TEMPORAL EXAMINATION OF NOVEL TRANSCRIPTION FACTORS IN THE CELL BODY RESPONSE OF SENSORY NEURONS TO INJURY

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Abstract

Primary sensory neurons in dorsal root ganglia (DRG) undergo a cell body response after injury, where the neurons activate genetic growth programs with the goal of regenerating new axons. Transcriptional regulators are key to this response and the role of cell stress mediated transcription factors including FOXO3a, Luman and Zhangfei (ZF) are not fully understood. FOXO3a is proapoptotic and implicated in many neuronal pathologies. Recently, Luman, a regulator of the unfolded protein response, was identified as a retrograde injury signal essential for intrinsic regenerative axon growth, while ZF is a known inhibitor of Luman in other cell types. This thesis focused on the cell body response of DRG neurons to injury and whether expression patterns of these stress related transcription factors were affected by axotomy. A rat unilateral spinal nerve transection time course was employed and temporal protein and mRNA changes evaluated. Ipsilateral observations were as follows: FOXO3a protein decreased in injured neurons, while mRNA levels remained relatively constant, suggesting changes were secondary to post-translational modifications; while there was an initial decline in ZF expression post-injury, both ZF and Luman protein and mRNA were upregulated in ipsilateral neurons in a biphasic manner. Brain-derived neurotrophic factor (BDNF) is a known regulator of the regeneration response in DRG neurons. Its impact on these factors was determined by reducing endogenous BDNF with small interfering RNAs (siBDNF) or applying brief electrical stimulation to injured nerves, the latter upregulating BDNF. SiBDNF diminished injury triggered FOXO3a mRNA and ZF protein alterations, while stimulation enhanced the responses of somal FOXO3a and axonal Luman. A striking finding was that unilateral injury resulted in a mostly parallel, albeit lower biphasic response in contralateral DRG for all three transcription factors, with similar impacts on FOXO3a expression observed in cervical DRG remote from injury. Such dramatic contralateral biphasic changes are novel and support the existence of a systemic injury response. The findings of this thesis expand on the importance of transcription factors in the cell body response of DRG neurons, the impact of BDNF on regeneration and enforces the reality of contralateral and systemic effects to injury that cannot be ignored.

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- AAR - amino acid response
- AARE – amino acid response element
- ADP - adenosine diphosphate
- ATF3 - activating transcription factor 3
- ATF4 – activating transcription factor 4
- ATF6 - activating transcription factor 6
- BDNF – brain-derived neurotrophic factor
- Bip - binding immunoglobulin protein
- BrCA – breast cancer
- Brn3a - brain-specific homeobox/POU domain protein 3A
- BSA - bovine serum albumin
- bZIP - basic leucine zipper protein
- C4 – cervical ganglion 4
- cAMP - cyclic adenosine monophosphate
- CAP-23 - cytoskeleton-associated protein 23
- CCK - cholecystokinin
- Cdc- cyclin-dependent kinase 1
- CGRP - calcitonin-gene related peptide
- CHOP - CCAAT/enhancer-binding protein homologous protein
- CLK2 - cdc2-like kinase 2
- CNS – central nervous system
- CNTF - ciliary neurotrophic factor
- CRE - cAMP response elements
- CREB - cAMP response-element binding protein
- CREB3 – cAMP response element binding protein-3
- CREB3L1 - cAMP responsive element binding protein 3-like 1
- CREBH - cAMP-responsive element-binding protein 3-like protein 3
- CREBZF- cAMP response element binding protein zhangfei
- Cy3 – cyanine -3
- DAF-16 - abnormal DAuer Formation-16
- DAPI - 4',6-diamidino-2-phenylindole
- DC - dendritic cell
- *dilp-2* – drosophila IGF-1 homologue insulin-like peptide
- DRG – dorsal root ganglion
- DTT - dithiothreitol
- EDEM - ER degradation-enhancing α- mannosidase-like protein
- eIF2 - eukaryotic translation initiation factor 2
- ER – endoplasmic reticulum
- ERAD - ER-associated protein degradation

- ERdj4 - ER-resident DnaJ - 4
- ERp29 - endoplasmic reticulum protein-29
- ERSE - ER stress response element
- ERSR-II - ER stress response element II
- ES - electrical stimulation
- FasL - fas-ligand
- FGF – fibroblast growth factor
- FKHRL1 - forkhead in rhabdomyosarcoma like 1
- FOXO - forkhead box transcription factors class O
- FOXO3a - forkhead box transcription factors class O 3a
- GADD34 - growth arrest and DNA damage-inducible protein 34
- Gal4 - galactose-responsive transcription factor 4
- GAP-43 – growth associated protein - 43
- GAPDH - glyceraldehyde 3-phosphate dehydrogenase
- GDNF - glial cell line–derived neurotrophic factor
- GR - glucocorticoid receptors
- GRp58 - 58 kDa glucose regulated protein
- GRP78 - 78 kDa glucose regulated protein
- HbA1C – hemoglobin A1C
- HCF-1 - host cell factor-1
- HDAC5 - histone deacetylase 5
- Herp - homocysteine-induced ER protein
- HEY1 - hes related family BHLH transcription factor
- HPA - hypothalamic-pituitary-adrenal
- HSPA5 - heat shock 70 kDa protein 5
- HSV - herpes simplex virus
- IGF-1- insulin-like growth factor 1
- IGF-1R - insulin-like growth factor-1 receptor
- IR - insulin receptor
- IRDye – infrared fluorescent dye
- IRE1 - inositol-requiring protein-1
- JAB1 - Jun activation domain-binding protein 1
- JNK - c-Jun N-terminal kinase
- L4-6 - lumbar 4-6
- LZIP – leucine zipper protein
- MAP - multiple microtubule-associated proteins,
- MDCK – Madin-Darby canine kidney
- MDM2 - mouse double minute 2 homolog
- MnSOD - manganese superoxide dismutase
- MST-1 - mammalian sterile 20-like kinase-1

- NAD+ - Nicotinamide adenine dinucleotide+
- NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells
- NGF – nerve growth factor
- NT3 – neurotrophin-3
- NT4/5 – neurotrophin-4/5
- OCT - optimal cutting temperature compound
- OligoDNA - Oligodeoxyribonucleotide
- p-Akt - phosphorylated Akt
- $p75^{NTR}$ - p75 (the common neurotrophin receptor)
- PACAP - pituitary adenylate cyclase activating polypeptide
- PBS – phosphate buffered saline
- PDIA3 - protein disulfide isomerase family A member 3
- PERK - protein kinase RNA-like ER kinase
- PI3K - phosphoinositide 3-kinase
- PKB – protein kinase B/Akt
- PLCγ - phospholipase C-γ
- PNS – peripheral nervous system
- RAG - regeneration associated gene
- REDOX - reduction–oxidation reaction
- RIP - regulated intramembrane proteolysis
- RIPA - radioimmunoprecipitation assay
- RISC - RNA-induced silencing complex
- ROS – reactive oxygen species
- S1P - site-1 protease
- S2P - site-2 protease
- SCG10 - superior cervical ganglion-10
- SCI - spinal cord injury
- SDS - sodium dodecyl sulfate
- SEM – standard error of the mean
- siRNA – small interfering RNA
- SKP2 - s-phase kinase-associated protein 2
- SMILE - small heterodimer partner-interacting leucine zipper protein
- SP - substance P
- SREBP - sterol regulatory element-binding protein
- SRP54 - signal recognition particle 54kDa
- SSC - saline sodium citrate
- STAT3 - signal transducer and activator of transcription 3
- TNF-alpha – tumour necrosis factor - alpha
- trk - tropomyosin related kinase
- TTX - tetrodotoxin

- UPR – unfolded protein response
- UPRE – unfolded protein response element
- VIP - vasoactive interstitial polypeptide
- VP16 – viral protein -16
- XBP1 - X-box binding protein 1
- XBP1s - spliced XBP1 mRNA
- XBP1u - unspliced XBP1 mRNA
- ZF – Zhangfei

1. Introduction

Traumatic nerve injury imposes a tremendous stress on the axotomized nerve cell as it attempts to survive the insult and repair. Effective peripheral nerve repair requires a coordinated series of cellular and molecular events many of which are largely driven by the nerve cell body response to injury, where the transcriptional and translational changes contributing to repair are induced. Understanding how gene expression patterns are temporally altered in response to the stress induced by axotomy and under conditions that optimize regeneration will hopefully identify novel pathways that can be therapeutically targeted and manipulated to optimize recovery.

1.1. Primary sensory neuron biology

The cell type focused on in this thesis is the primary sensory neuron. These neurons are located in dorsal root ganglia lateral to the dorsal horn of the spinal cord and are ideal models to explore neurobiology, as they stand up well to harvesting, sectioning and staining under standard protocols. Further, the spinal and peripheral nerves containing their axons are easily accessible for multiple surgical manipulations with high experimental reproducibility.

Our group has extensive documented experience in utilizing sensory neurons to answer pivotal neurobiological questions and test novel hypotheses. Past studies have garnered excellent results and laid the foundation upon which the current hypotheses were formulated and tested, yielding the findings presented in this thesis.

1.1.1. Anatomical organization

The nervous system is comprised of a group of specialized cells, tissue types, and organs responsible for an organism's ability to sustain life and respond to the external environment. This includes the regulation of physiologically required cardiac and pulmonary mechanisms, sensory and motor output, cognition, and the ability to communicate with the surroundings. This system is functionally and anatomically divided into the central nervous system (CNS) and the peripheral nervous system (PNS) both with specific cell types, including neurons and glial support cells. The CNS contains the brain and the central spinal cord which are responsible for receiving sensory input, integration, appropriation and finally formalizing an organized, purposeful motor output in response to a stimulus. The PNS includes sensory, motor,

sympathetic and parasympathetic neurons as well as associated peripheral nerve fibres. This system works in tandem with the CNS, detecting changes in the environment and relaying motor signals from the CNS to target tissues and organs. Although the cellular types and processing are similar in both, there are distinct differences resulting in obvious physiological consequences; particularly with regards to regrowth, response to injury, and repair.

As stated, sensory neurons are a major component of the PNS, responsible for input and signal transduction. These pseudounipolar neurons project a single axon from the cell body that bifurcates into a peripheral branch innervating peripheral tissues, and a central branch which sends electrophysiological signals to the spinal cord for further processing. DRG neurons detect a variety of specialized stimuli which include tactile mechanical sensation, proprioception of limbs in space and nociception from either thermal, chemical or mechanical triggers. These sensations are transmitted distally from target tissues by electro-chemical signal transduction through long axons which have their own unique intercellular relationships. Primary sensory neurons are a heterogeneous population with highly specialized cell types differing in function, physiology, and growth factor responsiveness.

1.1.2. Axon diameter and physiology

As a heterogeneous collection of cell types, DRG neurons have distinct subpopulations that govern specific sensory modalities and are largely biochemically unique. These neurons are typically classified by a number of criterion including axon or cell body diameter, conduction velocity and growth factor responsiveness [reviewed in(Lindsay 1996)]. Arguably the most common subdivision of these cells is based on neuronal diameter where size most often associates with function; however, a precise correlation may not exist in all instances. Small diameter sensory neurons with unmyelinated axons are termed C-fibres, which are slow-conducting nerves and transmit nociceptive information including dull pain, heat and chemical stimuli [reviewed in (Millan 1999)]. Aδ-fibers are medium sized, thinly myelinated neurons that also sense a certain degree of nociception such as sharp or pricking pain. A subset of Aδ-fibers also are triggered by low threshold mechanosensation. The largest of these neurons are the heavily myelinated, rapidly conducting Aβ-fibers, whose medium to large diameter cell bodies process crude touch, proprioception and vibratory sensation [reviewed in (Lewin and Mendell 1993)].

Besides physiological function, these neurons have unique protein and membrane receptor profiles with infinite overlap. Numerous biochemical markers can identify distinct subsets of these sensory neurons; however, the function of many of these markers is not known (Carr and Nagy 1993). Nevertheless, the expression of these biomarkers is altered when the cell body changes from an intact/native phenotype to an injured phenotype. The consequence of this genetic switch will be discussed further in this chapter.

1.1.3. Neurotrophins

The growth factor family of neurotrophins have been widely described as essential regulators of differentiation, maintenance and survival of a variety of neuronal cell types. Their role in primary sensory neuron and motoneuron biology has been further documented; these include the neurotrophins nerve growth factor (NGF) (Levi-Montalcini and Hamburger 1951, Levi-Montalcini, Meyer et al. 1954, Cohen 1960), brain-derived neurotrophic factor (BDNF) (Barde, Edgar et al. 1982, Leibrock, Lottspeich et al. 1989) Neurotrophin-3 (NT3) (Maisonpierre, Belluscio et al. 1990, Verge, Gratto et al. 1996) and Neurotrophin-4/5 (NT4/5) (Funakoshi, Belluardo et al. 1995). These peptide ligands generated by neurons, glia and peripheral target tissues as proneurotrophin molecules, can undergo proteolytic cleavage to its mature isoform and bind to either $p75^{NTR}$ (the common neurotrophin receptor) or to the tropomyosin related kinase (trk) family of receptors [reviewed in (Reichardt 2006)]. There is specific affinity of the trk receptor types to each neurotrophin; NGF preferentially binds to trkA, BDNF to trkB, NT-3 to trkC and with less affinity to both trkA and trkB, and NT4/5 to trkB. The trk receptors, as intrinsic receptor tyrosine kinases whose dimerization leads to the activation of a host of signal transduction pathways, are shown to regulate many cellular processes including axonal growth and guidance, synapse formation and assembly of the neuronal cytoskeleton [reviewed in (Huang and Reichardt 2003)]. While activation of trks occurs primarily through the binding of ligands and homodimerization, secondarily each receptor can heterodimerize to the low affinity $p75^{NTR}$, demonstrating the multivalent mechanisms neurotrophins can utilize to influence neurons (Chao and Hempstead 1995). Intracellular pathways that are downstream of neurotrophin/trk signaling include but are not limited to PLCγ, the kinase pathways extracellular related-kinase (Wilson-Gerwing, Dmyterko et al.), PI3K, p38 mitogen-activated protein kinase and others (Segal 2003).

Our lab has done extensive analysis of the expression pattern of trk receptors within the DRG neuron population (Karchewski, Kim et al. 1999). Approximately 40% of primary sensory neurons express mRNA for TrkA, 33% for TrkB, 43% for TrkC and 79% of the total population express $p75^{NTR}$. There is marked overlap with neurons responsive to multiple neurotrophins and number of neurons showing no detectable expression of any trk receptor. Further work has shown TrkA and TrkC co-localization in 20%, TrkA with TrkB in 10% and TrkB with TrkC in 15% of DRG neurons.

The $p75^{NTR}$ is the common receptor with lower affinity to the mature neurotrophin molecules but higher affinity to the unprocessed proneurotrophin protein. It is expressed in up to 80% of DRG neurons (Zhou, Gai et al. 1993, Wetmore and Olson 1995, Zhou, Rush et al. 1996, Karchewski, Kim et al. 1999). Signaling through this receptor has been shown to augment axonal growth (Bentley and Lee 2000) and significantly influence cell survival and apoptosis (Rabizadeh, Oh et al. 1993, Soilu-Hanninen, Ekert et al. 1999, Roux and Barker 2002). $p75^{NTR}$ does not possess a catalytic domain to initiate downstream events; therefore, its activity depends on docking of proteins with kinase activity or its heterodimerization with trk receptors (Dobrowsky and Carter 2000, Wang, Bauer et al. 2001). Regardless, the receptor is significant in sensory neuron neurotropic signaling as its presence enhances the activity of trk receptors for their native ligands (Hempstead, Martin-Zanca et al. 1991, Urra, Escudero et al. 2007).

1.1.3.1. Nerve growth factor

The discovery of the neurotrophin family of growth factors has been an important contribution to cellular neurobiology in the last half century. Initially, Levi-Montalcini and Hamburg found that sarcoma extracts had growth stimulating properties for sympathetic and sensory embryonic neurons (Levi-Montalcini and Hamburger 1951, Levi-Montalcini, Meyer et al. 1954). The critical growth molecule in these isolates was identified and termed nerve growth factor (NGF) due to its dramatic effects on axon outgrowth in both in vitro and in vivo CNS and PNS neurons (Levi-Montalcini and Calissano 1979). Further studies with genetic knockout mice reveled the important role of this target-derived neurotrophin in development, particularly in the survival of small diameter nociceptive DRG neurons. Deletion of NGF or anti-NGF antibody administration in utero prevents the development of small diameter primary sensory neurons (Ruit, Elliott et al. 1992, Crowley, Spencer et al. 1994). Their importance to this subpopulation

was further shown when NGF treatment rescued small nociceptive neurons in axotomized neonatal DRG but did not improve survival of larger presumed proprioceptive neurons (Miyata, Kashihara et al. 1986).

Despite the shown dependency of embryonic and early postnatal nociceptive neurons on NGF, adult small diameter DRG neurons do not require NGF for survival (Lewin, Ritter et al. 1992). However, their native physiologic and molecular functioning appears to rely heavily on proper NGF signaling (Lewin and Mendell 1993) which maintains the phenotype of these small diameter primary nociceptive neurons (Verge, Richardson et al. 1989, Verge, Riopelle et al. 1989, Mandelzys, Cooper et al. 1990, Verge, Tetzlaff et al. 1990, Verge, Tetzlaff et al. 1990, Verge, Richardson et al. 1995). For example, nearly all NGF-responsive neurons express calcitonin-gene related peptide (CGRP), a protein shown to play a role in pain transmission (Verge, Richardson et al. 1989, Averill, McMahon et al. 1995). Expression of CGRP and the neuropeptide substance P (SP) are markedly elevated with exogenous NGF infusion in intact DRG neurons (Inaishi, Kashihara et al. 1992, Verge, Richardson et al. 1995), while these and other neuropeptide levels are decreased with infusion of NGF antibody (Schwartz, Pearson et al. 1982). Even though only 40-45% of adult DRG neurons, primarily nociceptors, are responsive to NGF (Karchewski, Kim et al. 1999), the identified role of this neurotrophin in this subpopulation revealed the potential of these molecules as therapeutic targets for peripheral nerve pathologies.

1.1.3.2. Brain-derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) stands out in the family of neurotrophins as it is produced not only by axonal tissue targets but also at appreciable levels by the neurons themselves (Barde, Edgar et al. 1982). BDNF knockout mice have a reduced amount of DRG neurons compared to wildtypes, but not the degree of loss seen in those mice lacking NGF or NT3 (Ernfors, Lee et al. 1994, Ernfors, Lee et al. 1994). The role governed by BDNF in primary sensory neuron biology is not clear cut as subpopulations that are BDNF-responsive also respond to other neurotrophins (McMahon, Armanini et al. 1994, Farinas, Wilkinson et al. 1998, Karchewski, Kim et al. 1999). A subset of medium-large sized mechanoceptive neurons (approximately 33% of DRG neurons) depend on BDNF for maturation and functional performance but not for survival (Mu, Silos-Santiago et al. 1993, Carroll, Lewin et al. 1998). However, early studies demonstrated that adult sensory neurons *in vitro* can survive without

exogenous NGF support which suggests neuron viability is somewhat independent of target-derived factors (Lindsay 1988). In theory, peripheral trophic support is less reliable, as skin or organ derived sources are exposed to the external environment and are at risk of damage. Therefore, alternative neurotrophic factors must exist so neuronal death does not ensue when there is separation from its target (Lindsay 1996). The capability of these neurons to grow isolated from their targets pointed to BDNF and possibly other neuronal produced trophic factors as autocrine and paracrine modulators of cell function and survival (Acheson and Lindsay 1996, Lindsay 1996). In fact, disruption of BDNF with antisense oligonucleotide causes neuronal death in ~33% of single cell isolates of adult DRG neurons *in vitro* which supports the above claim (Acheson and Lindsay 1996).

In the naïve DRG, BDNF mRNA is expressed most highly in small to medium sized neurons (Ernfors, Wetmore et al. 1990, Wetmore and Olson 1995, Verge, Gratto et al. 1996). However, the populations that express the neurotrophin are distinctly separate from those expressing the high affinity TrkB BDNF receptor (Kashiba, Ueda et al. 1997). Regardless, BDNFs ability to act through the p75 common neurotrophin receptor which is present on ~80% of neurons of all sensory modalities points to its significant role in intracellular DRG neuron regulation.

For the purpose of this work, the role for BDNF in growth and regeneration of injured axons is of great interest. Peripheral nerve transection leads in essence to the loss of target derived BDNF; however, there is noted upregulation of BDNF in the nerve stump distal to injury and the denervated muscle (Funakoshi, Frisen et al. 1993) suggesting that its signaling may be necessary for newly growing axons. As stated, in the intact state, BDNF is predominantly synthesized in small to medium sized TrkA-positive neurons and at lower levels in TrkB positive neurons (Karchewski, Gratto et al. 2002). After nerve injury, there is a transient increase in anterograde transport of BDNF to the periphery (Tonra, Curtis et al. 1998). At the cell body, sciatic nerve transection results in a biphasic response in BDNF expression where levels are universally up-regulated in all size ranges of neurons at 1-day post-injury, then decline in small-medium size neurons but remains high only in medium-large size neurons after 1 week (Michael, Averill et al. 1999, Zhou, Chie et al. 1999, Karchewski, Gratto et al. 2002). The incidence of BDNF co-expression with TrkB is low in intact neurons, but increases dramatically following injury, signifying a phenotypic shift where those producing BDNF are now more sensitive to its action (Karchewski, Gratto et al. 2002). The increased production of BDNF in injured DRG neurons

could be neuroprotective (Murphy, Borthwick et al. 2000) but evidence from the Verge lab suggests that the upregulation is critical for induction of regeneration associated gene (RAG) expression and the intrinsic ability of an axon to regenerate.

Brain-derived neurotrophic factor is the only neurotrophin whose expression is significantly increased in peripheral neurons after injury. In both motoneurons and primary sensory neurons, peripheral nerve injury increases expression of neuronal BDNF and TrkB as well as peripheral sources of the neurotrophin. (Funakoshi, Frisen et al. 1993, Koliatsos, Clatterbuck et al. 1993, Karchewski, Gratto et al. 2002) Delivery of BDNF into the proximal nerve end post-injury was shown to improve functional recovery after sciatic nerve transection suggesting a growth-promoting role. (Utley, Lewin et al. 1996, Lewin, Utley et al. 1997). Furthermore, therapies known to enhance regeneration of injured neurons, specifically electrical stimulation, has been shown to augment BDNF expression (Al-Majed, Brushart et al. 2000, Geremia, Gordon et al. 2007). Extensive work by Geremia and colleagues showed that brief electrical stimulation of femoral nerves after transection resulted in elevated levels of BDNF, TrkB and regenerated associated genes in all DRG neurons, particularly in small-medium sized neurons (Geremia, Gordon et al. 2007). This initial work, lead to the hypothesis that BDNF acted as an important induction signal for the regenerative and cell body injury response. Disruption of BDNF action by delivery of either anti-BDNF antibody or targeted small-interfering RNA suppressed the intrinsic activation of growth associated genes in injured sensory neurons *in vivo* and *in vitro* (Geremia, Pettersson et al. 2010). Additionally, injury-induced increases of pituitary adenylate cyclase activating polypeptide (PACAP), a modulator of nerve repair and survival, were reduced using the same immunoneutralization or downregulation of endogenous BDNF approaches (Pettersson, Geremia et al. 2014); while administration of exogenous BDNF enhanced the expression of growth associated genes in injured but not intact sensory neurons, suggesting the neurotrophin can amplify regeneration (Geremia 2005). This collective evidence of the neurotrophin's role in augmenting injured sensory neurons' regeneration-associated gene (RAG) expression and their intrinsic ability to regrow an axon infers that therapies used to alter BDNF action would likely affect transcriptional regulators of regeneration.

1.2. Peripheral nerve injury and regeneration

The neurons of the PNS are significantly unique as they are able to regrow injured axons following injury; a characteristic not shared by their CNS counterparts. This growth capacity of the PNS has long been recognized and appreciated; however, repair is slow and often incomplete resulting in poor clinical outcomes for patients afflicted with nerve pathologies.

The propensity for these neurons to regenerate have been studies since the 2nd century where Galen of Pergamon (131-201 AD) first studied severed nerves and noticed the incredible findings in his experiments [reviewed in (Terzis, Sun et al. 1997)]. Throughout the scientific revolution; other academics and philosophers continued to examine the anatomical, physiological biochemical action of these growing nerves. In the 1600s, animal studies revealed that bilateral severance of the vagus nerves lead to death after a delay period (Willis T 1681, reprinted in 1966). Interestingly, the British chemist William Cumberland Cruikshank, sometime later observed that unilateral vagus nerve transection did not lead to animal death; furthermore, dissection of experimental tissue showed that the proximal and distal stumps were "firmly united; having their extremities covered with a kind of callous substance" (Cruickshank 1795). This and other findings lead to a period of discovery, where our understanding of the nervous system, brain and nerve pathologies and therapeutics grew exponentially. Despite the expanding discipline, it was not clear whether regeneration of the injured axons was facilitated solely by the rejoining of the severed nerve ends and/or if the neurons had an intrinsic ability for outgrowth of new fibres.

Augustus Waller in 1850, published his findings that injury to the frog hypoglossal nerve resulted in the loss or "resorption" of the distal nerve and Schwann cells but a mass of "amorphous medulla" or new axons appeared to develop (Waller 1850). The events he described have been termed Wallerian degeneration, which refers to the cellular processes proceeding a PNS injury event, where an inflammatory mediated process leads to the degradation of the distal nerve and the formation of a permissive growth environment for new axons [reviewed in (Burnett and Zager 2004, Rotshenker 2011)]. Surrounding myelin is broken down, mast cell degranulation and phagocytosis clears nerve tissue leaving rows of Schwann cells known as bands of Büngner. These remaining Schwann cells act as a guiding platform for newly synthesized axons to their previous targets (Snell 2005). Waller's observations were a window into the potential for injured nerves to recover from damage. However, this regrowth and regain

of function is frequently imperfect and clinical evidence shows the frequent failure of injured nerves to reinnervate target tissue after surgical repair (Noble, Munro et al. 1998).

Injury of nerves results in drastic morphological, molecular and physiologic changes at the level of the neuron cell body, proximal stump and the distal nerve. These changes are numerous and are governed by extracellular and intracellular mechanisms leading to clear genetic and phenotypical changes. Many neuronal and glial gene products including growth factors, signal transduction proteins and transcription factors have been extensively studied in the hopes their action after injury (or lack thereof) is the answer to improving nerve regeneration and functional outcomes. This essential cellular factor has yet to be identified.

1.2.1. Multiphasic injury signal and cell body response in peripheral neurons

Severance of the axon generates early signaling events which communicates to the cell body that injury has occurred. Firstly, the integrity of the plasma membrane is lost, causing a disruption in the ionic concentration gradient between the axon and the extracellular matrix and an influx of cations (Yoo, Nguyen et al. 2003). This causes depolarization and the transmission of injury-mediated action potentials, known as injury discharge, which leads to increases in intracellular second messenger molecules, including calcium and cAMP and activation of downstream signaling pathways (Berdan, Easaw et al. 1993). These early events can also drive activation of enzymes and transcription critical to induction of a robust regenerative state. Secondly, there is interference with retrograde trophic (i.e. NGF) signaling from the periphery within 12-24 hours following injury, disinhibiting the suppressed regeneration response (Raivich, Hellweg et al. 1991). Notably, injury-induced increases in neuronal BDNF expression observed in over 80% of sensory neurons is one of these early changes in gene expression. This elevated BDNF expression has been shown to be critically linked to induction of robust RAG expression and the intrinsic ability of injured sensory neurons to regenerate an axon (Geremia, Petterssen et al. 2010; Petterssen, Geremia et al. 2014). While a major inductive signal for regeneration, in these studies BDNF did not appear to be responsible for maintaining RAG expression one week after injury expression as 3-day neutralization of endogenous BDNF initiated at this time point as opposed to immediately at the time of injury had no discernible impact on RAG expression. Thirdly, the proximal injured nerve tip is exposed to the intracellular content of other axons and Schwann cells that express multiple growth factors including CNTF, NGF, FGF and BDNF

(Elde, Cao et al. 1991, Sendtner, Gotz et al. 1997, Karchewski, Gratto et al. 2002, Kirsch, Terheggen et al. 2003), that can have dramatic impacts on axonal guidance (Webber, Xu et al. 2008). Subsequently, the trophic and cytokine levels are altered locally due to the developing inflammatory environment (Lindholm, Heumann et al. 1987).

Logically, one of the primary responses of the injured sensory neuron must be linked to neuronal survival. The chance of neuronal death following peripheral nerve axotomy is directly related to the proximity of the cut site to the perikaryon (Gordon, Gillespie et al. 1991, Xu, Forden et al. 2010). No motoneurons are lost unless the injury is very proximal to the cell body (Vanden Noven, Wallace et al. 1993); however, peripheral sciatic nerve transection can result in elevation of pro-apoptotic markers in primary sensory neurons as soon as 1-day post-axotomy (McKay Hart, Brannstrom et al. 2002). Nevertheless, significant neuronal loss is only seen after approximately 4 weeks after injury, effecting at most 35% of the DRG neuron population with the small to medium size sensory neurons being most vulnerable (Otto, Unsicker et al. 1987). This tendency of prolonged survival is likely attributed to the robust trophic support for primary sensory neurons and their high-responsiveness to these signaling pathways (Verge, Gratto et al. 1996).

Axon injury also results in a process of morphological changes at the level of the cell body collectively known as chromatolysis [(Lieberman 1971), reviewed in (Gordon, Sulaiman et al. 2009)]. The gross histological changes include the shifting of the nucleus to a decentralized location within the soma, increased size of the nucleolus, swelling of the cell and partial loss of the distinctive Nissl body staining of the rough endoplasmic reticulum. These alterations are likely due to the molecular sequelae of the injury response and changes to the expression of countless genes including neuropeptides, transcription factors, and structural proteins (Fu and Gordon 1997, Stam, MacGillavry et al. 2007, Patodia and Raivich 2012). The surviving neuron undergoes a phenotypic shift from a “transmitting” or intact cell connected to its peripheral targets; to the genetic profile of a “growing” or regenerating one (Watson 1974). The genes whose expression levels are altered after injury have been collectively referred to as regeneration associated genes (RAGs) and include cytoskeletal proteins such as tubulin and actin, which are transported down to the growing axon tip where they provide support and create the ultrastructure of the newly synthesized nerve (Tetzlaff, Bisby et al. 1988, Tetzlaff and Bisby 1990, Tetzlaff, Alexander et al. 1991, Dent, Gupton et al. 2011). Other scaffolding protein levels

such as neurofilament are reduced in response to injury (Verge, Tetzlaff et al. 1990). Expression of synaptic-transmission proteins such as acetylcholine, acetylcholine esterase and choline acetyltransferase are also downregulated (Friedman, Kleinfeld et al. 1995). Other categories of RAGs that are upregulated include the immediate early genes such as the transcription factor c-jun (Leah, Herdegen et al. 1991), neurotrophins including BDNF in both sensory and motor neurons and their respective trk and p75 receptors in motor neurons (Friedman, Kleinfeld et al. 1995, Al-Majed, Brushart et al. 2000, Karchewski, Gratto et al. 2002), and growth associated proteins including GAP-43 (Verge, Tetzlaff et al. 1990, Schreyer and Skene 1993), CAP-23 and SCG10 (Mason, Lieberman et al. 2002). A number of other transcription factors are also known RAGs and will be discussed later in this chapter.

Elevations in neuronal RAGs expression following axotomy peak at around 1-week after injury (Gordon and English 2016). This RAG increase eventually dissipates, falling back to baseline levels after approximately 6 months in chronically axotomized motoneurons (Gordon and Tetzlaff 2015, Gordon, You et al. 2015). Interestingly, a re-injury of these chronically injured neurons proximal to the original cut site results in another up-regulation of RAGs but to a lesser extent than seen in acutely injured states (Gordon and Tetzlaff 2015, Gordon, You et al. 2015). This second RAGs increase is not triggered by loss of retrograde target trophic support, suggesting that signals emanating from the transection site itself are sufficient enough to produce a regeneration response.

1.3. Axonal mechanisms: transport

The ultrastructure of the neuron is complex. No other cells require such an extensive cytoskeletal intracellular network, with cellular compartments and axons often placed a great distance from the cell body. Axonal transport allows for delivery of newly synthesized protein, RNA, lipids or organelles to distal regions such as synapses to maintain active cell-to-cell connections [reviewed in (Perlson, Maday et al. 2010)]. Undoubtedly, with regards to regeneration, necessary cell products including cell membrane must be sent to the growth cone to build new axons. Conversely, axonal misfolded proteins or aggregates must be shuttled back to the cell body for better degradation and clearance (Chevalier-Larsen and Holzbaur 2006). To meet the demand of energy needs, mitochondria are also bi-directionally transported (Sheng 2014). Finally, axonal trafficking between the axon and the soma allows for retrograde

intracellular communication whereby the neuron can respond to changes in the environment. Transport along the axon occurs by both fast and slow speeds and is achieved with anterograde, retrograde, bi-directional machinery requiring multiple protein components (Maday, Twelvetrees et al. 2014).

Cytoskeletal filaments including microtubules, actin and intermediate filaments all play roles in neuron morphology; however, fast transport down the axon is dependent on the microtubules which act at tracks for motor proteins. Microtubules are rigid polymers formed by multiple tubulin molecules arranged in a polarized direction where the fast growing, dynamic plus end extend down the axon center while the stable minus end is anchored at the soma (Burton and Paige 1981, Stepanova, Slemmer et al. 2003). Dendritic microtubule organization is more complicated as these cytoskeletal tracks are arranged in mixed polarity directions (Baas, Deitch et al. 1988). Multiple microtubule-associated proteins, (MAPs) are bound to the structure along the length of the axon and promote microtubule polymerization and stabilization. The high expression level of MAPs in neurons suggests that neuronal microtubules are more stable than in other cell types and the interaction of MAPs with protein motors *in vitro* points to the possible involvement with transport (Vershinin, Carter et al. 2007, Dixit, Ross et al. 2008).

The two main motor protein types that transverse the axonal microtubules are kinesins and dynein. These motors interact with other associated proteins and are responsible for the travel of cargo along the axon. The kinesin superfamily contains 38 neuron gene products with kinesis-1, kinesis-2 and kinesis-3 families known to contribute to axonal transport (Miki, Setou et al. 2001, Lawrence, Dawe et al. 2004). Kinesin homo or heterodimers are the main drivers of anterograde transport; carrying a wide range of cargo including vesicles, organelles, RNA and proteins at velocities of ~1 um/s (Hirokawa, Niwa et al. 2010). Cytoplasmic dynein is the primary microtubule motor responsible for minus-end directed or retrograde transportation. This large protein complex consists of two heavy chains forming the motor domains and other intermediate and light chains responsible for cargo recognition and binding. Dynein is a fast motor, with velocities from 0.5 to 1 um/s. Contrary to the great diversity seen with the kinesin motors, the dynein motor domain is encoded by a single gene while there are two genes encoding for dynein light intermediate-cargo associated chains suggesting some variability [reviewed in (Roberts, Kon et al. 2013). The activity of this motor requires the dynein activator *dynactin*, a multiprotein complex essential both tubulin and cargo binding, whose disruption impairs normal neuron

function (Waterman-Storer, Karki et al. 1995, Schroer 2004, Moughamian and Holzbaur 2012, Yeh, Quintyne et al. 2012). Kinesin and dynein are the major motors which drive axonal transport; however, multiple MAPs, motor adaptors, regulators and components of cytoskeleton are required for efficient binding, proper cargo attachment and are essential components of the transport machinery [reviewed in (Maday, Twelvetrees et al. 2014). While these highly conserved mechanisms are utilized in many neuronal cell types, the role of axonal transport in sensory neurons is well established and plays a significant part in neuronal development, neurotrophin signaling and nerve regeneration.

1.3.1. Axonal mechanisms: local protein synthesis

The historical view has been that the soma provides distal axons with all the proteins and cellular structures necessary for maintenance and plasticity. The paradigm shift began to occur in the 1960s when large amounts mRNA were found localized to vertebrate axons, implying that these transcripts are independently translated away from the cell body (Edstrom, Eichner et al. 1962). Further justifying this hypothesis, it was discovered that axons possess ribosomal RNA (Rapallino, Cupello et al. 1988), ribosomal subunits (Tcherkezian, Brittis et al. 2010) and can translate endogenous and exogenous mRNA without interacting with the cell body (Van Minnen, Bergman et al. 1997, Eng, Lund et al. 1999). Not only does local translation occur in axoplasm, but local protein processing and trafficking takes place as ER and Golgi equivalents and associated proteins are present in axons (Jung, Yoon et al. 2012, Gonzalez and Couve 2014). Furthermore, isolated axons must have a high functioning protein trafficking network as locally synthesized proteins are readily deposited in plasma membranes (Merianda, Lin et al. 2009).

Axonal protein synthesis allows for rapid intracellular alterations in response to the extracellular milieu. Axons separated from the soma can still grow towards targets and respond to guidance cues (Harris, Holt et al. 1987, Campbell and Holt 2001, Ming, Wong et al. 2002). These and other studies have implied the potential involvement of axonal protein translation in regeneration following injury. In fact, blocking protein synthesis in axons inhibits regeneration (Verma, Chierzi et al. 2005) and there is an association of decreased axonal protein synthesis with reduced axon outgrowth in mature neurons (Jung, Yoon et al. 2012). Nerve injury increases axon protein translation in *in vitro* and *in vivo* sensory neuron models (Zheng, Kelly et al. 2001, Verma, Chierzi et al. 2005) and blocking axonal mRNA localization inhibits regeneration after

injury (Donnelly, Willis et al. 2011). The pattern of axon growth is also affected by axonally synthesized proteins and specific axon mRNA content. Increased translation of beta-actin leads to highly branched axons, while increased GAP-43 production causes the growth of more elongated and lesser branched axons (Donnelly, Park et al. 2013). The role of local axon protein synthesis in regeneration is shown by this collective evidence, where injury appears to up-regulate certain axonal transcripts associated with the growth response. It also leads to increased levels of transcriptional regulators that may serve to further regulate RAG expression (Ying, Misra et al. 2014). Thus, when examining alterations in somal protein levels following injury in DRG neurons, one must always consider that dynamic axonal synthesis and transport after injury may alter cell body protein quantity, in absence of increased translation at the somal level.

1.3.2. Axonal mechanisms: the injury signal

Proper anterograde and retrograde transport mechanisms are utilized for intracellular neuron signal transduction pathways. Neurotrophins from peripheral targets bind trk or p75 receptors causing internalization of the ligand-receptor complex forming the signaling endosome (Bronfman, Escudero et al. 2007). The neurotrophin containing vesicles are retrogradely transported by dynein-mediated processes [Reviewed in (Chowdary, Che et al. 2012, Cosker and Segal 2014). Studies show that disruption of the trk-dynein transport system leads to lost neuronal viability and that dynein based transport is required for retrograde survival of DRG neurons (Heerssen, Pazyra et al. 2004). NGF action on nerve terminals leads to NGF-TrkA endosome signaling which has been shown to increase axonal translation of the transcription factor cAMP response-element binding protein (CREB) in developing neurons (Cox, Hengst et al. 2008). Endosomes associated with activated CREB are retrogradely shuttled to the cell body where CREB activates target genes promoting survival.

While loss of trophic signaling endosomes transported by the microtubule-dynein mechanism occurs following injury of the axon, further positive injury signals from the site of damage are sent via axonal transport, inducing transcriptional change (Murphy, Borthwick et al. 1999; Ying, Misra and Verge 2014; Ying, Zhai et al. 2015). Some injury stimuli are rapid, such as calcium influx at the site of injury and soma (Cho and Cavalli 2012) and depolarization propagation causing direct genetic alterations including nuclear export of histone deacetylase 5 (HDAC5) in a protein kinase C-dependent manner, leading to enhanced histone acetylation (Cho, Sloutsky et al.

2013). Conversely, dynein associated retrograde signals initiated at the injury site are slower at effecting cell body changes. Their impact on axon growth take hours to days after injury but there is an emerging understanding of their importance in the induction and maintenance of the injury response (Ying, Misra and Verge 2014).

1.3.3. Transcriptional regulators of the cell body response to injury

Transection of peripheral nerves causes an injury signal that leads to the activation of various intracellular pathways with downstream targets that include many transcription factors whose post-translational state, cellular localization and activity are affected (reviewed in (Patodia and Raivich 2012)). These transcriptional regulators alter the genotype of sensory neurons including the expression of RAG; thus initiating and maintaining the axon outgrowth of injured neurons. While the injury-induced expression and activity levels have been described for multiple transcription factors, many regulators that are expressed in DRG neurons have yet to have their response to injury characterized.

Of particular interest to this thesis are transcription factors linked to how the sensory neuron responds to the stress induced by the nerve injury and to the demands of regeneration. To gain insight into how expression and nuclear translocation of these transcription factors might be temporally altered following injury, I chose to focus on regulation of transcription factors implicated in neuronal survival (FOXO3A) and regulation of the unfolded protein response (UPR; Luman/CREB3 and Zhangfei/CREBZF), the UPR being involved in the neuron's ability to meet the protein and sterol demands of regeneration and also linked to apoptosis.

1.3.3.1. Forkhead-transcription factors

Members of the forkhead box transcription factors class O (FOXO) family of proteins modulate the cellular processes of survival, differentiation, proliferation and stress resistance (reviewed in (van der Horst and Burgering 2007). These evolutionarily conserved transcription factors were first identified as essential to *Drosophila* development (Weigel, Jurgens et al. 1989). Since that time, our knowledge of FOXOs has grown exponentially with a protein catalogue of over 100 subtypes that are implicated in a variety of mammalian and non-mammalian cellular processes. They are heavily regulated by epigenetic (Peng, Zhao et al. 2015) and various post-translational modifications including phosphorylation, acetylation and poly- and

monoubiquitination (Huang and Tindall 2007, Chong, Hou et al. 2011, Wang, Yan et al. 2013). Nucleocytoplasmic shuttling of FOXOs is primarily controlled by an Akt/protein kinase B dependent phosphorylation mechanism, where phosphorylated protein remains cytoplasmic and dephosphorylation of serine-threonine residues facilitates nuclear localization. In the mammalian adult nervous system, FOXO1, FOXO3/FOXO3a, FOXO4 and FOXO6 proteins have been identified and are generally characterized as stress-related, pro-apoptotic transcription factors significant in neuronal survival (Maiese 2015).

1.3.3.1.1. FOXO3a

Of all the forkheads, FOXO3a (FOXO3/FKHRL1 (forkhead in rhabdomyosarcoma like 1)) has emerged as a target in multiple disease states, particularly neurodegenerative disorders (reviewed in (Maiese, Chong et al. 2007)). The sequence of the FOXO3a gene contains 3 exons and produces a 673 amino acid protein with the well conserved and defining forkhead domain (Anderson, Viars et al. 1998). FOXO3a protein is present in a variety of human and other mammalian cell types, including those in the nervous system, such as hippocampal and cortical neurons, the cerebellum and motor, sympathetic and primary sensory neurons (Gilley, Coffer et al. 2003, Barthelemy, Henderson et al. 2004, Hoekman, Jacobs et al. 2006, Wang, Liu et al. 2009). FOXO3a knockout mice (Foxo3a-/-) are viable but the development of certain organs are impaired (Hosaka, Biggs et al. 2004). Absence of FOXO3a leads to lymphoproliferative disease, organ inflammation and increased activity of helper T-cells, suggesting a role for FOXO3a in the Immune response (Lin, Hron et al. 2004). These FOXO3a deficient mice are also noted to have elevated levels of reactive oxygen species and altered expression in genes which regulate the REDOX cycle, pointing to its involvement in the oxidative stress response (Tothova and Gilliland 2007).

As stated, FOXO proteins are tightly regulated at the post-translational level and these are primarily mediated through the Akt/PKB pathway, a known downstream target of the insulin pathway and neurotrophins (Brunet, Bonni et al. 1999, Zheng, Kar et al. 2000, Kawano, Morioka et al. 2002, Zheng, Kar et al. 2002, Gilley, Coffer et al. 2003, Zhu, Bijur et al. 2004, Fukunaga, Ishigami et al. 2005). However, the regulation of FOXO in the nervous system is mediated through multiple intracellular pathways and is present in all tissue types (reviewed in (Maiese 2015). The main transcriptional outputs for FOXO activity are associated with stabilizing the cell

during times of homeostatic compromise and then determination of cell fate. These outputs include regulators of the cell cycle and apoptosis, glucose metabolism and oxidative stress resistance. As the information on the forkheads spans every mammalian cell type and multiple pathological states, this review will focus on the finding pertinent to FOXO3a, the nervous system and sensory neurons in particular.

1.3.3.1.2. Apoptosis and the cell cycle

Neuronal FOXO3a has classically been considered a pro-apoptotic transcription factor, which is accredited to its close relationship with the PI3K/Akt survival pathway. Phosphorylation by Akt inhibits its activity as phosphorylated FOXO3a associates with 14-3-3 scaffolding proteins, which leads to cytoplasmic sequestering (reviewed in Maiese 2015). Decreased Akt signaling or increased dephosphorylation by phosphatases results in nuclear translocation and the activation of forkhead-responsive genes, including the apoptotic genes *fas-ligand* (FasL) (Brunet, Bonni et al. 1999) and *bim* (Dijkers, Medema et al. 2000). Many growth factor signal transduction mechanisms are mediated through Akt and trophic availability can modulate FOXO3a activity. Insulin-like growth factor-1 treatment increases Akt phosphorylation of the transcription factor in PC12 cells (Zheng, Kar et al. 2000), while neurotrophin deprivation induces nuclear localization of FOXO3a, FasL gene expression and death of cultured motoneurons (Barthelemy, Henderson et al. 2004). In general, any conditions associated with neuronal loss; such as development (Srinivasan, Anitha et al. 2005) aging (Jackson, Rani et al. 2009) or pathological ischemic events, (Kawano, Morioka et al. 2002, Fukunaga, Ishigami et al. 2005, Maiese, Chong et al. 2007) have a connection to decreased Akt activity and FOXO3a activation.

FOXOs are transcriptional activators of genes involved in cell cycle inhibition, including $p27^{kip1}$ (Medema, Kops et al. 2000) and cyclin G2 (Martinez-Gac, Marques et al. 2004), as well as suppressors of the cycle progression protein cyclin D (Schmidt, Fernandez de Mattos et al. 2002). Insulin and other growth factors promote cell proliferation by inhibiting FOXO activation of cell-cycle arrest genes in both Alzheimer's and diabetic models (Sajan, Hansen et al. 2016). Depletion of cellular regulators including Cdc2-like kinase 2 (CLK2) lead to dephosphorylation of AKT and decreased phosphorylation of FOXO3a, which in turn increases p27 expression and slows glioblastoma growth (Park, Piao et al. 2016).

1.3.3.1.3. Glucose metabolism

Insulin is the main regulator of glucose uptake, glycolysis and glycogen synthesis. The forkheads regulate enzymes associated with the insulin pathway, glucose metabolism and gluconeogenesis such as phosphoenolpyruvate carboxykinase and glucose-6-phosphate (Barthel, Schmoll et al. 2001, Nakae, Kitamura et al. 2001, Nakae, Kitamura et al. 2001). FOXO1 was also shown to suppress genes involved in glycolysis (Zhang, Patil et al. 2006), suggesting that FOXO activity may induce a metabolic switch in low glucose conditions. However, this family of transcription factors has been implicated in diabetes mellitus onset and diabetic complications; for example, a prospective population based study indicated that patient carrying a specific FOXO1a haplotype had higher HbA1C levels and likely impaired glucose tolerance (Kuningas, Magi et al. 2007). With regards to FOXO3a, experimental animals models showed that high-fat diet induced hyperinsulinemic resistant obesity showed increased FOXO3a in cardiac myocytes (Relling, Esberg et al. 2006). It has also been linked to diabetic nephropathy as renal cortical tissue in diabetic rats has increased levels of phosphorylated FOXO3a (Kato, Yuan et al. 2006). Furthermore, enteric neurons are protected from hyperglycemia by glial-derived neurotrophic factor and signaling through the Akt/PKB pathway which decreased FOXO3a nuclear localization (Anitha, Gondha et al. 2006). FOXOs conserved relationship with glucose metabolism offers clues to FOXO3a's potential role in sensory neurons. Small neurons of the DRG have high levels of hexokinase, which initially phosphorylates glucose prior to the start of glycolysis (Gardiner, Wang et al. 2007). The elevated amounts of this kinase suggest higher concentrations of glucose in this population and the necessity for rigorous metabolic management. Small-medium neurons also express high levels of the insulin receptor (IR) (Sugimoto, Murakawa et al. 2002), insulin-like growth factor-1 receptor (IGF-1R) and insulin-like growth factor 1 (IGF-1) (Craner, Klein et al. 2002). As stated above, FOXO3a activation is induced in a low-glucose situation and activates genes associated with glucose conservation, such as glucose-6-phosphatase (Onuma, Vander Kooi et al. 2006). It also increases sensitivity to insulin by up-regulating IR and insulin receptor substrate proteins (Puig and Tjian 2005).

1.3.3.1.4. Ischemia and oxidative stress

There is a well-established connection between the forkheads and oxidative stress, where increased levels of cellular reactive oxygen species (Storlazzi, Mertens et al.) regulate FOXO

post-translational modification and thus regulate oxidative stress resistance (reviewed in (van der Horst and Burgering 2007).To mitigate the ROS-induced injury to cellular organelles, FOXO3a in particular, has been shown to have a protective tendency against oxidative damage where it upregulates several antioxidant enzymes such as catalase (Nemoto and Finkel 2002) and Manganese superoxide dismutase (MnSOD) (Kops, Dansen et al. 2002). However, a body of evidence suggests that FOXO3a might play an important role in oxidative stress-mediated damage and apoptosis of neuronal cells in models of cerebral ischemic stroke (Chong, Kang et al. 2003, Chong, Lin et al. 2003, Chong, Kang et al. 2004, Chong, Li et al. 2005, Chong, Li et al. 2005). In animal studies, increased FOXO3a activity is associated with altered c-jun N-terminal kinase 3 signaling and stroke promotion effects (Pirianov, Brywe et al. 2007). Other ischemic models show that inhibitory phosphorylation of FOXO3a may be associated with the neuroprotective effects of estradiol after stroke (Won, Ji et al. 2006). Inhibition or knockdown of FOXO3a in neuronal culture cells can assist in the ischemic protective effects of metabotropic glutamate receptors and improve neuronal survival through NAD+ precursor treatment (Chong, Lin et al. 2004, Chong, Li et al. 2006). Furthermore, inhibited FOXO3a activity increases trophic protection of neurons during oxidative stress via insulin-like growth factor-1 and neurotrophins (Zheng, Kar et al. 2000, Zheng, Kar et al. 2002, Zheng, Kar et al. 2002).

In clinical work, vitiligo patients with a polymorphism for FOXO3a had decreased levels of catalase enzyme activity suggesting a possible protective role of FOXO3a activity (Ozel Turkcu, Solak Tekin et al. 2014). However, examination of endometrial decidua showed that constitutively active FOXO3a conferred a tendency to oxidative stress mediated apoptosis (Kajihara, Jones et al. 2006) and patients with two FOXO3a haplotypes have increased risk of stroke (Kuningas, Magi et al. 2007). FOXO3a action in certain cell types appears to decrease levels of ROS; however, in neuronal cells its activity is associated with oxidative stress mediated cell death and decreased FOXO3a levels or increased degradation may be a survival mechanism and thus be desirable.

1.3.3.2. FOXO3a in primary sensory neurons

DRG neurons and associated glial cells express FOXO3a, and neuronal proteins levels are decreased following sciatic nerve transection (Wang, Liu et al. 2009). As previously stated, FOXOs are transcriptional activators of genes involved in cell cycle inhibition, including $p27^{kip1}$

(Medema, Kops et al. 2000) and cyclin G2 (Martinez-Gac, Marques et al. 2004), as well as suppressors of the cycle progression protein cyclin D (Schmidt, Fernandez de Mattos et al. 2002). The role of cell cycle proteins in post-mitotic neurons is under investigation and results indicate that induction of the cell cycle precedes neuronal apoptosis (Freeman, Estus et al. 1994, Park, Levine et al. 1997, Shirvan, Ziv et al. 1997, Liu and Greene 2001, Becker and Bonni 2004, Herrup, Neve et al. 2004). Nerve regeneration and axon outgrowth is associated with cell cycle inhibition events such as the up-regulation of cyclin G (Morita, Kiryu et al. 1996) and p21 [the cyclin dependent kinase (cdk) inhibitor](Tanaka, Yamashita et al. 2004), and the suppression of the anaphase promoting complex (Konishi, Stegmuller et al. 2004). Recently, the cdk inhibitor p27kip1 has been implicated in the regenerative response as the protein is down-regulated in motoneurons, axons and associated glia in response to peripheral nerve injury (Shi, Cheng et al. 2007, Shen, Shi et al. 2008). In these instances, the loss of p27kip1 is accredited to the upregulation of the ubiquitin ligase SKP2 and the ensuing proteasomal degradation. DRG neurons and satellite cells also have diminished levels of the protein after injury but this follows an initial reduction in FOXO3a, suggesting p27kip1 transcription is altered in these cell types (Wang, Liu et al. 2009). As FOXO3a activates the p27kip1 gene (Dijkers, Medema et al. 2000, Hu, Wang et al. 2005), the transcription factor's degradation and the decreased expression of the cdk inhibitor may play a key role in either axon growth or satellite cell proliferation. Although commencement of the cell cycle is typically linked to neuronal apoptosis, perhaps events that advance the cycle, such as the suppression of cdk inhibitors, are critical to the regeneration of postmitotic neurons (Krishnan, Duraikannu et al. 2016) .

