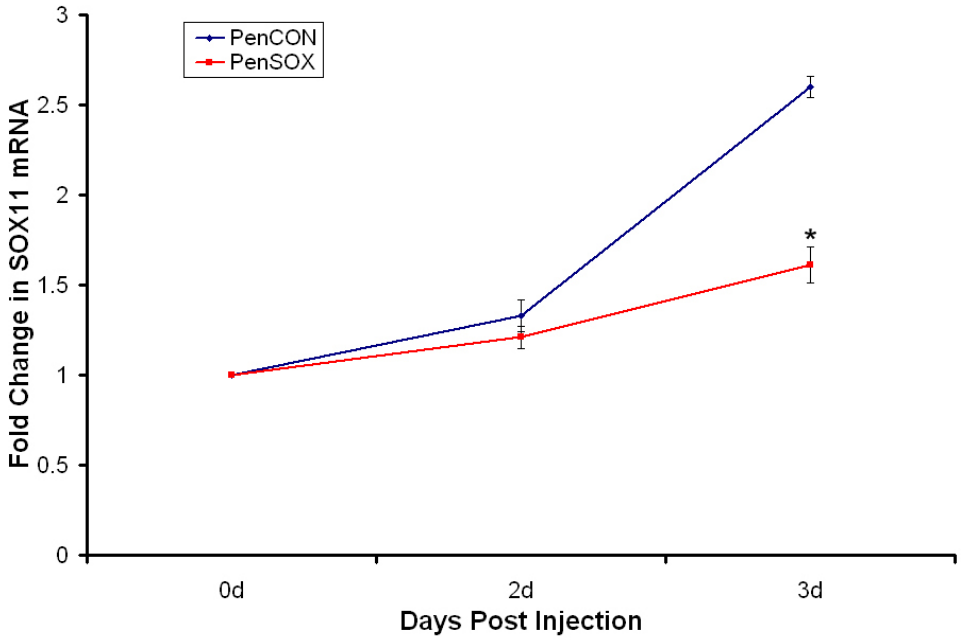

Fig. 22: Fold change in Sox11 mRNA two and three days after injection of either control (PenCON) or Sox11 targeting (PenSOX) siRNAs directly into the saphenous nerve. No detectable knockdown in expression is detected between PenCON (blue line) and PenSOX (red line) treated mice two days after injection. After three days however, PenSOX injected mice show a reduction in Sox11 expression relative to PenCON injected mice. *p value < 0.05, relative to controls.

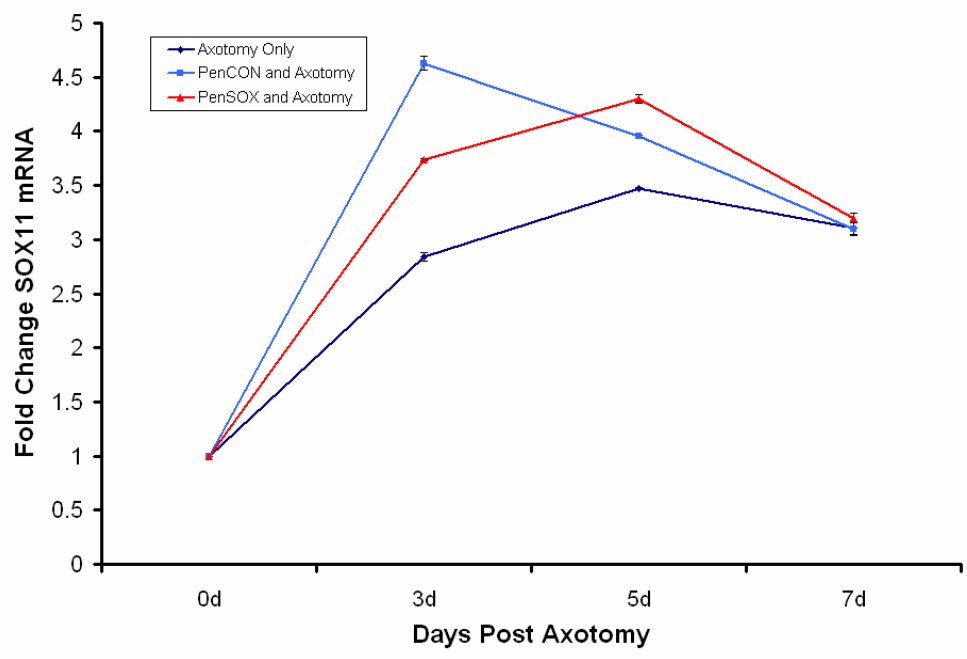

Fig. 23: Fold Change in Sox11 mRNA in L2/L3 DRGs from mice injected with non-targeting control (PenCON) or Sox11 targeting (PenSOX) siRNAs with or without axotomy of the saphenous nerve. Injection of PenCON (light blue line) and PenSOX (red line) siRNAs plus axotomy of the saphenous nerve both cause a larger increase in Sox11 expression compared to axotomy alone (dark blue line) at three days (4.63-fold vs. 3.7-fold vs 2.84-fold increase respectively). However, PenSOX injection plus axotomy induces a reduction in Sox11 expression relative to PenCON injection plus axotomy. Five days after axotomy and injection, PenSOX treated mice show higher levels of Sox11 mRNA (4.33-fold) than both PenCON treated mice (3.95-fold) and mice that received an axotomy of the saphenous nerve alone (3.47-fold). After seven days, all three conditions show similar expression for Sox11 (PenCON: 3.1-fold increase; PenSOX: 3.2-fold increase; axotomy alone: 3.1-fold increase).

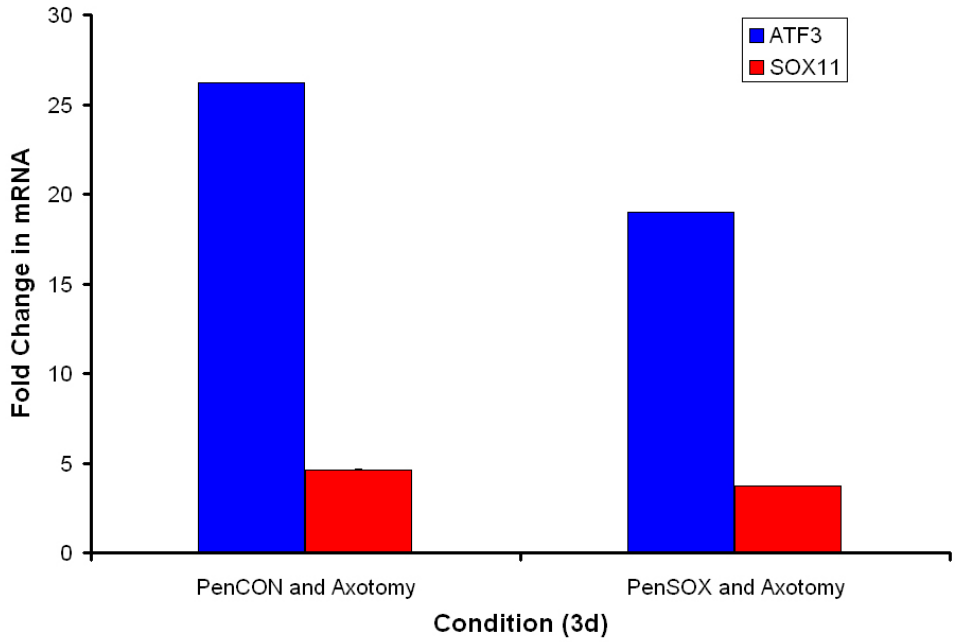

Fig. 24: Fold Change in ATF3 and Sox11 mRNA three days post saphenous nerve axotomy and injection of control (PenCON) or Sox11 targeting (PenSOX) siRNAs. PenCON injection plus axotomy induces a 26.2-fold increase in ATF3 and a 4.63-fold increase in Sox11. Surprisingly, PenSOX injection plus axotomy not only reduced the increase in Sox11 mRNA to a 3.7-fold, but it also only permitted a 19-fold increase in ATF3 expression.

Based on our previous data that showed that Sox11 is necessary for neurite outgrowth *in vitro*, and that inhibition of Sox11 could alter the levels of ATF3 induced by axotomy, we measured how PenSOX siRNA injection altered the rate of regenerating saphenous nerves. The saphenous nerve typically takes 2-4 weeks to functionally regenerate from a nerve transection or nerve crush (McIlwrath et al., 2005). We therefore determined the numbers of axons that regenerated after saphenous nerve crush in PenCON and PenSOX injected animals at three, seven and 14 days after nerve crush at the electron microscopic (EM) level. EM is necessary for this type of analysis since small diameter fibers cannot be seen at the light microscopic level. We first verified that siRNA injection and nerve *crush* induced similar changes in Sox11 and ATF3 as injection of siRNAs and axotomy. Injection of PenSOX siRNAs inhibited the increase in Sox11 and ATF3 induced by injection of PenCON siRNAs in animals that experienced nerve crush at three days and seven days. Both Sox11 and ATF3 mRNA levels returned to control levels after 14 days (Fig. 25). Pattern and levels of mRNA expression detected between axotomized and nerve crushed mice were found to be similar and therefore allowed us to continue our analysis of the anatomical consequences of Sox11 inhibition after nerve crush. Interestingly, we were able to maintain a knockdown of Sox11 and ATF3 for seven days when performing a nerve crush versus only three days after a complete axotomy.

