IDENTIFICATION OF TRANSCRIPTIONAL TARGETS OF THE NERVE INJURY-INDUCED TRANSCRIPTION FACTOR SOX11

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ABSTRACT

Transcriptional regulation is an important component of the peripheral sensory neuronal response to injury. The transcription factor Sox11 is upregulated in injured dorsal root ganglion (DRG) neurons. Sox11 expression has been linked to neuronal survival and improved regeneration following nerve injury; however, its transcriptional targets are largely unknown. Towards understanding the pro-regenerative effects of Sox11, this dissertation focused on the identification of novel Sox11 transcriptional targets. Targets were initially identified via microarray screening and were validated by approaches including real-time PCR, luciferase reporter assays, western blot and chromatin immunoprecipitation (ChIP). Brain-derived neurotrophic factor (BDNF), adenomatous polyposis coli (APC) and TRAF-associated activator of NF-kappa B (TANK) were identified as putative Sox11 transcriptional targets. BDNF and APC have previously been linked to pro-regenerative pathways in DRG neurons. BDNF is a growth factor that has been shown to increase the intrinsic growth state of neurons (Geremia et al., 2010). APC is a modulator of Wnt signaling pathways and a plus-end microtubule stabilizing protein and been shown to be essential for neurite extension following peripheral axotomy (Zhou et al., 2006). TANK is a novel gene in the context of nerve regeneration signaling; my experiments show that TANK is highly expressed in dissociated DRG neuronal cultures, that TANK knockdown increased apoptosis in Neuro-2a cells following TNF-alpha stimulation and that TANK expression may modulate activation of c-Jun N-terminal kinase (JNK).

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PREFACE

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1.0 INTRODUCTION

1.1. Role of transcriptional regulation in nerve regeneration.

The successful regeneration of nerve fibers following a crush or transection injury is highly dependent on the coordinated response of cellular pathways that are modulated by extrinsic and intrinsic factors (**Figure 1**). Extrinsic factors can be broadly defined as conditions external to the cell that lead to changes in cell signaling and therefore ultimately control the intrinsic mechanisms of a neuronal response to injury. These factors include ion influx through damaged membranes, changes in the level of signaling molecules such as growth factors and cytokines and the nature of the surrounding tissue environment.

Following a nerve injury in which the cell membrane is disrupted (e.g. axotomy or crush injuries), there is a brief window of time before the damaged axon re-seals and becomes more resistant to current flow and molecular diffusion (Berdan et al., 1993). During this time there is no separation between the intracellular and extracellular environments and ions are free to travel down their electrochemical gradients. This results in massive calcium and sodium influxes and discharges of injury-evoked action potentials (Berdan et al., 1993). Calcium and sodium influx also occur following milder forms of membrane disruption, including stretch injuries and other nondisruptive trauma (Maxwell et al., 1995, Wang et al., 2009a). This calcium influx and depolarization immediately after nerve injury is essential for neurite extension following peripheral nerve injury; neurons fail to extend neurites when deprived of extracellular calcium at the time of injury, as well as when depolarization and/or repolarization is prevented by manipulating extracellular potassium levels (Berdan et al., 1993, Chu and Tator, 2001).

The concentration and identities of growth factors, cytokines, chemokines, and other signaling peptides forms another important subset of extrinsic factors affecting neuronal

regeneration. Immediately following axotomy, neurons are severed from their target-derived source of trophic support. Compensatory changes in the local abundance of signaling peptides appears to be essential for maintaining neuronal survival and facilitating regeneration after axotomy. In addition to the increase in local synthesis of neurotrophins and GDNF family-member growth factors (Funakoshi et al., 1993, Hammarberg et al., 1996, Tonra et al., 1998, Shen et al., 1999, Zhou et al., 1999, Lee et al., 2001, Obata et al., 2003a), other injury-associated peptides that increase in DRG tissue following axotomy and have been implicated in regeneration signaling in injured peripheral sensory neurons include members of the fibroblast growth factor (FGF) family (Ji et al., 1995, Ji et al., 1996, Huber et al., 1997, Li et al., 2002b), transforming growth factor-beta (Kiefer et al., 1995, Taskinen et al., 2004), ciliary neurotrophic factor and the related leukemia-inhibitory factor (CNTF/LIF) (Ji et al., 1995, Zigmond et al., 1996, Murphy et al., 1997, Ekstrom et al., 2000), and inflammatory cytokines including interleukins 1-alpha, 1-beta, and 6, as well as tumor necrosis factor-alpha (TNF-alpha) (Thompson et al., 1998, Taskinen et al., 2000, Shamash et al., 2002, Ahmed et al., 2005, Cheepudomwit et al., 2008, Miao et al., 2008).

Furthermore, axonal regeneration may only occur in a sufficiently permissive tissue environment. This is well demonstrated by the successful regeneration of peripheral nerves compared to the lack of regeneration in the central nervous system (CNS) where repressive signaling through myelin-associated factors occurs. Schwann cells myelinate peripheral nerves, as opposed to oligodendrocytes which myelinate CNS structures. Following injury and macrophage infiltration, Schwann cells dedifferentiate, proliferate and form Bands of Bunger at the site of injury, which are permissive for neuronal regeneration (Fawcett and Keynes, 1990). Schwann cells do not express some inhibitory factors, such as Nogo-A, which are expressed in

the central nervous system and inhibit regeneration (Pot et al., 2002); furthermore, some inhibitory cues which exist in peripheral myelin, such as semaphorin III, are downregulated following injury whereas their expression is maintained in injured CNS tissue (Pasterkamp et al., 1998). Peripheral nerve also contains important extracellular matrix proteins, including laminin-2 and laminin-8, which are upregulated after nerve injury and appear to facilitate neurite extension. Although peripheral myelin still contains some inhibitory factors, such as myelin-associated glycoprotein (MAG) and oligodendrocyte myelinating glycoprotein (OMG) (reviewed in (Filbin, 2003)), the combination of growth factor stimulation and intrinsic growth potential of injured peripheral neurons are sufficient to overcome these inhibitory cues (Cai et al., 2002, Gao et al., 2003, Gao et al., 2004, Domeniconi and Filbin, 2005).

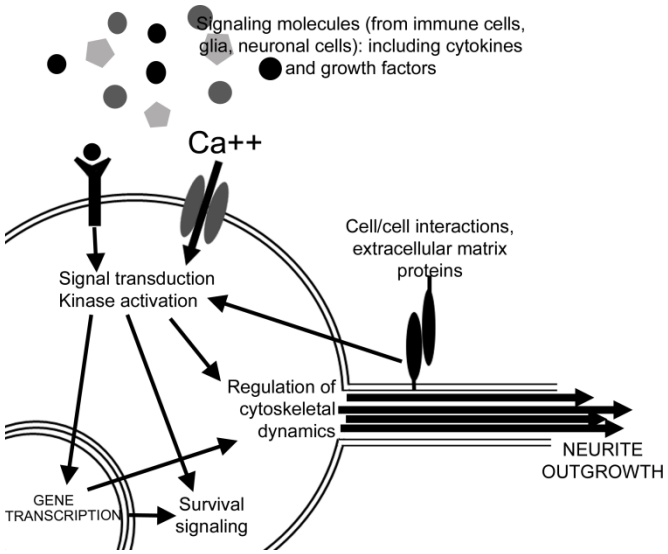

Figure 1: Schematic of factors influencing regeneration signaling in injured neurons. Successful regeneration depends on a combination of extrinsic factors (including ions, signaling molecules and cell-cell interactions) in the external environment of the injured nerve, coupled with the expression of appropriate intrinsic factors within the cell that permit neuronal survival and regeneration of neurites.

Also of critical importance to successful nerve regeneration are intrinsic factors that control the survival and regrowth of nerve fibers. Intrinsic factors may be broadly described as the molecules and signaling processes within an injured neuron that control intracellular mechanisms by which an injured neuron undergoes regeneration, assuming the presence of a sufficiently permissive environment. These factors include signaling receptors and their downstream cell signaling mediators, as well as proteins that regulate transcription of regeneration-associated genes.

Signals from the injured neuron's external environment can only stimulate or inhibit axonal regeneration if the neuron itself expresses the necessary receptors and intracellular machinery to transduce the signal. Thus, one of the major intrinsic factors governing peripheral neuronal regeneration is the expression of receptors that bind extracellular pro-regenerative factors, including the receptors for cytokines, growth factors, and extracellular matrix proteins that are implicated as extrinsic factors in nerve injury (Funakoshi et al., 1993, Sebert and Shooter, 1993, Bradbury et al., 1998, Ito et al., 1998, Kashiba et al., 1999, Xian and Zhou, 1999, Bennett et al., 2000, Lee et al., 2001, Mizusawa et al., 2003). Furthermore, receptor-bound signals must be transduced via cell signaling pathways, meaning that regulation of cell signaling is a major component of the intrinsic neuronal regenerative response. Major pathways implicated in the early activation of peripheral neuronal growth programs following nerve injury include cAMP-dependent signaling, activation of STAT3 downstream of cytokine signaling and activation of c-Jun (Leah et al., 1991, Broude et al., 1997, Kenney and Kocsis, 1998, Soares et al., 2001, Cai et al., 2002, Gao et al., 2003, Gao et al., 2004, Lindwall et al., 2004, Lindwall and Kanje, 2005a, Wang et al., 2009b, Barnat et al., 2010).

cAMP has an effect on cytoskeletal organization, and hence neurite extension, via protein

kinase A (PKA)-dependent inactivation of Rho kinase, as well as via Rho-independent pathways (Cai et al., 1999, Cai et al., 2002, Gao et al., 2004, Murray and Shewan, 2008). Additionally, cAMP-dependent CREB activation can activate immediate early genes (IEGs), including the transcription factors c-Fos, c-Jun, and activating transcription factor 3 (ATF3) (Liang et al., 1996, Sanyal et al., 2002, Kawasaki et al., 2004, Lu et al., 2007, Li et al., 2009).

Transcriptional regulation is an essential component of regeneration because neurons must upregulate production of proteins to rebuild cell membranes and cytoskeletal structures as well as reestablishing cellular homeostasis. Furthermore, growth factor and neurotransmitter receptors, transporters, ion channels and intracellular scaffolds that facilitate intrinsic and extrinsic signaling between neurons, glial cells and target tissues of innervation must be reestablished. Microarray studies have shown that thousands of genes are differentially regulated following peripheral nerve injury; in general, upregulated genes include genes coding for the above-mentioned classes of proteins as well as proteins involved in inflammatory responses, whereas downregulated genes tend to be pro-apoptotic in function (Costigan et al., 2002, Kubo et al., 2002, Rabert et al., 2004, Kuo et al., 2011).

Many transcription factors themselves are differentially regulated following injury. In addition to the early-response, activity-dependent transcription factors described above, other transcription factors are differentially expressed and/or activated following injury as late-response genes. Transcription factor activity increases as retrograde transport of injury signals continue in the initial hours and days following injury. Discrete time intervals following injury are therefore not readily identified since a continuum of target-derived signal loss, change in tissue milieu (e.g., inflammatory cell infiltration) and change in transcriptional activation persist over time. Indeed, changes are likely to continue even after completion of anatomic regeneration

since regenerated sensory neurons frequently display altered response properties (e.g., lower thresholds, different pattern of channel expression). In this study I have focused on events that occur on days 1-3 post nerve injury. This time frame was chosen to expand our understanding of the alterations in gene transcription that underlie the initial response to nerve injury and regeneration. In addition I was specifically interested in understanding the role of the transcription factor Sox11, which we previously found to be increased by one day after nerve injury (Jankowski et al., 2006, Jankowski et al., 2009).

1.2. Overview of Sox family transcription factors

The Sry-related HMG box (Sox) transcription factors belong to a super-family of genes characterized by the presence of the high mobility group (HMG)-box domain (Laudet et al., 1993). The HMG boxes of all Sox factors have at least 50% homology to the HMG box of Sry (sex-determining region Y protein, the first-identified Sox family member), and through this domain they bind the minor groove of DNA (Soullier et al., 1999, Nagai, 2001). The structure of the HMG-box/DNA complex has been solved, and studies using molecular modeling as well as crystallography have determined that DNA binding via the HMG domain induces a significant conformational change in the DNA whereby the major groove is compressed, the minor groove widens, and the DNA helix is significantly bent (Lefebvre et al., 2007). These conformational changes induced in the DNA helix are thought to increase the accessibility of the DNA to other proteins, and to facilitate the formation of complexes of transcription factors upon gene enhancer sequences. **Figure 2** displays a schematic of the HMG-box/DNA complex.

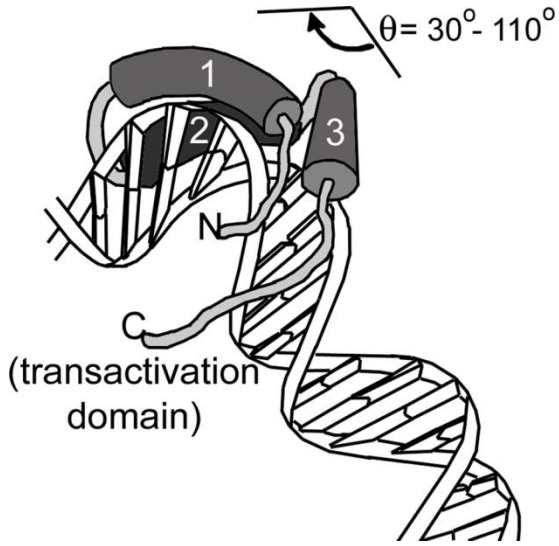

Figure 2. Sox proteins contain an HMG box, which induces a conformational change in DNA. The HMG box contains three alpha-helices (labeled 1, 2 and 3 in figure) that interact with the minor groove of DNA. Sox-DNA binding typically results in a significant conformational change to the DNA strand: the DNA helix is widened and bent between 30-110 degrees. This may result in increased opportunity for other DNA-binding proteins to interact with the DNA strand. In Sox proteins that are transcriptional activators, the C-terminal serves as the transactivating domain. (Adapted from LeFebvre et al., 2007)

The closest related protein family to the Sox family is the HMG-box-containing TCF/LEF family of transcription factors (Laudet et al., 1993, Soullier et al., 1999, Nagai, 2001), which are the effectors of canonical Wnt signaling (Kikuchi et al., 2006). Sox proteins and TCF/LEF proteins bind similar DNA sequences and, in some cases, there can be overlapping binding sites for Sox proteins and TCF/LEF proteins on the same promoter or enhancer region (Kuwabara et al., 2009). Furthermore, Sox proteins may interact with TCF/LEF proteins and modulate their transcriptional activity, either as coactivators or corepressors of TCF-mediated transcription (Zorn et al., 1999, Haremaki et al., 2003, Sinner et al., 2004, Chao et al., 2007, Sinner et al., 2007, Bernard and Harley, 2010, Kormish et al., 2010, Marfil et al., 2010, Martinez-Morales et al., 2010).

The Sox family consists of 20 genes in mouse and human. Up to 30 Sox proteins had been identified before whole-genome sequencing determined that only 20 were Sox genes (Schepers et al., 2002, Lefebvre et al., 2007). Later research confirmed that certain orthologous Sox proteins had been given different acronyms, e.g., Sox22 and Sox12 are, respectively, the human and mouse orthologues of the gene now known as Sox12 (Schepers et al., 2002). The Sox family is further subdivided into groups A-H, based on protein sequence homology (Soullier et al., 1999, Lefebvre et al., 2007). Different Sox groups exhibit different molecular properties and abilities to transactivate or repress gene expression. Groups A, B1, C, E, F, and H interact with co-activators to bind the basal transcriptional machinery, thereby directly participating in transactivation (Kuhlbrodt et al., 1998, Wiebe et al., 2003, Wissmuller et al., 2006, Dy et al., 2008, Kondoh and Kamachi, 2010). Groups B2, D, and G interact with a co-repressor to directly participate in transrepression (Uchikawa et al., 1999, Sinner et al., 2004, Lefebvre et al., 2007), and some Sox proteins in groups A and D act solely in a DNA-remodeling capacity, without interacting with transcriptional co-activators or –repressors meaning that they have the potential to act in either a transactivation or a transrepression complex (Lefebvre et al., 2007). Some Sox proteins are known to function as dimers, particularly the group D proteins with a DNA-remodeling function (Lefebvre et al., 1998, Bernard et al., 2003). Many Sox proteins have been shown to form functional interactions with Pit-Oct-Unc (POU) transcription factors (Kuhlbrodt et al., 1998, Remenyi et al., 2003, Tanaka et al., 2004). Different Sox and POU factors display differing affinities for each other, implying the presence of a partner code dependent on the expression patterns of various members of both families (Kuhlbrodt et al., 1998, Kamachi et al., 2000, Remenyi et al., 2003, Tanaka et al., 2004, Bernard and Harley, 2010, Kondoh and Kamachi, 2010). **Table 1** summarizes Sox binding partners by group.

Group	Members	Function	Known Binding Partners
A	Sry	Transactivator	KRAB (KRAB-O) POU (Oct4) (Oh and Lau, 2006, Jin et al., 2007)
B1	Sox1 Sox2 Sox3	Transactivators	POU (Oct3/4, Brn-2) paired (Pax6) (Remenyi et al., 2003, Kondoh et al., 2004, Tanaka et al., 2004)
B2	Sox14 Sox21	Transrepressors	Unknown
C	Sox4 Sox11 Sox12	Transactivators	POU (Brn-1, Brn-2) (Tanaka et al., 2004, Kim et al., 2008)
D	L-Sox5 Sox5 Sox6 Sox13	Architectural	Homeobox (Hhex, Pdx1) Homodimerization Other (beta-catenin, CtBP2, HDAC1, Prtb) (Lefebvre et al., 1998, Cohen-Barak et al., 2003, Iguchi et al., 2007, Hattori et al., 2008, Marfil et al., 2010)
E	Sox8 Sox9 Sox10	Transactivators	bHLH (Ngn-2, Reb, Olig2) bZip (c-Jun, c/EBPa) zinc finger (Krox-20, Sp1, ER, T3Rb, ZAS1) homeobox (Dlx5, Hhex, Hoxa3, Hoxc4, Meox1) paired (Pax3, Pax6, Prrx1, Prrx2, Alx4) POU (Oct3/4, Brn-1) Other (SF-1, UTF1) (De Santa Barbara et al., 1998, Wissmuller et al., 2006)
F	Sox7 Sox17 Sox18	Transactivators	MADS box (Mef2c) (Hosking et al., 2001)
G	Sox15	Transrepressor	Unknown
H	Sox30	Transactivator	Unknown

Table 1: Sox proteins, their functional roles and known binding partners. Sox family members, overall, are capable of interacting with a wide variety of binding partners. It is thought that specificity of Sox transcriptional targets may depend on the presence of a binding partner code. This dissertation focuses on Sox11, a member of the SoxC group, which is known to interact with POU transcription factors.

In general, Sox proteins regulate large panels of genes. This is likely due to the degenerate nature and short size (7 bp) of the Sox binding site (5'-WWCAAWG-3'), where W = A or T; (Harley et al., 1994). However, different Sox proteins appear to prefer different forms of the consensus Sox-binding sequence (Mertin et al., 1999, Badis et al., 2009). Furthermore, some researchers have proposed slightly longer motifs as particularly high-affinity Sox-binding targets (Mertin et al., 1999). The differences in transcriptional target specificity between different Sox proteins may be dependent on an individual Sox family member's preferential DNA-binding site and preferred binding partners; consequences of Sox gene expression can therefore be expected to vary depending not just on the presence of Sox binding sites but also depending on cellular context and which possible Sox binding partners are present in the nucleus. The functional consequences of Sox protein-mediated gene transcription therefore tend to be wide-ranging. Developmentally, Sox proteins are linked to specification of cell identities (neuronal vs. glial, osteocyte vs. chondrocyte, etc.); and to tissue remodeling, especially epithelial to mesenchymal transitions (Lefebvre et al., 2007).

1.3. SoxC transcription factors

The SoxC subfamily of Sox proteins consists of three members: Sox4, Sox11, and Sox12. All Group C Sox proteins are transactivators (Dy et al., 2008, Hoser et al., 2008, Brennan et al., 2009). The general structure of Group C Sox proteins includes an HMG-box domain and a C-terminal transactivation domain. These proteins are not known to require post-translational modifications for activation; furthermore, Sox11 contains a sequence in its N-terminal which is known to act as a nuclear localization sequence (NLS) in members of other Sox subfamilies.

Sox4 and Sox11 are more homologous to each other than Sox12, which has a shorter N-terminal than the other two Group C members (Dy et al., 2008).

The expression patterns of Sox4, Sox11, and Sox12 show considerable overlap during development, and some reciprocity of function, implying that these proteins have a degree of redundancy (Dy et al., 2008, Hoser et al., 2008). SoxC proteins are widely expressed during embryonic development, and are of particular importance in specifying neuronal fate (Jay et al., 1995, Cheung et al., 2000, Hong and Saint-Jeannet, 2005, Bergsland et al., 2006, Potzner et al., 2007, Dy et al., 2008, Hoser et al., 2008, Bhattaram et al., 2010, Penzo-Mendez, 2010, Potzner et al., 2010, Thein et al., 2010). Sox4 and Sox11 coordinate to specify neuronal identity vs. oligodendrocyte identity during development (Bergsland et al., 2006, Potzner et al., 2007); additionally, Sox4, Sox11, and Sox12 are implicated in non-neuronal roles including tissue remodeling and the normal development of heart, lungs, pancreas, spleen, and the immune system (Penzo-Mendez, 2010). Sox4- and Sox11-null mice are non-viable due to cardiac deficits; however, the Sox12-null mouse develops normally, due to compensation for lost Sox12 function by Sox4 and Sox11 (Hoser et al., 2008). Similarly, Sox4 and Sox11 display some extent of reciprocal functionality in CNS development: only combined deletion of Sox4 and Sox11 results in obvious abnormalities in brain and spinal cord development (Potzner et al., 2010, Thein et al., 2010).

1.4. Role of Sox11 in development and disease

Of the three Group C Sox proteins, Sox11 is the most potent activator of transcription; this appears to be entirely dependent on its C-terminal transactivation domain (Dy et al., 2008). It has also been suggested that Sox11 may contain an autoinhibitory element on its C-terminus,

which may mean that presence of a Sox11-binding partner is required for Sox11-mediated transactivation (Dy et al., 2008). Specific binding partners have not been fully characterized for Group C Sox proteins. However, Sox11 is known to be capable of associating with the Brn family of transcription factors (see **Table 1**).

Sox11 is expressed throughout the neuroepithelium during development and functions in concert with Sox4 to specify neuronal fate in neural progenitors that have ceased proliferation (Jay et al., 1995, Uwanogho et al., 1995, Hargrave et al., 1997, Azuma et al., 1999, Bergsland et al., 2006). At later times in development (up to 15.5 dpc in mouse), Sox11 is expressed at high levels at specific sites in the central nervous system, including the forebrain, hindbrain, and caudal end of the spinal cord (Hargrave et al., 1997). Sox11 is strongly expressed in the peripheral nervous system, including the trigeminal and dorsal root ganglia, from 11.5-18 dpc in mouse (Hargrave et al., 1997). The expression of Sox11 in the peripheral nervous system is crucial to the normal survival, maturation, and outgrowth of both primary sensory and sympathetic neurons. Recent studies of a Sox11-null mouse show that development of dorsal root ganglion (DRG) neurons as well as development of sympathetic neurons in the superior cervical ganglion (SCG) is severely perturbed in the absence of Sox11 (Potzner et al., 2010, Lin et al., 2011).

In addition to neuronal fate specification, Sox11 also functions in non-neuronal tissues during development. For example, Sox11 is expressed in the heart where it has an essential role in morphogenesis, i.e., the Sox11 knockout mouse is embryonic-lethal due to malformations of the cardiac outflow tract (Sock et al., 2004).

1.5. Sox11 in peripheral nerve injury

In the adult mouse, Sox11 is expressed at low levels in dorsal root ganglion (DRG) neurons. However, following peripheral nerve injury Sox11 expression rises rapidly, and this increase correlates with neuronal survival and regeneration (Tanabe et al., 2003, Jankowski et al., 2006, Jankowski et al., 2009). Blocking the upregulation of Sox11 in injured neurons results in an increase in apoptosis and decrease in neurite extension in cultured DRG neurons (Jankowski et al., 2006). A delay in nerve regeneration also occurs if Sox11 is transiently knocked down *in vivo* prior to nerve axotomy (Jankowski et al., 2009). In addition, herpes simplex virus (HSV)-mediated expression of Sox11 in DRG neurons prior to saphenous nerve crush injury enhances the rate of regeneration (Jing et al., unpublished). HSV-Sox11 treated mice have faster nerve regeneration of myelinated and unmyelinated axons as indicated by an increase in toluidine blue stained myelinated axon profiles, an increase in the area of peripherin-positive Remak bundles, faster recovery of behavioral responses to a nerve or skin pinch test and faster reinnervation of the foot skin.

1.6. Transcriptional targets of Sox11

The working hypothesis of this dissertation is that the neuroprotective and pro-regeneration effects of Sox11 are due to the ability of Sox11 to enhance transactivation of specific genes that function in the injured neuron to turn the overall direction of cell signaling away from apoptotic pathways and towards survival/regeneration pathways. Potential transcriptional targets of Sox11 were identified by gene expression array analysis of cDNAs isolated from cultured DRG neurons treated with small interfering RNAs (siRNAs) to Sox11. Sequence analysis allowed identification of potential Sox binding sites in the putative 5' proximal promoter region of genes

which were downregulated following Sox11 knockdown. This approach identified potential targets of Sox11 regulation that included the TRAF-interacting protein TANK (TRAF family member-associated activator of NF-kappa B), the neurotrophin brain-derived neurotrophic factor (BDNF) and the adenomatous polyposis coli (APC) gene. These are all novel transcriptional targets of Sox11 with possible relationships to survival and neurite-outgrowth pathways, summarized in **Figure 3**.

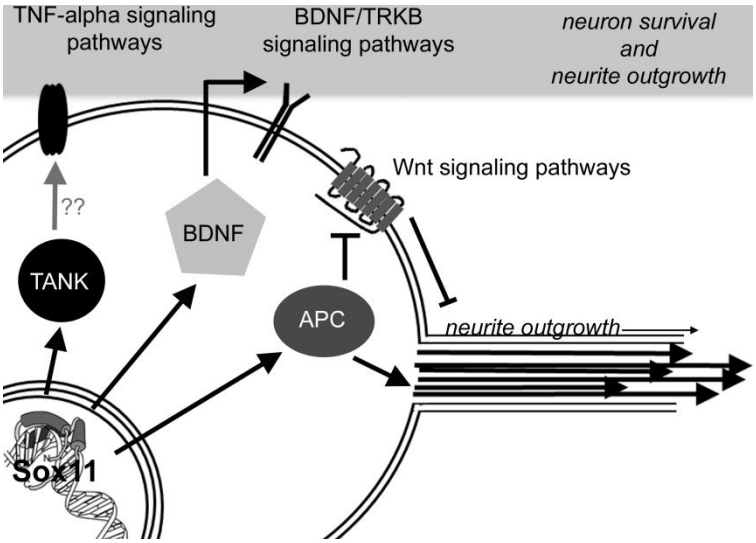

Figure 3. Putative transcriptional targets of Sox11. Sox11 may facilitate transcription of genes encoding TANK, BDNF and APC. TANK is a modulator of the TNF-alpha signaling pathway, which has demonstrated effects on neuronal survival and neurite extension. BDNF is a neurotrophin that has been shown to have pro-regenerative effects. APC is a microtubule-binding protein and Wnt signaling regulator, which is thought to be essential for neurite regeneration.