1.4. ER stress

There is established significance of protein misfolding in the ER as it relates to human disease (Wang and Kaufman 2016). The regenerative process of the growing axon requires a multitude of newly made proteins, utilizing highly conserved protein synthesis and processing mechanics of the mammalian cell. A significant amount of cellular protein is processed through the secretory pathway where genome products are translated and transported to intracellular and extracellular locations. The endoplasmic reticulum serves as a quality control organelle of this pathway where proper protein maturation and folding takes place [Reviewed in (Pluquet, Pourtier et al. 2015)]. Synthesized polypeptides enter the lumen of the ER where they undergo

folding, posttranslational modification and oligomerization which is facilitated by many chaperones including binding immunoglobulin protein/78 k-Da glucose regulated protein (Bip/GRP78) or GRP94 (Tu and Weissman 2004). During times of altered cellular activity, such as mutations, increases in production of nascent proteins, nutrient deprivation, hypoxia, or oxidative stress; an imbalance of misfolded or unfolded proteins accumulate in the ER leading to a toxic and detrimental cellular environment. This state, known as ER stress, requires cells to adjust their capacity for protein management, triggering mechanisms to eliminate and avoid further accumulation of misfolded products. A mechanism to eliminate misfolded proteins is via ER-associated protein degradation (ERAD), where proteins are retrotranslocated to the cytosol and further degraded by the proteasome (Hetz, Chevet et al. 2013). The intracellular signalling pathway that mediates the overall process is known as the unfolded protein response (UPR). The products of this response attempt to increase the proper folding of proteins, decrease the rate of protein synthesis and reduce translocation to the ER lumen. If executed appropriately, this will stabilize protein processing, establish homeostasis and suppress the UPR; however, failure will lead to programmed cell death, thus protecting tissues from a myriad of pathological states that occur with excessive amounts of malfolded proteins.

1.4.1. Transcriptional regulator pathways of the UPR

Unfolded protein response activation is primarily mediated via three extensively studied signal pathways with unique transmembrane proteins whose ER luminal domains sense the ER environment. These transducers include inositol-requiring protein-1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6).

1.4.1.1. IRE-1

Inositol-requiring protein-1 (IRE1) was the first signal transducer discovered in yeast and found to play a significant role in the UPR (Cox, Shamu et al. 1993). This type 1 ER- resident transmembrane protein cytoplasmic region contains both a protein kinase and an RNase domain, while its ER-lumenal region binds to a molecular chaperone protein, BiP/GRP78 and directly to misfolded proteins. The IRE1 is activated by the direct binding of unfolded proteins to its ER lumenal domain in the yeast model (Gardner and Walter 2011); however, in mammalian cell lines, IRE1 activation is triggered by the dissociation of GRP78 as these chaperones are called

upon to cope with the unfolded protein load (Zhou, Liu et al. 2006). Activated IRE-1 oligomerizes and the cytoplasmic kinase domain undergoes auto-phosphorylation, enabling the RNAse domain to cleave and decay a specific number of cytoplasmic mRNA (Hollien and Weissman 2006). This leads to a reduction in overall cellular protein synthesis and attempts to decrease the protein load within the ER.

The RNAse activity of IRE-1 specifically targets and cleaves X-box binding protein 1 (XBP1) precursor mRNA. The spliced XBP1 mRNA (XBP1s) product is an activator of the UPR and its translation is increased when IRE-1 is activated (Calfon, Zeng et al. 2002). Conversely, the unspliced XBP1 mRNA (XBP1u) product silences the UPR and its translation occurs when IRE-1 is suppressed (Yoshida, Oku et al. 2006). XBP1u readily binds to XBP1s and the complex is degraded. Furthermore, XBP1u interferes with the transcription of XBP1s target UPR genes during the recovery phase of the UPR (Yoshida, Oku et al. 2006). The transcriptional regulator XBP1s is a basic leucine zipper protein (bZIP) which binds and regulates genes containing either the UPR element (UPRE) or the ER stress response element (Cox and Walter 1996, Yoshida, Haze et al. 1998). These genes include SRP54 which facilitates entry of proteins into the ER (Rapiejko and Gilmore 1997), those which regulate ER and phospholipid biosynthesis (Sriburi, Jackowski et al. 2004), and gene products which assist in degradation of misfolded proteins including ERdj4 and EDEM (Hosokawa, Wada et al. 2001, Lai, Otero et al. 2012).

1.4.1.2. PERK

Another well described pathway is that mediated by PERK, a type I ER transmembrane protein similar in structure to IRE-1, and one that contains an ER localized domain with stress sensing capability and a cytoplasmic protein kinase catalytic domain (Harding, Zhang et al. 1999). PERK activation is triggered by the release of binding immunoglobulin protein (BiP or 78 kDa glucose-regulated protein (GRP-78) or heat shock 70 kDa protein 5 (HSPA5) chaperone protein from the ER-lumen domain during increased protein malfolding; leading to PERK dimerization, autophosphorylation and signal transduction (Bertolotti, Zhang et al. 2000). Downstream, activated PERK phosphorylates eukaryotic translation initiation factor 2 (eIF2) (Harding, Zhang et al. 1999), a regulator of protein translation. Phosphorylated eIF2 inhibits protein translation initiation, decreases total cellular protein production, thus mitigating the misfolded protein load.

Even though protein production is suppressed through PERK/eIF2 pathway, a number of proteins tasked with maintaining cell homeostasis continue to be translated. These include the key bZIP transcription factor ATF4, whose mRNA translation can occur even when eIF2 is phosphorylated (Lu, Harding et al. 2004). Two target genes for ATF4 are CCAAT/enhancer-binding protein homologous protein (CHOP) (Fawcett, Martindale et al. 1999) and the growth arrest and DNA damage-inducible protein 34 (GADD34) (Ma and Hendershot 2003). CHOP is a transcriptional regulator of pro-apoptotic genes (Sano and Reed 2013), suggesting PERK signaling is protective in slowing general protein production and further activating organized cell-death when the ER stress is not overcome. GADD34 binds to type I protein phosphatase (PP1) (Connor, Weiser et al. 2001) and induces dephosphorylation of eIF2 (Novoa, Zeng et al. 2001), leading to increase protein synthesis, counteracting the initial effects of the PERK pathway when cellular processing is able to recover.

1.4.1.3. ATF6

ATF-6 is a transcription factor integral to the third branch of the known UPR regulatory pathways. This type II ER located protein contains the bZIP transcription regulator element on the cytosolic domain and an ER-lumenal domain which binds to the GRP78 chaperone (Hai, Liu et al. 1989, Haze, Yoshida et al. 1999). When ER stress is sensed, ATF6 dissociates from GRP78, revealing the Golgi localization signal and translocates to the Golgi complex (Shen, Chen et al. 2002). Further processing occurs within the Golgi where cleavage by site-1 protease (S1P) and site-2 protease (S2P) releases the cytosolic/catalytic transcriptional activator (Ye, Rawson et al. 2000).

ATF6 regulates expression of genes in the ERAD pathway (Lee, Tirasophon et al. 2002) and other target genes containing the ERSE (Yoshida, Okada et al. 2000). These UPR associated genes include GRP78, Derlin-3 (an ER-associated degradation system-associated protein) (Adachi, Yamamoto et al. 2008), CHOP and XBP1 (Yoshida, Okada et al. 2000). ATF6 has also been shown to regulate ER component synthesis and phospholipid production (Bommiasamy, Back et al. 2009) (Maiuolo, Bulotta et al. 2011).

Other ER bound transcription factors appear to have structural similarities and the same processing as ATF6; furthermore, they also appear to be involved in the UPR in some capacity (Asada, Kanemoto et al. 2011). These include OASIS/CREB3L1 (Honma, Kanazawa et al.

1999), CREB3L2 (Storlazzi, Mertens et al. 2003), CREB3L4 (Nagamori, Yabuta et al. 2005), CREBH (Zhang, Shen et al. 2006) and Luman/CREB3 (Lu, Yang et al. 1997).

1.4.1.4. Luman/CREB3

A more recently discovered ER-stress transducer is Luman (CREB3/ Human LZIP), a member of the CREB/ATF family, homologous to the herpes simplex virus (HSV) protein VP16 (Lu, Yang et al. 1998) and identified as the first cellular ligand for host cell factor-1 (HCF-1) (Lu, Yang et al. 1997). Its association with HSV machinery suggests an involvement in viral latency and reactivation (Freiman and Herr 1997, Lu, Yang et al. 1997, Lu, Yang et al. 1998, Lu and Misra 2000). Protein processing resembles that of ATF6, where Luman bound to the ER contains an S1P and partial S2P cleavage sequence (Raggo, Rapin et al. 2002). The ER-domain undergoes regulated intracellular proteolysis (RIP) and the bZIP domain is then free to activate target genes (Raggo, Rapin et al. 2002). Despite the fact that the cleaved form of the transcription factor is extremely unstable and *in vitro* detection of the activated protein requires proteasomal inhibitors, studies have shown the protein to be an activator of transcription (Misra, Rapin et al. 2005). The bZIP proteins Zhangfei (Misra, Rapin et al. 2005) and Luman recruitment factor (LRF) (Audas, Li et al. 2008) potently and specifically inhibit Luman transactivation by possibly affecting protein stability. The Luman transcription activation domain has 3 motifs; two LxxLL areas that are highly conserved on many transcription factors and mediate protein-protein interactions, while the third binds HCF (Luciano and Wilson 2000, Luciano and Wilson 2002) .

Once translocated to the nucleus, Luman can bind cAMP response elements (CRE), including CRE promoter regions of HSV immediate early genes (Lu and Misra 2000), and the UPRE in a similar capacity to XBP1 (DenBoer, Hardy-Smith et al. 2005). As mentioned above, this transcription factor is inhibited by Luman recruitment factor (Audas, Li et al. 2008) and Jun activation domain-binding protein 1, which promote its degradation (DenBoer, Iyer et al. 2013). Artificial overexpression of Luman is cytoprotective against ER-stress, reducing the amount of ER-induced apoptosis (Liang, Audas et al. 2006). Induction of Luman expression and proteolytic cleavage in response to ER stress results in the transcriptional activation of the ERAD-related Herp (homocysteine-induced ER protein) (Kim, Kim et al. 2008) protein via the ER stress response element II (ERSR-II) (Liang, Audas et al. 2006). It also activates the promoter region

for the ER-degradation enhancing mannosidase (Kops, Dansen et al.), another participant in the ERAD pathway (DenBoer, Hardy-Smith et al. 2005).

Even though the bulk of the evidence points to Luman as a potent regulator of the UPR and protective action against ER-stress through its target genes, it has been connected to other cellular processes in a variety of cell types. Induction of monocytes migration is facilitated through Luman-mediated increased expression of C-chemokine receptor 1 and 2 (Sung, Kim et al. 2008), enhancing activity of NF-κB (Jang, Kim et al. 2007, Jang, Kim et al. 2007). Furthermore, Luman has been linked to dendritic cell (DC) maturation through its interaction with a DC-specific transmembrane protein (Eleveld-Trancikova, Sanecka et al. 2010) and interplay between Luman and ADP ribosylation factor 4 is required for the Golgi stress response (Reiling, Olive et al. 2013). While these findings open the possibility of Luman as a potential target to many pathologies and cell types outside of the nervous system, its importance to the UPR/ER stress response and impact on nerve regeneration has been our focus.

1.4.1.5. ER stress and peripheral nerve damage

Proper protein folding, chaperone function and mitigation of ER-stress are critical cellular processes that have been implicated in a variety of neurodegenerative and traumatic nervous system diseases (Garcia-Huerta, Bargsted et al. 2016, Valenzuela, Onate et al. 2016). Dysregulation of ER-stress and protein misfolding will eventually lead to activation of cell death pathways, irreparable neural tissue damage and poor recovery (Ogen-Shtern, Ben David et al. 2016, Valenzuela, Martinez et al. 2016). Injury to peripheral nerves by either traumatic, toxic or metabolic causes leads to a sequelae of events in the neuron cell body, distal and proximal nerve stumps and associated glia requiring increased protein loading and alterations to the ER network. Earlier studies show a correlation between PNS disease states and elevated levels of ER stress markers, as damage to sciatic nerves increased expression of BiP/GRP78 chaperone protein in the soma of non-degenerating neurons and the transcription factors XBP1s and ATF4 in degenerating motoneurons (Saxena, Cabuy et al. 2009, Penas, Font-Nieves et al. 2011). There are also reports of peripheral nerve damage upregulating other chaperone proteins and ER foldases, which assist in protein folding via forming non-covalent bonds and include calreticulin, endoplasmic reticulum protein-29 (ERp29) and ERp57 (also known as GRp58 or PDIA3) (Noel, Frost et al. 1995, Willis, Li et al. 2005, Castillo, Onate et al. 2015). Our lab described how

injury leads to upregulation of the UPR in the DRG cell body and axonal localized UPR elements including BiP/GRP78 and CHOP, which are retrogradely transported to the cell body of injured DRG neurons (Ying, Zhai et al. 2015). However, the need for a coordinated and controlled UPR/ER stress response for axon regeneration had not been proven. Onate et al, with a sciatic nerve injury model found that ablation of the ER stress regulator XBP1, but not ATF4, delayed motor recovery, decreased macrophage recruitment, reduced myelin removal and axonal regeneration (Onate, Catenaccio et al. 2016). Conversely, transgenic mice overexpressing XBP1 have enhanced regenerative events after nerve crush injuries. They assessed the therapeutic advantage of altering the UPR *in vivo* by local gene transfer experiments with XBP1s into DRG neurons which increased axon regeneration post-injury (Onate, Catenaccio et al. 2016). The response of associated glia is key to regeneration and they appear to initiate a UPR as there is activation of ER stress markers in dedifferentiated Schwann cells following nerve damage and in perineuronal presumably satellite glial cells in the DRG (Mantuano, Henry et al. 2011). These glial cells and the distal nerve segment showed elevated levels of the protein chaperone BiP/GRP78 and XBP1s after a crush injury; however, there appeared to be no upregulation of the PERK pathway with no induction of the proapoptotic ATF4 or its targets CHOP or GADD43 (Onate, Catenaccio et al. 2016). These correlated to injured neuronal cell body findings as CHOP was not elevated in DRG neurons or motoneurons of the ventral horn as far as 8-days post injury. These findings pointed to a more predominating role of the IREα/XBP1 pathway in the ER stress reaction of injured neuronal and Schwann cells in nerve degeneration and regeneration.

As stated above, the therapeutic potential of either inhibiting or enhancing components of the UPR has been explored. Besides the pro-regeneration effect of XBP1s adenoviral vectors in DRG neurons, ERp57 overexpression in transgenic mice showed similar results with increases in myelin clearance, macrophage infiltration and axonal regeneration (Castillo, Onate et al. 2015). Furthermore, the adenoviral vector mediated overexpression of BiP/GRP78 appears to increase motoneuron survival in spinal root avulsion models (Penas, Font-Nieves et al. 2011). However, while PERK/ATF4 pathway is a known regulator of the UPR, ATF4 knockouts had no difference in axonal outgrowth following injury compared to wildtypes (Onate, Catenaccio et al. 2016). These results show that some UPR elements maybe essential to the events of regeneration and others not, and their manipulation is a potential target to improve functional recovery. Another pathological state where ER stress is likely to cause deleterious effects is diabetic

peripheral neuropathy, where progressive degeneration of peripheral axons leads to decreased tactile sensation and altered pain states (Wu, Li et al. 2013, O'Brien, Hinder et al. 2014). In streptozotocin diabetic rat models, elevated CHOP expression is found in nerve tissue, with the levels correlating with severity of disease (Wu, Li et al. 2013). These and pre-diabetic models also exhibit higher levels of ER stress markers including Phosphorylated PERK, eIF2a, IRE1a and BiP/GRP78 in sciatic nerve (Lupachyk, Watcho et al. 2013, Lupachyk, Watcho et al. 2013). These studies looking at injury and other disease states demonstrate the wide implication of ER stress and its likely involvement in all sensory neuron pathologies.

1.4.1.6. Luman: A regulator of the UPR and regeneration

As stated above, Luman is a known transcriptional regulator of the UPR, binding promoter regions containing CRE and UPRE (Lu, Yang et al. 1997, Lu and Misra 2000) and is expressed by a variety of rat tissue types (Ying, Zhang et al. 2015). The highest levels seen in nervous tissue and DRG in particular. The participation of Luman in the ER stress response led to the hypothesis that it and UPR components are up-regulated in axotomized DRG neuron. Previous work has shown that injured neurons necessitate a coordinated UPR to enhanced axonal regeneration (Onate, Catenaccio et al. 2016). Studies from our lab demonstrated increased expression of ER stress markers including CHOP and BiP/GRP78 in both DRG soma, the axon, and non-neuronal components, where axonal elements were retrogradely transported back to the cell body (Ying, Zhai et al. 2015). Further seminal work by Ying et al identified Luman as an axonally localized transcription factor that co-localized and interacted with the transport molecule Importin (Ying, Misra et al. 2014). Axon-derived Luman is rapidly synthesized from axonally confined transcripts in response to axotomy and serves as an injury signal where the transcriptional activation domain is retrogradely transported back to the DRG soma nuclei by an importin-dynein mediated mechanism. Remarkably, reduction of Luman using small interfering RNAs (siRNA) impaired neurite outgrowth of injured sensory neurons. This implies that Luman can modulate the capacity for sensory neurons to regenerate by either its action on UPR or other growth-related targets. Such targets include components of the cholesterol biosynthesis pathway and the UPR as Luman knockdown reduces both free and total cholesterol levels in injured sensory neurons and downregulates a number of genes involved in the regulation of cholesterol synthesis and the UPR (Ying, Zhai et al. 2015). Cholesterol is an essential component of plasma

membrane, that is required in abundance for the growth of regenerating axons. The potential importance of Luman in the regulation of the regeneration response via cholesterol biosynthesis or induction of the UPR was supported by enhanced neurite outgrowth in Luman knockdown neurons with cholesterol supplementation or addition of tunicamycin which induces the UPR in the absence of a notable impact on Luman expression (Ying, Zhai et al. 2015). The identification of Luman as an axon-localized sensor of injury was a principal finding, where loss of axon integrity leads to an elegant signaling loop, where injury-induced Luman is transported back to the soma where it regulates regeneration-associated events. However, all these studies were done employing one or 2-day injury time points, with a major focus on the importance of the axon-derived Luman signal. What is not known, is whether an intrinsic upregulation of Luman occurs at the level of soma as part of the very early inductive and more protracted maintenance phases of the cell body response to injury associated with regenerative axon growth.

1.4.1.7. Zhangfei/CREBZF

As stated, Luman was initially recognized as the first host ligand for host cell factor-1 (HCF-1) (Lu, Yang et al. 1997) and was associated with reactivation of latent herpes virus. Only later was its importance with regards to the UPR identified (DenBoer, Hardy-Smith et al. 2005) . Further elucidation of the function and inherent role of this neuronal transcription factor lead to the discovery of Zhangfei, a potent and efficient inhibitor of Luman activity (Lu and Misra 2000, Misra, Rapin et al. 2005). First experiments revealed that Zhangfei (ZF aka CREBZF/SMILE), another basic leucine zipper transcription factor, when co-expressed with Luman in Vero (kidney epithelial cells) and Hep2 cells could potently and effectively inhibit the host cell factor (HCF)-dependent transactivation function by the Gal4-Luman fusion protein of a UPR element containing promoter in a dose dependent manner (Misra et al., 2005). Zhangfei, like Luman was discovered due to its interaction with the Herpes Simplex Virus (HSV) -1 related HCF-1, binding to HCF in a similar manner to HSV viral protein – 16 (VP16) (Lu, Yang et al. 1997, Lu and Misra 2000). ZF is expressed in sensory neurons and its overexpression *in vitro* competitively inhibits the VP16 associated transactivation complexes on HSV-1 immediate-early genes and disrupts viral replication (Akhova, Bainbridge et al. 2005). The fact that Luman facilitates the reactivation of latent HSV and ZF acts as a suppressant, further argues that their roles in native cells are likely in opposition to each other.

More recent work has revealed ZF as a potential regulator of the UPR, where its ectopic expression in osteosarcoma cell lines lead to decreased levels of Xbp1, HERP, CHOP and Bip, thus suppressing the UPR (Bergeron, Zhang et al. 2013). ZF appears to specifically suppress the action of the Xbp1 by a leucine zipper (LZIP) mediated interaction, where XPB1 transcriptional activity is inhibited and the protein is rapidly degraded (Zhang, Rapin et al. 2013). Even though ZF is a LZIP regulator of transcription, its basic LZIP domain lacks a specific amino acid residue that is critical to protein-gene promoter interaction; therefore, a ZF homodimer is unable to bind response elements known to recognize other LZIP proteins (i.e. Luman) (Lu and Misra 2000). The transactivation function of ZF is accomplished through its interaction with intermediary proteins including the tumor suppressor protein p53 (Lopez-Mateo, Villaronga et al. 2012) and ATF4 (Hogan, Cockram et al. 2006), where ZF binding or heterodimerization promotes their transcriptional activity and enhances binding to their target promoters, including cAMP response elements (Hogan, Cockram et al. 2006). Contrary to this, ZF has suppressive properties as it inhibits the action of many transcription factors including Luman (Misra, Rapin et al. 2005), nuclear estrogen receptors (Xie, Lee et al. 2008), CREBH (Misra, Chanda et al. 2011), the UPR regulator XBP1 (Zhang, Rapin et al. 2013) and members of the SMAD family (Lee, Lee et al. 2012). The specific inhibitory mechanism appears to combine decreasing the transcription factors affinity to known promoter regions and increasing its proteasomal degradation.

ZF interaction with p53 is of particular interest, as ZF overexpression in both human and canine osteosarcoma cells inhibits growth and triggers apoptosis through p53 (Zhang and Misra 2014, Zhang, Thamm et al. 2015). ZF co-localizes and stabilizes nuclear p53 by displacing a ubiqutin ligase, thus protecting from proteasomal degradation (Zhang and Misra 2014). Suppression of the UPR by ZF is also dependent on p53, as siRNA disruption of p53 prevents the ZF-mediated UPR response and ectopic expression of both the proteins shows synergistic UPR upregulation (Zhang and Misra 2014). The action of ZF exerted through the tumor suppressor protein p53 has multiple consequences not only for tumor cell biology, but also nerve regeneration, as p53 is also clearly implicated in this process (reviewed in (Krishnan, Duraikannu et al. 2016).

Other stress pathways are also associated with ZF, including the amino acid response (AAR), a cellular mechanism that protects cells from amino acid deprivation which is associated with multiple pathologies including malnutrition, sepsis and trauma (Kilberg, Pan et al. 2005). ATF4

is known regulator of AAR, whose activity is enhanced by ZF, with downstream targets of ATF4, such as CHOP, being induced by amino acid deprivation (Averous, Bruhat et al. 2004, Hogan, Cockram et al. 2006). Zhang et al discovered that amino acid deprivation upregulated ZF in Madin-Darby canine kidney epithelial cells (MDCK) cells though an AAR Element -like gene in the ZF promoter region, which is also found in the CHOP promoter (Zhang, Jin et al. 2010). The loss of this AARE promoter region in the ZF gene resulted in complete loss of ZF AAR activation. These results point to ZF as a stress sensor and a possible regulator of AAR pathways and suggests that pathological states may alter ZF activity at the gene level.

Interestingly, ZF has also been implicated in nerve growth factor (NGF) signaling through its regulation of expression of the NGF receptor TrkA signaling (Valderrama, Rapin et al. 2008) . The role of ZF in TrkA regulation is complex. During development, TrkA triggers pathways for differentiation and in an undifferentiated cell ZF can activate the expression of the receptor, thus facilitating NGF mediated maturation or apoptosis (Valderrama, Rapin et al. 2009). But ZF also has a suppressive action on TrkA, as its interaction with Brn3a (Valderrama and Misra 2008) inhibits transcription of the receptor in non-neuronal cells (Valderrama, Rapin et al. 2008). As stated, DRG neurons are highly regulated by neurotrophins though trk signaling and peripheral nerve injury alters TrkA expression in adult sensory neurons (Verge, Merlio et al. 1992). With the knowledge that ZF regulates TrkA expression, it's plausible that the injury associated expression changes of trk receptors may be facilitated via a ZF related process.

ZF appears to suppress the UPR and likely is a counter to Luman activation, by inhibiting Luman and perhaps turning off the UPR through this route. With the importance of the UPR in nerve injury noted and with ZF's known involvement with the regeneration related tumor suppressor p53 and TrkA, the expression of ZF is presumably important in injured neurons. However, the expression pattern of ZF in sensory neurons has not been characterized and its response to injury is not known.

1.5. Hypothesis and specific aims

Peripheral nerve injury induces a complex series of cellular events in sensory neurons, many of which are linked to the stress induced by axotomy. Successful regeneration of these sensory neurons can be bolstered by an understanding of how sensory neurons are sensing and responding to stress at the various stages of the cell body response and whether this aids the neuron to survive axotomy and mount a strong regeneration response. One way to gain insight is to examine how expression of known stress-associated transcription factors are altered by axotomy. Thus, I chose to examine one transcription factor linked to neuronal survival and stabilizing the cell during times of homeostatic compromise, FOXO3A; and two transcription factors which we and others have shown to be involved in regulation of one of the earliest responses to cellular stress, the unfolded protein response, Luman/CREB3 and Zhangfei/CREBZF, the former of which has also been shown to be an important axon-derived retrograde signal linked to the intrinsic ability of an injured neuron to intrinsically regenerate an axon; while the latter has been shown to be a repressor of Luman transcriptional activity. More specifically, I set out to test the hypothesis "Sensory neurons respond to peripheral axotomy by mounting a cell body response involving a coordinated regulation of stress-associated transcription factors linked to distinct phases of regeneration". My doctoral research was designed to test this hypothesis by addressing the following aims:

(i) to gain insight into the temporal expression patterns of transcriptional regulators associated with cellular stress responses of axotomized sensory neurons;

(ii) to ascertain whether the responses of these transcription factors are coordinately regulated; and finally

(iii) to gain insight into whether BDNF, a known regulator of the inductive signal of regeneration responses of sensory neurons is involved in this regulation.

2. Materials and Methods

2.1. Nerve injury animal model

Male Wistar Rats (Charles River Laboratories, Willmington, MA) weighing between 200-300g housed at room temperature on a 12-h light-dark cycle with access to food and water were used in all experiments. Animal procedures were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Saskatchewan Animal Research Ethics Board.

Animals were given buprenorphine (Temgesic; 0.05- 0.1 mg/kg) analgesic subcutaneously pre- and postoperatively. For peripheral nerve axotomy, animals were deeply anesthetized with inhalational isoflurane (2% delivered at a rate of 2L/min). A dorsal incision was made exposing the lumbar and sacral spinal column followed by careful dissection of surrounding bone and muscle to reveal the Lumbar segment spinal nerves. The right sciatic nerve was transected at its origins from the lumbar 4-6 (L4-6) spinal nerves and a small 5mm segment resected to prevent regeneration. This anatomical injury site was selected as it ensures nearly 100% injury of the L4-6 DRG neurons. Axotomized animals were sutured closed in layers and placed in individual cages until tissue harvesting.

The injury time course was conducted by sacrificing the animals after the pre-determined post-injury time points of 1 hour, 1 day, 2 days, 4 days and 1 week. Naïve animals underwent anesthetic procedures but had no surgical intervention. Sham animals were surgically exposed and tissues were dissected out as with the experimental models with the exception that no spinal nerve was handled or injured. The sham time course was conducted as described and the animals were sacrificed after the post-surgical time points of 1 hour, 1 day, 2 days, 4 days and 1 week. Each time point/time course had an N=3 minimum. A total of 7 time courses were generated during the course of this thesis and included naïve animals for direct comparison and to rule out confounders such as anesthetic technique and animal batch variability. Three time courses were analyzed for quantitative data and the others showed qualitative reproducibility that matched the quantitative results.

Prior to tissue harvesting, animals were anesthetized with a Euthanyl Forte overdose (Bimeda-MTC, Cambridge, ON). The internal thorax was exposed each subject was perfused via the left ventricle with 100 ml of warm phosphate buffered saline then 500 ml of ice cold 4% paraformaldehyde with 0.2% picric acid for tissue fixation. The L4-L6 ipsilateral and

contralateral DRGs, corresponding spinal cord segments and nerve tissue was dissected out promptly and stored in the same fixation solution until further processing.

2.2. Nerve crush and electrical stimulation model

A nerve crush injury was performed in conjunction with the electrical stimulation experiments. Prior to brief stimulation, the exposed mid-thigh sciatic sustained a crush injury by applying closed fine 5.0 forceps (at the same scored level on the forceps) for 10 seconds. In some animals one hour of brief electrical stimulation was applied proximal to the crush site (Fig. 2.1) immediately following the crush injury. Electrical stimulation experiments were done similar to published protocols (McLean, Popescu et al. 2014). The distal sciatic nerve of male Wistar rats were exposed at the mid-thigh level. Two insulated stainless steel wires (Cooner A5632) free of insulation at the ends for 2-3mm and twisted to from a loop were used; the cathode end was looped around the sciatic nerve proximal to the crush injury site and sutured in place. The anode was inserted between skin and muscle distal from the exposed nerve. The wires were connected to an external Grass (Quincy, MA) SD-9 stimulator. Brief electrical stimulation was delivered at a continuous 20 Hz train of supramaximal pulses (100 msec; 3V) for one hour. Epineural 10-0 suture marked the stimulation site. These stimulation parameters employed in this study were selected as they closely mimic neuronal firing patterns.

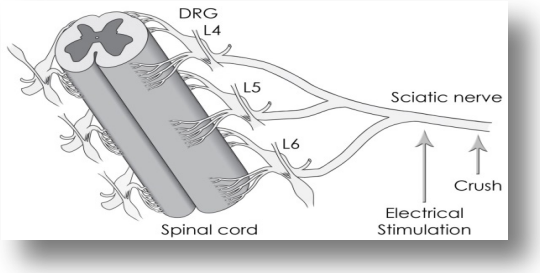

Figure 2.1 Nerve injury model and stimulation. For electrical nerve stimulation experiments, nerves were first crushed followed by immediate electrical stimulation at 20 Hz proximal to the crush site for one hour continuous.

2.3. siRNA infusion treatments

In order to suppress endogenous BDNF levels in vivo, an siRNA infusion specific for BDNF was used in a similar manner to previous published methods (Baker-Herman et al., 2004;

Geremia, Pettersson et al. 2010). Small interfering RNAs (siRNA) directed against rat BDNF mRNA (accession number – M012513), a control non-targeting scrambled sequence (siCONTROL Non-targeting siRNA Pool #D-001206-13-20; 5′-AUGUAUUGGCC UGUAUUAG-3′; 5′UAGC- GACUAAACACACAUCAA-3′) or a fluorescently labeled control scrambled siRNA (siGLO – RISC-free siRNA #D-001206-13-20; proprietary sequences) were obtained from Dharmacon, Inc. BDNF siRNA consisted of four pooled 21-nucleotide duplexes with symmetrical 3′ over-hangs and a 5 phosphate on the antisense strand (SMARTpool – #M-080046-00):1) 5′-PUCAUCCAGCAGCUCUUCGAUU, 2) 5′- PUUAAUGGUCAGUG UACAUAUU 3) 5′-PAAUACUGUCACACGCUCUU and 4) 5′-PACAUACGAUUG GGUAGUUCUU. Scrambled siRNA and BDNF siRNA and siRNA GLO were suspended in siRNA Universal Buffer (Dharmacon) to yield a concentration of 100 μM. siRNA stocks were aliquoted and stored at − 80 °C.

On the day of injection, 15 μl of siRNA (100 μM stock) was combined with 2 μl of Oligofectamine (Invitrogen) and incubated for 15 min at room temperature. A laminectomy was performed and a sterile indwelling catheter was inserted into the subarachnoid space at the lumbar/sacral junction so the end of the catheter lay at the level of the L5 DRG. 17 μl (~20 μg) of siRNA/Oligofectamine solution is injected over 5 min followed by 3 μl of sterile PBS into the indwelling catheter to push the remaining siRNA through. Three days later a second injection of BDNF siRNA (n=3 at 3 days) or control non-targeting siRNA (n = 3 at 3 days) was administered via the catheter and immediately followed by unilateral transection of the L4,5,6 spinal nerves. An additional 4 control animals received unilateral transection of the L4,5,6 spinal nerves alone. Animals were perfused 3 days later and L4,5,6 DRG removed and processed for immunohistochemistry to detect changes in BDNF and GAP-43 expression and BDNF mRNA levels or in situ hybridization to assess the effectiveness of BDNF silencing and impact on RAG protein and mRNA expression [shown previously by (Geremia, Pettersson et al. 2010)]. Prior to conducting the BDNF siRNA experiments, fluorescently labeled control scrambled siRNA–siGLO was delivered intrathecally in the same manner and concentration to first ensure that the siRNA was effectively reaching the neuronal cell bodies in the L5 DRG.

2.4. Tissue processing

Animals were deeply anesthetized and perfused via the left ventricle with cold phosphate buffered saline (PBS, 0.1M, pH 7.4) followed by 4% paraformaldehyde (Apfel, Wright et al.) and 0.2% picric acid in phosphate buffer (PB, 0.1 M, pH 7.4). Dissected tissues (L4, 5,6 DRGs, sciatic nerve, C4 ganglia) were postfixed (1–1.5 hours) and cryoprotected in 20% sucrose. Control and experimental tissues were embedded in the same cryomolds to ensure processing under identical conditions, covered in OCT and frozen in cooled isopentane prior to storing at -80°C until processing.

2.5. Western blot

Proteins were extracted from tissue and cell samples with RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). These samples included DRG from naïve and spinal nerve injured animals as stated and other tissue samples to assess the specificity of the antibodies employed in the studies. For FOXO3a, samples from a BrCA cell line and rat liver were used as positive controls with known expression of FOXO3a. For Luman and ZF, antibody specificity controls Vero cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), with 10% newborn calf serum, 100 unit/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified incubator with 5 % CO2. The day prior to transfection, Vero cells were seeded into six-well plates at a density of $5X10^5$cells/collagen-coated well. Cells were transfected with one microgram of pcDNA3.1, pcLuman or pcZhangfei, using Lipofectamine (Canadian Life Technologies) in six-well plates as per the manufacturer's instructions.

For DRG samples, 20 μg of protein were electrophoresed on a 12% SDS polyacrylamide gel, along with a protein molecular size marker (Licor, #928-40000) and then transferred onto a polyvinylidene fluoride membrane (Bio-rad) by semi-dry electroblotting for 15 minutes in cold transfer buffer (25mM Tris, 192 mM Glycine, 20% methanol) at 15V using a Bio-Rad Trans-Blot apparatus. Membranes were blocked with blocking buffer (LI-COR Biosciences) at room temperature for 1 h. Membranes were then incubated with primary antibodies (Foxo3A 1:2,000 (Cell Signaling #9467) in 5% BSA; Luman 1:4,000; Zhangfei 1:4,000) in LI-COR Odyssey blocking buffer with 0.1% Tween 20 overnight at 4 °C, followed by incubation with Goat Anti-Rabbit LI-COR IRDye 680 (1:10,000) and Goat anti-Mouse LI-COR IRDye 680 (1:10,000)

secondary antibodies for 1 h at room temperature. Proteins were visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences). Mouse anti-GAPDH (1:10,000) was used to detect GAPDH loading control. Membranes were then rinsed in distilled water and scanned on the Licor Odyssey 9120 infrared scanning system. For quantification, the density of each band on the immunoblot was estimated by densitometry and normalized to the density of the loading control band in the sample. Samples contained an N=3 animals per set condition and each sample was analyzed in triplicate.

2.6. Immunohistochemistry

Injured neurons in DRG sections were identified using an antibody against activating transcription factor 3 (ATF-3), a specific marker induced in injured DRG neurons (figure 2.2) (Tsujino, Kondo et al. 2000). The tissues were sectioned serially at 6 μm (DRG) or 10 μm (nerves) in a cryostat and thaw mounted onto cooled ProbeOn Plus slides (Fisher Scientific). Immunohistochemistry slides were air-dried, washed in phosphate buffered saline (PBS, 3 x 10 minutes) and blocked with 10% donkey serum in 0.25% Triton-X in PBS for 1 hour at room temperature. After blocking, tissues were incubated with primary antibody, either rabbit anti-Luman (diluted 1:400 in 2% donkey serum+0.25% Triton X-100, Misra Lab), rabbit anti-ZF (1:400, Misra Lab), rabbit anti-FOXO3a (1:200, Cell Signaling, Cat# - 24975) or rabbit anti-ATF-3 (1:2,000 Santa Cruz Biotech Inc, sc-188) overnight in air sealed, humidified containers at 4°C. All slides were also incubated with goat anti-LaminB (1:50, Santa Cruz) and were DAPI stained with mounting medium (Prolong Gold with DAPI; Life technologies) to ensure accurate nuclear localization. For nerve sections, mouse anti-βIII tubulin (1:100, Millipore) was applied as a marker of axons. The following day, slides were washed in PBS (3 x 10 minutes) and incubated with secondary antibodies, donkey anti- rabbit Cy3 (1:600, Jackson ImmunoResearch) donkey anti-rabbit Alexa Fluor 350 (1:500, Life Technologies), donkey anti-goat Alexa Fluor 680 (1:10000, Life Technologies) and donkey anti-mouse Alexa Fluor 488 (1:100, Jackson ImmunoResearch), for 1 hour in the dark at room temperature. Finally, slides were washed in PBS (3 x 10 minutes) and coverslipped with Prolong Gold with DAPI (Life technologies). To establish the specificity of the immunostaining, additional slides were incubated with the omission of primary antibody and processed as above. Specificity of the Luman and ZF

antibodies were assessed with antibody aliquots pre-absorbed with cell protein isolates from Vero cells transfected with either Luman or Zhangfei.

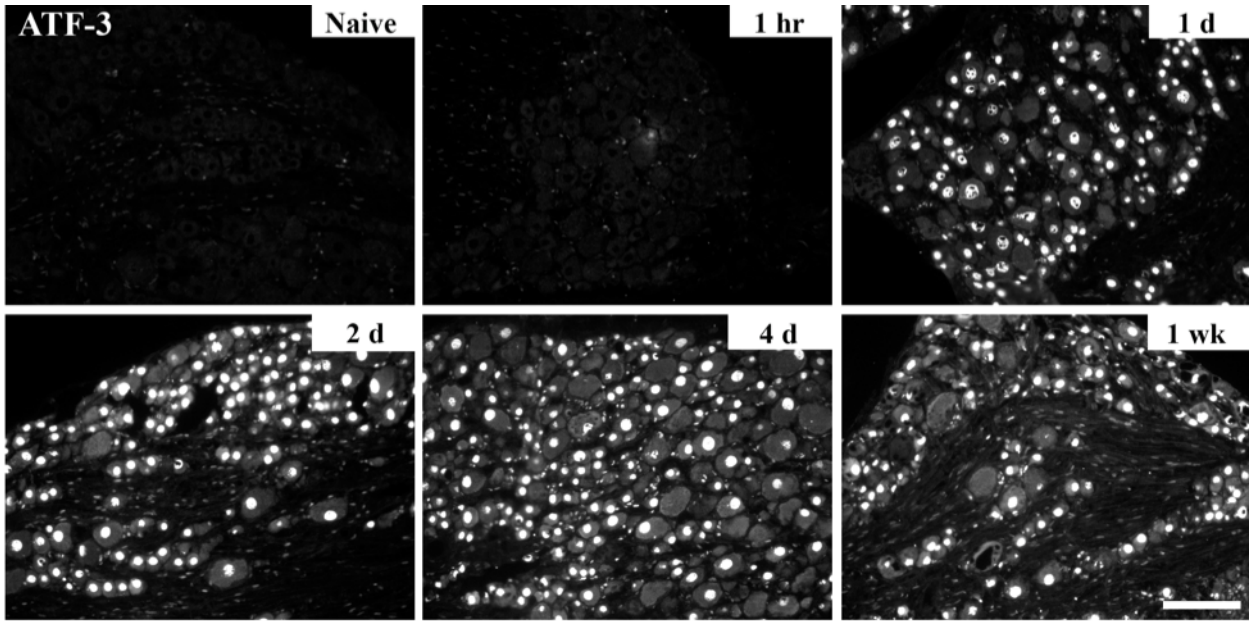

Figure 2.2. Effective injury of DRG neurons confirmed with ATF-3 staining. Immunofluorescence photomicrographs of L5 DRG sections (6 μm) processed to detect ATF3 protein. Ipsilateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day and 1week. Scale bar = 100 μm. All injured animals used in these studies had positive ATF-3 staining detectable by the 1-day post-injury time point.

2.7. *In situ* hybridization

Oligodeoxyribonucleotide (OligoDNA) 48mer probes were synthesized complementary to and selective for mRNA for FOXO3a (5'CCAACAACGTTCTGTGTGGAGATGAGGGAGTC AAAGTTAAAATCCAAC-3', University of Calgary DNA services, Alberta, Canada) ZF (5'-CGCCGAGCAGAACTCCACCGACACGTTATCCTTGTCCACATGGAGACA-3', University of Calgary DNA services, Alberta, Canada) and Luman (5'-TATCTCAATCACCATGGCTTGA AGCTTCCTCAGTTGATCTAGAAGGGA-3', University of Calgary DNA services, Alberta, Canada). All cDNA regions used were checked against the Genbank database (NIH, at the Internet site www.ncbi.nlm. nih.gov); no greater than 60% homology were found to sequences other than the selected transcript. Labeling of probe with ^{35}S–dATP (Perkin Elmer, USA) and terminal transferase enzyme (Amersham, Canada) was performed in a terminal transferase

buffer, containing sodium cacaodylate 500 mM, CoCl2 (pH 7.2) 10 mM, mercaptoethanol 1 mM, for 1.5–2 hours at 37uC. The reaction was stalled by adding 500 μl, 0.1 M Tris HCl (pH 8.0), after which probe was purified through a NENSORB-20 column (New England Nuclear, USA), and dithiothreitol added to a final concentration of 10 mM. The radioactivity was measured and the labeled probe was stored at 4°C.

All steps prior to hybridization were performed under RNase free conditions, and all dilutions were performed in autoclaved double distilled water. The slides were air-dried and postfixed in 4% PF (20 minutes), washed in PBS (3x5 minutes), treated with proteinase K at 37°C (20 μg/ml; 7–8 minutes), rinsed in PBS (5 minutes), fixed in 4% PF (5 minutes), rinsed in PBS (2x 5 minutes), rinsed in diethyl pyrocarbonate (0.1%) -H2O (5 minutes), and dehydrated in increasing ethanol concentrations (70%, 90%, 100%; approximately 1 minute in each). Sections were hybridized with radiolabeled probe at a concentration of 10^7 cpm/ ml in a hybridization solution consisting of 50% formamide, 4x saline sodium citrate (SSC; 1xSSC = 0.15 M NaCl, 0.015 M sodium citrate), 1xDenhardts solution (0.02% bovine serum albumin (BSA), 0.02% Ficoll and 0.02% polyvinylpyrrolidone), 10% dextran sulphate, 0.5 mg/ml salmon sperm DNA, 1% sarcosyl and 0.2 M DTT. Hybridization, with approximately 100 μl hybridization solution/slide, was conducted over night at 43°C in air sealed, humidified chambers to prevent evaporation. Following hybridization, the slides were washed in 1xSSC (4x15 minutes, 55°C, and an additional 30 minutes, room temperature), dipped twice in distilled water, dehydrated in ascending ethanols and air dried. Slides were dipped in Kodak NTB2 photoemulsion (diluted 1:1 in distilled water), to generate autoradiograms. After 4–8 weeks exposure the slides were developed in Kodak D19 (3–5 minutes), rinsed in water, fixed in Kodak rapid fix (5 minutes) and rinsed in water (20 minutes). For darkfield viewing and photography slides were left unstained, whereas slides for brightfield examination were counterstained with 0.5 % toluidine blue (in an acetate buffer; pH 4–4.5), and mounted with Permount (Fisher, Canada). The specificity of hybridization signal for the individual probes was determined by hybridization of adjacent 6 micron sections with labeled probe with the addition of either 1000-fold excess corresponding unlabeled probe which abolished the signal, or 1000-fold excess of a dissimilar unlabeled probe of the same length and similar G-C content which left the signal unchanged from that observed with labelled probe alone.

2.8. Quantification and analysis

Fluorescence photographs of prepared tissue samples were taken using Zeiss Axio Imager M.1 fluorescence microscope. Digital images were taken under identical exposure conditions using Northern Eclipse v7.0 software (EMPIX Imaging Inc) for all experimental groups in the same imaging session. Intensity of the immunofluorescence signal (average grey/micron^2) for each neuron and neuronal diameter was measured using Northern Eclipse v7.0 software (EMPIX Imaging Inc.). Scatter plots and line graphs were made with Graphpad Prism v5.0 and statistical significance between time points and conditions were assessed using the Kruskal-Wallis one-way ANOVA with Dunn's post-test analysis or the Mann-Whitney t-test, with statistical significance at p values <0.05.

3. FOXO3a as a BDNF-regulated systemic sensor of nerve injury in dorsal root ganglion neurons

3.1. Abstract

The regulation of neuronal transcription factors is critically important during pathological events, as their activation or repression may benefit or hinder a successful recovery. Forkhead class box O3a (FOXO3a)'s functional reaction to stress events has been characterized in a variety of neuronal cell types, where induction of this transcription factor leads to the activation of apoptotic genes and cell death. Recent studies however suggest a more involved role for FOXO3a in the cellular stress and injury response of sensory neurons. Thus, this study examined the temporal impact of chronic nerve transection on FOXO3a expression and nuclear localization in dorsal root ganglion (DRG) neurons. L4-L6 spinal nerves of male Wistar rats were unilaterally axotomized and corresponding ipsilateral and contralateral DRGs from 1-hour, 1-day, 2-day, 4-day and 1-week injured male Wistar rats were processed for immunofluorescence analysis, with naïve animals serving as controls. Immunohistochemical protein analysis revealed that in naive neurons, cytoplasmic and nuclear FOXO3a levels were higher in small to medium sized neurons representing the presumably nociceptive subpopulation, as compared to large size neurons. At just 1-hour post-injury significantly increased FOXO3a nuclear localization was observed in small size injured neurons. This trend however rapidly reversed, with significant declines in ipsilateral FOXO3a protein levels and nuclear localization occurring by 1-day post injury and at subsequent time points examined relative to 1-hour post-injury. By 1-week, while sensory neurons ipsilateral to injury now displayed slightly elevated cytoplasmic levels compared to earlier injury time points, the levels still did not approach the 1-hour injury levels. Interestingly, FOXO3a levels and sub-cellular localization in DRG neurons directly contralateral to injury were dramatically altered, as nuclear levels showed a biphasic response; peaking at both the 1-day and 1-week post-injury, with a dramatic decline at 4 days. Furthermore, L4-L6 spinal nerve transection also altered FOXO3a protein levels and nuclear localization in uninjured C4 DRG neurons, in a manner consistent with the neurons contralateral to injury. These findings in contralateral L4-L6 DRG and C4 DRG remote from the injury support that unilateral nerve injury exerts a systemic impact on regulation of FOXO3a expression and its nuclear translocation. Brief 1-hour electrical stimulation (20Hz) of the sciatic

nerve at the time of crush injury, a treatment known to enhance BDNF expression and regeneration responses, further reduced FOXO3a protein levels in the injured sensory neurons. Investigations utilizing intrathecal siBDNF treatment resulted in increased FOXO3a mRNA levels both ipsilateral and contralateral to a 3d L4-6 spinal nerve lesion implicating endogenous BDNF in FOXO3a regulation. Collectively, suppression of FOXO3a after injury and further decline with electrical stimulation indicates that inhibition of the protein maybe required for a proper injury repair response. By identifying a suppressive action of BDNF on FOXO3a expression, this further signifies the important role of this neurotrophin in induction of the injury phenotype. Finally, the observed systemic changes support the existence of stress/injury-induced humeral factor(s) influencing transcriptional events in uninjured DRG neurons.

3.2. Introduction

Injury of the peripheral branch of primary sensory neurons induces a myriad of molecular changes as the cell attempts to stay viable with the loss of axoplasm integrity and without connection to its target tissue. Furthermore, as neurons of the peripheral nervous system have the propensity to regenerate severed fibres, axotomy leads to the induction of repair/regeneration programs with the main goals being survival, growth of injured axons and reestablishment of the homeostatic state. The phenotype of an injured sensory neuron differs greatly from an intact one. A regenerating neuron has elevated expression of regenerative associated genes (RAGs), neuropeptides such as galanin and neuropeptide Y (Verge, Richardson et al. 1995), brain-derived neurotrophic factor (BDNF) (Karchewski, Gratto et al. 2002) and cell stress proteins including heat shock protein-27 (Costigan, Mannion et al. 1998, Benn, Perrelet et al. 2002, Willis, Li et al. 2005, Williams and Mearow 2011). In contrast, spinal nerve injury leads to the down regulation of markers associated with the homeostatic state such as neurofilament proteins, the peptides calcitonin gene-related peptide and substance P, and the neurotrophin receptors tropomyosin-related kinase (trk) A, B and C and the common neurotrophin receptor p75 (Verge, Tetzlaff et al. 1990, Verge, Merlio et al. 1992, Verge, Richardson et al. 1995, Verge, Gratto et al. 1996, Karchewski, Kim et al. 1999). Stress and injury associated transcription factors also undergo expressional and post-translational changes as many transcription factors such as activating transcription factor 3 (ATF-3) (Tsujino, Kondo et al. 2000, Seijffers, Mills et al. 2007), c-jun (Ruff, Staak et al. 2012), STAT3 (Sheu, Kulhanek et al. 2000, Miao, Wu et al. 2006, Quarta,

Baeumer et al. 2014) and Luman/CREB3 (Ying, Misra et al. 2014) are up-regulated, have increased nuclear localization in sensory neurons following axotomy and are linked to axon regeneration. The induction and maintenance of regrowth and repair mechanisms depends on the modification of certain transcription factors; therefore, the characterization of novel regulators and their response to stress and injury has the potential to critically impact our understanding of peripheral neuron disorders and their repair.

Members of the forkhead box transcription factors class O (FOXO) family of proteins modulate the cellular processes of survival, differentiation, proliferation and stress resistance (reviewed in (van der Horst and Burgering 2007). These evolutionarily conserved transcription factors were first identified as essential to *Drosophila* development (reviewed in (Wang, Karpac et al. 2014). Since that time, our knowledge of FOXOs has grown exponentially with a protein catalogue of over 100 subtypes, implicated in a variety of mammalian and non-mammalian cellular processes. They are heavily regulated by various post-translational modifications including phosphorylation, and poly- and monoubiquitination (reviewed in (Xie, Chen et al. 2012). Nucleocytoplasmic shuttling of FOXOs is primarily controlled by an Akt/protein kinase B dependent phosphorylation mechanism, whereby the phosphorylated protein remains cytoplasmic and dephosphorylation of serine-threonine residues facilitates nuclear localization. In the mammalian adult nervous system, only the FOXO1, FOXO3/FOXO3a and FOXO4 species have been identified and are generally characterized as stress-sensing, pro-apoptotic transcription factors (Barthelemy, Henderson et al. 2004, Wen, Wang et al. 2012). FOXO3a has traditionally fit this description in a variety of neuronal cell types; however, studies suggest its function in dorsal root ganglion neurons is not restricted to cell death. In recently published work, peripheral nerve crush injuries appeared to down-regulate FOXO3a levels in both sensory neurons and associated glial cells, which coincided with the ensuing axonal regeneration and satellite cell proliferation that accompanies the pathology (Wang, Liu et al. 2009). This suggests the protein plays a stronger role in the transcriptional events of intact neurons, and its suppression may be a result of the injury response.

The initial trigger of the injury/regeneration response of sensory neurons is not fully known but strong evidence implicates altered neurotrophin signaling as a candidate for the inducing signal. Severing of the neural axon removes DRG neurons from their primary source of the peripherally derived neurotrophins nerve growth factor (NGF) and Neurotrophin-3 (NT-3);

(reviewed in (Verge, Gratto et al. 1996). Brain-derived neurotrophic factor (BDNF) is expressed in small diameter c-fibre neurons at moderate levels under normal conditions. After injury, BDNF is rapidly elevated in this population followed by up-regulation in medium-large diameter neurons and down-regulation in small neurons at later time points (Karchewski, Gratto et al. 2002). Geremia et al (Geremia, Pettersson et al. 2010) found that antagonizing endogenous BDNF signaling immediately at the time of injury, but not one week after injury lead to diminished RAG expression in injured neurons and decreased neurite outgrowth, implicating BDNF in the induction but not maintenance of the regeneration response. Neurotrophins are known regulators of FOXOs in neurons, including FOXO3a (Zhu, Bijur et al. 2004, Gan, Zheng et al. 2005, Biswas, Shi et al. 2007, Wen, Duan et al. 2011). Therefore, the changes in BDNF or other trophin levels during nerve injury events may be responsible for FOXO3a down-regulation in injured sensory neurons.

The objectives of this study were to gain further insight into injury-associated alterations in FOXO3a expression in sensory neurons and determine whether injury-associated changes in BDNF expression are implicated in this regulation. The data reveal a temporal decrease in FOXO3a protein in acutely injured lumbar DRG neurons in response to sciatic nerve transection, confirming work by Wang et al (Wang, Liu et al. 2009), including a slight resurgence of FOXO3a expression at the more protracted 1 week time point examined. Brief electrical stimulation of the sciatic nerve at the time of injury, an experimental design that leads to rapid up-regulation of BDNF and increased axonal regeneration (Geremia, Gordon et al. 2007), led to a further reduction of FOXO3a mRNA expression than the crush only controls. A role for endogenous BDNF in the FOXO3a injury response was confirmed by knocking down BDNF expression through the intrathecal delivery of BDNF specific siRNAs, leading to increased FOX3a mRNA expression.

Finally, because unilateral peripheral nerve injury can result in molecular and physiological alteration to uninjured contralateral neurons (reviewed in (Koltzenburg, Wall et al. 1999), close examination of FOXO3a expression in ganglia contralateral and remote to nerve injury was performed revealing distinct expression patterns, implicating FOXO3a in both local and global responses to injury.

3.3. Results

To address the effect of peripheral nerve injury on the spatial and temporal expression pattern of FOXO3a in DRG neurons, the right L4-L6 spinal nerves were unilaterally transected. Nerve transection at this high level results in almost 100% axotomy of L4 and L5 DRG neurons (Swett, Torigoe et al. 1991). Furthermore, regenerating axons are unlikely to fully reinnervate the distal nerve stump, thus prolonging the injury response. This contrasts a nerve crush model where the nerve epineurium remains intact and the injured axons can better regenerate across the lesioned area. Assessment of FOXO3a protein levels was accomplished using immunofluorescence histochemistry with the specificity of the antibody employed to recognize FOXO3a confirmed by Western blot analysis (Fig. 3.1). The histological examination and quantitative computer-assisted analysis of injury-induced alterations in FOXO3a expression in DRG neurons allows for both quantification of shifts in subcellular compartment localization (i.e. nucleus versus cytoplasm) and shifts expression within distinct size ranges of neurons, generally associated with specific sensory modalities.