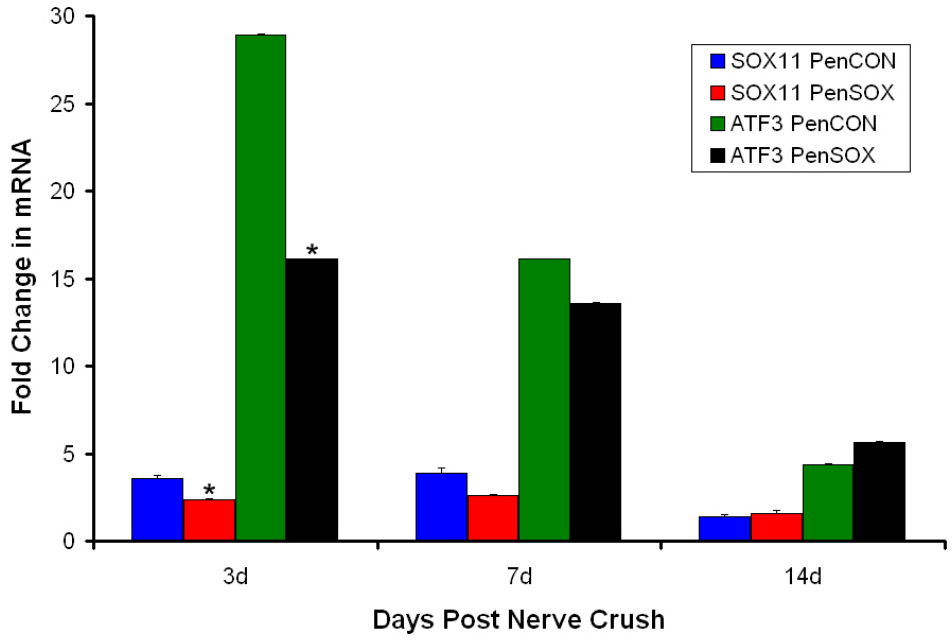

Fig. 25: Fold change in ATF3 and Sox11 mRNA at three, seven and 14 days after saphenous nerve crush and injection of either control (PenCON) or Sox11 targeting (PenSOX) siRNAs. After three days, PenCON injection plus nerve crush induces a 3.6-fold increase in Sox11 and a 28.9-fold increase in ATF3. PenSOX injection plus crushing the saphenous nerve only induces an increase in Sox11 mRNA to 2.36-fold and ATF3 16.1-fold relative to the contralateral side. Seven days after nerve crush, PenCON injected mice have a 3.9-fold increase in Sox11 and a 16.1-fold increase in ATF3. Injection of PenSOX siRNAs prior to nerve crush only permitted a 2.6-fold increase in Sox11 and a 13.1-fold increase in ATF3. After 14 days however, both PenCON and PenSOX injected mice showed similar levels of Sox11 and ATF3 expression (Sox11: 1.4-fold increase vs 1.57-fold increase; ATF3: 4.37-fold increase vs. 5.63-fold increase, respectively).

To determine if *in vivo* delivery of Sox11 siRNAs to the saphenous nerve blocked regeneration from nerve crush, we analyzed saphenous nerve regeneration in mice that received PenCON or PenSOX siRNA injection prior to a nerve crush. The saphenous nerve is comprised of both myelinated and unmyelinated axons (Fig. 26A). EM analysis of saphenous nerves at the injection site and crush site three days after nerve crush shows that injection of siRNAs into the nerve induces some damage to both myelinated and unmyelinated fibers (Fig. 26B). However, crushing the saphenous nerve causes complete damage to all fibers (Fig. 26C). Occasionally, some regenerating heavily myelinated fibers are present 1mm distal to the crush, but most fibers have not yet begun to regenerate at this time point in either PenCON or PenSOX injected conditions (Fig 27).

We therefore waited for mice to recover from saphenous nerve injury for one week after injection of siRNAs and nerve crush to analyze regeneration. At 2 mm distal to the site of the nerve crush both regenerating myelinated and unmyelinated fibers were identified. In Sox11 siRNA injected nerves, several myelinated fibers were present though appeared smaller and less myelinated than in PenCON injected mice. Unmyelinated fibers were also visible in Remak bundles. Animals that received PenSOX siRNAs prior to nerve crush also displayed similar regeneration patterns. Both myelinated and unmyelinated fibers were present 2mm distal to the nerve crush (not shown). Initial quantification of regeneration 2mm distal to the nerve crush showed no

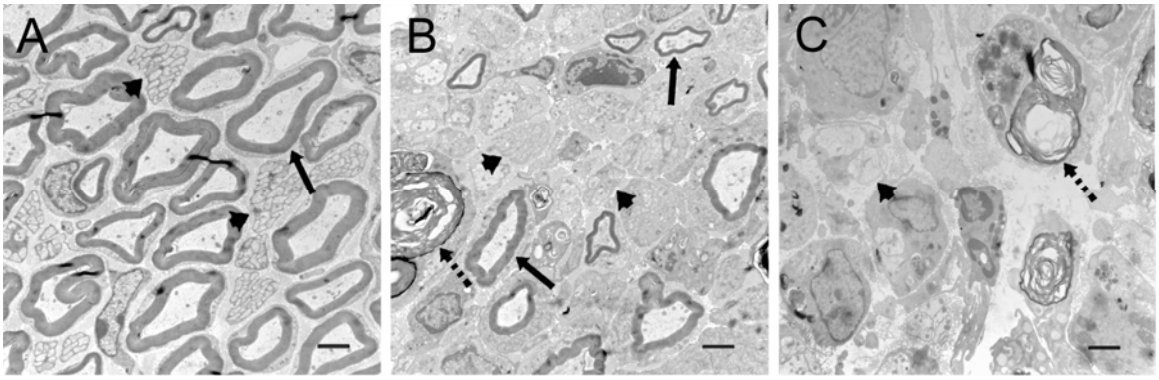

Fig. 26: Electron micrographs of the saphenous nerve at the site of siRNA injection, the site of nerve crush and under normal conditions. A: Uninjured saphenous nerves have multiple myelineated (arrow) and unmyelinated (arrowheads) fibers. B: Injection of siRNAs into the saphenous nerve induces some damage to myelinated (dashed arrow) and unmyelinated fibers. The majority of myelinated axons however appear normal (arrows) and unmyelinated fibers are still packaged into Remak bundles (arrowheads). C: Crushing the saphenous nerve damages all fibers. Myelinated fibers have degenerated and myelin sheaths have unraveled (dashed arrow). Remak bundles are largely disrupted and appear to be undergoing degeneration (arrowhead). Scale Bar: 2μm.

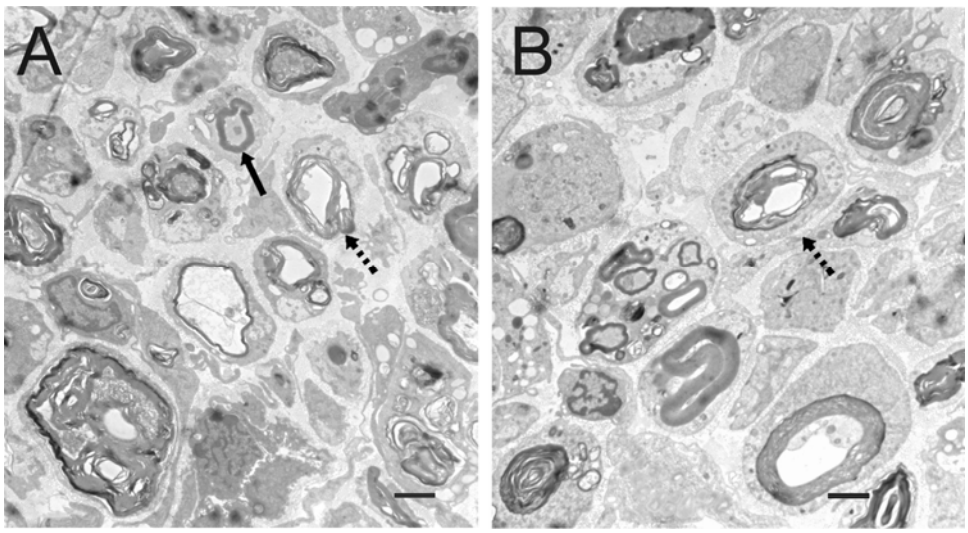

Fig. 27: Electron micrographs of the saphenous nerve 1mm distal to the nerve crush three days after injury. Extensive degeneration is present in saphenous nerves and most myelinated fibers are absent and show unraveling of their myelin (dashed arrows) in both control siRNA injected (Panel A) and Sox11 targeting siRNA injected mice (Panel B). Occasionally, some myelinated fibers appeared to have begun regeneration (arrow). Scale Bar: 2μm.

difference in the numbers of myelinated or unmyelinated axons (not shown). We therefore analyzed regeneration 4mm distal to the nerve crush. Several myelinated and unmyelinated fibers were present 4mm distal to the crush in PenCON injected mice (Fig. 28A); however, there was a significant reduction in the number of myelinated and unmyelinated fibers that regenerated 4mm distal to the nerve crush in PenSOX injected mice (Figs. 28B). Sox11 targeted siRNA injection decreased the number of myelinated fibers that had regenerated by 36% and the number of unmyelinated fibers that had regenerated by 51% (Fig. 29). 14d after nerve crush when Sox11 and ATF3 mRNA levels begin to return to baseline, both PenCON and PenSOX injected mice showed similar extent of regeneration at the most distal segment (6mm) from the nerve crush (Fig. 30).