Previously described targets of Sox11 activation include the neuronal cytoskeletal protein beta-tubulin III (Tubb3) (Dy et al., 2008). Twenty-six additional Sox11 transcriptional targets were most recently identified by a group working on Sox11 in the context of mantle cell

lymphoma (Wang et al., 2010); however, these targets were identified using the microarray approach and the majority of them were not validated by other methods.

1.7. BDNF and nerve injury

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors. Neurotrophin signaling is critical for the survival, development, neurite extension, and axonal guidance of peripheral sensory neurons (Ernfors et al., 1995a, Ernfors et al., 1995b, Liu et al., 1995, LeMaster et al., 1999, Ernsberger, 2009, Valdes-Sanchez et al., 2010). In the adult, BDNF is a key factor involved in synapse formation and synaptic plasticity (Cowansage et al., 2010), as well as acting as a target-derived factor involved in the maintenance of neuronal survival and neuronal connectivity to synaptic targets (Yoshii and Constantine-Paton, 2010). Additionally, neurotrophins including nerve growth factor (NGF) and BDNF are important modulators of the injury response in primary sensory afferents. BDNF is rapidly upregulated following injury (Funakoshi et al., 1993) and has been shown to activate intrinsic neuronal growth pathways after injury (Tonra et al., 1998, Geremia et al., 2010). Additionally, BDNF is retrogradely transported to the spinal cord dorsal horn following nerve injury; this transport of BDNF contributes to the sensitization of dorsal horn neurons and the development of chronic pain following nerve injury (Funakoshi et al., 1993, Sterne et al., 1998, Tonra et al., 1998, Shen et al., 1999, Lee et al., 2001, Ahmed et al., 2005, Jiang et al., 2005, Zhou et al., 2005).

The primary receptor for BDNF is the receptor tyrosine kinase family member TrkB. Upon binding of BDNF, TrkB dimerizes and autophosphorylates, leading to the activation of diverse downstream signaling pathways including the PI3K/Akt pathway, multiple MAP kinase signaling cascades, Rho GTPases, and the phospholipase C pathway (Reichardt, 2006). Activation of

these signaling pathways results in the transcription of several immediate-early genes, including CREB and Fos (Reichardt, 2006). BDNF/TrkB-mediated CREB activation in particular has been linked to neurite outgrowth (Gao et al., 2003, Ji et al., 2005). BDNF also has a low-affinity receptor, the p75 neurotrophin receptor (p75 NTR), which is a TNF-alpha receptor superfamily member. This receptor is capable of binding all neurotrophin ligands and is primarily implicated in survival vs. apoptosis signaling. Neurotrophin activation of p75 is thought to be largely pro-apoptotic following nerve injury (Lowry et al., 2001, Sorensen et al., 2003, Boyle et al., 2005, Zhou et al., 2005, Lebrun-Julien et al., 2009, Jiang and Jakobsen, 2010); however, this signal is mitigated dependent on which other intrinsic factors are present in an injured neuron; p75 can potentiate Trk receptor-mediated survival and neurite outgrowth (Barrett and Bartlett, 1994, Hantzopoulos et al., 1994, Hartmann et al., 2004, Diolaiti et al., 2007, Fobian et al., 2010, Michaelsen et al., 2010), and p75-induced apoptosis is dependent on expression of its coreceptor, sortilin (Nykjaer et al., 2004, Teng et al., 2005). Additionally, p75 is required for normal survival and differentiation of sensory neurons during development (Lee et al., 1992, Barrett and Bartlett, 1994, Lee et al., 1994, Stucky and Koltzenburg, 1997). Abrogation of p75-mediated apoptotic signaling in adult DRG neurons is thought to be one of the major reasons for their high resistance to axotomy-induced apoptosis compared to neonatal DRG neurons (Zhou et al., 2005); in injured DRG, the balance of BDNF-mediated signaling is shifted towards TrkB-dependent signaling.

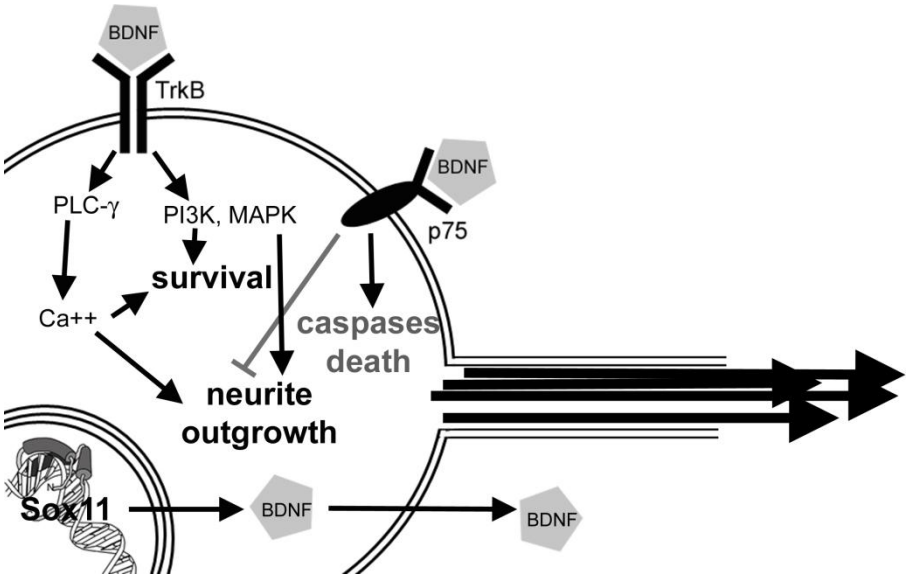

Figure 4. BDNF pathways in the context of nerve injury. Secreted BDNF may bind to either the high-affinity TrkB receptor or the low-affinity p75 receptor. TrkB receptor activation leads to survival signaling and neurite extension downstream of the PI3K/Akt signaling pathway as well as via activation of MAP kinases. Additionally, TrkB activation leads to PLC-mediated increases in intracellular calcium, which are also essential for neurite outgrowth. Activation of the p75 receptor may lead to inhibition of neurite outgrowth as well as activation of caspases.

1.8. APC and nerve injury

Adenomatous polyposis coli (APC) is a microtubule plus end binding protein that is downstream of phosphatidylinositol-3-kinase (PI3K) and glycogen synthase kinase 3-beta (GSK-3β) activation (Barth et al., 2008). The association of APC with the plus end of microtubules stabilizes microtubules and prevents their collapse (Zumbrunn et al., 2001, Kroboth et al., 2007). APC has been shown to be an essential component of axonal extension during development (Votin et al., 2005). APC and GSK-3b form a scaffold that is associated with cytoskeletal receptors including neurotrophin receptors (Li et al., 2005a, Zhou et al., 2006, Katoh, 2007,

Matsumoto et al., 2010). The activation of APC downstream of PI3K allows APC to bind microtubules, thereby locally stabilizing the microtubule cytoskeleton and permitting targeted neurite outgrowth in the direction of neurotrophin ligands (Zhou et al., 2006).

APC and GSK-3b also function as modulators of noncanonical and canonical Wnt signaling pathways (McCartney et al., 1999, Ahmed et al., 2002, Akong et al., 2002, Votin et al., 2005, Farias et al., 2007, Hayden et al., 2007, Katoh, 2007, Purro et al., 2008, Takacs et al., 2008, Ivaniutsin et al., 2009, Paridaen et al., 2009, Matsumoto et al., 2010). Noncanonical Wnt signaling pathways include the divergent canonical Wnt pathway, mediated by the Frizzled receptors, as well as the Ryk receptor-dependent planar cell polarity pathway. Activation of these pathways leads to alterations in microtubule stability and therefore cause changes in cell polarity, extension of cell processes and migration. APC-mediated microtubule stabilization is a target of stimulatory Wnt signals (Matsumoto et al., 2010); loss of APC from microtubule plus-ends is a primary driver of the microtubule-destabilizing function of inhibitory Wnt signals such as Wnt3a (Purro et al., 2008). Furthermore, APC is a negative regulator of canonical Wnt signaling (Ahmed et al., 2002, Takacs et al., 2008, Paridaen et al., 2009). The APC/GSK-3b scaffold inactivates beta-catenin, causing it to become ubiquitinated and degraded in the proteosome (Easwaran et al., 1999). This prevents the beta-catenin dependent activation of the TCF/LEF family of transcription factors, ordinarily the downstream effectors of Wnt/Frizzled signaling. Canonical Wnt signaling is known to participate in signaling crosstalk with several pathways important in neuronal regeneration, most notably JNK/c-Jun signaling.

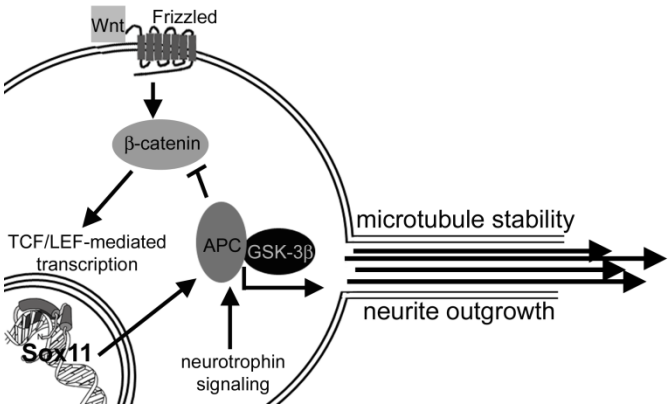

Figure 5. APC signaling in the context of nerve injury. APC is a microtubule-stabilizing protein that is essential for neurotrophin-induced neurite extension. Additionally, APC binds to beta-catenin and prevents it from activating TCF/LEF transcription factors, thereby altering Wnt signaling pathway dynamics.

1.9. TANK and nerve injury

TRAF family member-associated activator of NF-kappa B (TANK) is an intracellular signaling adaptor downstream of TNF-alpha receptor superfamily (TNFR-SF) members. TANK was discovered in 1996 following yeast two-hybrid screens for proteins associating with TRAF2 (Cheng and Baltimore, 1996, Rothe et al., 1996). TNFR-SF members are activated following nerve injury (Stoll et al., 1993, Taskinen et al., 2000, Schafers et al., 2003a, Boyle et al., 2005, Fernyhough et al., 2005, Miao et al., 2008, Schafers et al., 2008), and the balance of TNF receptor signaling is thought to be critical in maintaining cell survival (Baker and Reddy, 1998, Silke and Brink, 2010). TNF receptors activate multiple signaling pathways and have multiple effectors, including caspases, NF-kappa B, p38 MAP kinase and c-Jun N-terminal kinase (JNK) (Malek et al., 1998, Arron et al., 2002, Ha et al., 2009, Silke and Brink, 2010). The relative

abundance of TANK may alter the dynamics of TRAF association with other downstream components of TNF signaling (Cheng and Baltimore, 1996, Kaye et al., 1996, Chin et al., 1999, Chariot et al., 2002, Bonif et al., 2006, Guo and Cheng, 2007, Manna et al., 2010, Zhang et al., 2010), possibly shifting the balance of TNFR-SF signaling in favor of activation of pro-survival effectors.

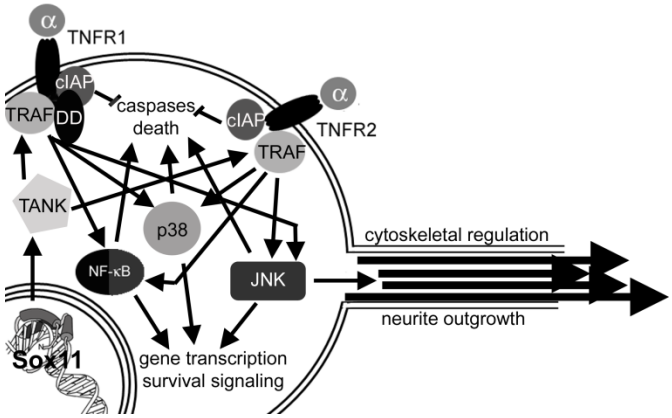

Figure 6. Possible effects of TNF-alpha pathway signaling on nerve injury. TNF alpha receptors have wide-ranging effects on cell survival, gene transcription and cytoskeletal regulation. Cell death may be regulated via activation of death domains on TNF receptors or via TRAF-mediated activation of signaling pathways including JNK, p38 and NF-kappa B. However, TRAF-mediated signaling may also function in a neuroprotective and proregenerative role. The putative Sox11 transcriptional target TANK may interact with TRAF family members, thereby altering TRAF-mediated signaling downstream of TNF receptors.

1.10. Goals of dissertation

The goals of this dissertation are to further investigate the relationship between Sox11 expression and transcriptional activation of the putative gene targets TANK, BDNF and APC and to relate these data to cell signaling pathways activated during peripheral nerve regeneration.

My approach has been to determine whether putative Sox11 transcriptional targets are upregulated during injury, to determine whether overexpression of Sox11 in a neuronal-like cell line can mimic injury-induced upregulation of putative transcriptional targets and finally to determine whether Sox11 is capable of activating the promoter region of putative transcriptional targets through use of luciferase assay reporter vectors. In the case of TANK, which has never previously been described in connection with peripheral nerve regeneration, I have also made efforts to determine TANK protein localization in neuronal cells and to determine whether TANK plays a role in cell survival and in the activation of JNK downstream of TNF receptor activation.

2.0 MATERIALS AND METHODS

2.1 Animal models and sciatic nerve cut surgery. Experiments were conducted on male C57BL/6J mice between 6-8 wks of age (Jackson Laboratory, Bar Harbor, ME). Animals were housed in group-cages and maintained in a temperature-controlled environment on a 12h light/dark cycle with food and water provided *ad libitum*. Sciatic nerve cut surgeries were performed on the left flank of mice anesthetized using 2-3% isoflurane anesthesia (Abbott Laboratories, Abbott Park, IL). All studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

2.2 Neuro-2a 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 were used before the fourth passage after being thawed.

2.3 Dissociated dorsal root ganglion cell preparation. Mice were deeply anesthetized using isoflurane then perfused with Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY). DRGs from all spinal cord levels were collected into ice-cold HBSS and cultures prepared according to the method described in (Malin et al., 2007). Briefly, DRGs were treated with L-cysteine and papain and connective tissue further degraded with collagenase and dispase.

Ganglia were washed and resuspended in F12 medium supplemented with 10% fetal bovine serum, gently dissociated by 5-10 rounds of trituration with a Pasteur pipette and plated on laminin and poly-lysine treated surfaces in 12-well culture plates. 2h after plating cell preparations were flooded with 1 mL of F12 medium per well unless otherwise specified.

2.4 RNA isolation and real-time polymerase chain reaction (RTPCR) assays. To analyze transcript expression in DRG the left L3-L5 DRGs were removed and immediately frozen on dry ice. Frozen ganglia were homogenized in RLT buffer (Qiagen, Gaithersburg, MD) using a Polytron mechanical tissue homogenizer. RNA was extracted using Qiagen RNeasy columns and reverse-transcribed using the Invitrogen cDNA kit according to manufacturer's protocol. One uL cDNA was used as template in a 25-uL reaction in an ABI7000 thermocycler, which measures the relative fluorescence of SYBR Green dye bound to double-stranded DNA. The Ct value, described as the cycle number at which SYBR green fluorescence rises above background level, was recorded as a readout for relative mRNA abundance. All results were normalized to GAPDH mRNA level. Primers against GAPDH, TANK, Sox11, the nine noncoding BDNF exons BDNF I – BDNF IX, the BDNF protein-coding region and APC were designed using MacVector 9.0, or their sequences were obtained from the primary literature (**Table 2**). Fold changes were calculated for the (target gene – GAPDH) values, according to the $\Delta\Delta$Ct formula (Livak and Schmittgen, 2001). For TANK, APC, and Sox11, statistical significance was determined at 1 day and 3 days post-nerve cut, using single-variable analysis of variance (ANOVA) with Bonferroni post-hoc testing. For BDNF exons, statistical significance was determined at the same time points using two-way ANOVA with Bonferroni post-hoc testing.

To isolate RNA from cultured cells, the medium was removed from the wells and wells

were washed with sterile PBS, pH 7.4. Qiagen Buffer RLT was added directly to the wells. Wells were scraped with a rubber cell scraper and the lysates were collected and further homogenized using the Polytron homogenizer. RNA isolation and real-time PCR proceeded as described for real-time PCR on nerve-injury samples.

2.5 Protein isolation and western blot analysis. Mice were deeply anesthetized using isoflurane inhalation and perfused with 0.9 % saline. Ipsilateral L3-L5 DRGs were removed at 3 days following sciatic nerve injury. For controls, naïve DRGs were used. DRGs were homogenized in SDS/Tris lysis buffer containing PMSF, aprotinin, pepstatin, leupeptin and activated sodium orthovanadate, using a hand-held mechanical grinder. For western blots run on lysates from cultured cells, culture dishes were washed with PBS, treated with lysis buffer, cells were scraped from the plates using a disposable cell scraper and then further homogenized using a hand-held mechanical grinder. Lysates were centrifuged to remove insoluble material and the supernatant was collected. 20 ug protein per lane was typically used for SDS-PAGE. Gels were transferred to Hybond-P membrane (Amersham, Piscataway, NJ) at 35 V, 4^{o}C, overnight or at 100V, 4^{o}C, for 2 h. Unless otherwise indicated in Table 2, membranes were blocked in 5% milk in TBST (Tris-HCl buffer + 0.1% Tween-20), incubated overnight at 4^{o}C with primary antibody diluted in blocking buffer and thoroughly washed with TBST. Membranes were then incubated in secondary antibody diluted in blocking buffer for 1 hour at room temperature, washed with TBST and developed using the Pierce ECL reagent. Western blots were run using antibodies against TANK protein, activated JNK, total JNK, Sox11, and GAPDH (**Table 3**). For normalization to GAPDH, membranes were stripped by incubation in 0.2M glycine/0.1% SDS, pH 2.0, for 10 minutes at room temperature, then blocked and probed using the GAPDH

antibody. Relative protein abundance was determined by creating digital images of the developed films using a Canon LIDE scanner, and using Image J software (NIH) to determine the intensity of each band. Band intensities were normalized to GAPDH band intensity. Statistical analysis was carried out using Student's t-test, one-way ANOVA with Bonferroni post-hoc testing, or two-way ANOVA with Bonferroni post-hoc testing.

2.6 Immunocytochemistry Cells used for immunocytochemistry were grown on coated coverslips or pre-treated chamber slides (Nunc). Cells were stained with antibodies against TANK and the neuronal marker beta-III tubulin (see **Table 3**). Coverslips were washed with 0.1 M phosphate buffer (PB) and fixed in -20^{o}C acetone for two minutes or in 4% paraformaldehyde for 10 minutes. Coverslips were then washed 3 x 5' in 0.1 M PB and blocked for 30' in 0.1 M PB + 0.025% Triton-X + 2% normal serum. After blocking, coverslips were incubated in primary antibody for 2h at room temperature and then washed 3 x 10' in 0.1 M PB. Secondary antibodies were added at a dilution of 1:1000 in 0.1 M PB and incubated for 1 hour at room temperature. If nuclear visualization was desired, a stock solution of DAPI was diluted to 1:300 in PB and added onto coverslips for 2' at room temperature. Coverslips were then washed 2 x 15' in PB and mounted using 80% glycerol made in PB.

2.7 Fluorescent microscopic analysis of cultured cells. Immunolabeled cells were examined using a Leica epifluorescent microscope equipped with the Leica Automated Software suite digital imaging system. In order to eliminate bias in overall fluorescence intensity, the same camera settings were used for each set of slides in a given experiment. Additionally, in the event that color balances needed to be adjusted, the same adjustment was made to every image

captured during the experiment. Fields for image capture and cell counting were chosen based on beta-III tubulin staining to eliminate biased selection based on TANK expression and/or morphology of TANK-expressing cells.

2.8 HSV-Sox11 viral infection of Neuro-2a cells. HSV constructs were generated following procedures described in (Mata et al., 2002). The control vector (HSV-GFP) contains two copies of a hCMVp:EGFP cassette targeted to the deleted (Δ) infected cell protein 4 (ICP4) loci of the backbone vector (vHG) (see Fig. 4A). An insert containing the human CMV promoter driving Sox11 (hCMV:Sox11) was also targeted to the ICP4 loci. Propagation and purification of high titer stocks of the HSV-Sox11 vector were produced as previously described and stored at -80^{o}C until used (Goins et al., 2002, Goins et al., 2008). Neuro2a cells were incubated for 2h with HSV vectors at a multiplicity of infection (MOI) of 2.5 after which fresh medium was added.

2.9 siRNA transfection. siRNA directed against the mouse TANK gene and nontargeting control siRNA was obtained from Dharmacon (Lafayette, CO) and resuspended according to the manufacturer's directions to produce a 20 uM stock solution. siRNAs were aliquoted in 10-uL aliquots and stored at -20^{o}C until needed.

siRNA was transfected into Neuro2a cells using Mirus TransIT-TKO reagent (Mirus Biosciences, Madison, WI). The day before transfection Neuro2a cells were plated in 12-well plates at 1 x 10^5 cells/well (a sufficient density to ensure 60-75% confluence on the day of transfection). The culture medium was changed immediately before starting the transfection protocol. TransIT-TKO reagent was diluted in serum-free medium and siRNA was added according to the manufacturer's protocol. The TransIT-TKO and siRNA mixture was added

dropwise to each well to yield a final concentration of 15 nM siRNA. Cells were stimulated with TNF-alpha, and/or harvested for RNA and protein analysis, 24h post-transfection.

2.10 Plasmid transfection. For luciferase assays in the site-directed mutagenesis study cells were transfected using the calcium-phosphate precipitatation method. Cells were plated in 6-well plates at 5 x 10^5 cells/well the day before transfection. This density resulted in approximately 30% confluence on the day of transfection. Transfection by calcium-phosphate precipitation was performed according to the protocol described in (Kingston et al., 2001).

For all other experiments plasmid transfection was performed using Mirus TransIT-Neural transfection reagent (Mirus Biosciences, Madison, WI). Cells were plated in 6-well plates at 1 x 10^6 cells/well on the day before transfection. This density resulted in approximately 60-75% confluence on the day of transfection. Cells were transfected with TransIT-Neural according to the manufacturer's protocol, using 2 uL of transfection reagent per microgram of transfected DNA and changing the culture medium approximately 30 minutes prior to transfection.

2.11 Plasmid construction. pCMV-Sox11 was made by subcloning mouse Sox11 (from IMAGE clone #63299841) into pCMV-IRES-EGFP (Jankowski et al., 2006). The IRES-EGFP sequence was removed by restriction digest to create pCMV-Sox11 plasmid, which contains 166 bp of 5'UTR and 1,093 bp of 3'UTR plus the SV40 polyA cassette from the pCMV-IRES-EGFP vector. (A pCMV-IRES-EGFP plasmid lacking the IRES-EGFP sequence was used as a control in cotransfection experiments.) Putative proximal promoter regions of target genes were cloned from C57BL/6J mouse genomic DNA using the TOPO-TA cloning system (Invitrogen,

Carlsbad, CA). The resulting promoter fragment orientation was determined using restriction analysis. Inserts in the correct orientation were sequenced to confirm identity, then subcloned into the pGL2-Basic vector (Promega, Madison, WI), generally using the KpnI/XhoI restriction sites which both vectors have in common on their multiple cloning regions. The following regions were cloned: BDNF exon I proximal promoter (3,027 bp), BDNF exon IV (2,844 bp), TANK (2,273 bp), and APC (1,887 bp). Primer sequences for these cloned regions are listed in **Table 2**. The Renilla luciferase expression vector pRL-TK (Promega, Madison, WI) was used for normalizing expression in luciferase assays. All vectors used in these studies are summarized in **Table 4**.

2.12 Site-directed mutagenesis. Vectors containing mutated Sox consensus binding sequences were created by designing site-directed mutagenesis primers using the online PrimerX software. The Sox consensus binding sequence mutation AACAAAG → CCCAAAG was generated to specifically remove Sox-binding ability from the mutated heptamer while, theoretically, preserving the ability of other HMG box-containing transcription factors (such as the TCF/LEF family members) to bind the DNA. Mutations were generated using the Stratagene Quik-Change site-directed mutagenesis kit, which is a PCR-based method using oligonucleotide primers containing mismatched base pairs to generate point mutations of the original DNA sequence. Site-directed mutagenesis primers are listed in **Table 2**. The double mutant TANKmut1mut2-pGL2 was generated by sequential iterations of site-directed mutagenesis, using the mut2 primer set to mutate the previously-generated TANKmut1-pGL2.

2.13 Luciferase reporter assays. The day before transfection cells were plated in 6-well plates at 2 x 10^5 cells per well. The following morning culture medium was changed and cells were transfected using Mirus TransIT-Neural transfection reagent (Mirus Biosciences, Madison, WI). The following plasmid dosages were used: pGL-BDNF promoter vectors: 1450 ng; pRL-TK: 50 ng; pCMV-Sox11 or empty pCMV vector: 150 ng. Cells were harvested 24h after transfection using Promega Passive Lysis Buffer: the culture medium was removed from the wells, each well was washed twice with 1 mL PBS pH 7.4 and 250 uL Passive Lysis Buffer was added to each well. The wells were briefly frozen to assist with cell lysis, thawed and cells were scraped from the well using a rubber cell scraper. Lysates were centrifuged and the supernatant was removed to a clean tube. Luciferase assays were performed using the Promega Dual-Luciferase Assay kit, with a Turner $20/20^n$ automatic luminometer with dual injectors. Firefly luciferase activity was used as a measure of promoter activity and Renilla (sea pansy) luciferase was used as a normalization signal. Renilla luciferase activity did not change significantly following Sox11 transfection at the dosages used in experimental luciferase assays. The normalized value ($RLU_{pGL2}/RLU_{pRL\text{-}TK}$) was used for statistical analysis. For luciferase assay experiments involving dose-response curves and for luciferase assays involving multiple mutagenized vectors, data were analyzed using two-way ANOVA with Bonferroni post-hoc testing. For luciferase assay experiments involving single vectors, data were analyzed using Student's t-test. Each experiment was performed in triplicate and repeated at least three times.

2.14 Electrophoretic mobility shift assays (EMSA). Short complementary oligonucleotides corresponding to areas of the TANK promoter containing Sox11 sites were designed and then ordered from Integrated DNA Technologies (Skokie, IA) (**Table 2**). Complementary oligos were

5' end-labeled with ^{32}P, run through a gel filtration column (Roche) to remove unincorporated radioisotope and annealed by denaturation in boiling water followed by slow cooling to 4^{o}C. Radiation counts were done on the resulting probes to ensure that each probe was equally radioactive and probes were stored overnight at -80^{o}C.

Neuro-2a cells were transfected with CMV-empty vector or CMV-Sox11 and nuclear extracts were prepared from the transfected cells. A Bradford assay was performed on the nuclear extracts (Bio-Rad, Hercules, CA) to determine protein concentration. Lysates were incubated at room temperature with 10fmol of probe for 1 h and run on a non-denaturing polyacrylamide gel in 1x TBE at 300V for approximately 2h. The gel was dried onto filter paper using a vacuum gel dryer, placed against photographic film which was exposed to the dried gel at -80 degrees C in a cassette with an intensifying screen.