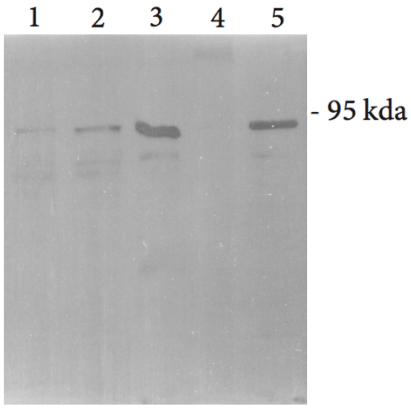

Figure 3.1 Anti-FOXO3a monoclonal antibody specifically recognizes protein band at the expected molecular weight of FOXO3a. Western blot analysis of monoclonal anti-FOXO3a treated membrane of electrophoresed protein extracts from 2d-injured ipsilateral L4-L6 DRG (lane 1), 2d-injured contralateral L4-L6 DRG (lane 2), control BrCA cell line (lane 3), and control rat liver (lane 5). Lane 4 empty. Western blots were performed in triplicate. Note: The FOXO3a antibody recognizes a band of approximately 85-90 kDa, consistent with the expected molecular weight for FOXO3a and nerve injury results in reduced levels of FOXO3a.

3.3.1. FOXO3a expression in naïve DRG neurons and DRG neurons ipsilateral to injury

Immunofluorescence analysis of tissue sections from naïve L5 DRG revealed immunostaining throughout the cell bodies of the neurons (Fig. 3.2) as well as staining within neuronal fibre tracts (data not shown). Quantitative fluorescence analysis and scatter-plot representation of the naïve ganglia indicated FOXO3a staining to be the highest in small to medium-sized, presumably nociceptive neurons with perikaryal diameters between 20-40 μm. Furthermore, a number of these smaller diameter neurons had highly stained neuronal nuclei whereas nuclear FOXO3a staining in larger diameter neurons was less prevalent.

To examine the effect of nerve transection on FOXO3a expression, the L4-L6 spinal nerves of male Wistar rats were unilaterally transected and both the ipsilateral and contralateral DRGs were collected at predetermined time points. Nerve injury was verified by ATF3 positive staining (Fig 2.2, methods chapter). Immunofluorescence analysis of ipsilateral DRGs 1-hour after injury showed increased FOXO3a nuclear staining in the small-med diameter neuron population relative to naïve (Fig. 3.2). Changes in cellular localization at this early time point implies rapid protein alterations in response to acute injury.

At later time points, axotomy of the peripheral branch lead to further changes in FOXO3a. After 1-day, the intensity of FOXO3a immunofluorescence decreased in DRG neurons ipsilateral to lesion, especially in the small to medium-sized neurons where staining was usually high. Both nuclear and cytoplasmic labelling intensities at 1-day post-injury were significantly lower compared to those at 1-hour and naïve (Fig. 3.2). Western blot analysis performed from 2-day post injured DRG confirms the immunofluorescence results of this time point (Fig. 3.1). This lower level of FOXO3a expression in the small to medium-sized was evident in 1-day and continued in 4-day injured DRG neurons (Fig. 3.2). These observations confirm the previously documented results that FOXO3a is down-regulated in response to semi-acute peripheral nerve injury (Wang et al., 2009).

To assess the effect of a more chronic injury state, the DRG from 1-week injured animals were also examined and the down-regulation of FOXO3a protein was less significant in these injured DRG neurons, as levels appeared slightly higher than the 4 day time point (Fig. 3.2), also consistent with the findings of Wang et al (Wang, Liu et al. 2009). The impact of nerve injury

on the expression pattern of FOXO3a appears to depend on the type of peripheral nerve lesion and the duration of the injury and are summarized in Figure 3.4.

Sections of both the ipsilateral injured and contralateral uninjured DRG tissue was processed for *in situ* hybridization with ^{35}S-labelled oligoDNA probes specific for FOXO3a mRNA (Fig 3.9) to visualize whether changes observed at the protein level were seen at the level of transcription. No appreciable qualitative difference in neuronal FOXO3a mRNA is noticed between the DRG neurons ipsilateral and contralateral to injury at each individual time point. While subtle differences between time points are noted, these do not correlate to the significant changes seen with neuronal FOXO3a protein levels in response to injury. This finding suggests that alterations in FOXO3a protein are likely secondary to post-translational modifications.

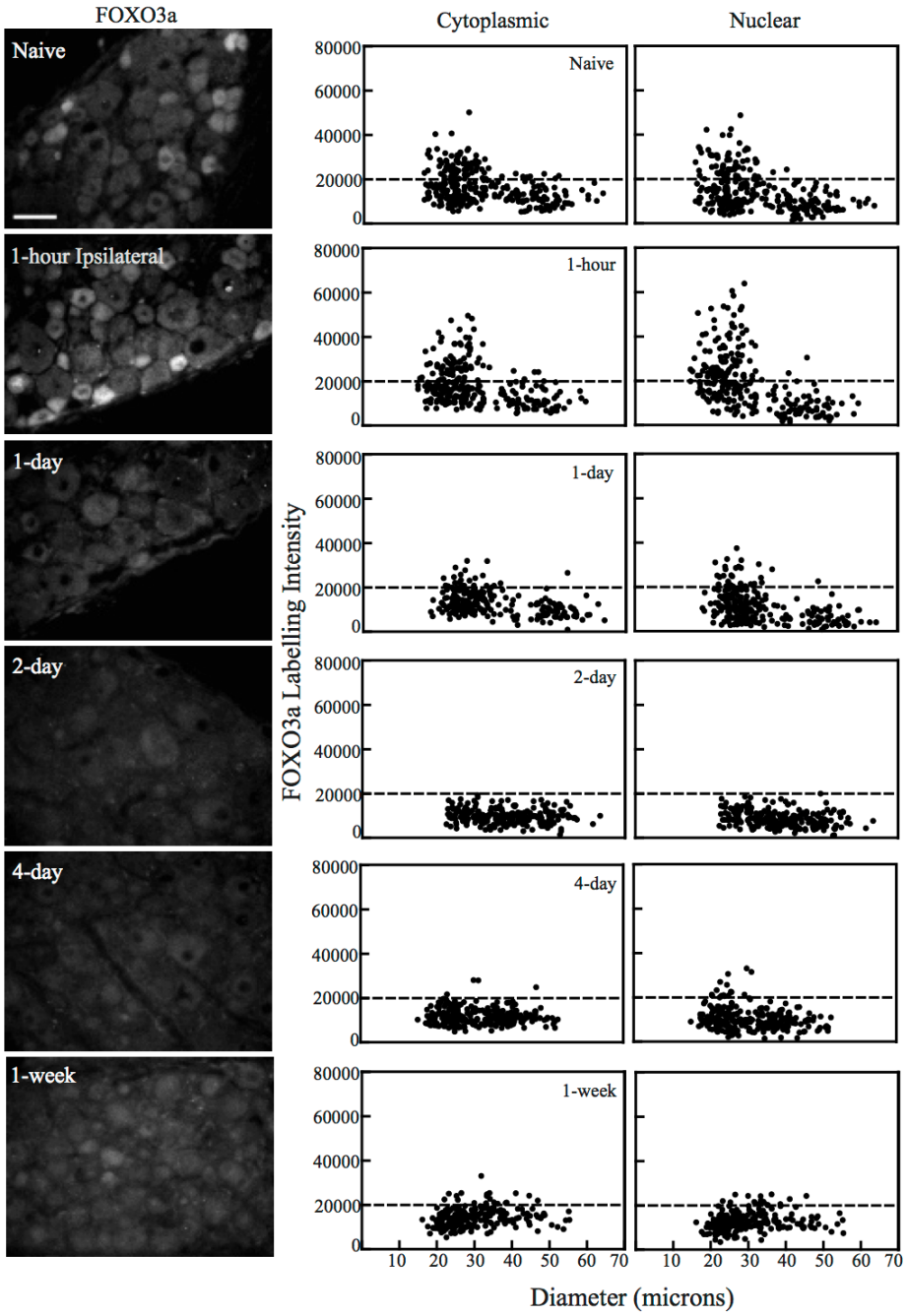

Figure 3.2. Peripheral nerve injury alters FOXO3a protein levels in DRG neurons ipsilateral to axotomy. *Left Column*. Immunofluorescence photomicrographs of L5 DRG sections (6 µm) processed to detect FOXO3a protein. Ipsilateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day and 1week. Scale bar = 50 µm. Naïve animals served as controls. *Right Column*. Representative

scatterplots depicting relative changes in FOXO3a immunofluorescence signal over individual cytoplasmic and nuclear regions as related to cell size from sections all processed under identical conditions in parallel. Experimental states as indicated. Dashed lines divide the plots into low versus heavily labelled populations. N= 217 to 294 neurons analyzed per condition.

3.3.2. FOXO3a protein expression pattern in uninjured DRG neurons contralateral to injury

Immunofluorescence analysis on sections from DRG contralateral to the injury indicate that unilateral nerve injury has an effect on FOXO3a protein expression in contralateral uninjured DRG. By 1-day, nuclear and cytoplasmic intensity levels were elevated compared to the contralateral 1-hr and naïve neurons (Fig. 3.3). At 2 and 4-days post-injury, staining intensities were decreased and nuclear and cytoplasmic intensity reached the lowest levels (Fig 3.3). However, 1-week following unilateral injury, contralateral neurons had elevated FOXO3a staining to levels similar to those seen in small to medium size neurons at 1-day and exceeding levels in seen at 1 day in large neurons (Fig. 3.3). Summary line plots clearly reveal this biphasic cytoplasmic and nuclear response in the contralateral uninjured neurons (Fig. 3.4).

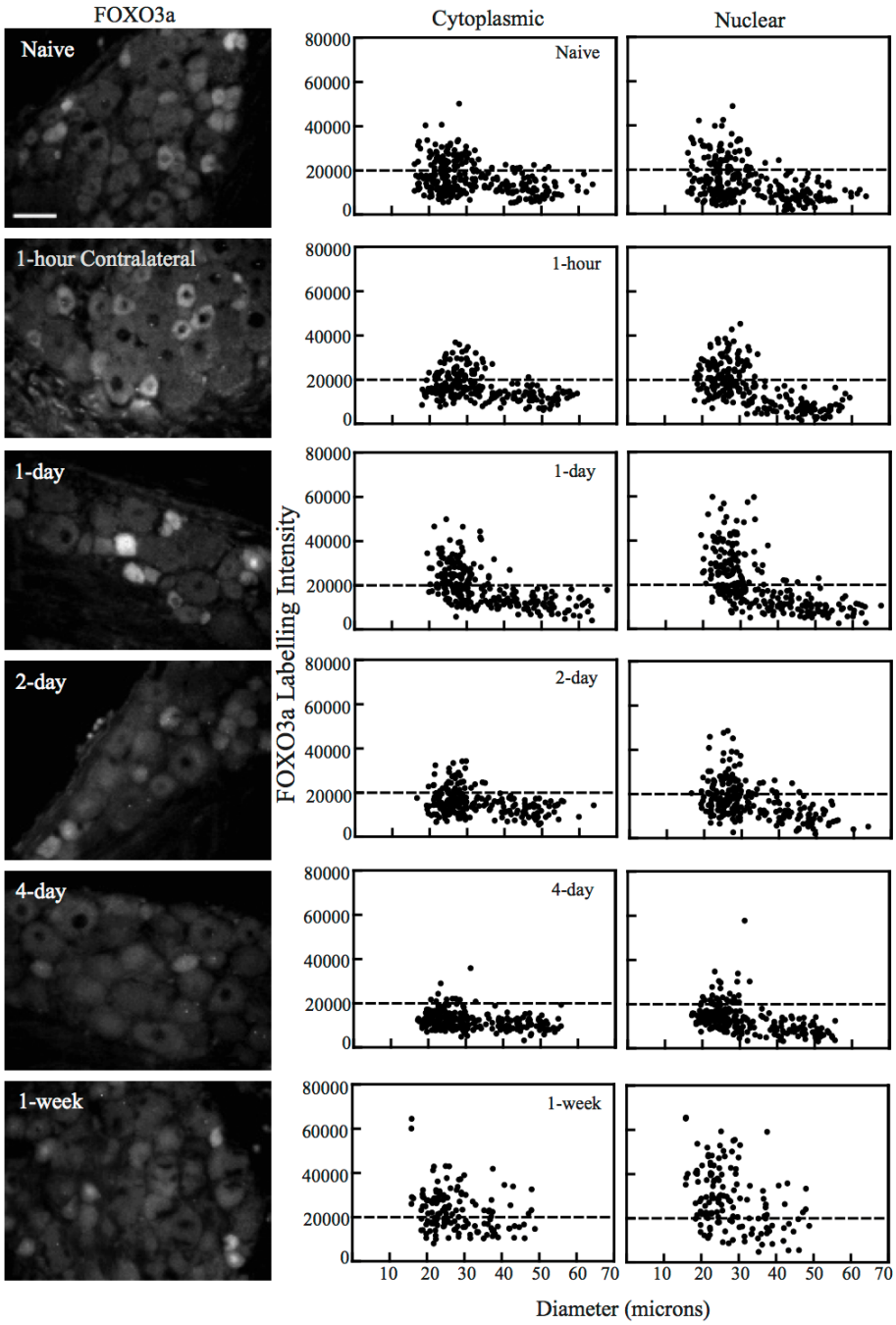

Figure 3.3. Peripheral nerve injury alters FOXO3a protein levels in DRG neurons contralateral to axotomy. *Left Column.* Fluorescence photomicrographs of L5 DRG sections (6 µm) processed for immunohistochemistry to detect cell FOXO3a protein. Contralateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day and 1week. Scale bar = 50 µm. Naïve animals served as controls. *Right*

Column. Representative scatterplots depicting relative changes in FOXO3a immunofluorescence signal over individual cytoplasmic and nuclear regions as related to cell size from sections all processed under identical conditions in parallel. Experimental states as indicated. Dashed lines divide the plots into low versus moderate to heavily labelled populations. N= 178 to 294 neurons analyzed per condition.

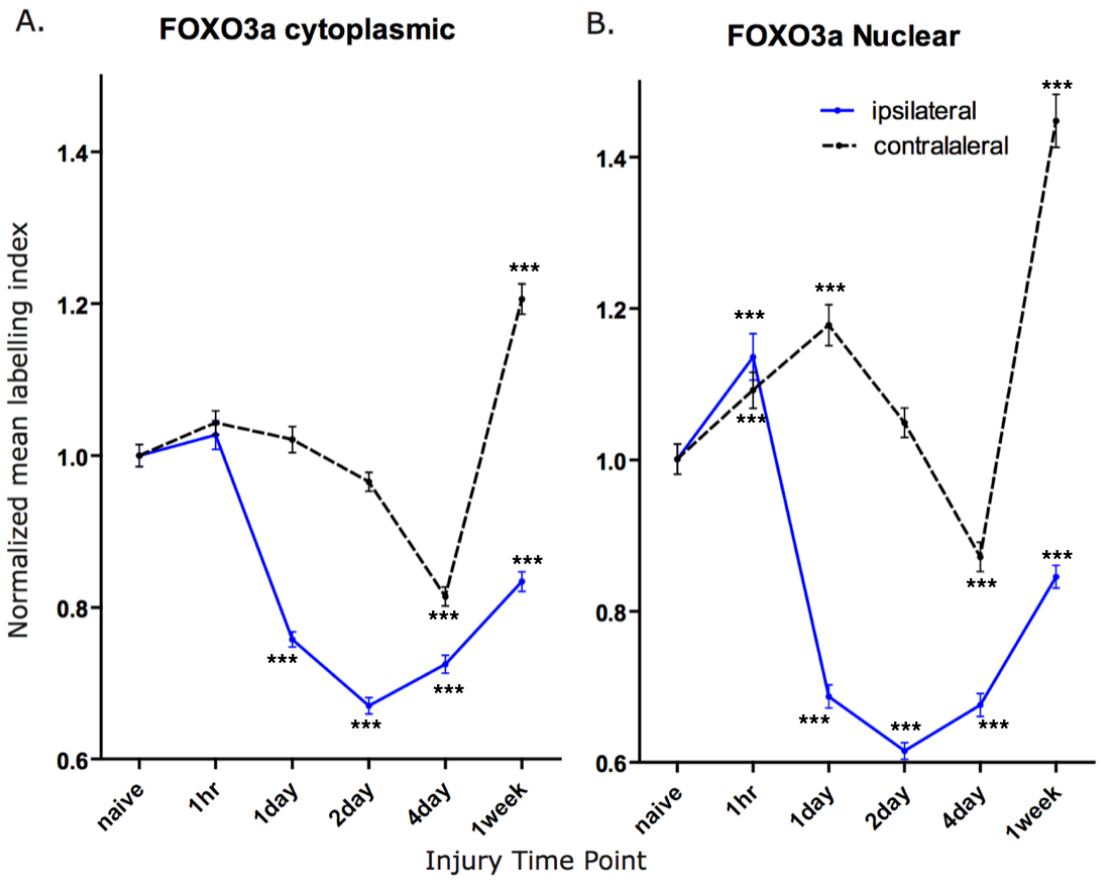

Figure 3.4 Summary line graphs of alterations in the mean labeling index of FOXO3a. Cytoplasmic (A) and nuclear (B) FOXO3a immunofluorescence relative mean intensity levels ± S.E.M. observed in DRG neurons ipsilateral and contralateral to injury at time points as indicated. Each graph point represents a quantitative analysis of N=600 or more neurons from 3 animals in total per condition. ***p value<0.001 ANOVA with Dunn's post-test analysis signifies significant difference from the naïve state.

3.3.3. Brief electrical nerve stimulation and experimental knockdown of BDNF regulate neuronal FOXO3a levels in injured neurons.

Electrical stimulation (ES) just proximal to nerve injury heightens the regenerative capacity of sensory neurons following injury, increases expression of RAGs and elevates somal levels of BDNF (Geremia, Gordon et al. 2007). In order to assess the effect of this therapy on FOXO3a protein levels in injured primary sensory neurons, sciatic nerves sustained a crush injury at the mid-thigh level. Immediately after crush injury, brief 1 hr 20 Hz continuous (alternating) electrical stimulation was applied to the nerve proximal to the crush site in one half of the animals. Tissue was collected 1-hour, 1 day and 4 days post injury +/- ES. The midthigh sciatic nerve crush injury paradigm, unlike the spinal nerve transection injury, results in only ~80% of L4,5 DRG neurons being injured as opposed to virtually all neurons in the latter spinal nerve transection model.

Confirming previously data (Geremia, Gordon et al. 2007), nerve injury plus ES increased BDNF mRNA expression in DRG neurons ipsilateral to injury at the 4 day time point (Fig. 3.5A). Crush injury alone caused a decrease in FOXO3a levels at the 1-day time point; however, this decrease appears to reverse slightly at 4-days post crush. Brief ES further reduces FOXO3a levels in 4-day post lesion animals compared to 4-day injury alone controls (Fig. 3.5B,C). This suggests the ES treatment further suppresses FOXO3a in the 4-day ipsilaterally injured neurons. Implying that inhibition of the transcription factor in the injured/regenerating phenotype of DRG neurons may improve growth and that the mechanism of this inhibition is either electrical or neurotrophin-based, potentially BDNF mediated.

To investigate whether endogenous neuronal BDNF is implicated in the suppression of FOXO3a expression in injured sensory neurons, BDNF specific siRNA intrathecally delivered at the level of the L4 and L5 DRG was employed to selectively knock down neuronal BDNF levels. Sections processed for *in situ* hybridization to detect FOXO3a mRNA expression were taken from the same tissue generated for experiments in Geremia et al (Geremia, Pettersson et al. 2010), where the intrathecally delivered siRNA was shown to target DRG neurons (Fig. 8 in (Geremia, Pettersson et al. 2010)). In addition, the BDNF siRNA effectively knocked down BDNF mRNA and protein expression, leading to reduced regeneration–associated gene expression (Fig. 8 in (Geremia, Pettersson et al. 2010)).

Sections from this tissue processed for FOXO3a mRNA *in situ* hybridization revealed that siRNA knockdown of BDNF neuronal expression resulted in increased FOXO3a mRNA in L5 DRG both ipsilateral and contralateral to a 3d L4-6 spinal nerve injury, supporting a role for endogenous BDNF in suppression of FOXO3a expression (Fig. 3.6C,D) in both intact and injured neurons. In situ hybridization control experiments confirmed that addition of 100X excess cold (unlabeled) FOXO3a to the hybridization solution containing ^{35}S-labeled FOXO3a effectively competed away the radio-labelled hybridization signal (Fig. 3.6E,F). In siRNA control experiments, infusion of nontargeting control siRNAs resulted in FOXO3a expression levels that were not discernibly different from non-infused control 3 day injured animals (Fig. 3.6A, B).

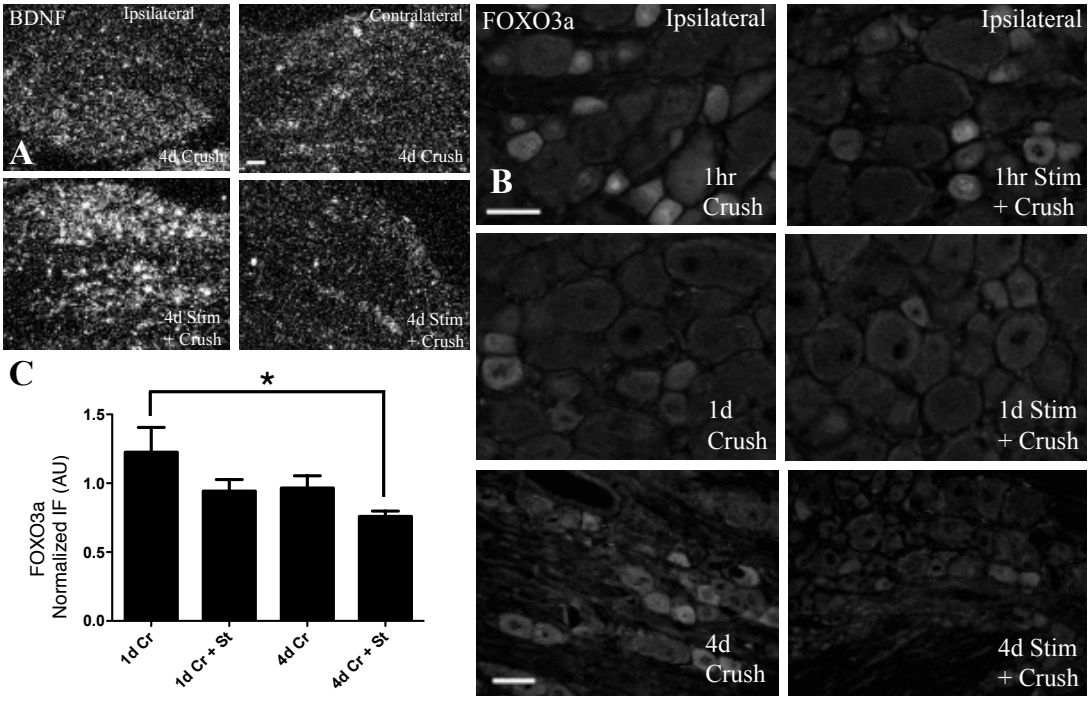

Figure. 3.5. Brief electrical stimulation elevates BDNF expression in DRG neurons and reduces FOXO3a levels in DRG ipsilateral to injury. A. Dark field photomicrographs of L5 DRG sections (6 microns) processed for in situ hybridization to detect BDNF mRNA expression. Scale bar = 100 microns. The sciatic nerve of animals was crushed at the mid-thigh level and brief electrical stimulation of 20Hz was applied for 1hr proximal to the crush site. Crush only animals served as controls. Experimental groups are indicated. B. Ipsilateral L5 DRG sections processed for FOXO3a immunohistochemistry from crush injured (left column) or crush + stim treated (right column) animals after the stated time points of 1 hour, 1 day and 4 days post-

injury. Scale bars = 50 microns. C. Bar graph from analysis of DRG protein extracts from the stated conditions (N=3, repeated in triplicate) processed by Western blot representing FOXO3a IF densitometry (arbitrary units) normalized to the GAPDH loading controls. *p value<0.05 (Mann-Whitney, t-test).

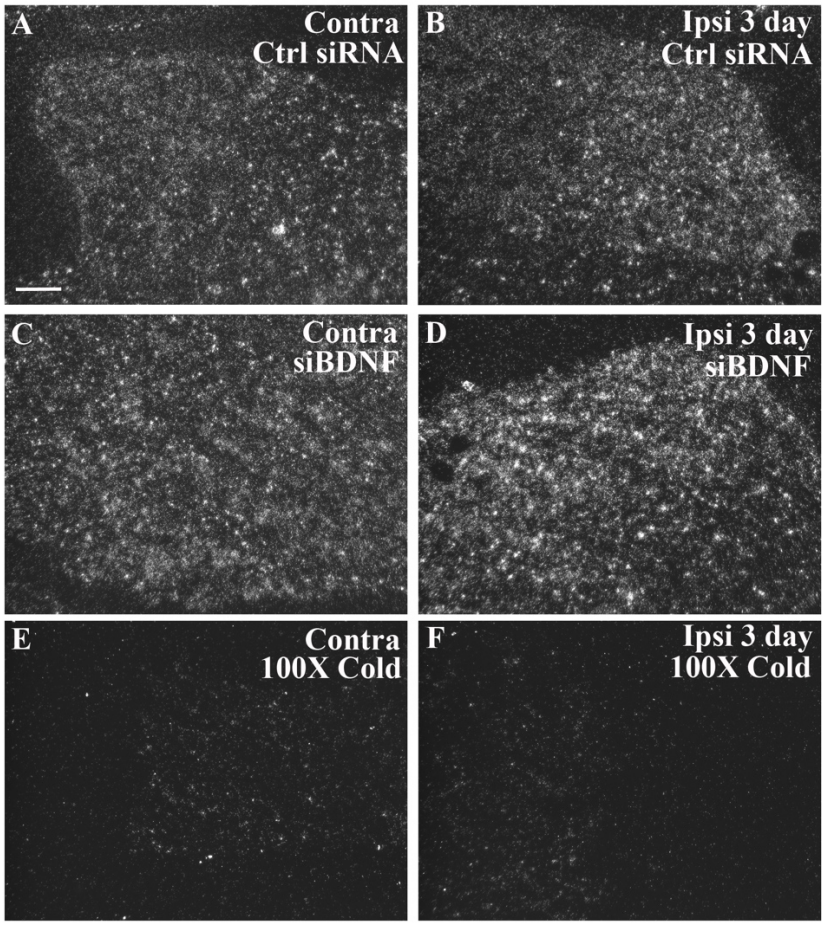

Figure. 3.6. Endogenous BDNF suppresses FOXO3a expression. Representative darkfield photomicrographs of 6 micron L5 DRG sections processed for in situ hybridization to detect FOXO3a mRNA. Bolus intrathecal delivery of 20 µg BDNF siRNA 3 days prior to injury and again at time of injury results in increased levels of FOXO3a mRNA both ipsilateral (D) and contralateral (C) to 3 day L4-6 spinal nerve lesion relative to non-targeting control siRNA (A,B). In situ hybridization probe specificity control experiments reveal addition of 100X excess unlabeled FOXO3a oligonucleotide probe effectively competes away the ^{35}S-labelled FOXO3a probe. Scale bar = 100 microns (N= 3 animals per experimental group).

3.3.4. FOXO3a protein levels are altered in uninjured cervical DRG neurons

The interesting impacts of nerve injury on FOXO3a expression in L4,5 DRG neurons contralateral to injury might be attributed to either a systemic, likely humoral response to the

injury, or to a response mediated contralaterally through the spinal cord. To test for the involvement of a systemic factor, cervical ganglia remote from the injury site, were taken from 1-day L4-L6 spinal nerve transected and naïve rats and assessed for FOXO3a expression. Qualitative immunofluorescence analysis of 6 animals per condition revealed that C4 DRG neurons from 1-day sciatic injury animals had elevated nuclear and cytoplasmic staining relative to naïve C4 DRG, especially in the small-med diameter neurons (Fig. 3.7A). Quantitative analysis of neurons from 3 animals confirmed the qualitative observations, revealing significant differences between the two conditions (Fig. 3.7,B,C). Although a humoral mechanism best explains the effect seen in the cervical ganglia, a neuronal signal through the spinal cord that affects contralateral FOXO3a processing may still exist.

To discern whether the systemic impacts on FOXO3a protein levels were a result of the spinal nerve injury itself and/or as a result of the surgical exposure to perform the injury, sham surgeries were performed on 3 animals for each injury time point. The L4-L6 spinal nerves of these anesthetized rats were exposed but not transected and the animals were left to recover after 1-hour, 1-day, 2-days, 4-days and 1-week. FOXO3a protein levels and cellular localization appeared unchanged (Fig. 3.8). The sham experiments verify that the contralateral effect observed at the protein level is due to injury of the nerve and not a byproduct of the surgical exposure.

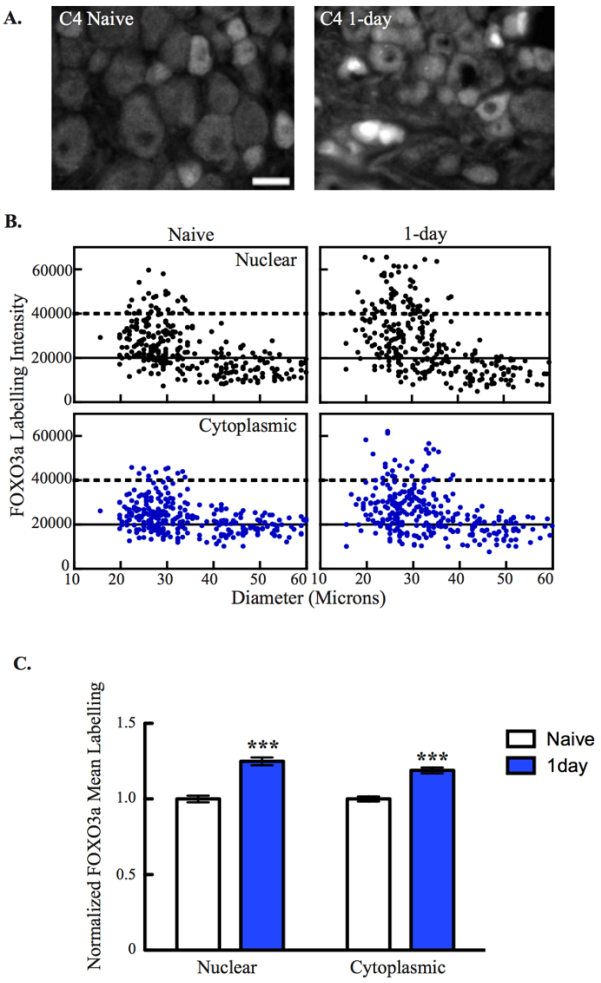

Figure 3.7 L4-L6 spinal nerve transection alters DRG neuronal FOXO3a expression and localization in uninjured C4 ganglia remote from the injury site. A. Fluorescence photomicrographs of FOXO3a immunostained C4 DRG sections collected from naïve and rats that underwent 1 day unilateral L4-L6 spinal nerve injury. Scale bar = 50µm. Note: L4-L6 spinal nerve injury results in elevated nuclear and cytoplasmic FOXO3a staining in small to medium sized neurons of the uninjured C4 DRG. B. Scatterplots whereby each point represents the relationship between the FOXO3a nuclear (top) and cytoplasmic (bottom) labelling index and cell body diameter. Solid lines divide the plots into least labelled and moderately labelled populations; dotted lines separate moderately labelled from heavily labelled populations of FOXO3a expressing neurons. C. Bar graphs representing normalized nuclear (left) and cytoplasmic (right) FOXO3a mean labeling index ± SEM. (***p-value < 0.0001, Mann-Whitney t-test). Each graph bar represents a quantitative analysis of N=600 or more neurons from 3 animals in total per condition. Qualitative analysis of N=6 animals processed per condition was performed and quantitative analysis confirmed qualitative observations.

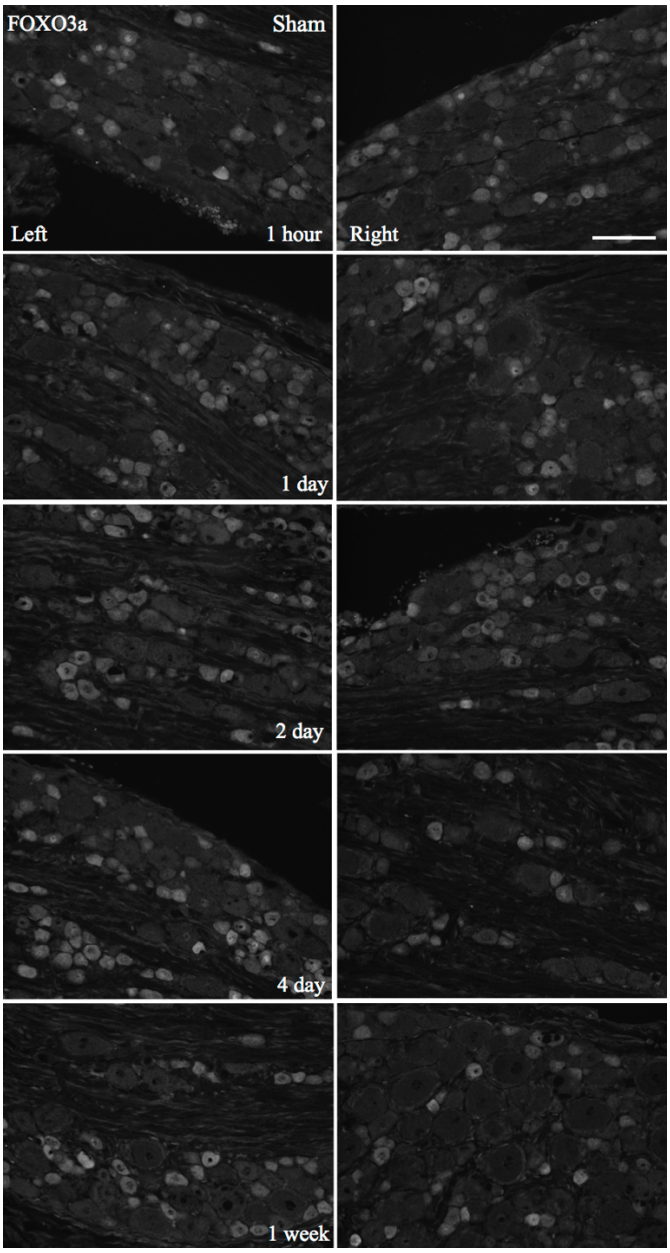

Figure 3.8. Sham surgery time course did not result in discernibly altered FOXO3a immunostaining. Representative fluorescence photomicrographs of right and left L5 DRG (6 μm sections) from sham animals with intact peripheral spinal nerves 1-hour, 1day, 2days, 4days and 1-week post sham surgeries processed for immunohistochemistry to detect cell FOXO3a protein. Scale bar = 100 μm. Note: Sham procedures involved exposure and handling of the spinal nerves without transection.

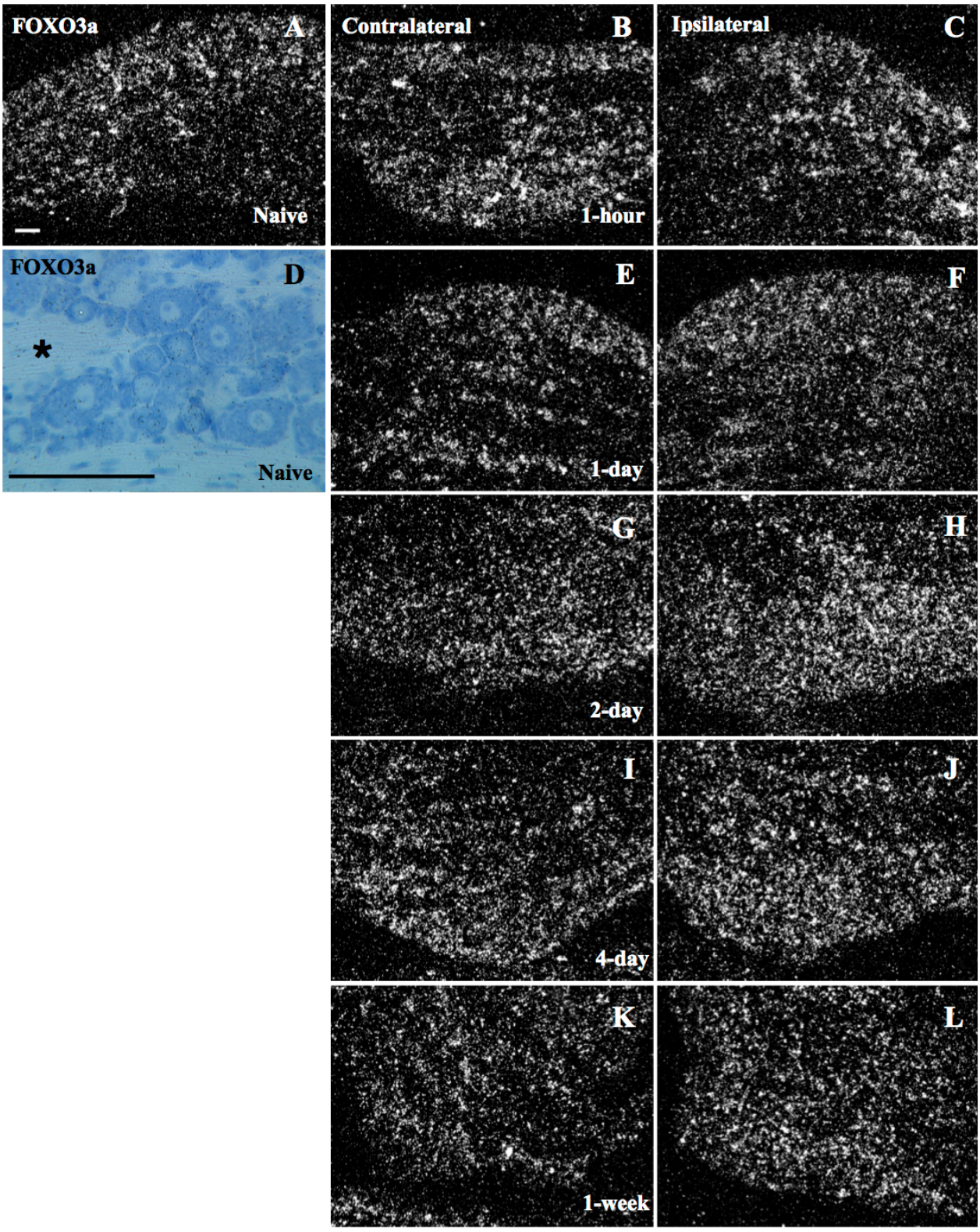

Figure 3.9 Unilateral sciatic nerve injury did not cause dramatic changes is FOXO3a mRNA levels between ipsilateral and contralateral DRG. Darkfield (A-C, E-L) and lightfield (D) photomicrographs of L5 DRG (6 micron sections) ipsilateral (C,F,H,J,L) and contralateral

(B,E,G,I,K) to injury from L4-L6 unilateral spinal nerve transected rats processed for *in situ* hybridization with an ^{35}S-labelled oligonucleotide 48mer probe specific for FOXO3a mRNA. DRGs were harvested after the predetermines time points of 1-hout (B,C), 1-day (E,F), 2-days (G,H), 4days (I,J), and 1-week (K,L) post-injury. Naïve animals served as controls (A, D). D. FOXO3a mRNA were highly expressed in DRG cell bodies but not so in nerve fibres. N = 3 for each group. Scale bars = 100 microns. Note: no appreciable qualitative difference in neuronal FOXO3a mRNA is noticed between the DRGs ipsilateral and contralateral to injury at each individual time point. While subtle differences between time points are noted, these do not correlate to the significant changes seen with neuronal FOXO3a protein levels in response to injury.

3.4. Discussion

These experiments shed novel insights into the regulation of FOXO3a expression in DRG neurons. The early initial rapid up-regulation and then profound down-regulation in response to peripheral nerve injury implies a connection to both the induction and maintenance phases of the regeneration process that may include protection from injury-associated cell death. In addition, the initial increase in nuclear localization observed at one hour after nerve transection suggest it is capable of sensing alterations from the homeostatic state. Although this scenario is plausible, constitutive expression in the naïve animal supports a role for this transcription factor outside of the realms of neuronal death and injury, with its high level of protein expression in small to medium size uninjured neurons implicating it in the nociception. Nevertheless, our study also supports that the decrease in FOXO3a in injured neurons as a result of nerve transection might be partly due to the elevation in BDNF, a molecule critical to the induction of the injury/regeneration response in sensory neurons (Geremia, Pettersson et al. 2010) and also linked to their survival (Acheson and Lindsay 1996; Murphy, Borthwick et al. 2000) . Finally, the alterations of FOXO3a protein expression in sensory neurons contralateral and remote to injury raises questions we cannot yet answer, but supports an ability of this molecule to sense very subtle alterations in systemic states associated with nerve injury. Further examination of this phenomenon is essential to the understanding of the sensory neurons response to stress and disease.

3.4.1. Role of FOXO3a in the nervous system and DRG neurons

The FOXO proteins are a highly conserved family of transcriptional regulators and are homologues to DAF-16 (abnormal DAuer Formation-16) in *C. elegans* (Lin, Dorman et al. 1997). This invertebrate transcription factor primarily regulates metabolic processes as well as survival and lifespan (Ogg, Paradis et al. 1997, Clancy, Gems et al. 2001). In mammals, their involvement with multiple cellular pathways is centered on maintaining homeostasis under stressful conditions and directing the cell towards survival or controlled cell death. FOXOs are associated with insulin-mediated events via the insulin-PI3K-Akt pathway, where loss of insulin signaling leads to nuclear translocation of the protein (reviewed in (Gross, van den Heuvel et al. 2008) and altered glucose metabolism (Zhang, Patil et al. 2006). Insulin and other growth factors promote cell proliferation by inhibiting FOXO activation of cell-cycle arrest genes, including $p27^{kip1}$. To protect cells from oxidant production associated with the loss of metabolic integrity, antioxidant enzymes catalase and manganese superoxide dismutase (MnSOD) are up-regulated by FOXOs to assist in radical scavenging (Yun, Park et al. 2014, Maiese 2015, Rangarajan, Karthikeyan et al. 2015). Although FOXO-mediated stress resistance and cell stabilization are well documented, the transcription factors are closely linked to apoptosis. Indeed, the regulation of cellular events can be complex with recent evidence describing an initial protective role for FOXO3a in nervous system tissue that switches to neurodegenerative with prolonged exposure to oxidative stress (Shi, Viccaro et al. 2016).

As previously stated, neuronal FOXO3a (FOXO3, FKHR-L1) has classically been considered a pro-apoptotic transcription factor, which is accredited to its close relationship with the PI3K/Akt survival pathway. Phosphorylation by Akt inhibits its activity as phosphorylated FOXO3a associates with 14-3-3 proteins, which leads to cytoplasmic sequestering (Maiese 2015). Decreased Akt signaling or increased dephosphorylation by phosphatases results in nuclear translocation and the activation of forkhead-responsive genes, including the apoptotic genes *fas-ligand* (FasL) (Brunet, Bonni et al. 1999) and *bim* (Dijkers, Medema et al. 2000). Many growth factor signal transduction mechanisms are mediated through Akt, and trophic availability modulates FOXO3a activity. Insulin-like growth factor-1 treatment increases Akt phosphorylation of the transcription factor in PC12 cells (Zheng, Kar et al. 2000), while neurotrophin deprivation induces nuclear localization of FOXO3a, FasL gene expression and death of cultured motoneurons (Barthelemy, Henderson et al. 2004). In general, any conditions

associated with neuronal loss; such as development (Srinivasan, Anitha et al. 2005) aging (Jackson, Rani et al. 2009) or pathological ischemic events, (Kawano, Morioka et al. 2002, Fukunaga, Ishigami et al. 2005, Maiese, Chong et al. 2007) have a connection to decreased Akt activity and FOXO3a activation. In spite of this evidence, FOXO3a's functional importance in naïve intact DRG neurons does not appear to be cell death related. Furthermore, peripheral nerve injury does not overtly lead to apoptosis. Long-term sciatic transection studies suggest that retrograde loss of L4-L5 sensory neurons is only detectable 1-month post-axotomy (Groves, Schanzer et al. 2003, Kuo, Simpson et al. 2005). This resistance to death is attributed to the high level of autocrine/paracrine trophic support from associated glia and the neurons themselves (Acheson and Lindsay 1996, Xian and Zhou 1999, Karchewski, Gratto et al. 2002, Vigneswara, Berry et al. 2013, Nadeau, Wilson-Gerwing et al. 2014). As the current study does not examine injury events past the 1-week time point, FOXO3a's influence on apoptosis in this instance is debatable.

Primary sensory neurons of the DRG are heterogeneous in both structure and function. Distinct populations exist with specific sensory modalities (proprioception, mechanoception, nociception, etc.) and common morphological, biochemical, and physiological characteristics (Carr and Nagy 1993). Nociceptor neurons with unmyelinated c-fibres or thinly myelinated Aδ-fibres generally have smaller cell bodies. It is these small-diameter presumably nociceptive DRG neurons that exhibit the highest levels of FOXO3a protein in the naïve animal and undergo FOXO3a down-regulation in response to injury. This high expression and nuclear localization in small-medium diameter naïve neurons is perplexing, as activity of this protein coincides with cellular stress states. However, FOXOs conserved relationship with glucose metabolism offers clues to FOXO3a's potential role in these nociceptors. Small neurons of the DRG have high levels of hexokinase, which initially phosphorylates glucose prior to the start of glycolysis (Gardiner, Wang et al. 2007). Elevated amounts of this kinase suggest higher concentrations of glucose in this population and the necessity for rigorous metabolic management. Small-medium neurons also express high levels of the insulin receptor (IR) (Sugimoto, Murakawa et al. 2002), insulin-like growth factor-1 receptor (IGF-1R) and insulin-like growth factor 1 (IGF-1) (Craner, Klein et al. 2002). As stated above, FOXO3a activation is induced in a low-glucose situation and activates genes associated with glucose conservation, such as glucose-6-phosphatase (Onuma, Vander Kooi et al. 2006). It also increases sensitivity to insulin by up-regulating IR and insulin

receptor substrate proteins (Puig and Tjian 2005). Although naïve sensory neurons are not glucose deprived, they appear to be highly sensitive to alterations in insulin circulation. In injury experiments, axotomy caused the down-regulation of IGF-1, whereas a diabetic neuropathic state significantly reduced both IGF-1 and its receptor in smaller neurons (Craner, Klein et al. 2002). Interestingly, the Drosophila FOXO3a homolog (dFOXO) suppresses transcription of the IGF-1 homologue insulin-like peptide *dilp-2* in neurons (Hwangbo, Gershman et al. 2004). Our findings do not support this scenario, as injury appears to effect downregulation both the transcription factor and the insulin-like molecule. The extent of FOXO3a's involvement in the glucose regulation of these small DRG neurons before or after injury is unknown and requires further study.

Mitochondrial dysfunction and reactive oxygen species production are consequences of elevated glucose concentrations (Nishikawa, Edelstein et al. 2000). The antioxidant properties of FOXOs are well understood and the overexpression of FOXO3a can protect mammalian cells from oxidative stress through the upregulation of MnSOD (Kops, Dansen et al. 2002, Li, Chiu et al. 2006). Oxidant regulation of FOXO nuclear localization is not Akt-dependent, rather the process is mediated through the oxidative-stress-regulated mammalian sterile 20-like kinase-1 (MST-1) (Lehtinen, Yuan et al. 2006) or the c-Jun N-terminal kinase (JNK) (Essers, Weijzen et al. 2004) pathways where phosphorylation activates the protein by an unknown mechanism. This processing is consistent with the FOXO-Akt regulation, as JNK has been shown to phosphorylate 14-3-3, which leads to decreased 14-3-3 binding to FOXOs and increased nuclear localization of FOXOs (Sunayama, Tsuruta et al. 2005). This information taken together with glucose and insulin regulation of DRG neurons suggests that naïve small sensory neurons may be susceptible to oxidative stress, thus resulting in elevated nuclear levels of FOXO3a.

3.4.2. FOXO3a response to unilateral nerve injury

Axotomy of the sciatic nerve resulted in down-regulation of FOXO3a protein, supporting observations seen in previous finding (Wang, Liu et al. 2009). There is no convincing data suggesting that injury suppresses FOXO3a at the gene level, and our studies show that mRNA expression appeared relatively similar throughout the injury time course. With presumed little change in FOXO3a gene transcription, any observed changes in protein levels are likely a consequence of post-translational events. FOXOs are heavily controlled by post-translational

modifications with a number of studies highlighting the importance of FOXO polyubiqutination and protein degradation (Matsuzaki, Daitoku et al. 2003, Plas and Thompson 2003, Huang, Regan et al. 2005, Fu, Ma et al. 2009). FOXO1 and FOXO3a are ubiquitinated and targeted to the proteasome in response to insulin or growth factor treatment, which is accredited to activation of the PI3K/Akt pathway and phosphorylation of the FOXOs at known Akt sites (Matsuzaki, Daitoku et al. 2003, Plas and Thompson 2003). Genetic transfection of active PI3K or Akt leads to phosphorylation-dependent, proteasome mediated FOXO1 degradation (Aoki, Jiang et al. 2004). SKP2 and MDM2 have been identified as E3 ubiquitin ligases that polyubiquitinate FOXO1 and FOXO3a respectively (Huang, Regan et al. 2005, Fu, Ma et al. 2009). Increased PI3K signaling and Akt phosphorylation results in FOXO nuclear exclusion as well as ubiquitination and degradation of the cytoplasmic protein. Therefore, fluctuations in the Akt activity is likely responsible for FOXO3a's suppression in injured DRG neurons.

The significance of Akt in adult sensory neurons has been explored through a variety of neuropathic pain models where elevated amounts of activated or phosphorylated Akt (p-Akt) are associated with certain pain states (Zhuang, Xu et al. 2004, Sun, Tu et al. 2006, Xu, Tu et al. 2007). Recently, peripheral nerve axotomy was shown to dramatically increase p-Akt in both mouse spinal cord and DRG neurons (Shi, Huang et al. 2009). Furthermore, phosphorylation of Akt is activity-dependent as electrical stimulation of the sciatic nerve also increases p-Akt levels (Pezet, Spyropoulos et al. 2005). These observations strengthen the argument that a rise in p-Akt and subsequent substrate phosphorylation and ubiquitination may underlie the axotomy and electrical stimulation induced changes in FOXO3a expression. However, in naïve animal p-Akt immunoreactivity is highest in small and medium sized nociceptive neurons (Pezet, Spyropoulos et al. 2005), a population we show to have the highest constitutive expression of the transcription factor. This suggests a cell specific threshold whereby heightened Akt activity can exist without inducing proteasomal degradation of the protein.

A distinctive feature of primary sensory neurons is their ability to regenerate in response to injury. The spatial loss of the FOXO3a transcription factor and changes in forkhead responsive gene expression during the injured state may be crucial to the regenerative process. As previously stated, FOXOs are transcriptional activators of genes involved in cell cycle inhibition, including $p27^{kip1}$ (Medema, Kops et al. 2000) and cyclin G2 (Martinez-Gac, Marques et al. 2004), as well as suppressors of the cycle progression protein cyclin D (Schmidt, Fernandez de

Mattos et al. 2002). The role of cell cycle proteins in post-mitotic neurons is under investigation and results indicate that induction of the cell cycle precedes neuronal apoptosis (Freeman, Estus et al. 1994, Park, Levine et al. 1997, Shirvan, Ziv et al. 1997, Liu and Greene 2001, Becker and Bonni 2004, Herrup, Neve et al. 2004). Nerve regeneration and axon outgrowth is associated with cell cycle inhibition events such as the up-regulation of cyclin G (Morita, Kiryu et al. 1996) and p21 [the cyclin dependent kinase (cdk) inhibitor](Tanaka, Yamashita et al. 2004), and the suppression of the anaphase promoting complex (Konishi, Stegmuller et al. 2004). Recently, the cdk inhibitor p27kip1 has been implicated in the regenerative response as the protein is down-regulated in motoneurons, axons and associated glia in response to peripheral nerve injury (Shi, Cheng et al. 2007, Shen, Shi et al. 2008). In these instances, the loss of p27kip1 is accredited to the upregulation of the ubiquitin ligase SKP2 and the ensuing proteasomal degradation. DRG neurons and satellite cells also have diminished levels of the protein after injury but this follows an initial reduction in FOXO3a, suggesting p27kip1 transcription is altered in these cell types (Wang, Liu et al. 2009). As FOXO3a activates the p27kip1 gene (Dijkers, Medema et al. 2000, Hu, Wang et al. 2005), the transcription factors degradation and the decreased expression of the cdk inhibitor may play a key role in either axon growth or satellite cell proliferation. Although commencement of the cell cycle is typically linked to neuronal apoptosis, perhaps events that advance the cycle, such as the suppression of cdk inhibitors, are critical to the regeneration of postmitotic neurons.

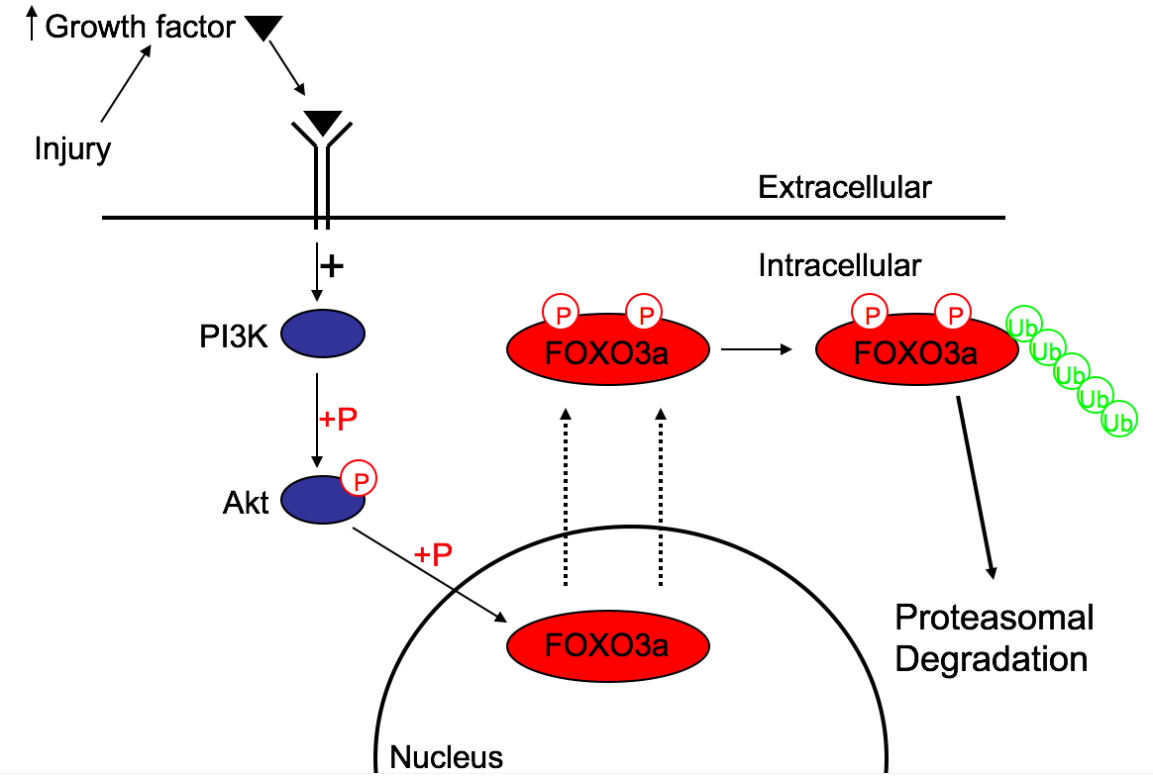

Figure 3.10. Potential mechanism for growth factor mediated FOXO3a degradation in injured DRG neurons. Diagrammatic representation of a potential mechanism whereby peripheral nerve axotomy results in the elevated production of local growth factors (including BDNF) in DRG which in turn trigger well established intracellular conduction cascades including the PI3k/Akt pathway. Activation of this pathway would lead to hyperphosphorylation of intranuclear FOXO3a, enhanced nuclear export and subsequent ubiquitination and proteasomal degradation. This process could eventually lead to the decreased protein levels seen in injured DRG neurons, described in this study.