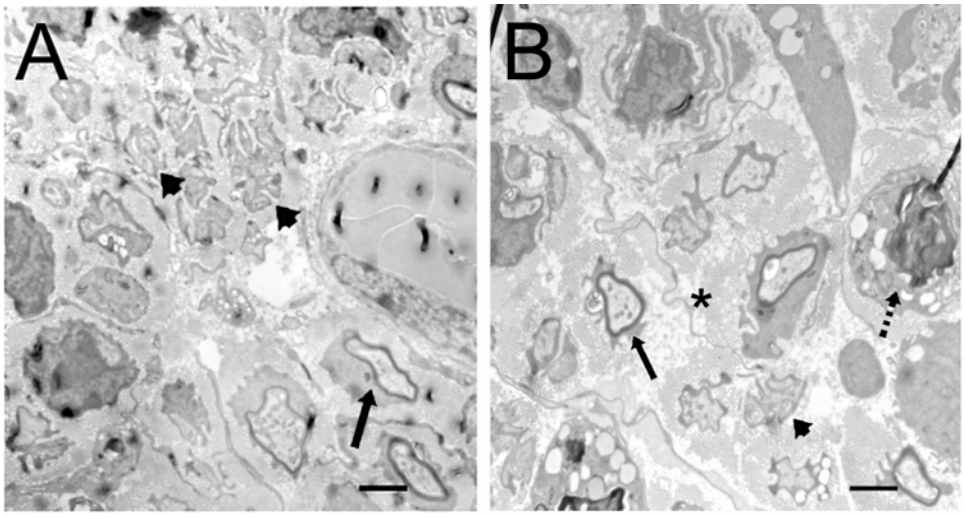

Fig. 28: Electron micrographs of the saphenous nerve 4mm distal to the nerve crush seven days after injury. Panel A: Control siRNA (PenCON) injected mice show extensive regeneration of myelinated (arrow) and unmyelinated (arrowheads) axons. Panel B: Sox11 targeting siRNA (PenSOX) injected mice show some regeneration of myelinated fibers (arrow) but fewer unmyelinated axons (arrowhead) and larger interaxonal space (asterisk). Scale Bar: 2μm.

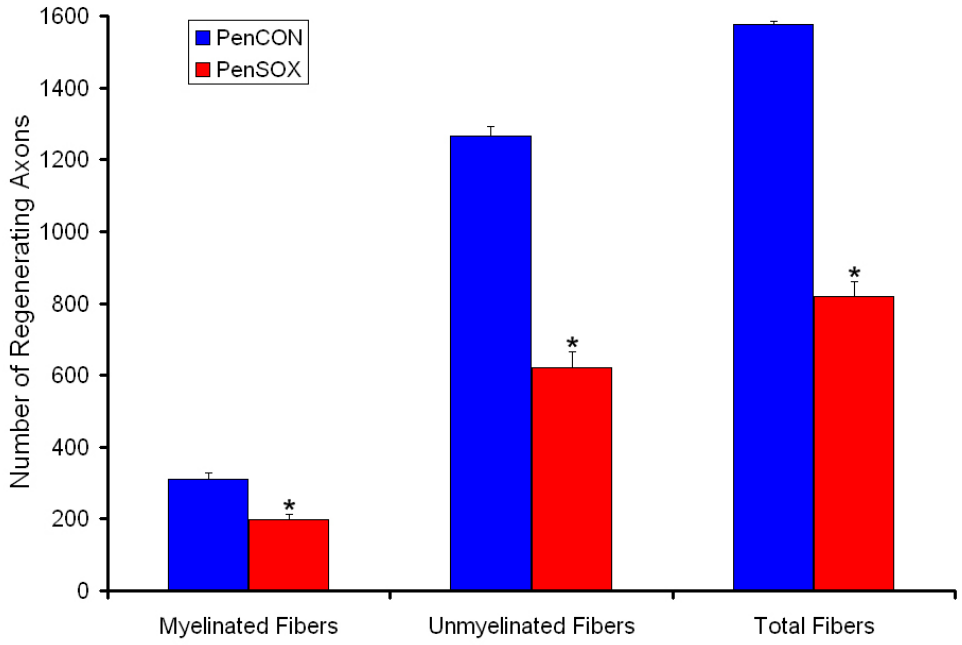

Fig. 29: Quantification of saphenous nerve regeneration in mice that underwent nerve crush and injection of either control (PenCON) or Sox11 targeting (PenSOX) siRNAs. There was a significant difference in the number of regenerating myelinated fibers detected between PenCON (310 +/- 17) and PenSOX (198 +/- 14) injected mice (p value < 0.02). There was also a significant difference in the number of regenerating unmyelinated fibers. PenSOX injection reduced the number of unmyelinated axons that had regenerated 4mm distal to the nerve crush from 1267 +/- 26 to 622 +/- 43 (p value < 0.001). Overall there was a 48% decrease in the number of fibers that regenerated 4mm in PenSOX siRNA injected mice compared to PenCON siRNA injected mice (1577 +/- 9 vs. 820 +/- 40; p value < 0.001).

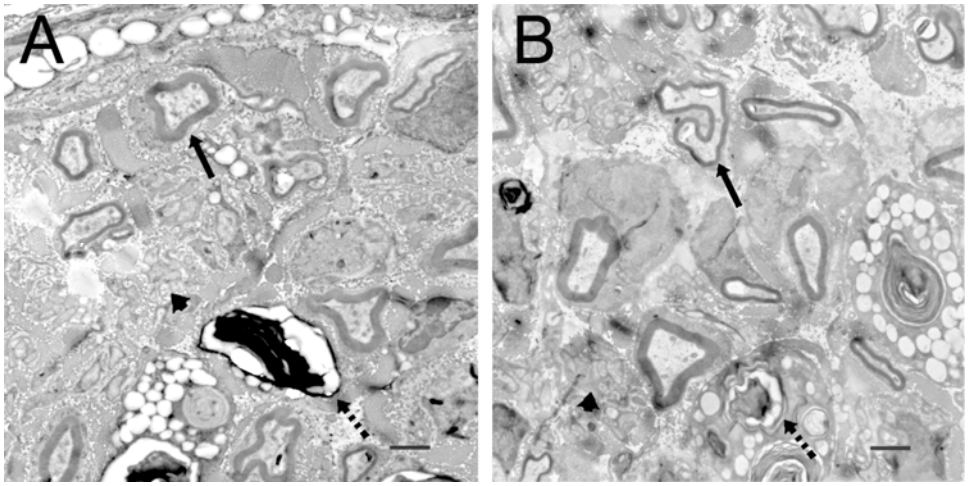

Fig. 30: Electron micrographs of the saphenous nerve 6mm distal to the nerve crush 14 days after injury. Both non-targeting siRNA injected (PenCON, panel A) and Sox11 targeting siRNA injected (PenSOX; panel B) mice show similar patterns of regeneration of both myelinated (arrows) and unmyelinated fibers (arrowheads). There still is evidence of degeneration in some fibers (dashed arrows) at this distance and time point. Scale Bar, 2μm.

5.4 DISCUSSION

We have used a new approach to deliver siRNAs to peripheral nerves that allows the knockdown of gene expression *in vivo.* Nerve injection of Penetratin-linked siRNAs allowed us to target cutaneous neurons that innervate the leg and foot specifically. Injected siRNAs were taken up by axons and retrogradely transported back to the soma where they produced knockdown in gene expression. Since the successful performance of RNA interference *in vivo* is dependent on targeted and efficient delivery, this approach offers a powerful means by which to regulate gene expression *in vivo*. In this study, detectable knockdown of Sox11 mRNA was achieved within three days after injection. The effect of this knockdown *in vivo* was an inhibition of ATF3 expression and peripheral nerve regeneration.

Using Neuro2A cells, Sox11 was shown to be necessary to prevent cells from entering an apoptotic death pathway as cells were induced to differentiate (Jankowski et al., 2006). Whether a similar mechanism operates in primary DRGs lacking Sox11 (Jankowski et al., 2005, 2006; CHAPTER FOUR) and in DRG neurons *in vivo* after nerve injury is unclear. No cell death was detected three days after saphenous nerve injury as measured by immunolabeling for activated caspase3 (not shown) in the L2/L3 DRGs of PenCON or PenSOX injected and axotomized mice. Thus, the effects of Sox11 siRNA knockdown does not produce the same effect of knockdown in cultured cells *in vitro.* It is likely that the effect of Sox11 upregulation from axotomy initiated cell survival pathways prior to a functionally significant Sox11 knockdown, which could be

detected on the mRNA level by day three. The knockdown obtained in Sox11 mRNA was also transient in the injected and *axotomized* mice (Sox11 mRNA was inhibited for less than five days post axotomy; Fig. 24), in which case we may have been able to see casp3* immunostaining if we were able to knockdown expression for an extended period. Performing activated caspase3 labeling experiments in the L2/L3 DRGs of animals that receive PenSOX injection and nerve *crush* may allow us to detect neuroprotective affects of Sox11 after injury since some studies have reported cell death in injured DRG neurons only after at least a week (Jiang et al., 2005; Kuo et al., 2005; Thippeswamy et al., 2001). However, since it is known that Sox11 can regulate cell death at early stages of cell differentiation (Jankowski et al., 2006), it seems likely that Sox11 is acting in a neuroprotective manner early after injury. Intrathecal delivery of PenSOX siRNAs prior to nerve injury, which would allow us to induce a faster knockdown, may have enabled us to discover apoptotic cells in nerve injured mice within this time frame.