2.15 Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed according to the Upstate Biotechnology protocol with minor modifications. Neuro2a cells (4 x 10^7) transiently transfected with pFlag-Sox11 were crosslinked with 1% formaldehyde for 10 min at 37°C followed by addition of glycine to 125 mM to quench formaldehyde. Cells were lysed in buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO) and chromatin sheared by sonication. Extracts were pre-cleared with protein G-agarose beads preadsorbed with salmon sperm DNA, diluted in IP buffer and incubated with 5 μg anti-FLAG M2 antibody (Sigma-Aldrich, Saint Louis, MO). Immunocomplexes were collected on preadsorbed protein G-agarose beads, washed, eluted and cross-linked complexes reversed by heating at 65^{o}C overnight. No antibody and non-immune mouse IgG (Sigma-Aldrich, Saint Louis, MO) were used as controls. DNA from

immunoprecipitates was purified using spin columns and incubated with PCR primers designed to amplify regions specific to the predicted Sox binding sites on the TANK promoter (see **Table 2** for primer sequences). Amplification was done using the following conditions: one cycle at 95°C 5 min followed by 35 cycles of 95°C 30s, 62°C 30s and 72°C 30s followed by one 72°C for 5 min incubation.

2.16 TNF-alpha stimulation. Neuro2a cells were stimulated using mouse recombinant TNF-alpha (Sigma-Aldrich, Saint Louis, MO), using a modified version of the protocol described in (Mielke et al., 1999). 24h after plating or transfection, stock TNF-alpha (resuspended in sterile water at a concentration of 1 ug/mL) was diluted to 2x final concentration in culture medium. 2x TNF-alpha was added to cultured cells to yield a 1x final concentration of TNF-alpha.

2.17 Trypan blue cell viability assay. Trypan blue assays were conducted using a modified version of (Strober, 2001). Culture medium was removed from wells and each well was gently washed twice with sterile PBS. 200 uL ice-cold sterile PBS was added to each well and the cells were detached by gentle scraping with a rubber cell scraper, removed to a sterile 1.5-mL microcentrifuge tube and placed on ice. 22 uL 10x trypan blue dye was added to tubes which were then incubated for 5 minutes to allow the dye to fully penetrate non-viable cells. 20 uL of the trypan blue-treated cell mixture was loaded onto a hemacytometer for counting. In order to eliminate bias in field selection, cells were always counted from the same region of the hemacytometer's counting grid. At least 100 cells from each individual sample were counted.

Primer Name	**Purpose**	**Sequence (5' → 3')**	**Source**
APC-6641	Real-time PCR	AACAACCCCTCCCGACAAAC	Lab-designed
APC-6816R	Real-time PCR	CTGACTTTCCTGCTGGCTTAGTTC	Lab-designed
APC-1140F	Luciferase vector	ATGCGGCTTACTGTTTCCTTTTC	Lab-designed
APC-3027R	Luciferase vector	TCCTTGGCTACCCTTGGACCTA	Lab-designed
BDNF-545F	Real-time PCR	AGACTGCAGTGGACATGTCT	Lab-designed
BDNF-790R	Real-time PCR	GGTCAGTGTACATACACAGG	Lab-designed
BDNFexon1F	Real-time PCR	CCTGCATCTGTTGGGGAGAC	(Zajac et al., 2010)
BDNFexon1R	Real-time PCR	GCCTTGTCCGTGGACGTTTA	(Zajac et al., 2010)
BDNFexon2F	Real-time PCR	CTAGCCACCGGGGTGGTGTAA	(Zajac et al., 2010)
BDNFexon2R	Real-time PCR	AGGATGGTCATCACTCTTCTC	(Zajac et al., 2010)
BDNFexon3F	Real-time PCR	CTTCCTTGAGCCCAGTTCC	(Zajac et al., 2010)
BDNFexon3R	Real-time PCR	CCGTGGACGTTTACTTCTTTC	(Zajac et al., 2010)
BDNFexon4F	Real-time PCR	CAGAGCAGCTGCCTTGATGTT	(Zajac et al., 2010)
BDNFexon4R	Real-time PCR	GCCTTGTCCGTGGACGTTTA	(Zajac et al., 2010)
BDNFexon5F	Real-time PCR	CCATAACCCCGCACACTCTG	Lab-designed
BDNFexon5R	Real-time PCR	TGGTCATCACTCTTCTCACCTGG	Lab-designed
BDNFexon6F	Real-time PCR	CTGGGAGGCTTTGATGAGAC	(Zajac et al., 2010)
BDNFexon6R	Real-time PCR	GCCTTCATGCAACCGAAGTA	(Zajac et al., 2010)
BDNFexon7F	Real-time PCR	CTTACTTACAGGTCCAAGGTCAACG	Lab-designed
BDNFexon7R	Real-time PCR	CAGAGGGTCGGATACAGGCTG	Lab-designed
BDNFexon8F	Real-time PCR	TCCCATCTACCCACACACTTTTATG	Lab-designed
BDNFexon8R	Real-time PCR	TGTTCGGCTCCACTGAGGCG	Lab-designed
BDNFexon9F	Real-time PCR	GCCACATGCTGTCCCCGAG	(Zajac et al., 2010)
BDNFexon9R	Real-time PCR	GCCAAGTTGCCTTGTCCGTG	(Zajac et al., 2010)
BDNFpro1-408F	Luciferase vector	TTCAGAAAGAGGTTAGAGCCTG	Lab-designed
BDNFpro1-3435R	Luciferase vector	GCAGTAAATCCAGTGTTGCG	Lab-designed

BDNFpro4-12192F	Luciferase vector	CATCCTTGTCACTCTGCTCATCG	Lab-designed
BDNFpro4-15036R	Luciferase vector	CCAGTCCTAGCAAATTCACGCAC	Lab-designed
GAPDH-756F	Real-time PCR	ATGTGTCCGTCGTGGATCTGA	Lab-designed
GAPDH-904R	Real-time PCR	GCTGTTGAAGTCGCAGGAGACA	Lab-designed
Sox11F	Real-time PCR	ATCAAGCGGCCCATGAAC	Lab-designed
Sox11R	Real-time PCR	TGCCCAGCCTCTTGGAGAT	Lab-designed
TANK-2724F	Luciferase vector	CTCAAGCTACCCCATGTGACAC	Lab-designed
TANK-4997R	Luciferase vector	AGGCACGACCCATTCTGGAAGC	Lab-designed
TANK-812F	Real-time PCR	GTTTCCGCCTATGGACAATGAC	Lab-designed
TANK-913R	Real-time PCR	AATCGGTCCTGGCACAGTGT	Lab-designed
TANKmut1F	Site-directed mutagenesis	CCATGTGACACACACAAAACCCC GGCACAAAAGTGGAAGATGG	Lab-designed
TANKmut1R	Site-directed mutagenesis	CCATCTTCCACTTTTGTGCCGGG GTTTTGTGTGTGTCACATGG	Lab-designed
TANKmut2F	Site-directed mutagenesis	GGACATTTCCATTTTATAACCCC GAAAAATGCCCGGATATTGC	Lab-designed
TANKmut2R	Site-directed mutagenesis	GCAATATCCGGGCATTTTTCGGG GTTATAAAATGGAAATGTCC	Lab-designed
TANK-chip-2255F	ChIP assay	TCTCACCCAAGTTCACTCAGGCT A	Lab-designed
TANK-chip-2002R	ChIP assay	GGCTGGTATGAATGGCTGGAGAA T	Lab-designed
TANK-chip-701F	ChIP assay	ACCGAAGAGATCGTGTCTAAGGA C	Lab-designed
TANK-chip-484R	ChIP assay	GGTACTAGTGAGAGAGGTAAAG CA	Lab-designed
TANK-site2-WT-F	EMSA probe	CCATTTTATAACAAAGAAAAATG CC	Lab-designed
TANK-site2-WT-R	EMSA probe	GGCATTTTTCTTTGTTATAAAATG G	Lab-designed
TANK-site2-mut-F	EMSA probe	CCATTTTATAACCCCGAAAAATG CC	Lab-designed
TANK-site2-mut-R	EMSA probe	GGCATTTTTCGGGGTTATAAAAT GG	Lab-designed

Table 2. Sequences of oligonucleotides used in these studies, including all PCR primers and EMSA probes.

Host	Antigen, type	Source	Application	Dilution	Tag
rabbit	TANK, polyclonal	Santa Cruz	Western blot	1:2000	n/a
rabbit	TANK, polyclonal	Abcam	Immunochemistry	1:1200 to 1:1600	n/a
rabbit	GAPDH, polyclonal	Santa Cruz	Western blot	1:5000	n/a
mouse	Beta-tubulin, monoclonal	Upstate	Immunochemistry	1:1000	n/a
rabbit	Phospho-JNK, polyclonal	Cell Signaling	Western blot	1:2000	n/a
rabbit	JNK, polyclonal	Cell Signaling	Western blot	1:2000	n/a
donkey	Rabbit IgG, polyclonal	Jackson	Western blot	1:5000	HRP
goat	Mouse IgG, polyclonal	Jackson	Western blot	1:5000	HRP
donkey	Rabbit IgG, polyclonal	Jackson	Immunochemistry	1:500	Cy3
donkey	Mouse IgG, polyclonal	Jackson	Immunochemistry	1:500	Cy2
n/a	DNA-binding stain	Sigma-Aldrich	Immunochemistry	1:300	DAPI

Table 3: Antibodies used in these studies.

Plasmid	Use	Vector Backbone	Promoter	Coding Sequence	Antibiotic Resistance
pCMV-Sox11	Sox11 overexpression	pIRES-EGFP	CMV	Sox11 CDS (mouse)	Kanamycin
pCMV-Sox11-HA	HA-tagged Sox11 overexpression	pIRES-EGFP	CMV	Sox11 CDS (mouse)	Kanamycin
pCMV-empty	Weight control	pIRES-EGFP	CMV	n/a	Kanamycin
pGL2-Basic	Firefly luciferase vector	n/a	n/a	Firefly luciferase	Ampicillin
pGL2-BDNF1	Luciferase reporter vector	pGL2-Basic	Mouse BDNF exon 1	Firefly luciferase	Ampicillin
pGL2-BDNF4	Luciferase reporter vector	pGL2-Basic	Mouse BDNF exon 4	Firefly luciferase	Ampicillin
pGL2-APC	Luciferase reporter vector	pGL2-Basic	Mouse APC	Firefly luciferase	Ampicillin
pGL2-TANK	Luciferase reporter vector	pGL2-Basic	Mouse TANK	Firefly luciferase	Ampicillin
pGL2-TANKmut1	Luciferase reporter vector	pGL2-Basic	Mouse TANK	Firefly luciferase	Ampicillin
pGL2-TANKmut2	Luciferase reporter vector	pGL2-Basic	Mouse TANK	Firefly luciferase	Ampicillin
pGL2-TANKmut1mut2	Luciferase reporter vector	pGL2-Basic	Mouse TANK	Firefly luciferase	Ampicillin

Table 4: Plasmids used in this dissertation. Columns from left to right: Name of the plasmid, purpose for which the vector was used, backbone of each vector, promoter driving expression of coding sequence on each vector, identity of coding sequence on each vector and antibiotic resistance of each construct as applicable.

3.0 SOX11 CONTRIBUTES TO THE DIFFERENTIAL EXPRESSION OF BDNF TRANSCRIPTS AFTER NERVE INJURY

3.1 Introduction

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, is critical for the survival, differentiation, and neurite outgrowth of sensory neurons (Ernfors et al., 1995a, Ernfors et al., 1995b, Liu et al., 1995, LeMaster et al., 1999, Ernsberger, 2009, Valdes-Sanchez et al., 2010). In mature neurons, BDNF is important as a mediator of synaptic plasticity (Cowansage et al., 2010) and as a molecule of interest in peripheral nerve injury.

BDNF expression in dorsal root ganglion (DRG) neurons is increased following nerve injury (Cho et al., 1998, Ha et al., 2001), and this increase serves as a pro-regenerative signal (Geremia et al., 2010). BDNF has two receptors, the low-affinity p75 neurotrophin receptor (p75 NTR) which is a member of the TNF-alpha receptor superfamily, and the high-affinity TrkB receptor which is a member of the receptor tyrosine kinase family. TrkB and p75 are frequently expressed in the same populations of cells and work together to coordinate neurotrophin signaling; however, TrkB receptors are thought to be most important in terms of conveying pro-regenerative signals following nerve injury.

TrkB has a full-length isoform (TrkB.FL) which includes a tyrosine kinase domain, as well as truncated isoforms (TrkB.t1 and TrkB.t2) which does not include a tyrosine kinase domain (Allendoerfer et al., 1994) but may still participate in signaling cascades (Baxter et al., 1997). Both full-length and truncated TrkB isoforms are expressed in DRG neurons (Sebert and Shooter, 1993). In overexpression studies, TrkB.t1 has an inhibitory effect on TrkB.FL-mediated

survival signaling via competition for BDNF (De Wit et al., 2006, Tervonen et al., 2006); however, endogenous TrkB.t1 is an important mediator of neurite extension (Haapasalo et al., 1999, Hartmann et al., 2004, Carim-Todd et al., 2009, Michaelsen et al., 2010). TrkB.t1 may regulate neurite extension in conjunction with p75NTR signaling (Hartmann et al., 2004, Michaelsen et al., 2010); overexpression of TrkB.t1 results in the formation of dendritic filopodia in cultured neurons in a p75NTR-dependent manner (Hartmann et al., 2004, Michaelsen et al., 2010), deletion of TrkB.t1 results in abnormal neurite morphology in the brain (Carim-Todd et al., 2009), and overexpression of BDNF in skin results in increased neurite innervation density, and increased expression of TrkB.t1 (LeMaster et al., 1999). The precise signaling mechanism is unknown; however, TrkB.t1 associates with the Rho GDP dissociation inhibitory protein GDI1 and can regulate Rho signaling cascades in a BDNF-dependent manner, which can alter cell morphology (Ohira et al., 2006).

Following binding of BDNF to dimerized full-length TrkB, the TrkB dimer activates via autophosphorylation of tyrosine residues. Activation of TrkB receptors downstream of BDNF binding results in the activation of numerous intracellular signaling cascades, including the PLC-γ pathway, the MAP kinase pathway, and the PI3K/Akt pathway, which ultimately result in the activation of survival signals and the extension of neurites (Reichardt, 2006) (**Figure 7**).

PLC-γ activation results in the production of the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activates the protein kinase C (PKC) signaling pathway, and IP3 binds to intracellular receptors resulting in the release of intracellular calcium stores (Reichardt, 2006). This branch of the BDNF/TrkB signaling cascade is of critical importance in synaptic plasticity-related events (Korte et al., 1995, Tyler and Pozzo-Miller, 2001, Kovalchuk et al., 2002, Lang et al., 2007); additionally, the intracellular rise in calcium contributes to the

extension of growth cones by facilitating transient receptor potential channel (TRP channel)-induced calcium increases at the site of growth cones (Li et al., 2005b, Amaral et al., 2007, Amaral and Pozzo-Miller, 2007a, b) . PLC-γ induced intracellular calcium release also results in increases in adenylyl cyclase (AC) activity and in cAMP-dependent gene transcription, which supports the cAMP-dependent gene transcription that occurs via cAMP-response element binding protein (CREB) downstream of MAP kinase signaling (Nguyen et al., 1994, Shaywitz and Greenberg, 1999).

MAP kinases are activated downstream of TrkB receptors via TrkB/Shc/Ras signaling. MAPK/ERK signaling is of critical importance in transcription-dependent mechanisms of BDNF/TrkB effects on survival and regeneration: MAPK signaling not only activates CREB-dependent gene transcription resulting in the transcription of immediate early genes which are involved in survival signaling and growth cone formation/neurite extension (Finkbeiner et al., 1997, Shaywitz and Greenberg, 1999, West et al., 2001, Gao et al., 2004), but also activates multiple components of transcriptional machinery including eukaryotic initiation factor 4E (eIF4E) and the ribosomal protein S6 (Bonni et al., 1999, Kelleher et al., 2004, Klann et al., 2004).

The third main branch of the BDNF/TrkB signaling pathway is the phosphatidylinositol 3-kinase (PI3K) pathway. PI3K is also activated downstream of TrkB-mediated Shc activation. This results in the translocation of Akt/Protein Kinase B to the cytoplasm. Akt signaling drives transcription dependent on mammalian target of rapamycin (mTOR), a major facilitator of neurite outgrowth and of neuronal survival signaling (Sarbassov et al., 2005, Okada et al., 2011); however, this pathway also facilitaties peripheral regeneration via mTOR-independent mechanisms (Christie et al., 2010). Additionally, this pathway is of major importance in

maintaining long-term synaptic plasticity, which is highly pertinent to the study of mechanisms of neuropathic pain following peripheral nerve injury (Tartaglia et al., 2001, Groth and Aanonsen, 2002, Cowansage et al., 2010, Geng et al., 2010).

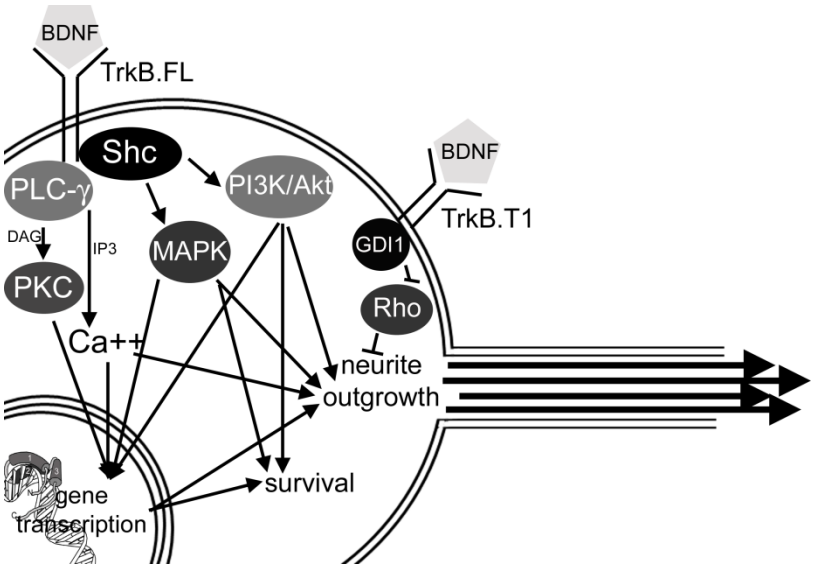

Figure 7: BDNF-TrkB signaling pathways leading to survival and neurite outgrowth. BDNF-TrkB signaling uses transcription-dependent and –independent mechanisms to increase neuronal survival and neurite outgrowth.

In addition to the activation of intrinsic neuronal growth pathways in DRG neurons downstream of BDNF-TrkB receptor signaling, BDNF is also retrogradely transported in DRG neurons and released onto the spinal dorsal horn (Tonra et al., 1998). This leads to increased activation of MAP kinases, which contribute to the development of neuropathic pain (Obata et al., 2003a, Obata et al., 2003b, Obata et al., 2004a, Obata et al., 2004b). BDNF release onto central terminals is one of the factors driving central sensitization of dorsal horn synapses, an NMDA-receptor-dependent phenomenon which results in long-lasting LTP-like changes at the synaptic level, ultimately resulting in increased firing rates of dorsal horn neurons and the

development of chronic neuropathic pain (Kerr et al., 1999, Groth and Aanonsen, 2002, Garraway et al., 2003, Kawasaki et al., 2004, Geng et al., 2010).

The regulation of BDNF expression following nerve injury is of special interest because of the complex structure of the BDNF gene. In both humans and rodents, the BDNF gene has multiple promoter elements (Aid et al., 2007, Pruunsild et al., 2007), suggesting BDNF is differentially regulated in a contextual and temporal manner. In the rodent the BDNF gene has eight noncoding exons (exons I-IXA) and one coding exon (exon IX). Mature BDNF transcripts typically have a unique noncoding 5' exon spliced to the common BDNF encoding exon IX (although exon IX can also be independently transcribed) (Aid et al., 2007). Furthermore, exon II has multiple splice junctions, meaning that there are three possible transcriptional variants of BDNF transcripts transcribed using the exon II promoter; these transcripts are denoted as IIA/B/C. The regions immediately flanking most BDNF exons contain the bulk of potential BDNF regulatory sequences, such as TATA boxes (Liu et al., 2006); however, regulation of the BDNF gene has not yet been extensively detailed and relatively little is known about the precise mechanisms which regulate exon-specific BDNF expression. Figure 8 details the structure of the rodent BDNF gene (Aid et al., 2007).

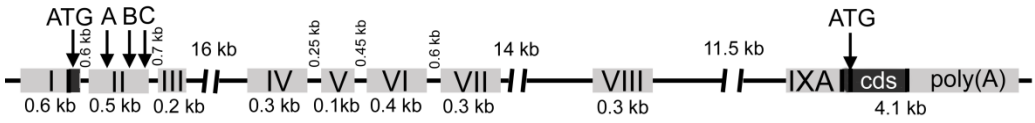

Figure 8: Rodent BDNF gene structure (adapted from Aid et al., 2007). Noncoding exons I-VIII, or the noncoding region of Exon IX, are spliced to the coding region of Exon IX to form a mature BDNF transcript. Arrows denote alternative translational start sites (ATG) or alternate splice junctions (A, B, C). Gray areas denote protein coding regions.

Although each mature BDNF transcriptional variant codes for the same peptide (Aid et al., 2007), different transcript variants may have different functional effects on neurons based on their intracellular transport and local translation: transcripts 1 and 4 are contained within the cytoplasm and proximal dendrites, whereas all other transcripts are transported more distally on the dendrites (Pattabiraman et al., 2005, Chiaruttini et al., 2009).

Previous studies of injured peripheral nerves of rat and mouse have focused on the role of BDNF in promoting regeneration and the consequences of its continued expression on the development of neuropathic pain (Funakoshi et al., 1993, Timmusk et al., 1995, Cho et al., 1998, Tonra et al., 1998, Ha et al., 2001, Kim et al., 2001, Willis et al., 2005, Obata and Noguchi, 2006, Matsuoka et al., 2007, Ng et al., 2007, Kobayashi et al., 2008, Geremia et al., 2010). As a result, the majority of studies of BDNF following nerve injury have focused on total BDNF mRNA expression, or on BDNF at the protein level.

BDNF was initially identified as a putative transcriptional target of Sox11 using a microarray screen of DRG neurons treated with siRNA targeted against Sox11. A 2-fold difference in expression was used as a cutoff for identification of putative transcriptional targets of Sox11. Following Sox11 knockdown, BDNF expression was reduced compared with neurons treated with nontargeting siRNA (Jankowski, unpublished). Additionally, Neuro-2a cells infected with HSV-Sox11 upregulate expression of BDNF ($n = 4$, fold change = 7.4, $p < 0.05$, Student's t-test) (**Figure 9**).

Some studies have been performed on BDNF transcript-specific expression following nerve injury in the rat, or in cultured neurons stimulated with neurotrophins (an in vitro model of nerve injury) (Funakoshi et al., 1993, Timmusk et al., 1995, Kim et al., 2001, Matsuoka et al., 2007,

Kobayashi et al., 2008). However, exon-specific expression of BDNF in mouse DRG following peripheral nerve injury has not been reported. Additionally, all recent studies of BDNF transcript expression were done at two weeks after sciatic nerve injury (Matsuoka et al., 2007, Kobayashi et al., 2008). Although later time points are highly relevant to the study of BDNF function, assessment of BDNF transcriptional regulation at earlier times after nerve injury is also valuable given its predicted modulation of survival and growth pathways. The aims of this study were therefore to determine the expression pattern of BDNF exons in mouse lumbar DRGs at early times (1 and 3 days) after sciatic nerve injury, to determine whether Sox11 overexpression can upregulate BDNF exons, and to determine whether Sox11 can activate injury-activated BDNF promoters.

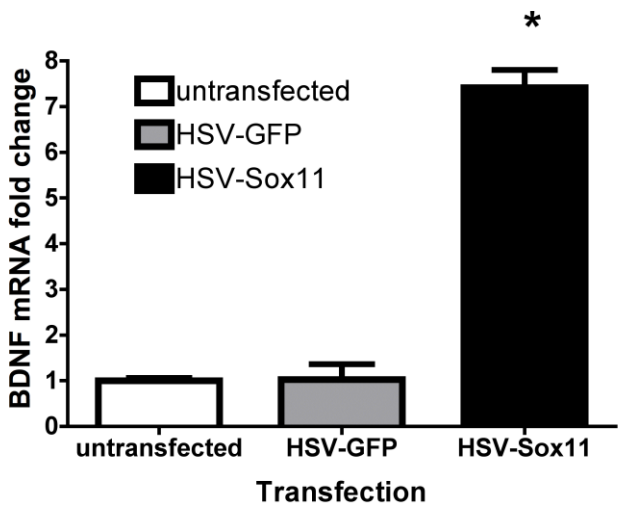

Figure 9: Sox11 overexpression increases BDNF expression in Neuro-2a cells. Compared with both untransfected and HSV-GFP transfected cells, HSV-Sox11 transfected cells increased total BDNF mRNA 7.4-fold (n = 4 per group, $p < .05$, ANOVA with Bonferroni post-hoc test).

3.2 BDNF transcripts are differentially regulated following nerve injury.

BDNF has been shown to increase in DRG neurons by 1d following nerve injury (Kim et al., 2001). Previous results in rat showed that exon I was primarily responsible for this upregulation (Kim et al., 2001). To investigate how this early, injury-induced rise in BDNF is controlled on a transcriptional level in the mouse, we used real time PCR assays to compare the relative expression of each BDNF exon at 1d and 3d post sciatic nerve injury. PCR primers were designed to amplify regions within each exon of the mouse BDNF gene (see **Table 2**). Compared with naïve mice, transcripts containing each BDNF exon were present in mouse lumbar ganglia with the exception of exon V (**Fig. 10**). At 1d post-injury, transcripts I, IV, VII, and VIII were significantly increased compared with naïve controls ($n = 4$ mice per group, $p < .05$, two-way ANOVA with Bonferroni post-hoc test). At 3d post-injury, expression of transcripts IV, VII, and VIII remained at control levels but BDNF transcript I level remained significantly increased ($n = 4$ mice per group, $p < .05$, two-way ANOVA with Bonferroni post-hoc test). BDNF transcripts II, III, VI, and IX did not show significant change in expression at 1d and 3d post-sciatic nerve injury.

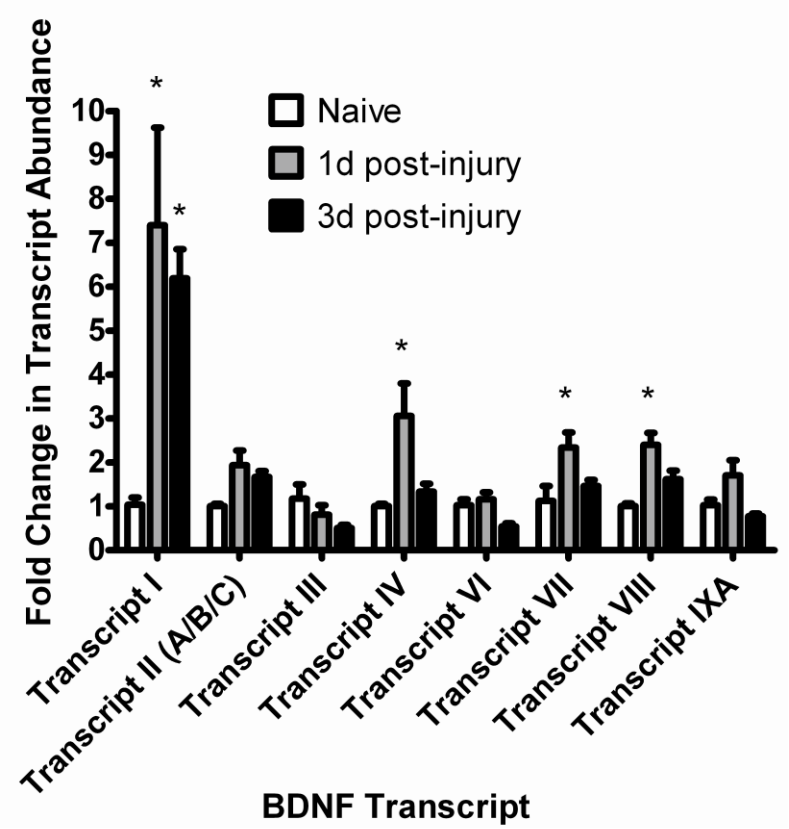

Figure 10: BDNF exons are differentially regulated following sciatic nerve cut injury. Left L3-L5 DRGs were harvested from four naïve mice, four mice one day post-nerve cut, and four mice three days post-nerve cut. Real-time PCR was performed on RNA extracted from these DRGs. BDNF transcripts I, IV, VII, and VIII were upregulated one day post-injury, whereas only BDNF transcript I was upregulated three days post-injury. All values mean +/- SEM, *$p < .05$ vs. naïve.