An interesting aspect of this study was the effect of unilateral axotomy on neuronal FOXO3a expression in contralateral L4,5 DRG and cervical ganglia located far from the injury level. Contralateral neurons showed a biphasic response where nuclear and cytoplasmic staining was increased by 1-day post injury, reduced by 2 and 4-days and elevated again by 1-week. Neurons of cervical DRG from 1-day sciatic nerve transected rats had higher FOXO3a immunostaining in small to medium neurons as compared to naïve controls. Few connections exist between neurons that innervate opposite sides of the body; however, a collection of clinical evidence confirms contralateral deficits in patients with one-sided injuries (Kozin, Genant et al.

1976, Oaklander, Romans et al. 1998). The bilateral impact of nerve injury has been reported in a number of animal models where sensory, sympathetic or motoneurons opposite to the lesion site differ morphologically and/or biochemically from naïve controls (Koltzenburg, Wall et al. 1999). In addition to these changes, neurons of intact DRG contralateral or segmentally adjacent to the injured ganglia have altered nerve sprouting in unaffected limb areas (Devor, Schonfeld et al. 1979, Navarro, Verdu et al. 1997, Oaklander and Brown 2004). In general, these observations are considered a result of a neural mechanism with a propagating signal through the spinal cord and not due to a global effect. However, our results support the existence of a humeral response to peripheral nerve axotomy whereby injury signals originating from the lesion site and/or systemically released may circulate via spinal fluid or bloodstream and influence uninjured sites. Systemic administration of glucocorticoids and adrenalectomy was shown to affect neuropeptide levels in DRG neurons (Smith, Seckl et al. 1991, Covenas, DeLeon et al. 1994), which express the glucocorticoid receptor (DeLeon, Covenas et al. 1994). The changes seen in FOXO3a of uninjured DRG neurons might be caused by such circulating factors, but a relationship between nerve injury and steroidal hormone release has not been identified. As FOXO3a appears primarily regulated by post-translational modifications, its plausible a global stress event in the nervous system such as trauma could extrinsically lead to altered protein degradation or nuclear shuttling. The reality of contralateral and humeral effects of nerve injury illustrates the importance of using naïve animals and not uninjured tissue as controls.

3.4.3. Potential neurotrophin regulated mechanism

Neurotrophins are known to influence the phosphorylation and cellular localization of FOXO transcription factors *in vitro* (Gan, Zheng et al. 2005). NGF action through the high affinity trkA receptor induces Akt-mediated phosphorylation of FOXO3a (Zheng, Kar et al. 2002). Withdrawal of NGF results in FOXO3a nuclear translocation in cultured sympathetic neurons leading to *bim* gene expression and apoptosis (Gilley, Coffer et al. 2003). BDNF can also regulate the FOXO3a transcription factor, as trophin treatment causes a rapid decrease in nuclear levels with a corresponding increase in cytosolic protein (Zhu, Bijur et al. 2004). The PI3K/Akt cascade is again responsible for this response but whether BDNF is acting through the high-affinity trkB receptor or the common neurotrophin receptor p75 is unknown. Each can modulate

the Akt pathway (Lachyankar, Condon et al. 2003) and are suitable receptor candidates for the BNDF mediated phosphorylation of FOXO3a.

For adult sensory neurons, BDNF stands alone in the neurotrophin family as both a target-derived and a neuron-derived trophic factor. In the naïve ganglia, small to medium sized nociceptive neurons express BDNF (Wetmore and Olson 1995, Verge, Gratto et al. 1996, Karchewski, Gratto et al. 2002); however, in the intact state this subpopulation is not highly responsive to the neurotrophin (Kashiba, Ueda et al. 1997). These nociceptors are responsive to NGF as they exhibit high levels of trkA (McMahon, Armanini et al. 1994). Consequently, BDNF gene expression is strongly stimulated by NGF/trkA signaling (Apfel, Wright et al. 1996, Verge, Gratto et al. 1996). The low affinity neurotrophin receptor p75 is present in the majority of DRG neurons, including all trkA and trkB positive neurons and those expressing trkC (Verge, Merlio et al. 1992, Wright and Snider 1995, Karchewski, Kim et al. 1999). Upon injury to the peripheral branch of sensory neurons, there is a marked shift in neurotrophin content and neurotrophin receptor expression. Although an initial increase in glia-derived NGF occurs in the proximal nerve stump immediately following axotomy (Abrahamson, Bridges et al. 1987), retrograde axonal transport of the target derived neurotrophin decreases dramatically ((Raivich, Hellweg et al. 1991). Conversely, ~80% of injured neurons up-regulate BDNF 1-day post-injury, including all small nociceptors and a number of trkB neurons (Karchewski, Gratto et al. 2002). The transient increase in NGF in the proximal nerve stump may account for this BDNF upregulation in the trkA responsive neurons. After 1-week, BDNF levels are reduced in this small diameter population (Michael, Averill et al. 1999, Zhou, Chie et al. 1999) and elevated in larger trkB and trkC positive, medium to large sized neurons (Karchewski, Gratto et al. 2002). Another consequence of sciatic nerve transection is down-regulation of the trk and p75 receptors (Verge, Riopelle et al. 1989, Verge, Merlio et al. 1992, Zhou, Rush et al. 1996, Karchewski, Gratto et al. 2002), further compounding an already altered trophin environment. Neuroprotection is the putative role of increased BDNF (Murphy, Borthwick et al. 2000) but the neurotrophin also serves as the induction signal for the regenerative response (Geremia, Pettersson et al. 2010).

FOXO3a amounts declined in lumbar DRG neurons after transection of the sciatic nerve. Injury amplifies BDNF expression, and lead to the theory that elevated trophin signaling contributes to the protein loss. One drawback to this notion is the already robust levels of

FOXO3a in small diameter nociceptive neurons, which produce the highest quantities of BDNF in the intact DRG. This quandary is similar to the p-Akt pattern seen in DRG, where constitutively high activity of the kinase in small naïve neurons does not induce significant FOXO3a degradation. These situations suggest that moderate BDNF signaling or Akt activity can occur in these neurons without interfering with the protein's stability. However, in the event of injury or stimulation, BDNF and p-Akt levels may rise past this threshold such that their effects on FOXO3a cannot be inhibited.

We speculate, that during the injury response of sensory neurons, increased production of BDNF and heightened signaling through p75 results in elevated activity of Akt (Lachyankar, Condon et al. 2003). As a result, FOXO3a is persistently phosphorylated at key serine/threonine residues, leading to nuclear exclusion, polyubiquitination and proteasomal degradation. Low FOXO3a expression in larger neurons might be a consequence of higher trkB levels in this population and an increased sensitivity to BDNF. While other factors are most certainly involved, the unique role BDNF in injured neurons designates the neurotrophin as the possible signaling molecule of this mechanism. This line of thinking is further supported by our observations that modulating BDNF expression in sensory neurons through electrical stimulation which upregulates neuronal BDNF expression or knocking down sensory neuron BDNF with selective siRNAs led to reduced or increased levels of FOXO3a expression respectively.

3.4.4. Conclusion and significance

Cellular stress and trauma has a profound effect on cell phenotype, as epitomized by DRG neurons that regenerate after injury. Transcription factor regulation during this compromised state is essential to the activation of repair and regeneration programs. The likely impact of peripheral nerve axotomy on FOXO3a and its post-translational state emphasizes the importance of these modification events, which are critical to injury pathophysiology and a promising target area for therapeutics. The apparent systemic response of the protein begs questioning as to whether a global response to injury exists and if so, can DRG neurons sense these subtle changes? The identity of the underlying mechanisms responsible for contralateral alterations is still in question.

4. Biphasic expression of the ER stress/UPR-associated transcription factor Luman/CREB3 in sensory neurons ipsilateral and contralateral to nerve injury

4.1. Abstract

Peripheral nerve injury induces dorsal root ganglion neurons to regenerate severed axons, thus requiring increased protein production. The elevated protein load leads to a high incidence of misfolding and ER stress. Proper activation of the unfolded protein response during this state, via specific transcription factors and their gene products, assists in stress alleviation and is essential to regeneration following injury. Luman has been identified as an ER localized transcriptional regulator of the UPR and ER stress and is constitutively expressed in sensory neurons. Previously, we have shown nerve injury induces axonal Luman mRNA translation. These new proteins along with Luman already present in the axonal ER equivalent are activated, then retrogradely transported to the cell body as an injury signal necessary for neurite outgrowth at very early time points following crush injury. However, it is not known whether somal production of Luman is similarly altered following complete spinal nerve transection injuries quite proximal to the cell bodies over a more protracted time course, nor whether there is evidence of systemic responses manifested by altered expression in DRG neurons contralateral to injury. To investigate this, a peripheral axotomy time course model was employed where the L4-L6 spinal nerves are fully transected. The corresponding ipsilateral and contralateral DRGs were examined after the predetermined time points of 1-hr, 1-day, 2-days, 4-days and 1-week. Injury resulted in the time dependent biphasic up- and down-regulation of Luman protein in injured sensory neuron cell bodies and perineuronal satellite glial cells, while mRNA levels peaked at 2-days post-lesion then remained relatively unchanged for the remainder of the time course. Immunofluorescence histochemistry showed highest levels to be localized in the cytoplasm with nuclear staining appearing less intense. This increase was initially observed in all size ranges of neurons, albeit highest in small to medium sized neurons, peaking in the latter at the 2-day time point, declining in all size ranges by 4 days to pre-injury levels and then followed by greatly elevated levels at 1-week post-injury. Notably, there a was a largely parallel response in the DRG neurons contralateral to injury, albeit generally at lower levels suggesting a global or trans-spinal component to the response which was not discernible in the sham surgery time course. Axonal Luman levels were also elevated after a crush injury detectable just

proximal to the injury site as early as 3-hours post-injury, peaking at 1-day and then decreasing; supporting previous work that the protein is retrogradely transported. Electrical stimulation appeared to augment axonal levels and retrograde transport as stimulated nerves beyond the 3-hour time point showed less Luman staining. These findings support a role for Luman in distinct phases of the regeneration/cell body response including protective mechanisms utilized by these cells as they attempt to regenerate injured axons and also respond to systemic changes.

4.2. Introduction

A characteristic of peripheral nervous system neurons is their capacity for regeneration and self-repair. This property is essential to survival following injury; however, deleterious sequelae can occur that may hinder a successful recovery. Severance of the axon alters neuron phenotype as the priority changes from signal transmission to the creation of new fibre tracts. Regenerating axons require lipid membrane replenishment (Vance, Campenot et al. 2000) and cytoskeletal proteins (Galbraith and Gallant 2000), which can originate from cell body or axoplasmic endoplasmic reticulum (ER) (Koenig, Martin et al. 2000). Furthermore, nerve growth cones have a high metabolic demand as they explore the extracellular environment in search of trophic support and guidance cues. Necessary proteins and lipids are packaged in vesicles and sent to the distal axon tip via slow (Hoffman, Lopata et al. 1992) or fast (Ochs 1972) anterograde axonal transport. Augmented ER protein production and vesicular trafficking during regeneration can result in elevated levels of unfolded or misfolded proteins and ER stress can ensue (Saxena, Cabuy et al. 2009). Cellular mechanisms associated with an unfolded protein response (UPR) ameliorate this stress state and reestablish ER homeostasis (Schroder and Kaufman 2005). ER stress is linked to a number of neurological diseases where prolonged stress due to protein misfolding or an insufficient UPR leads to neuronal loss (Naidoo 2009).

ER localized processes regulate protein conformation and ensure proper folding. Excessive misfolding and accumulation triggers the UPR or ER stress response, which involves translational attenuation and the recruitment of molecular chaperones to assist with ER management (Ron 2002, Schroder and Kaufman 2005). ER-associated degradation (ERAD) can also alleviate this load, as malformed proteins are translocated to the cytoplasm and undergo ubiquitination and proteasomal degradation (Kopito 1997). These mechanisms are not mutually exclusive events, as gene products of the UPR are involved in the protein degradation pathway

(Schroder and Kaufman 2005); however, ERAD is the ideal route as apoptosis is a consequence of the UPR (Rao, Peel et al. 2002, Oyadomari and Mori 2004).

Three distinct ER signaling cascades are triggered during the UPR and all result in the downstream modulation of transcription factors. Each pathway involves a specific ER-membrane bound protein: pancreatic eukaryotic initiation factor subunit 2α (eIF2α) kinase (PERK), inositol requiring 1 (IRE1), or activating transcription factor 6 (ATF6). ER stress induced PERK phosphorylation of eIF2α leads to the translational activation of ATF4 (Harding, Novoa et al. 2000, Scheuner, Song et al. 2001), an activator for the pro-apoptotic gene CHOP (Ma, Brewer et al. 2002). The kinase endoribonuclease IRE1 is activated during the UPR and mediates the alternative splicing of the XBP1 mRNA (Yoshida, Matsui et al. 2001, Calfon, Zeng et al. 2002). The XBP1 transcript encodes a transcription factor with gene target specificity for ER chaperone molecules containing the unfolded protein response element (UPRE) (Yoshida, Matsui et al. 2001). ATF6 is bound to the ER and in response to ER stress undergoes regulated intramembrane proteolysis (RIP) (Haze, Yoshida et al. 1999), a process first identified with SREBP (Eberle, Hegarty et al. 2004). After two separate cleavage events, the released ATF6 catalytic domain translocates to the nucleus where it primarily activates ER chaperone genes (Yoshida, Okada et al. 2000). These are well-characterized ER stress response pathways; however, other mechanisms exist and evidence points to the involvement of additional transcription factors.

Luman (CREB3/LZIP), a member of the CREB/ATF family, is homologous to the herpes simplex virus (HSV) protein VP16 (Lu, Yang et al. 1998) and was identified as the first cellular ligand for host cell factor-1 (HCF-1) (Lu, Yang et al. 1997). Its association with HSV machinery suggests an involvement in viral latency and reactivation (Freiman and Herr 1997, Lu, Yang et al. 1997, Lu, Yang et al. 1998, Lu and Misra 2000). Protein processing resembles that of ATF6, where Luman bound to the ER undergoes RIP and the basic leucine zipper (bZIP) domain is free to activate target genes (Raggo, Rapin et al. 2002). The cleaved form of the transcription factor is extremely unstable and *in vitro* detection of the activated protein requires proteasomal inhibitors; however, studies have shown the protein to be an activator of transcription (Misra, Rapin et al. 2005). The bZIP proteins Zhangfei (Misra, Rapin et al. 2005) and Luman recruitment factor (LRF) (Audas, Li et al. 2008) potently and specifically inhibit Luman transactivation by possibly affecting protein stability. Once translocated to the nucleus, Luman can bind cAMP response

elements (CRE), including CRE promoter regions of HSV immediate early genes (Lu and Misra 2000), and the UPRE in a similar capacity to XBP1 (DenBoer, Hardy-Smith et al. 2005). Induction of Luman expression and proteolytic cleavage in response to ER stress results in the transcriptional activation of the ERAD-related Herp (homocysteine-induced ER protein) (Kim, Kim et al. 2008) protein via the ER stress response element II (ERSR-II) (Liang, Audas et al. 2006). The transcription factor is expressed in multiple rat tissue types, the highest levels seen in nervous tissue and DRG in particular (Ying, Zhang et al. 2015). The participation of Luman in the ER stress response led to the hypothesis that these cellular proteins are UPR components and are up-regulated in axotomized *in vivo* DRG neuron cell bodies. Previous work has shown that injured neurons necessitate a coordinated UPR and improving the response by increasing levels of certain ER stress regulators; in particular XPB1s, leads to increased myelin degradation, macrophage infiltration and enhanced axonal regeneration (Onate, Catenaccio et al. 2016). Studies from our lab demonstrated increased expression of ER stress markers including CHOP and BiP in both DRG soma and the axon in response to one day injury, with axonal elements being retrogradely transported back to the cell body (Ying, Zhai et al. 2015). Further seminal work by Ying et al identified Luman as an axonally localized transcription factor that co-localized and interacted with the transport molecule Importin-α (Ying, Misra et al. 2014). Axon-derived Luman is rapidly synthesized from axonally confined transcripts in response to axotomy and serves as an injury signal where the transcriptional activation domain is translocated retrogradely back to the DRG soma nuclei by an importin-dynein mediated mechanism. Remarkably, reduction of Luman with the use of small interfering RNA impaired neurite outgrowth of injured sensory neurons. This implies that the transcription factor can modulate the capacity for sensory neurons to regenerate, either by its action on the UPR or on other growth-related targets. Such targets include components of the cholesterol biosynthesis pathway as Luman knockdown reduces free and total cholesterol levels in injured sensory neurons and downregulates a number of genes involved in the regulation of cholesterol synthesis (Ying, Zhai et al. 2015). Cholesterol is an essential component of plasma membrane that is acquired in abundance for the growth of regenerating axons. The potential importance of Luman in the regulation of the regeneration response via cholesterol biosynthesis was supported by enhanced neurite outgrowth in Luman knockdown neurons with cholesterol supplementation (Ying, Zhai et al. 2015).

Our initial studies focused on early (one and two day) alterations in Luman expression, its subcellular localization in neurons and axons ipsilateral to crush injury and its role in the inductive phase of the cell body response to injury. Recent work has revealed there to be distinct transcriptional phases to the cell body response that include an early stress response phase that is over by 6-hours post-injury, followed by a pre-regeneration phase that lasts until 4 days post-injury and finally a regeneration phase that is clearly on by 7 days (Li, Xue et al. 2015). This coupled with my findings that the transcription factors FOXO3a and Zhangfei also appear to have distinct phasic cell body responses to the injury that extend beyond those observed in ipsilateral neurons to include phasic impacts on neurons contralateral and remote to the injury (Thesis chapters 3&5), lead me to hypothesize that Luman expression also undergoes distinct phasic responses to nerve injury. Thus, this study examined the effect of nerve transection on the spatial and temporal expression patterns of Luman, primarily at the level of the sensory neuron cell body in DRG ipsilateral and contralateral to injury. The findings reveal distinct biphasic responses for Luman in both ipsilateral and contralateral DRG neurons, supporting a role for Luman not only as a sensor of injury but also one implicated in all transcriptional phases of the cell body response associated with axon regeneration. Furthermore, the apparent involvement of this regulator of the UPR in the injured state highlights the significance of ER stress in this pathology.

4.3. Results

4.3.1. Luman expression pattern in DRG neurons

The specificity of the anti-Luman antibody employed in my studies was assessed immunohistochemically on frozen sectioned DRG tissue samples incubated with anti-Luman or anti-Luman serum absorbed with cell protein isolates from Luman transfected Vero cells (Fig. 4.1A). Absorption of the antibody resulted in near elimination of immunostaining as compared to tissue samples processed with anti-Luman alone. Western blot analysis was performed to further characterize the specificity of the anti-serum, where membranes of electrophoresed lysates of cells transfected with Luman (lane 2, Fig 4.1B) and Zhangfei (lane 3, Fig. 4.1B) were treated with anti-Luman serum. In the Luman transfected cell extract, Anti-Luman recognizes a single band of approximately 60 kDa, the suspected molecular weight of unprocessed Luman; while unable to detect any identifiable antigen in the Zhangfei transfected cell extract at its expected molecular weight of ~30 kDa. Luman anti-serum also recognized 2 identifiable bands in rat DRG extract of approximately 37 and 15 kDa, which are predicted molecular weights of Luman protein cleavage products (Fig. 4.1C).

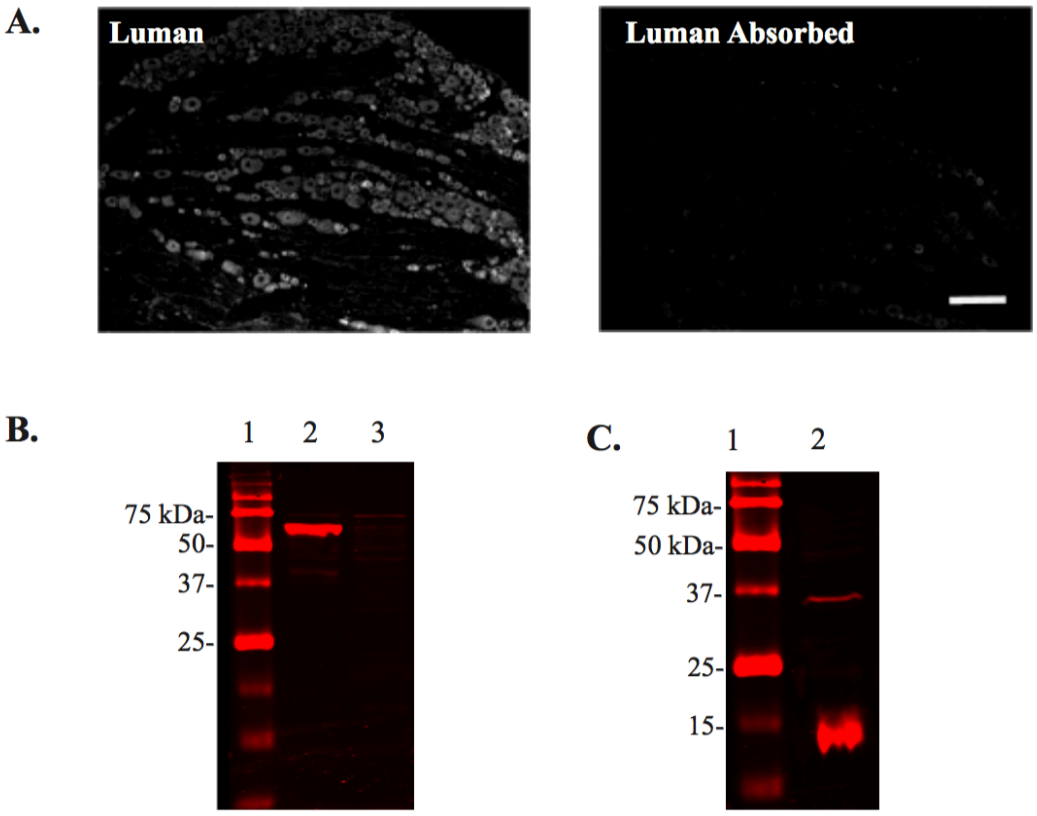

Figure 4.1 Luman antibody specificity controls. A. Fluorescence photomicrographs of L5 DRG (6µm sections) processed for immunohistochemistry detect Luman protein with anti-Luman rabbit serum (left) and anti-Luman absorbed with cell protein isolates from Luman transfected vero cells. Scale bar = 100 µm. Note: Absorption of anti-Luman abolishes immunofluorescence staining. B. Western blot analysis of anti-Luman rabbit serum treated membrane of electrophoresed protein extracts from Vero cells transfected with Luman (lane 2) and Zhangfei (lane 3). Molecular weight marker (lane 1). Note: In the Luman transfected cell extract, Anti-Luman recognizes a single band of approximately 60 kDa, the suspected molecular weight of unprocessed Luman; while unable to detect any identifiable antigen in the Zhangfei transfected cell extract at its expected molecular weight of ~30 kDa C. Western blot analysis of anti-Luman serum treated membrane of electrophoreses protein extracts from normal L4-L5 DRG (lane 2). Molecular weight marker (lane 1) Note: Anti-Luman rabbit serum recognizes 2 additional bands of approximately 37 and 15 kDa, the predicted molecular weights of Luman protein cleavage products.

As the unstable catalytic domain of Luman is cleaved and translocates to the nucleus in response to stress events, the nuclear envelope protein Lamin B was used to assure accurate

visualization of the nucleus to allow for quantification of alterations in Luman nuclear levels. Only neurons with complete and distinct Lamin B staining around the nucleus were considered for further analysis (Fig. 4.2).

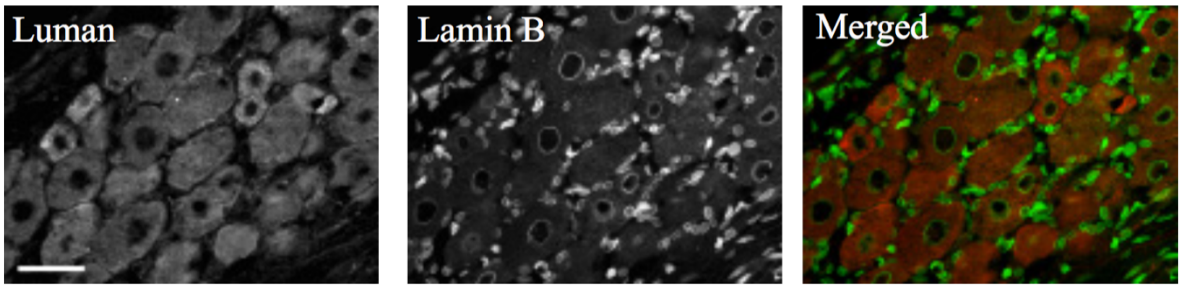

Figure 4.2. Double staining for Luman and the nuclear envelope cytoskeletal protein Lamin B allows for accurate analysis of nucleus-associated immunofluorescence. Fluorescence photomicrographs of L5 DRG (6 µm sections) processed for immunohistochemistry with a Luman and Lamin B specific antibody. Scale bar = 100 microns. To ensure proper cellular localization, all tissue was dually processed for Luman (red) and LaminB (green) immunofluorescence for more accurate differentiation of nuclear and cytoplasmic staining.

4.3.2. Sciatic spinal nerve transection induces biphasic changes in DRG Luman expression

The effect of nerve transection on Luman sensory neuron expression was investigated by axotomizing the sciatic nerve of male Wistar rats at the level of the L4-6 spinal nerves to assure complete transection of virtually all axons within the ganglion. *In situ* hybridization and immunofluorescence histochemistry of sectioned DRG were used to assess alterations in mRNA and protein levels respectively. Luman protein levels in naïve ganglia were higher in small-medium diameter neurons, with low-moderate nuclear staining in a small fraction of these cells and in perineuronal cell nuclei. At 1-day post axotomy (Figs. 4.3; 4.5), ipsilateral neurons had slightly higher protein and mRNA (Fig. 4.6) levels compared to naïve controls. Peak Luman protein expression was seen at 2-days post-injury, where high cytoplasmic and nuclear immunoreactivity was clearly evident in the subset of small-medium sized neurons, with larger neurons demonstrating only slightly increased levels (Figs. 4.3; 4.5). Parallel changes were also observed for nuclear Luman levels in the perineuronal, presumably satellite glial cells.

At 4 days, protein levels were dramatically decreased in all size ranges of neurons and the perineuronal cell nuclei with only a few neurons appearing moderately labelled, similar to the

naïve state in both cytoplasmic and nuclear staining (Figs. 4.3; 4.5); however, Luman mRNA remained high in DRG ipsilateral to 4-day spinal nerve lesion (Fig. 4.6). Thus, the decrease in protein levels at 4-days are then likely attributed to regulation at the post transcriptional level. By 1week after injury, neuronal protein levels had risen dramatically, with elevated protein expression in both neurons and perineuronal cell nuclei (Fig. 4.3; 4.5). These observations suggest that as result of injury and/or regeneration, ER stress may be initially higher in small presumably nociceptive neurons and only rise to higher levels in larger proprioceptive or mechanoreceptive cells at later time-points.

Our previous studies focused primarily on increases in axonal Luman synthesis, retrograde transport of the activated Luman signal back to the cell body following injury, and the significance of this retrograde signal in regulation of early axon regeneration through its regulation of the UPR and cholesterol (Ying, Misra et al. 2014, Ying, Zhai et al. 2015). Present data using temporal analysis of the axotomy response reveals clear biphasic changes in Luman expression at the neuronal and perineuronal cell levels, both ipsilateral and contralateral to injury. This suggests that in addition to axonal Luman "sensing" an injured state and encoding a retrograde signal, alterations in neuronal and perineuronal Luman expression likely serve as part of an adaptive response to help mitigate regeneration-associated ER-stress or UPR challenges.

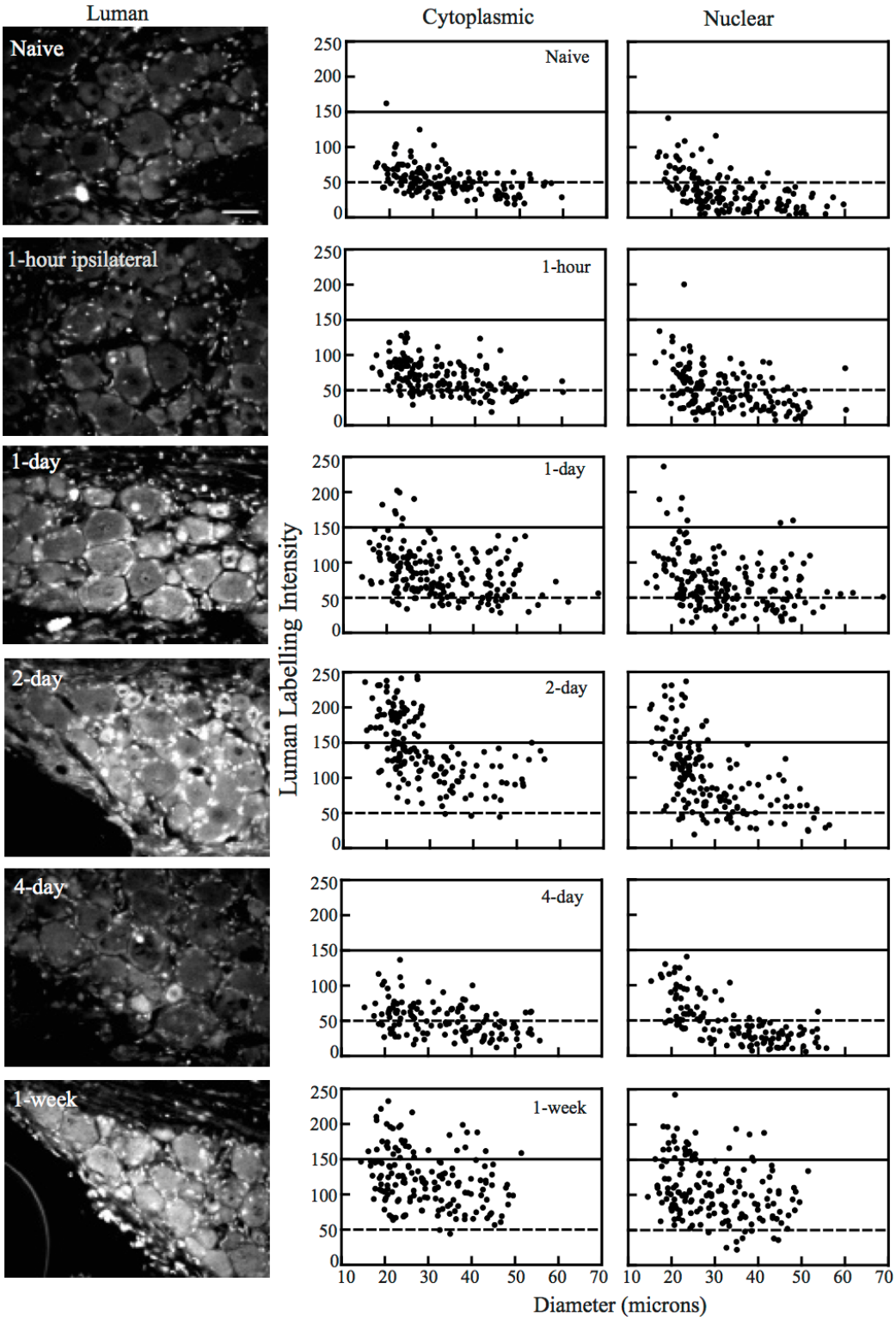

Figure 4.3. Peripheral nerve injury alters Luman protein levels in DRG neurons ipsilateral to axotomy. *Left Column*. Fluorescence photomicrographs of L5 DRG (6 µm sections) processed for immunohistochemistry to detect cell Luman protein. Ipsilateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day

and 1week. Scale bar = 50 μm. Naïve animals served as controls. *Right Column*. Representative scatterplots depicting relative changes in Luman immunofluorescence signal over individual cytoplasmic and nuclear regions as related to cell size from sections all processed under identical conditions in parallel. Experimental states as indicated. Dashed lines divide the plots into low versus moderate to heavily labelled populations. N= 181 to 206 neurons analyzed per condition.

4.3.3. Unilateral nerve transection has contralateral effects on Luman protein expression

Phenomena of altered protein expression occurring on the uninjured/contralateral side are well documented. Here, severance of the right sciatic spinal nerves caused changes to left-side (contralateral) Luman protein concentrations (Figs. 4.4; 4.5), whereas mRNA expression was relatively stable (Fig. 4.6). While the first peak in Luman expression ipsilateral to lesion occurred at 2-days post-lesion, the first peak in Luman expression contralateral to lesion was evident at the 1-hour time point, with elevated neuronal cytoplasmic and nuclear Luman immunoreactivity. In addition, there was elevated Luman in the nuclei of perineuronal satellite glial cells at this early time point. This heightened expression pattern was still evident at 1-day (Figs. 4.4; 4.5). By 2 days a slight decline in Luman levels was discernible with once again significantly lower levels found in the 4-day injured DRGs with levels similar to that in naïve neurons (Figs. 4.4; 4.5). However, unlike the ganglia ipsilateral to lesion, the Luman detected in the perineuronal presumably satellite cell nuclei at 4-days post-injury were still quite elevated, albeit lower than 2-days post-injury (Fig. 4.4).

Evidence exists claiming “unstressed” cells undergo a UPR (Schroder and Kaufman 2005); therefore, as contralateral neurons are not injured, the source appearing to trigger an ER stress response likely originates either systemically or from the site of injury. The increased immunofluorescence at 1 hour is not mirrored in mRNA levels (Fig. 4.6), as expression of Luman mRNA does not appear to change contralaterally, although one cannot rule out an impact on mRNA stability. The elevated Luman levels in the neuronal cell bodies may arise from increases in Luman retrogradely transported from distal axons in the periphery or from the central axon branch, which might be sensing trans-spinal disturbances from the ipsilateral injured side, although the former is unlikely in the contralateral neurons. However, repression of Luman protein at 4-days both ipsilaterally and contralaterally suggests that the systemic response also

has distinct phases of transcriptional regulation as that recently described for DRG ipsilateral to lesion (Li, Xue et al. 2015).

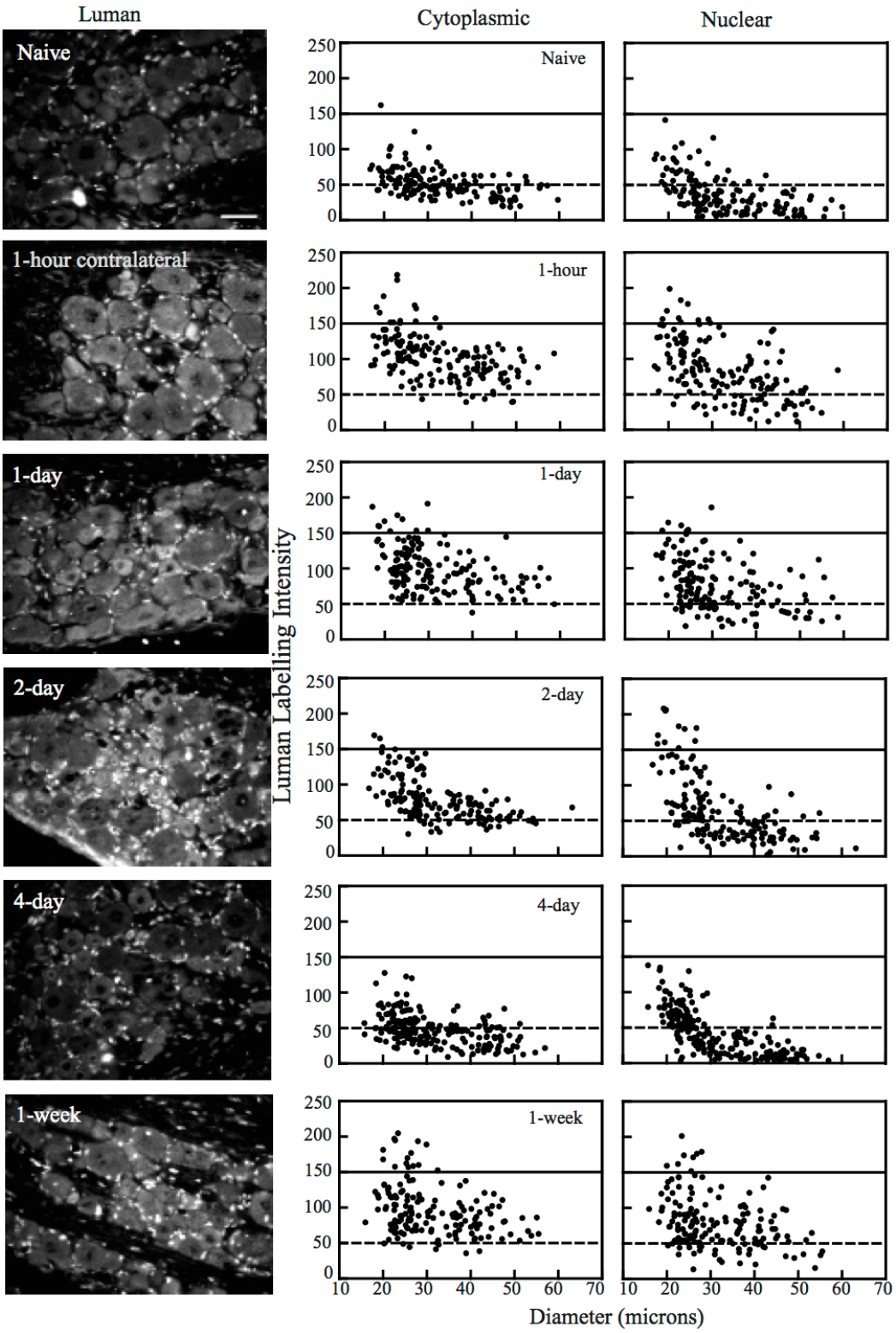

Figure 4.4. Peripheral nerve injury alters Luman protein levels in DRG neurons contralateral to axotomy. *Left Column*. Fluorescence photomicrographs of L5 DRG sections (6 µm) processed for immunohistochemistry to detect Luman protein. Contralateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day and 1week. Scale bar = 50 µm. Naïve animals served as controls. *Right Column*. Representative scatterplots depicting relative changes in individual neuronal Luman immunofluorescence signal over cytoplasmic and nuclear regions as related to cell size from sections processed under identical conditions and in parallel. Experimental states as indicated. Dashed lines divide the plots into low versus moderate to heavily labelled populations. N= 181 to 204 neurons analyzed per condition.

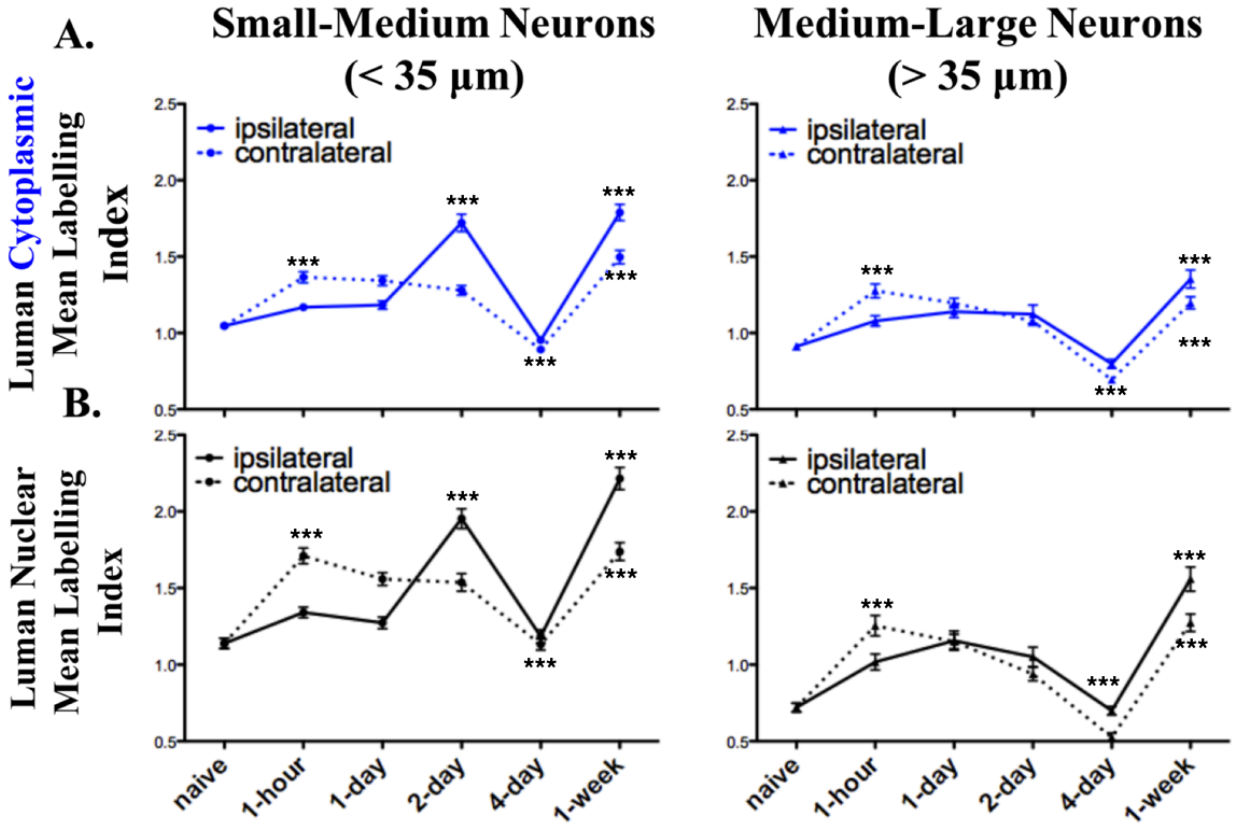

Figure 4.5. Peripheral unilateral axotomy causes bilateral biphasic alterations in Luman protein immunoreactivity in both injured and contralateral uninjured DRG neurons. Luman summary line graphs of alterations in the mean + s.e.m. cytoplasmic (A, blue) and nuclear (B, black) immunofluorescence intensity levels observed in small-medium sized (< 35 µm, column 1) and medium-large sized (>35 µm, column 2) DRG neurons ipsilateral (solid line) and contralateral (dashed line) to injury at time-points as indicated. N=3 animals for each data point. ***p value<0.001 ANOVA with Dunn's post-test analysis. Note: relative changes in nuclear localization parallel that observed for the cytoplasmic staining.

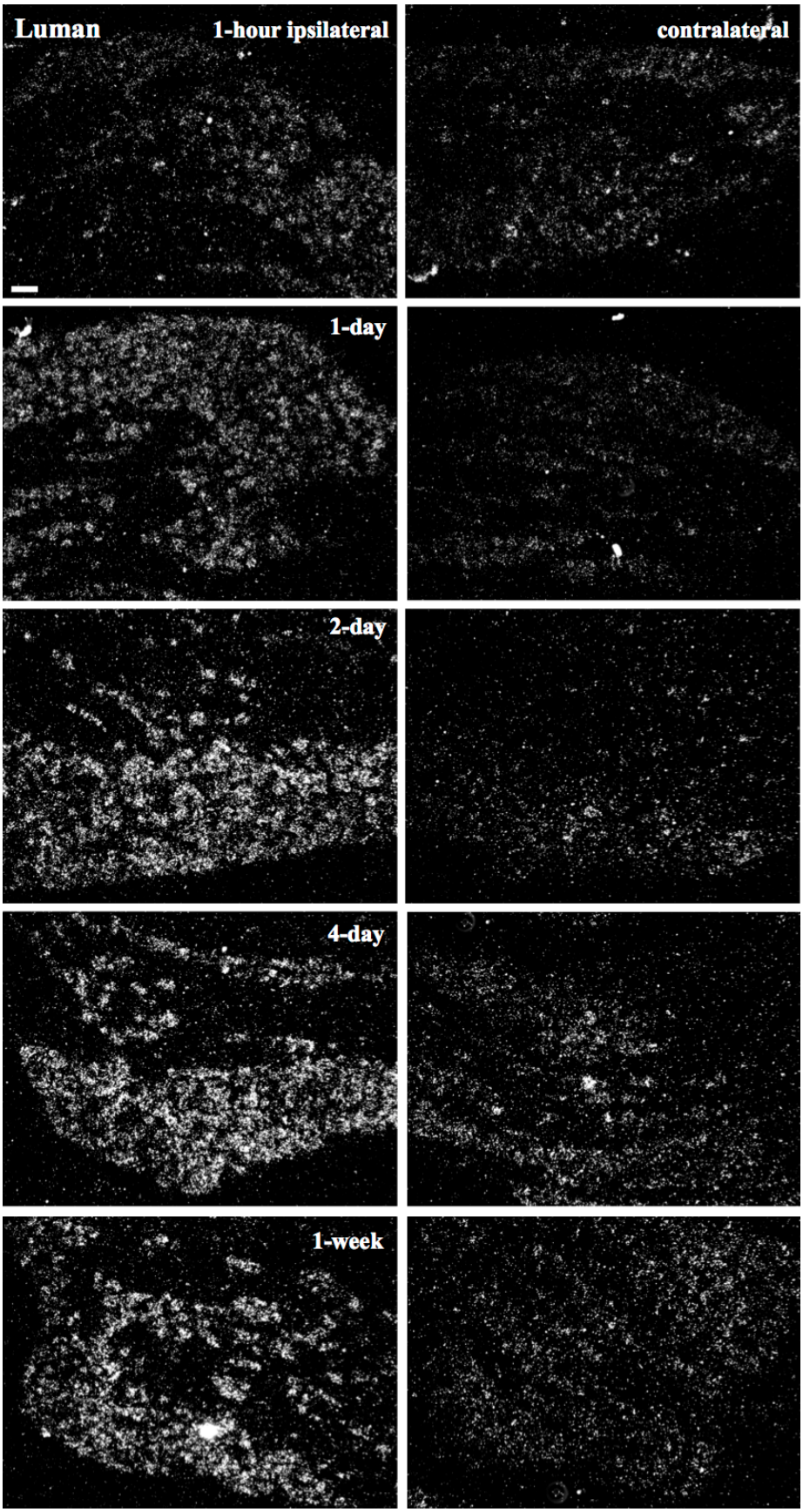
Luman
1-hour ipsilateral
contralateral
1-day
2-day
4-day
1-week

Figure. 4.6. Unilateral peripheral axotomy alters Luman mRNA expression levels in cell bodies of DRG neurons ipsilateral to injury. Representative darkfield photomicrographs of L5 DRG sections (6μm) processed for *in situ* hybridization with a 48mer oligonucleotide radiolabeled probe specific for Luman mRNA in order to visualize transcript localization and expression following injury. Ipsilateral (*Left column)* and contralateral (*Right column*) DRG sections from L4-L6 unilateral spinal nerve transected rats harvested 1-hour, 1-day, 2-days, 4-days and 1-week after injury. Naïve animals served as controls (image not shown). Scale bar = 100 μm. N =3 for each condition. Note; Peripheral nerve axotomy causes a relative increase in Luman mRNA levels in DRG neuron cell bodies ipsilateral to injury gradually throughout the time course, peaking around the 2-day – 4-day post lesion time point. Luman oligonucleotide probe specificity controls were previously published (Ying, Zhang et al. 2015).

4.3.4. Luman levels are not discernibly altered by sham surgery

Because there is a robust increase in Luman detected in the nuclei and cytoplasm of neurons and perineuronal cells in contralateral ganglia across all size ranges of neurons, we had to ascertain whether these contralateral changes in expression were due to systemic stress response associated with the surgical exposure or the actual spinal nerve lesions. Thus, sham surgeries were performed for the entire time course and qualitatively assessed for marked responses in L4,5 DRG ipsilateral or contralateral to the surgical exposure site. Qualitative analysis of ipsilateral or contralateral L5 DRG sections processed for Luman immunofluorescence (N= 3 animals/time point), did not reveal any significant changes from naïve controls, suggesting that the changes observed in the axotomy time course were due to the nerve injury imposed and not the surgical stress-state (Fig. 4.7).

4.3.5. Sciatic nerve crush injury and brief electrical nerve stimulation result in altered Luman levels in nerve fibres proximal to crush site

Previous work by our group showed that Luman protein and mRNA resides in axons of DRG neurons, the former in the axonal ER equivalent, while the latter can be translated locally from axonal Luman mRNA (Ying, Misra et al. 2014). Indeed, axonal synthesis of the transcription factor is upregulated following injury in *in vitro* models where the neurons were injury-conditioned for 1 day prior to assay. ER-resident axonal Luman is cleaved upon axon injury and the N-terminal active portion is then retrogradely transported back to the cell body in an importin mediated manner with increased levels of the N-terminal seen at the level of the cell body and nucleus (Ying, Misra et al. 2014). Further, axonal siRNA knockdown of Luman expression

greatly diminishes neurite outgrowth following injury. The results from Ying et al. advocate the role of Luman as a retrograde injury signal and an inducer of the injury response. The current work has shown that axotomy causes a rapid upregulation of Luman protein and mRNA at the cell body level with modulation of Luman expression over a week time course being biphasic in nature. While we speculated that the neuronal increases in Luman protein levels could potentially reflect increased retrograde delivery of the injury signal from the axon, it is also possible that the decrease observed 4-days post-lesion represents increased anterograde delivery to the axon or potentially enhanced degradation. To assess the potential impacts of axonal Luman in the injury-associated changes observed at the cell body level, we temporally examined changes in Luman levels in injured axons using an *in vivo* time course model to determine if the axonal changes also exhibited a biphasic expression pattern. Further, we also examined whether brief electrical nerve just proximal to the injury site, a therapeutic intervention shown to improve axon regeneration (Al-Majed, Neumann et al. 2000, Geremia, Gordon et al. 2007) might alter the nerve-derived Luman signal, as Luman is recognized as a pro-regeneration retrograde signal.

Thus, the sciatic nerves of rats sustained a crush injury at the mid-thigh level and nerve samples immediately proximal to the crush site were examined for Luman immunostaining at 3-hours, 1-day, 3-days and 7-days post-injury with naïve animals serving as control (Fig 4.8). Axonal localization of Luman was accomplished by βIII-tubulin co-staining, a nerve cytoskeletal marker (Fig 4.8 A). After only 3-hours of the crush injured state, protein levels are markedly increased in proximal nerve fibres compared to naïve tissue (Figure 4.8 A, B). As Luman is readily synthesized in injured axons, these protein products are likely rapidly produced from already present axonal transcripts. Nerve protein levels continue to rise at the 1-day time point and retrograde transport of its activated/cleaved N-terminal likely contributes to elevated Luman observed at the neuronal cell body level (Fig 4.8, D; Fig. 4.3). This time point correlates to increases in protein and mRNA at the cell body that was seen in the axotomy model. After crush injury alone this rise is transient as Luman axonal immunofluorescence is decreased by the 3-day time point. (Fig 4.8, F). This decreased immunostaining is due to either increased retrograde transport back to the cell body, but is rather most likely secondary to a degradation process as protein levels begin to lower in ipsilateral injured DRG neuron cell bodies around the 3 to 4 days. At 7-days, axonal Luman levels are again elevated, possible coinciding with a delayed growth phase taking place post injury after the initial regeneration response (Fig 4.8. H). One

hour of electrical stimulation (ES) proximal to the crush site immediately after injury results in initially increased levels at 3 hours post crush+ES, even relative to crush alone (Fig 4.8, C,B) followed by decreased levels at 1-day and 3-days postlesion (Fig 4.8, C, E, G). ES is known to augment the injury/regeneration response for injured sensory neurons; upregulating RAGs and increasing axon outgrowth. Stimulation appears to decrease levels of axonal Luman possibly by inducing increased retrograde transport of the injury signal. At 7 days (Fig 4.8, H, I) crush and crush plus ES groups have very similar Luman staining, as the impact of the one hour ES at the time of crush is likely dissipated with respect to augmenting the cell body response.