5.4.1 Sox11 is enhanced by axotomy and necessary for the induction of ATF3 after injury.

The increase in Sox11 mRNA after peripheral axotomy of the sciatic nerve has been reported previously (Jankowski et al., 2004; Tanabe et al., 2003). In this study, we show that a similar pattern of Sox11 expression occurs in response to saphenous nerve crush and axotomy. The rise of Sox11 mRNA expression coincides with the time in which injured nerves are initiating Wallerian degeneration, "dying back" and subsequent regeneration (Baba et al., 1982; Kury et al., 2001; Mi et al., 2005; Sebille, 1982; Thomas, 1982). It is also known that functional regeneration of the saphenous nerve occurs four weeks after nerve transection (McIlwrath et al., 2005) and the levels of Sox11 mRNA have returned to baseline by this time. Since we have

shown previously that Sox11 is required for proper neurite growth of DRG neurons *in vitro*, this *in vivo* evidence would suggest that Sox11 plays a role in functional nerve regeneration.

Because Sox11 was shown *in vitro* to modulate ATF3 expression, we examined if ATF3 expression could be regulated by Sox11 *in vivo*. In animals whose peripheral nerve was injected with PenSOX siRNAs and then crushed or axotomized, ATF3 expression was inhibited relative to injection of control siRNA. This knockdown in ATF3 does not appear to be due to an off-target effect of the Sox11 siRNAs since the PenSOX siRNA sequence has almost no homology to the ATF3 gene. Furthermore, we have shown previously that siRNA-mediated knockdown of Sox11 does not affect the levels of other transcription factors with higher sequence homology (Chapter Three, Fig. 4). In addition, preliminary studies using luciferase reporter assays indicate a direct regulation of Sox11 on the ATF3 promoter (Kathleen Solerno, personal communication). ATF3 has been linked to neurite outgrowth (Seijfers et al., 2006) and nerve regeneration (Campbell et al., 2003; Isacsson et al., 2005; Lindwall et al., 2004) and many act through c-jun (Lindwall et al., 2004; Pearson et al., 2003). Our data suggests that the role that Sox11 plays in nerve regeneration may therefore be, in part, via upstream regulatory action on ATF3 expression.

5.4.2 Sox11 upregulation after nerve injury is essential for proper peripheral nerve regeneration.

We have shown that inhibition of Sox11 significantly blocks the number of fibers that regenerate after crushing the saphenous nerve. The functional affect of inhibition could not be quantified within three days of the crush but Sox11 inhibition does become significant after seven days. Transfection of primary DRG neurons with PenSOX siRNAs reduces neurite

growth in all cell sizes (Chapter Four). This correlates with nerve regeneration *in vivo* since both myelinated and unmyelinated fibers were affected by Sox11 inhibition. Light microscopic analysis suggests that there is an equal uptake and retrograde transport of siRNAs in all fibers (Fig. 22); however, a detailed quantification of this feature would be necessary to rule out differential uptake of siRNAs among different fiber types. Sox11 inhibition affected unmyelinated axon regeneration more than the myelinated axons. This may be related to the fact that myelinated fibers regenerate faster than unmyelinated fibers (Lozeron et al. 2004). In addition, it is possible that Sox11 inhibition more significantly hindered myelinated fiber regeneration prior to seven days and this was not measured. Additional studies to analyze regeneration in PenCON and PenSOX injected mice between three and seven days after nerve crush will be necessary to determine the degree to which Sox11 regulates early myelinated fiber regeneration.

Taking into consideration the *in vitro* evidence presented previously, it is possible that Sox11 plays a role in proper *functional* regeneration of nerves as well. Branching indices of DRG neurons *in vitro* are greatly compromised by lack of sufficient levels of Sox11. After saphenous nerves regenerate to the skin, it is reasonable to suggest that these axons will not be heavily branched in their peripheral targets. A detailed analysis of axon arborzation in the skin after nerve regeneration in PenCON and PenSOX injected mice that underwent axotomy of the saphenous nerve will be necessary to test this hypothesis. Nevertheless, we have shown that developmentally regulated transcription factor Sox11 is necessary for proper *in vivo* nerve regeneration, most likely through its regulatory actions on ATF3.

6.0 GENERAL DISCUSSION

Individual populations of DRG neurons display unique protein expression patterns that contribute to their anatomical and functional properties. During development, many DRG neurons express trkB and trkC receptors at embryonic day 10.5 (E10.5). By E11.5, trkA becomes detectable and soon after becomes the most prevalent neurotrophin receptor present in the ganglion. At approximately E13, the GDNF co-receptor, ret is upregulated, and later during embryogenesis, the GDNF family receptors (GFRs) are upregulated. Throughout this time, the level of each of the neurotrophins in the skin is changing and this in turn regulates the survival and differentiation of particular populations of sensory neurons (Huang et al. 1999). Presence of neurotrophins and responsiveness of certain neurons to different neurotrophins are requisite for cell survival during the period of rapid cell death in the DRG (Oppenheim, 1991). In fact, overexpression of different neurotrophins in the periphery can enhance the survival of different populations of sensory neurons. Overproduction of NGF in the skin causes an increase in the survival of trkA positive neurons (Albers, 1994; 1996) while overexpression of GDNF results in the survival of the IB4 population of neurons (Zwick et al. 2002). Furthermore, excess neurotrophin in the skin results in enhanced branching in the target tissue (Albers et al., 1994; LeMaster et al., 1995; Zwick et al., 2002). This supports the hypothesis that neurotrophins are necessary for sensory neuron survival (Oppenheim, 1991) and for differentiation of different types of neurons (Lewin et al., 1992). Peripheral projections become more elaborate and developed as neurons survive the period of programmed cell death (Berg and Farel, 2000).

However, the molecular mechanism that underlies how neurotrophic growth factors regulate survival and differentiation remains to be fully elucidated.

Preliminary data suggested that overexpresion of NGF could enhance the survival of trkA/ CGRP neurons at the expense of the IB4 population. Conversely, overproduction of GDNF in the skin resulted in a selective survival of ret and IB4 (Zwick et al. 2002) positive neurons while decreasing the expression of trkA in the trigeminal ganglia (TG). This may suggest that phenotypic expression is dependent on the neurotrophic responsiveness while survival may be dependent on common factors that can be activated by all neurotrophins. The degree to which the neurotrophins can activate this mechanism may determine the level of survival. In order to determine what factors may be regulating this phenomenon we performed microarray analysis on E16.5 and adult TGs from wild type, GDNF- and NGF-overexpressor transgenic mice (OEs). This analysis revealed that many families of genes are differentially regulated in the sensory ganglia as a result of overproduction of neurotrophins in the periphery. Certain families of genes are likely candidates to regulate a common survival pathway from a wide range of signaling molecules.

Microarray data from DRG and trigeminal neurons suggested that one transcription factor regulated by neurotrophic factor production in the skin was Sox11. Sox11 is an HMG transcription factor thought to be associated with neuronal maturation due to its localization in sensory ganglia and the fact that it is upregulated during development (Hargrave et al., 1997; Uwanogho et al., 1995). Overexpression of both NGF and GDNF resulted in enhanced levels of Sox11 in the trigeminal ganglia at E16.5 as verified by RT-PCR. It was further determined that

Sox11 was localized to the neurons during development, and although not readily detectable in the adult DRG using immunocytochemistry, was significantly upregulated after peripheral nerve transection for several weeks. The change in expression was found to be specific to the sensory ganglia as no change in Sox11 was found in the spinal cord dorsal horn after sciatic nerve axotomy (not shown). The HMG superfamily of DNA-binding proteins can either activate or repress transcription of target genes and regulate multiple processes (Kamachi et al., 1999). Therefore, based on these data, the transcription factor Sox11 was chosen for further analysis to determine whether it had a role in regulating neuronal survival and differentiation and if it played a role in an animal's response to injury in the adult.