3.3 All upregulated BDNF transcripts in injured DRG share the common Sox-binding site AACAAAG

To examine if Sox11 could potentially bind BDNF exons and control transcript expression we screened up to 5 Kbp of genomic sequence upstream of the transcriptional start site of each exon for the SRY binding motif 5'-WWCAAWG-3' (Harley et al., 1994, Badis et al., 2009). **Table 5** summarizes the presence or absence of Sox binding sites immediately upstream of each murine BDNF exon. The 5' flanking regions of all BDNF exons excepting exons III, V, and VI contained at least one potential Sox binding site in the forward (5' → 3') direction. All BDNF exons contained at least one potential Sox binding site in the reverse (3' → 5') direction excepting exons V and VI. However, only those exons with at least one 5' instance of the Sox-binding motif 5'-AACAAAG-3' demonstrated upregulation at days 1 and 3 after nerve injury. Interestingly, exon IXA contains 8 potential Sox-binding sites, of which 4 are in the forward and 4 in the reverse direction; however, it was not significantly regulated following nerve injury.

3.4 Sox sites are conserved in BDNF promoters 1 and 4 in the human genome.

It is difficult to directly compare the promoter regions of rodent and primate BDNF exons; although the human and rodent BDNF locus are similarly constructed overall, the human locus is slightly more complex, featuring one additional exon (exon Vh) and reverse transcription of antiBDNF RNA (Pruunsild et al., 2007). For this reason, only the Sox binding sites on the promoter regions of relatively-homologous and highly-upregulated exons I and IV were analyzed. 300-nt stretches of genomic sequence surrounding each Sox binding site from the human and mouse BDNF promoters were analyzed. MacVector 9.0 software was used to run a ClustalW alignment on all sequences. While some Sox site-containing sequences were highly

similar to each other within species, likely as a result of gene reduplication, no significant homologies were seen in Sox site-containing regions between species (not shown). However, the human BDNF exons 1 and 4 also contain a large number of Sox binding sites (**Table 6**), and each of these exons contains at least one forward binding site with the AACAAAG motif.

Exon #	**# Sox Sites (+ strand)**	**Location (sequence)**	**# Sox Sites (- strand)**	**Location (sequence)**
Exon I	3	**-2927 (AACAAAG)** -4422 (AACAATG) -4973 (TTCAAAG)	5	-1632 (TTCAAAG) -1685 (TACAAAG) -2822 (TTCAAAG) -2953 (ATCAATG) -4138 (ATCAAAG)
Exon II	1	-326 (TTCAAAG)	0	
Exon III	0		1	-253 (TACAAAG)
Exon IV	5	-172 (TACAAAG) **-479 (AACAAAG)** **-1606 (AACAAAG)** **-3434 (AACAAAG)** -3915 (TTCAAAG)	4	**-1051 (AACAAAG)** **-1397 (AACAAAG)** -2238 (ATCAATG) -3514 (AACAATG)
Exon V	All Sox sites on 5kb promoter also upstream of Exon IV			
Exon VI	All Sox sites on 5kb promoter also upstream of Exon IV			
Exon VII	4	-550 (AACAATG) -2145 (TACAAAG) **-2452 (AACAAAG)** **-4280 (AACAAAG)**	4	-649 (ATCAAAG) **-3024 (AACAAAG)** **-3190 (AACAAAG)** -4157 (ATCAATG)
Exon VIII	5	**-862 (AACAAAG)** -1872 (TACAAAG) -3071 (TTCAATG) -3951 (TACAAAG) -4539 (TTCAAAG)	2	-2468 (TTCAAAG) -3333 (TTCAATG)
Exon IXA	4	-416 (ATCAAAG) -3020 (TTCAAAG) -4626 (TACAATG) -4760 (ATCAAAG)	4	-303 (AACAATG) -371 (ATCAATG) -560 (TACAAAG) -2006 (TTCAAAG)

Table 5: All upregulated BDNF exons in mouse contain multiple Sox binding sites. The 5kb region upstream of each BDNF transcript's transcriptional start site was analyzed for the presence of Sox sites. Exons I, IV, VII, and VIII were upregulated after nerve cut injury and after HSV-Sox11 infection. Each of these exons contains at least three Sox binding sites in the forward direction, at least two Sox sites in the reverse direction, and at least one forward 5'-AACAAAG-3' site. Exon IXA was upregulated after HSV-Sox11 infection, but not after nerve cut injury. This exon contains four Sox binding sites each in the forward and reverse directions.

Exon	# of Sox sites on (+) strand	Positions relative to transcriptional start	# of Sox sites on (-) strand	Positions relative to transcriptional start
I	**5**	-1463 (ATCAATG) -1931 (TACAAAG) -1946 (**AACAAAG**) -2957 (ATCAATG) -3039 (TTCAATG)	**2**	-849 (AACAATG) -3673 (**AACAAAG**)
IV	**3**	-1052 (TACAAAG) -2307 (**AACAAAG**) -2724 (AACAATG)	**2**	-1052 (TACAAAG) -3281 (**AACAAAG**)

Table 6: Human BDNF exons I and IV also contain Sox binding sites in their proximal promoters. The 5kb region upstream of the injury-regulated exons I and IV, on the human BDNF locus, was analyzed using MacVector 9.0 software for the presence of Sox sites. The presence of Sox sites is conserved between the two species, although the regions which contain Sox sites are not highly homologous. Additionally, the AACAAAG binding site is conserved in both mouse and human BDNF promoter regions.

3.5 Overexpression of Sox11 leads to BDNF upregulation in Neuro-2a cells.

To determine whether the link between Sox11 upregulation and BDNF upregulation may be causal, Neuro-2a cells were infected with the HSV-Sox11 viral vector or the HSV-GFP control viral vector (n = 4 cultures per group). RNA was collected for semi-quantitative real-time PCR to determine relative expression of each BDNF transcript; the ΔΔCt values were analyzed using two-way ANOVA with Bonferroni post-hoc testing. Transcripts that were not detected by real-time PCR were not included in the ANOVA analysis: transcripts I, IV, V, and VII were analyzed, while transcripts II, III, VI, VIII, and IXA were excluded. **Figure 11** shows the results of this analysis, with ΔΔCt values converted into fold change. Significant upregulation of BDNF transcripts were seen for transcripts IV and VII (transcript IV fold change = 2.00 [vs. GFP], 1.84 [vs. untransfected], $p < 0.05$; transcript VII fold change = 3.41 [vs. GFP], 3.59 [vs.

untransfected], $p < 0.05$). Both these transcripts were also upregulated following sciatic nerve injury. However, BDNF transcript I, which was strongly upregulated after injury, was not significantly upregulated following Sox11 overexpression (fold change 1.46 [vs. GFP], 1.59 [vs. untransfected], $p > 0.05$).

This result is likely to reflect differences in BDNF gene regulation in Sox11-transfected Neuro2a cells as opposed to differences in transcript-specific BDNF expression after nerve injury, possibly reflecting the presence of different panels of transcription factors in Neuro-2a cells vs. injured neurons. While both Neuro-2a cells and DRG neurons express Sox11, there are likely to be many differences in the expression of other transcription factors. As Sox11 transcriptional targeting depends strongly on the presence of a partner code, it is probable that Sox11 targets and/or binding sites are slightly altered in Neuro-2a cells.

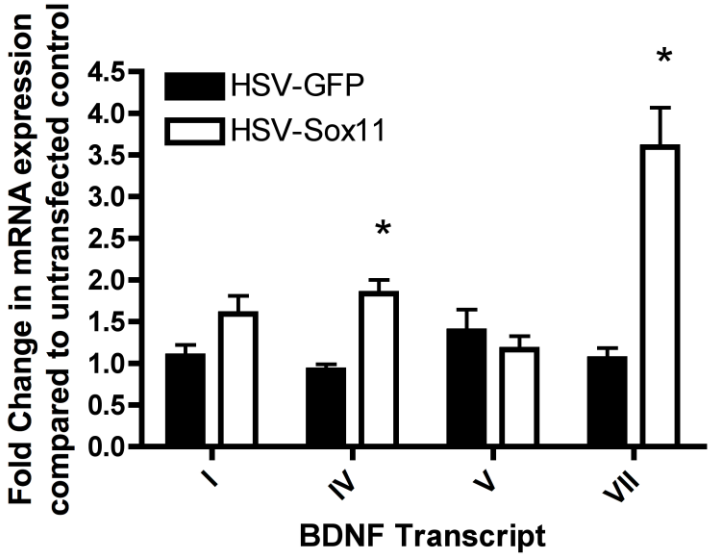

Figure 11: BDNF transcripts are differentially regulated following Sox11 overexpression. Neuro-2a cells were infected with HSV-GFP (control virus) or HSV-Sox11. Compared with control, BDNF transcripts IV and VII were upregulated following Sox11 overexpression (n = 4 per group, fold change at least 2.00-fold for all upregulated exons [HSV-Sox11 vs. HSV-GFP], $p < 0.05$ (two-way ANOVA with Bonferroni post-hoc testing).

3.6 Cotransfection of BDNFI-pGL2 and pRL-TK in a 25:1 ratio is sufficient to detect Sox11-driven regulation of promoter regions without altering expression of Renilla luciferase.

To determine whether Sox11 may play a role in increasing BDNF exon I expression after injury by causing activation of the Exon I promoter, a 3027-bp fragment directly proximal to the Exon I transcriptional start site was cloned into the pGL2-Basic vector to produce pGL2-BDNF1 (see **Section 2.11**, Luciferase Vector Creation). **Figure 12** shows the areas of the BDNF gene used to produce pGL2-BDNF1 and a second vector, pGL2-BDNF4 (see **Section 3.7**). pGL2-BDNF1 was transfected into Neuro-2a cells along with pRL-TK and either pCMV-Sox11 or pCMV-empty vector, and luciferase assays were performed. Because the normalization vector must remain at a consistent level of activation to ensure that the luciferase assay results reflect changes in activation of the experimental vector, it was critical to first ensure that Sox11 did not activate the HSV-TK promoter, which drives expression of Renilla luciferase from the pRL-TK vector. Optimization of the pGL2:pRL vector ratio is critical to achieving adequate expression of the normalization signal without unintended alterations in Renilla luciferase expression.

To optimize pGL2:pRL vector ratios, pGL2-BDNF1 and pRL-TK were cotransfected in ratios varying between 5:1 and 50:1, with the total amount of transfected luciferase vector held constant at 1500 ng. pCMV or pCMV-Sox11 were also cotransfected at a dosage of 150 ng per well of a 6-well plate. Data from these experiments are shown in **Figure 13**. The 25:1 ratio of pGL2:pRL-TK was sufficient to adequately drive expression of both firefly and Renilla luciferase without significantly altering Renilla luciferase with Sox11 cotransfection ($n = 3$ per group, $p > 0.05$, two-way ANOVA with Bonferroni post-hoc testing).

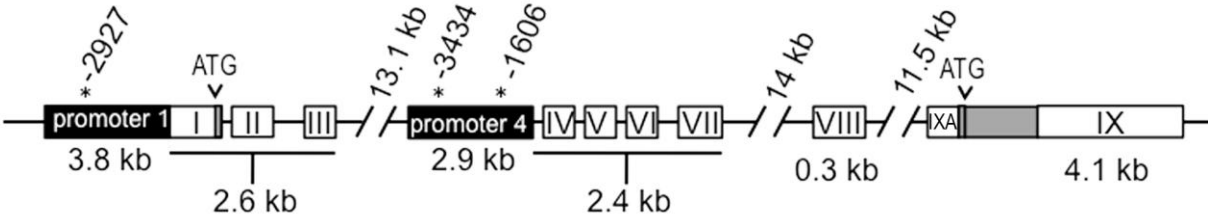

Figure 12. BDNF gene structure diagram (adapted from Aid et al., 2007), showing promoter regions and forward-direction Sox binding sites used in the pGL2-BDNF promoter vectors. A 3.8-kb region of the BDNF exon I promoter, containing one forward Sox binding site, was cloned into the pGL2-Basic vector, in order to produce pGL2-BDNF1. A 2.9-kb region of the BDNF exon IV promoter, containing two forward Sox binding sites, was cloned into the pGL2-Basic vector, in order to produce pGL2-BDNF4.

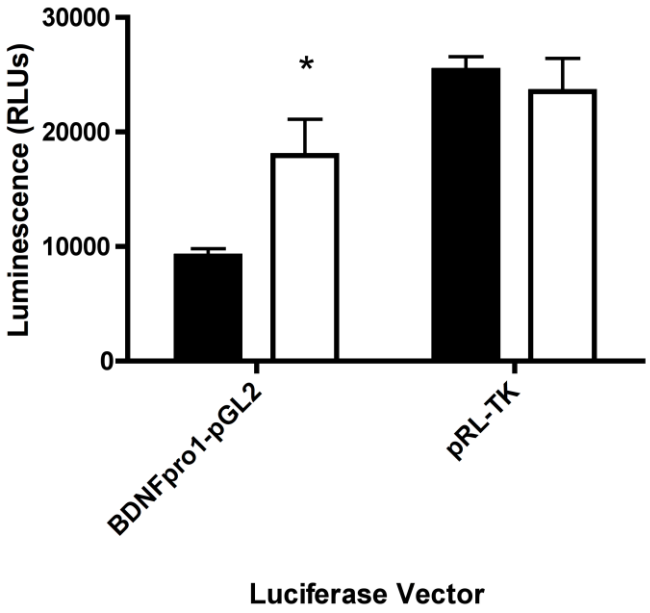

Figure 13. Cotransfection of BDNFpro1-pGL2 and pRL-TK in a 25:1 ratio permits Sox11 to drive the BDNF exon I promoter without affecting HSV-TK promoter activity. BDNFpro1-pGL2 and pRL-TK were cotransfected in a 25:1 ratio (total amount of luciferase vectors transfected = 2500 ng), with additional cotransfection of 150 ng pCMV-Sox11 (white) or empty pCMV plasmid (black). Sox11 significantly upregulated the BDNF exon I promoter (left, also see Figure 14) ($n = 3$ per group, $p < 0.01$, two-way ANOVA with Bonferroni post-hoc test), but did not affect expression of Renilla luciferase downstream of the HSV-TK promoter (right) ($n = 3$ per group, $p > 0.05$, two-way ANOVA with Bonferroni post-hoc test).

3.7 Sox11 increases activation of the BDNF exon I promoter

To determine whether Sox11 may play a role in increasing BDNF exon I expression after injury by causing activation of the Exon I promoter, a 3027-bp fragment directly proximal to the Exon I transcriptional start site was cloned into the pGL2-Basic vector to produce pGL2-BDNF1 (see **Section 2.11**, Luciferase Vector Creation). pGL2-BDNF1 was transfected into Neuro-2a cells along with pRL-TK and either pCMV-Sox11 or pCMV-empty vector, and luciferase assays were performed.

Figure 14 shows the results of luciferase assays performed following cotransfection of pCMV-Sox11 with pGL2-BDNF1 and pRL-TK. At baseline, pGL2-BDNF1 can weakly drive luciferase expression when transfected into Neuro-2a cells, whereas cotransfection of pGL2-BDNF1with pCMV-Sox11 caused a significant increase in activation (fold change = 2.5-fold over baseline, n = 3 per group, $p < 0.0001$, Student's t-test). This data indicates that Sox11 overexpression is sufficient to activate the BDNF exon I promoter, consistent with the hypothesis that Sox11 is a transcriptional mediator of BDNF transcript I following nerve injury.

Figure 14: Sox11 activates the BDNF exon I promoter. Neuro-2a cells were cotransfected with pGL2-BDNF1 luciferase reporter vector, pCMV-Sox11 or pCMV weight control vector, and pRL-TK (normalization signal). Cotransfection with Sox11 caused a 2.5-fold activation over baseline of the BDNF exon I promoter (n = 3 per group, $p < 0.0001$, Student's t-test).

3.8 Sox11 increases activation of the BDNF exon IV promoter

To determine whether Sox11 might increase BDNF exon IV expression following nerve injury via activation of the Exon 4 promoter, a 2844-bp fragment proximal to the exon IV transcriptional start site of the mouse BDNF gene was cloned into the pGL2-Basic vector to produce pGL2-BDNF4 (see Chapter 2.11). This construct was transfected into Neuro-2a cells along with pRL-TK and either pCMV-Sox11 or pCMV-empty vector. **Figure 15** summarizes the results of these assays. Exon IV promoter demonstrated very little activity when transfected into Neuro-2a cells in the absence of Sox11, whereas cotransfection of pCMV-Sox11 and pGL2-BDNFpro4 increased promoter activity 23-fold over baseline (n = 3, $p < 0.0001$, Student's t-test). This demonstrates that Sox11 is also sufficient to activate the BDNF exon IV promoter, consistent with the hypothesis that Sox11 regulates BDNF IV expression following nerve injury.

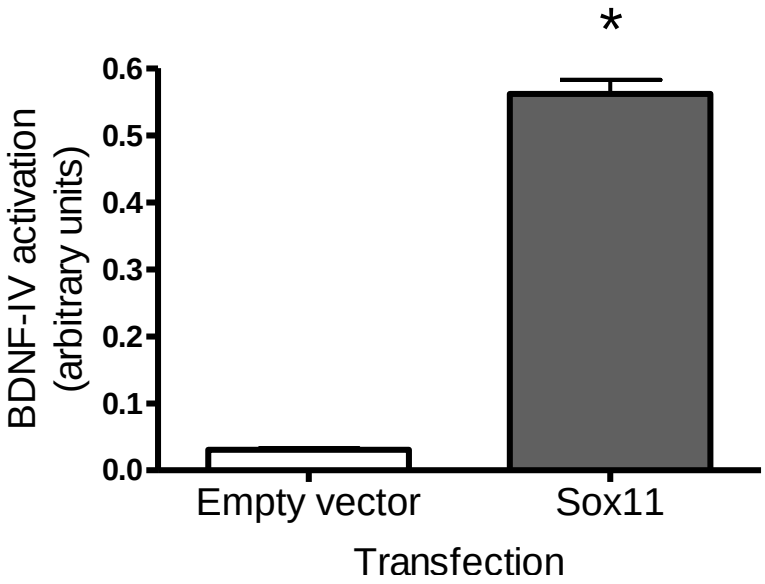

Figure 15: Sox11 activates the BDNF exon IV promoter. Neuro-2a cells were cotransfected with pGL2-BDNF4 luciferase reporter vector, pCMV-Sox11 or pCMV weight control vector, and pRL-TK (normalization signal). Cotransfection with Sox11 caused a 23-fold activation over baseline of the BDNF exon IV promoter ($n = 3$ per group, $p < 0.0001$, Student's t-test).

4.0 SOX11 MODULATES APC FOLLOWING NERVE INJURY

4.1 Introduction

Adenomatous polyposis coli (APC) is a scaffold protein which associates directly with the microtubule cytoskeleton, and is also capable of altering Wnt signaling pathway dynamics (Barth et al., 2008). Microtubule regulation is key to axonal extension following nerve injury: microtubule plus-end extension into the growth cone is required for both axonal extension and for the turning of the growth cone that is an essential part of axon guidance (Gallo, 1998, Avwenagha et al., 2003, Bouquet et al., 2004, Jones et al., 2006, Rajnicek et al., 2006). APC is a microtubule-associated protein (MAP) which interacts with the plus ends of microtubules and is capable of bundling microtubules, increasing their stability and facilitating their coordinated extension (Zumbrunn et al., 2001, Votin et al., 2005, Barth et al., 2008). APC is inactivated via phosphorylation by its binding partner GSK-3β (Zumbrunn et al., 2001). Signaling downstream of the APC- GSK-3β pathway is a required component of axon guidance during development, particularly neurite extension (Ahmed et al., 2002, Votin et al., 2005, Zhou et al., 2006, Hayden et al., 2007, Kroboth et al., 2007, Purro et al., 2008, Fancy et al., 2009, Ivaniutsin et al., 2009, Paridaen et al., 2009). The microtubule-stabilizing capability of APC and GSK-3β is also required for axonal regeneration following injury (Zhou et al., 2006). Removal of APC gene function via either transgenic approaches or RNAi, in model systems including fruit fly, mouse, and human-derived cells, has resulted in clear deficits in cell process extension, neuronal morphology, and nervous system development (Hayden et al., 2007, Kroboth et al., 2007, Purro et al., 2008, Ivaniutsin et al., 2009, Paridaen et al., 2009). The major pathways that have been described as regulators of APC-mediated microtubule stabilization and growth cone direction in neuronal cells, are non-canonical Wnt pathway signaling, extracellular matrix (ECM)/integrin

interactions, and neurotrophin signaling. **Figure 16** summarizes the major pathways which regulate APC in neuronal cells.

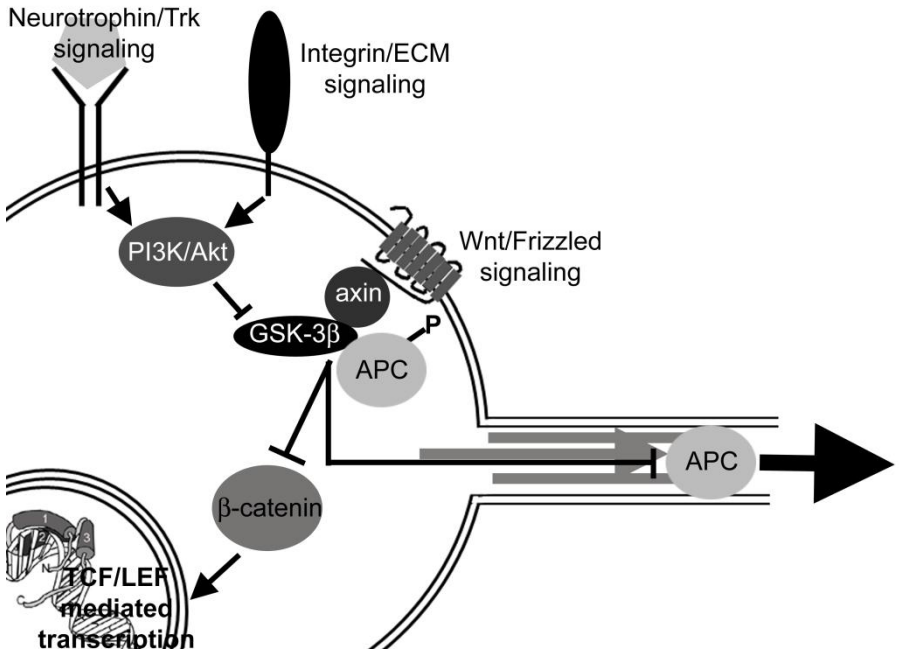

Figure 16. APC acts as a microtubule stabilizer and a modulator of Wnt signaling dynamics. Neurotrophin/Trk receptor interactions, and integrin/extracellular matrix interactions, function through the PI3K/Akt pathway to increase APC activation. PI3K/Akt activation results in GSK-3b inhibition and a net increase in APC binding of microtubules to increase microtubule stability and neurite outgrowth. The divergent canonical Wnt signaling pathway may also increase APC activation and microtubule stability via a cascade that requires Disheveled and axin. At the same time, the APC/GSK-3b/axin complex is capable of binding and inhibiting beta-catenin, preventing it from activating the TCF/LEF transcription factors, which are the effectors of the canonical Wnt signaling pathway.

Wnt signaling affects APC localization and activation. The Wnt signaling pathway known to modulate APC activity is the divergent canonical signaling pathway which functions downstream of the Frizzled receptor. Activation of Frizzled by binding of stimulatory Wnts

results in the activation of Dishevelled (Dvl) and, subsequently, the inhibition of GSK-3β in an axin-dependent manner (Lucas et al., 1998, Orme et al., 2003, Ciani et al., 2004, Chiang et al., 2009). Although an increase in APC binding of microtubules following activation of this pathway has not yet been demonstrated, inhibition of GSK-3β, specifically by axin, has been shown to be essential to Wnt-mediated neurite extension (Orme et al., 2003). Furthermore, inhibitory Wnts such as Wnt3a signal via a divergent pathway that results in increased activation of GSK-3β, facilitating microtubule collapse and axonal turning (Purro et al., 2008).

Integrin/ECM interactions are a second regulator of the GSK-3β /APC pathway. Integrins are a class of receptors which bind extracellular matrix proteins of the laminin family. The formation of focal adhesion complexes following laminin/integrin interactions are important regulators of the actin and microtubule cytoskeletons; in addition, focal adhesion complexes may transduce survival signals. One signaling cascade activated following laminin/integrin interaction involves integrin-linked kinase (ILK), which functions in the PI3K/Akt pathway downstream of PI3K but upstream of Akt (Guo et al., 2007). ILK and neurotrophin signaling are both required for initiation of neurite extension in developing neurons (Naska et al., 2006), and ILK signaling appears to facilitate neurite extension mediated by neurotrophins (Mills et al., 2003). The effects of ILK activation on neurite extension are dependent on GSK-3β inhibition (Mills et al., 2003, Naska et al., 2006, Guo et al., 2007, Oinuma et al., 2007), and can be enhanced by activation of integrin (Gibson et al., 2005) and blocked by deletion of laminin subunits (Chen et al., 2009), indicating that ILK's effect on neurite outgrowth reflects ILK-induced inhibition of GSK-3β downstream of laminin/integrin binding.

The third major regulator of APC-mediated microtubule extension is neurotrophin signaling. Zhou and Snider demonstrated that PI3K activation downstream of neurotrophin-Trk

receptor interaction results in GSK-3β inhibition and increased neurite extension (Zhou et al., 2006); furthermore, this work demonstrated that APC is a required component for the well-demonstrated effects of neurotrophins as facilitators of neurite extension and as chemotropic factors (Zhou et al., 2006). While laminin/integrin interaction appears to be required during development to sufficiently activate the PI3K/ILK/Akt pathway to facilitate survival and neurite extension (Chen et al., 2009), neurotrophin signaling alone appears to be sufficient to drive axonal regeneration in adult peripheral sensory neurons (Zhou et al., 2006).

APC also affects transcriptional regulation within cells, due to its additional function as a negative regulator of canonical Wnt signaling. APC and GSK-3b, together with the scaffolding proteins Dishevelled and axin, form a complex associated with the Frizzled receptor (Barth et al., 2008). This complex binds beta-catenin, an essential constituent of the canonical Wnt pathway . The association of beta-catenin with the APC/ GSK-3β scaffold results in the ubiquitination and subsequent degradation of beta-catenin, thereby preventing its nuclear translocation and activation of the TCF/LEF family of transcription factors (Li et al., 2005a). Beta-catenin-mediated pathways are essential in neuronal fate specification (Hari et al., 2002, Lee et al., 2004, Kormish et al., 2010), as well as in neurite outgrowth and neural regeneration (Votin et al., 2005, Maro et al., 2009, Paridaen et al., 2009), and the APC- GSK-3β signaling pathway is a key regulator of beta-catenin signaling during neuronal development (Ahmed et al., 2002, Li et al., 2005a, Kroboth et al., 2007, Takacs et al., 2008, Ivaniutsin et al., 2009, Paridaen et al., 2009).