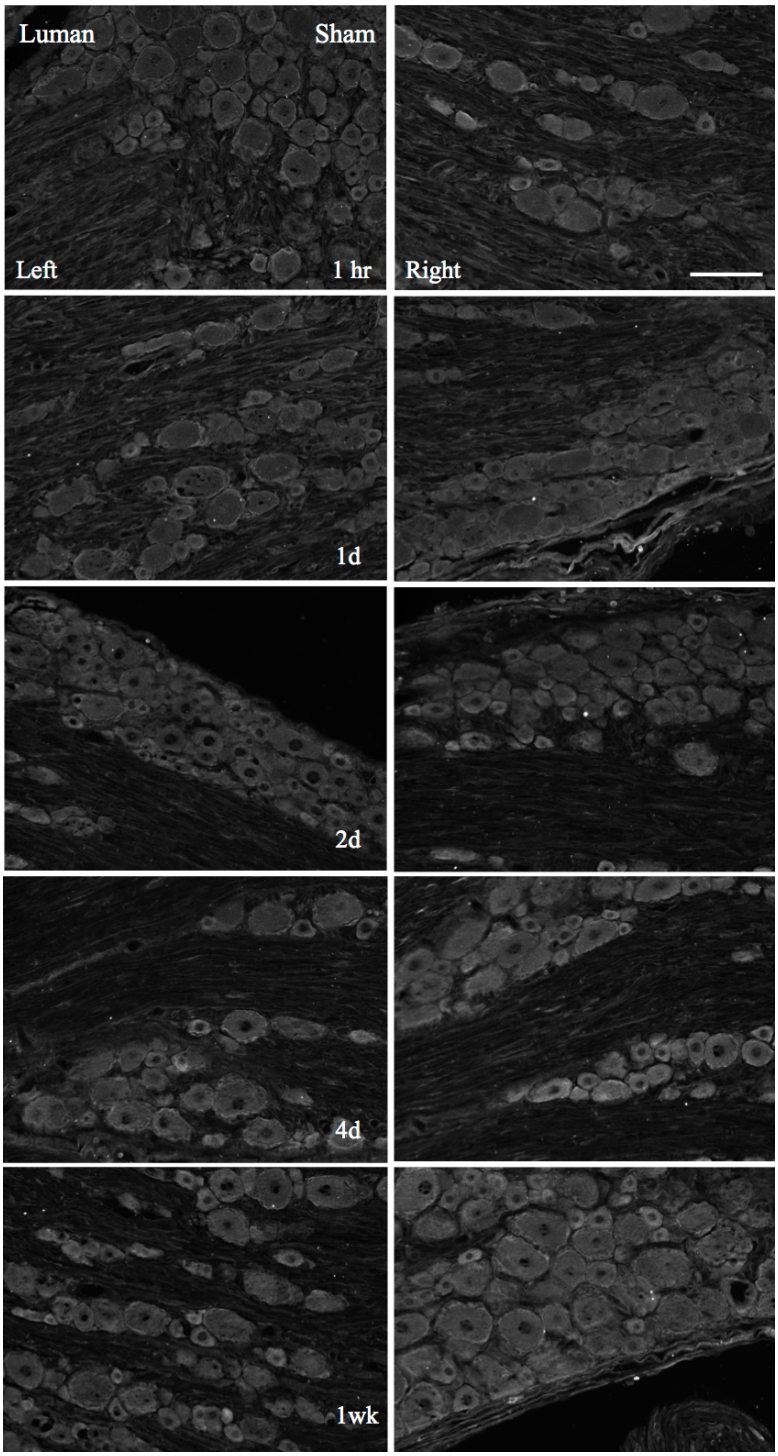

Figure 4.7 Sham surgeries did not change Luman immunofluorescence levels in DRG neurons. Fluorescence photomicrographs of L5 DRG (6 µm sections) processed for immunohistochemistry to detect cell Luman protein from animals that underwent sham surgeries where the right L4-L6 spinal nerves were exposed but not injured. Right and left dorsal root ganglia were harvested from each animal at post-surgical times 1-hour, 1-day, 2-days, 4-days and 1-week (as indicated). Scale bar = 100 µM. Note: Luman immunostaining does not discernibly

change between each time point and the right versus left side examined. Naïve animals served as controls and did not differ from that observed at the different sham time points examined (data not shown). N=3 animals/time point.

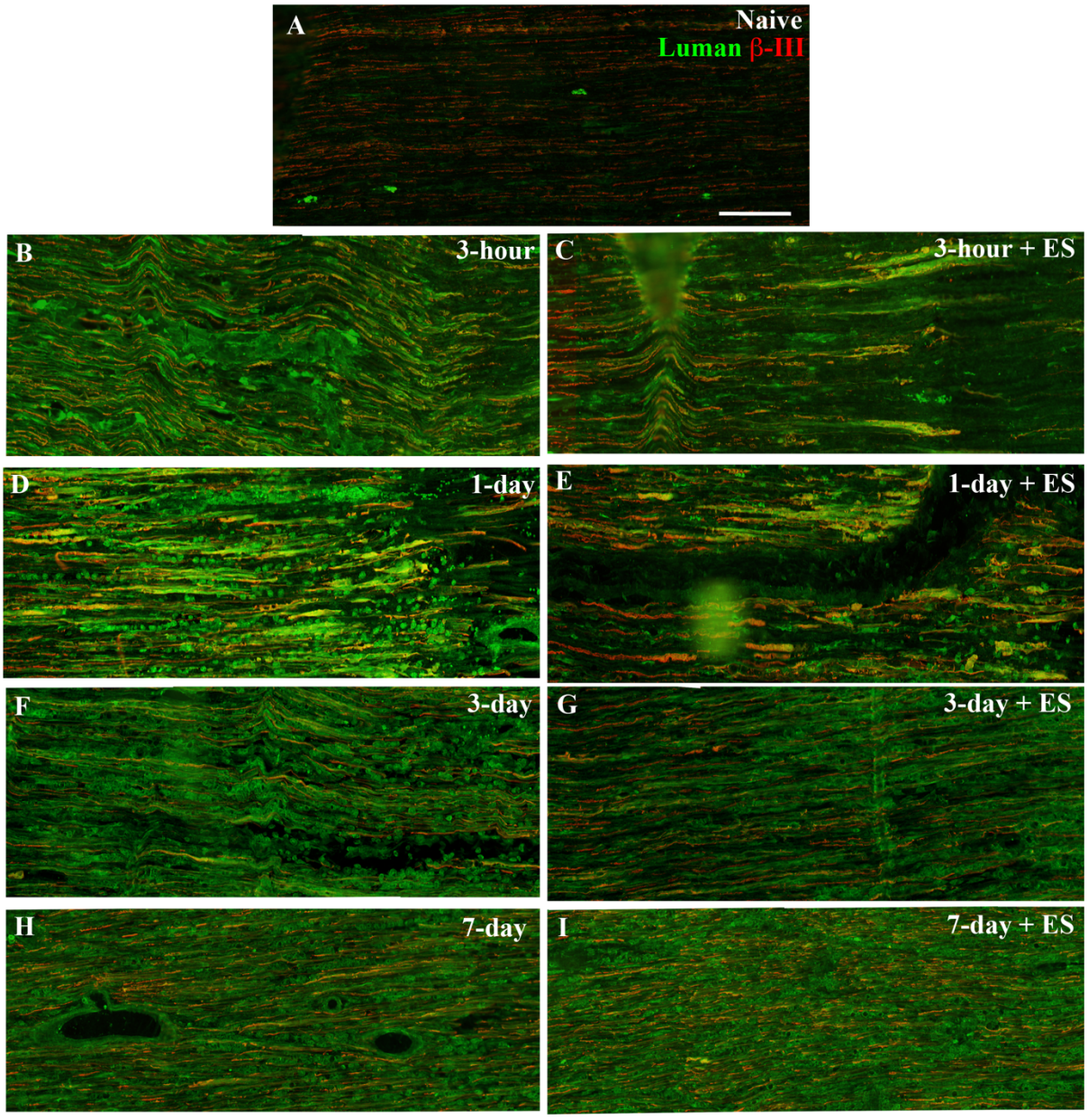

Figure 4.8 Axonal Luman levels are altered by crush injury and brief electrical stimulation. A. Representative fluorescence photomicrographs of ipsilateral sciatic nerves just proximal to the crush injury site (located at the far right of each photomicrograph; 10 μm sections) processed for immunohistochemistry to detect axonal Luman (green), the axon marker βIII tubulin (red) and their co-localization (yellow) from animals that underwent sciatic nerve crush injuries at the mid-

thigh level 3-hours, 1-day, 3-days, and 7-days post injury (B,D,F,H). Immediately following injury, brief 1-hour 20 Hz electrical stimulation (ES) was applied proximal to the crush site (C,E,G,I). N= 3 for each time point; naïve animals served as controls. Scale bar = 100 μm. Note: Crush alone appeared to increase Luman axonal staining as early as 3 hours post injury and peaking at 1 day. By 3 days this is decreased, however slightly elevated again at 7 days. ES initially increased (3 hour), then decreased Luman staining as early as 1 day post-injury, suggesting possibility of increased cleavage and clearance of the protein by increased retrograde transport of the injury signal. By 7 days post-injury Luman levels are again detectable at comparable levels in axons from both experimental groups.

4.4. Discussion

These studies on Luman expand previous work by our group, where we discovered it to be an early injury-associated and axon-localized UPR and axon regeneration regulator. My focus was on expanding our understanding of its potential role in the cell body response of DRG neurons to acute and prolonged axotomy. I sought to determine whether Luman protein and mRNA were coordinately altered in the injured sensory neurons at the somal level by employing the same temporal injury paradigm, utilized previously to study expression of another stress-associated transcription factor, FOXO3a, after injury. In addition, given ours and others' findings that brief electrical stimulation (ES) of injured peripheral nerves can be employed as an adjunct therapy to positively modulate the injury response, increase regeneration of axons and improve function in both animal and human models (Gordon 2016), I sought to determine whether alterations in Luman expression correlate with these events. My findings indicate that spinal nerve axotomy causes a marked upregulation of Luman mRNA and protein in the cell bodies of DRG neurons. Luman protein levels show a biphasic response with temporarily heightened levels early after injury peaking at 2-days post-lesion, decreasing to below baseline levels by 4 days and then increasing at the later time points. These levels were most robustly elevated in small to medium sized, presumably nociceptive, neurons initially but with parallel biphasic albeit lower increases in large diameter neurons which were most notable by one week. Similar to the cell body findings, nerves showed marked axonal Luman expression as soon as 3-hours post crush, confirming previous findings at the one day time point (Ying, Misra et al. 2014). Axonal increases were also biphasic in nature; treatment with brief electrical stimulation led to an initial increase at the 3 hours followed by a decreased axonal levels at 1 and 3 days relative to crush alone until one-week post-injury, when they were elevated and detectable at levels comparable to

crush injury alone. The most surprising finding was the dramatic impact of injury on Luman protein in neurons contralateral to injury that mirrored the injury response to some degree and paralleled that which we observed for FOXO3A and Zhangfei (Chapter 5). This response was not seen in sham animals, suggesting that surgical stress in itself was not a cause of the alteration but rather was due to responses to the injured state.

4.4.1. Luman expression in sensory neurons and its role in the nerve injury response

Luman is a known regulator of the UPR (Liang, Audas et al. 2006, Audas, Li et al. 2008), a role recently described for injured sensory neurons and one that was linked to the intrinsic ability of an injured axon to regrow (Ying, Zhai et al. 2015). Further, this association with ER stress is the likely explanation for the robust expression changes seen in injured neurons. Peripheral nerve damage causes a phenotypic shift in sensory neurons, from a "transmitting" cell to one of "regeneration" with goal of producing new fibres and growth back to previously innervated areas (Watson 1974). Sensory neurons upregulate a myriad of regenerative associated genes (RAGs) critical to axon growth including other transcription factors such as activating transcription factor-3 (ATF-3) (Tsujino, Kondo et al. 2000, Seijffers, Allchorne et al. 2006) c-Jun (Raivich, Bohatschek et al. 2004) and growth associated proteins (Bomze, Bulsara et al. 2001). This regeneration response causes a significant protein burden to the cell and evidence suggests that proper protein processing, ER stress and the UPR are critical especially during this growth phase. Studies show a correlation between PNS disease states and elevated levels of ER stress markers. For example damage to sciatic nerves increased expression of the chaperone protein BiP/GRP78 in the soma of non-degenerating neurons and the transcription factors XBP1s and ATF4 in degenerating motoneurons (Saxena, Cabuy et al. 2009, Penas, Font-Nieves et al. 2011). There are also reports of peripheral nerve damage upregulating other chaperone proteins and ER foldases, types of chaperones that assist in protein folding via forming non-covalent bonds, including calreticulin, endoplasmic reticulum protein-29 (ERp29) and ERp57 (also known as GRp58 or PDIA3) (Noel, Frost et al. 1995, Willis, Li et al. 2005, Castillo, Onate et al. 2015). Our lab described how injury leads to upregulation of DRG cell body and axonal UPR elements including BiP/GRP78 and CHOP, which are retrogradely transported to the cell body of injured DRG neurons (Ying, Zhai et al. 2015). However, the need for a coordinated and controlled

UPR/ER stress response for axon regeneration had not been proven. Onate et al, with a sciatic nerve injury model found that ablation of the ER stress regulator XBP1, but not ATF4, delayed motor recovery, decreased macrophage recruitment, reduced myelin removal and axonal regeneration (Onate, Catenaccio et al. 2016). Conversely, transgenic mice overexpressing XBP1 have enhanced regenerative events after nerve crush injuries. They assessed the therapeutic advantage of altering the UPR *in vivo* by local gene transfer experiments with XBP1s into DRG neurons which increased axon regeneration post-injury (Onate, Catenaccio et al. 2016). These and other findings are evidence that certain aspects of the UPR are necessary for the injury response of sensory neurons and our findings, along with the axonal Luman response, support the theory that the primary importance of upregulating Luman in injured neurons is to mitigate ER stress. However, with the discovery that Luman is pro-regeneration through transcriptional activation of the cholesterol biosynthesis pathway widens its potential as a sentinel regulator of other injury associated genes, including RAGs.

Primary sensory neurons of the DRG are heterogeneous in both structure and function. Distinct populations exist with specific sensory modalities (proprioception, mechanoception, nociception, etc.) and common morphological, biochemical, and physiological characteristics (Lindsay 1996). Nociceptor neurons with unmyelinated c-fibres or thinly myelinated Aδ-fibres generally have smaller cell bodies. It is these small diameters presumably nociceptive DRG neurons that exhibited the highest upregulation of Luman after spinal axotomy. Small to medium neurons are highly metabolically active with regards to glucose metabolism (Gardiner, Wang et al. 2007) and growth potential, as these nerve growth factor (NGF) responsive neurons have high baseline levels of growth associated protein (Verge, Tetzlaff et al. 1990). They also produce the highest amounts of brain-derived neurotrophic factor (BDNF) in the intact state and this production is upregulated very early post injury, playing a key role in the induction of the regeneration response (Karchewski, Gratto et al. 2002, Geremia, Pettersson et al. 2010), perhaps due to the enhanced ability to respond to local cues (Webber, Xu et al. 2008). Evidence suggests that small-medium sized neurons are more primed for growth compared to their larger diameter counterparts (Andersen and Schreyer 1999) and the rise in Luman levels predominantly in these cell types correlates to a greater amount of ER stress they may face. Peripheral nerve injury causes a phenotypic switch in DRG sensory neurons, dramatically evidenced with BDNF expression, whereby BDNF levels in smaller neurons gradually declines over time and

expression increases in larger sized neurons (Cho, Kim et al. 1998, Tonra, Curtis et al. 1998, Karchewski, Gratto et al. 2002). This shift in neurotrophin production and change in phenotype likely signifies increased growth capability of these larger neurons at later time points when Luman levels were increased. As with the small diameter neurons, higher levels in medium-large sized DRG neurons at 1-week corresponds to the need for a heightened UPR with the change in phenotype.

A temporal biphasic response to injury was seen with Luman with protein immunoreactivity peaking 2 days after injury, falling by 4 days and elevated again in all neuron populations by 1 week. Multiple transcriptional changes take place as a homeostatic DRG neuron transitions to a regenerating one (Costigan, Befort et al. 2002, Christie, Webber et al. 2010) . A recent study used cDNA micro array analysis to identify the different genes expressed by DRG neurons after sciatic nerve transection over time (Li, Xue et al. 2015). These expressed genes belonged to multiple biological processing including the detection of stimulus, signaling transduction, the response to stimulus, transcription mechanics, regeneration and growth and various others. Interestingly, they found 3 distinct transcriptional phases throughout the injury time course where the upregulation or down-regulation of genes serve a common purpose. These phases included (Li, Xue et al. 2015); the "stress-response" phase occurring at 0.5 hrs to 6 hrs post-injury and was enriched with transcription events associated with detection of stimulus and signal transduction, the "pre-generation phase" at approximately 9hrs to 1 day corresponded to increased regulation of DNA replication and transcription and elevated molecular functions such as "transcription factor activity", and finally a "regeneration phase" beginning at 4-days showed processed related to cell proliferation, growth and growth factor activity. The groups' molecular function analysis showed a multiphasic trend with regards to transcription factor activity post injury with a peak during the early phase (~9hour mark) and a marked decrease at the 4-day time point, the transition point from a "pre-regeneration" to a regeneration phase, and a gradual increase thereafter. This trend shows a close similarity to the finding with Luman as levels are significantly shut down in both the ipsilateral and contralateral neurons at 4 days and this trend has been noted by our group with other transcription factors (FOXO3A, Zhangfei) which we have shown to also be injury regulated (Hasmatali, Noyan-Ashraf et al. 2009, Hasmatali, De Guzman et al. 2012).

What drives the downregulation in Luman expression, evident at 4 days, especially in light of no discernible decline in mRNA expression is not yet known. However, the Luman protein transactivation domain is very unstable upon cleavage, rapidly degraded and only readily visualized *in vitro* with the use of protease inhibitors (Lu, Yang et al. 1997). Recently, Jun activation domain-binding protein 1 (JAB1) was identified as a specific inhibitor of Luman by increasing its degradation (DenBoer, Iyer et al. 2013) which builds an argument that a primary regulatory mechanism for Luman occurs at the protein level. Thus at 4-days, the possibility exists that there is elevation of its degradation, a cellular event that needs to be further elucidated. Regardless, the temporal pattern of Luman immunoreactivity in injured neurons appears to coordinate nicely with the transition point of the identified transcriptional phases where there is likely a protein burden and increased ER stress.

Examination of Luman in nerves ipsilateral to crush injury also showed a biphasic response similar to what was seen in the cell body. In preliminary studies we have observed a similar biphasic regulation of two ER stress-associated target genes of Luman immediately proximal to the crush injury site, CHOP and BiP/GRP78 in axons (data not shown). This suggests that there are downstream consequences to the observed injury-associated regulation of Luman expression, the relevance of which also needs to be determined. In addition to reduced levels of Luman protein potentially contributing to the similar impacts on expression of target genes, the downregulation of UPR gene expression at 4 days may be due to increased expression of proteins known to negatively repress Luman-mediated activation of genes with UPR element-containing promoters, such as Luman/CREB3 recruitment factor which interacts with Luman and recruits it to discrete nuclear foci, repressing Luman transactivation activity (Audas, Li et al. 2008).

The very early post-injury increases in Luman corroborate findings that axotomy induces a rapid translation of already present axon-localized Luman transcripts that along with existing Luman protein can be activated and retrogradely transported to the neuronal cell body (Ying, Misra et al. 2014). To assess the effect of a pro-regenerating therapy, brief electrical stimulation (ES) was applied proximal to the crush site. Injured neurons undergo a latent period with no axon regeneration of approximately 1-3 days that proceeds outgrowth; however, afterwards the axons extend from the proximal nerve stump into the distal site at a rate of 1-3mm/day in both rat and human model (Danielsen, Lundborg et al. 1986, Gordon 2016, Gordon and English 2016).

One hour of brief ES of transected nerves that have been surgically repaired, reduces this latent period and increases axon growth across the repair site (Al-Majed, Neumann et al. 2000). This also leads to increase pro-growth neurotrophin signaling, increasing neuronal production of BDNF which is a likely inducer of the enhanced injury response (Al-Majed, Brushart et al. 2000, Geremia, Gordon et al. 2007, Geremia, Pettersson et al. 2010). However, the boost in regeneration appears to be due to both enhanced neurotrophin signaling and ES dependent rise in cAMP levels (Udina, Ladak et al. 2010), leading to increased expression of RAGs. With the findings that the growth capacity of DRG neurons are regulated by axon-derived increases in retrogradely delivered Luman (Ying, Misra et al. 2014, Ying, Zhai et al. 2015), we hypothesized that ES would alter Luman expression and/or protein processing. Furthermore, ES is a known reactivation trigger of latent HSV in sensory neurons (Chan, Beck et al. 1989) and Luman's known transcriptional regulation of latent HSV may point to a possible link between stimulation and the proteins' activity. Following a very early rise in Luman axonal expression, treatment with stimulation lead to decreased Luman staining in nerves proximal to the crush site. Altered levels could be due to enhanced protein transport mechanisms, but evidence suggests that electrical stimulation does not affect the rate of axonal transport in injured motoneuron models with the use of radiolabeled tracers (Brushart, Hoffman et al. 2002). Nevertheless, a study examining the effect of stimulation on trigeminal ganglia found no change to the rate of axonal transport but did increase the amount of protein undergoing transport (Chan, Beck et al. 1989). Thus, the decreased amount of axonal Luman post stimulation could be attributed to increased protein packaging and augmentation of the retrograde transport mechanics while the rate of transport was unchanged at the one and 3-day post-injury time points. This finding still suggests a possibility that Luman action is intensified by electrical stimulation, contributing to the alteration of growth programs and UPR induction. Indeed, at the level of the cell body, preliminary data reveals increased cytoplasmic and nuclear Luman levels at one-day post crush + ES (YZ, JCDH, data not shown) which may be attributable to increased delivery of axonally-derived Luman and/or increased synthesis of Luman. These results also beg the question of whether injury/stimulation rises in BDNF signaling in response to ES influences Luman activity, particularly after the 1-day time point when endogenous BDNF begins to rise in DRG neurons (Karchewski, Gratto et al. 2002). Experiments manipulating the neurotrophin's activity would assist in answering this question, but it's expected that any anti-growth state such as BDNF

disruption would decrease the regenerative response, lessening the need for UPR and suppressing injury induced Luman expression.

4.4.2. Implication of the contralateral effect

An interesting aspect of this study was the effect of unilateral axotomy on neuronal Luman expression in uninjured contralateral DRG whereby contralateral neurons showed a similar biphasic, but less robust response without the excessive 2-day increase in Luman expression seen in the ipsilateral injured state. Few connections exist between neurons that innervate opposite sides of the body; however, a collection of clinical evidence confirms contralateral deficits in patients with one-sided injuries (Kozin, Genant et al. 1976, Oaklander, Romans et al. 1998). The bilateral impact of nerve injury has been reported in a number of animal models where sensory, sympathetic or motoneurons opposite to the lesion site differ morphologically and/or biochemically from naïve controls (Koltzenburg, Wall et al. 1999). In addition to these changes, neurons of intact DRG contralateral or segmentally adjacent to the injured ganglia have altered nerve sprouting in unaffected limb areas (Devor, Schonfeld et al. 1979, Navarro, Verdu et al. 1997, Oaklander and Brown 2004). Furthermore, contralateral DRG neurons may have altered neurotrophin signaling as sciatic nerve injury causes increased p75 receptor expression in perineuronal glial cells in contralateral DRGs (Zhou, Rush et al. 1996). In general, these observations are considered a result of a neural mechanism with a propagating signal through the spinal cord and not due to a global effect. However, preliminary results with other possible injury associated transcription factors shows support of the existence of a humeral response to peripheral nerve axotomy, whereby injury signals originating from the lesion site and/or systemically released may circulate via spinal fluid or bloodstream and influence uninjured sites (Hasmatali, Noyan-Ashraf et al. 2009). Systemic administration of glucocorticoids and adrenalectomy was shown to affect neuropeptide levels in DRG neurons (Smith, Seckl et al. 1991, Covenas, DeLeon et al. 1994), which express the glucocorticoid receptor (DeLeon, Covenas et al. 1994). The changes seen in Luman of uninjured DRG neurons might be caused by such circulating factors, but a relationship between nerve injury and steroidal hormone release has not been identified. There are findings showing that “unstressed” motoneurons do have elevated ER-stress markers (Saxena, Cabuy et al. 2009) therefore, its plausible a global stress event in the nervous system such as trauma could extrinsically lead to

altered protein misfolding and the need for UPR at distal sites. The reality of contralateral and humeral effects of nerve injury illustrates the importance of using naïve animals and not uninjured tissue as controls.

4.4.3. Conclusion

While peripheral neurons do grow axons after nerve injury, repair is often slow and functional recovery is poor. Evidence supports the necessity of a coordinated UPR and the induction of certain UPR regulators for optimal regeneration to occur. We had previous identified axonal Luman as a sensor of injury, regulating the UPR in DRG neurons and impacting their growth capacity. Here, we show Luman as part of the injury cell body response where it is markedly upregulated following axotomy. Its upregulation coordinates with recently identified time points of increased transcriptional activity in injured neurons. Increased Luman at these points and in general is likely associated with increased ER stress and UPR activation. The upstream signaling pathway responsible for both the cell body response and axonal alteration are not known. A neurotrophin mediated mechanism is likely involved, more so in somal Luman responses as our previous work suggests more rapid injury mediated signaling may contribute to changes in the axon. Results from the nerve crush and electrical stimulation experiments confirms previous findings and further explores the likely impact of stimulation on intracellular protein processing, transcription factor activation and regeneration programs.

5. Nerve injury induces multiphasic alterations in Zhangfei/CREBZF expression in sensory neurons ipsilateral and contralateral to injury.

5.1. Abstract

Nerve transection imposes intracellular stress in DRG neurons that leads to adaptive changes driving the alterations in phenotype required for the injured axons to regenerate. These include mounting the unfolded protein response (UPR), which we have shown is regulated by injury-associated changes in the ER-resident transcription factor Luman/CREB3/LZIP expression and activity (Ying, Misra et al. 2014, Ying, Zhai et al. 2015). Because the transactivation properties of Luman and the UPR have been shown to be regulated by the transcription factor Zhangfei (ZF) in other cell types, we sought to see if it too might be involved in the injury response of sensory neurons. Thus, the impact of injury on the sensory neuron ZF expression and nuclear localization was temporally evaluated using a sciatic spinal nerve transection model and histological techniques. Immunostaining of naïve ganglia revealed ZF to have possible transcriptional significance in intact large diameter neurons, as nuclear levels were high in this population. Axotomy led to an acute bilateral reduction of nuclear ZF localization in this neuronal subset; followed by a biphasic response with elevated expression by 2-days post injury of both mRNA and protein levels, most evident in small to medium size neurons followed by a decline at 4 days and a second increase by 1week post injury. The fluctuating responses in contralateral neurons at the different time points were not evident in sham experiments suggesting potential systemic effects resulting from the contralateral spinal nerve transections. Finally, the involvement of the neurotrophin BDNF (a molecule critical to induction of the regeneration response in sensory neurons) in the regulation of ZF was examined with the use of small interfering (si) RNA. Intrathecal administration of siRNA targeted to BDNF attenuated ZF's early response to injury. Collectively, the early response of ZF to injury and its regulation by BDNF supports that it may play a role in induction of the regeneration response in sensory neurons. Further, its bilateral biphasic response from two days post-injury on, similar to that observed for two additional stress-associated transcription factors, Luman and FOXO3a, suggests an emerging complex pattern of transcriptional stress responses in sensory neurons that includes a systemic component in addition to those on the injured neuron.

5.2. Introduction

The consequences of peripheral nerve injury are dramatic as affected neurons attempt to survive and regrow lost axons. During episodes of stress and axonal injury, sensory neurons can undergo rapid alterations in gene expression due to their highly plastic nature. Modulation of transcription factor expression and activity are some of the early events by which cells mount adaptive responses to cope with the pathological event and set up effective repair programs. The Verge lab has recently revealed that the ER-resident stress-related transcription factor, Luman/CREB-3/LZIP (herein called Luman) plays a pivotal role in early regenerative events such as the intrinsic ability of sensory neurons to regenerate an axon through its regulation of the unfolded protein response (UPR) and cholesterol biosynthesis (Ying, Misra et al. 2014, Ying, Zhai et al. 2015). In the previous chapter we examined temporal alterations in Luman expression to better predict beyond the one-day injury time point assessed in Ying et al., (2014; 2015), when cells are mounting adaptive responses in the acute versus chronic phases of regeneration and whether these responses are also indicative of a more global systemic stress response.

Little is known about regulators of Luman function and elucidating this could have important implications for modulating regenerative responses. However, *in vitro* studies have revealed that another basic leucine zipper transcription factor (bLZip), Zhangfei (ZF aka CREBZF/SMILE) when co-expressed with Luman in Vero and Hep2 cells could potently and effectively inhibit the host cell factor (HCF)-dependent transactivation function by the Gal4-Luman fusion protein of a UPR element containing promoter in a dose dependent manner (Misra, Rapin et al. 2005). Interestingly, Zhangfei, like Luman was discovered due to its interaction with the Herpes Simplex Virus-1 related HCF protein and like Luman, is also expressed in trigeminal sensory neurons (Lu and Misra 2000). More recent work has revealed a role for ZF in the UPR exerted through the tumor suppressor protein p53 (Zhang, Rapin et al. 2013, Zhang and Misra 2014, Zhang, Thamm et al. 2015), a molecular pathway implicated in peripheral nerve regeneration (reviewed in (Krishnan, Duraikannu et al. 2016)).

An additional regulator of repair programs is the neurotrophin family of growth factors which has a longstanding relationship with PNS neurons and their regenerative action (Verge, Gratto et al. 1996). Neurotrophins are mostly target derived; however, BDNF is found in innervated tissue and produced by neurons themselves. In the naïve DRG, small to medium sized nociceptive neurons express BDNF (Wetmore and Olson 1995, Verge, Gratto et al. 1996), and as

these neurons have high amounts of the TrkA receptor, their BDNF levels are regulated by NGF signaling (Apfel, Wright et al. 1996). BDNF stands alone as the sole neurotrophin up-regulated in sensory neurons in response to injury. This characteristic has made it the candidate molecule for the induction of the injury response (Geremia and Verge 2001, Geremia, Gordon et al. 2007).

The distinct trophin environment in the injured state accounts for the altered expression and activation of transcription factors in DRG neurons. Activating transcription factor 3 (ATF-3), c-jun, and STAT3 are up-regulated in sensory neurons following axotomy and their expression and post-translational process are mediated through neurotrophin governed pathways (Averill, Michael et al. 2004, Lindwall and Kanje 2005, Hyatt Sachs, Schreiber et al. 2007). Interestingly, ZF has also been implicated in nerve growth factor (NGF) signaling through its regulation of expression of the NGF receptor trkA signaling (Valderrama, Rapin et al. 2008). The role of ZF in trkA regulation is complex. During development, trkA triggers pathways for differentiation and in an undifferentiated cell ZF can activate the expression of the receptor, thus facilitating NGF mediated maturation or apoptosis (Valderrama, Rapin et al. 2009). But ZF also has a suppressive action on TrkA, as its interaction with Brn3a inhibits transcription of the receptor in non-neuronal cells (Valderrama, Rapin et al. 2008). In NGF-differentiated PC-12 cells, stress events can cause down-regulation of ZF and its target gene trkA (Valderrama, Rapin et al. 2008), paralleling a response seen in injured sensory neurons where neurotrophin trk and p75 receptor expression at the level of the cell body is reduced (Verge, Riopelle et al. 1989, Zhou, Rush et al. 1996).

Established links between ZF and the NGF receptor trkA, Luman's actions and the UPR cellular stress response suggests that it may play a role in regeneration of sensory neurons following axotomy. Thus, we hypothesize that transection of the sciatic nerve will impact ZF expression and/or cellular localization in DRG neurons. Our results demonstrate a neuron population-dependent response where nuclear ZF initially rapidly decreases in large neurons, then undergoes a biphasic response that parallels other stress-associated transcription factors analyzed in this thesis. Additional experiments utilizing siRNA for BDNF suggests the trophin plays a role in regulation of injury-associated changes in ZF expression.

5.3. Results

5.3.1. Peripheral nerve injury results in bilateral alterations in ZF expression and nuclear localization

Prior to beginning studies examining the temporal changes in Zhangfei expression in response to peripheral nerve lesion the validity of the antibody and oligonucleotide probes employed were examined using several specificity controls. First, the rabbit ZF antibody was preabsorbed with ZF protein prior to processing tissue with anti-ZF, and anti-ZF preabsorbed with protein isolates from Vero cells transfected with ZF expression plasmids. Processing of naïve L5 DRG sections for immunofluorescence revealed that pre-absorbed anti-Zhangfei results in near abolishment of the immunofluorescence staining discerned with anti-ZF alone (Fig. 5.1A). Further, Western blot analysis of electrophoresed protein extracts from Vero cells transfected with Luman or Zhangfei was conducted employing anti-ZF. In the lane running protein isolate extract from Zhangfei transfected cells, anti-Zhangfei recognized a single band of approximately 37 kDa, the predicted molecular weight of Zhangfei protein; while unable to detect any identifiable antigen in the lane where protein isolates from Luman transfected cells were run (Fig. 5.1.1B3). Finally, Western blot analysis of anti-Zhangfei rabbit serum treated membrane of electrophoresed protein extracts from naïve L4-L5 DRG (lane 2) revealed that anti-Zhangfei recognized only a single band of approximately 37 kDa (Fig. 5.1.1C). For *in situ* hybridization experiments, the specificity of the radiolabelled oligonucleotide probe to selectively detect ZF mRNA was examined by hybridizing tissue sections with a hybridization cocktail containing the radiolabelled probe with or without the addition of a 100X excess unlabeled cold ZF probe. Addition of the excess cold probe was effective in competing away the ability of the radiolabelled probe to hybridize to the mRNA (Fig. 5.2).

Immunohistochemical analysis of section L5 DRG from naïve animals shows ZF to be expressed in all sensory neurons with high nuclear localization in a number of small neurons and medium-large diameter population (> 35 μm; Fig. 5.2). The ratio of nuclear to cytoplasmic staining appears slightly higher in these medium-large diameter neurons compared to the small-medium diameter cells (1.04 vs. 1.01, based on cumulative naïve DRG N=3) implying a possible greater transcriptional significance.

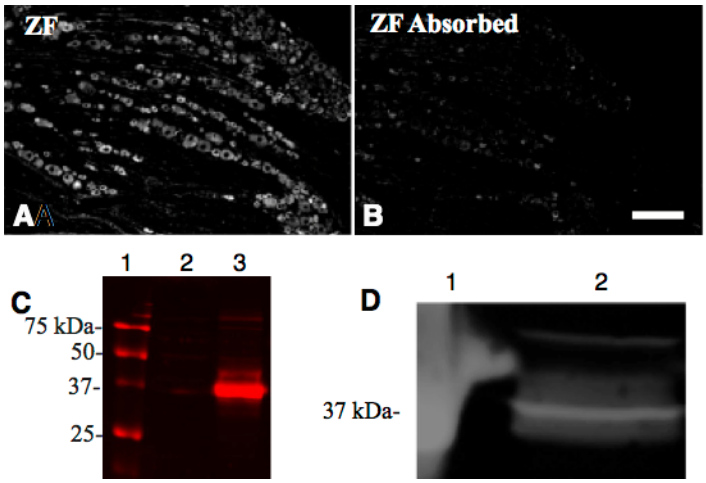

Figure 5.1 Zhangfei antibody specificity controls. Fluorescence photomicrographs of DRG (6µm sections) processed for immunohistochemistry to detect cell Zhangfei protein with anti-Zhangfei rabbits serum (A) and anti-Zhangfei absorbed with cell protein isolates from vero cells transfected with Zhangfei (B) . Scale bar = 100µm. Note: absorption of anti-Zhangfei results in near abolishment of immunofluorescence staining. C. Western blot analysis of anti-Zhangfei rabbit serum treated membrane of electrophoresed protein extracts from vero cells transfected with Luman (lane 2) and Zhangfei (lane 3). Lane 1, Molecular weight marker. Note: In the Zhangfei transfected cell extract, Anti-Zhangfei recognizes a single band of approximately 37 kDa, the predicted molecular weight of Zhangfei protein; while unable to detect any identifiable antigen in the Luman transfected cell extract. D. Western blot analysis of anti-Zhangfei rabbit serum treated membrane of electrophoreses protein extracts from normal DRG (Lane 2). Lane 1 -Molecular weight marker. Note: anti-Zhangfei recognizes band of approximately 37 kDa.

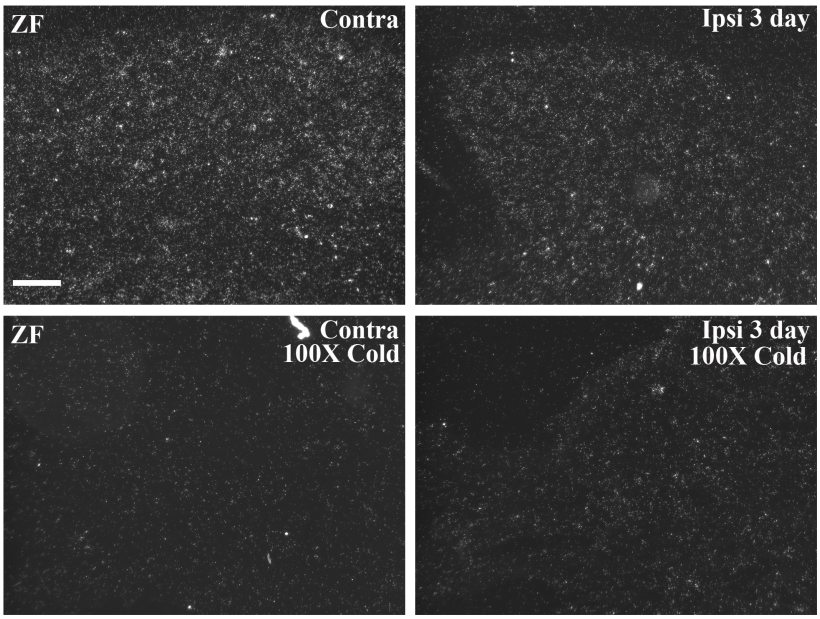

Figure 5.2. Zhangfei oligonucleotide probe specificity controls. Representative darkfield photomicrographs of 6 μm L5 DRG cryosections contralateral (Contra) or ipsilateral (Ipsi) to 3 day L4-6 spinal nerve transection (as indicated) and processed for in situ hybridization with a 48mer oligonucleotide radiolabeled probe specific for Zhangfei mRNA reveals a diffuse level of hybridization signal that is competed away when 100X unlabelled ZF oligonucleotide probe is added to mixture containing the radiolabeled probe. Scale bar = 100 μm.

To examine the repercussions of injury on ZF expression in sensory neurons, an injury time course model was used where the L4-L6 spinal nerves are unilaterally transected. In an identical manner to that utilized to determine nuclear levels of Luman and FOXO3a, nuclear ZF staining was assessed using dually immunostained sections that localized ZF and Lamin B, the latter a component of the nuclear envelope (Fig. 4.2). Only neurons with complete and distinct Lamin B staining around the nucleus were considered for analysis. Quantitative immunofluorescence protein analysis reveals an initial bilateral loss in high ZF nuclear staining as early as 1-hour following acute injury with a further decline by 1day post-lesion. This decrease in nucleus-localized protein is observed primarily in the medium-large size neuron subpopulation (Figs. 5.3, 5.4, 5.7). However, by 2-days post injury, ZF protein and mRNA (Figs. 5.3; 5.5) staining has risen significantly across all size ranges of neurons, with the small-medium sized population having stronger immunoreactivity in the cytoplasm and nucleus of neurons ipsilateral to injury (Figs 5.3,5.5, 5.7). This increase peaks at the 2-day time point.

However, by 4-days, ipsilateral levels have decreased to pre injury levels in all size ranges of neurons, followed by a significant rise in expression 1-week post injury in all size ranges of neurons, but most evident in the small-medium size neurons (Figs. 5.4 and 5.7). In agreement with the multiphasic changes in neuronal nuclear ZF levels following injury, the nuclear ZF levels in perineuronal, presumably satellite glial cells undergo similar changes, dramatically decreasing 1 hour after injury rising thereafter to high levels at 2 days, declining at 4 days, then rising again to high levels one week after injury (Fig. 5.3).

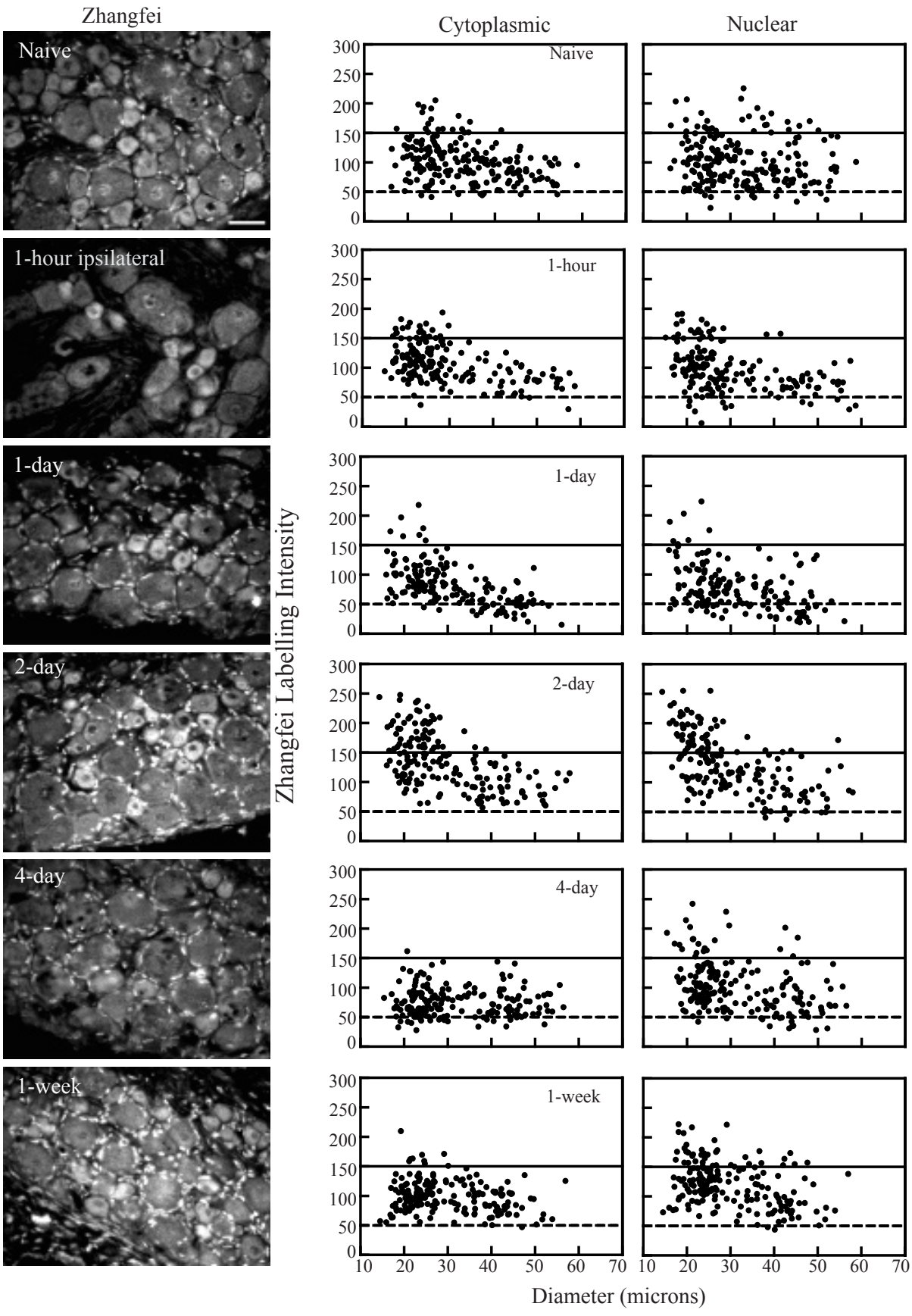
Zhangfei
Cytoplasmic
Nuclear
Naive
1-hour ipsilateral
1-day
2-day
4-day
1-week
Zhangfei Labelling Intensity
Diameter (microns)
0
50
100
150
200
250
300
10
20
30
40
50
60
70

Figure 5.3. Peripheral nerve injury alters Zhangfei protein levels in DRG neurons ipsilateral to axotomy. Left Column. Fluorescence photomicrographs of L5 DRG (6 μm sections) processed for immunohistochemistry to detect cell Zhangfei protein. Ipsilateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day and 1week (as indicated). Scale bar = 50 μm. Naïve animals served as controls. Right Column. Representative scatterplots depicting relative changes in Zhangfei immunofluorescence signal over individual cytoplasmic and nuclear regions as related to cell size from sections all processed under identical conditions in parallel. Experimental states as indicated. Dashed lines divide the plots into low versus moderate to heavily labelled populations. N= 181 to 206 neurons analyzed per condition.

Nerve injury also impacts ZF expression and localization in uninjured contralateral neurons. Besides the initial bilateral loss of nuclear ZF immunostaining in medium to large size neurons at 1 hour to 1-day post injury, cytoplasmic levels remain relatively stable in the medium to large size contralateral neurons, while rising slightly in the contralateral small to medium size neurons (Fig.5.4). Contralateral neuronal cytoplasmic ZF levels then remain stable in the medium to large size neurons at 2 days, followed by a decline across all size ranges of neurons to levels lower than pre-injury levels by 4 days, with a subsequent rise by 7 days. In contrast to the early general downward trend in the cytoplasm, nuclear intensity undergoes a clear biphasic response across all size ranges of intact contralateral neurons following the initial decline in nuclear levels at 1-hour post-injury (Fig.5.4). Two-day post injury, contralateral ganglia have elevated nuclear levels most notable in small-medium size neurons. This strong signal diminishes significantly across all size ranges of neurons by 4 days, to just above pre-injury levels in the small-medium size neurons, while decreasing dramatically to below pre-injury, practically undetectable levels in the medium to large size neurons. There is however, a significant rise back to pre-injury levels in this latter population of neurons by 7 days, while overall levels in the small to medium size neurons remain stable. ZF mRNA (Fig. 5.5) expression remains relatively unchanged in contralateral neurons throughout the time course, suggesting that regulation of some aspects of the ZF injury response occur at the post-transcriptional level.

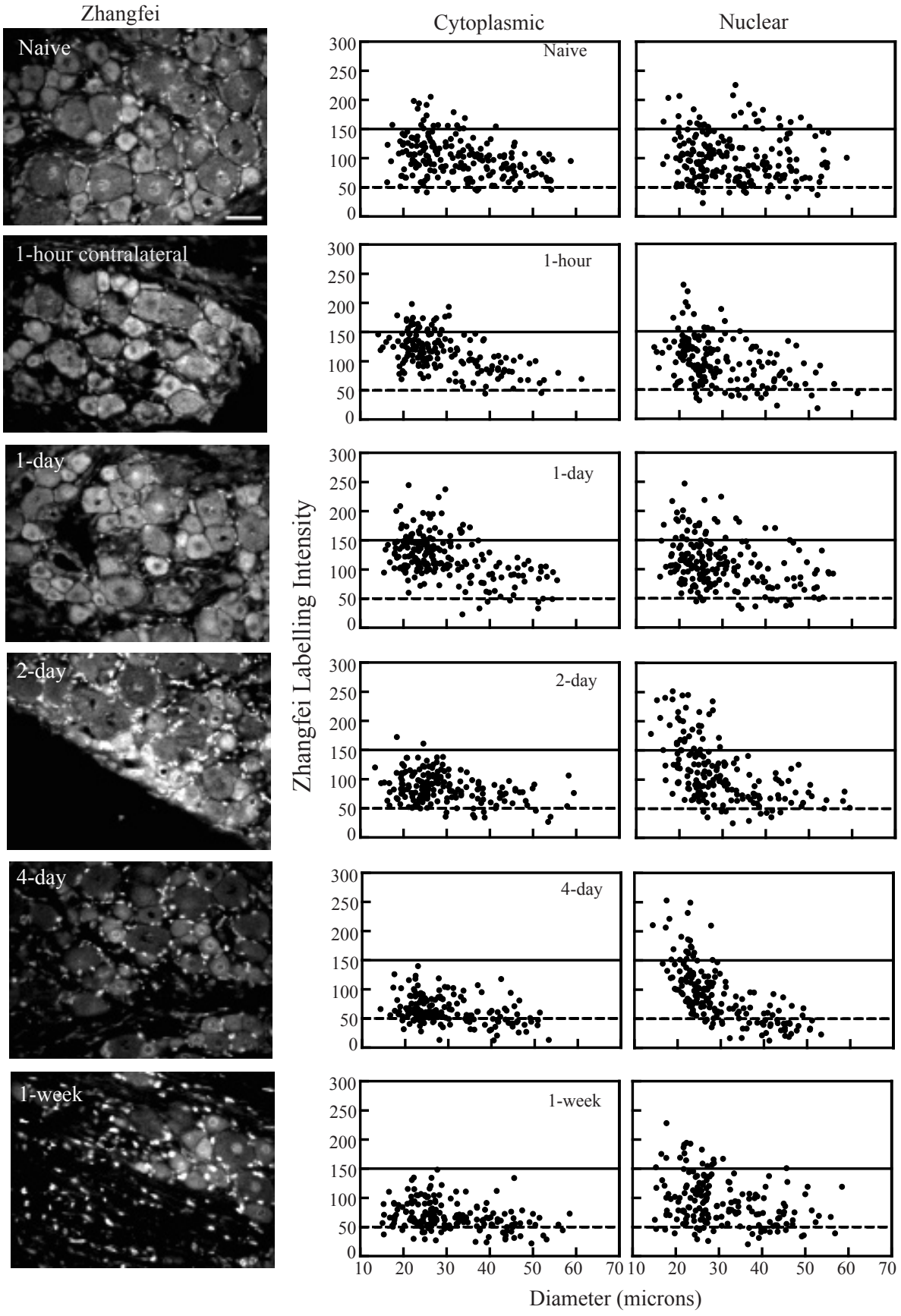
Zhangfei
Cytoplasmic
Nuclear
Naive
1-hour contralateral
1-day
2-day
4-day
1-week
Zhangfei Labelling Intensity
Diameter (microns)
0
50
100
150
200
250
300
10
20
30
40
50
60
70

Figure 5.4. Peripheral nerve injury alters Zhangfei protein levels in DRG neurons contralateral to axotomy. *Left Column.* Fluorescence photomicrographs of L5 DRG (6 μm sections) processed for immunohistochemistry to detect cell Zhangfei protein. Contralateral DRG from L4-L6 spinal nerve transected rats were collected after the predetermined time points of 1hr, 1day, 2day, 4day and 1week. Scale bar = 50 μm. Naïve animals served as controls. *Right Column*. Representative scatterplots depicting relative changes in Zhangfei immunofluorescence signal over individual cytoplasmic and nuclear regions as related to cell size from sections all processed under identical conditions in parallel. Experimental states as indicated. Dashed and solid lines divide the plots into low versus moderate to heavily labelled populations. N= 185 to 206 neurons analyzed per condition.

5.3.2. Zhangfei levels are not discernibly altered by sham surgery

Because there was such a robust increase in ZF detected in the nuclei of small to medium size neurons in contralateral ganglia and a decline in cytoplasmic levels across all size ranges and nuclear levels in medium to large size neurons, we had to ascertain whether these contralateral changes in expression were due to systemic stress response associated with the surgical exposure or the actual lesion of the nerve. Thus, sham surgeries were performed for the entire time course and qualitatively assessed for marked responses in L4,5 DRG ipsilateral or contralateral to the surgical exposure site. Qualitative analysis of ipsilateral or contralateral L5 DRG sections processed for ZF immunofluorescence (N=3 animals/time point), did not reveal any significant changes from naïve controls, suggesting that the changes observed in the axotomy time course were due to the nerve injury imposed (Fig. 5.6).

5.3.3. Knockdown of BDNF suppresses injury-associated ZF up-regulation

Injury drives changes in BDNF expression in peripherally axotomized DRG that play a critical role in the induction of the injury response in sensory neurons (Geremia, Pettersson et al. 2010). What is not known however, is whether this also includes regulation of injury-associated changes in ZF expression. Thus, to test the role of endogenous BDNF in regulation of the ZF, tissue generated for the Geremia et al., (2010) study was employed to examine alterations in injury-associated changes in neuronal ZF expression in response to reductions in neuronal BDNF effected by intrathecal infusion of BDNF selective small interfering RNA (siRNA). The strategy employed involved delivering an intrathecal bolus injection of siRNA into the spinal cord at the

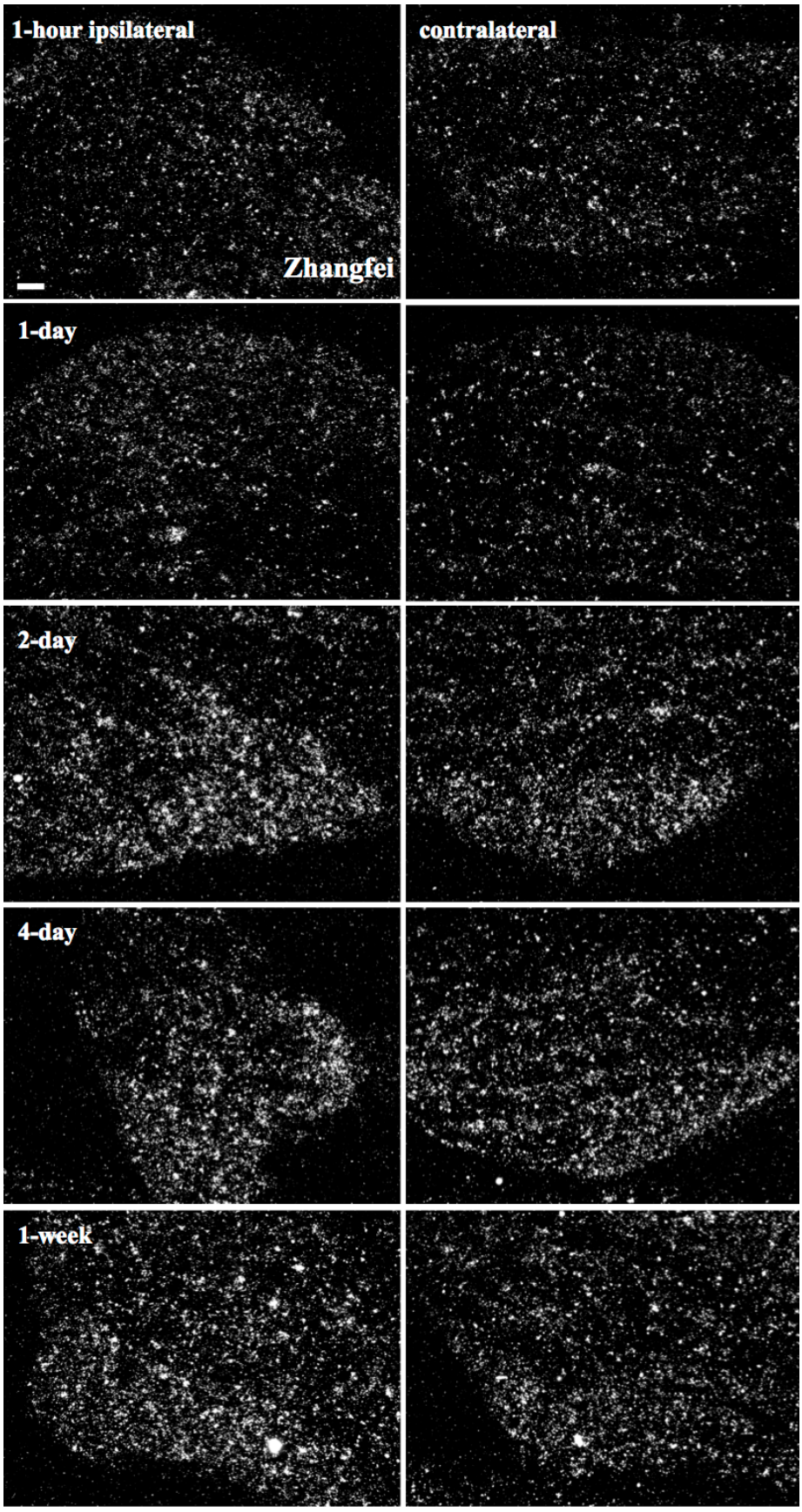
1-hour ipsilateral
contralateral
Zhangfei
1-day
2-day
4-day
1-week

Figure. 5.5. Unilateral peripheral axotomy alters Zhangfei (ZF) mRNA expression levels in DRG bilaterally. Representative darkfield photomicrographs of L5 DRG sections (6µm) processed for in situ hybridization with a 48mer oligonucleotide radiolabeled probe specific for ZF mRNA in order to visualize transcript localization and expression following injury. Ipsilateral (left column) and contralateral (right column) DRG sections from L4-L6 unilateral spinal nerve transected rats harvested 1-hour, 1-day, 2-days, 4-days and 1-week after injury (as indicated). Scale bar = 100 µm. Note: Peripheral nerve axotomy results in a modest decline in ZF mRNA expression by 1 hr and 1 day, followed by a bilateral increase mRNA levels that is higher in the DRG ipsilateral to injury and peaking at the 2-day post lesion.