Initial studies using a neuroblastoma cell line showed that Sox11 increases as Neuro2A cells differentiate *in vitro*. Upon addition of RA to the cultures, Neuro2A cells cease dividing and begin to extend neurites and undergo differentiation (Shea et al., 1984). Sox11 is increased 100% by addition of RA to Neuro2A cells suggesting that the increase in Sox11 may underlie in part, Neuro2A differentiation in response to RA. To test this hypothesis, cells were transfected with siRNAs directed against Sox11 in the presence or absence of RA. Sox11 siRNAs alone appear to have no affect on Neuro2A survival or differentiation; however, it is unclear if inhibition of Sox11 is blocking cell division since fewer cells were present after addition of Sox11 siRNAs. Future studies will be necessary to determine if Sox11 plays a role in exiting the cell cycle. Surprisingly, after addition of RA, Sox11 siRNAs induced significant cell death suggesting that Sox11 may be necessary for survival when cells are undergoing differentiation. The percentage of cells that normally differentiate in the presence of RA is similar to the percentage of cells that undergo programmed cell death in the presence of RA and Sox11

siRNAs. Normal Sox11 function may be to promote neurite outgrowth as both untreated cells and RA treated cells both enhance their levels of Sox11 in correlation with the extent of differentiation/ neurite growth. The fact that Neuro2A cells undergo apoptosis when sufficient levels of Sox11 are not attained as they differentiate suggests that Sox11 may have a dual role, supporting neurite growth and differentiation. All of the aforementioned phenomena occur within 24h. This would suggest that Sox11 is highly important for the early stages of differentiation and lack of this transcription factor during this critical period results in apoptosis.

To investigate how Sox11 functions, microarray screening of genes linked to the apoptosis pathway were used to analyze changes in gene expression in Neuro2A cells grown with RA and transfected with or without Sox11 siRNAs. Multiple genes were differentially regulated by inhibition of Sox11. We focused our analysis on genes that contained Sox11 binding sites in their upstream promoter regions. Two survival related genes, BNIP3 and TANK were found to be increased and decreased respectively by lack of Sox11 in RA treated cells. Cells transfected with BNIP3 have been shown to rapidly undergo apoptosis (Chen et al., 1999) and TANK has been linked to enhanced proliferation (Conti et al., 2005) and stress responses (Chin et al., 1999; Coyle et al., 2003). An increase in a pro-apoptosis gene such as BNIP3 in conjunction with downregulation of a survival/ stress activated gene like TANK may underlie how Sox11 is mediating its affects on cell death. Although it is unclear at this time if Sox11 is directly binding to the promoters of these genes and regulating their transcription, lack of Sox11 does alter the mRNA levels of these genes, which may in turn result in the induction of apoptosis seen in Neuro2A cells treated with RA and Sox11 siRNAs.

The experiments performed thus far suggest that Sox11 may play a role in neuron survival and differentiation; however, it is not clear if this gene has functional significance in peripheral sensory neurons specifically and whether similar features could be detected in the adult after peripheral nerve injury. Totest this, we used adult mouse dissociated DRG neurons to analyze Sox11 function. Although another way to analyze Sox11 function in sensory neurons would have been to use a knockout strategy, these mice exhibit early postnatal death (Sock et al., 2001). Assay of Sox11 expression in cultured DRG neurons showed however a very similar significant upregulation in Sox11 following plating. Thus it allowed us to use primary cultures to study the importance of Sox11 expression following axotomy.

Transfection of adult neurons can be problematic when designing *in vitro* experiments. On average, adult neurons are only transfected at an efficiency of 1%. Performing experiments on an entire neuron culture thus would be severely confounded when using this method. Lipophillic reagents can greatly enhance the ability of adult cells to be transfected, but have been shown to induce cell death (Davidson et al., 2000). Therefore to both mimick studies performed in Neuro2A cells and use adult primary DRG neurons, we modified the siRNAs against Sox11 with Penetratin-1. Penetratin-1 has been shown to be a highly lipophillic protein that is able to cross cell membranes (Derossi et al., 1998) and when conjugated to siRNAs via a disulfide bond, can greatly enhance the transfection efficiency of adult primary neurons without inducing cell death and without inhibiting the function of the siRNAs (Davidson et al., 2000).

siRNA knockdown of Sox11 mRNA in adult DRG neurons *in vitro* was transient but significant and induced a protein knockdown that lasted for several days. Quantification of cell

death in these neurons showed that there was a 2-fold increase in casp3* positive neurons at 24h and 4d *in vitro*. This correlated with what was shown in Neuro2A cells induced to differentiate with RA without sufficient levels of Sox11 suggesting that similar functions of Sox11 found in Neuro2A cells relating to apoptosis may also be present in injured DRG neurons. Since the majority of adult DRG neurons do not readily undergo programmed cell death from a peripheral axotomy (Jiang et al., 2005; Kuo et al., 2005), the function of Sox11 upregulation after nerve injury may be to initiate neuroprotective and anti-apoptotic mechanisms. Genetic screens of programmed cell death-related genes in primary DRGs transfected with Sox11 siRNAs shows that many apoptosis associated genes are regulated in the absence of Sox11. TANK was again found to be regulated by Sox11 in primary DRGs along with other apoptosis inducing factors such as Traf1. Thus, Sox11 may indeed be protecting sensory neurons from injury induced cell death.

Another feature of RA treated Neuro2A cells was that neurite branching and differentiation correlated with an increase in Sox11. Evidence in adult DRG neurons shows that as neurons grow *in vitro* into intricate neuronal networks, Sox11 is subsequently upregulated. We therefore wanted to investigate whether Sox11 also played a role in neurite growth. Quantification of neurite growth and branching in DRG neurons shows that there is a gradual but significant increase in neurite length over time as well as an increase in branching. Inhibition of Sox11 not only inhibited branching for several days, but it also blocked the normal increase in neurite length. All populations of DRG neurons appeared to be affected (albeit to different degrees) by Sox11 inhibition. These data imply that along with a potential neuroprotective

function of Sox11, this transcription factor may also be important for neurite growth and nerve regeneration of adult sensory axons.

To determine if experiments designed to analyze the role of Sox11 in nerve regeneration were reasonable, we first performed RT-PCR on numerous neurite growth and regeneration associated genes in primary DRG neurons transfected with Sox11 siRNAs. Inhibition of Sox11 significantly altered the expression of growth associated genes ATF3, Arpc3 and Sprr1a. ATF3 is a classic marker of injured neurons and has been linked to neurite ourtgrowth and nerve regeneration (Seijfers et al., 2006; Lindwall et al., 2004; Issacson et al., 2005; Tsujino et al., 2000). Arpc3 is not characterized as well as ATF3, but is known to be important for actin cytoskelatal stability (Beltzner and Pollard, 2004; Zhang et al., 2005). Sprr1a has been identified as a gene that is upregulated by nerve injury and important for nerve regeneration and axon elongation (Bonilla et al., 2002). These results suggest that Sox11 may be mediating its actions on neurite growth by regulating these pro-axon growth genes. Although luciferase assays would be necessary to confirm a direct promoter regulation of these genes by Sox11, multiple Sox11 binding sites in the promoter regions of these genes suggests that Sox11 has the potential for direct regulation. The fact that Sox11 can regulate the expression of more than one gene linked to the axon growth pathway, allows one to speculate that this transcription factor may be a master regulator of axon regeneration in the adult after nerve damage. Previous work on other Sox genes like Sox10 have shown that these transcription factors bind to DNA tightly and make it sterically feasible for other transcription factors to bind nearby. This feature of the ability of Sox genes to enhance the affinity of other DNA binding proteins for DNA may increase the specificity as well as diversity of Sox gene function (Schlierf et al., 2002; Wegner, 1999).

These results promoted us to investigate whether Sox11 was functionally relevant in adult sensory neurons after injury *in vivo*. We therefore, analyzed the extent of nerve regeneration and expression of one neurite growth/ regeneration associated gene, ATF3, after a nerve crush in the presence or absence of Sox11. In order to perform this type of analysis, we needed to determine how to deliver siRNAs directly to the cells in the DRGs that would be injured. Peri-DRG or intrathecal delivery is known to deliver siRNAs successfully to the ganglia; however, not all axons that make up a nerve originate in a single ganglion. We therefore attempted to knockdown Sox11 expression by directly injecting the Penetratin-1 conjugated siRNAs into the saphenous nerve and allowed them to retrogradely transport back to the somas that make up that nerve. This is a previously uncharacterized method of siRNA delivery to neurons but has proven to be a successful and efficient way to analyze gene function *in vivo* specifically in neurons that make up a single nerve. Our data shows that not only do we obtain a knockdown in Sox11 mRNA *in vivo*, but we also knock down the expression of ATF3, similar to what we have shown *in vitro*. We therefore proceeded with anatomical analysis of regenerating nerves at the electron microscopic level. No change in the rate of regeneration could be detected three days after nerve crush. This is not surprising since peripheral nerves undergo a period of "dying back" and initiation of Wallerian degeneration during the first several days after nerve transection or crush (Baba et al., 1982; Mi et al., 2005; Sebille, 1982; Thomas, 1982). The degenerating myelin and nerves need to be cleaned of debris by infiltrating macrophages and prepared for regeneration. Seven days after nerve crush however, control injected mice show extensive regeneration 4mm distal to the nerve crush. Inhibition of Sox11 resulted in a decrease in the number of myelinated and unmyelinated regenerating saphenous nerve fibers that reached the 4mm distance; a phenomenon that subsides when Sox11 mRNA levels return to control levels after 14 days. *In*

vivo inhibition of Sox11 results in a decrease in ATF3 expression and blockade of nerve regeneration supporting the hypothesis that Sox11 is a transcription factor that is responsible for inducing pro-regeneration genes necessary for proper nerve regeneration.