Although pathways that involve cytoskeletally-associated APC activity, as well as APC inhibitory effects on beta-catenin, are known to be required for regeneration following nerve injury, little is known about the extent of APC regulation after injury, or about the molecular mechanisms governing APC expression in neurons; the majority of the research on this pathway

has focused on the regulation of the APC inhibitor GSK-3β. The goals of this portion of my dissertation research were to determine whether APC is transcriptionally-regulated at early time points following nerve injury and, if so, whether APC may be a transcriptional target of Sox11.

4.2 APC is upregulated following nerve injury.

Previous research indicates that the APC/GSK-3β pathway is essential for neurite specification, neurite extension, and neurite regeneration (Avwenagha et al., 2003, Mills et al., 2003, Votin et al., 2005, Naska et al., 2006, Zhou et al., 2006, Oinuma et al., 2007, Purro et al., 2008, Paridaen et al., 2009, David et al., 2010). However, there are no data regarding whether APC is upregulated following nerve injury in the adult rodent. To determine whether APC is upregulated following nerve injury, mice were sacrificed at 1 or 3 days following sciatic nerve cut injury and RNA was isolated from the L3-L5 DRGs. Semi-quantitative real-time PCR was performed on the RNA; results were compared to naïve controls. **Figure 17** shows the results of the real-time PCR analysis. In nerve-injured mice 1 day following injury, APC was significantly upregulated compared to naïve mice ($n = 4$ mice per condition, $\Delta\Delta Ct = -0.355$, fold change = 1.278, $p < .01$ [one-way ANOVA with Bonferroni post-hoc testing]). APC expression was not significantly altered compared with naïve mice by three days post-sciatic nerve cut.

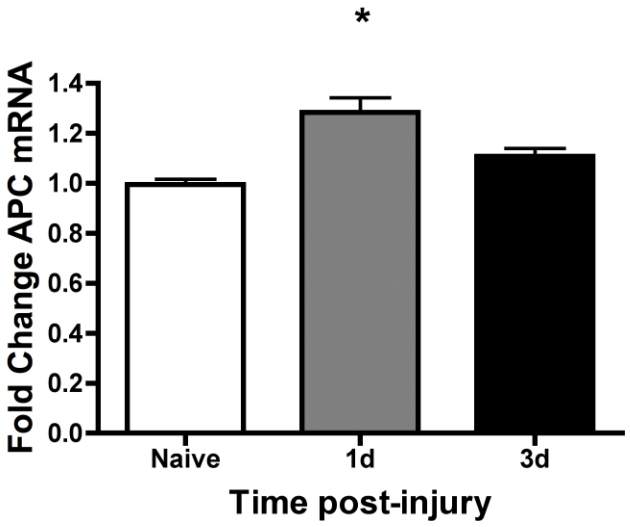

Figure 17: APC is transiently upregulated following nerve injury. Left L3-L5 DRGs were harvested from naïve mice, as well as mice one day or three days post-sciatic axotomy (n = 4 per group, 12 animals total). Real-time PCR was performed to assess APC mRNA expression. APC mRNA was significantly increased 24 hours post-axotomy ($p < 0.01$ vs. naïve mouse, ANOVA with Bonferroni post-hoc test).

4.3 APC is upregulated following Sox11 overexpression in neurons.

To determine whether the transient upregulation in APC expression following sciatic nerve injury might be at least partially due to Sox11-mediated transcription of APC, dissociated DRG cultures were prepared and infected with either HSV-GFP (control virus) or HSV-Sox11 (Sox11 overexpressing virus). RNA was isolated from these cultures and used for real-time PCR analysis. **Figure 18** shows the results of this analysis. Compared with control cultures, Sox11-overexpressing cultures also expressed significantly more APC mRNA (n = 4 cultures per condition, $\Delta\Delta Ct = -0.837$, fold change = 1.78, $p < 0.05$ [one-tailed t-test]). Overexpression of Sox11 is sufficient to induce transcription of APC mRNA, indicating that APC may be a transcriptional target of Sox11.

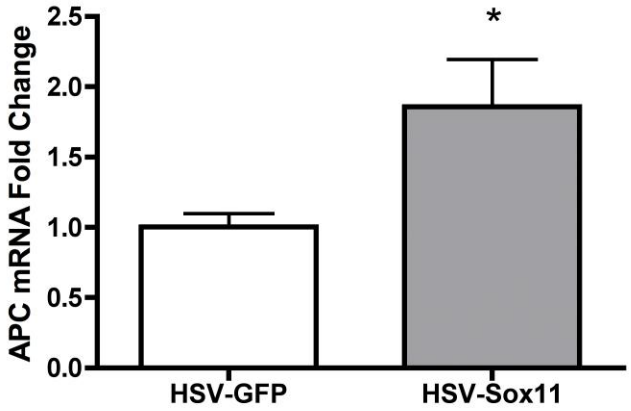

Figure 18: Sox11 overexpression increases APC transcription. Cultured DRG neurons were infected with HSV-GFP (control) or HSV-Sox11, and real-time PCR analysis was performed to determine relative APC mRNA abundance. Sox11 overexpression significantly increased APC expression compared with control viral vector ($n = 4$ per group, $p < 0.05$, one-tailed t-test).

4.4 Sox binding sites on APC promoter

To determine whether Sox11 may possibly bind to and directly regulate the APC promoter, the 5 kb proximal to the APC transcriptional start site of both mouse and human genome was analyzed for Sox consensus binding sites using MacVector 9.0 software. The human APC gene contains two transcriptional variants transcribed from two separate promoters; each promoter was separately analyzed (the promoters are more than 5 kb apart, so analysis of overlapping sequences was not a concern).

Both mouse and human APC genes contain multiple Sox binding sites in their promoter regions (see **Table 7**). The mouse APC promoter contains seven forward-orientation Sox sites and three Sox sites in the reverse orientation. The first human APC promoter contains one forward Sox site and four reverse-direction sites, while the second human APC promoter contains five Sox sites in the forward direction and five in the reverse direction. While there are no instances of the AACAAAG motif in the mouse APC promoter, there are two forward-

direction sites with the similar motif TACAAAG. Both human APC promoters contain the AACAAAG motif in the 5' → 3' orientation: the first APC promoter has one instance of this motif, while the second APC promoter contains two AACAAAG motifs.

Species	**# of Sox sites on (+) strand**	**Positions relative to transcriptional start**	**# of Sox sites on (-) strand**	**Positions relative to transcriptional start**
Mouse	**7**	-1029 (TACAAAG) -1468 (AACAATG) -1779 (TACAATG) -1841 (TACAATG) -3372 (TTCAAAG) -4855 (TACAAAG) -4904 (TTCAAAG)	**3**	-2549 (ATCAATG) -4121 (TTCAAAG) -4670 (TACAAAG)
Human (pro 1)	**1**	-3218 (**AACAAAG**)	**4**	-163 (TACAATG) -2699 (TACAATG) -2846 (AACAATG) -3414 (TTCAAAG)
Human (pro 2)	**5**	-1064 (**AACAAAG**) -1568 (**AACAAAG**) -2755 (TACAATG) -3477 (TTCAATG) -4995 (TACAAAG)	**5**	-518 (TACAATG) -1596 (TACAATG) -1938 (**AACAAAG**) -2329 (ATCAAAG) -3670 (TACAATG)

Table 7. Sox binding sites on mouse and human APC promoters. Both mouse and human APC promoters contain multiple Sox binding sites. Human APC promoters contain multiple instances of the AACAAAG motif, but not mouse APC promoters. However, the mouse APC promoter contains the similar motifs TACAAAG and AACAATG.

To determine whether Sox-containing regions of the APC promoter are conserved between human and mouse, 300-bp stretches of APC promoter surrounding Sox binding sites were subjected to ClustalW alignment in MacVector. Following sequence alignment, the aligned sequences were used as a basis for phylogenetic reconstruction to determine relatedness. Several

sequences which appear in the mouse APC promoter were conserved in human APC promoter regions (**Figure 19**). Phylogenetic reconstruction shows that there are two main clusters of Sox-containing regions which are conserved in human and mouse APC promoters (**Figure 19, top**). A consensus sequence was generated from the two most highly-homologous Sox site-containing regions of human and mouse APC genes (**Figure 19, bottom**): this sequence contains binding sites for numerous other transcription factors, including POU family transcription factors from the Brn and Oct families, as well as TCF/LEF transcription factor binding sites.

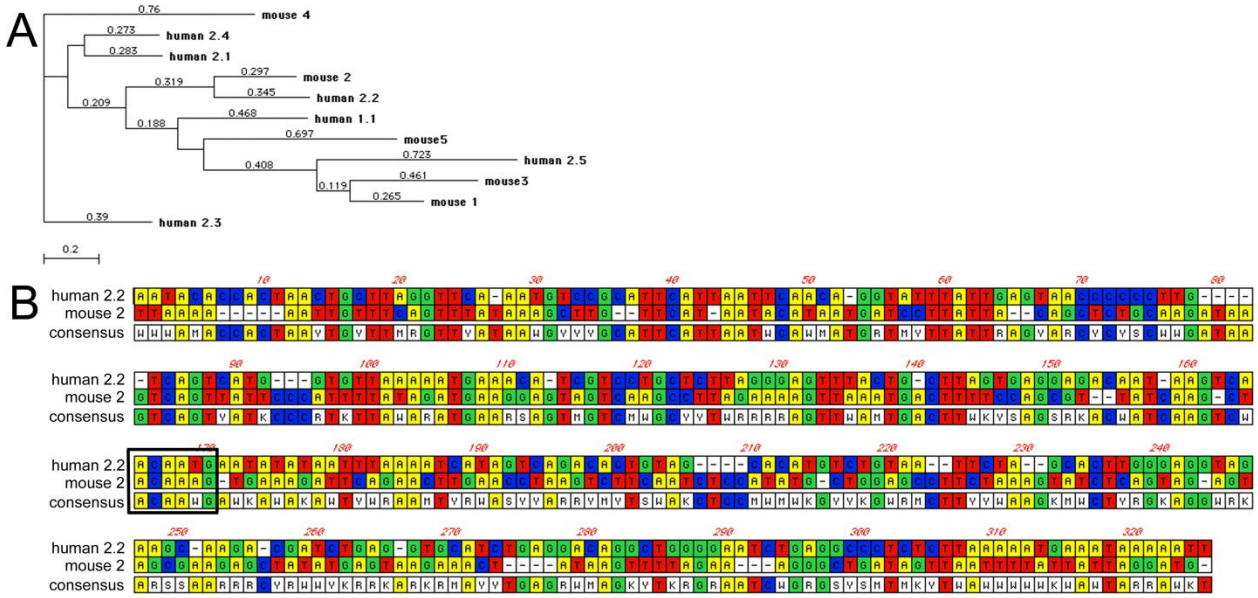

Figure 19: Sox binding sites are conserved on the APC promoter in human and mouse. Short (300-nt) Sox binding site-containing sequences, in the APC promoter of both mouse and human, were subjected to ClustalW sequence alignment. Each binding site on the promoter was separately analyzed: numbers are given in increasing order of proximity to the APC transcriptional start site (i.e. mouse sequence 1 is the most distal sequence on the mouse promoter). **(A)** Relatedness of APC sites on human and mouse promoters. **(B)** Construction of a consensus sequence for the two most homologous sequences from the mouse and human APC promoter regions. The consensus sequence contains multiple binding sites for many different transcription factors, including transcription factors in the POU family.

4.5 Sox11 increases activation of APC proximal promoter

To determine whether Sox11 might increase APC transcription via activation of the APC promoter, a 2.2 kilobase fragment proximal to the APC transcriptional start site was cloned into the pGL2-Basic vector to produce pGL2-APC (see **Section 2.11**). **Figure 20** diagrams the pGL2-APC vector. The resulting construct was transfected into Neuro-2a cells along with pRL-TK and either pCMV-Sox11 or pCMV-empty vector. **Figure 21** summarizes the results of these assays. Cotransfection of pCMV-Sox11 and pGL2-APC increased promoter activity 11.6-fold over baseline ($n = 3$, $p < 0.0001$, Student's t-test). This demonstrates that Sox11 is sufficient to activate the APC promoter, consistent with the hypothesis that Sox11 regulates APC expression following nerve injury.

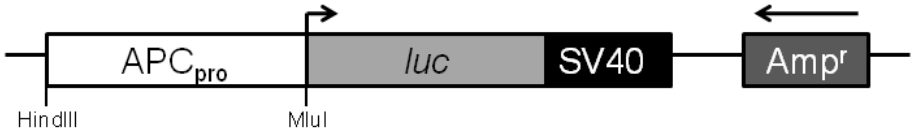

Figure 20: Construction of the pGL2-APC reporter vector. A 1.9-kb fragment of the mouse APC promoter containing 2 Sox binding sites was cloned from DNA obtained from the liver of a male C57/Blk6 mouse using the TOPO-TA cloning system (Invitrogen). The fragment was subcloned into the pGL2-Basic vector to form pGL2-APC. APCpro: APC promoter region (Sox sites are marked in terms of distance from the APC transcriptional start site); luc: luciferase coding sequence; SV40: SV40 polyadenylation sequence.

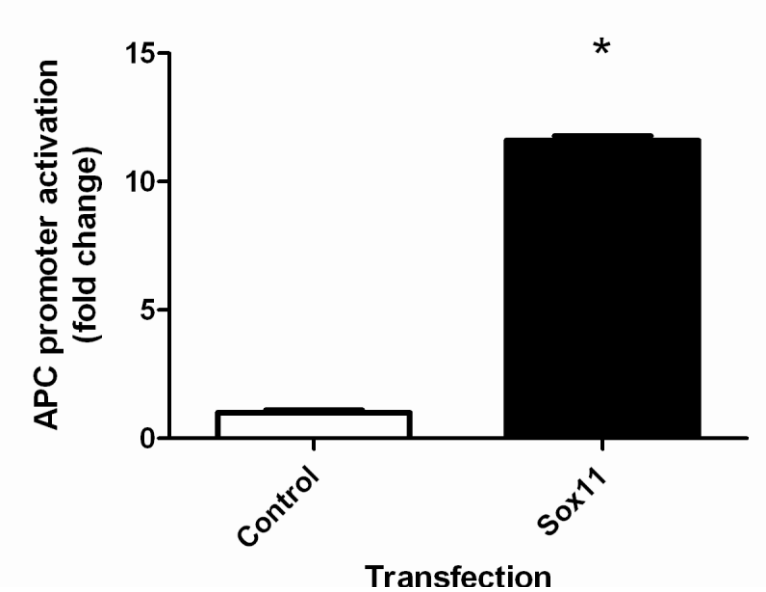

Figure 21. Sox11 activates the APC promoter. A 1.9-kb region of the APC promoter, containing two forward Sox binding sites, was cloned into the pGL2-Basic vector. Neuro-2a cells were cotransfected with pGL2-APC luciferase reporter vector, pCMV-Sox11 or pCMV weight control vector, and pRL-TK (normalization signal). Cotransfection with Sox11 caused a 11.6-fold activation over baseline of the APC promoter. All values mean +/- SEM, *P < .0001 vs. WT.

5.0 SOX11 MODULATES EXPRESSION OF THE GENE ENCODING TRAF FAMILY MEMBER-ASSOCIATED ACTIVATOR OF NF-KAPPA B (TANK)

5.1 INTRODUCTION

TANK protein was first identified in 1996 by two groups working independently to find binding partners of TNF-receptor associated factor (TRAF) family member proteins. Cheng and Baltimore found that TANK was a net activator of TRAF2-mediated NF-kappa B activation whereas Rothe et al. found that TANK inhibited TRAF2-dependent signaling (Cheng and Baltimore, 1996, Rothe et al., 1996). This early difference in proposed function for TANK suggests TANK can influence cell signaling in different ways, dependent on the cellular context.

TANK can associate with diverse TNF receptor-associated family proteins (TRAFs), including TRAF1, TRAF2, TRAF3, and TRAF6 (Arron et al., 2002). The TRAF proteins are intracellular signaling mediators that function downstream of many receptor proteins, including those in the TNF (tumor necrosis factor) receptor superfamily (TNFR-SF). The TNFR-SF proteins are a large and diverse family, some members of which include the TNF-alpha receptors TNFRI and TNFRII, as well as the p75 neurotrophin receptor (p75 NTR). TRAF proteins associate with the cytoplasmic tail of these receptors, dissociating from the receptor when the receptor is activated by ligand binding (the cytokine TNF-alpha in the case of the TNFRs, or the neurotrophins NGF or BDNF in the case of the p75 NTR) (Arron et al., 2002). TRAFs are capable of activating numerous kinases in sensory neurons, including IKK family members, p38 MAP kinase, and JNK (c-Jun terminal kinase) (Hehlgans and Mannel, 2002, Park et al., 2002, Pollock et al., 2002, Dempsey et al., 2003, Schafers et al., 2003b, Fernyhough et al., 2005). The net effect of TNFR-TRAF signaling depends on cell type and environmental factors both inside and outside the cell. Activation of TNFR-SF receptors can lead to the activation of survival

signaling pathways, death pathways, or pathways associated with cell fate specification and differentiation (Yoshida et al., 1992, Ware et al., 1996, Baker and Reddy, 1998, Arnett et al., 2001, Robertson et al., 2001, Gupta, 2002, Mielke and Herdegen, 2002, Park et al., 2002, Cha et al., 2003, Lee and Kim, 2003, Conti et al., 2005, Fernyhough et al., 2005, Heldmann et al., 2005, Quintana et al., 2005, Murata et al., 2006, Chung et al., 2007, Soni et al., 2007, Angileri et al., 2008, Lee et al., 2008, Ajmone-Cat et al., 2010, Kanno et al., 2010, Chen et al., 2011).

TNFR-SF receptors are expressed in primary sensory neurons, both in the uninjured state and following axotomy (Schafers et al., 2003a, Schafers et al., 2008). Both TNFRI and TNFRII are expressed in DRG neurons of all sizes, indicating that they are both likely expressed in nociceptive and non-nociceptive sensory neurons (Schafers et al., 2003a, Schafers et al., 2008). Both TNFRI and TNFRII participate in TRAF-mediated signaling, and TANK may therefore function downstream of either receptor. A major difference between TNFRI and TNFRII is that TNFRI contains a death domain which TNFRII lacks. Activation of the death domain typically results in activation of pro-apoptotic proteins (including many caspases) and cell death (Baker and Reddy, 1998, Harry et al., 2008, Silke and Brink, 2010). However, activation of TNFRI does not automatically entail pro-apoptotic signaling; inhibitors of apoptosis protein (cIAP) are also activated downstream of TNF-alpha receptors (Kanno et al., 2010, Silke and Brink, 2010). Furthermore, TRAF activation downstream of TNF-alpha receptors can activate many neuroprotective cell signals, including transcription of pro-survival signaling molecules downstream of NF-kappa B activation (Ha et al., 2009).

Many neurons in the DRG also express the p75 NTR, although this receptor is significantly downregulated following axotomy (Krekoski et al., 1996). The p75 NTR is upregulated in primary sensory neurons grown in vitro, however, and application of BDNF to

these cultured neurons results in programmed cell death (Zhou et al., 2005), indicating that neurotrophin-p75 signaling has a deleterious effect on survival of these neurons, possibly via death domain signaling. Additionally, the availability of TNFR-SF receptors' ligands increases during nerve injury. The cytokine TNF-alpha is upregulated in both cut nerve and in DRG at time points immediately following nerve injury (Taskinen et al., 2000, Ohtori et al., 2004, Miao et al., 2008), while BDNF and NGF are both upregulated within 24 hours of injury and remain upregulated for weeks following axotomy (Funakoshi et al., 1993, Lee et al., 2001). **Figure 22** summarizes the three main effectors of TRAF-dependent (i.e. non-death-domain-dependent) TNFR-SF signaling and their possible implications for neuronal survival and/or regeneration following injury.

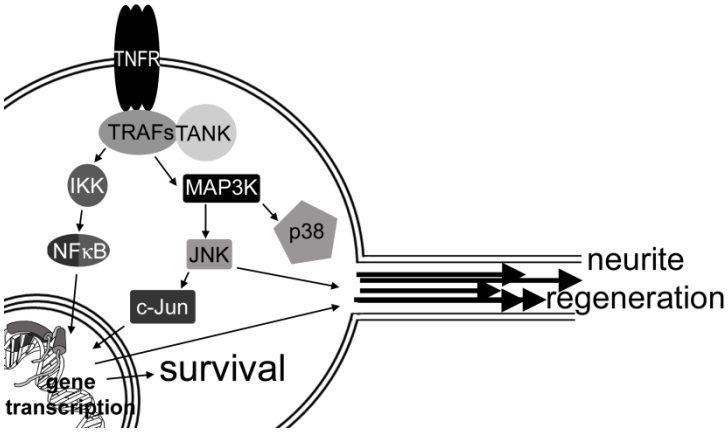

Figure 22. TRAF-dependent TNF receptor signaling and its possible implications for neuronal survival and regeneration. TRAFs activate the transcription factor NF-kB via I-kappa kinase signaling. Furthermore, TRAFs activate JNK and p38 MAP kinase via MAP3K signaling pathways. JNK has been linked to both survival and regeneration via c-Jun dependent transcription as well as c-Jun-independent pathways. p38 is not known to be implicated in pro-regenerative signaling in the injured DRG.

The three main effectors of TRAF-dependent TNFR-SF signaling are the transcription factor NF-kappa B, the p38 MAP kinase, and the kinase JNK (c-Jun N-terminal kinase) (Arron et al., 2002). Previous research demonstrates that it is unlikely that NF-kappa B activation in neurons contributes to the early stages of regeneration in adult mice. While injured neurons frequently display activated NF-kappa B (Fernyhough et al., 2005), these neurons do not upregulate their transcription of genes activated by NF-kappa B (Gushchina et al., 2009). This appears to be due to the activity of histone deacetylases (HDACs), which remove acetyl groups from the areas of chromatin containing NF-kappa B transcriptional targets, thereby inactivating those genes (Gushchina et al., 2009). (Activation of NF-kappa B in Schwann cells and in immune cells, however, appears to be essential for remyelination and other processes involved in successful neuronal regeneration (Camara-Lemarroy et al., 2010).) The two other main effectors of TNFR-SF signaling, p38 MAP kinase and JNK, are both implicated in the neuronal response to injury.

p38 MAPK is upregulated in the DRG following peripheral inflammation and peripheral nerve injury (Kikuchi et al., 2000, Kim et al., 2002, Obata et al., 2004b, Jiang et al., 2005, Yamanaka et al., 2007). The consequences of p38 MAPK activation have been very well-studied in terms of the contribution that p38 MAPK makes to the hyperexcitability and increased conductance of injured neurons, and it has been demonstrated multiple times that p38 MAPK is a major contributor to the development of neuropathic pain, both at the level of the DRG and the spinal dorsal horn (Kim et al., 2002, Pollock et al., 2002, Schafers et al., 2003b, Obata and Noguchi, 2004, Obata et al., 2004a, Obata et al., 2004b, Svensson et al., 2005, Zelenka et al., 2005, Jin and Gereau, 2006, Obata et al., 2006, Ito et al., 2007, Xu et al., 2007, Yamanaka et al.,

2007). However, there is relatively little evidence suggesting that p38 MAPK is involved as a positive regenerative signal. In fact, p75 NTR-mediated p38 MAPK activation may actually inhibit neuronal regeneration (Myers et al., 2003). One paper shows that p38 MAPK activation can stimulate neurite outgrowth; however, this p38 MAPK-dependent neurite outgrowth was mediated by the proinflammatory cytokine IL1-beta, whose receptor is a member of the Toll-like receptor superfamily (TLR-SF), not the TNFR-SF (Temporin et al., 2008).

The final major effector of TNFR-SF signaling is JNK, which is extremely strongly linked to both peripheral nerve injury and regeneration of injured peripheral axons (Leah et al., 1991, Kenney and Kocsis, 1998, Mielke et al., 1999, Lindwall et al., 2004, Lindwall and Kanje, 2005b, Ciani and Salinas, 2007, Barnat et al., 2010). There are three different isoforms of JNK: JNK1, JNK2, and JNK3. All three isoforms are expressed in neurons (Barnat et al., 2010). JNK proteins are important regulators of neuronal regeneration by both c-Jun-dependent and c-Jun-independent mechanisms (Leah et al., 1991, Lindwall et al., 2004, Barnat et al., 2010). In injured primary sensory neurons from the trigeminal ganglion, JNK1 is the main activator of the transcription factor c-Jun (Wu et al., 2008). c-Jun activity is required for peripheral regeneration (Leah et al., 1991, Jenkins et al., 1993, Lindwall et al., 2004). JNK is also an upstream regulator of the transcription factor ATF3, via JNK-dependent phosphorylation of ATF2 (Lindwall et al., 2004). ATF3 is so frequently expressed in injured sensory neurons that it is widely used as a marker of neuronal injury (Tsujino et al., 2000). Furthermore, ATF3 activation is also required for normal regeneration of injured primary sensory neurons (Seijffers et al., 2007). Chin et al. have previously demonstrated that TANK may activate JNK via TRAF2-dependent signaling (Chin et al., 1999); however, this was in a non-neuronal cell line, and TRAF signaling dynamics may significantly vary between cell types.

JNK has additional cellular functions besides activation of transcription factors: recent studies have shown that JNK pool is also implicated in synaptic plasticity (Costello and Herron, 2004, Etter et al., 2005, Li et al., 2007, Kenney et al., 2010, Pavlowsky et al., 2010, Liu et al., 2011) and in glutamate receptor trafficking (Thomas et al., 2008). Furthermore, JNK activation is required for neuritogenesis and neurite extension; although all three isoforms differentially participate in the regenerative response, they appear to do so via a common mechanism of increasing microtubule stability (Neidhart et al., 2001, Chang et al., 2003, Tararuk et al., 2006, Ciani and Salinas, 2007, Barnat et al., 2010, Podkowa et al., 2010). Pharmacological inhibition of specific JNK isoforms demonstrated that JNK2 and JNK3 were required for neuritogenesis, whereas JNK1 and JNK2 were required for the elongation of neurites (Barnat et al., 2010). This later function appears to be related related to JNK's ability to phosphorylate microtubule-associated proteins (Neidhart et al., 2001, Chang et al., 2003, Tararuk et al., 2006, Ciani and Salinas, 2007).

TANK was identified as a possible target of Sox11 in our microarray pilot study. Downregulation of TANK mRNA following Sox11-targeted siRNA treatment of cultured Neuro-2a cells was further validated using real-time PCR ((Jankowski et al., 2006); **Figure 23**). The aims of this portion of my dissertation were to determine, 1) whether TANK mRNA and protein expression is modulated following nerve injury, 2) If TANK protein is expressed in DRG neurons, 3) If an injury-induced change in TANK expression is mediated by Sox11-dependent activation of the TANK promoter, to determine whether TANK protein is expressed in DRG neurons, and 4) If alterations in TANK protein expression affect the balance of signaling downstream of TNF-alpha receptors, using endpoints of JNK activation and cell survival.

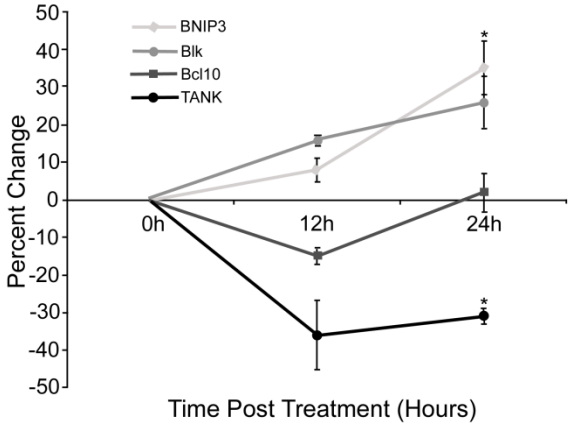

Figure 23. TANK mRNA is downregulated following Sox11 knockdown. Neuro-2a cells were treated with siRNA targeted against Sox11. At 24h following knockdown, TANK mRNA was significantly reduced relative to cells treated with a nontargeting control siRNA. Figure adapted from (Jankowski et al., 2006).