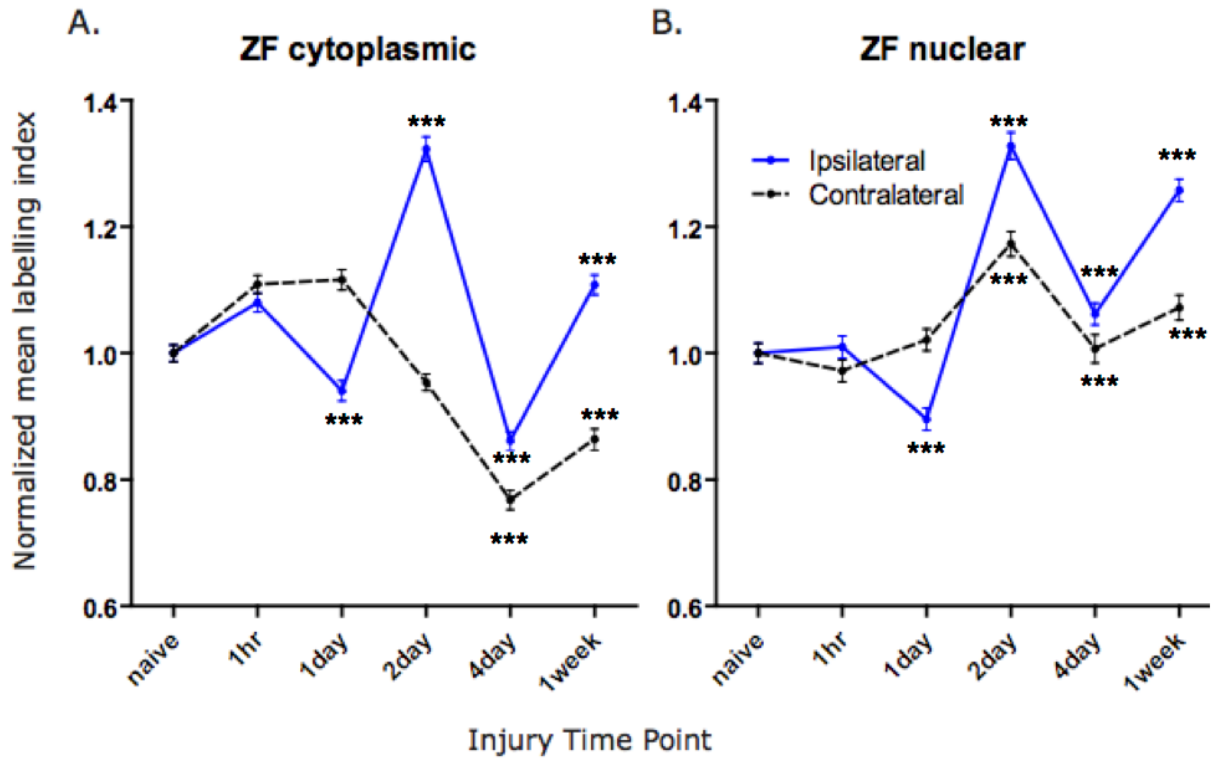

Figure 5.6. Summary line graphs of Zhangfei (ZF) labelling index of grouped DRG neuron populations. Summary line graphs of alterations in the mean labeling index ± s.e.m. of cytoplasmic (A) and nuclear (B) ZF immunofluorescence intensity levels observed in DRG neurons ipsilateral and contralateral to injury at time points as indicated. Each graph point represents a quantitative analysis of N=600 or more neurons from 3 animals in total per condition. ***p value<0.001 ANOVA with Dunn's post-test analysis.

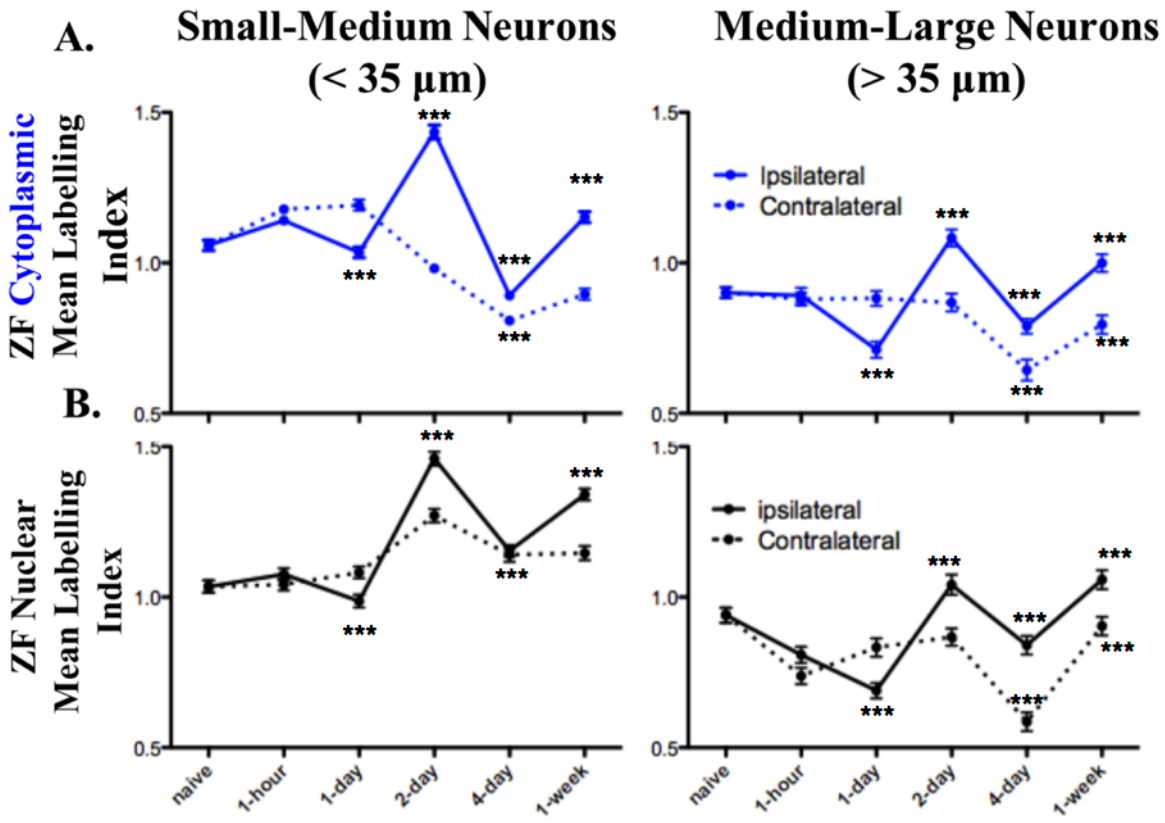

Figure 5.7. Summary line graphs of Zhangfei (ZF) labelling index of DRG neurons subdivided into the small-medium and medium-large subpopulations. ZF summary line graphs of alterations in the mean + s.e.m. cytoplasmic (A; blue) and nuclear (B; black) immunofluorescence intensity levels normalized to the naïve control and as a function of neuronal size. Data is summarized for small-medium sized (< 35 µm, column 1) and medium-large sized (>35 µm, column 2) DRG neurons ipsilateral (solid lines) and contralateral (dashed lines) to injury at time points as indicated. N=3 animals for each data point. Note: relative changes in nuclear localization parallel that observed for the cytoplasmic staining. Small to medium sized ipsilateral neurons show elevated levels of ZF particularly at the 2-day time point. This response is transient as staining is decreasing by 4-days. Interestingly, ZF immunostaining in medium to large size neurons is transiently decreased at the 1-day time point and then elevated again by 2 days. ***p value<0.001 ANOVA with Dunn's post-test analysis.

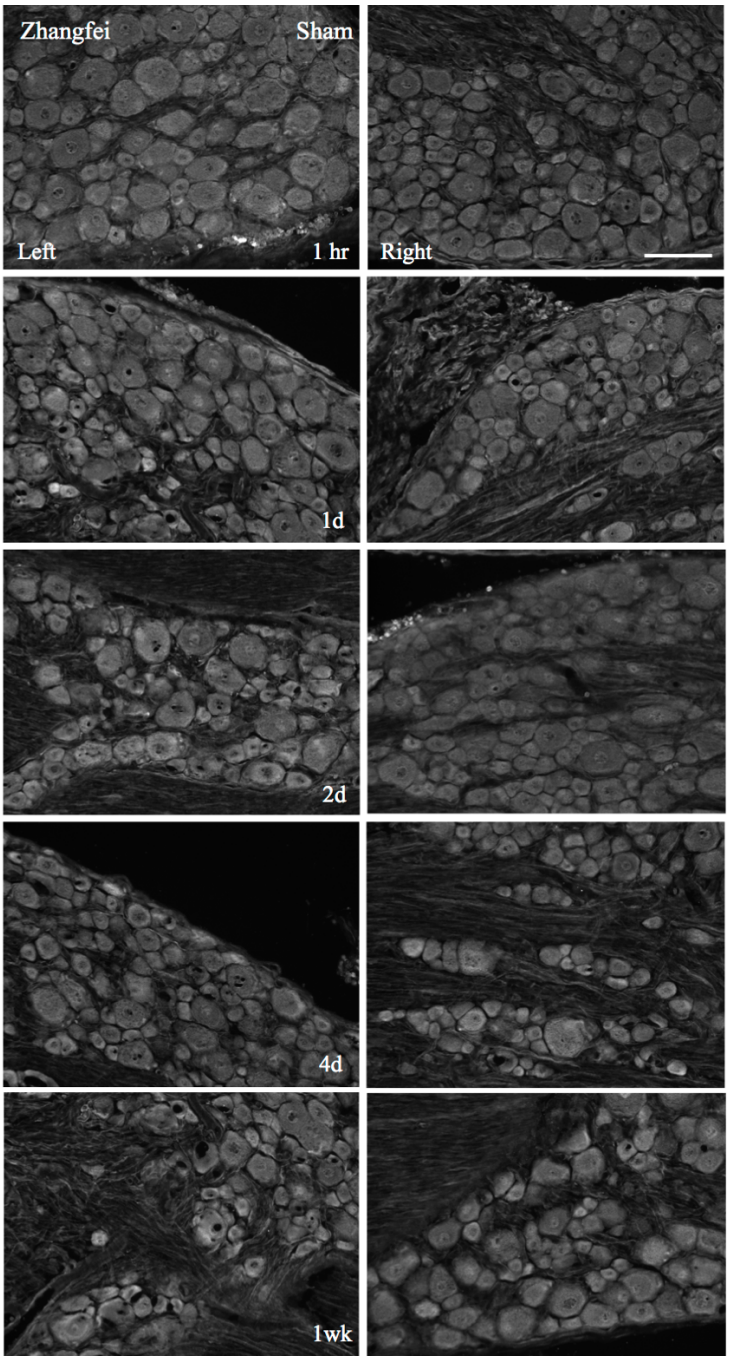

Figure 5.8. Temporal study of the impact of sham surgery on neuronal Zhangfei (ZF) expression Fluorescence photomicrographs of L5 DRG (6 μm sections) processed for immunohistochemistry to detect cell ZF protein from animals that underwent sham surgeries where the right L4-L6 spinal nerves were exposed but not injured. Right and left dorsal root ganglia were harvested at post-surgical times 1-hour, 1-day, 2-days, 4-days and 1-week (as indicated). Scale bar = 100 μm. Note: ZF immunostaining does not discernibly change between each injury time point nor between the right versus left sides. Naïve animals served as controls

and did not differ from that observed at the different sham time points examined (data not shown). N=3 animals/time point.

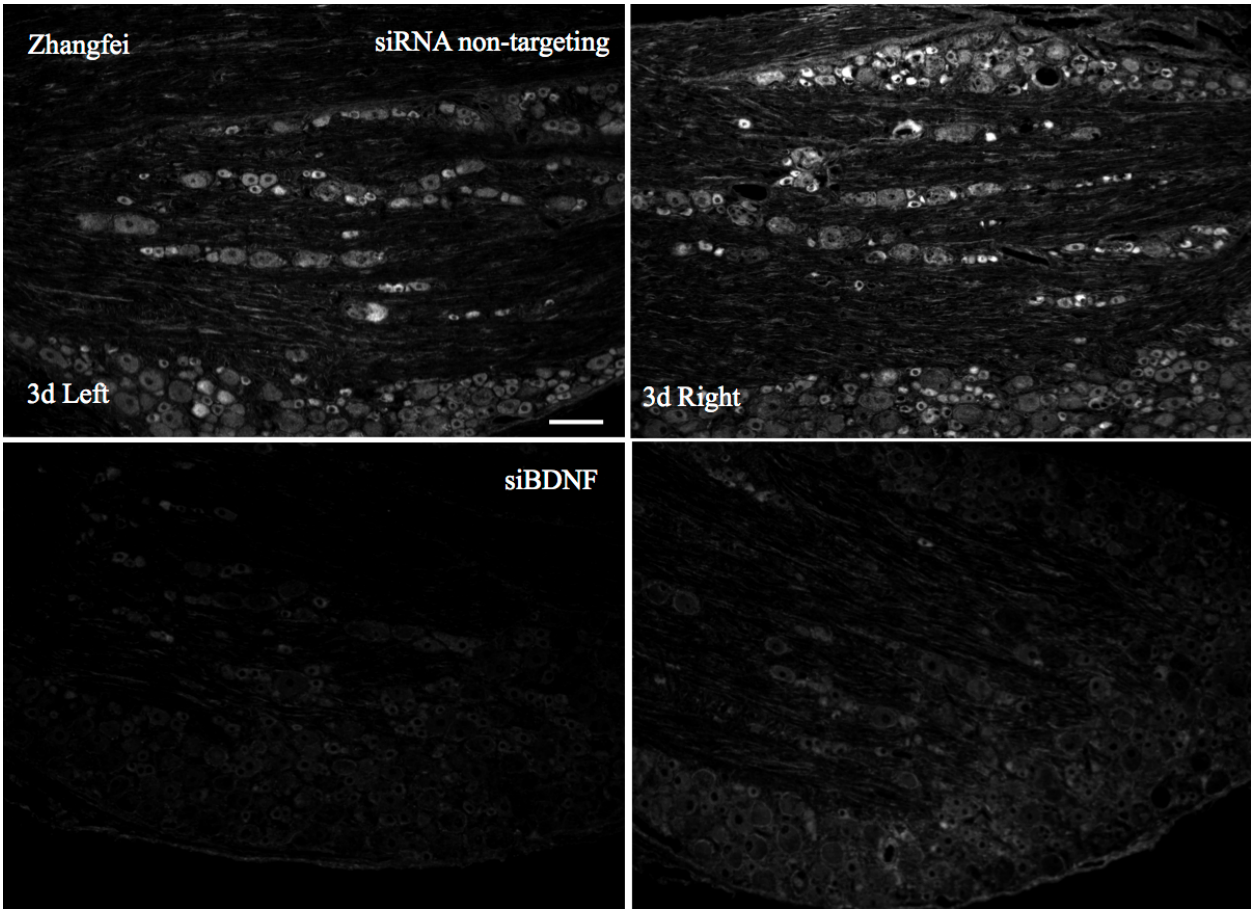

Figure 5.9. Role of endogenous BDNF in regulation of early Zhangfei (ZF) injury responses in sensory neurons. L5 DRG sections processed for ZF immunofluorescence reveal that siBDNF treatment results in reduced expression of ZF protein in 3d injured neurons relative to 3d injury alone. Note: ZF expression in the 3d injury + non-targeting siRNA control is not discernibly different from that of 3d injury alone. Scale bars=100 μm.

level of the L5 DRG 1 or 3 days prior to nerve injury to reduce endogenous stores prior to injury, and thus reduce the amount released at the time of injury. This was followed by an additional infusion at the time of the 3 day spinal nerve transection. Infusion of fluorescently tagged non-targeting control siRNA demonstrated that the infused siRNA accumulated in L5 DRG neurons both ipsilateral and contralateral to lesion, but did not alter BDNF expression, while infusion of

BDNF selective siRNA effectively reduced expression of neuronal BDNF mRNA and protein in intact and injured L5 DRG neurons (Fig. 8 in (Geremia, Pettersson et al. 2010)).

Administration of the siBDNF muted the ZF injury response, as immunofluorescence staining of the transcription factor in treatment DRGs was lower than those given non-targeting control siRNA (Fig. 5.7). This result suggests a BDNF-mediated mechanism, whereby increased production of the neurotrophin in response to injury may regulate corresponding injury-associated changes in ZF protein levels.

5.4. Discussion

In this study I sought to determine whether ZF expression is coordinately regulated with Luman in the injured sensory neurons by employing the same temporal injury paradigm utilized to study Luman expression after injury and also examined whether endogenous BDNF is implicated in regulation of this response. The role of ZF in cellular responses has not been extensively elucidated, with our study the first to link it to the cell body response of adult sensory neurons to injury. My findings indicate that this response is multiphasic ipsilateral to injury. There is an initial decline in ZF expression most evident in the nuclei of medium to large size injured neurons 1-hour and 1 day after injury, followed by a biphasic response largely paralleling that observed for Luman, with a rise in all size ranges of neurons at 2 days, a decline at 4 days and then another rise at 7 days in both cytoplasmic and nuclear ZF levels, with endogenous BDNF implicated in its increased expression observed after injury. These changes are also accompanied by biphasic changes in ZF expression in contralateral neurons that largely parallel those observed for Luman and FOXO3a in this thesis, once again supporting a global stress response to nerve injury.

5.4.1. ZF is implicated in homeostasis and the cell body response to injury

The strong nuclear presence of ZF primarily in medium to large intact neurons, typically mechanosensitive/proprioceptive in function and trkA negative, suggests that ZF serves a role in regulating genes associated with homeostasis or proteostasis in this subpopulation. Alternatively, ZF could be active repressing an injury/plasticity-associated phenotype with the decrease in both cytoplasmic nuclear levels, evident as early as 1-hour post-injury, supporting that its rapid removal may be involved in the switching neurons to a regenerating state. The low levels of

plasticity associated markers such as GAP43 in this subpopulation before injury suggests that a plasticity state may be actively repressed in the intact state, and stands in contrast to the NGF-responsive nociceptive trkA expressing subpopulation of primarily small to medium size uninjured neurons which are nociceptive in function, appear to have relatively high basal rates of GAP43 synthesis and are highly plastic (Verge, Tetzlaff et al. 1990, Sommervaille, Reynolds et al. 1991, Diamond, Holmes et al. 1992, Karchewski, Kim et al. 1999).

ZF plays a complex role in regulating expression of the NGF receptor tyrosine kinase trkA (Valderrama, Rapin et al. 2008). In undifferentiated medulloblastoma cells or NGF-differentiated sympathetic neuron-like PC12 cells, ectopic expression of ZF can activate trkA expression, facilitating NGF-mediated maturation or apoptosis in the former while promoting differentiation in the latter (Valderrama, Rapin et al. 2009). But ZF also have a dose dependent suppressive action on Brn3a activity and its ability to activate the trkA promoter in non-neuronal Vero cells (Valderrama, Rapin et al. 2008). The precise role that ZF plays in expression or repression of trkA and other properties of sensory neurons remains to be investigated in experiments that manipulate endogenous levels of ZF.

5.4.2. Cellular stress and regulation of ZF expression

A link between ZF expression and stress responses exists in other cell types. Twenty four hour exposure of NGF-differentiated PC12 cells to the cellular stressor capsaicin caused down-regulation of ZF and its target gene trkA (Valderrama, Rapin et al. 2008). The latter is in agreement with what we and others observe in regards to trkA expression in sensory neurons in response to another stressor, nerve injury (Verge, Riopelle et al. 1989, Zhou, Rush et al. 1996). In the present study, using the same injury model as the aforementioned Verge et al., and Karchewski et al., I have also observed a decrease in ZF expression across all size ranges of neurons by one day, with expression in the nuclei of medium to large size neurons already decreased by one hour. There was also a marked reduction in the levels of ZF detected in the perineuronal cells at one hour post-injury that appears to recover by one day and then parallels the changes observed here in neuronal ZF and Luman expression (Chapter 4), once again highlighting the robust axis of communication between these glial cells and intraganglionic neurons and/or in response to systemic influences (Lu and Richardson 1993, Nadeau, Wilson-Gerwing et al. 2014, Christie, Koshy et al. 2015). It is possible that these rapidly induced

alterations in ZF expression, especially those observed in the nuclei are part of a response to the initial acute stress that axotomy imposes.

With the exception of the very early decline in ZF expression observed up to one-day post-injury, the subsequent biphasic changes parallel those observed for Luman ipsilateral and contralateral to injury. These multiphasic responses, observed both ipsilaterally and contralaterally to injury for ZF align closely with the recently described distinct transcriptional phases associated with cell body response to peripheral nerve injury examined in DRG ipsilateral to injury (Li, Xue et al. 2015). In particular, the initial early decline seen in ZF expression corresponds well with the "stress-response" phase occurring within hours of the insult. The next phase, the "pre-regeneration phase" is in place by 1-day post-injury and represents a time of great transcriptional activity, such as I observed with increasing levels of ZF expression until the DRG transition into the "regeneration phase" at 4 days post-injury, a time where I see an abrupt decline in stress transcription factor ZF expression both ipsilaterally and contralateral to injury. Finally, I observe an increase in expression at 7-days post-injury, a time point when regeneration is well underway.

5.4.3. Potential mechanisms of how ZF might participate in nerve regeneration

Successful regeneration depends on a robust intrinsic neuronal cell body response that involves the coordinated expression of regeneration-associated genes regulated by a host of transcription factors. ZF belongs to the CREB (cAMP-response-element-binding protein)/ATF (activating transcription factor) family of basic region-leucine zipper (b-LZIP) transcription factors (Lu and Misra 2000) that are implicated in the regeneration response of sensory neurons. The actions of ZF are complex. At a transcriptional level ZF is not like other members of the bLZip family in that it appears to be incapable of binding to bLZip response elements as a homodimer, but it can heterodimerize with or modulate other transcription factors, some of which have been directly implicated in axon/nerve degeneration or regeneration, mostly through the ER stress induced as a result of nerve injury(Penas, Font-Nieves et al. 2011, Quadrato and Di Giovanni 2012, Ying, Zhai et al. 2015, Krishnan, Duraikannu et al. 2016, Onate, Catenaccio et al. 2016, Onate, Court et al. 2016). This includes heterodimerization with Luman (Misra, Rapin et al. 2005), ATF4 (Hogan, Cockram et al. 2006) , Xbp1 (Zhang, Rapin et al. 2013) and p53 (Lopez-Mateo, Villaronga et al. 2012). Like many of these transcription factors, the alterations in

ZF expression in sensory neurons following nerve injury supports that it likely serves as a multifunctional sensor of a number of cellular events and through heterodimerization with multiple transcription factors is poised to integrate a number of extracellular signals that ultimately may have pathogenic or regenerative functions.

As mentioned above, ZF may heterodimerize with other transcription factors to impact peripheral nerve repair. Two potential stress-associated transcriptional partners for ZF that come to the forefront are p53 and Luman. With respect to p53, in a yeast two-hybrid screen using HEY1, an indirect activator of the tumor suppressor p53 as bait, ZF was shown to interact with both Hey1 and the transcription factor p53. ZF was also shown to stabilize p53 and to cooperate synergistically with Hey1 to enhance p53 transcriptional activity (Lopez-Mateo, Villaronga et al. 2012). Interestingly, part of this effect may include induction of posttranslational modifications in p53 that prevents its degradation, thereby potentiating its actions. Whether such an interaction occurs in sensory neurons is not yet known, but it is known that acetylated p53 is a positive regulator of peripheral nerve regeneration-associated gene expression (Tedeschi, Nguyen et al. 2009) and neurite outgrowth (reviewed in (Krishnan, Duraikannu et al. 2016)). It likely does so via post-translational modifications as there were no changes in p53 transcript levels in response to 3,7 or 14 day sciatic nerve injury (Renno, Al-Maghrebi et al. 2013).

In contrast to p53's positive links to regeneration, when it interacts with two E3 ubiquitin ligases, MDM2 and MDM4 to form a triad, it can impede regeneration in the CNS. If this interaction between the ubiquitin ligases and p53 is interrupted, then p53 becomes activated and it can promote axonal growth through the IGF1 signaling pathway (Joshi, Soria et al. 2015). Of interest, Zhang and Misra (Zhang and Misra 2014) revealed that ZF can displace MDM2 from its association with p53 in U2OS cells, a potential mechanism for its role in injured sensory neurons. Whether an interaction between ZF and p53 occurs in sensory neurons and whether it serves to promote regeneration needs to be explored.

Peripheral nerve repair necessitates tremendous protein production and correct protein folding. Thus, it is not surprising that induction of an UPR in sensory neurons following injury has recently been described and shown to be critically linked to the ability of axons to regenerate (Onate, Catenaccio et al. 2016) and which we have shown to be regulated by Luman (Ying, Misra et al. 2014, Ying, Zhai et al. 2015). Recent work in the Misra lab supports a potential role for injury-induced changes in ZF expression in the positive impact that the UPR has on nerve

repair. They reveal an interaction between p53 and ZF in regulation of the unfolded protein response (UPR) in osteosarcoma cells, a response which is lost in the absence of p53 (Zhang, Thamm et al. 2015). Furthermore, amino acid deprivation, another ER stressor and a situation that likely arises in sensory neurons as they attempt to meet the protein synthesis demands of regeneration has been shown to induce ZF expression (Zhang, Jin et al. 2010).

Finally, we also observed an association between injury-induced changes in endogenous BDNF expression and ZF expression. We have shown that injury-associated changes in BDNF expression in sensory neurons are critical for induction of regeneration-associated gene expression and the intrinsic ability of a sensory neuron to regenerate an axon (Geremia, Pettersson et al. 2010). I have shown that intrathecal delivery of BDNF-specific siRNAs can attenuate ZF's early response to injury. Thus, collectively, the early response of ZF to injury, namely its initial decreased expression, coupled with its regulation by BDNF supports that it may play a role in induction of the regeneration response in sensory neurons by potentially de-repressing gene expression. Further, its bilateral biphasic response from two days post-injury on, similar to that observed for two additional stress-associated transcription factors, Luman and FOXO3a, suggests an emerging complex pattern of transcriptional stress responses in sensory neurons that includes a systemic component in addition to those on the injured neuron.

In conclusion, it is clear that a deeper understanding of the complex regulation of ZF and its transcriptional partners in homeostasis and cellular stress responses of injured sensory neurons is needed. Future studies will explore direct interactions between ZF and nerve regeneration by preventing the upregulation in ZF expression at 2days using siRNA or by creating an adenoviral vector that will allow overexpression of ZF in the one day injured neurons to see how this impacts induction of regeneration programs in sensory neurons. Learning how to exploit and manipulate the transcriptional programs driving effective nerve regeneration will benefit many.

6. General Discussion

6.1. Summary of findings

The regrowth potential of the peripheral nervous system has been extensively studied. Upon injury, DRG neurons undergo a sequelae of genetic events resulting in a phenotypic shift from a quiescent cell to a regenerating one. This shift is accomplished by distinct transcriptional phases to first sense the stress event, then start the injury response and finally, to build and sustain newly constructed axons. The expression and post-translational state of multiple transcription factors is altered by the intracellular signaling cascades initiated after axotomy (Patodia and Raivich 2012). But, little is known about the how regulators of stress responses are altered in these different phases of the cell body response of sensory neurons. Here, I investigated further, 3 unique transcriptional regulators, all previously shown to respond to select cellular stress events in either sensory neurons or other cell types to determine how changes in their expression patterns correlate with the response to nerve injury. We further identified the possibility that the expression of these transcription factor might be regulated by either BDNF, a neurotrophin that we have shown regulates induction of the regeneration response or by brief electrical stimulation, a therapeutic intervention that enhances the regeneration response. Either modulation of endogenous BDNF signaling or brief electrical stimulation led to alterations in the expression of the three stress transcription factors examined, suggesting that these proteins are involved in the cell body response of sensory neurons to injury. The specific findings were:

6.1.1. FOXO3a

The family of forkhead box class O family are a group of highly conserved transcription factors responsible for a number of cellular processes including glucose metabolism dysfunction, cell cycle arrest, inflammation and apoptosis (van der Horst and Burgering 2007). Specifically, FOXO3a activation is the downstream target of these stress states in many neuronal cell types (Maiese, Chong et al. 2007). Furthermore, FOXO3a protein levels were shown to decrease in DRG neurons in response to sciatic nerve injury (Wang, Liu et al. 2009). However, despite being shown to be regulated by nerve injury by Western blot analysis in the Wang, Lui et al. 2009 study, this is a transcription factor that I found is heterogeneously expressed in both the cytoplasmic and nuclear neuronal compartments and how this expression is altered temporally in distinct subpopulations and neuronal compartments has never been characterized.

To further examine the temporal impact of injury on FOXO3a expression, a unilateral spinal nerve transection model was used and both ipsilateral and contralateral DRGs were harvested after the predetermined time points. Immunohistochemical analysis showed that FOXO3a is most highly expressed in small-medium presumed nociceptive neurons in intact DRG and that axotomy leads to a significant time dependent decrease in transcription factor protein levels ipsilateral to lesion beginning at 1 day with a further decline by 2days. This change was most obvious in the small to medium sized population; however, levels began to rise by 4 days and 1 week in all DRG neurons including the medium to large sized. While there were definitive changes in protein levels, FOXO3a mRNA levels between ipsilateral and contralateral DRG proceeds for in situ hybridization were relatively similar with only mild differences between time points. It is well established that FOXO transcription factors are heavily regulated at the post translational level (van der Horst and Burgering 2007) and therefore we hypothesized that the injury induced alterations might be secondary to alterations in neurotrophin signaling. DRG neurons are highly responsive to neurotrophin manipulation in both development and pathological states (Lindsay 1996, Verge, Gratto et al. 1996). Extensive work by our lab has suggested that BDNF is a candidate for the induction of the injury response as it is the only neurotrophin whose expression is upregulated in after injury, with its suppression of its expression or actions decreasing intrinsic axon growth capabilities and RAG levels (Karchewski, Kim et al. 1999, Karchewski, Gratto et al. 2002, Geremia 2005, Geremia, Pettersson et al. 2010). With this knowledge, we ascertained the effect of endogenous BDNF manipulation on FOXO3a activity in injured DRG neurons by using siBDNF and conversely brief electrical stimulation, which upregulates the neurotrophin and enhances the regeneration response (Geremia, Gordon et al. 2007). Disruption of BDNF with siBDNF intrathecal injections lead to increased expression of FOXO3a mRNA in ipsilateral and contralateral DRG neurons, suggesting that baseline endogenous levels of BDNF potentially regulate transcriptional changes of FOXO3a. Crush injury lead to a temporary decrease in FOXO3a immunoreactivity; however, electrical stimulation appeared to further suppress FOXO3a in crush plus stimulation animals compared to crush alone. It is suspected that increased BDNF signaling during this therapy was responsible for this observation.

These results helped to theorize a potential mechanism of FOXO3a in injured DRG neurons where increased BDNF leads to hyperphosphorylation of nuclear FOXO3a through trk signaling;

thus facilitating its cytoplasmic translocation and subsequent ubiquitination and degradation, but this remains to be tested. The biological consequence of suppressed FOXO3a after injury is not clear; however, we theorized that its transcriptional regulation of cell cycle regulators, including $p27^{kip1}$, is critical. Evidence suggests that peripheral nerve injury alters the activity of multiple tumor suppressor genes and that a trigger for regeneration might be unlocking cell cycle arrest (Krishnan, Duraikannu et al. 2016).

In the past, it has been generally accepted that genetic observation seen in contralateral DRG were comparable to baseline expression patterns seen in naïve animals and contralateral samples were used as controls. However, there have been frequent reports of unilateral injury causing changes to "uninjured" neurons with various suspected mechanisms. This work was the first to examine the effect of unilateral nerve injury on FOXO3a expression in contralateral DRG neurons. We observed a biphasic response in neurons contralateral to injury where FOXO3a protein nuclear staining was up by 1 day, down at 4 days and up by 1 week with staining in medium to large neurons higher than naïves. Again, these changes were primarily seen at the protein level. It was unclear whether these changes were due to propagating signals through the spinal cord or from a systemic source. Uninjured C4 ganglia from L4-L6 spinal nerve transected rats showed elevated FOXO3a levels compared to naïves, suggesting an unknown humeral injury signal triggered by peripheral axotomy likely exists.

6.1.2. Luman

The injury response and subsequent regeneration of DRG neurons involves the upregulation of numerous RAGs, an increase in protein synthesis and packaging and undoubtedly elevated levels of ER stress as the cells attempt to regrow injured axons. The unfolded protein response is activated in times of ER stress where transcriptional regulators are employed and activate target genes to assist in protein folding, degradation and triggering apoptotic pathways if necessary (Ron and Walter 2007). The importance of a complete and coordinated UPR to peripheral nerve recovery after injury has been recently described where inhibition or knockout of certain UPR regulators leads to delayed motor recovery, reduced myelin removal and axonal regeneration (Onate, Catenaccio et al. 2016).

Luman is a known transcriptional regulator of the UPR, binding promoter regions containing CRE and UPRE (Lu, Yang et al. 1997, Lu and Misra 2000) and is expressed by a variety of rat

tissue types (Ying, Zhang et al. 2015). Ying et al demonstrated that axonal Luman is rapidly activated in the axonal ER equivalent, translated from axon-derived transcripts and is retrogradely transported by an importin-dynein mediated mechanism to the sensory neuron soma (Ying, Misra et al. 2014). In addition, they established a link between Luman activation and the regenerative response as disruption with siRNA impaired neurite outgrowth (Ying, Misra et al. 2014). This association with regeneration was further examined as the transcription factors was shown to regulate components of the cholesterol biosynthetic pathway, which is critical to the production of plasma membrane for growth axons (Ying, Zhai et al. 2015). These finding were remarkable, as they pointed to Luman as not only a regulator of the UPR in sensory neurons but as a sensor of injury and potential modulator of the regenerative response.

Even though early axonal Luman responses were characterized at 1 day after injury and also examined at the somal level, it was unknown how expression of the transcription factor was temporally regulated in response to injury at times outside the one-day point as part of the cell body response to injury. The spinal nerve transection model showed a temporal increase in cell body Luman protein and RNA levels in ipsilateral injured primary sensory neurons. This was a biphasic response where expression levels peaked at 2-days post injury, were decreased by 4 days and up again by 1 week. Luman protein immunoreactivity was seen highest in small to medium sized neurons. Interestingly, we again saw a contralateral response to injury as uninjured DRG neurons also had a biphasic response minus the significant rise at 2 days seen in the injured group. The suppression of Luman at the 4-day time point was initially perplexing; however, recent seminal work by Li et al described the transcriptional landscape of DRG neurons post-injury with three distinct transcriptional phases: The stress sensing, early regeneration and late regeneration phases (Li, Xue et al. 2015). The 4-day point is the transition point for the early to later regeneration phases and was shown to have a nadir in transcription factor activity; coinciding to the reduced expression seen with Luman at this post lesion time point. Finally, to further explore the impact of injury on axonal Luman, brief electrical stimulation proximal to a crush site caused decreased protein levels in nerve fibres proximal to the crush site. Administration of this pro-regenerative therapy likely heightens the injury response of the transcription factor, increasing Luman's activation and association with the importin complex machinery for ease of retrograde transport.

6.1.3. Zhangfei

The initial studies that elucidated the role of Luman as a neuronal transcription factor lead to the discovery of Zhangfei, a potent and efficient inhibitor of Luman activity (Lu and Misra 2000, Misra, Rapin et al. 2005). Experiments revealed that ZF was another basic leucine zipper transcriptional regulator expressed in sensory neurons (Akhova, Bainbridge et al. 2005). Its overexpression *in vitro* competitively inhibits the VP16 associated transactivation complexes on HSV-1 immediate-early genes and disrupts viral replication, in opposition to the actions of Luman (Akhova, Bainbridge et al. 2005). The transactivation function of ZF is accomplished through its interaction with intermediary proteins including the tumor suppressor protein p53 (Lopez-Mateo, Villaronga et al. 2012) and ATF4 (Hogan, Cockram et al. 2006) where ZF binding or heterodimerization promotes their transcriptional activity and enhances binding to their target promoters, including cAMP response elements with the later (Hogan, Cockram et al. 2006). While Luman is clearly an inducer of the UPR, ZF suppresses the UPR where its ectopic expression in osteosarcoma cell lines lead to decreased levels of Xbp1, HERP, CHOP and Bip (Bergeron, Zhang et al. 2013). With the work of Ying et al showcasing Luman as not only a regulator of the UPR and a role as an injury signal, I hypothesized that ZF expression would be altered in atomized DRG neurons with a potential role in the regenerative response.

With the previously described L4-L6 spinal nerve transection time course model and immunohistochemistry, I observed that peripheral transection led to a multiphasic phasic response in ZF protein levels with an initial decrease at the 1-day, transient robust increase at 2-days then another elevation in levels at 1-week. This was seen in both the small-medium and medium-large sized population with higher overall staining in the former. Again, a contralateral effect was observed in a biphasic pattern as well with a significant decline in staining seen at the 4-day time point. This was a bilateral observation with both sides showing decreased immunofluorescence staining at 4-days compared to the prior time point. ZF mRNA levels were also bilaterally altered in a pattern similar to those seen at the protein level. As with the previous studies, a sham time course did not show any significant qualitative changes in ZF protein staining, suggesting that the contralateral changes were not secondary to the surgical stress related to the nerve exposure, but rather are associated with the actual injury response.

To examine whether BDNF, as an inducer of the regenerative response, is a potential regulator of injury-associated changes in ZF expression, we downregulated endogenous levels of

BDNF in neurons after injury (Geremia, Pettersson et al. 2010). Infusion of siBDNF lead to dramatic decrease in both contralateral and ipsilateral ZF mRNA levels and prevented the injury responses seen in the controlled groups. This suggests two possible mechanisms; either the BDNF signaling pathway is directly responsible for ZF expression at either the gene or protein level or inhibiting the regeneration response secondarily mitigates the need for ZF upregulation. The second hypothesis is very plausible as a decreased regenerative response would lead to depressed axonal outgrowth and less need for UPR suppression via ZF.

6.2. Contralateral effect of nerve injury

The innervation of muscle, skin and other structures by peripheral nerves is largely distinct for each half of the body; where distal sensory afferents, motor axons and sympathetic fibres of the right are anatomically separated from the left. Historically, this fact has allowed nerve injury researchers to compare observations in injured ipsilateral neurons to the contralateral "control" side. As the neurobiology of peripheral nerve injury advanced, there was a growing body of evidence that unilateral nerve damage led to bilateral changes in a wide variety of measured outcomes including gene expression and anatomical organization (reviewed in (Koltzenburg, Wall et al. 1999)). Clinically, "mirror-pain" or "mirror image pain" is a documented phenomenon where unilateral injury results in bilateral changes in pain sensation in certain patient populations (Huge, Lauchart et al. 2008, de la Llave-Rincon, Fernandez-de-las-Penas et al. 2009, Fernandez-de-las-Penas, de la Llave-Rincon et al. 2009, Konopka, Harbers et al. 2012, Werner, Ringsted et al. 2013); however, the incidence in humans is not known and there is no consensus on effective treatment strategies (Drinovac Vlah, Bach-Rojecky et al. 2016).

The contralateral effect of injury has been demonstrated in motor neurons which includes; increased mRNA of neuropeptides including CGRP, cholecystokinin (CCK), and RAGs such as GAP43, and increased release of acetylcholine (Herrera and Grinnell 1981, Herrera, Grinnell et al. 1985, Piehl, Arvidsson et al. 1991, Linda, Piehl et al. 1992, Booth and Brown 1993, Verge, Wiesenfeld-Hallin et al. 1993). Similar findings are seen in autonomic neurons as injury to sympathetic nerves causes contralateral upregulation in the p75 neurotrophin receptor, increased sprouting into DRG and spinal roots and decreased acetylcholine (Viana and Kauffman 1984, Dornay, Gilad et al. 1985, Smith, Reddy et al. 1990, Kuchel and Zigmond 1991, Kuchel, Hellendall et al. 1992, McLachlan, Janig et al. 1993). However, the bulk and strongest evidence

suggesting the existence of a contralateral injury response are with DRG neurons and sciatic injury models, specifically the nociception subpopulation. Unmyelinated sensory neurons when electrically stimulated release neuropeptides SP and CGRP into peripheral tissues causing increased blood flow, vascular permeability and extravasation of plasma proteins which is reduced after injury (Lembeck and Holzer 1979, Lembeck and Gamse 1982, Brain and Cambridge 1996). Injury and subsequent regeneration of saphenous nerves significantly inhibits the ability for peripheral plasma extravasation in contralateral nerve muscle targets (Allnatt, Dickson et al. 1990). This suggests that a bilateral effect is not only isolated to the neuron itself but to innervated tissues with likely behavioral and clinical significance.

Several of axotomy induced alteration in sensory neuron expression patterns have been observed in contralateral dorsal root and other sensory ganglia. There is bilateral change in mRNA for CCK and other neuropeptides including vasoactive interstitial polypeptide (VIP), neuropeptide Y and galanin (Verge, Wiesenfeld-Hallin et al. 1993, Zhang, Ji et al. 1996). There is a trend that many of these bilateral changes are observed in inflammatory and neuropathic pain models and with known potential pain mediating molecules; for instance, with the pain and injury models such as chronic constriction injury there is increased expression of bradykinin receptors B1 and B2, and decreases in tetrodotoxin-resistant Na+ channels which both are implicated in neuropathic hypersensitivity following injury (Oaklander and Belzberg 1997, Petersen, Eckert et al. 1998, Levy and Zochodne 2000) . Unilateral injury is observed to cause a bilateral activation of endogenous μ-opioid receptors which attenuates bilateral pain states in animal behavior models (Mansikka, Zhao et al. 2004). Sciatic nerve and spinal nerve transection also leads to increases TNF-alpha and TNF receptor immunoreactivity in L4-L5 DRG ipsilateral and contralateral to injury compared to sham and naïve animals, supporting this effect plays a role in inflammatory states (Dubovy, Jancalek et al. 2006). Neurotrophin signaling may also be altered in contralateral ganglia, as there is a significant increase in p75 neurotrophin receptor in DRG perineuronal satellite cells (Zhou, Rush et al. 1996). Furthermore, the neurotrophin NGF has been implicated as a signaling factor in these bilateral changes, such that unilateral injection of NGF increased the levels of substance P and CGRP in similar neuron subpopulations in both ipsilateral and contralateral DRG (Amann, Sirinathsinghji et al. 1996, Donnerer, Amann et al. 1996). In addition, the involvement of neurotrophins is also implicated in p75 knockout mice which have decreased sprouting of contralateral sympathetic fibres (Ramer and Bisby 1997).

These finding taken together show that contralateral changes are frequently observed in multiple cell types but also with different experimental designs and outcome measures including immunohistochemistry and *in situ* hybridization. However, the interpretation of these results should be readily scrutinized as most are incidental findings where the original research question and design was not aimed at elucidating whether contralateral changes were different from controls. One commonality is that a vast majority of these positive contralateral effects are to a lesser degree than those seen ipsilaterally and have a more delayed time course. Despite this, published reports with multiple injury markers including RAGs, neuropeptides, the neurotrophin BDNF (Karchewski, Gratto et al. 2002) have shown no evidence to suggest that their contralateral expression patterns are affected in injured states. In order to fully understand the bilateral implications of peripheral nerve transection, the researcher may need to augment experimental numbers to compensate for this less robust response.

6.2.1. Potential mechanisms: neuronal versus humoral

There are two main mechanisms that are hypothesized to contribute to the contralateral changes; firstly, that signals propagated from the injured neurons are transmitted through the spinal cord and effect the neurons directly. Secondly, this injury signal originates from the injured tissue or from a central source which is transmitted systemically by the blood to act on the opposite side. While I observed consistent largely biphasic contralateral responses for all three transcription factors examined in contralateral DRG, the fact that I also observed similar impacts on FOXO3A in DRG at the cervical level, remote from the site of lesion, supports a that a humoral source contributes to the observed response. There is little to no evidence in the literature for the existence of either of these mechanisms; however, there are some examples in the literature that can be used to support both (reviewed in (Koltzenburg, Wall et al. 1999). Regardless, the neuronal mediated process is generally the more accepted mechanism.

6.2.2. Humoral/systemic mechanism

Systemic inflammatory responses are well-defined clinical entities, where a severe insult to the organism results in a systemic cascade of inflammatory mediators causing hemodynamic instability, end organ damage and potentially multisystem organ failure (Balk 2014). This pathophysiologic response is most commonly associated with infection, severe sepsis, trauma

and a variety of other injuries (Angus and van der Poll 2013, Balk 2014, Binkowska, Michalak et al. 2015). Spinal cord injury (SCI) is one such case where evidence suggests pathological connections emanating from the injured spinal cord result in a profound and sustained intraspinal and systemic inflammatory response with increased circulation of immune cells and pro-inflammatory mediators resulting in distal end organ dysfunction [reviewed in (Sun, Jones et al. 2016)]. Following transection of a peripheral nerve, the severed distal segment undergoes Wallerian degeneration where an inflammatory driven process leads to macrophage invasion and clearance of the damaged axons and associated glia (Rotshenker 2011). While release of breakdown products and inflammatory markers from the lesion site into the circulation is likely to occur, studies investigating the impact of peripheral nerve injury on systemic inflammatory states is markedly absent in the literature. It is unknown whether these signals exist at a level to potentially contribute to a contralateral affect. Even though SCI and central nervous system trauma is a pathologically and biochemically distinct process as compared to PNS disease, the data pointing to the role of systemic inflammation post-SCI and its deleterious consequences implies that peripheral nerve injury or other neurotraumatic events may cause humoral inflammatory changes that are potentially significant.

There is also dysregulation of the neuroendocrine system in SCI subjects with increased activation of the hypothalamic-pituitary-adrenal (HPA) axis, a potent endocrine regulator of stress and inflammation with elevated levels of glucocorticoids as the end result (Lerch, Puga et al. 2014). Glucocorticoids including corticosterone and cortisol are steroid hormones produced in the zona fasciculate of the adrenal cortex, which bind to glucocorticoid receptors (GR) that then translocate to the nucleus and regulate the transcription of genes containing the GR response element (Purves D 2001). Circulating levels of endogenous corticosterone (rodents) and cortisol (humans) increase after SCI (Cruse, Keith et al. 1996, Lucin, Sanders et al. 2007), undoubtedly affecting multiple GR-regulated cellular functions; however, specifics of GR signaling is not well defined in the context of nervous system injury. The impact of peripheral nerve damage on the HPA axis is not well understood but dorsal root ganglion neurons express GR and steroid or HPA axis manipulation has been shown to alter DRG neuron expression patterns (Smith, Seckl et al. 1991, Covenas, DeLeon et al. 1994, DeLeon, Covenas et al. 1994). Adrenalectomy and exogenous glucocorticoid administration leads to changes in DRG neuropeptide levels including SP, CGRP and somatostatin (Smith, Seckl et al. 1991, Covenas, DeLeon et al. 1994). As these

steroid hormones can directly affect DRG phenotype, its plausible to consider glucocorticoids as a potential contributor to either bilateral, contralateral or systemic sensory nerve injury changes but the response of the HPA in the injured state needs to be characterized.

As stated above, altered neurotrophin signaling has been a suspected contributor to the contralateral effect. Most recently, Shakhbazau et al discovered that unilateral sciatic nerve transection resulted in elevation of NFG and NT3, but not GDNF or BDNF, in the uninjured contralateral nerve (Shakhbazau, Martinez et al. 2012). This evidence suggests that unilateral injury can regulate systemic neurotrophin levels and therefore enhance neurotrophin signaling in uninjured DRG neurons, a population highly sensitive to these growth factors.

There have been problems with the humoral explanation for the contralateral effect. Firstly, contralateral findings have traditionally been very specific to the DRG or spinal segments corresponding to the levels effected by the ipsilateral injury. Its argued that a generalized non-specific systemic mediated response could not cause the precise spatial distribution of findings in structures on the opposing side. Furthermore, no strong evidence exists to suggest that circulating factors have a role in ipsilateral regulation of the injury response in DRG neurons. This area of study requires better understanding of the systemic implications of PNS pathologies before they can be attributed to changes in contralateral neurons.

6.2.3. Neuronal/trans-spinal mechanism

The other possibility that is theorized is that damage to axons leads to generation of an anterograde signal into the spinal cord and ultimately acts upon contralateral homonymous neurons. Changes in electrical neuronal activity is a potential signaling candidate, but with regards to sensory neurons and the significance of electrical activity propagating into the spinal cord the data is conflicting. Severance of the axon does lead to retrograde electrical signaling to injured DRG neurons as calcium influx at the site of injury (Cho and Cavalli 2012) causes depolarization leading to direct genetic alterations. One study utilizing peripheral administration of TTX showed that chronic depression of sensory neuron activity did not produce central changes in the dorsal horn of the spinal cord, particularly with neuropeptide levels which often exhibit contralateral changes (Wall, Mills et al. 1982). However, other data suggest dorsal horn neurons alter activity dependent enzyme levels following either physical (axotomy) or functional (TTX) disconnection (Carr, Haftel et al. 1998), which counters the above claim.

Spinal cord glia are now more recognized as important mediators in a variety of pathological conditions, particularly certain pain states, and have shown the propensity for intraspinal electrical signaling (Watkins, Milligan et al. 2001, DeLeo, Tanga et al. 2004). Inflammatory mediated neuropathic pain models can lead to increased allodynia (pain to nocuous stimuli that was previously benign) bilaterally or mirror image pain (Chacur, Milligan et al. 2001, Milligan, Twining et al. 2003). The ipsilateral and mirror image (contralateral) pain is blocked by preventing spinal glial activation and blocking the release of glial derived proinflammatory cytokines (Chacur, Milligan et al. 2001). These contralateral communications are thought to be mediated through gap junctions that connect spinal glia into widespread networks (Verkhratsky, Orkand et al. 1998). Activation of glia at one site leads to propagation of calcium waves via gap junctions, activating distant glia and releasing pain mediating substances (Newman 2001). Treatment with a gap junction decoupler, carbenoxolone, reversed mirror pain in inflammatory neuropathic pain models but left ipsilateral allodynia unaffected (Spataro, Sloane et al. 2004).

Finally, commissural interneurons may have the capacity to convey signal messages to the contralateral side. It has long been known neurons convey axons from one dorsal horn to the other via the dorsal commissure (Ramón y Cajal 1895). Electrophysiological work showed that neurons in the superficial dorsal horn respond to electrical stimulus of unmyelinated c-fibres in the contralateral nerve (Fitzgerald 1982, Fitzgerald 1982, Fitzgerald 1983). This transmedian signaling through interneurons would require bidirectional signals across synapse and in theory electrical activity could accomplish this. Some have hypothesized that the trans-spinal mediators are neurotrophins, particularly BDNF and NT3, as their expression is upregulated after injury and could signal both anterogradely and retrogradely (Koltzenburg, Wall et al. 1999). While there is a lack of evidence supporting this mechanism, more recent data suggests that neurotrophin signaling is likely altered systemically and peripherally (Shakhbazau, Martinez et al. 2012) and this possibly leads to contralateral changes. While little research has been done in this area, the existence of both central and systemic influences is likely. The spinal mechanism is more strongly supported by the neuropathic pain data and the reality of mirror pain states.

6.2.4. Significance of new contralateral findings

This work was the first to show that unilateral sciatic nerve transection can cause contralateral changes in transcription factor expression and thereby potentially altering the

phenotype of the “uninjured” side. All three of the stress related transcriptional activators, FOXO3a, Luman and ZF, showed contralateral changes in expression and localization in the injury time course models but not in sham experiments, inferring that these changes are secondary to unilateral injury and not surgical stress. FOXO3a expression in contralateral DRG showed a biphasic response with an initial increase that was opposite to the ipsilateral response. This finding is not in keeping with previous documented bilateral responses, as the contralateral effect usually mirrors the ipsilateral but to a lesser degree. A significant finding for FOXO3a was that uninjured C4 ganglia from L4-L5 spinal nerve injured rates had increased levels of the protein, similar to what was seen contralaterally. As with the ipsilateral findings, these are possibly secondary to post-translational modifications of FOXO3a. This unexpected result builds the argument that a systemic response to peripheral injury likely exists in some capacity and may be biochemically significant.

The effects on Luman and ZF were also biphasic with a significant drop in expression at the 4-day time point. Any changes in these regulators of the UPR would imply that the contralateral DRG neurons are experiencing varying degrees of ER stress. As explained, the 4-day time point corresponds to a significant transition point in the injury response of sensory neurons when transcription factor activity and transcriptional activation is depressed and less need for a coordinated UPR. This contralateral effect could be attributed to both humoral or trans-spinal communication, but due to the close spatial and temporal relationship a neuronal mechanism is more plausible but still speculative. That these findings potentially correlate with altered plasticity in the affected contralateral ganglia is supported by the Ryoke, Ochi et al. (Ryoke, Ochi et al. 2000) study showing that a lesion can serve to condition contralateral neurons to regenerate more robustly should they become injured.