This report is the first evidence that HMG-box transcription factor Sox11 is necessary for peripheral nerve regeneration. Sox11 may be necessary to activate genes associated with regeneration while repressing those involved in apoptosis. It will be important in the future to determine how Sox11 itself is upregulated in response to nerve injury. Studies have shown that during development, Sox11 is upregulated in response to neurotrophins NGF and GDNF (Jankowski et al., 2004). Evidence in adult primary DRG neurons shows that within the first 24h *in vitro*, the presence of NGF actually inhibits the upregulation of Sox11 mRNA. However, after five days *in vitro*, NGF enhances the levels of Sox11 (Fig 31). It is tempting to speculate that the neurotrophins have a dual function after injury; one that initially is inhibitory to pro-regeneration genes followed by a period of axon growth promotion. This is plausible since delivery of NGF or GDNF to the DRG neurons before nerve transection reduces the expression of ATF3 (Averill et al., 2002). This study suggested that nerve axotomy removes the DRG

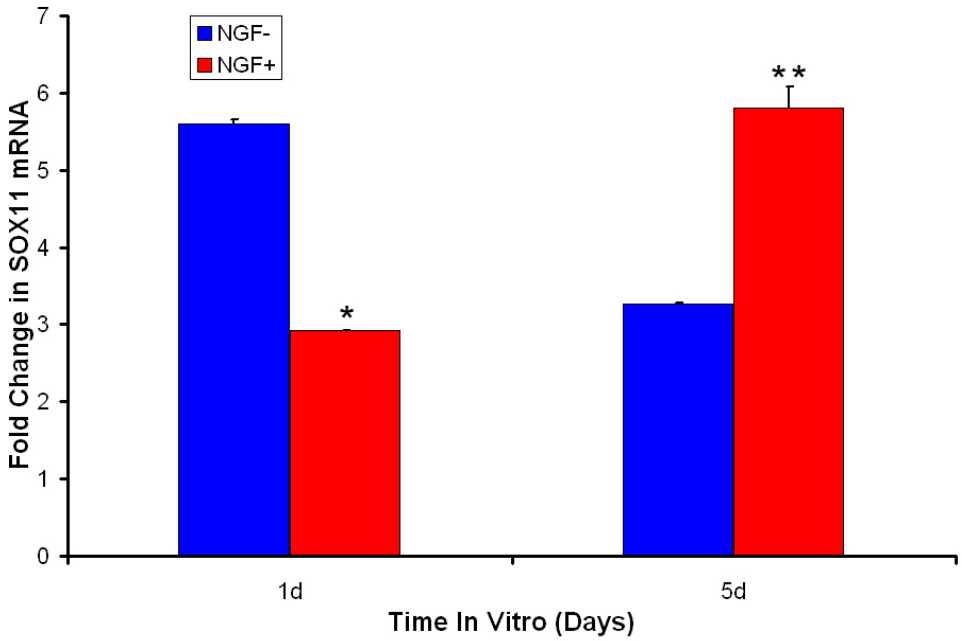

Fig. 31: Fold Change in Sox11 mRNA after one day and five days *in vitro* in the presence or absence of NGF. Sox11 mRNA normally increases 5.6-fold after one day and falls to 3.3-fold after five days (blue bars; NGF-). The presence of NGF prevents the initial increase in Sox11 expression (2.92-fold increase) but induces more Sox11 mRNA expression after five days *in vitro* (5.8-fold increase) relative to cells grown without NGF (red bars; NGF+). * p value < 0.05; ** p value < 0.08, relative to NGF- cells.

neurons from their peripheral source of neurotrophins and replenishing this source prior to axotomy prevents normal upregulation of injury markers and pro-regeneration genes like ATF3. Our *in vitro* evidence supports this hypothesis since NGF can inhibit Sox11 expression at 24h. However, neurotrophins are known to be upregulated in the *targets* of sensory neurons after various peripheral injuries (Ji et al., 2003; Jin et al., 2002) and at the site of injury in support cells (Omura et al., 2005; Lee et al., 2001; Syroid et al., 2000) *after 48h* (Zhou et al., 1999). These data suggest that an upregulation of peripheral neurotrophins after injury serves to prepare the regenerating axons for re-innervation or to induce survival promoting mechanisms and programs of axon growth maintenance. The timing of the different effects of neurotrophins after axotomy also suggests that neurotrophins could have a dual function. So it is reasonable to suggest that peripheral axotomy induces the expression of pro-survival and pro-regeneration genes like Sox11 and ATF3 and immediately replenishing the neurotrophic supply induces a pathway of signaling in the cells that prevents this upregulation. However, after the initial upregulation of Sox11 and ATF3 has occurred, neurotrophins can be necessary for axon growth as shown during development (Arumae et al., 1993; Yan et al., 2003). Maintenance of Sox11 expression which we have shown is necessary for neurite growth and regeneration after the initial stage of nerve injury, may therefore involve neurotrophic signaling.

The fact that Sox11 is differentially regulated by NGF at 24h and 5d *in vitro* suggests a dual function for the neurotrophic regulation of axon outgrowth. Studies involving other Sox genes and signaling pathways have described phenomena similar to our data. Bone morphogenetic protein (BMP) signaling in early stages of limb chondrogenesis is pro-apoptotic while expression of Sox8 and 9 are necessary for induction of chondrogenesis. After the initial

stages of chondrogenesis, BMP signaling was found to be necessary for maintaining the expression and function of Sox9 (along with Sox5 and 6) in late stage chondrocyte differentiation (Chimal-Monroy et al., 2003). Future studies designed to determine if the neurotrophins truly play two roles during injury responses and subsequent regeneration/ neurite growth will be necessary to establish these hypotheses.

The downstream targets of the neurotrophins or upstream signaling pathways that may be responsible for changes in Sox11 are currently unknown. Mitogen activated protein kinase (MAPK) p38 is activated at the site of injury within three minutes after nerve transection (Zrouri et al., 2004) and is one possible candidate for the upregulation of Sox11 after injury. Sox11 also contains multiple protein kinase C (PKC) phosphorylation sites, and PKC is known to be upregulated after peripheral injury as well (Wiklund et al., 1996). Understanding how Sox11 is regulated after injury, whether it is via neurotrophic signaling or some other means is an important question that needs to be addressed in the future to fully determine the functional significance of Sox11 after injury. Future endeavors should include experiments to determine if MAPKs, protein kinases or neurotrophins are responsible for the upregulation of Sox11 after nerve regeneration to decipher the mechanism of Sox11 actions.

DRG neurons are known to regenerate their peripheral axons but not their central axons (Snider et al., 2002) and it is known that certain transcriptional mechanisms are initiated after peripheral but not central nerve injury (Kenny and Koscis, 1997). One example of a gene that is significantly increased after peripheral axotomy but not central axotomy is ATF3 (Seijfers et al., 2006; Shortland et al., 2006). We have suggested that Sox11 may be necessary for the

upregulation of ATF3 after peripheral nerve injury; therefore, it is reasonable to hypothesize that Sox11 will not be altered as a result of central nerve injury. Our data shows that L5 rhizotomy does not induce a significant increase in Sox11 in the L5 DRGs. In fact, there is a significantly larger increase in Sox11 mRNA after sciatic nerve axotomy compared to L5 rhizotomy (Fig. 32). Since we have shown that Sox11 is necessary for proper neurite growth and saphenous nerve regeneration, possibly through its regulation of ATF3, is it reasonable to suggest that the lack of ATF3 from central injury is due to the lack of Sox11. In this regard, Sox11 is a likely candidate gene to describe the transcriptional discrepancies that are present in DRG neurons between central and peripheral injuries. A detailed analysis of the role of Sox11 after rhizotomy will be necessary to elucidate the function of Sox11 after central nerve injury and whether artificial induction of Sox11 after injuries of this kind enhances central axon regeneration of the dorsal columns.