5.2 TANK is upregulated following nerve injury.

RTPCR was performed to assess Sox11 and TANK expression in cDNA samples prepared from L3-L5 DRGs of uninjured mice (n = 4), and the L3-L5 DRGs of sciatic nerve-injured mice at the 1d and 3d post injury (n = 4 for each group). At 1 day following nerve injury, Sox11 expression had risen to 800% of baseline (**Figure 24;** $p < 0.001$). TANK expression also increased to 220% of baseline ($p < 0.01$; all mRNA statistical analysis conducted using one-way ANOVA with Bonferroni post-hoc test). At 3d post injury, Sox11 expression was still increasing, with expression at 950% of baseline ($p < 0.001$) whereas TANK expression remained elevated over baseline (130%; $p < 0.025$), although this marked a decrease in TANK expression from the 1d time point.

To determine whether the injury-induced increase in TANK mRNA expression occurred at the protein level, western blot analysis was performed on naïve and 3d injury DRG lysates (n = 4 per group with each sample composed of DRGs from two mice). **Figure 24** shows a

representative western blot from naïve vs. injured mice, as well as the quantification of western blot band intensity from all western blots following normalization to GAPDH. At 3d following injury TANK band intensity was increased by approximately 36% ($p < 0.05$, Student's t-test), indicating that TANK is increased at the protein level.

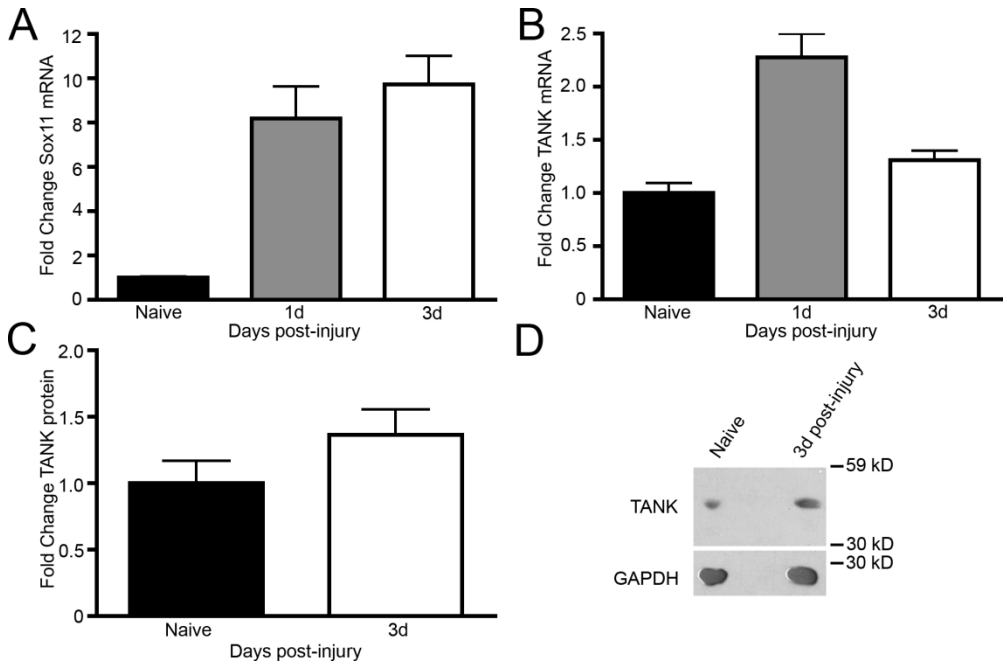

Figure 24. TANK is upregulated following nerve injury. (A) Sox11 mRNA is significantly upregulated one day and three days post-sciatic nerve axotomy ($n = 4$ animals per group, $p < 0.001$ vs. naïve, ANOVA with Bonferroni post-hoc test). (B) TANK mRNA is significantly upregulated one day post-sciatic nerve axotomy ($n = 4$ animals per group, $p < 0.05$ vs. naïve, ANOVA with Bonferroni post-hoc test). (C) TANK protein is significantly upregulated three days post-sciatic nerve axotomy ($n = 4$ animals per group, $p < 0.05$ vs. naïve, Student's t-test). (D) Representative western blot of TANK protein upregulation post-sciatic nerve cut.

Together, these data indicate that TANK mRNA is increased at early time points following nerve injury, that the increase in TANK mRNA occurs at a time point consistent with the possibility of Sox11-mediated TANK upregulation, and that the increase in TANK mRNA

levels also occurs at the protein level. Thus, TANK protein may affect cellular function following nerve injury.

5.3 TANK is expressed by DRG neurons.

To determine if DRG neurons express TANK protein, dissociated DRG cultures were prepared on laminin- and poly-D-lysine-coated coverslips and grown in complete medium for 24 hours. The cultures were then triple-labeled with the nuclear stain DAPI and antibodies against TANK and beta-III-tubulin (a neuronal marker). **Figure 25(A)** shows representative immunostaining results from these colabeled DRG cultures; **Table 8** summarizes the results of cell counts.

All tubulin-labeled cells (105 cells; 28.6% of all cells counted), stained positive for TANK, indicating that TANK is expressed in virtually all dissociated DRG neurons. Some TuJ3-negative cells, which are likely glial cell types, also exhibited TANK immunolabeling (123 cells; 33.5% of all cells counted). TANK-positive glia frequently exhibit morphologies characteristic of Schwann cells, i.e., elongated spindle-shaped, bipolar cells. Another population of cells was TuJ3- and TANK-negative (139 cells; 37.9% of all cells counted), indicating that not all glial cells express TANK at detectable levels.

To insure the specificity of TANK labeling a blocking experiment was performed. TANK antibody was preincubated with a three-fold excess of TANK blocking peptide (Abcam) at 4 degrees overnight on a shaker platform and the immunolabeling performed the next day. Preincubation with blocking peptide resulted in elimination of TANK staining from neuronal and glial cells in 24h DRG cultures (**Figure 25, A and B**). After 48h, TANK immunostaining intensity was diminished but not eliminated by the blocking peptide (**Figure 25B**). After 72 hours in culture, TANK immunostaining was only slightly diminished after preincubation with

blocking peptide (**Figure 24B**). This may reflect an upregulation in TANK protein levels as time spent in culture increases, which would increase the favorability of antibody binding to endogenous TANK rather than to the blocking peptide.

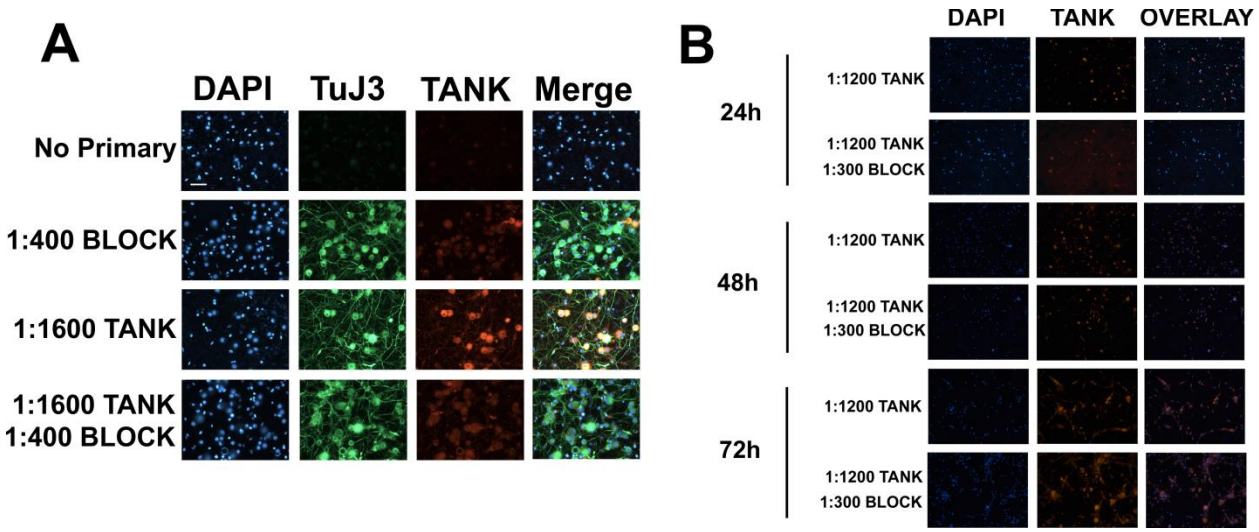

Figure 25. TANK protein is expressed in DRG neurons. A. Triple labeling of dissociated DRG cultures with anti-TANK antibody (red), anti-beta-III-tubulin antibody (neuronal marker, green) and DAPI (nuclear stain, blue). TANK was expressed in all DRG neurons and in a population of non-neuronal cells. Some non-neuronal cells did not exhibit TANK staining. Preincubation of TANK antibody with a three-fold excess of TANK blocking peptide caused loss of TANK staining in DRG neurons, indicating antibody binding specificity. **B**. Time course of TANK expression in DRG neurons. Four-fold excess of blocking peptide removes TANK staining after 24h in culture, diminishes TANK staining after 48h in culture but does not remove TANK staining after 72h in culture. This may reflect upregulation/stabilization of TANK protein with time.

	TuJ3 (+)	TuJ3(-)	TOTAL
TANK (+)	105	123	228
TANK (-)	0	139	139
TOTAL	105	262	367

Table 8. Counts of TANK immunostaining results from cultured DRG neurons. All neurons counted (105/105) expressed TANK protein. Non-neuronal (TuJ3-negative) cells were roughly evenly distributed between TANK-positive and TANK-negative (123/262 and 139/262, respectively).

5.4 Overexpression of Sox11 leads to TANK upregulation in Neuro-2a cells.

The possible interaction of Sox11 and TANK was further investigated to determine if increased Sox11 expression altered TANK level. Neuro2a cells transfected with pCMV-Sox11, a plasmid vector in which the CMV promoter drives expression of the mouse Sox11 cDNA, caused a 1.2-fold increase in TANK mRNA at 24h post-transfection ($n = 4$ per group, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test) (**Fig. 26).** An increase in TANK protein also occurred in transfected cells at 36h post-transfection ($n = 3$ per group, $p < 0.05$, Student's t-test) (**Fig. 26**). These data suggested that Sox11 could transcriptionally regulate *TANK* gene expression.

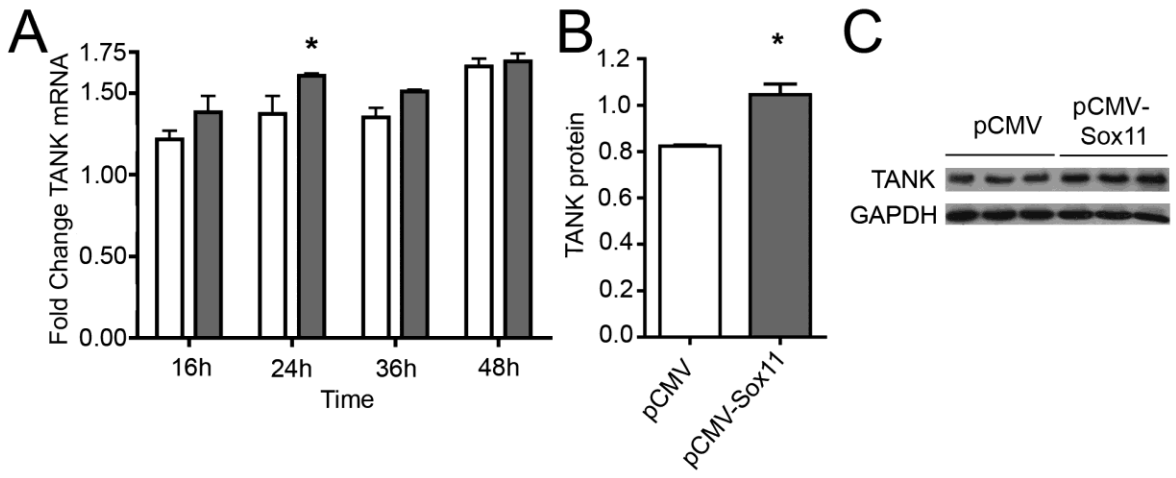

Figure 26. TANK is upregulated following Sox11 transfection in Neuro-2a cells. Neuro-2a cells were transfected with pCMV or pCMV-Sox11. (A) TANK mRNA expression was assessed at varying time points following transfection. Fold changes are shown relative to 0h baseline Neuro-2a cultures. TANK mRNA was upregulated significantly in pCMV-Sox11 samples relative to pCMV-transfected samples at the 24h time point ($n = 4$ per group, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). TANK expression increased overall as a function of time ($n = 8$ per time point, main effect using two-way ANOVA, $p < 0.05$). (B) TANK protein was upregulated at 36h in Sox11-transfected samples ($n = 3$ per group, $p < 0.05$, Student's t-test). (C) Western blot quantified in (b).

5.5 The TANK promoter contains multiple Sox-binding sites that are conserved in vertebrates.

To further investigate whether TANK upregulation following Sox overexpression may be due to direct activation of the TANK promoter by Sox11, we screened the proximal promoter (5 Kbp) of *TANK* for the degenerate SRY binding motif 5'-(A/T)(A/T)CAA(A/T)G-3' (Harley et al., 1994, Badis et al., 2009). Across several vertebrate species with the exception of the common marmoset, at least one Sox binding sequence was present (**Fig. 27, top**). A sequence alignment analysis on short (300 bp) stretches of sequence containing each Sox binding site was then performed to further determine relatedness. Most species have multiple Sox binding sites but not all are homologous to one other (data not shown). One cluster within 1 kb of the *TANK* transcriptional start site (in rodent and primate TANK genes) contains one binding site found in mouse and rat and two binding sites each in human, chimpanzee and macaque (the second binding site shows several deletions in comparison to the first, suggesting this site may be due to reduplication of this section of the genome in an early primate). An alignment of this region is shown in **Fig. 27, bottom.** The conserved nucleotides in this region, in addition to the conserved AACAAAG Sox binding site, include binding sites for various other transcription factors, including TCF/LEF transcription factors, and POU homeodomain family transcription factors including Oct-4, Brn family members, and Ngn-1.

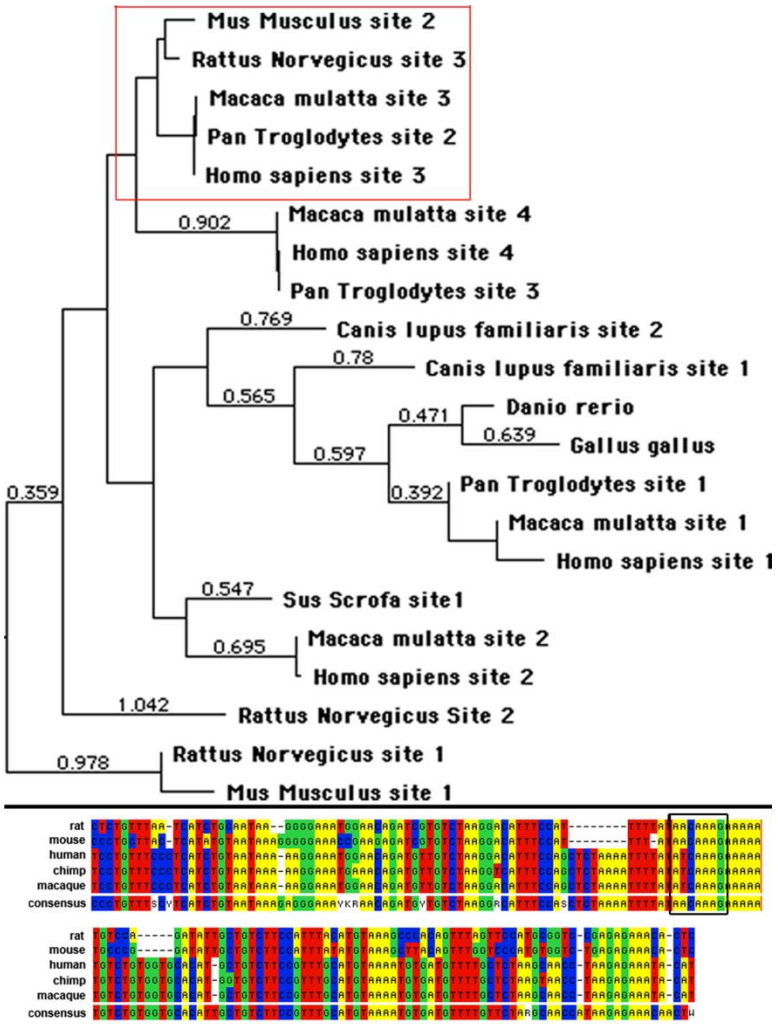

Figure 27. Sox sites are conserved in the TANK promoter across vertebrate species. (Top) Phylogenetic analysis of Sox site-containing TANK promoter fragments in vertebrates. One Sox site-containing region is strongly conserved in rodents and primates (red box). (Bottom) Nucleotide alignment of the strongly-conserved region indicated in red box, top figure. The Sox-binding motif AACAAAG is outlined in a black box. Other conserved regions include binding sites for possible Sox binding partners Oct-4, Ngn1, and Brn family members.

5.6 Construction and site-directed mutagenesis of the pGL2-TANK luciferase reporter vector.

Luciferase assays were carried out to determine whether Sox11 could regulate the TANK promoter. The pGL2-TANK promoter vector was created by cloning the 2.2 kb of mouse genomic sequence directly 5' to the TANK transcriptional start site, and inserting it into the pGL2-Basic vector (see **Chapter 2.11**). **Figure 28** shows a diagram of the pGL2-TANK vector, and sequences targeted for site-directed mutagenesis in later experiments.

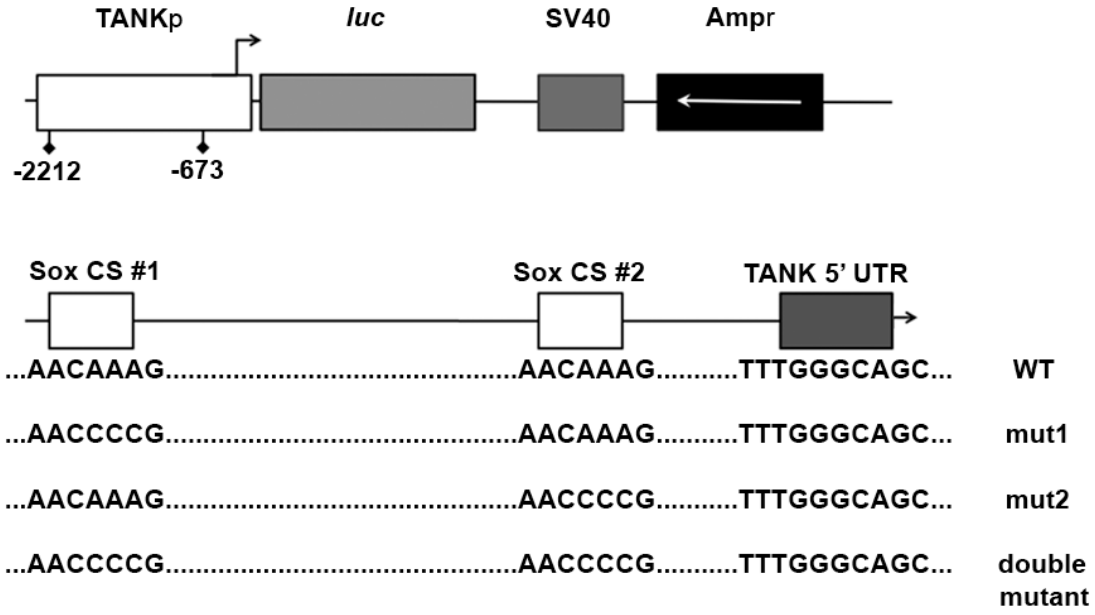

Figure 28. Diagram of pGL2-TANK luciferase vector. (Top) A 2.3-kb fragment of the TANK promoter was cloned into the pGL2-basic vector. Forward-direction Sox binding sites are indicated relative to TANK transcriptional start site. (Bottom) Site-directed mutagenesis constructs. pGL2-TANK was mutated to selectively inactivate one or both Sox binding sites.

5.7 TANK promoter activation is dose-dependently regulated by Sox11.

To determine the dose-dependency of TANK promoter activation by Sox11, 1450 ng TANK-pGL2 plasmid and 50 ng pRL-TK Renilla luciferase reporter vector were cotransfected with varying doses of pCMV-Sox11 or pCMV (in nanograms: 0, 25, 50, 150, 250, 500). Cells were harvested and lysed at 24h post-transfection and luciferase assays performed on lysates. Data were analyzed by two-way ANOVA with Bonferroni post-hoc tests. **Figure 29** shows that cotransfection with pCMV-Sox11 significantly upregulated TANK promoter activity for all doses of pCMV-Sox11. The activation of the TANK promoter was dose-dependent, ranging from 2.1-fold (after transfection with 25 ng plasmid) to 5.5-fold (after transfection with 500 ng pCMV-Sox11). No activation of the TANK promoter was seen following transfection with varying doses of pCMV plasmid and HSV-TK promoter activity remained constant for all combinations of vector + dosage.

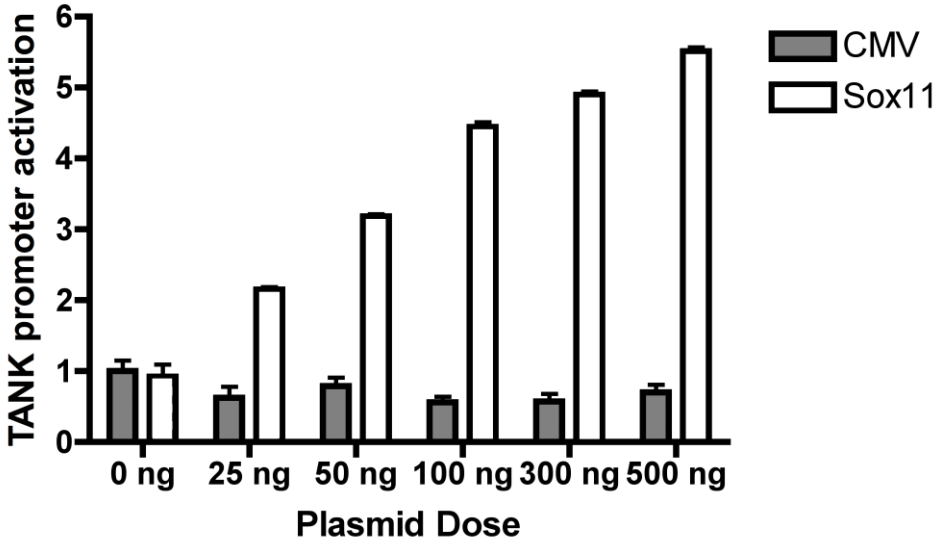

Figure 29. Sox11 dose-dependently activates the TANK promoter. Neuro-2a cells were transfected with 1450 ng pGL2-TANK, 50 ng pRL-TK, and varying quantities of pCMV (empty vector) or pCMV-Sox11. All doses of Sox11 activated the TANK promoter relative to empty vector transfection ($n = 4$ per group, $p < 0.001$ for all non-zero dosages, two-way ANOVA with Bonferroni post-hoc test).

5.8 Sox11 activation of the TANK proximal promoter is site-dependent.

Mutagenized TANK promoter constructs were generated to investigate the ability of Sox11 to activate the TANK proximal promoter (**Figure 28).** TANK-pGL2 contains the firefly luciferase gene driven by the wild-type TANK proximal promoter. TANKmut1-pGL2 contains the luciferase gene driven by the TANK proximal promoter that lacks the distal Sox binding site whereas TANKmut2-pGL2 lacks the proximal Sox binding site. TANKmut1mut2-pGL2 contains the firefly luciferase gene driven by the TANK proximal promoter with both Sox binding sites mutagenized.

Figure 30 compares luciferase reporter data from the wildtype and mutagenized TANK promoter vectors transfected into Neuro-2a cells (n = 4 per group). Cotransfection of TANK-pGL2 with pCMV-Sox11 caused a 17.9-fold increase in TANK promoter activity ($p < 1 \times 10^{-8}$, ANOVA with Bonferroni post-hoc test). Mutagenesis of the first binding site resulted in a 50% decrease in Sox11-mediated TANK promoter activation ($p < 1 \times 10^{-4}$, ANOVA with Bonferroni post-hoc test). Mutation of the second binding site resulted in an 85% decrease in Sox11-mediated TANK promoter activation ($p < 1 \times 10^{-6}$, ANOVA with Bonferroni post-hoc test), while ablation of both binding sites resulted in a 75% decrease in Sox11-mediated TANK promoter activation ($p < 1 \times 10^{-6}$, ANOVA with Bonferroni post-hoc test).

These data indicate that Sox11 is capable of activating the TANK promoter and that this activation is dependent on the presence of intact Sox binding sites on the TANK promoter.

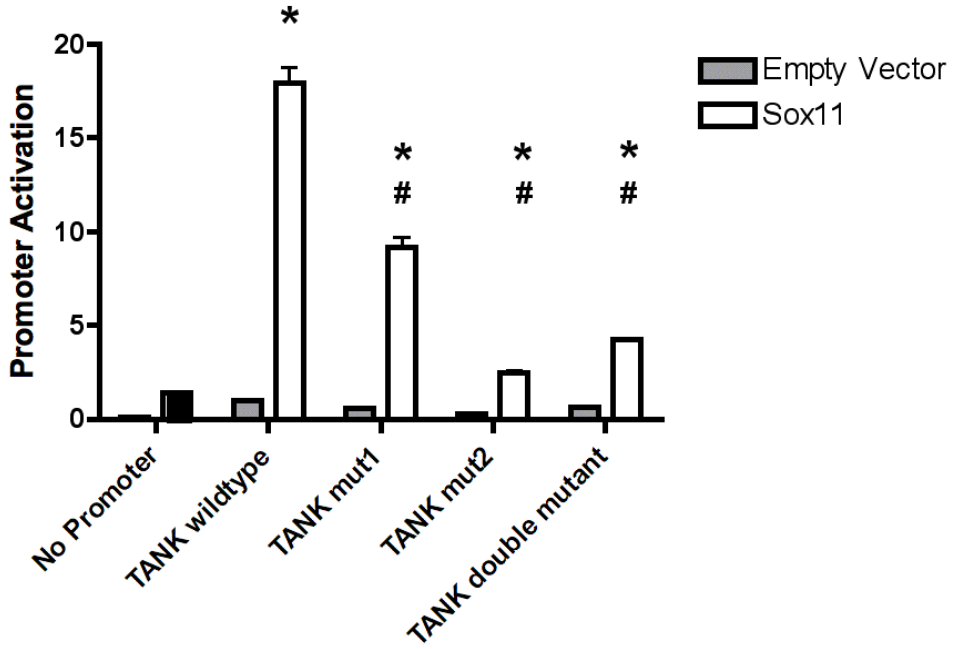

Figure 30. Sox binding sites are required for full Sox-mediated activation of the TANK promoter. Mutagenesis of Sox binding sites resulted in a 50%-85% loss of Sox11-mediated TANK promoter activation relative to the wild-type promoter construct (n = 4 per group, *P < 0.05 vs. empty vector cotransfection, #P < 0.05 vs. wild-type promoter, ANOVA with Bonferroni post-hoc test).