6.3. Potential therapeutics

The work of Ying et al was significant in identifying the UPR associated transcription factor Luman as a retrograde injury signal essential for the regrowth of injured axons (Ying, Misra et al. 2014, Ying, Zhai et al. 2015, Ying, Zhang et al. 2015). In continuing that work, we showed that Luman and the UPR repressor ZF are upregulated in soma of DRG neurons after axotomy and associated with the cell body response of injured neurons. This further strengthens the argument that a coordinated UPR is essential for the regeneration process. Despite the years of

study and the development of multiple therapies that have shown biochemical and clinical evidence of repair, recovery from PNS injury is still slow and minimal. While Luman's mechanism is better understood at this point, the importance of ZF with regards to either the hindrance or enhancement of repair is not known. If the UPR is essential to either the initiation or the maintenance of the regeneration response, its postulated that the action of this UPR suppressor may inhibit the early stages of axon growth. However, a prolonged UPR could eventually lead to apoptosis. Therefore, the timing of ZF expression appears critical and therapies that might streamline its response could lead to better functional recovery. These could include target direct therapy with a monoclonal antibody to ZF, administered early hours after injury to better enhance the UPR during the early regeneration phase.

We continued to examine the role endogenous BDNF plays in the injury response of DRG neurons and saw that its disruption reversed the injury associated changes seen with ZF and FOXO3a. These finding continue to support the claim of BDNF as the candidate induction molecule of the regeneration response and the changes seen with these transcriptional regulators are likely secondary to blunted outgrowth of axons when BDNF is inhibited. The bulk of evidence from our lab points to importance of this neurotrophin in sensory neuron biology and its potential as a target to improve repair.

Brief electrical stimulation has successfully moved from animal models to the clinical world and will soon be readily available to patients suffering from traumatic nerve injures as a first-line treatment. This therapy was shown to enhance the injury responses of FOXO3a and Luman in DRG cell bodies and nerves respectively. These results further advocate for the use of electrical stimulation in these pathologies and its mechanism of action may entail direct or indirect transcription factor regulation

6.4. Future directions

Expression of these stress related transcription factors in DRG neurons appears to be altered in by nerve injury as observed in the spinal nerve transection model. Their role in sensory neurons likely exceeds the scope of regeneration and should be explored with other peripheral nerve pathology models, including chronic inflammation/neuropathic pain and demyelination. Recent data shows that ER stress is impaired in spinal cord neurons of neuropathic pain models (Zhang, Yi et al. 2015). Further exploration into the expression pattern of Luman and ZF during

neuropathic pain would lead to the hypothesis that endogenous disruption of these regulators with intrathecal injections of targeted small interfering RNAs (siRNAs) might significantly alter behavioral pain states.

Ying et al demonstrated that treatment with siLuman decreased neurite outgrowth of injured DRG neurons *in vitro* (Ying, Misra et al. 2014). As I have demonstrated here, Luman expression is associated with the cell body injury response. The next step is the intrathecal administration of siLuman *in vivo* to peripherally injured animals and observe whether regeneration is inhibited by examining the expression of RAGs and the use of behavioral studies assessing functional recovery. These experiments should be duplicated with siZF *in vitro* and *in vivo* with the hypothesis that axon growth will also be inhibited, arguing further for the need of a coordinated UPR for proper regeneration. ZF is also expressed in the axons of sensory neurons and it's unknown whether it has axonal localized mRNA that undergoes local protein synthesis similar to Luman. The idea of ZF as potential retrograde injury signal to turn off the UPR initiated by Luman is an enticing theory.

It was revealed that Luman potentially regulates axon growth not only through regulating the UPR but by activating mediators in the cholesterol biosynthesis pathway (Ying, Zhai et al. 2015). Whereby the inhibitory effect of Luman disruption with siLuman were partially rescued by the administration of cholesterol. Statins are one of the most frequently prescribed medications in the western world with retrospective and prospective evidenced showing that these lipid lowering agents decrease the incidence of cardiovascular and cerebrovascular events and improve outcomes after these events have occurred (Anderson, Gregoire et al. 2016). However, statins are noted for side effects such as myopathies that can lead to significant functional symptoms for patients (Collins, Reith et al. 2016). More recently, statins have been linked to sensory and autonomic ganglionopathies and small fibre neuropathies (Novak, Pimentel et al. 2015). This has led to the hypothesis that statin therapy might alter sensory neuron homeostasis in either native or nerve transected subjects and heighten Luman activity due to its association with cholesterol synthesis.

6.5. Conclusion

The findings of this thesis demonstrate that the expression of stress related transcription factors associated with either neuronal survival (FOXO3a) or the unfolded protein response

(Luman and ZF) is temporally altered in axotomized DRG neurons. These 3 regulators of transcription are now attributed as part of the cell body regeneration response. Furthermore, their activity and expression appears partly regulated by a BDNF-associated mechanism. The most striking discovery is the noticeable contralateral effect of these injury markers, and the first noted impact on contralateral transcription factors. While the ipsilateral findings were predictable due to the noted phenotypic switch of injured sensory neurons, the interesting and dramatic contralateral changes brings the topic of this effect to the forefront. Besides the further work needed to decipher which mechanisms are responsible, the question remains: Are these contralateral changes harmful or helpful? Experimentation with key questions pointing to the nature of the contralateral effect and not merely as an incidental finding is required.

7. References

Abrahamson, I. K., D. Bridges and R. A. Rush (1987). "Transport of endogenous nerve growth factor in the proximal stump of sectioned nerves." J Neurocytol **16**(3): 417-422.

Acheson, A. and R. M. Lindsay (1996). "Non target-derived roles of the neurotrophins." Philos Trans R Soc Lond B Biol Sci **351**(1338): 417-422.

Adachi, Y., K. Yamamoto, T. Okada, H. Yoshida, A. Harada and K. Mori (2008). "ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum." Cell Struct Funct **33**(1): 75-89.

Akhova, O., M. Bainbridge and V. Misra (2005). "The neuronal host cell factor-binding protein Zhangfei inhibits herpes simplex virus replication." J Virol **79**(23): 14708-14718.

Al-Majed, A. A., T. M. Brushart and T. Gordon (2000). "Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons." Eur J Neurosci **12**(12): 4381-4390.

Al-Majed, A. A., C. M. Neumann, T. M. Brushart and T. Gordon (2000). "Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration." J Neurosci **20**(7): 2602-2608.

Allnatt, J. P., K. E. Dickson and S. J. Lisney (1990). "Saphenous nerve injury and regeneration on one side of a rat suppresses the ability of the contralateral nerve to evoke plasma extravasation." Neurosci Lett **118**(2): 219-222.

Amann, R., D. J. Sirinathsinghji, J. Donnerer, I. Liebmann and R. Schuligoi (1996). "Stimulation by nerve growth factor of neuropeptide synthesis in the adult rat in vivo: bilateral response to unilateral intraplantar injections." Neurosci Lett **203**(3): 171-174.

Andersen, L. B. and D. J. Schreyer (1999). "Constitutive expression of GAP-43 correlates with rapid, but not slow regrowth of injured dorsal root axons in the adult rat." Exp Neurol **155**(2): 157-164.

Anderson, M. J., C. S. Viars, S. Czekay, W. K. Cavenee and K. C. Arden (1998). "Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily." Genomics **47**(2): 187-199.

Anderson, T. J., J. Gregoire, G. J. Pearson, A. R. Barry, P. Couture, M. Dawes, G. A. Francis, J. Genest, Jr., S. Grover, M. Gupta, R. A. Hegele, D. C. Lau, L. A. Leiter, E. Lonn, G. B. Mancini,

R. McPherson, D. Ngui, P. Poirier, J. L. Sievenpiper, J. A. Stone, G. Thanassoulis and R. Ward (2016). "2016 Canadian Cardiovascular Society Guidelines for the Management of Dyslipidemia for the Prevention of Cardiovascular Disease in the Adult." Can J Cardiol.

Angus, D. C. and T. van der Poll (2013). "Severe sepsis and septic shock." N Engl J Med **369**(21): 2063.

Anitha, M., C. Gondha, R. Sutliff, A. Parsadanian, S. Mwangi, S. V. Sitaraman and S. Srinivasan (2006). "GDNF rescues hyperglycemia-induced diabetic enteric neuropathy through activation of the PI3K/Akt pathway." J Clin Invest **116**(2): 344-356.

Aoki, M., H. Jiang and P. K. Vogt (2004). "Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins." Proc Natl Acad Sci U S A **101**(37): 13613-13617.

Apfel, S. C., D. E. Wright, A. M. Wiideman, C. Dormia, W. D. Snider and J. A. Kessler (1996). "Nerve growth factor regulates the expression of brain-derived neurotrophic factor mRNA in the peripheral nervous system." Mol Cell Neurosci **7**(2): 134-142.

Asada, R., S. Kanemoto, S. Kondo, A. Saito and K. Imaizumi (2011). "The signalling from endoplasmic reticulum-resident bZIP transcription factors involved in diverse cellular physiology." J Biochem **149**(5): 507-518.

Audas, T. E., Y. Li, G. Liang and R. Lu (2008). "A novel protein, Luman/CREB3 recruitment factor, inhibits Luman activation of the unfolded protein response." Mol Cell Biol **28**(12): 3952-3966.

Averill, S., S. B. McMahon, D. O. Clary, L. F. Reichardt and J. V. Priestley (1995). "Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons." Eur J Neurosci **7**(7): 1484-1494.

Averill, S., G. J. Michael, P. J. Shortland, R. C. Leavesley, V. R. King, E. J. Bradbury, S. B. McMahon and J. V. Priestley (2004). "NGF and GDNF ameliorate the increase in ATF3 expression which occurs in dorsal root ganglion cells in response to peripheral nerve injury." Eur J Neurosci **19**(6): 1437-1445.

Averous, J., A. Bruhat, C. Jousse, V. Carraro, G. Thiel and P. Fafournoux (2004). "Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation." J Biol Chem **279**(7): 5288-5297.

Baas, P. W., J. S. Deitch, M. M. Black and G. A. Banker (1988). "Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite." Proc Natl Acad Sci U S A **85**(21): 8335-8339.

Balk, R. A. (2014). "Systemic inflammatory response syndrome (SIRS): where did it come from and is it still relevant today?" Virulence **5**(1): 20-26.

Barde, Y. A., D. Edgar and H. Thoenen (1982). "Purification of a new neurotrophic factor from mammalian brain." EMBO J **1**(5): 549-553.

Barthel, A., D. Schmoll, K. D. Kruger, G. Bahrenberg, R. Walther, R. A. Roth and H. G. Joost (2001). "Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells." Biochem Biophys Res Commun **285**(4): 897-902.

Barthelemy, C., C. E. Henderson and B. Pettmann (2004). "Foxo3a induces motoneuron death through the Fas pathway in cooperation with JNK." BMC Neurosci **5**(1): 48.

Becker, E. B. and A. Bonni (2004). "Cell cycle regulation of neuronal apoptosis in development and disease." Prog Neurobiol **72**(1): 1-25.

Benn, S. C., D. Perrelet, A. C. Kato, J. Scholz, I. Decosterd, R. J. Mannion, J. C. Bakowska and C. J. Woolf (2002). "Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival." Neuron **36**(1): 45-56.

Bentley, C. A. and K. F. Lee (2000). "p75 is important for axon growth and schwann cell migration during development." J Neurosci **20**(20): 7706-7715.

Berdan, R. C., J. C. Easaw and R. Wang (1993). "Alterations in membrane potential after axotomy at different distances from the soma of an identified neuron and the effect of depolarization on neurite outgrowth and calcium channel expression." J Neurophysiol **69**(1): 151-164.

Bergeron, T., R. Zhang, K. Elliot, N. Rapin, V. MacDonald, K. Linn, E. Simko and V. Misra (2013). "The effect of Zhangfei on the unfolded protein response and growth of cells derived from canine and human osteosarcomas." Vet Comp Oncol **11**(2): 140-150.

Bertolotti, A., Y. Zhang, L. M. Hendershot, H. P. Harding and D. Ron (2000). "Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response." Nat Cell Biol **2**(6): 326-332.

Binkowska, A. M., G. Michalak and R. Slotwinski (2015). "Current views on the mechanisms of immune responses to trauma and infection." Cent Eur J Immunol **40**(2): 206-216.
Biswas, S. C., Y. Shi, A. Sproul and L. A. Greene (2007). "Pro-apoptotic Bim induction in response to nerve growth factor deprivation requires simultaneous activation of three different death signaling pathways." J Biol Chem **282**(40): 29368-29374.
Bommiasamy, H., S. H. Back, P. Fagone, K. Lee, S. Meshinchi, E. Vink, R. Sriburi, M. Frank, S. Jackowski, R. J. Kaufman and J. W. Brewer (2009). "ATF6alpha induces XBP1-independent expansion of the endoplasmic reticulum." J Cell Sci **122**(Pt 10): 1626-1636.
Bomze, H. M., K. R. Bulsara, B. J. Iskandar, P. Caroni and J. H. Skene (2001). "Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons." Nat Neurosci **4**(1): 38-43.
Booth, C. M. and M. C. Brown (1993). "Expression of GAP-43 mRNA in mouse spinal cord following unilateral peripheral nerve damage: is there a contralateral effect?" Eur J Neurosci **5**(12): 1663-1676.
Brain, S. D. and H. Cambridge (1996). "Calcitonin gene-related peptide: vasoactive effects and potential therapeutic role." Gen Pharmacol **27**(4): 607-611.
Bronfman, F. C., C. A. Escudero, J. Weis and A. Kruttgen (2007). "Endosomal transport of neurotrophins: roles in signaling and neurodegenerative diseases." Dev Neurobiol **67**(9): 1183-1203.
Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis and M. E. Greenberg (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." Cell **96**(6): 857-868.
Brushart, T. M., P. N. Hoffman, R. M. Royall, B. B. Murinson, C. Witzel and T. Gordon (2002). "Electrical stimulation promotes motoneuron regeneration without increasing its speed or conditioning the neuron." J Neurosci **22**(15): 6631-6638.
Burnett, M. G. and E. L. Zager (2004). "Pathophysiology of peripheral nerve injury: a brief review." Neurosurg Focus **16**(5): E1.
Burton, P. R. and J. L. Paige (1981). "Polarity of axoplasmic microtubules in the olfactory nerve of the frog." Proc Natl Acad Sci U S A **78**(5): 3269-3273.

Calfon, M., H. Zeng, F. Urano, J. H. Till, S. R. Hubbard, H. P. Harding, S. G. Clark and D. Ron (2002). "IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA." Nature **415**(6867): 92-96.

Campbell, D. S. and C. E. Holt (2001). "Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation." Neuron **32**(6): 1013-1026.

Carr, P. A., V. Haftel, F. J. Alvarez, T. C. Cope and R. E. Fyffe (1998). "Effect of sciatic nerve transection or TTX application on enzyme activity in rat spinal cord." Neuroreport **9**(2): 357-361.

Carr, P. A. and J. I. Nagy (1993). "Emerging relationships between cytochemical properties and sensory modality transmission in primary sensory neurons." Brain Res Bull **30**(3-4): 209-219.

Carroll, P., G. R. Lewin, M. Koltzenburg, K. V. Toyka and H. Thoenen (1998). "A role for BDNF in mechanosensation." Nat Neurosci **1**(1): 42-46.

Castillo, V., M. Onate, U. Woehlbier, P. Rozas, C. Andreu, D. Medinas, P. Valdes, F. Osorio, G. Mercado, R. L. Vidal, B. Kerr, F. A. Court and C. Hetz (2015). "Functional Role of the Disulfide Isomerase ERp57 in Axonal Regeneration." PLoS One **10**(9): e0136620.

Chacur, M., E. D. Milligan, L. S. Gazda, C. Armstrong, H. Wang, K. J. Tracey, S. F. Maier and L. R. Watkins (2001). "A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats." Pain **94**(3): 231-244.

Chan, K. Y., T. W. Beck, P. Mullen and W. K. Dong (1989). "Increased axonal transport of trigeminal ganglion proteins in an electrode model." Invest Ophthalmol Vis Sci **30**(9): 2050-2055.

Chao, M. V. and B. L. Hempstead (1995). "p75 and Trk: a two-receptor system." Trends Neurosci **18**(7): 321-326.

Chevalier-Larsen, E. and E. L. Holzbaur (2006). "Axonal transport and neurodegenerative disease." Biochim Biophys Acta **1762**(11-12): 1094-1108.

Cho, H. J., J. K. Kim, H. C. Park, J. K. Kim, D. S. Kim, S. O. Ha and H. S. Hong (1998). "Changes in brain-derived neurotrophic factor immunoreactivity in rat dorsal root ganglia, spinal cord, and gracile nuclei following cut or crush injuries." Exp Neurol **154**(1): 224-230.

Cho, Y. and V. Cavalli (2012). "HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration." EMBO J **31**(14): 3063-3078.

Cho, Y., R. Sloutsky, K. M. Naegle and V. Cavalli (2013). "Injury-induced HDAC5 nuclear export is essential for axon regeneration." Cell **155**(4): 894-908.
Chong, Z. Z., J. Hou, Y. C. Shang, S. Wang and K. Maiese (2011). "EPO relies upon novel signaling of Wnt1 that requires Akt1, FoxO3a, GSK-3beta, and beta-catenin to foster vascular integrity during experimental diabetes." Curr Neurovasc Res **8**(2): 103-120.
Chong, Z. Z., J. Q. Kang and K. Maiese (2003). "Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt1, Bad, and caspase-mediated pathways." Br J Pharmacol **138**(6): 1107-1118.
Chong, Z. Z., J. Q. Kang and K. Maiese (2004). "Essential cellular regulatory elements of oxidative stress in early and late phases of apoptosis in the central nervous system." Antioxid Redox Signal **6**(2): 277-287.
Chong, Z. Z., F. Li and K. Maiese (2005). "Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury." Histol Histopathol **20**(1): 299-315.
Chong, Z. Z., F. Li and K. Maiese (2005). "Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease." Prog Neurobiol **75**(3): 207-246.
Chong, Z. Z., F. Li and K. Maiese (2006). "Group I metabotropic receptor neuroprotection requires Akt and its substrates that govern FOXO3a, Bim, and beta-catenin during oxidative stress." Curr Neurovasc Res **3**(2): 107-117.
Chong, Z. Z., S. H. Lin, J. Q. Kang and K. Maiese (2003). "Erythropoietin prevents early and late neuronal demise through modulation of Akt1 and induction of caspase 1, 3, and 8." J Neurosci Res **71**(5): 659-669.
Chong, Z. Z., S. H. Lin and K. Maiese (2004). "The NAD+ precursor nicotinamide governs neuronal survival during oxidative stress through protein kinase B coupled to FOXO3a and mitochondrial membrane potential." J Cereb Blood Flow Metab **24**(7): 728-743.
Chowdary, P. D., D. L. Che and B. Cui (2012). "Neurotrophin signaling via long-distance axonal transport." Annu Rev Phys Chem **63**: 571-594.
Christie, K., D. Koshy, C. Cheng, G. Guo, J. A. Martinez, A. Duraikannu and D. W. Zochodne (2015). "Intraganglionic interactions between satellite cells and adult sensory neurons." Mol Cell Neurosci **67**: 1-12.

Christie, K. J., C. A. Webber, J. A. Martinez, B. Singh and D. W. Zochodne (2010). "PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons." J Neurosci **30**(27): 9306-9315.

Clancy, D. J., D. Gems, L. G. Harshman, S. Oldham, H. Stocker, E. Hafen, S. J. Leevers and L. Partridge (2001). "Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein." Science **292**(5514): 104-106.

Cohen, S. (1960). "Purification of a Nerve-Growth Promoting Protein from the Mouse Salivary Gland and Its Neuro-Cytotoxic Antiserum." Proc Natl Acad Sci U S A **46**(3): 302-311.

Collins, R., C. Reith, J. Emberson, J. Armitage, C. Baigent, L. Blackwell, R. Blumenthal, J. Danesh, G. D. Smith, D. DeMets, S. Evans, M. Law, S. MacMahon, S. Martin, B. Neal, N. Poulter, D. Preiss, P. Ridker, I. Roberts, A. Rodgers, P. Sandercock, K. Schulz, P. Sever, J. Simes, L. Smeeth, N. Wald, S. Yusuf and R. Peto (2016). "Interpretation of the evidence for the efficacy and safety of statin therapy." Lancet.

Connor, J. H., D. C. Weiser, S. Li, J. M. Hallenbeck and S. Shenolikar (2001). "Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1." Mol Cell Biol **21**(20): 6841-6850.

Cosker, K. E. and R. A. Segal (2014). "Neuronal signaling through endocytosis." Cold Spring Harb Perspect Biol **6**(2).

Costigan, M., K. Befort, L. Karchewski, R. S. Griffin, D. D'Urso, A. Allchorne, J. Sitarski, J. W. Mannion, R. E. Pratt and C. J. Woolf (2002). "Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury." BMC Neurosci **3**: 16.

Costigan, M., R. J. Mannion, G. Kendall, S. E. Lewis, J. A. Campagna, R. E. Coggeshall, J. Meridith-Middleton, S. Tate and C. J. Woolf (1998). "Heat shock protein 27: developmental regulation and expression after peripheral nerve injury." J Neurosci **18**(15): 5891-5900.

Covenas, R., M. DeLeon, G. Chadi, A. Cintra, J. A. Gustafsson, J. A. Narvaez and K. Fuxe (1994). "Adrenalectomy increases the number of substance P and somatostatin immunoreactive nerve cells in the rat lumbar dorsal root ganglia." Brain Res **640**(1-2): 352-356.

Cox, J. S., C. E. Shamu and P. Walter (1993). "Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase." Cell **73**(6): 1197-1206.

Cox, J. S. and P. Walter (1996). "A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response." Cell **87**(3): 391-404.
Cox, L. J., U. Hengst, N. G. Gurskaya, K. A. Lukyanov and S. R. Jaffrey (2008). "Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival." Nat Cell Biol **10**(2): 149-159.
Craner, M. J., J. P. Klein, J. A. Black and S. G. Waxman (2002). "Preferential expression of IGF-I in small DRG neurons and down-regulation following injury." Neuroreport **13**(13): 1649-1652.
Crowley, C., S. D. Spencer, M. C. Nishimura, K. S. Chen, S. Pitts-Meek, M. P. Armanini, L. H. Ling, S. B. McMahon, D. L. Shelton, A. D. Levinson and et al. (1994). "Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons." Cell **76**(6): 1001-1011.
Cruickshank, W. (1795). "Experiments on the nerves, particularly on their reproduction and on the
spinal marrow of living animals." Philosophical Transactions of the Royal Society of London **85**: 177-189.
Cruse, J. M., J. C. Keith, M. L. Bryant, Jr. and R. E. Lewis, Jr. (1996). "Immune system-neuroendocrine dysregulation in spinal cord injury." Immunol Res **15**(4): 306-314.
Danielsen, N., G. Lundborg and M. Frizell (1986). "Nerve repair and axonal transport: outgrowth delay and regeneration rate after transection and repair of rabbit hypoglossal nerve." Brain Res **376**(1): 125-132.
de la Llave-Rincon, A. I., C. Fernandez-de-las-Penas, J. Fernandez-Carnero, L. Padua, L. Arendt-Nielsen and J. A. Pareja (2009). "Bilateral hand/wrist heat and cold hyperalgesia, but not hypoesthesia, in unilateral carpal tunnel syndrome." Exp Brain Res **198**(4): 455-463.
DeLeo, J. A., F. Y. Tanga and V. L. Tawfik (2004). "Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia." Neuroscientist **10**(1): 40-52.
DeLeon, M., R. Covenas, G. Chadi, J. A. Narvaez, K. Fuxe and A. Cintra (1994). "Subpopulations of primary sensory neurons show coexistence of neuropeptides and glucocorticoid receptors in the rat spinal and trigeminal ganglia." Brain Res **636**(2): 338-342.

DenBoer, L. M., P. W. Hardy-Smith, M. R. Hogan, G. P. Cockram, T. E. Audas and R. Lu (2005). "Luman is capable of binding and activating transcription from the unfolded protein response element." Biochem Biophys Res Commun **331**(1): 113-119.

DenBoer, L. M., A. Iyer, A. R. McCluggage, Y. Li, A. C. Martyn and R. Lu (2013). "JAB1/CSN5 inhibits the activity of Luman/CREB3 by promoting its degradation." Biochim Biophys Acta **1829**(9): 921-929.

Dent, E. W., S. L. Gupton and F. B. Gertler (2011). "The growth cone cytoskeleton in axon outgrowth and guidance." Cold Spring Harb Perspect Biol **3**(3).

Devor, M., D. Schonfeld, Z. Seltzer and P. D. Wall (1979). "Two modes of cutaneous reinnervation following peripheral nerve injury." J Comp Neurol **185**(1): 211-220.

Diamond, J., M. Holmes and M. Coughlin (1992). "Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat." J Neurosci **12**(4): 1454-1466.

Dijkers, P. F., R. H. Medema, J. W. Lammers, L. Koenderman and P. J. Coffer (2000). "Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1." Curr Biol **10**(19): 1201-1204.

Dijkers, P. F., R. H. Medema, C. Pals, L. Banerji, N. S. Thomas, E. W. Lam, B. M. Burgering, J. A. Raaijmakers, J. W. Lammers, L. Koenderman and P. J. Coffer (2000). "Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1)." Mol Cell Biol **20**(24): 9138-9148.

Dixit, R., J. L. Ross, Y. E. Goldman and E. L. Holzbaur (2008). "Differential regulation of dynein and kinesin motor proteins by tau." Science **319**(5866): 1086-1089.

Dobrowsky, R. T. and B. D. Carter (2000). "p75 neurotrophin receptor signaling: mechanisms for neurotrophic modulation of cell stress?" J Neurosci Res **61**(3): 237-243.

Donnelly, C. J., M. Park, M. Spillane, S. Yoo, A. Pacheco, C. Gomes, D. Vuppalanchi, M. McDonald, H. H. Kim, T. T. Merianda, G. Gallo and J. L. Twiss (2013). "Axonally synthesized beta-actin and GAP-43 proteins support distinct modes of axonal growth." J Neurosci **33**(8): 3311-3322.

Donnelly, C. J., D. E. Willis, M. Xu, C. Tep, C. Jiang, S. Yoo, N. C. Schanen, C. B. Kirn-Safran, J. van Minnen, A. English, S. O. Yoon, G. J. Bassell and J. L. Twiss (2011). "Limited

availability of ZBP1 restricts axonal mRNA localization and nerve regeneration capacity." EMBO J **30**(22): 4665-4677.
Donnerer, J., R. Amann, R. Schuligoi and G. Skofitsch (1996). "Complete recovery by nerve growth factor of neuropeptide content and function in capsaicin-impaired sensory neurons." Brain Res **741**(1-2): 103-108.
Dornay, M., V. H. Gilad and G. M. Gilad (1985). "Compensatory changes in contralateral sympathetic neurons of the superior cervical ganglion and in their terminals in the pineal gland following unilateral ganglionectomy." J Neurosci **5**(6): 1522-1526.
Drinovac Vlah, V., L. Bach-Rojecky and Z. Lackovic (2016). "Antinociceptive action of botulinum toxin type A in carrageenan-induced mirror pain." J Neural Transm (Vienna).
Dubovy, P., R. Jancalek, I. Klusakova, I. Svizenska and K. Pejchalova (2006). "Intra- and extraneuronal changes of immunofluorescence staining for TNF-alpha and TNFR1 in the dorsal root ganglia of rat peripheral neuropathic pain models." Cell Mol Neurobiol **26**(7-8): 1205-1217.
Eberle, D., B. Hegarty, P. Bossard, P. Ferre and F. Foufelle (2004). "SREBP transcription factors: master regulators of lipid homeostasis." Biochimie **86**(11): 839-848.
Edstrom, J. E., D. Eichner and A. Edstrom (1962). "The ribonucleic acid of axons and myelin sheaths from Mauthner neurons." Biochim Biophys Acta **61**: 178-184.
Elde, R., Y. H. Cao, A. Cintra, T. C. Brelje, M. Pelto-Huikko, T. Junttila, K. Fuxe, R. F. Pettersson and T. Hokfelt (1991). "Prominent expression of acidic fibroblast growth factor in motor and sensory neurons." Neuron **7**(3): 349-364.
Eleveld-Trancikova, D., A. Sanecka, M. A. van Hout-Kuijer, M. W. Looman, I. A. Hendriks, B. J. Jansen and G. J. Adema (2010). "DC-STAMP interacts with ER-resident transcription factor LUMAN which becomes activated during DC maturation." Mol Immunol **47**(11-12): 1963-1973.
Eng, H., K. Lund and R. B. Campenot (1999). "Synthesis of beta-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures." J Neurosci **19**(1): 1-9.
Ernfors, P., K. F. Lee and R. Jaenisch (1994). "Mice lacking brain-derived neurotrophic factor develop with sensory deficits." Nature **368**(6467): 147-150.
Ernfors, P., K. F. Lee, J. Kucera and R. Jaenisch (1994). "Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents." Cell **77**(4): 503-512.

Ernfors, P., C. Wetmore, L. Olson and H. Persson (1990). "Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family." Neuron **5**(4): 511-526.

Essers, M. A., S. Weijzen, A. M. de Vries-Smits, I. Saarloos, N. D. de Ruiter, J. L. Bos and B. M. Burgering (2004). "FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK." Embo J **23**(24): 4802-4812.

Farinas, I., G. A. Wilkinson, C. Backus, L. F. Reichardt and A. Patapoutian (1998). "Characterization of neurotrophin and Trk receptor functions in developing sensory ganglia: direct NT-3 activation of TrkB neurons in vivo." Neuron **21**(2): 325-334.

Fawcett, T. W., J. L. Martindale, K. Z. Guyton, T. Hai and N. J. Holbrook (1999). "Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response." Biochem J **339 (Pt 1)**: 135-141.

Fernandez-de-las-Penas, C., A. I. de la Llave-Rincon, J. Fernandez-Carnero, M. L. Cuadrado, L. Arendt-Nielsen and J. A. Pareja (2009). "Bilateral widespread mechanical pain sensitivity in carpal tunnel syndrome: evidence of central processing in unilateral neuropathy." Brain **132**(Pt 6): 1472-1479.

Fitzgerald, M. (1982). "Alterations in the ipsi- and contralateral afferent inputs of dorsal horn cells produced by capsaicin treatment of one sciatic nerve in the rat." Brain Res **248**(1): 97-107.

Fitzgerald, M. (1982). "The contralateral input to the dorsal horn of the spinal cord in the decerebrate spinal rat." Brain Res **236**(2): 275-287.

Fitzgerald, M. (1983). "Influences of contralateral nerve and skin stimulation on neurones in the substantia gelatinosa of the rat spinal cord." Neurosci Lett **36**(2): 139-143.

Freeman, R. S., S. Estus and E. M. Johnson, Jr. (1994). "Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death." Neuron **12**(2): 343-355.

Freiman, R. N. and W. Herr (1997). "Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP." Genes Dev **11**(23): 3122-3127.

Friedman, B., D. Kleinfeld, N. Y. Ip, V. M. Verge, R. Moulton, P. Boland, E. Zlotchenko, R. M. Lindsay and L. Liu (1995). "BDNF and NT-4/5 exert neurotrophic influences on injured adult spinal motor neurons." J Neurosci **15**(2): 1044-1056.

Fu, S. Y. and T. Gordon (1997). "The cellular and molecular basis of peripheral nerve regeneration." Mol Neurobiol **14**(1-2): 67-116.

Fu, W., Q. Ma, L. Chen, P. Li, M. Zhang, S. Ramamoorthy, Z. Nawaz, T. Shimojima, H. Wang, Y. Yang, Z. Shen, Y. Zhang, X. Zhang, S. V. Nicosia, Y. Zhang, J. W. Pledger, J. Chen and W. Bai (2009). "MDM2 acts downstream of p53 as an E3 ligase to promote FOXO ubiquitination and degradation." J Biol Chem **284**(21): 13987-14000.

Fukunaga, K., T. Ishigami and T. Kawano (2005). "Transcriptional regulation of neuronal genes and its effect on neural functions: expression and function of forkhead transcription factors in neurons." J Pharmacol Sci **98**(3): 205-211.

Funakoshi, H., N. Belluardo, E. Arenas, Y. Yamamoto, A. Casabona, H. Persson and C. F. Ibanez (1995). "Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons." Science **268**(5216): 1495-1499.

Funakoshi, H., J. Frisen, G. Barbany, T. Timmusk, O. Zachrisson, V. M. Verge and H. Persson (1993). "Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve." J Cell Biol **123**(2): 455-465.

Galbraith, J. A. and P. E. Gallant (2000). "Axonal transport of tubulin and actin." J Neurocytol **29**(11-12): 889-911.

Gan, L., W. Zheng, J. G. Chabot, T. G. Unterman and R. Quirion (2005). "Nuclear/cytoplasmic shuttling of the transcription factor FoxO1 is regulated by neurotrophic factors." J Neurochem **93**(5): 1209-1219.

Garcia-Huerta, P., L. Bargsted, A. Rivas, S. Matus and R. L. Vidal (2016). "ER chaperones in neurodegenerative disease: Folding and beyond." Brain Res.

Gardiner, N. J., Z. Wang, C. Luke, A. Gott, S. A. Price and P. Fernyhough (2007). "Expression of hexokinase isoforms in the dorsal root ganglion of the adult rat and effect of experimental diabetes." Brain Res **1175**: 143-154.

Gardner, B. M. and P. Walter (2011). "Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response." Science **333**(6051): 1891-1894.

Geremia, N. M. (2005). "Exogenous brain-derived neurotrophic factor enhances the intrinsic cell body response of injured sensory neurons." University of Saskatchewan Thesis: 88-106.

Geremia, N. M., T. Gordon, T. M. Brushart, A. A. Al-Majed and V. M. Verge (2007). "Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression." Exp Neurol **205**(2): 347-359.
Geremia, N. M., L. M. Pettersson, J. C. Hasmatali, T. Hryciw, N. Danielsen, D. J. Schreyer and V. M. Verge (2010). "Endogenous BDNF regulates induction of intrinsic neuronal growth programs in injured sensory neurons." Exp Neurol **223**(1): 128-142.
Geremia, N. M. and V. M. Verge (2001). "Role of BDNF in inducing an injury phenotype in intact sensory neurons." Abstr.-Soc. Neurosci. **595.20**.
Gilley, J., P. J. Coffer and J. Ham (2003). "FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons." J Cell Biol **162**(4): 613-622.
Gonzalez, C. and A. Couve (2014). "The axonal endoplasmic reticulum and protein trafficking: Cellular bootlegging south of the soma." Semin Cell Dev Biol **27**: 23-31.
Gordon, T. (2016). "Electrical Stimulation to Enhance Axon Regeneration After Peripheral Nerve Injuries in Animal Models and Humans." Neurotherapeutics **13**(2): 295-310.
Gordon, T. and A. W. English (2016). "Strategies to promote peripheral nerve regeneration: electrical stimulation and/or exercise." Eur J Neurosci **43**(3): 336-350.
Gordon, T., J. Gillespie, R. Orozco and L. Davis (1991). "Axotomy-induced changes in rabbit hindlimb nerves and the effects of chronic electrical stimulation." J Neurosci **11**(7): 2157-2169.
Gordon, T., O. A. Sulaiman and A. Ladak (2009). "Chapter 24: Electrical stimulation for improving nerve regeneration: where do we stand?" Int Rev Neurobiol **87**: 433-444.
Gordon, T. and W. Tetzlaff (2015). "Regeneration-associated genes decline in chronically injured rat sciatic motoneurons." Eur J Neurosci **42**(10): 2783-2791.
Gordon, T., S. You, S. L. Cassar and W. Tetzlaff (2015). "Reduced expression of regeneration associated genes in chronically axotomized facial motoneurons." Exp Neurol **264**: 26-32.
Gross, D. N., A. P. van den Heuvel and M. J. Birnbaum (2008). "The role of FoxO in the regulation of metabolism." Oncogene **27**(16): 2320-2336.
Groves, M. J., A. Schanzer, A. J. Simpson, S. F. An, L. T. Kuo and F. Scaravilli (2003). "Profile of adult rat sensory neuron loss, apoptosis and replacement after sciatic nerve crush." J Neurocytol **32**(2): 113-122.

Hai, T. W., F. Liu, W. J. Coukos and M. R. Green (1989). "Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers." Genes Dev **3**(12B): 2083-2090.

Harding, H. P., I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira and D. Ron (2000). "Regulated translation initiation controls stress-induced gene expression in mammalian cells." Mol Cell **6**(5): 1099-1108.

Harding, H. P., Y. Zhang and D. Ron (1999). "Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase." Nature **397**(6716): 271-274.

Harris, W. A., C. E. Holt and F. Bonhoeffer (1987). "Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibres in vivo." Development **101**(1): 123-133.

Hasmatali, J. C., J. De Guzman, Z. Ying, V. Misra and V. M. Verge (2012). "Transcription factors Luman and Zhangfei expression is altered in primary sensory neurons ipsilateral and contralateral to peripheral nerve injury." Neuroscience 2012 Abstracts **234.04**.

Hasmatali, J. C., M. H. Noyan-Ashraf, B. H. Juurlink and V. M. Verge (2009). "Regulation of FOXO3a/FOXO3 transcription factor expression and nuclear localization in sensory neurons in response to nerve injury." Neuroscience 2009 Abstracts **442.14**.

Haze, K., H. Yoshida, H. Yanagi, T. Yura and K. Mori (1999). "Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress." Mol Biol Cell **10**(11): 3787-3799.

Heerssen, H. M., M. F. Pazyra and R. A. Segal (2004). "Dynein motors transport activated Trks to promote survival of target-dependent neurons." Nat Neurosci **7**(6): 596-604.

Hempstead, B. L., D. Martin-Zanca, D. R. Kaplan, L. F. Parada and M. V. Chao (1991). "High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor." Nature **350**(6320): 678-683.

Herrera, A. A. and A. D. Grinnell (1981). "Contralateral denervation causes enhanced transmitter release from frog motor nerve terminals." Nature **291**(5815): 495-497.

Herrera, A. A., A. D. Grinnell and B. Wolowske (1985). "Ultrastructural correlates of experimentally altered transmitter release efficacy in frog motor nerve terminals." Neuroscience **16**(3): 491-500.

Herrup, K., R. Neve, S. L. Ackerman and A. Copani (2004). "Divide and die: cell cycle events as triggers of nerve cell death." J Neurosci **24**(42): 9232-9239.
Hetz, C., E. Chevet and H. P. Harding (2013). "Targeting the unfolded protein response in disease." Nat Rev Drug Discov **12**(9): 703-719.
Hirokawa, N., S. Niwa and Y. Tanaka (2010). "Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease." Neuron **68**(4): 610-638.
Hoekman, M. F., F. M. Jacobs, M. P. Smidt and J. P. Burbach (2006). "Spatial and temporal expression of FoxO transcription factors in the developing and adult murine brain." Gene Expr Patterns **6**(2): 134-140.
Hoffman, P. N., M. A. Lopata, D. F. Watson and R. F. Luduena (1992). "Axonal transport of class II and III beta-tubulin: evidence that the slow component wave represents the movement of only a small fraction of the tubulin in mature motor axons." J Cell Biol **119**(3): 595-604.
Hogan, M. R., G. P. Cockram and R. Lu (2006). "Cooperative interaction of Zhangfei and ATF4 in transactivation of the cyclic AMP response element." FEBS Lett **580**(1): 58-62.
Hollien, J. and J. S. Weissman (2006). "Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response." Science **313**(5783): 104-107.
Honma, Y., K. Kanazawa, T. Mori, Y. Tanno, M. Tojo, H. Kiyosawa, J. Takeda, T. Nikaido, T. Tsukamoto, S. Yokoya and A. Wanaka (1999). "Identification of a novel gene, OASIS, which encodes for a putative CREB/ATF family transcription factor in the long-term cultured astrocytes and gliotic tissue." Brain Res Mol Brain Res **69**(1): 93-103.
Hosaka, T., W. H. Biggs, 3rd, D. Tieu, A. D. Boyer, N. M. Varki, W. K. Cavenee and K. C. Arden (2004). "Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification." Proc Natl Acad Sci U S A **101**(9): 2975-2980.
Hosokawa, N., I. Wada, K. Hasegawa, T. Yorihuzi, L. O. Tremblay, A. Herscovics and K. Nagata (2001). "A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation." EMBO Rep **2**(5): 415-422.
Hu, Y., X. Wang, L. Zeng, D. Y. Cai, K. Sabapathy, S. P. Goff, E. J. Firpo and B. Li (2005). "ERK phosphorylates p66shcA on Ser36 and subsequently regulates p27kip1 expression via the Akt-FOXO3a pathway: implication of p27kip1 in cell response to oxidative stress." Mol Biol Cell **16**(8): 3705-3718.

Huang, E. J. and L. F. Reichardt (2003). "Trk receptors: roles in neuronal signal transduction." Annu Rev Biochem **72**: 609-642.

Huang, H., K. M. Regan, F. Wang, D. Wang, D. I. Smith, J. M. van Deursen and D. J. Tindall (2005). "Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation." Proc Natl Acad Sci U S A **102**(5): 1649-1654.

Huang, H. and D. J. Tindall (2007). "Dynamic FoxO transcription factors." J Cell Sci **120**(Pt 15): 2479-2487.

Huge, V., M. Lauchart, S. Forderreuther, W. Kaufhold, M. Valet, S. C. Azad, A. Beyer and W. Magerl (2008). "Interaction of hyperalgesia and sensory loss in complex regional pain syndrome type I (CRPS I)." PLoS One **3**(7): e2742.

Hwangbo, D. S., B. Gershman, M. P. Tu, M. Palmer and M. Tatar (2004). "Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body." Nature **429**(6991): 562-566.

Hyatt Sachs, H., R. C. Schreiber, S. E. Shoemaker, A. Sabe, E. Reed and R. E. Zigmond (2007). "Activating transcription factor 3 induction in sympathetic neurons after axotomy: response to decreased neurotrophin availability." Neuroscience **150**(4): 887-897.

Inaishi, Y., Y. Kashihara, M. Sakaguchi, H. Nawa and M. Kuno (1992). "Cooperative regulation of calcitonin gene-related peptide levels in rat sensory neurons via their central and peripheral processes." J Neurosci **12**(2): 518-524.

Jackson, T. C., A. Rani, A. Kumar and T. C. Foster (2009). "Regional hippocampal differences in AKT survival signaling across the lifespan: implications for CA1 vulnerability with aging." Cell Death Differ **16**(3): 439-448.

Jang, S. W., Y. S. Kim, Y. R. Kim, H. J. Sung and J. Ko (2007). "Regulation of human LZIP expression by NF-kappaB and its involvement in monocyte cell migration induced by Lkn-1." J Biol Chem **282**(15): 11092-11100.

Jang, S. W., Y. S. Kim, Y. H. Lee and J. Ko (2007). "Role of human LZIP in differential activation of the NF-kappaB pathway that is induced by CCR1-dependent chemokines." J Cell Physiol **211**(3): 630-637.

Joshi, Y., M. G. Soria, G. Quadrato, G. Inak, L. Zhou, A. Hervera, K. I. Rathore, M. Elnaggar, M. Cucchiarini, J. C. Marine, R. Puttagunta and S. Di Giovanni (2015). "The MDM4/MDM2-

p53-IGF1 axis controls axonal regeneration, sprouting and functional recovery after CNS injury." Brain **138**(Pt 7): 1843-1862.

Jung, H., B. C. Yoon and C. E. Holt (2012). "Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair." Nat Rev Neurosci **13**(5): 308-324.

Kajihara, T., M. Jones, L. Fusi, M. Takano, F. Feroze-Zaidi, G. Pirianov, H. Mehmet, O. Ishihara, J. M. Higham, E. W. Lam and J. J. Brosens (2006). "Differential expression of FOXO1 and FOXO3a confers resistance to oxidative cell death upon endometrial decidualization." Mol Endocrinol **20**(10): 2444-2455.

Karchewski, L. A., K. A. Gratto, C. Wetmore and V. M. Verge (2002). "Dynamic patterns of BDNF expression in injured sensory neurons: differential modulation by NGF and NT-3." Eur J Neurosci **16**(8): 1449-1462.

Karchewski, L. A., F. A. Kim, J. Johnston, R. M. McKnight and V. M. Verge (1999). "Anatomical evidence supporting the potential for modulation by multiple neurotrophins in the majority of adult lumbar sensory neurons." J Comp Neurol **413**(2): 327-341.

Kashiba, H., Y. Ueda, T. Ueyama, K. Nemoto and E. Senba (1997). "Relationship between BDNF- and trk-expressing neurones in rat dorsal root ganglion: an analysis by in situ hybridization." Neuroreport **8**(5): 1229-1234.

Kato, M., H. Yuan, Z. G. Xu, L. Lanting, S. L. Li, M. Wang, M. C. Hu, M. A. Reddy and R. Natarajan (2006). "Role of the Akt/FoxO3a pathway in TGF-beta1-mediated mesangial cell dysfunction: a novel mechanism related to diabetic kidney disease." J Am Soc Nephrol **17**(12): 3325-3335.

Kawano, T., M. Morioka, S. Yano, J. Hamada, Y. Ushio, E. Miyamoto and K. Fukunaga (2002). "Decreased akt activity is associated with activation of forkhead transcription factor after transient forebrain ischemia in gerbil hippocampus." J Cereb Blood Flow Metab **22**(8): 926-934.

Kilberg, M. S., Y. X. Pan, H. Chen and V. Leung-Pineda (2005). "Nutritional control of gene expression: how mammalian cells respond to amino acid limitation." Annu Rev Nutr **25**: 59-85.

Kim, T. Y., E. Kim, S. K. Yoon and J. B. Yoon (2008). "Herp enhances ER-associated protein degradation by recruiting ubiquilins." Biochem Biophys Res Commun **369**(2): 741-746.

Kirsch, M., U. Terheggen and H. D. Hofmann (2003). "Ciliary neurotrophic factor is an early lesion-induced retrograde signal for axotomized facial motoneurons." Mol Cell Neurosci **24**(1): 130-138.
Koenig, E., R. Martin, M. Titmus and J. R. Sotelo-Silveira (2000). "Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons." J Neurosci **20**(22): 8390-8400.
Koliatsos, V. E., R. E. Clatterbuck, J. W. Winslow, M. H. Cayouette and D. L. Price (1993). "Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo." Neuron **10**(3): 359-367.
Koltzenburg, M., P. D. Wall and S. B. McMahon (1999). "Does the right side know what the left is doing?" Trends Neurosci **22**(3): 122-127.
Konishi, Y., J. Stegmuller, T. Matsuda, S. Bonni and A. Bonni (2004). "Cdh1-APC controls axonal growth and patterning in the mammalian brain." Science **303**(5660): 1026-1030.
Konopka, K. H., M. Harbers, A. Houghton, R. Kortekaas, A. van Vliet, W. Timmerman, J. A. den Boer, M. M. Struys and M. van Wijhe (2012). "Bilateral sensory abnormalities in patients with unilateral neuropathic pain; a quantitative sensory testing (QST) study." PLoS One **7**(5): e37524.
Kopito, R. R. (1997). "ER quality control: the cytoplasmic connection." Cell **88**(4): 427-430.
Kops, G. J., T. B. Dansen, P. E. Polderman, I. Saarloos, K. W. Wirtz, P. J. Coffer, T. T. Huang, J. L. Bos, R. H. Medema and B. M. Burgering (2002). "Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress." Nature **419**(6904): 316-321.
Kozin, F., H. K. Genant, C. Bekerman and D. J. McCarty (1976). "The reflex sympathetic dystrophy syndrome. II. Roentgenographic and scintigraphic evidence of bilaterality and of periarticular accentuation." Am J Med **60**(3): 332-338.
Krishnan, A., A. Duraikannu and D. W. Zochodne (2016). "Releasing 'brakes' to nerve regeneration: intrinsic molecular targets." Eur J Neurosci **43**(3): 297-308.
Kuchel, G. A., R. Hellendall and M. Blum (1992). "Transynaptic regulation of low-affinity p75 nerve growth factor receptor mRNA precedes and accompanies lesion-induced collateral neuronal sprouting." Exp Neurol **118**(1): 73-84.
Kuchel, G. A. and R. E. Zigmond (1991). "Functional recovery and collateral neuronal sprouting examined in young and aged rats following a partial neural lesion." Brain Res **540**(1-2): 195-203.

Kuningas, M., R. Magi, R. G. Westendorp, P. E. Slagboom, M. Remm and D. van Heemst (2007). "Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age." Eur J Hum Genet **15**(3): 294-301.

Kuo, L. T., A. Simpson, A. Schanzer, J. Tse, S. F. An, F. Scaravilli and M. J. Groves (2005). "Effects of systemically administered NT-3 on sensory neuron loss and nestin expression following axotomy." J Comp Neurol **482**(4): 320-332.

Lachyankar, M. B., P. J. Condon, M. C. Daou, A. K. De, J. B. Levine, A. Obermeier and A. H. Ross (2003). "Novel functional interactions between Trk kinase and p75 neurotrophin receptor in neuroblastoma cells." J Neurosci Res **71**(2): 157-172.

Lai, C. W., J. H. Otero, L. M. Hendershot and E. Snapp (2012). "ERdj4 protein is a soluble endoplasmic reticulum (ER) DnaJ family protein that interacts with ER-associated degradation machinery." J Biol Chem **287**(11): 7969-7978.

Lawrence, C. J., R. K. Dawe, K. R. Christie, D. W. Cleveland, S. C. Dawson, S. A. Endow, L. S. Goldstein, H. V. Goodson, N. Hirokawa, J. Howard, R. L. Malmberg, J. R. McIntosh, H. Miki, T. J. Mitchison, Y. Okada, A. S. Reddy, W. M. Saxton, M. Schliwa, J. M. Scholey, R. D. Vale, C. E. Walczak and L. Wordeman (2004). "A standardized kinesin nomenclature." J Cell Biol **167**(1): 19-22.

Leah, J. D., T. Herdegen and R. Bravo (1991). "Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: evidence for a role in the regeneration process." Brain Res **566**(1-2): 198-207.

Lee, J. H., G. T. Lee, S. J. Kwon, J. Jeong, Y. S. Ha, W. J. Kim and I. Y. Kim (2012). "CREBZF, a novel Smad8-binding protein." Mol Cell Biochem **368**(1-2): 147-153.

Lee, K., W. Tirasophon, X. Shen, M. Michalak, R. Prywes, T. Okada, H. Yoshida, K. Mori and R. J. Kaufman (2002). "IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response." Genes Dev **16**(4): 452-466.

Lehtinen, M. K., Z. Yuan, P. R. Boag, Y. Yang, J. Villen, E. B. Becker, S. DiBacco, N. de la Iglesia, S. Gygi, T. K. Blackwell and A. Bonni (2006). "A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span." Cell **125**(5): 987-1001.

Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen and Y. A. Barde (1989). "Molecular cloning and expression of brain-derived neurotrophic factor." Nature **341**(6238): 149-152.

Lembeck, F. and R. Gamse (1982). "Substance P in peripheral sensory processes." Ciba Found Symp(91): 35-54.

Lembeck, F. and P. Holzer (1979). "Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation." Naunyn Schmiedebergs Arch Pharmacol **310**(2): 175-183.

Lerch, J. K., D. A. Puga, O. Bloom and P. G. Popovich (2014). "Glucocorticoids and macrophage migration inhibitory factor (MIF) are neuroendocrine modulators of inflammation and neuropathic pain after spinal cord injury." Semin Immunol **26**(5): 409-414.

Levi-Montalcini, R. and P. Calissano (1979). "The nerve-growth factor." Sci Am **240**(6): 68-77.

Levi-Montalcini, R. and V. Hamburger (1951). "Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo." J Exp Zool **116**(2): 321-361.

Levi-Montalcini, R., H. Meyer and V. Hamburger (1954). "In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo." Cancer Res **14**(1): 49-57.

Levy, D. and D. W. Zochodne (2000). "Increased mRNA expression of the B1 and B2 bradykinin receptors and antinociceptive effects of their antagonists in an animal model of neuropathic pain." Pain **86**(3): 265-271.

Lewin, G. R. and L. M. Mendell (1993). "Nerve growth factor and nociception." Trends Neurosci **16**(9): 353-359.

Lewin, G. R., A. M. Ritter and L. M. Mendell (1992). "On the role of nerve growth factor in the development of myelinated nociceptors." J Neurosci **12**(5): 1896-1905.

Lewin, S. L., D. S. Utley, E. T. Cheng, A. N. Verity and D. J. Terris (1997). "Simultaneous treatment with BDNF and CNTF after peripheral nerve transection and repair enhances rate of functional recovery compared with BDNF treatment alone." Laryngoscope **107**(7): 992-999.

Li, M., J. F. Chiu, B. T. Mossman and N. K. Fukagawa (2006). "Down-regulation of manganese-superoxide dismutase through phosphorylation of FOXO3a by Akt in explanted vascular smooth muscle cells from old rats." J Biol Chem **281**(52): 40429-40439.

Li, S., C. Xue, Y. Yuan, R. Zhang, Y. Wang, Y. Wang, B. Yu, J. Liu, F. Ding, Y. Yang and X. Gu (2015). "The transcriptional landscape of dorsal root ganglia after sciatic nerve transection." Sci Rep **5**: 16888.

Liang, G., T. E. Audas, Y. Li, G. P. Cockram, J. D. Dean, A. C. Martyn, K. Kokame and R. Lu (2006). "Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element." Mol Cell Biol **26**(21): 7999-8010.

Lieberman, A. R. (1971). "The axon reaction: a review of the principal features of perikaryal responses to axon injury." Int Rev Neurobiol **14**: 49-124.

Lin, K., J. B. Dorman, A. Rodan and C. Kenyon (1997). "daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans." Science **278**(5341): 1319-1322.

Lin, L., J. D. Hron and S. L. Peng (2004). "Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a." Immunity **21**(2): 203-213.

Linda, H., F. Piehl, A. Dagerlind, V. M. Verge, U. Arvidsson, S. Cullheim, M. Risling, B. Ulfhake and T. Hokfelt (1992). "Expression of GAP-43 mRNA in the adult mammalian spinal cord under normal conditions and after different types of lesions, with special reference to motoneurons." Exp Brain Res **91**(2): 284-295.

Lindholm, D., R. Heumann, M. Meyer and H. Thoenen (1987). "Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve." Nature **330**(6149): 658-659.