In conclusion, the data described herein suggests that the HMG transcription factor Sox11 is necessary for neuron survival and neurite growth after injury (Fig. 33). Sox11 appears to be implementing its effects on survival and axon regeneration through regulation of genes linked to apoptosis and neurite growth. Although the direct mechanism of how Sox11 itself is regulated is currently unknown, evidence points to a complex regulation of Sox11

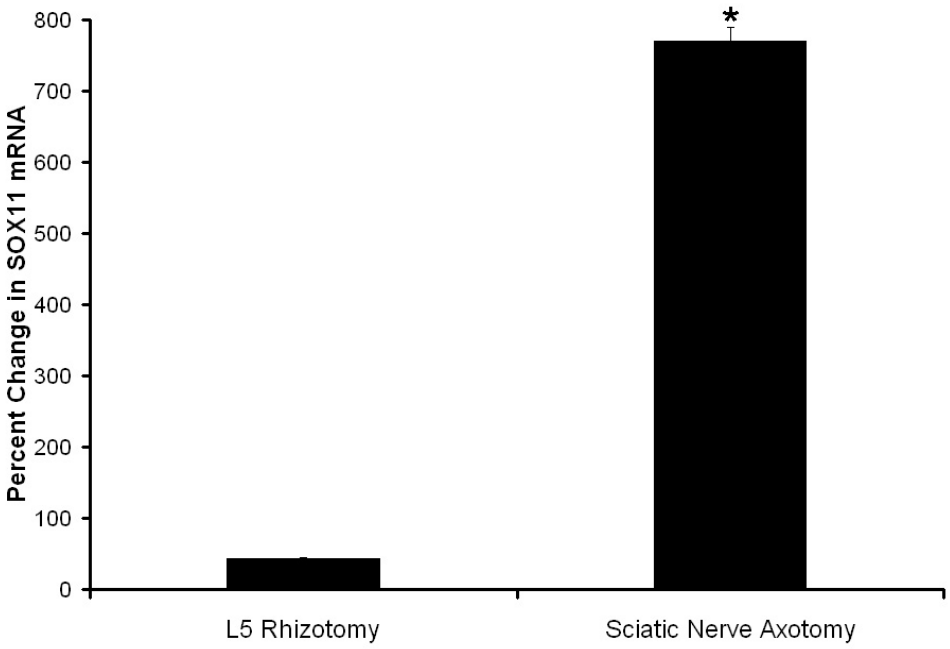

Fig. 32: Fold change in Sox11 mRNA after L5 rhizotomy and sciatic nerve axotomy. Three days after L5 rhizotomy, there is only a small increase in Sox11 mRNA in the L5 DRGs relative to the contralateral ganglia. Four days after sciatic nerve axotomy, there was a large increase in Sox11 mRNA relative to the contralateral ganglia. Axotomy was found to significantly increase the levels of Sox11 compared to rhizotomy (*p value < 0.001).

expression through neurotrophic signaling. The molecule(s) downstream of neurotrophin receptors and upstream of Sox11 remains to be determined. Nevertheless, these studies are the first evidence that developmentally regulated transcription factor Sox11 can regulate two separate pathways of apoptosis and neurite growth (and possibly other pathways linked to the general injury response) is induced after peripheral nerve injury in the adult. These experiments may provide evidence to develop new therapies for patients that experience poor nerve regeneration or receovery following nerve injury. These data may also provide insight to the differences that arise between peripheral and central nerve injuries and whether genes like Sox11 can be induced to promote both peripheral and central nerve regeneration.

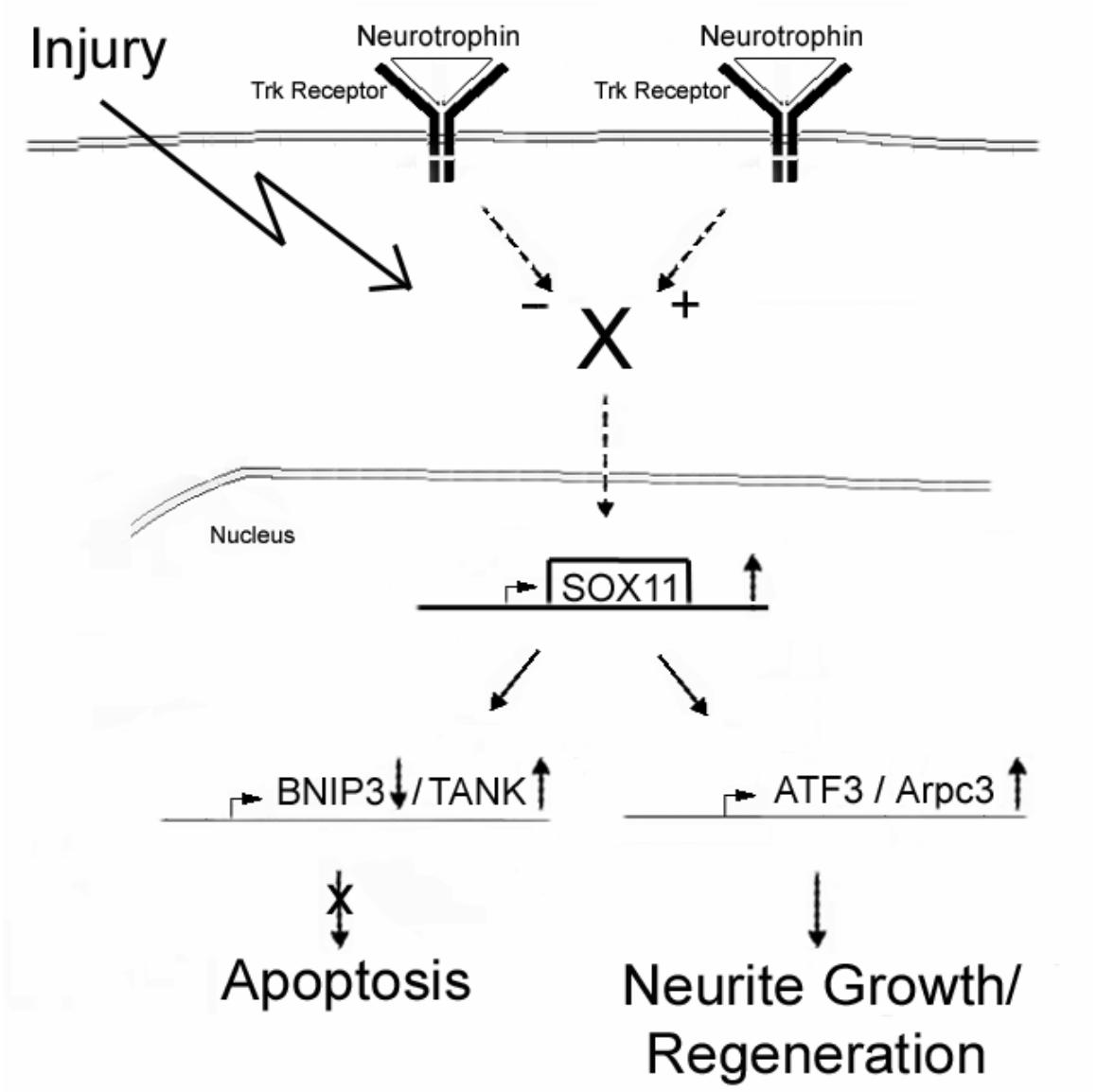
Injury
Neurotrophin
Trk Receptor
Neurotrophin
Trk Receptor
−
X
+
Nucleus
SOX11
BNIP3 / TANK
ATF3 / Arpc3
Apoptosis
Neurite Growth/
Regeneration

Fig. 33: Summary diagram of the sox11 regulation of the injury response**.** Injury to sensory neurons in conjunction with both positive and negative neurotrophic signaling (through an undetermined factor) results in a balanced increase in Sox11 expression. The Sox11 transcription factor then has the function of turning off the transcription of pro-death genes like BNIP3 while it promotes the transcription of pro-survival genes like TANK and pro-regeneration genes like ATF3 and Arpc3. The overall effect of enhanced levels of Sox11 in response to nerve injury is to promote neurite growth and regeneration while inhibiting apoptosis.

APPENDIX A: ADDITIONAL INFORMATION

Sox11 may also have an uncharacterized role in the general injury response or injury induced pain states. Sox11 regulates the expression of many genes known to be associated with nerve injuries *and* inflammation (Fig. 34; Molliver et al. 2005; Costigan et al., 2002) and thus may underlie the means to which changes in gene expression are associated with peripheral inflammation, nerve transection or pain in general. RT-PCR analysis of 12 genes linked to peripheral inflammation revealed that seven of them were differentially regulated by overexpresion of Sox11. N-methyl-d aspartate (NMDA) receptor subunits NR1 and NR2 are both changed in the DRGs after injection of complete Freund's adjuvant (CFA) into the hindpaw (Molliver et al., 2005; Wang et al., 1999). Transfection of Neuro2A cells with a plasmid vector containing Sox11 driven by a CMV promoter shows that there is a decrease in NR1 and an increase in NR2 receptor subunit expression similar to what has been shown *in vivo* after CFA injection. We also found that receptors mGluR1, mGluR5 and GFRα1 which have been linked to sensory neuron sensitivity (Huang et al., 2005; Crawford et al., 2000; Pollock et al., 1999; Bennett et al., 2000) were also altered as a result of enhanced Sox11 expression. Transfected Neuro2A cells also show that transient receptor potential vanilloid 1 (TrpV1) and TrpV2 were downregulated 24h after transfection. TrpV1 has been linked to noxious heat responses in mice (Caterina et al., 1997) and is known to be upregulated in response to inflammation in the DRGs (Bar et al., 2004; Ji et al., 2002; Molliver et al., 2005; Wilson-Gerwing et al., 2005) but downregulated by axotomy (Michael and Priestley, 1999). TrpV2 displays similar features after inflammation as TrpV1 (Shimosato et al., 2005) but does not appear to be heavily regulated by