5.9 Sox binding sites on the TANK promoter are required for the formation of DNA-protein complexes.

Electrophoretic mobility shift assay (EMSA) was performed using probes constructed to mimic Sox binding site-containing regions of the TANK promoter. We chose to focus our experiments on the second Sox binding site, as our mutagenesis experiment showed that binding site to be most critical to Sox11-mediated TANK promoter activation. **Figure 31** shows a representative EMSA experiment, demonstrating the Sox binding site-dependent formation of DNA-protein complexes on the TANK promoter.

Incubation of nuclear extracts from Sox11-transfected Neuro2a cells with a 22-bp double-stranded oligonucleotide (corresponding to the wild-type second Sox binding site on the TANK promoter) resulted in the formation of numerous DNA-protein complexes (**Figure 31, lane 2**). Complex formation could be reduced by addition of a binding competitor in the form of a non-radiolabeled (cold) wild-type probe (**Figure 31, lane 3**); however, complex formation was also reduced by addition of a cold mutant probe (corresponding to the Sox-null mutant luciferase vector's sequence) (**Figure 31, lane 4**). These results were replicated with a HA-tagged version of the Sox-11 overexpression plasmid (**Figure 31, lanes 8, 9, 11**). Complex formation was dependent on the presence of the intact Sox binding site; far fewer complexes formed when nuclear extracts from Sox11-HA overexpressing Neuro-2a cells were incubated with an oligo corresponding to the Sox-null mutant (**Figure 31, lanes b-e**). These results indicate that protein-DNA complex formation on this region of the TANK promoter is dependent on the presence of the intact Sox binding site.

In order to attempt to determine whether Sox11 was present in any of the DNA-protein complexes on the TANK promoter, we performed a supershift assay using an anti-Sox11

antibody; theoretically, the antibody should bind to a Sox11-DNA complex and cause a shift in at least one band on the gel as compared with an antibody-free control. However, no band shift resulted (**Figure 31, lanes 5, 6, 10, 12**). We repeated the EMSA using an HA-tagged version of Sox11 and an antibody directed against the HA tag; nuclear extracts prepared from samples transfected with the Sox11-HA fusion plasmid produced an indistinguishable pattern of DNA-protein complexes as compared with extracts prepared using the wild-type Sox11, indicating that the HA tag did not affect the formation of DNA-protein complexes (**Figure 31, lanes 8, 9, 11**). However, addition of the HA antibody again failed to produce a supershift (**Figure 31, lanes 13, 14**). In a final experiment to attempt to determine whether Sox11 directly binds to this short stretch of the TANK promoter, we affinity-purified a small quantity of Sox11-HA protein using a hemagglutanin affinity column and estimated its purity and concentration using a silver-stained gel (data not shown). We ran a final EMSA using 500 ng of purified Sox11-HA. No complexes whatsoever were seen on this gel. This result however, is not unexpected since existing research indicates that Sox proteins function in the presence of binding partners (Dy et al., 2008). It is possible that Sox11 requires a binding partner in order to form a stable interaction with the DNA strand, and that Sox11 on its own cannot bind DNA for a protracted period of time.

From these experiments, we can conclude only that the intact Sox11 binding site is necessary for nuclear proteins to bind to a small area of the TANK promoter. We can conclude neither that Sox11 binds to this site, nor that Sox11 does not directly bind to this site – it is possible that the supershift assay did not work because Sox11 was not stably integrated in the observed DNA-protein complexes, but it is also possible that no band shift was observed because the epitopes on Sox11 are masked during DNA-protein interactions, either by the DNA strand or by interactions with other proteins as it forms the architecture of the transcriptional complex.

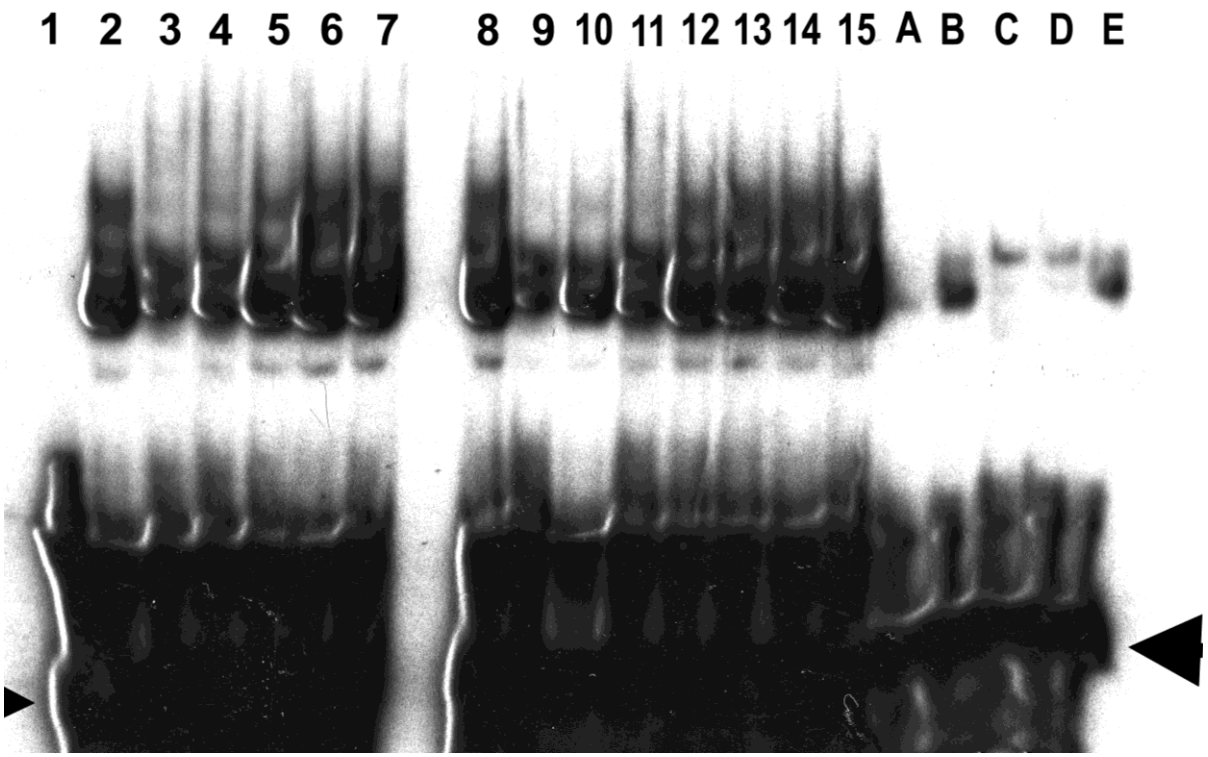

Figure 31. Sox binding sites are required on the TANK promoter for DNA-protein complexes to form. (1-7) Neuro-2a cells were transfected with CMV-Sox11. Nuclear extracts were produced from the cells, and EMSA was run with a wild-type probe corresponding to the second Sox binding site on the TANK promoter. (1) Wild-type probe only (left-hand arrow indicates band size of probe). (2) Wild-type probe + 5 ug nuclear extract. (3) Wild-type probe + 5 ug nuclear extract + 10-fold excess cold wild-type probe. (4) Wild-type probe + 5 ug nuclear extract + 10-fold excess cold mutant probe. (5) Wild-type probe + 5 ug nuclear extract + preincubation with anti-Sox11 antibody. (6) Wild-type probe + 5 ug nuclear extract + post-incubation with anti-Sox11 antibody. (7) Wild-type probe + post-incubation with anti-GAPDH antibody. **(8-15) Neuro-2a cells were transfected with CMV-Sox11-HA.** Nuclear extracts were produced from the cells, and EMSA was run with a wild-type probe corresponding to the second Sox binding site on the TANK promoter. (8) Wild-type probe + 5 ug nuclear extract. (9) Wild-type probe + 5 ug nuclear extract + 10-fold excess cold wild-type probe. (10) Wild-type probe + 5 ug nuclear extract + pre-incubation with anti-Sox11 antibody. (11) Wild-type probe + 5 ug nuclear extract + 10-fold excess cold mutant probe. (12) Wild-type probe + 5 ug nuclear extract + post-incubation with anti-Sox11 antibody. (13) Wild-type probe + 5 ug nuclear extract + preincubation with anti-HA antibody. (14) Wild-type probe + 5 ug nuclear extract + post-incubation with anti-HA antibody. (15) Wild-type probe + 5 ug nuclear extract + post-incubation with anti-GAPDH antibody. **(a-e) Neuro-2a cells were transfected with CMV-Sox11 or CMV-Sox11-HA.** Nuclear extracts were produced from the cells, and EMSA was run with a probe corresponding to the second Sox binding site on the TANK promoter with the Sox binding site mutation AACAAAG → AACCCCG. (a) Mutant probe only (right-hand arrow indicates band size of probe). (b) Mutant probe + 5 ug CMV-Sox11 nuclear extract. (c) Mutant probe + 5 ug CMV-Sox11-HA nuclear extract. (d) Mutant probe + 5 ug CMV-Sox11-HA nuclear extract + 10-fold excess wild-type probe. (e) Mutant probe + 5 ug CMV-Sox11-HA nuclear extract + 10-fold excess mutant probe.

5.10 ChIP assays show Sox11 can bind directly to the TANK promoter.

To further assess whether Sox11 may modulate the mouse TANK promoter we used chromatin immunoprecipitation (ChIP) assays to analyze the 2.27 Kbp proximal region that contains the two Sox binding sites that were conserved across species. ChIP assays are more sensitive than EMSAs and more directly indicate whether a particular transcription factor binds a defined genomic region within the context of the cell. Cross-linked protein was obtained from Neuro-2a cells expressing FLAG-tagged Sox11 and immunoprecipitated using an anti-FLAG antibody (**Fig. 32**). Purified chromatin/protein complexes were then assayed by PCR using primer sets designed to amplify regions bounding the two Sox11 sites on the TANK promoter. Results indicate that the FLAG-tagged Sox11 protein is capable of directly interacting with both binding sites on the TANK promoter.

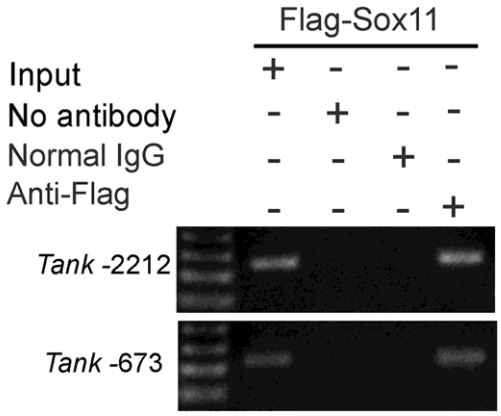

Figure 32. Sox11 binds to the TANK promoter. Primers were designed against the two most proximal Sox binding sites on the TANK promoter region, and ChIP was performed on Flag-Sox11 transfected Neuro-2A cells. Lanes from left to right: Input DNA only (no IP), PCR performed after IP with no precipitating antibody, PCR after IP with control IgG antibody, PCR performed after IP with anti-FLAG IgG. Bands were seen corresponding to both putative Sox binding sites on the TANK promoter, indicating that Sox11 binds to the TANK promoter.

5.11 TANK siRNA reduces TANK mRNA and protein expression in Neuro-2a cells 24h post-transfection

Evidence from nonneuronal cell lines has suggested that TANK, through interaction with TNFRs and TRAF proteins, may modulate TNF signaling pathways. If TANK does modulate TNF signaling a prediction, based on the literature, is that this modulation could impact downstream JNK activity. To further explore if TANK interacts with TNF/JNK signaling we first developed procedures that would allow knockdown of TANK expression in cultured neuronal cells. Neuro-2a cells were transfected with 15nM siRNA (nontargeting or anti-TANK) and harvested 24h post-transfection protein analysis. Transfection of Neuro-2a cells with anti-TANK siRNA resulted in a 66% knockdown of TANK protein at 24h post-transfection (**Figure 33**).

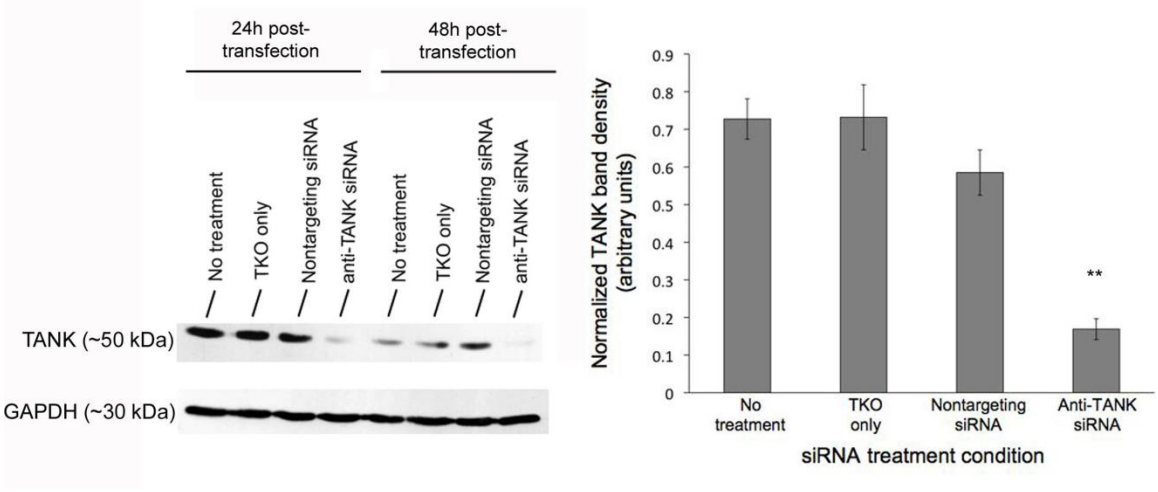

Figure 33: 15 nM TANK siRNA reduces TANK protein abundance at 24h and 48h post-transfection. (Left) Representative Western blot. TANK protein was reduced following administration of siRNA directed against TANK. (Right) Quantification of TANK knockdown 24h following siRNA transfection. TANK siRNA produced a 66% knockdown in TANK protein compared with nontargeting siRNA ($p < 0.05$, ANOVA with Bonferroni post-hoc test). The 24h time point was used for all subsequent siRNA experiments.

5.12 Stimulation of Neuro-2a cells with TNF-alpha results in a dose-dependent activation of JNK protein, without causing increased cell death at baseline.

We next developed a cell culture system in which to examine the interaction of TANK, TNF and JNK activation. The goal here was to identify a concentration of TNF that caused stimulation of JNK phosphorylation but did not cause cell death. Neuro-2a cells were stimulated with TNF-alpha at varying concentrations (0, 10, 50, and 100 ng/mL). Dosages were chosen based on previous studies of JNK activation in Neuro-2a cells (Mielke et al., 2000). Cells were harvested at 30 min or 1h post-stimulation (n = 3 per group: total n = 24) and western blots against JNK were performed on the lysates. To determine relative JNK activation levels, phospho-JNK bands were normalized to total JNK bands. To determine total JNK levels, total JNK bands were normalized to GAPDH. Normalized data were analyzed using two-way ANOVA with Bonferroni post-hoc test. A further group of TNF-alpha stimulated cells were harvested 6h after stimulation and used for trypan blue assay to ensure that cell viability was not compromised at baseline (n = 4 per group).

Our results showed that 50 ng/mL TNF is sufficient to produce increased phosphorylation of both JNK1 and the JNK2/3 by 30 min after TNF-alpha stimulation in Neuro-2a cells (**Figure 34A**, **B**). 10 ng/mL produced a non-significant increase in JNK activation, as did 100 ng/mL. These results are consistent with previous findings (Mielke et al., 2000).

Analysis of cell viability showed that TNF-alpha stimulation had no significant effect on viability 6h after stimulation; again, consistent with previous results. Unstimulated cells and cells stimulated with either 10 ng/mL or 50 ng/mL TNF-alpha had virtually identical viability (roughly 67% viable). At the 100 ng/mL dose, there was a slight decrease in cell viability; however, this decrease in cell viability failed to approach statistical significance (**Figure 34C**).

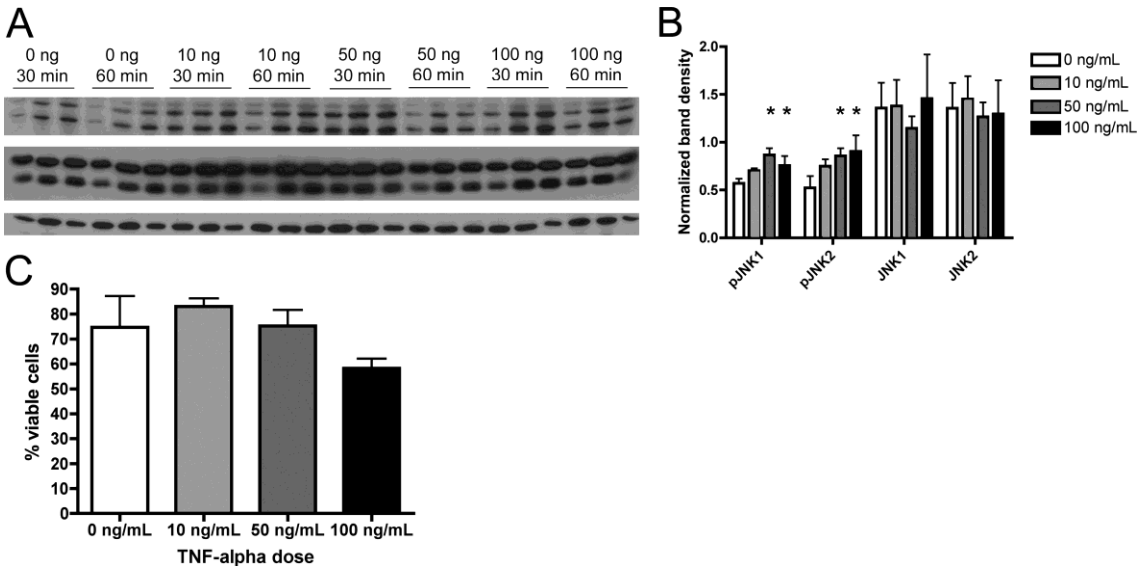

Figure 34: 50 ng/mL TNF-alpha activates JNK isoforms without increasing total JNK abundance or death in Neuro-2a cells. (A) Western blot of dose-response experiment (top to bottom: activated JNK, total JNK, GAPDH). (B) Quantification of all dosages at the 30-minute time point. Addition of 50 ng/mL or 100 ng/mL TNF-alpha increased activation of JNK1 and of JNK2/3 without increasing JNK expression ($n = 3$ per group, $p < 0.05$ compared to 0 ng/mL dosage, two-way ANOVA with Bonferroni post-hoc test). (C) Quantification of trypan blue assay for cell viability. TNF-alpha stimulation did not significantly affect Neuro-2A cell viability after 6 hours ($n = 4$ per group, $p > 0.05$ compared with 0 ng/mL dosage, one-way ANOVA with Bonferroni post-hoc test).

5.13 siRNA-mediated TANK knockdown reduces JNK activation in Neuro-2a cells.

To determine if the level of TANK modulated the state of JNK activation, transfection assays were carried out in Neuro-2a cells treated with TANK siRNAs. Neuro-2a cells treated with transfection reagent alone, transfection reagent + 15 nM nontargeting siRNA or transfection reagent + 15 nM TANK siRNA were compared. At 24 hours post-transfection, cells lysates were harvested and analyzed by western blots using antibodies to phospho-JNK, total JNK and GAPDH (**Figure 35**).

TANK knockdown produced a significant reduction in JNK1 activation compared with both untransfected Neuro-2a cells and cells transfected with nontargeting siRNA ($n = 3$, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). No changes in total JNK1 or JNK 2/3 abundance were seen.

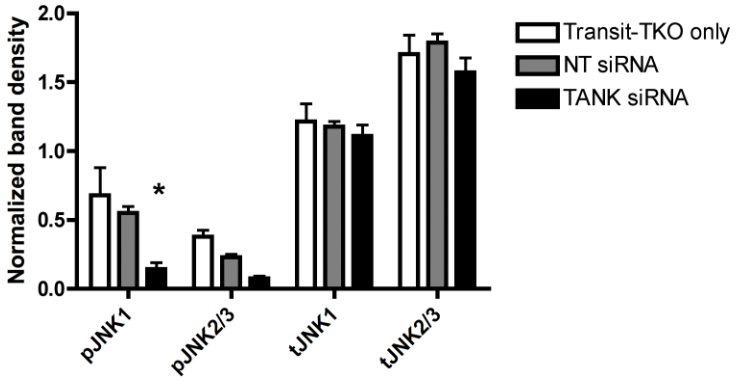

Figure 35: TANK siRNA significantly reduced JNK1 activation at baseline. Neuro-2a cells were transfected with TANK siRNA, and western blots were run for JNK1 24 hours post-transfection. TANK knockdown significantly reduced activation of JNK1 compared with nontargeting siRNA ($n = 3$ per group, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). Although a trend towards reduction of JNK2/3 activation was also seen following TANK knockdown, the effect failed to reach statistical significance. Total JNK expression was unaffected following TANK knockdown.

5.14 TANK expression correlates with TNF-alpha dependent JNK activation.

We next determined if a reduction in TANK protein would impact the level of JNK activation that occurs in response to TNF stimulation. Neuro-2a cells were transfected with nontargeting siRNA or TANK siRNA, or treated with transfection reagent only. At 24h after transfection cells were stimulated with 50 ng/mL TNF-alpha or left as an unstimulated control group. For each group, 6 replicate wells were transfected (total n = 36 samples). At 30 min after TNF-alpha stimulation, cells were harvested and western blots were performed probing for TANK, phospho-JNK, total JNK and GAPDH. Relative expression of TANK protein and JNK protein were calculated by normalizing to GAPDH. Relative activation of JNK protein was calculated by normalizing phospho-JNK to total JNK. (**Figure 36**)

TANK knockdown was ascertained using one-way ANOVA with Bonferroni post-hoc test. In these samples, TANK knockdown was statistically significant but not robust (knockdown = 55%; $p < .05$). Due to the lack of robust knockdown and the high variability of TANK expression between samples in the non-knockdown groups, all samples were pooled for regression analysis, to determine whether TANK expression correlated with JNK activation.

In the absence of TNF-alpha, TANK expression did not correlate with activation of any isoform of JNK, or with total levels of JNK1; however, TANK expression did correlate with expression of JNK2/3 at baseline (slope = 0.60, $r^2 = .40$, $F = 7.98$, $p < 0.05$). Following TNF-alpha stimulation, TANK expression was strongly negatively correlated with pJNK1 activation (slope = -3.12, $r^2 = .89$, $F = 85.49$, $p < 0.0001$) and with pJNK2/3 activation (slope = -2.19, $r^2 = .80$, $F = 44.46$, $p < 0.0001$). Following TNF-alpha stimulation, TANK expression was also positively correlated with total abundance of JNK1 (slope = .87, $r^2 = .72$, $F = 27.69$, $p < 0.001$) and JNK2/3 (slope = 1.05, $r^2 = .82$, $F = 50.48$, $p < 0.0001$).

Our results showed that TANK expression was only related to JNK activation following activation of TNF-alpha receptors. Samples that had lower TANK expression had relatively high activation of both JNK1 and JNK2/3 isoforms, but relatively low total expression of JNK. This indicates that TANK expression is associated with negative regulation of TNF-alpha-dependent JNK activation, but that TANK expression may not be associated with regulation of JNK activation from pathways other than the TNF-alpha signaling pathway.

Our results also showed that TANK expression and JNK expression were correlated. In unstimulated cells, samples that were low in TANK also expressed lower levels of JNK2/3. In TNF-alpha stimulated cells, samples that were low in TANK tended to express lower levels of both JNK1 and of JNK 2/3. It is possible that the decrease in JNK expression seen in TNF-stimulated, TANK-deficient cells may reflect a compensatory downregulation of JNK, secondary to abnormally high levels of JNK activation.

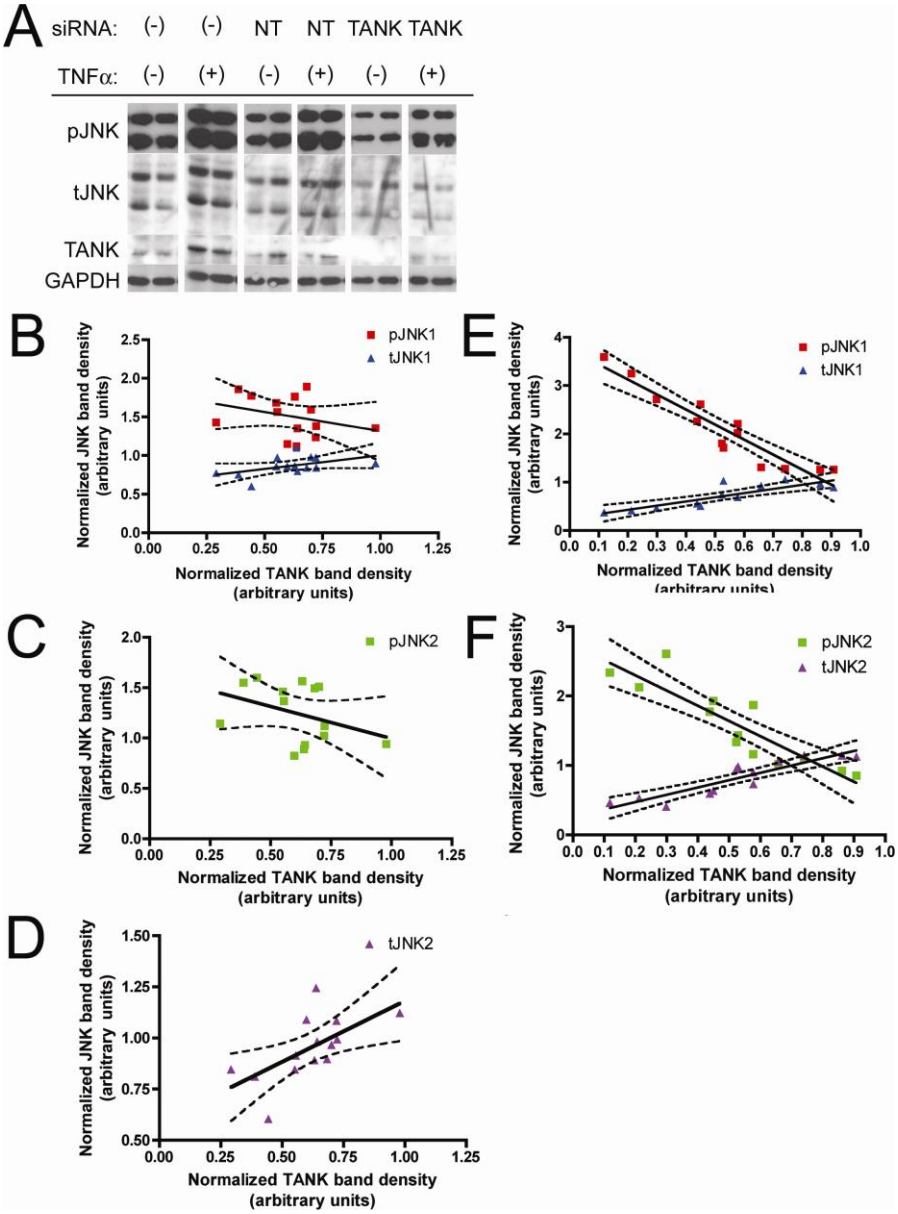

Figure 36: TANK protein expression level negatively correlates with JNK activation level after TNF-alpha stimulation. Neuro-2a cells were transfected with transfection reagent only, nontargeting siRNA, or TANK-directed siRNA. 24 hours post-transfection, cells were left unstimulated, or were stimulated with TNF-alpha for 30 minutes (n = 6 samples per group, total n = 36 samples). Western blots were run for TANK, phospho-JNK, total JNK, and GAPDH. (A) Representative Western blot of samples. Treatment with TANK siRNA produced a 55% knockdown of TANK protein; however, TANK expression varied dramatically within treatment groups. (B) In unstimulated Neuro-2a cells, there is no correlation between TANK expression and JNK1 activation or expression. (C) In unstimulated Neuro-2a cells, there is no correlation between TANK expression and JNK2/3 activation. (D) In unstimulated Neuro-2a cells, there is a significant correlation between TANK expression and JNK2/3 expression ($p < .05$). (E) In stimulated Neuro-2a cells, TANK expression correlates negatively with JNK1 activation and positively with total JNK1 expression. (F) In stimulated Neuro-2a cells, TANK expression correlates negatively with JNK2/3 activation and positively with total JNK2/3 expression.