Lindsay, R. M. (1988). "Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons." J Neurosci **8**(7): 2394-2405.

Lindsay, R. M. (1996). "Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: an overview." Philos Trans R Soc Lond B Biol Sci **351**(1338): 365-373.

Lindwall, C. and M. Kanje (2005). "The Janus role of c-Jun: cell death versus survival and regeneration of neonatal sympathetic and sensory neurons." Exp Neurol **196**(1): 184-194.

Liu, D. X. and L. A. Greene (2001). "Neuronal apoptosis at the G1/S cell cycle checkpoint." Cell Tissue Res **305**(2): 217-228.

Lopez-Mateo, I., M. A. Villaronga, S. Llanos and B. Belandia (2012). "The transcription factor CREBZF is a novel positive regulator of p53." Cell Cycle **11**(20): 3887-3895.

Lu, P. D., H. P. Harding and D. Ron (2004). "Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response." J Cell Biol **167**(1): 27-33.
Lu, R. and V. Misra (2000). "Potential role for luman, the cellular homologue of herpes simplex virus VP16 (alpha gene trans-inducing factor), in herpesvirus latency." J Virol **74**(2): 934-943.
Lu, R. and V. Misra (2000). "Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16." Nucleic Acids Res **28**(12): 2446-2454.
Lu, R., P. Yang, P. O'Hare and V. Misra (1997). "Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor." Mol Cell Biol **17**(9): 5117-5126.
Lu, R., P. Yang, S. Padmakumar and V. Misra (1998). "The herpesvirus transactivator VP16 mimics a human basic domain leucine zipper protein, luman, in its interaction with HCF." J Virol **72**(8): 6291-6297.
Lu, X. and P. M. Richardson (1993). "Responses of macrophages in rat dorsal root ganglia following peripheral nerve injury." J Neurocytol **22**(5): 334-341.
Luciano, R. L. and A. C. Wilson (2000). "N-terminal transcriptional activation domain of LZIP comprises two LxxLL motifs and the host cell factor-1 binding motif." Proc Natl Acad Sci U S A **97**(20): 10757-10762.
Luciano, R. L. and A. C. Wilson (2002). "An activation domain in the C-terminal subunit of HCF-1 is important for transactivation by VP16 and LZIP." Proc Natl Acad Sci U S A **99**(21): 13403-13408.
Lucin, K. M., V. M. Sanders, T. B. Jones, W. B. Malarkey and P. G. Popovich (2007). "Impaired antibody synthesis after spinal cord injury is level dependent and is due to sympathetic nervous system dysregulation." Exp Neurol **207**(1): 75-84.
Lupachyk, S., P. Watcho, A. A. Obrosov, R. Stavniichuk and I. G. Obrosova (2013). "Endoplasmic reticulum stress contributes to prediabetic peripheral neuropathy." Exp Neurol **247**: 342-348.
Lupachyk, S., P. Watcho, R. Stavniichuk, H. Shevalye and I. G. Obrosova (2013). "Endoplasmic reticulum stress plays a key role in the pathogenesis of diabetic peripheral neuropathy." Diabetes **62**(3): 944-952.

Ma, Y., J. W. Brewer, J. A. Diehl and L. M. Hendershot (2002). "Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response." J Mol Biol **318**(5): 1351-1365.

Ma, Y. and L. M. Hendershot (2003). "Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress." J Biol Chem **278**(37): 34864-34873.

Maday, S., A. E. Twelvetrees, A. J. Moughamian and E. L. Holzbaur (2014). "Axonal transport: cargo-specific mechanisms of motility and regulation." Neuron **84**(2): 292-309.

Maiese, K. (2015). "FoxO proteins in the nervous system." Anal Cell Pathol (Amst) **2015**: 569392.

Maiese, K., Z. Z. Chong and Y. C. Shang (2007). ""Sly as a FOXO": new paths with Forkhead signaling in the brain." Curr Neurovasc Res **4**(4): 295-302.

Maisonpierre, P. C., L. Belluscio, S. Squinto, N. Y. Ip, M. E. Furth, R. M. Lindsay and G. D. Yancopoulos (1990). "Neurotrophin-3: a neurotrophic factor related to NGF and BDNF." Science **247**(4949 Pt 1): 1446-1451.

Maiuolo, J., S. Bulotta, C. Verderio, R. Benfante and N. Borgese (2011). "Selective activation of the transcription factor ATF6 mediates endoplasmic reticulum proliferation triggered by a membrane protein." Proc Natl Acad Sci U S A **108**(19): 7832-7837.

Mandelzys, A., E. Cooper, V. M. Verge and P. M. Richardson (1990). "Nerve growth factor induces functional nicotinic acetylcholine receptors on rat sensory neurons in culture." Neuroscience **37**(2): 523-530.

Mansikka, H., C. Zhao, R. N. Sheth, I. Sora, G. Uhl and S. N. Raja (2004). "Nerve injury induces a tonic bilateral mu-opioid receptor-mediated inhibitory effect on mechanical allodynia in mice." Anesthesiology **100**(4): 912-921.

Mantuano, E., K. Henry, T. Yamauchi, N. Hiramatsu, K. Yamauchi, S. Orita, K. Takahashi, J. H. Lin, S. L. Gonias and W. M. Campana (2011). "The unfolded protein response is a major mechanism by which LRP1 regulates Schwann cell survival after injury." J Neurosci **31**(38): 13376-13385.

Martinez-Gac, L., M. Marques, Z. Garcia, M. R. Campanero and A. C. Carrera (2004). "Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead." Mol Cell Biol **24**(5): 2181-2189.

Mason, M. R., A. R. Lieberman, G. Grenningloh and P. N. Anderson (2002). "Transcriptional upregulation of SCG10 and CAP-23 is correlated with regeneration of the axons of peripheral and central neurons in vivo." Mol Cell Neurosci **20**(4): 595-615.

Matsuzaki, H., H. Daitoku, M. Hatta, K. Tanaka and A. Fukamizu (2003). "Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation." Proc Natl Acad Sci U S A **100**(20): 11285-11290.

McKay Hart, A., T. Brannstrom, M. Wiberg and G. Terenghi (2002). "Primary sensory neurons and satellite cells after peripheral axotomy in the adult rat: timecourse of cell death and elimination." Exp Brain Res **142**(3): 308-318.

McLachlan, E. M., W. Janig, M. Devor and M. Michaelis (1993). "Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia." Nature **363**(6429): 543-546.

McLean, N. A., B. F. Popescu, T. Gordon, D. W. Zochodne and V. M. Verge (2014). "Delayed nerve stimulation promotes axon-protective neurofilament phosphorylation, accelerates immune cell clearance and enhances remyelination in vivo in focally demyelinated nerves." PLoS One **9**(10): e110174.

McMahon, S. B., M. P. Armanini, L. H. Ling and H. S. Phillips (1994). "Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets." Neuron **12**(5): 1161-1171.

Medema, R. H., G. J. Kops, J. L. Bos and B. M. Burgering (2000). "AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1." Nature **404**(6779): 782-787.

Merianda, T. T., A. C. Lin, J. S. Lam, D. Vuppalanchi, D. E. Willis, N. Karin, C. E. Holt and J. L. Twiss (2009). "A functional equivalent of endoplasmic reticulum and Golgi in axons for secretion of locally synthesized proteins." Mol Cell Neurosci **40**(2): 128-142.

Miao, T., D. Wu, Y. Zhang, X. Bo, M. C. Subang, P. Wang and P. M. Richardson (2006). "Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons." J Neurosci **26**(37): 9512-9519.

Michael, G. J., S. Averill, P. J. Shortland, Q. Yan and J. V. Priestley (1999). "Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB

and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei." Eur J Neurosci **11**(10): 3539-3551.

Miki, H., M. Setou, K. Kaneshiro and N. Hirokawa (2001). "All kinesin superfamily protein, KIF, genes in mouse and human." Proc Natl Acad Sci U S A **98**(13): 7004-7011.

Millan, M. J. (1999). "The induction of pain: an integrative review." Prog Neurobiol **57**(1): 1-164.

Milligan, E. D., C. Twining, M. Chacur, J. Biedenkapp, K. O'Connor, S. Poole, K. Tracey, D. Martin, S. F. Maier and L. R. Watkins (2003). "Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats." J Neurosci **23**(3): 1026-1040.

Ming, G. L., S. T. Wong, J. Henley, X. B. Yuan, H. J. Song, N. C. Spitzer and M. M. Poo (2002). "Adaptation in the chemotactic guidance of nerve growth cones." Nature **417**(6887): 411-418.

Misra, J., D. Chanda, D. K. Kim, T. Li, S. H. Koo, S. H. Back, J. Y. Chiang and H. S. Choi (2011). "Curcumin differentially regulates endoplasmic reticulum stress through transcriptional corepressor SMILE (small heterodimer partner-interacting leucine zipper protein)-mediated inhibition of CREBH (cAMP responsive element-binding protein H)." J Biol Chem **286**(49): 41972-41984.

Misra, V., N. Rapin, O. Akhova, M. Bainbridge and P. Korchinski (2005). "Zhangfei is a potent and specific inhibitor of the host cell factor-binding transcription factor Luman." J Biol Chem **280**(15): 15257-15266.

Miyata, Y., Y. Kashihara, S. Homma and M. Kuno (1986). "Effects of nerve growth factor on the survival and synaptic function of Ia sensory neurons axotomized in neonatal rats." J Neurosci **6**(7): 2012-2018.

Morita, N., S. Kiryu and H. Kiyama (1996). "p53-independent cyclin G expression in a group of mature neurons and its enhanced expression during nerve regeneration." J Neurosci **16**(19): 5961-5966.

Moughamian, A. J. and E. L. Holzbaur (2012). "Dynactin is required for transport initiation from the distal axon." Neuron **74**(2): 331-343.

Mu, X., I. Silos-Santiago, S. L. Carroll and W. D. Snider (1993). "Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia." J Neurosci **13**(9): 4029-4041.

Murphy, P. G., L. A. Borthwick, M. Altares, J. Gauldie, D. Kaplan and P. M. Richardson (2000). "Reciprocal actions of interleukin-6 and brain-derived neurotrophic factor on rat and mouse primary sensory neurons." Eur J Neurosci **12**(6): 1891-1899.

Nadeau, J. R., T. D. Wilson-Gerwing and V. M. Verge (2014). "Induction of a reactive state in perineuronal satellite glial cells akin to that produced by nerve injury is linked to the level of p75NTR expression in adult sensory neurons." Glia **62**(5): 763-777.

Nagamori, I., N. Yabuta, T. Fujii, H. Tanaka, K. Yomogida, Y. Nishimune and H. Nojima (2005). "Tisp40, a spermatid specific bZip transcription factor, functions by binding to the unfolded protein response element via the Rip pathway." Genes Cells **10**(6): 575-594.

Naidoo, N. (2009). "The endoplasmic reticulum stress response and aging." Rev Neurosci **20**(1): 23-37.

Nakae, J., T. Kitamura, W. Ogawa, M. Kasuga and D. Accili (2001). "Insulin regulation of gene expression through the forkhead transcription factor Foxo1 (Fkhr) requires kinases distinct from Akt." Biochemistry **40**(39): 11768-11776.

Nakae, J., T. Kitamura, D. L. Silver and D. Accili (2001). "The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression." J Clin Invest **108**(9): 1359-1367.

Navarro, X., E. Verdu, G. Wendelschafer-Crabb and W. R. Kennedy (1997). "Immunohistochemical study of skin reinnervation by regenerative axons." J Comp Neurol **380**(2): 164-174.

Nemoto, S. and T. Finkel (2002). "Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway." Science **295**(5564): 2450-2452.

Newman, E. A. (2001). "Propagation of intercellular calcium waves in retinal astrocytes and Muller cells." J Neurosci **21**(7): 2215-2223.

Nishikawa, T., D. Edelstein, X. L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M. A. Yorek, D. Beebe, P. J. Oates, H. P. Hammes, I. Giardino and M. Brownlee (2000). "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage." Nature **404**(6779): 787-790.

Noble, J., C. A. Munro, V. S. Prasad and R. Midha (1998). "Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries." J Trauma **45**(1): 116-122.

Noel, F., W. N. Frost, L. M. Tian, M. A. Colicos and P. K. Dash (1995). "Recovery of tail-elicited siphon-withdrawal reflex following unilateral axonal injury is associated with ipsi- and contralateral changes in gene expression in Aplysia californica." J Neurosci **15**(10): 6926-6938.
Novak, P., D. A. Pimentel, B. Sundar, M. Moonis, L. Qin and V. Novak (2015). "Association of Statins with Sensory and Autonomic Ganglionopathy." Front Aging Neurosci **7**: 191.
Novoa, I., H. Zeng, H. P. Harding and D. Ron (2001). "Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha." J Cell Biol **153**(5): 1011-1022.
O'Brien, P. D., L. M. Hinder, S. A. Sakowski and E. L. Feldman (2014). "ER stress in diabetic peripheral neuropathy: A new therapeutic target." Antioxid Redox Signal **21**(4): 621-633.
Oaklander, A. L. and A. J. Belzberg (1997). "Unilateral nerve injury down-regulates mRNA for Na+ channel SCN10A bilaterally in rat dorsal root ganglia." Brain Res Mol Brain Res **52**(1): 162-165.
Oaklander, A. L. and J. M. Brown (2004). "Unilateral nerve injury produces bilateral loss of distal innervation." Ann Neurol **55**(5): 639-644.
Oaklander, A. L., K. Romans, S. Horasek, A. Stocks, P. Hauer and R. A. Meyer (1998). "Unilateral postherpetic neuralgia is associated with bilateral sensory neuron damage." Ann Neurol **44**(5): 789-795.
Ochs, S. (1972). "Rate of fast axoplasmic transport in mammalian nerve fibres." J Physiol **227**(3): 627-645.
Ogen-Shtern, N., T. Ben David and G. Z. Lederkremer (2016). "Protein aggregation and ER stress." Brain Res.
Ogg, S., S. Paradis, S. Gottlieb, G. I. Patterson, L. Lee, H. A. Tissenbaum and G. Ruvkun (1997). "The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans." Nature **389**(6654): 994-999.
Onate, M., A. Catenaccio, G. Martinez, D. Armentano, G. Parsons, B. Kerr, C. Hetz and F. A. Court (2016). "Activation of the unfolded protein response promotes axonal regeneration after peripheral nerve injury." Sci Rep **6**: 21709.
Onate, M., F. A. Court and C. Hetz (2016). "Bursting the unfolded protein response accelerates axonal regeneration." Neural Regen Res **11**(6): 892-893.

Onuma, H., B. T. Vander Kooi, J. N. Boustead, J. K. Oeser and R. M. O'Brien (2006). "Correlation between FOXO1a (FKHR) and FOXO3a (FKHRL1) binding and the inhibition of basal glucose-6-phosphatase catalytic subunit gene transcription by insulin." Mol Endocrinol **20**(11): 2831-2847.

Otto, D., K. Unsicker and C. Grothe (1987). "Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transectioned sciatic nerve on neuron death in adult rat dorsal root ganglia." Neurosci Lett **83**(1-2): 156-160.

Oyadomari, S. and M. Mori (2004). "Roles of CHOP/GADD153 in endoplasmic reticulum stress." Cell Death Differ **11**(4): 381-389.

Ozel Turkcu, U., N. Solak Tekin, T. Gokdogan Edgunlu, S. Karakas Celik and S. Oner (2014). "The association of FOXO3A gene polymorphisms with serum FOXO3A levels and oxidative stress markers in vitiligo patients." Gene **536**(1): 129-134.

Park, D. S., B. Levine, G. Ferrari and L. A. Greene (1997). "Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons." J Neurosci **17**(23): 8975-8983.

Park, S. Y., Y. Piao, C. Thomas, G. N. Fuller and J. F. de Groot (2016). "Cdc2-like kinase 2 is a key regulator of the cell cycle via FOXO3a/p27 in glioblastoma." Oncotarget **7**(18): 26793-26805.

Patodia, S. and G. Raivich (2012). "Role of transcription factors in peripheral nerve regeneration." Front Mol Neurosci **5**: 8.

Penas, C., M. Font-Nieves, J. Fores, V. Petegnief, A. Planas, X. Navarro and C. Casas (2011). "Autophagy, and BiP level decrease are early key events in retrograde degeneration of motoneurons." Cell Death Differ **18**(10): 1617-1627.

Peng, S., S. Zhao, F. Yan, J. Cheng, L. Huang, H. Chen, Q. Liu, X. Ji and Z. Yuan (2015). "HDAC2 selectively regulates FOXO3a-mediated gene transcription during oxidative stress-induced neuronal cell death." J Neurosci **35**(3): 1250-1259.

Perlson, E., S. Maday, M. M. Fu, A. J. Moughamian and E. L. Holzbaur (2010). "Retrograde axonal transport: pathways to cell death?" Trends Neurosci **33**(7): 335-344.

Petersen, M., A. S. Eckert, G. Segond von Banchet, B. Heppelmann, A. Klusch and K. D. Kniffki (1998). "Plasticity in the expression of bradykinin binding sites in sensory neurons after mechanical nerve injury." Neuroscience **83**(3): 949-959.

Pettersson, L. M., N. M. Geremia, Z. Ying and V. M. Verge (2014). "Injury-associated PACAP expression in rat sensory and motor neurons is induced by endogenous BDNF." PLoS One **9**(6): e100730.

Pezet, S., A. Spyropoulos, R. J. Williams and S. B. McMahon (2005). "Activity-dependent phosphorylation of Akt/PKB in adult DRG neurons." Eur J Neurosci **21**(7): 1785-1797.

Piehl, F., U. Arvidsson, H. Johnson, S. Cullheim, M. Villar, A. Dagerlind, L. Terenius, T. Hokfelt and B. Ulfhake (1991). "Calcitonin Gene-related Peptide (CGRP)-like Immunoreactivity and CGRP mRNA in Rat Spinal Cord Motoneurons after Different Types of Lesions." Eur J Neurosci **3**(8): 737-757.

Pirianov, G., K. G. Brywe, C. Mallard, A. D. Edwards, R. A. Flavell, H. Hagberg and H. Mehmet (2007). "Deletion of the c-Jun N-terminal kinase 3 gene protects neonatal mice against cerebral hypoxic-ischaemic injury." J Cereb Blood Flow Metab **27**(5): 1022-1032.

Plas, D. R. and C. B. Thompson (2003). "Akt activation promotes degradation of tuberin and FOXO3a via the proteasome." J Biol Chem **278**(14): 12361-12366.

Pluquet, O., A. Pourtier and C. Abbadie (2015). "The unfolded protein response and cellular senescence. A review in the theme: cellular mechanisms of endoplasmic reticulum stress signaling in health and disease." Am J Physiol Cell Physiol **308**(6): C415-425.

Puig, O. and R. Tjian (2005). "Transcriptional feedback control of insulin receptor by dFOXO/FOXO1." Genes Dev **19**(20): 2435-2446.

Purves D, A. G., Fitzpatrick D, Katz L, LaMantia A-S, McNamara J, (2001). Neuroscience. Sunderland, MA, Sinauer Associates.

Quadrato, G. and S. Di Giovanni (2012). "Gatekeeper between quiescence and differentiation: p53 in axonal outgrowth and neurogenesis." Int Rev Neurobiol **105**: 71-89.

Quarta, S., B. E. Baeumer, N. Scherbakov, M. Andratsch, S. Rose-John, G. Dechant, C. E. Bandtlow and M. Kress (2014). "Peripheral nerve regeneration and NGF-dependent neurite outgrowth of adult sensory neurons converge on STAT3 phosphorylation downstream of neuropoietic cytokine receptor gp130." J Neurosci **34**(39): 13222-13233.

Rabizadeh, S., J. Oh, L. T. Zhong, J. Yang, C. M. Bitler, L. L. Butcher and D. E. Bredesen (1993). "Induction of apoptosis by the low-affinity NGF receptor." Science **261**(5119): 345-348.

Raggo, C., N. Rapin, J. Stirling, P. Gobeil, E. Smith-Windsor, P. O'Hare and V. Misra (2002). "Luman, the cellular counterpart of herpes simplex virus VP16, is processed by regulated intramembrane proteolysis." Mol Cell Biol **22**(16): 5639-5649.

Raivich, G., M. Bohatschek, C. Da Costa, O. Iwata, M. Galiano, M. Hristova, A. S. Nateri, M. Makwana, L. Riera-Sans, D. P. Wolfer, H. P. Lipp, A. Aguzzi, E. F. Wagner and A. Behrens (2004). "The AP-1 transcription factor c-Jun is required for efficient axonal regeneration." Neuron **43**(1): 57-67.

Raivich, G., R. Hellweg and G. W. Kreutzberg (1991). "NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration." Neuron **7**(1): 151-164.

Ramer, M. and M. Bisby (1997). "Reduced sympathetic sprouting occurs in dorsal root ganglia after axotomy in mice lacking low-affinity neurotrophin receptor." Neurosci Lett **228**(1): 9-12.

Ramón y Cajal, S. R. (1895). Histology of the Nervous System, Oxford University Press.

Rangarajan, P., A. Karthikeyan, J. Lu, E. A. Ling and S. T. Dheen (2015). "Sirtuin 3 regulates Foxo3a-mediated antioxidant pathway in microglia." Neuroscience **311**: 398-414.

Rao, R. V., A. Peel, A. Logvinova, G. del Rio, E. Hermel, T. Yokota, P. C. Goldsmith, L. M. Ellerby, H. M. Ellerby and D. E. Bredesen (2002). "Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78." FEBS Lett **514**(2-3): 122-128.

Rapallino, M. V., A. Cupello and A. Giuditta (1988). "Axoplasmic RNA species synthesized in the isolated squid giant axon." Neurochem Res **13**(7): 625-631.

Rapiejko, P. J. and R. Gilmore (1997). "Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum." Cell **89**(5): 703-713.

Reichardt, L. F. (2006). "Neurotrophin-regulated signalling pathways." Philos Trans R Soc Lond B Biol Sci **361**(1473): 1545-1564.

Reiling, J. H., A. J. Olive, S. Sanyal, J. E. Carette, T. R. Brummelkamp, H. L. Ploegh, M. N. Starnbach and D. M. Sabatini (2013). "A CREB3-ARF4 signalling pathway mediates the response to Golgi stress and susceptibility to pathogens." Nat Cell Biol **15**(12): 1473-1485.

Relling, D. P., L. B. Esberg, C. X. Fang, W. T. Johnson, E. J. Murphy, E. C. Carlson, J. T. Saari and J. Ren (2006). "High-fat diet-induced juvenile obesity leads to cardiomyocyte dysfunction

and upregulation of Foxo3a transcription factor independent of lipotoxicity and apoptosis." J Hypertens **24**(3): 549-561.

Renno, W. M., M. Al-Maghrebi, A. Alshammari and P. George (2013). "(-)-Epigallocatechin-3-gallate (EGCG) attenuates peripheral nerve degeneration in rat sciatic nerve crush injury." Neurochem Int **62**(3): 221-231.

Roberts, A. J., T. Kon, P. J. Knight, K. Sutoh and S. A. Burgess (2013). "Functions and mechanics of dynein motor proteins." Nat Rev Mol Cell Biol **14**(11): 713-726.

Ron, D. (2002). "Translational control in the endoplasmic reticulum stress response." J Clin Invest **110**(10): 1383-1388.

Ron, D. and P. Walter (2007). "Signal integration in the endoplasmic reticulum unfolded protein response." Nat Rev Mol Cell Biol **8**(7): 519-529.

Rotshenker, S. (2011). "Wallerian degeneration: the innate-immune response to traumatic nerve injury." J Neuroinflammation **8**: 109.

Roux, P. P. and P. A. Barker (2002). "Neurotrophin signaling through the p75 neurotrophin receptor." Prog Neurobiol **67**(3): 203-233.

Ruff, C. A., N. Staak, S. Patodia, M. Kaswich, E. Rocha-Ferreira, C. Da Costa, S. Brecht, M. Makwana, X. Fontana, M. Hristova, P. Rumajogee, M. Galiano, M. Bohatschek, T. Herdegen, A. Behrens and G. Raivich (2012). "Neuronal c-Jun is required for successful axonal regeneration, but the effects of phosphorylation of its N-terminus are moderate." J Neurochem **121**(4): 607-618.

Ruit, K. G., J. L. Elliott, P. A. Osborne, Q. Yan and W. D. Snider (1992). "Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development." Neuron **8**(3): 573-587.

Ryoke, K., M. Ochi, A. Iwata, Y. Uchio, S. Yamamoto and H. Yamaguchi (2000). "A conditioning lesion promotes in vivo nerve regeneration in the contralateral sciatic nerve of rats." Biochem Biophys Res Commun **267**(3): 715-718.

Sajan, M., B. Hansen, R. Ivey, 3rd, J. Sajan, C. Ari, S. Song, U. Braun, M. Leitges, M. Farese-Higgs and R. V. Farese (2016). "Brain Insulin Signaling Is Increased in Insulin-Resistant States and Decreases in FOXOs and PGC-1alpha and Increases in Abeta1-40/42 and Phospho-Tau May Abet Alzheimer Development." Diabetes **65**(7): 1892-1903.

Sano, R. and J. C. Reed (2013). "ER stress-induced cell death mechanisms." Biochim Biophys Acta **1833**(12): 3460-3470.
Saxena, S., E. Cabuy and P. Caroni (2009). "A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice." Nat Neurosci **12**(5): 627-636.
Scheuner, D., B. Song, E. McEwen, C. Liu, R. Laybutt, P. Gillespie, T. Saunders, S. Bonner-Weir and R. J. Kaufman (2001). "Translational control is required for the unfolded protein response and in vivo glucose homeostasis." Mol Cell **7**(6): 1165-1176.
Schmidt, M., S. Fernandez de Mattos, A. van der Horst, R. Klompmaker, G. J. Kops, E. W. Lam, B. M. Burgering and R. H. Medema (2002). "Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D." Mol Cell Biol **22**(22): 7842-7852.
Schreyer, D. J. and J. H. Skene (1993). "Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons." J Neurobiol **24**(7): 959-970.
Schroder, M. and R. J. Kaufman (2005). "ER stress and the unfolded protein response." Mutat Res **569**(1-2): 29-63.
Schroder, M. and R. J. Kaufman (2005). "The mammalian unfolded protein response." Annu Rev Biochem **74**: 739-789.
Schroer, T. A. (2004). "Dynactin." Annu Rev Cell Dev Biol **20**: 759-779.
Schwartz, J. P., J. Pearson and E. M. Johnson (1982). "Effect of exposure to anti-NGF on sensory neurons of adult rats and guinea pigs." Brain Res **244**(2): 378-381.
Segal, R. A. (2003). "Selectivity in neurotrophin signaling: theme and variations." Annu Rev Neurosci **26**: 299-330.
Seijffers, R., A. J. Allchorne and C. J. Woolf (2006). "The transcription factor ATF-3 promotes neurite outgrowth." Mol Cell Neurosci **32**(1-2): 143-154.
Seijffers, R., C. D. Mills and C. J. Woolf (2007). "ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration." J Neurosci **27**(30): 7911-7920.
Sendtner, M., R. Gotz, B. Holtmann and H. Thoenen (1997). "Endogenous ciliary neurotrophic factor is a lesion factor for axotomized motoneurons in adult mice." J Neurosci **17**(18): 6999-7006.

Shakhbazau, A., J. A. Martinez, Q. G. Xu, J. Kawasoe, J. van Minnen and R. Midha (2012). "Evidence for a systemic regulation of neurotrophin synthesis in response to peripheral nerve injury." J Neurochem **122**(3): 501-511.
Shen, A. G., S. X. Shi, M. L. Chen, J. Qin, S. F. Gao and C. Cheng (2008). "Dynamic changes of p27(kip1) and Skp2 expression in injured rat sciatic nerve." Cell Mol Neurobiol **28**(5): 713-725.
Shen, J., X. Chen, L. Hendershot and R. Prywes (2002). "ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals." Dev Cell **3**(1): 99-111.
Sheng, Z. H. (2014). "Mitochondrial trafficking and anchoring in neurons: New insight and implications." J Cell Biol **204**(7): 1087-1098.
Sheu, J. Y., D. J. Kulhanek and F. P. Eckenstein (2000). "Differential patterns of ERK and STAT3 phosphorylation after sciatic nerve transection in the rat." Exp Neurol **166**(2): 392-402.
Shi, C., K. Viccaro, H. G. Lee and K. Shah (2016). "Cdk5-FOXO3a axis: initially neuroprotective, eventually neurodegenerative in Alzheimer's disease models." J Cell Sci.
Shi, S., C. Cheng, J. Zhao, M. Chen, J. Qin, S. Gao and A. Shen (2007). "Expression of p27kip1 and Skp2 in the adult spinal cord following sciatic nerve injury." J Mol Neurosci **32**(1): 64-71.
Shi, T. J., P. Huang, J. Mulder, S. Ceccatelli and T. Hokfelt (2009). "Expression of p-Akt in Sensory Neurons and Spinal Cord after Peripheral Nerve Injury." Neurosignals **17**(3): 203-212.
Shirvan, A., I. Ziv, A. Barzilai, R. Djaldeti, R. Zilkh-Falb, T. Michlin and E. Melamed (1997). "Induction of mitosis-related genes during dopamine-triggered apoptosis in sympathetic neurons." J Neural Transm Suppl **50**: 67-78.
Smith, G. D., J. R. Seckl, W. J. Sheward, J. G. Bennie, S. M. Carroll, H. Dick and A. J. Harmar (1991). "Effect of adrenalectomy and dexamethasone on neuropeptide content of dorsal root ganglia in the rat." Brain Res **564**(1): 27-30.
Smith, P. G., H. Reddy and P. Venkataraman (1990). "Sympathetic sprouting into neonatally denervated contralateral target: superior cervical ganglion neuronal numbers and sizes." Brain Res Dev Brain Res **53**(2): 208-214.
Snell (2005). Clinical Neuroanatomy, Lippincott Williams and Wilkins.
Soilu-Hanninen, M., P. Ekert, T. Bucci, D. Syroid, P. F. Bartlett and T. J. Kilpatrick (1999). "Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway." J Neurosci **19**(12): 4828-4838.

Sommervaille, T., M. L. Reynolds and C. J. Woolf (1991). "Time-dependent differences in the increase in GAP-43 expression in dorsal root ganglion cells after peripheral axotomy." Neuroscience **45**(1): 213-220.

Spataro, L. E., E. M. Sloane, E. D. Milligan, J. Wieseler-Frank, D. Schoeniger, B. M. Jekich, R. M. Barrientos, S. F. Maier and L. R. Watkins (2004). "Spinal gap junctions: potential involvement in pain facilitation." J Pain **5**(7): 392-405.

Sriburi, R., S. Jackowski, K. Mori and J. W. Brewer (2004). "XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum." J Cell Biol **167**(1): 35-41.

Srinivasan, S., M. Anitha, S. Mwangi and R. O. Heuckeroth (2005). "Enteric neuroblasts require the phosphatidylinositol 3-kinase/Akt/Forkhead pathway for GDNF-stimulated survival." Mol Cell Neurosci **29**(1): 107-119.

Stam, F. J., H. D. MacGillavry, N. J. Armstrong, M. C. de Gunst, Y. Zhang, R. E. van Kesteren, A. B. Smit and J. Verhaagen (2007). "Identification of candidate transcriptional modulators involved in successful regeneration after nerve injury." Eur J Neurosci **25**(12): 3629-3637.

Stepanova, T., J. Slemmer, C. C. Hoogenraad, G. Lansbergen, B. Dortland, C. I. De Zeeuw, F. Grosveld, G. van Cappellen, A. Akhmanova and N. Galjart (2003). "Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein)." J Neurosci **23**(7): 2655-2664.

Storlazzi, C. T., F. Mertens, A. Nascimento, M. Isaksson, J. Wejde, O. Brosjo, N. Mandahl and I. Panagopoulos (2003). "Fusion of the FUS and BBF2H7 genes in low grade fibromyxoid sarcoma." Hum Mol Genet **12**(18): 2349-2358.

Sugimoto, K., Y. Murakawa and A. A. Sima (2002). "Expression and localization of insulin receptor in rat dorsal root ganglion and spinal cord." J Peripher Nerv Syst **7**(1): 44-53.

Sun, R. Q., Y. J. Tu, J. Y. Yan and W. D. Willis (2006). "Activation of protein kinase B/Akt signaling pathway contributes to mechanical hypersensitivity induced by capsaicin." Pain **120**(1-2): 86-96.

Sun, X., Z. B. Jones, X. M. Chen, L. Zhou, K. F. So and Y. Ren (2016). "Multiple organ dysfunction and systemic inflammation after spinal cord injury: a complex relationship." J Neuroinflammation **13**(1): 260.

Sunayama, J., F. Tsuruta, N. Masuyama and Y. Gotoh (2005). "JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3." J Cell Biol **170**(2): 295-304.
Sung, H. J., Y. S. Kim, H. Kang and J. Ko (2008). "Human LZIP induces monocyte CC chemokine receptor 2 expression leading to enhancement of monocyte chemoattractant protein 1/CCL2-induced cell migration." Exp Mol Med **40**(3): 332-338.
Swett, J. E., Y. Torigoe, V. R. Elie, C. M. Bourassa and P. G. Miller (1991). "Sensory neurons of the rat sciatic nerve." Exp Neurol **114**(1): 82-103.
Tanaka, H., T. Yamashita, K. Yachi, T. Fujiwara, H. Yoshikawa and M. Tohyama (2004). "Cytoplasmic p21(Cip1/WAF1) enhances axonal regeneration and functional recovery after spinal cord injury in rats." Neuroscience **127**(1): 155-164.
Tcherkezian, J., P. A. Brittis, F. Thomas, P. P. Roux and J. G. Flanagan (2010). "Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation." Cell **141**(4): 632-644.
Tedeschi, A., T. Nguyen, R. Puttagunta, P. Gaub and S. Di Giovanni (2009). "A p53-CBP/p300 transcription module is required for GAP-43 expression, axon outgrowth, and regeneration." Cell Death Differ **16**(4): 543-554.
Terzis, J. K., D. D. Sun and P. K. Thanos (1997). "Historical and basic science review: past, present, and future of nerve repair." J Reconstr Microsurg **13**(3): 215-225.
Tetzlaff, W., S. W. Alexander, F. D. Miller and M. A. Bisby (1991). "Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43." J Neurosci **11**(8): 2528-2544.
Tetzlaff, W. and M. A. Bisby (1990). "Cytoskeletal protein synthesis and regulation of nerve regeneration in PNS and CNS neurons of the rat." Restor Neurol Neurosci **1**(3): 189-196.
Tetzlaff, W., M. A. Bisby and G. W. Kreutzberg (1988). "Changes in cytoskeletal proteins in the rat facial nucleus following axotomy." J Neurosci **8**(9): 3181-3189.
Tonra, J. R., R. Curtis, V. Wong, K. D. Cliffer, J. S. Park, A. Timmes, T. Nguyen, R. M. Lindsay, A. Acheson and P. S. DiStefano (1998). "Axotomy upregulates the anterograde transport and expression of brain-derived neurotrophic factor by sensory neurons." J Neurosci **18**(11): 4374-4383.
Tothova, Z. and D. G. Gilliland (2007). "FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system." Cell Stem Cell **1**(2): 140-152.

Tsujino, H., E. Kondo, T. Fukuoka, Y. Dai, A. Tokunaga, K. Miki, K. Yonenobu, T. Ochi and K. Noguchi (2000). "Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury." Mol Cell Neurosci **15**(2): 170-182.
Tu, B. P. and J. S. Weissman (2004). "Oxidative protein folding in eukaryotes: mechanisms and consequences." J Cell Biol **164**(3): 341-346.
Udina, E., A. Ladak, M. Furey, T. Brushart, N. Tyreman and T. Gordon (2010). "Rolipram-induced elevation of cAMP or chondroitinase ABC breakdown of inhibitory proteoglycans in the extracellular matrix promotes peripheral nerve regeneration." Exp Neurol **223**(1): 143-152.
Urra, S., C. A. Escudero, P. Ramos, F. Lisbona, E. Allende, P. Covarrubias, J. I. Parraguez, N. Zampieri, M. V. Chao, W. Annaert and F. C. Bronfman (2007). "TrkA receptor activation by nerve growth factor induces shedding of the p75 neurotrophin receptor followed by endosomal gamma-secretase-mediated release of the p75 intracellular domain." J Biol Chem **282**(10): 7606-7615.
Utley, D. S., S. L. Lewin, E. T. Cheng, A. N. Verity, D. Sierra and D. J. Terris (1996). "Brain-derived neurotrophic factor and collagen tubulization enhance functional recovery after peripheral nerve transection and repair." Arch Otolaryngol Head Neck Surg **122**(4): 407-413.
Valderrama, X. and V. Misra (2008). "Novel Brn3a cis-acting sequences mediate transcription of human trkA in neurons." J Neurochem **105**(2): 425-435.
Valderrama, X., N. Rapin and V. Misra (2008). "Zhangfei, a novel regulator of the human nerve growth factor receptor, trkA." J Neurovirol **14**(5): 425-436.
Valderrama, X., N. Rapin, V. M. Verge and V. Misra (2009). "Zhangfei induces the expression of the nerve growth factor receptor, trkA, in medulloblastoma cells and causes their differentiation or apoptosis." J Neurooncol **91**(1): 7-17.
Valenzuela, V., G. Martinez, C. Duran-Aniotz and C. Hetz (2016). "Gene therapy to target ER stress in brain diseases." Brain Res.
Valenzuela, V., M. Onate, C. Hetz and F. A. Court (2016). "Injury to the nervous system: A look into the ER." Brain Res.
van der Horst, A. and B. M. Burgering (2007). "Stressing the role of FoxO proteins in lifespan and disease." Nat Rev Mol Cell Biol **8**(6): 440-450.

Van Minnen, J., J. J. Bergman, E. R. Van Kesteren, A. B. Smit, W. P. Geraerts, K. Lukowiak, S. U. Hasan and N. I. Syed (1997). "De novo protein synthesis in isolated axons of identified neurons." Neuroscience **80**(1): 1-7.

Vance, J. E., R. B. Campenot and D. E. Vance (2000). "The synthesis and transport of lipids for axonal growth and nerve regeneration." Biochim Biophys Acta **1486**(1): 84-96.

Vanden Noven, S., N. Wallace, D. Muccio, A. Turtz and M. J. Pinter (1993). "Adult spinal motoneurons remain viable despite prolonged absence of functional synaptic contact with muscle." Exp Neurol **123**(1): 147-156.

Verge, V. M., K. A. Gratto, L. A. Karchewski and P. M. Richardson (1996). "Neurotrophins and nerve injury in the adult." Philos Trans R Soc Lond B Biol Sci **351**(1338): 423-430.

Verge, V. M., J. P. Merlio, J. Grondin, P. Ernfors, H. Persson, R. J. Riopelle, T. Hokfelt and P. M. Richardson (1992). "Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF." J Neurosci **12**(10): 4011-4022.

Verge, V. M., P. M. Richardson, R. Benoit and R. J. Riopelle (1989). "Histochemical characterization of sensory neurons with high-affinity receptors for nerve growth factor." J Neurocytol **18**(5): 583-591.

Verge, V. M., P. M. Richardson, Z. Wiesenfeld-Hallin and T. Hokfelt (1995). "Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons." J Neurosci **15**(3 Pt 1): 2081-2096.

Verge, V. M., R. J. Riopelle and P. M. Richardson (1989). "Nerve growth factor receptors on normal and injured sensory neurons." J Neurosci **9**(3): 914-922.

Verge, V. M., W. Tetzlaff, M. A. Bisby and P. M. Richardson (1990). "Influence of nerve growth factor on neurofilament gene expression in mature primary sensory neurons." J Neurosci **10**(6): 2018-2025.

Verge, V. M., W. Tetzlaff, P. M. Richardson and M. A. Bisby (1990). "Correlation between GAP43 and nerve growth factor receptors in rat sensory neurons." J Neurosci **10**(3): 926-934.

Verge, V. M., Z. Wiesenfeld-Hallin and T. Hokfelt (1993). "Cholecystokinin in mammalian primary sensory neurons and spinal cord: in situ hybridization studies in rat and monkey." Eur J Neurosci **5**(3): 240-250.

Verkhratsky, A., R. K. Orkand and H. Kettenmann (1998). "Glial calcium: homeostasis and signaling function." Physiol Rev **78**(1): 99-141.

Verma, P., S. Chierzi, A. M. Codd, D. S. Campbell, R. L. Meyer, C. E. Holt and J. W. Fawcett (2005). "Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration." J Neurosci **25**(2): 331-342.

Vershinin, M., B. C. Carter, D. S. Razafsky, S. J. King and S. P. Gross (2007). "Multiple-motor based transport and its regulation by Tau." Proc Natl Acad Sci U S A **104**(1): 87-92.

Viana, G. B. and F. C. Kauffman (1984). "Cholinesterase activity in the rat superior cervical ganglion: effect of denervation and axotomy." Brain Res **304**(1): 37-45.

Vigneswara, V., M. Berry, A. Logan and Z. Ahmed (2013). "Caspase-2 is upregulated after sciatic nerve transection and its inhibition protects dorsal root ganglion neurons from apoptosis after serum withdrawal." PLoS One **8**(2): e57861.

Wall, P. D., R. Mills, M. Fitzgerald and S. J. Gibson (1982). "Chronic blockade of sciatic nerve transmission by tetrodotoxin does not produce central changes in the dorsal horn of the spinal cord of the rat." Neurosci Lett **30**(3): 315-320.

Waller, A. (1850). "Experiments on the Section of the Glossopharyngeal and Hypoglossal Nerves of the Frog, and Observations of the Alterations Produced Thereby in the Structure of Their Primitive Fibres." Philosophical Transactions of the Royal Society of London **140**: 423-429.

Wang, L., J. Karpac and H. Jasper (2014). "Promoting longevity by maintaining metabolic and proliferative homeostasis." J Exp Biol **217**(Pt 1): 109-118.

Wang, M. and R. J. Kaufman (2016). "Protein misfolding in the endoplasmic reticulum as a conduit to human disease." Nature **529**(7586): 326-335.

Wang, W., C. Yan, J. Zhang, R. Lin, Q. Lin, L. Yang, F. Ren, J. Zhang, M. Ji and Y. Li (2013). "SIRT1 inhibits TNF-alpha-induced apoptosis of vascular adventitial fibroblasts partly through the deacetylation of FoxO1." Apoptosis **18**(6): 689-701.

Wang, X., J. H. Bauer, Y. Li, Z. Shao, F. S. Zetoune, E. Cattaneo and C. Vincenz (2001). "Characterization of a p75(NTR) apoptotic signaling pathway using a novel cellular model." J Biol Chem **276**(36): 33812-33820.

Wang, Y., Y. Liu, Y. Chen, S. Shi, J. Qin, F. Xiao, D. Zhou, M. Lu, Q. Lu and A. Shen (2009). "Peripheral nerve injury induces down-regulation of Foxo3a and p27kip1 in rat dorsal root ganglia." Neurochem Res **34**(5): 891-898.

Waterman-Storer, C. M., S. Karki and E. L. Holzbaur (1995). "The p150Glued component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1)." Proc Natl Acad Sci U S A **92**(5): 1634-1638.

Watkins, L. R., E. D. Milligan and S. F. Maier (2001). "Glial activation: a driving force for pathological pain." Trends Neurosci **24**(8): 450-455.

Watson, W. E. (1974). "Cellular responses to axotomy and to related procedures." Br Med Bull **30**(2): 112-115.

Webber, C. A., Y. Xu, K. J. Vanneste, J. A. Martinez, V. M. Verge and D. W. Zochodne (2008). "Guiding adult Mammalian sensory axons during regeneration." J Neuropathol Exp Neurol **67**(3): 212-222.

Weigel, D., G. Jurgens, F. Kuttner, E. Seifert and H. Jackle (1989). "The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo." Cell **57**(4): 645-658.

Wen, Q., X. Duan, R. Liao, P. Little, G. Gao, H. Jiang, S. Lalit, R. Quirion and W. Zheng (2011). "Characterization of intracellular translocation of Forkhead transcription factor O (FoxO) members induced by NGF in PC12 cells." Neurosci Lett **498**(1): 31-36.

Wen, Q., H. Wang, P. J. Little, R. Quirion and W. Zheng (2012). "Forkhead family transcription factor FoxO and neural differentiation." Neurogenetics **13**(2): 105-113.

Werner, M. U., T. K. Ringsted, H. Kehlet and K. Wildgaard (2013). "Sensory testing in patients with postthoracotomy pain syndrome: Part 1: mirror-image sensory dysfunction." Clin J Pain **29**(9): 775-783.

Wetmore, C. and L. Olson (1995). "Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions." J Comp Neurol **353**(1): 143-159.

Williams, K. L. and K. M. Mearow (2011). "Phosphorylation status of heat shock protein 27 influences neurite growth in adult dorsal root ganglion sensory neurons in vitro." J Neurosci Res **89**(8): 1160-1172.

Willis, D., K. W. Li, J. Q. Zheng, J. H. Chang, A. B. Smit, T. Kelly, T. T. Merianda, J. Sylvester, J. van Minnen and J. L. Twiss (2005). "Differential transport and local translation of cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons." J Neurosci **25**(4): 778-791.

Willis T, F. W. (1681, reprinted in 1966). "The anatomy of the brain and nerves. ." Montreal: McGill University Press.

Wilson-Gerwing, T. D., M. V. Dmyterko, D. W. Zochodne, J. M. Johnston and V. M. Verge (2005). "Neurotrophin-3 suppresses thermal hyperalgesia associated with neuropathic pain and attenuates transient receptor potential vanilloid receptor-1 expression in adult sensory neurons." J Neurosci **25**(3): 758-767.

Won, C. K., H. H. Ji and P. O. Koh (2006). "Estradiol prevents the focal cerebral ischemic injury-induced decrease of forkhead transcription factors phosphorylation." Neurosci Lett **398**(1-2): 39-43.

Wright, D. E. and W. D. Snider (1995). "Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia." J Comp Neurol **351**(3): 329-338.

Wu, Y. B., H. Q. Li, M. S. Ren, W. T. Li, X. Y. Lv and L. Wang (2013). "CHOP/ORP150 ratio in endoplasmic reticulum stress: a new mechanism for diabetic peripheral neuropathy." Cell Physiol Biochem **32**(2): 367-379.

Xian, C. J. and X. F. Zhou (1999). "Neuronal-glial differential expression of TGF-alpha and its receptor in the dorsal root ganglia in response to sciatic nerve lesion." Exp Neurol **157**(2): 317-326.

Xie, Q., J. Chen and Z. Yuan (2012). "Post-translational regulation of FOXO." Acta Biochim Biophys Sin (Shanghai) **44**(11): 897-901.

Xie, Y. B., O. H. Lee, B. Nedumaran, H. A. Seong, K. M. Lee, H. Ha, I. K. Lee, Y. Yun and H. S. Choi (2008). "SMILE, a new orphan nuclear receptor SHP-interacting protein, regulates SHP-repressed estrogen receptor transactivation." Biochem J **416**(3): 463-473.

Xu, J. T., H. Y. Tu, W. J. Xin, X. G. Liu, G. H. Zhang and C. H. Zhai (2007). "Activation of phosphatidylinositol 3-kinase and protein kinase B/Akt in dorsal root ganglia and spinal cord contributes to the neuropathic pain induced by spinal nerve ligation in rats." Exp Neurol **206**(2): 269-279.

Xu, Q. G., J. Forden, S. K. Walsh, T. Gordon and R. Midha (2010). "Motoneuron survival after chronic and sequential peripheral nerve injuries in the rat." J Neurosurg **112**(4): 890-899.

Ye, J., R. B. Rawson, R. Komuro, X. Chen, U. P. Dave, R. Prywes, M. S. Brown and J. L. Goldstein (2000). "ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs." Mol Cell **6**(6): 1355-1364.

Yeh, T. Y., N. J. Quintyne, B. R. Scipioni, D. M. Eckley and T. A. Schroer (2012). "Dynactin's pointed-end complex is a cargo-targeting module." Mol Biol Cell **23**(19): 3827-3837.

Ying, Z., V. Misra and V. M. Verge (2014). "Sensing nerve injury at the axonal ER: activated Luman/CREB3 serves as a novel axonally synthesized retrograde regeneration signal." Proc Natl Acad Sci U S A **111**(45): 16142-16147.

Ying, Z., R. Zhai, N. A. McLean, J. M. Johnston, V. Misra and V. M. Verge (2015). "The Unfolded Protein Response and Cholesterol Biosynthesis Link Luman/CREB3 to Regenerative Axon Growth in Sensory Neurons." J Neurosci **35**(43): 14557-14570.

Ying, Z., R. Zhang, V. M. Verge and V. Misra (2015). "Cloning and characterization of rat Luman/CREB3, a transcription factor highly expressed in nervous system tissue." J Mol Neurosci **55**(2): 347-354.

Yoo, S., M. P. Nguyen, M. Fukuda, G. D. Bittner and H. M. Fishman (2003). "Plasmalemmal sealing of transected mammalian neurites is a gradual process mediated by Ca(2+)-regulated proteins." J Neurosci Res **74**(4): 541-551.

Yoshida, H., K. Haze, H. Yanagi, T. Yura and K. Mori (1998). "Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors." J Biol Chem **273**(50): 33741-33749.

Yoshida, H., T. Matsui, A. Yamamoto, T. Okada and K. Mori (2001). "XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor." Cell **107**(7): 881-891.

Yoshida, H., T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi and K. Mori (2000). "ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response." Mol Cell Biol **20**(18): 6755-6767.

Yoshida, H., M. Oku, M. Suzuki and K. Mori (2006). "pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response." J Cell Biol **172**(4): 565-575.

Yun, H., S. Park, M. J. Kim, W. K. Yang, D. U. Im, K. R. Yang, J. Hong, W. Choe, I. Kang, S. S. Kim and J. Ha (2014). "AMP-activated protein kinase mediates the antioxidant effects of resveratrol through regulation of the transcription factor FoxO1." FEBS J **281**(19): 4421-4438.

Zhang, E., M. H. Yi, N. Shin, H. Baek, S. Kim, E. Kim, K. Kwon, S. Lee, H. W. Kim, Y. Chul Bae, Y. Kim, O. Y. Kwon, W. H. Lee and D. W. Kim (2015). "Endoplasmic reticulum stress impairment in the spinal dorsal horn of a neuropathic pain model." Sci Rep **5**: 11555.

Zhang, K., X. Shen, J. Wu, K. Sakaki, T. Saunders, D. T. Rutkowski, S. H. Back and R. J. Kaufman (2006). "Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response." Cell **124**(3): 587-599.

Zhang, R. and V. Misra (2014). "Effects of cyclic AMP response element binding protein-Zhangfei (CREBZF) on the unfolded protein response and cell growth are exerted through the tumor suppressor p53." Cell Cycle **13**(2): 279-292.

Zhang, R., N. Rapin, Z. Ying, E. Shklanka, T. W. Bodnarchuk, V. M. Verge and V. Misra (2013). "Zhangfei/CREB-ZF - a potential regulator of the unfolded protein response." PLoS One **8**(10): e77256.

Zhang, R., D. H. Thamm and V. Misra (2015). "The effect of Zhangfei/CREBZF on cell growth, differentiation, apoptosis, migration, and the unfolded protein response in several canine osteosarcoma cell lines." BMC Vet Res **11**: 22.

Zhang, W., S. Patil, B. Chauhan, S. Guo, D. R. Powell, J. Le, A. Klotsas, R. Matika, X. Xiao, R. Franks, K. A. Heidenreich, M. P. Sajan, R. V. Farese, D. B. Stolz, P. Tso, S. H. Koo, M. Montminy and T. G. Unterman (2006). "FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression." J Biol Chem **281**(15): 10105-10117.

Zhang, X., R. R. Ji, J. Arvidsson, J. M. Lundberg, T. Bartfai, K. Bedecs and T. Hokfelt (1996). "Expression of peptides, nitric oxide synthase and NPY receptor in trigeminal and nodose ganglia after nerve lesions." Exp Brain Res **111**(3): 393-404.

Zhang, Y., Y. Jin, T. A. Williams, S. M. Burtenshaw, A. C. Martyn and R. Lu (2010). "Amino acid deprivation induces CREBZF/Zhangfei expression via an AARE-like element in the promoter." Biochem Biophys Res Commun **391**(3): 1352-1357.

Zheng, J. Q., T. K. Kelly, B. Chang, S. Ryazantsev, A. K. Rajasekaran, K. C. Martin and J. L. Twiss (2001). "A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons." J Neurosci **21**(23): 9291-9303.

Zheng, W. H., S. Kar and R. Quirion (2000). "Insulin-like growth factor-1-induced phosphorylation of the forkhead family transcription factor FKHRL1 is mediated by Akt kinase in PC12 cells." J Biol Chem **275**(50): 39152-39158.

Zheng, W. H., S. Kar and R. Quirion (2002). "FKHRL1 and its homologs are new targets of nerve growth factor Trk receptor signaling." J Neurochem **80**(6): 1049-1061.

Zheng, W. H., S. Kar and R. Quirion (2002). "Insulin-like growth factor-1-induced phosphorylation of transcription factor FKHRL1 is mediated by phosphatidylinositol 3-kinase/Akt kinase and role of this pathway in insulin-like growth factor-1-induced survival of cultured hippocampal neurons." Mol Pharmacol **62**(2): 225-233.

Zhou, J., C. Y. Liu, S. H. Back, R. L. Clark, D. Peisach, Z. Xu and R. J. Kaufman (2006). "The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response." Proc Natl Acad Sci U S A **103**(39): 14343-14348.

Zhou, X. F., E. T. Chie, Y. S. Deng, J. H. Zhong, Q. Xue, R. A. Rush and C. J. Xian (1999). "Injured primary sensory neurons switch phenotype for brain-derived neurotrophic factor in the rat." Neuroscience **92**(3): 841-853.

Zhou, X. F., W. P. Gai and R. A. Rush (1993). "CGRP immunoreactive neurons in rat dorsal root ganglia do not all contain low-affinity NGF receptor immunoreactivity." Brain Res **612**(1-2): 322-325.

Zhou, X. F., R. A. Rush and E. M. McLachlan (1996). "Differential expression of the p75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection." J Neurosci **16**(9): 2901-2911.

Zhu, W., G. N. Bijur, N. A. Styles and X. Li (2004). "Regulation of FOXO3a by brain-derived neurotrophic factor in differentiated human SH-SY5Y neuroblastoma cells." Brain Res Mol Brain Res **126**(1): 45-56.

Zhuang, Z. Y., H. Xu, D. E. Clapham and R. R. Ji (2004). "Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization." J Neurosci **24**(38): 8300-8309.