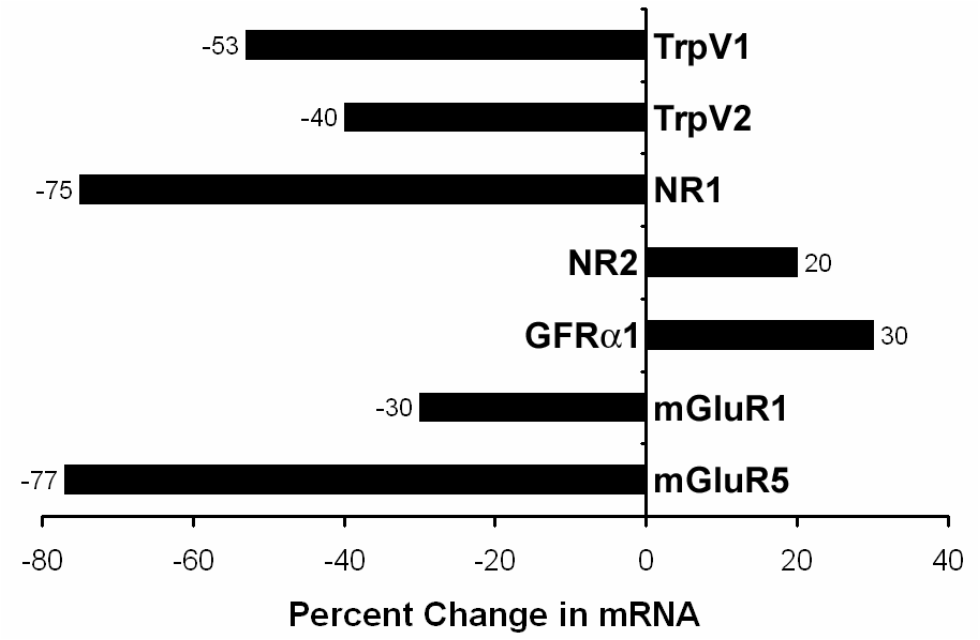

Fig. 34: Alternate changes in gene expression in Neuro2A cells transfected with plasmid vectors containing Sox11 and an eGFP reporter. Many genes linked to neuronal sensitivity are altered by enhanced levels of Sox11. Transient receptor potential vanilloid receptor 1 (TrpV1), TrpV2, the NR1 subunit of the N-methyl-d-aspartate (NMDA) receptor, metabotropic glutamate receptor 1 (mGluR1) and mGluR5 are all decreased by overproduction of Sox11. The NR2 subunit of the NMDA receptor and GDNF-family receptor α1 (GFRα1) are both increased by Sox11 overexpression.

axotomy (Gaudet et al., 2004). Thus Sox11 may also play a role in neuronal sensitivity or responses to inflammation.

To test whether Sox11 was regulated by inflammation, pilot studies were performed on wild type (WT), GDNF-OE and NGF-OE DRGs at multiple time points after injection of CFA into the hindpaw. Surprisingly, Sox11 was regulated to a small degree by peripheral inflammation. GDNF-OEs showed a larger increase in Sox11 four days after CFA injection than WT mice (Fig. 35); however, NGF-OEs not only had a lower baseline level of Sox11 in the DRGs, but Sox11 was not altered at any time point after injection of CFA. The results are intriguing since both NGF- and GDNF-OEs have increased numbers of nociceptors (Albers et al., 1994; Zwick et al., 2002). It will be interesting to investigate in the future whether the changes in gene expression that are induced by overexpression of Sox11 *in vitro* result in the changes in gene expression seen *in vivo* after CFA injection in both WT and transgenic animals.

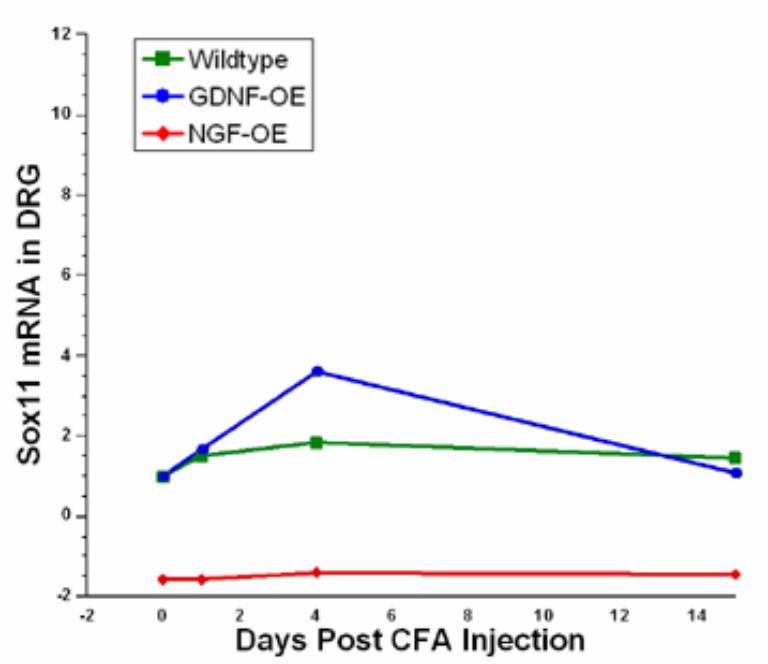

Fig. 35: Fold change in Sox11 mRNA in the L4/L5 DRGs after injection of complete freund's adjuvant (CFA) into the hindpaw of wildtype (WT), GDNF overexpressing (GDNF-OE) and NGF-overexpressing (NGF-OE) mice. CFA injection induces a small increase in the DRGs of WT mice that peaks four days after injection (1.85-fold increase) and returns to baseline after two weeks. GDNF-OEs show a similar pattern but the peak level of expression at four days shows a 3.6-fold increase. Surprisingly, NGF-OEs did not display an increase in Sox11 expression after injection of CFA into the hindpaw. In fact, NGF-OEs have a lower baseline level of Sox11 relative to their WT littermates (1.56-fold decrease).

APPENDIX B: LIST OF ABBREVIATIONS

ABC- avidin biotin peroxidase

Arpc3- actin related protein complex subunit 3

ATF3- activating transcription factor 3

Bad- Bcl-associated death promoter

Bax- Bcl-2 associated x protein

Bcl10- B-cell lymphoma-like 10

Bcl2- B-cell lymphoma-like 2

Bcl2a1d- Bcl2 associated protein 1d

Bcl2l10- Bcl-2-like 10

BDNF- brain derived neurotrophic factor

BI- branching index

Bid- BH3- interacting domain death agonist

Biklk- Bcl-2 interacting killer like

Birc6- Baculoviral IAP repeat-containing 6

Blk- B lymphoid kinase

BMP- bone morphogenetic protein

BNIP3- Bcl2 interacting protein NIP3

Bok- Bcl-2 related ovarian killer protein

BrdU- bromodeoxyuridine

Casp- caspase

Casp2- caspase 2

Casp3*- activated caspase 3

Casp8ap2- caspase 8 associated protein 2

Casp9- caspase 9

CFA- complete Freund's adjuvant

CGRP- calcitonin gene related peptide

CMV- cytomegalovirus

CNS- central nervous system

DAB- 3, 3-diaminobenzidine

DAPI- 4, 6-diamino-2-phenyl-indole, dihydrochloride

DIC- differential interference contrast

DIV- days *in vitro*

DRG- dorsal root ganglion

DUSP6- dual specificity phosphatase 6

EC- embryonic carcinoma

EM- electron microscopy

ERK- extracellular signal related kinase

Fas- TNF receptor

FGF- fibroblast growth factor

GABA- gamma amino butyric acid

GAPDH- glyceraldehyde-3-phosphate dehydrogenase

GDNF- glial cell line derived neurotrophic factor

GDNF-OE- GDNF overexpressor

GFR- GDNF family receptor

GPI- glycosyl phosphatidyl inositol

HBSS- Hank's balanced salt solution

HMG- high mobility group

IAP- inhibitor of apoptosis

IB4- isolectin B4

JNK- c-jun N-terminal kinase

MAPK- mitogen activated protein kinase

Mcl1- myeloid cell leukemia sequence 1

MEM- minimal essential media

MEMS- MEM containing fetal bovine serum

MEMS-RA- MEMS containing 20μM RA

mGluR- metabotropic glutamate receptor

N52- neurofilament 200

Neuro2A- neuroblastoma 2A

NF- neurofilament

NFκB- nuclear factor κB

NGF- nerve growth factor

NGF-OE- NGF overexpressor

NMDA- N-methyl-d-aspartate

NSE- neuronal specific enolase

NT-3- neurotrophin 3

PBS- phosphate buffered saline

PenCON/ Pen-siCON- Penetratin linked control siRNAs

PenSOX/ Pen-siSOX- Penetratin linked Sox11 targeting siRNAs

PGP- protein gene product

PKC- protein kinase C

PNS- peripheral nervous system

PTPro- protein tyrosine phosphatase receptor type O

RA- retinoic acid

RAR- retinoic acid receptor A

RISC- RNA induced silencing complex

RNAi- RNA interference

RT-PCR- reverse transcriptase polymerase chain reaction

RT- room temperature

RXR- retinoic acid receptor X

SC- spinal cord

siControl- non-targeting control siRNA

siRNA- small interfering RNA

Sox- SRY-box containing gene

Sprr1a- small proline rich protein 1a

SwC- Schwann cell

TANK- Traf member- associated NfκB activator

TG- trigeminal ganglion

TNF- tumor necrosis factor

Tnfrsf21- TNF receptor superfamily 21

Trk- tyrosine receptor kinase

TrpV- transient receptor potential vanilloid

TUNEL- terminal deoxynucleotidyl nick end labeling

WD- Wallerian degeneration

WT- wild type

β-ME- β mercaptoethanol

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