5.15 Reduction in TANK protein increases Neuro-2a cell death following TNF-alpha stimulation.

To determine whether TANK protein might function downstream of TNFR-SF receptors to promote survival following TNF receptor activation, Neuro-2a cells were transfected with anti-TANK siRNA, nontargeting siRNA or transfection reagent alone. 24h post-transfection, cells were stimulated with 50 ng/mL TNF-alpha. 6 hours after stimulation, cells were washed in PBS, scraped with a rubber cell scraper and stained with trypan blue for cell viability counts. Studies were performed blinded in order to avoid biased counts. Trypan blue is not membrane-permeable in viable cells, but can permeate membranes of injured, dying, and dead cells, so this assay provides a quick although non-specific measure of overall viability. (However, this assay does not distinguish between apoptotic vs. non-apoptotic cell death.)

Figure 37 shows a representative experiment detailing the effect of TANK knockdown on Neuro-2a cell viability. Before TNF-alpha stimulation, there is no significant effect of TANK siRNA on cell viability ($viability_{control}$ = 72%, $viability_{NT}$ = 75%, $viability_{TANK}$ = 65%; n = 3 per group; $p > 0.05$, two-way ANOVA with Bonferroni post-hoc test). Following TNF-alpha stimulation, TANK knockdown significantly reduces cell viability ($viability_{control}$ = 71%, $viability_{NT}$ = 74%, $viability_{TANK}$ = 48%; n = 3 per group; $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). Neuro-2a cells are known to be resistant to TNF-alpha induced cell death; the dramatic increase in cell death seen in TANK-deficient Neuro-2a cells treated with TNF-alpha suggests that TANK is an important intracellular mediator in facilitating cell survival responses downstream of TNF-alpha receptor-mediated signaling. TANK-deficient Neuro-2a cells showed no baseline decrease in viability, suggesting that TANK is important to cell survival only when its upstream signaling mediators are activated.

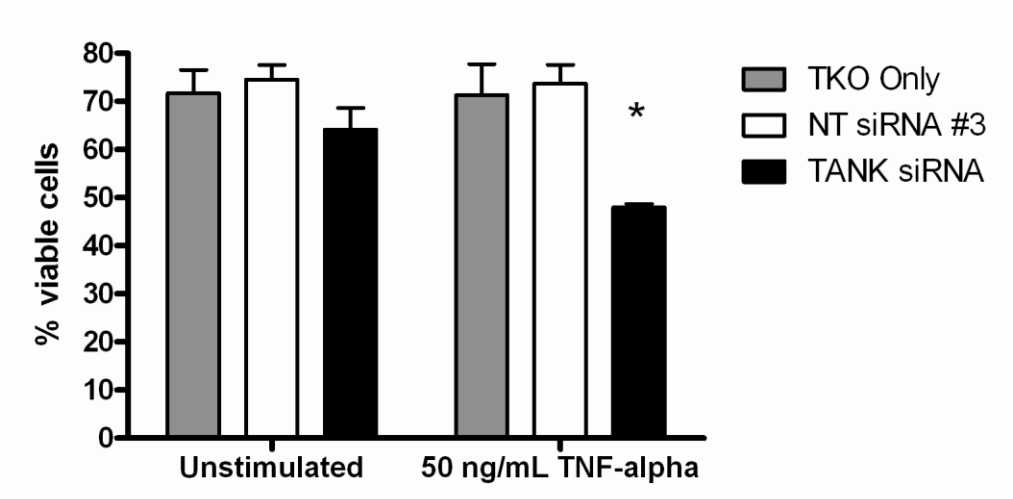

Figure 37. TANK knockdown decreases cell viability following TNF-alpha stimulation. Neuro-2a cells were treated with transfection reagent alone, nontargeting siRNA, or siRNA directed against TANK. 24h post-transfection, cells were stimulated with 50 ng/mL TNF-alpha, or vehicle alone, for 6h. Cell viability was assessed using trypan blue assay. Cell counts were performed blinded. TANK siRNA did not significantly diminish cell viability in the absence of TNF-alpha stimulation ($n = 3$ samples per group, $p > 0.05$, two-way ANOVA with Bonferroni post-hoc test). Following TNF-alpha stimulation, TANK siRNA significantly reduced cell viability ($n = 3$ samples per group, $p < 0.05$ vs. nontargeting siRNA, two-way ANOVA with Bonferroni post-hoc test).

6.0 DISCUSSION

6.1 Sox11 regulates three genes involved in cell signaling pathways active during nerve injury and regeneration

The above work has identified three possible targets of Sox11-mediated gene transcription: the neurotrophin BDNF, the Wnt inhibitor and microtubule-stabilizing protein APC and the TNF-receptor signaling modulator TANK. All three of these genes are capable of modulating diverse cell signaling pathways known to affect cell survival and regeneration following peripheral nerve injury. While roles for BDNF and APC in neuronal survival and/or neurite extension have already been well characterized, TANK has not been studied in the context of peripheral nerve regeneration. My findings indicate that Sox11-mediated regulation of TANK may be a neuroprotective mechanism that stimulates survival signaling and/or decreases death signaling downstream of TNF receptor activation.

6.2 BDNF: Relevance of Sox11-upregulated exons to nerve injury and regeneration

The complex gene structure and multiple promoters of BDNF are thought to provide multiple layers of transcriptional regulation. BDNF promoters are differentially activated across various brain areas (Malkovska et al., 2006), during development (Sathanoori et al., 2004, Pattabiraman et al., 2005), and in response to neuronal activity (Metsis et al., 1993, Timmusk et

al., 1995, Pattabiraman et al., 2005, Hara et al., 2009, Zheng and Wang, 2009, Cowansage et al., 2010). In addition to the fine-tuning of transcriptional regulation associated with BDNF's multiple promoters, the multiple untranslated exons appear to play an important role in the intracellular localization of BDNF mRNA: transcripts containing exon I or exon IV appear to be restricted to cell somata and proximal dendrites, whereas all other transcripts are dendritically-targeted (Chiaruttini et al., 2009).

Our results demonstrate that BDNF exons are differentially regulated in sensory neurons at two early time points following sciatic nerve axotomy, that upregulated exons contain Sox binding sites in their promoter regions, and that promoters upstream of the two most strongly-regulated BDNF exons may be activated by the transcription factor Sox11. Consistent with previous findings, we saw an early upregulation of BDNF exon I following injury. Exon I has been shown to be persistently upregulated in multiple models of sensory neuron injury and/or inflammation; recent work shows that activation of BDNF promoter I is associated with chromatin remodeling in neurons (Tian et al., 2009). Furthermore, chromatin remodeling following the activation of BDNF promoter I permits sustained upregulation of BDNF exon I (Hara et al., 2009). In addition to acting as traditional activators of transcription, SoxC proteins physically bend DNA strands, rendering the DNA more accessible to other DNA-binding proteins (Lefebvre et al., 2007). It is possible therefore, that Sox11-mediated activation of promoters, including BDNF promoter I, may prove to be important to the initiation of long-term epigenetic regulatory mechanisms coordinating the transcriptional response to injury in neurons.

Interestingly, BDNF exon IV was transiently upregulated within the first 24 hours of nerve injury. BDNF exon IV can be transcribed as an immediate early gene following NMDA receptor activation (Lauterborn et al., 1996); additionally, exon IV transcription may be self-

induced (Zheng and Wang, 2009). Further studies at earlier time points following injury may be necessary to determine to what extent Sox11-mediated regulation of BDNF exon IV contributes to upregulation of transcript IV following nerve injury; it is possible that at least a portion of the upregulation of transcript IV seen at early time points in the injured DRG is activity-dependent and reflects the overall increase in immediate early gene transcription seen following nerve injury.

The other BDNF exons (VII and VIII) upregulated following nerve injury have not been extensively characterized in terms of their regulation or in terms of the specific functions of their mature transcripts. It is likely that their contribution to BDNF signaling following nerve injury is relatively insignificant compared to the contributions of exons I and IV; transcripts I, II, III, IV, VI, and IXA are the dominantly-expressed BDNF mRNAs in mouse neural tissue, whereas transcripts V, VII, and VIII tend to be expressed at lower levels (Aid et al., 2007). However, it is interesting to note that recent studies have demonstrated that transcripts I and IV (the dominant upregulated exons) are specifically targeted to the cell body, whereas all other BDNF transcripts are targeted towards proximal dendrites (in bipolar neurons) (Pattabiraman et al., 2005, Chiaruttini et al., 2009). Further experiments must be performed in order to determine the localization of BDNF transcripts VII and VIII in DRG neurons (which are pseudo-bipolar and lack dendrites), and to determine whether these transcripts contribute in any substantial way to the intra-axonal translation of BDNF mRNA, which plays an important role in neurite regeneration.

BDNF transcript regulation appears to be substantially different at early time points following injury compared with the two-week time point. At early time points, only transcripts that may possibly be Sox11-regulated were upregulated. Other researchers studying later time

points have found increases in virtually every transcript (Funakoshi et al., 1993, Timmusk et al., 1995, Matsuoka et al., 2007, Kobayashi et al., 2008). This indicates that BDNF is regulated by multiple transcriptional mechanisms in a time-dependent manner. More research is needed in order to determine the exact time scale of BDNF regulation post-injury, what mechanisms contribute to the upregulation of which transcripts, and whether different transcripts may play different roles in the neuroregenerative response and/or the development of neuropathic pain after injury.

6.3 Sox11 regulates APC: Possible implications for neurite regeneration and gene transcription

This work has shown that APC is transiently upregulated following nerve injury, and that Sox11 is capable of activating the APC promoter and causing transcription of APC mRNA. The APC/GSK-3b pathway has previously been shown to be an essential component of neurite outgrowth during development and following injury (Votin et al., 2005, Zhou et al., 2006, Barth et al., 2008, Purro et al., 2008, Ivaniutsin et al., 2009, Paridaen et al., 2009); however, this is the first evidence that APC is actually upregulated following injury.

Sox11-mediated regulation of both APC and BDNF gene transcription is a possible example of Sox gene function as a "master regulator" which coordinates transcription of a panel of genes, thereby radically altering the properties of a cell. Neurotrophin signaling and the APC/GSK-3b pathway are thought to function together in a neuron to increase microtubule stability and therefore neurite outgrowth (Zhou et al., 2006). Thus, the upregulation of both BDNF and APC is one possible mechanism by which Sox11 increases neurite outgrowth following peripheral nerve injury.

Other functions of APC include inhibition of beta-catenin. Beta-catenin is an effector of numerous cell signaling pathways, including the Wnt signaling pathway. Wnt/beta-catenin signaling is required for development of the dorsal root ganglion (Hari et al., 2002, Lee et al., 2004) and for Schwann cell proliferation after nerve injury (Fancy et al., 2009, Tawk et al., 2011). However, beta-catenin-independent Wnt signaling is more important for the establishment of cell polarity, neurite outgrowth, and synaptic plasticity. Furthermore, Sox family proteins have been demonstrated to interact with Wnt signaling pathways (reviewed in (Kormish et al., 2010)); in particular, Sox4 (the most closely-related protein to Sox11) has been shown to enhance TCF/LEF transcriptional activity (Sinner et al., 2007); however, Sox proteins in the same group may have differential effects upon TCF/LEF activity (Bernard and Harley, 2010, Kormish et al., 2010). It is possible that regulation of APC may indicate a Wnt-b-catenin modulatory function for Sox11, but further research is needed to determine if this is the case and if so, whether Sox11 enhances or decreases TCF/LEF-mediated transcription.

6.4 TANK: Sox11-mediated regulation of a signal modulator.

The current work has shown that TANK protein is upregulated following nerve injury, and that lack of TANK synthesis has a deleterious effect on cell survival downstream of TNF-alpha receptor stimulation. However, determining a precise mechanism by which TANK may increase neuronal survival following injury is outside the scope of this work. It is likely that the relative abundance of TANK protein influences the ability of TRAF proteins to activate their normal targets, and that the transient upregulation of TANK during nerve injury serves to modulate the dynamics of TRAF-mediated signaling in directions that favor cell survival. Previous biochemical studies focusing on TANK/TRAF protein interactions have largely focused

on interactions between TANK and TRAF2 (Cheng and Baltimore, 1996, Kaye et al., 1996, Chin et al., 1999, Pomerantz and Baltimore, 1999, Nomura et al., 2000, Li et al., 2002a, Kanno et al., 2010, Manna et al., 2010); another interaction of special interest is the interaction between TANK and TRAF6 (Chin et al., 1999, Sato et al., 2003, Miranda et al., 2006, Kawagoe et al., 2009, Manna et al., 2010). The consensus of the research community at this point is that TANK appears to enhance TRAF2-mediated signaling, but might decrease TRAF6-mediated signaling by increasing the degradation rate of TRAF6.

The specific role of TANK in the modulation of TNFR and/or p75 NTR signaling has not been examined. Most of the work done on TANK protein has been to determine its function downstream of Toll receptor superfamily (TLR-SF) receptors in immune cell lines. However, TANK is capable of interacting with TRAF2, TRAF3, TRAF5, and TRAF6 (Chin et al., 1999, Pomerantz and Baltimore, 1999, Nomura et al., 2000, Li et al., 2002a). TRAF1 and TRAF2 are the main TRAFs associating with TNFRI and TNFRII (Arron et al., 2002). TRAF2, TRAF3, TRAF4, and TRAF6 associate with the p75 NTR (Arron et al., 2002). This means that TRAF2, TRAF3, and TRAF6 are the only TRAFs that could be involved in TANK modulation downstream of the TNF-alpha or p75 receptors in neurons. If TANK modulates TNFR1 or TNFR2 signaling, it must be via TRAF2. If TANK modulates p75 NTR signaling, it could act via TRAF2, TRAF3, or TRAF6. Furthermore, data from the TANK knockout mouse suggests that TANK inhibits the autoubiquitination of TRAF6, and therefore blocks TRAF6 activation (Kawagoe et al., 2009). This may indicate that TANK could be capable of diminishing the net effect of TRAF6-mediated p75 NTR signaling, while possibly potentiating the effect of TRAF2 signaling via TNFR1, TNFR2, and p75 NTR. Therefore, increasing intracellular levels of TANK may turn up the gain on TRAF2 signaling, resulting in consequences such as increased JNK-

mediated activation of c-Jun, while at the same time turning down the gain on TRAF6, blocking potentially lethal signals from the p75 NTR. It is also possible that TANK binding to TRAF2 might alter the ability of anti-apoptotic proteins such as cIAP to interact with TRAF2, switching the balance of TRAF2 signaling towards survival. Sox11-mediated activation of TANK could therefore serve as a mechanism to switch TNF receptor signaling, which is usually pro-apoptotic in neurons, away from pro-death signaling pathways and towards signaling pathways which favor survival and regeneration of sensory neurons.

In addition to the demonstrated pro-survival effect of TANK downstream of TNF receptor superfamily members, there is additional potential for TANK to modulate signaling downstream of several other transmembrane receptor superfamilies, most notably the Toll-like receptor (TLR) superfamily and the transforming growth factor-beta receptor (TGFβ-R) superfamily (Kawai and Akira, 2007, Yamashita et al., 2008). Receptors in both these superfamilies contain domains with which TRAF proteins interact, peripheral neurons express receptors belonging to both these families, and signaling downstream of both receptor superfamilies is implicated in the neuronal response to injury. It is possible that TANK upregulation may also alter the dynamics of these signaling pathways following nerve injury.

6.5 Possibility for crosstalk between pathways modulated by Sox11-mediated transcription.

Many recent studies in signal transduction have focused on the role of crosstalk in the coordination of multiple cell signaling pathways. Crosstalk between signaling pathways occurs when signaling pathways share common targets, or when signaling components initially activated by one signaling pathway regulate targets which are downstream of another signaling

pathway. A third way for crosstalk to occur between signaling pathways is for one signal pathway to control expression of either a signaling molecule itself, its receptor, or a downstream effector of cell signaling.

Crosstalk mechanisms have been identified between each of the cell signaling pathways identified as possibly modulated by Sox11-mediated gene transcription: interactions occur between cytokine and Wnt signaling pathways (Cantarella et al., 2008), as well as between BDNF signaling and TNF-alpha (Bayas et al., 2003, Blasing et al., 2005, Boyle et al., 2005, Osamura et al., 2005, Schulte-Herbruggen et al., 2005, Churchill et al., 2006, del Porto et al., 2006, Furuno and Nakanishi, 2006, Saha et al., 2006, Hong et al., 2008, Taishi et al., 2008, Fujino et al., 2009, Prakash et al., 2009, Jiang et al., 2010, Urshansky et al., 2010, Balkowiec-Iskra et al., 2011).

Signaling via inflammatory cytokines including TNF-alpha, as well as signaling downstream of BDNF and other growth factors, have a demonstrated capacity to result in the activation of beta-catenin (Hetman et al., 2000, Satoh and Kuroda, 2000, Bamji et al., 2006), which is an essential component of the canonical Wnt signaling pathway. In addition to this interaction, there is the previously-discussed ability of APC to alter Wnt signaling dynamics in favor of neurite extension; this interaction appears to be mediated by neurotrophin signaling.

The link between TNF-alpha and BDNF synthesis has been well-documented for many years. Multiple researchers working in multiple cell types have demonstrated that stimulation with pro-inflammatory cytokines such as TNF-alpha leads to increased transcription of neurotrophins including BDNF (Bayas et al., 2003, Blasing et al., 2005, Boyle et al., 2005, Osamura et al., 2005, Schulte-Herbruggen et al., 2005, Churchill et al., 2006, del Porto et al., 2006, Furuno and Nakanishi, 2006, Saha et al., 2006, Hong et al., 2008, Taishi et al., 2008,

Fujino et al., 2009, Prakash et al., 2009, Jiang et al., 2010, Urshansky et al., 2010, Balkowiec-Iskra et al., 2011). More recently, it has been demonstrated that co-stimulation with BDNF and TNF-alpha leads to an increase in NF-kappa B activation downstream of TNF receptors (Furuno and Nakanishi, 2006), and that BDNF and TNF-alpha when applied together increase intracellular calcium synergistically (Prakash et al., 2009).

6.6 Possible integration of multiple cell signaling pathways to modulate JNK/c-Jun/AP-1 by Sox11

One possible signaling effector that may be influenced by crosstalk between multiple cell-signaling pathways is JNK. JNK is activated by MAP3 kinase proteins, which are targets of many different signaling mechanisms. In particular, neurotrophin signaling, Wnt signal cascades, and TNF-alpha signaling have all been clearly linked to activation of JNK in multiple cell types, including neuronal cells.

JNK has diverse intracellular functions, all of which may contribute to the regeneration response of injured neurons (Kenney and Kocsis, 1998, Mielke et al., 1999, Neidhart et al., 2001, Mielke and Herdegen, 2002, Chang et al., 2003, Costello and Herron, 2004, Lindwall et al., 2004, Etter et al., 2005, Lindwall and Kanje, 2005b, Tararuk et al., 2006, Ciani and Salinas, 2007, Li et al., 2007, Thomas et al., 2008, Wu et al., 2008, Barnat et al., 2010, Kenney et al., 2010). Little is known about the molecular mechanisms underlying the isoform-specific expression of JNK, or of the mechanisms governing the intracellular localization and specific function of JNK proteins. It is possible that different signaling cascades preferentially activate different subcellular fractions of the total JNK pool, resulting in different isoform-specific effects of JNK activation. Survival and regeneration of injured neurons requires c-Jun-dependent as

well as c-Jun-independent JNK signaling pathways; it is possible that Sox11 is involved in coordinating the dynamics of intracellular signaling pathways which converge upon JNK. This may be a major mechanism by which Sox11 promotes neuronal survival and regeneration.

6.7 Conservation of Sox-binding sites in Sox11-regulated gene promoters and possible coregulation of promoters by Sox11 and TCF/LEF genes

Every Sox-regulated promoter region studied in this work contains Sox binding sequences in mouse and human. Furthermore, many of the Sox-binding regions display some level of homology between mouse and human sequences. These regions frequently contain binding sites for other transcription factors, including Brn family transcription factors (a known binding partner of Sox11). Even those Sox-binding regions of genomic DNA that are not significantly conserved between mouse and human, tend to contain binding sites for Brn or other POU-homeodomain transcription factors. This may indicate that Sox-mediated transcription of these genes is an important regulatory mechanism. Furthermore, it may be possible to identify other proteins that may be involved in the Sox11 transactivating complex using analysis of conserved promoter regions that are known to be regulated by Sox11. Identification of conserved sequence motifs near the Sox consensus binding site could allow researchers to identify DNA-binding proteins that bind these sequences, and which may possibly partner with Sox11.

Alternately, the presence of conserved sequence motifs near Sox binding sites on gene promoter regions could indicate the presence of proteins that compete or interfere with Sox11 binding of DNA. A particularly interesting potential competitor for these binding sites is the TCF/LEF family of transcription factors. These are the effectors of canonical Wnt signaling, and

the closest relatives of Sox-family transcription factors. Their core binding domain is the sequence 5'-CAAAG-3'. In my work, the binding sites that were most strongly regulated by Sox11 tended to contain the Sox-binding sequence 5'-AACAAAG-3'; this sequence is a TCF/LEF binding site as well as a Sox binding site. This is particularly interesting in that Tcf4 (Tcf7l2) appears to be upregulated following axotomy in DRG (unpublished microarray data); this gene has multiple splice variants and can function either as a transactivator or a transrepressor. It is possible that Tcf4-mediated transrepression might function as an inhibitor of Sox11-mediated transactivation by competing with Sox11 for available DNA binding sites, and that this might provide a partial explanation for the transient upregulation seen by many targets containing the 5'-AACAAAG'-3' binding site. Other targets identified as transcriptional targets of Sox11, such as Sprr1a, do not contain this binding site and are regulated at later time points after injury (Jing et al., unpublished). It is possible that, depending on the abundance of Sox11 and its binding partners, that there is a subset of higher-affinity Sox-binding sites which are activated at relatively low levels of Sox11 upregulation, but which are quickly shut down by negative feedback mechanisms; likewise, there may be another subset of lower-affinity Sox-binding sites that require a much higher abundance of Sox protein in order for transcription to be activated, but which are more resistant to competitive binding from transrepression complexes.

6.8 Limitations in the interpretation of results from this study

The current study has some limitations in the models and technical approaches employed. One limitation is the use of Neuro-2a cells as a model for many of the experiments performed using transfection of either plasmids or siRNA. Neuro2a cells were used because of the difficulty in transfecting primary sensory neurons; however, the Neuro-2a line is a

neuroblastoma line and is therefore intrinsically different from the DRG neuron. It is probable that the nuclear environment of Neuro-2a cells (expression of Sox binding partners, chromatin remodeling, etc.) is very different from that of DRG neurons. This has the potential to affect the subset of possible Sox11 transcriptional targets regulated in the Neuro-2a cell line vs. the DRG neuron. Therefore, it is difficult to interpret negative results of studies that were performed using Neuro-2a cells; we do not know whether a negative result reflects a global inability of Sox11 to activate the putative transcriptional target, or whether the ability of Sox11 to regulate a given target differs between Neuro-2a cells and DRG neurons. The use of recombinant herpesvirus (HSV) constructs for gene delivery to peripheral sensory neurons is a strategy that may prove useful in eliminating some of these potential confounds in future work.

A further limitation of the current study is the use of luciferase reporter assays to determine the extent to which promoters are activated by Sox11. Luciferase assays are a powerful technique; however, in addition to having the same limitations described above in relation to the Neuro-2a cell model in general, our luciferase assays also have the limitation of only examining a relatively small upstream region of any given gene. It is possible for gene regulatory regions to exist many thousands of base pairs upstream of the transcriptional start site of a gene; therefore, it is possible that activation of a luciferase reporter construct may not translate to upregulation of that same gene in the same circumstances. Use of ChIP assays and real-time PCR analysis in conjunction with luciferase assay has proven to be a useful strategy to circumvent this without needing costly and time-consuming transgenic approaches.

Finally, the BDNF and APC studies are limited by lack of evidence of direct Sox11 binding to the promoter regions of these genes. While RT-PCR approaches demonstrate the upregulation of both these genes following nerve injury as well as after Sox11 overexpression,

and while luciferase assays demonstrate that Sox11 is capable of activating the promoter regions of these genes, ChIP assay is still required to determine whether Sox11 activates transcription of these genes directly, or whether Sox11-mediated upregulation of BDNF and APC occurs indirectly via upregulation or increased activation of another transcription factor.

A final consideration that should be discussed is the use of statistical methodology in these experiments, in particular the methodology used in the BDNF study. For this study, exons were analyzed using two-way ANOVA, rather than the approaches used in most of the real-time PCR analysis in other chapters (t-tests and one-way ANOVA). This was done out of concern that expression of each BDNF exon may not be a totally independent variable in these studies. Although some researchers claim that BDNF exons are independently transcribed (Aid et al., 2007), other groups have used bioinformatics techniques to show that many essential components of transcriptional machinery are located upstream of BDNF exons I and IV and have claimed that these regions may be important in driving expression of other exons (Liu et al., 2006). Therefore, the more statistically-stringent two-way ANOVA test was used to reduce the incidence of Type I errors. The use of this test resulted in demonstration of upregulation of exons IV and VII. However, the power of the BDNF study was low enough that gene upregulations that were deemed statistically significant in other studies would not have been found significant in this study. In a theoretical large-n study using the magnitude of BDNF upregulation as a cutoff value for statistical significance, we would have demonstrated upregulation of exons I, II, IV, V, VII, VIII, and XI revised this; meanwhile, we would have demonstrated downregulation of exons III and VI revise. However, the AACAAAG motif-containing exons IV and VII would still have been the most highly-upregulated constructs, implying that this motif is involved in activation of Sox11-mediated transcription. Further

studies should be performed to determine the nature of BDNF exon upregulation following nerve injury, as well as to determine whether BDNF exons are truly independently transcribed: the trend towards upregulation of exons II and V does parallel the upregulation of the immediately-upstream exons I and IV in terms of both direction of regulation as well as time course of upregulation (peaking at one day, and declining by three days post-injury), which is an argument against the independence of exon-specific BDNF transcription.

Other questions that arose during the course of these experiments include the extent to which individual permutations of the Sox consensus binding site affect the ability of Sox11 to activate transcription, the possibility for interaction between Sox proteins and Wnt signaling during development and disease, and the possibility that one major function of Sox11 following peripheral nerve injury may be to coordinate the convergence of multiple pathways upon JNK activation.

Future studies may address the potential for interaction between TCF/LEF and Sox family members upon the same transcriptional targets, the extent to which the AACAAAG binding site is preferentially bound by Sox11, the use of bioinformatics methods and analysis of Sox-binding regions on Sox11 transcriptional targets to identify additional sequence motifs and/or binding partners implicated in Sox11-mediated transcription following nerve injury, the molecular mechanisms by which TANK is capable of increasing neuronal survival downstream of TNF-alpha stimulation, and the net result of Sox11 upregulation on activation of JNK isoforms.

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