Student Name: Alyssa Verrelli **Date:** 8/8/14

Project Title: Characterizing thymic stromal lymphopoietin (TSLP): a role in neurite outgrowth

Primary Supervisor Name: Dr. Paul Fernyhough

Department: Pharmacology, Division of Neurodegenerative Disorders

SUMMARY: (no more than 250 words single spaced)

Neuropathy is a serious complication of diabetes mellitus. In particular, sensory innervation is progressively lost as neurons degenerate distally. Many proinflammatory cytokines have been implicated in signalling through pathways that mediate nerve regeneration. These cytokines have also been associated with mitochondrial respiratory regulation. Mitochondrial dysfunction is increasingly being linked to diabetic sensory neuropathy. These cytokines, therefore, may play an important role in modulating mitochondrial function for neurite outgrowth. The aim of my work was to characterize the function of the cytokine thymic stromal lymphopoietin (TSLP) in relation to control of sensory neuron phenotype. I hypothesized that TSLP signals through JAK/STAT3 and AMPK pathways to modulate mitochondrial function and induce neurite outgrowth. My objectives were: to study the effect of cytokine TSLP on the phenotype of sensory neurons, to investigate signaling pathways activated by TSLP, and to explore effect of TSLP on mitochondrial bioenergetics. My results demonstrated that the effect of TSLP on normal and diabetic sensory neurons resulted in a dose-dependent induction of neurite outgrowth. TSLP was shown to signal through the AMPK pathway. TSLP was shown to also signal through JAK/STAT3 to induce neurite outgrowth. The mitochondrial bioenergetics of sensory neurons as influenced by TSLP, mediated in part via JAK/STAT3 and AMPK pathways, demonstrated an increased spare respiratory capacity meaning that TSLP enhances the ability of mitochondria to work under high demand for energy. Therefore, the role of TSLP in enhancing mitochondrial efficiency may be a mechanism worth targeting therapeutically, in the treatment of diabetic neuropathy and possibly other diseases of the peripheral nervous system.

ACKNOWLEDGEMENTS:
I gratefully acknowledge the support by one or more of the following sponsors;
H.T. Thorlakson Foundation
Dean, Faculty of Medicine
Manitoba Health Research Council
Manitoba Institute of Child Health
Kidney Foundation of Manitoba
Leukemia and Lymphoma Society of Canada
CancerCare Manitoba
Manitoba Medical Service Foundation
Associate Dean (Research), Faculty of Medicine
Heart and Stroke Foundation
Health Sciences Centre Research Foundation
Other: Dr. Paul Fernyhough, and St. Boniface Research Centre

INTRODUCTION

Diabetic Neuropathy

Diabetes mellitus is a disease of impaired insulin secretion, or function, or both. It is characterized by elevated blood glucose levels [1]. Diabetes is classified as either Type 1, a deficiency of insulin, or Type 2, a resistance to insulin—preventing insulin-dependent transport of glucose into cells. Diabetic neuropathy is a long-term complication of diabetes. The most common of the neuropathies is the diffuse symmetrical sensorimotor neuropathy. This is associated with progressive 'dying back' of nerve endings in the extremities [1]. Multiple mechanisms have been proposed in the pathogenesis of diabetic neuropathy. They are discussed in detail below.

The Nervous System

The nervous system is the conductor of sensory inputs from the body's environment; it enables the perception of that information by the brain, and effects the responses that follow [2]. It is divided into the central nervous system (CNS) which is composed of the brain and spinal cord, and the peripheral nervous system (PNS) which refers to the cranial and spinal nerves [3]. The PNS is composed of afferent and efferent nerves. Afferents are sensory nerves which begin peripherally and converge on the dorsal root ganglia (DRG) of the spinal column at each vertebral level. DRG are bundles of sensory cell bodies. The axons continue to the spinal cord, where they may innervate interneurons, or motor neurons (efferent nerves) [4].

The nervous system has two types of cells: the neurons and the glial cells. Glia are the support cells of the nervous system. They exhibit a number of functions including structural support for the neurons, debris removal, and promotion of efficient signaling [2-4]. Of note, the Schwann cells in the PNS and the oligodendrocytes of the CNS produce myelin, an insulating substance that promotes conduction in the neuronal fibers.

Neuronal cells are the main signaling units, and are generally composed of four regions: the cell body, dendrites, the axon, and presynaptic terminals. The cell body houses the organelles such as the nucleus which stores the cell's genes, the endoplasmic reticulum for protein synthesis, and the mitochondria for energy production. The dendrites and axons are processes of the cell body known as neurites. Several short dendrites branch out from the cell body, and receive incoming signals from other neurons. The single, long axon conducts the signal onwards [2].

The electrical signals that pass through neurons are known as action potentials. These are rapid, transient, reversals of the membrane polarity, above a certain threshold voltage [2, 4].The axon divides distally into presynaptic terminals, which transmit action potentials via small molecules known as neurotransmitters. They cross the synaptic cleft, a space between one neuron and the next, to exert an effect on the postsynaptic cell, thus propagating the signal.

If an axon becomes damaged the distal portion degenerates, the endoplasmic reticuli reduce in number (chromatolysis), and the cell body swells. Macrophages arrive at the site of injury to clear dead debris, while the distal Schwann cells proliferate (Wallerian degeneration). In the PNS, it is possible for the proximal axon to regenerate, send branches into the distal nerve stump, and restore function. The CNS, however, does not commonly regenerate axons after injury [3, 4].

Axon Regeneration-Neurotrophins

The regulation of neuronal plasticity and regeneration is complex. Two major classes of compounds that influence regeneration are a) cytokines, cell signaling molecules closely involved in immunomodulation, and b) neurotrophic factors. The latter are growth factors involved in neuronal development, growth, and maintenance [5, 6]. These include nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT3) among others. NGF is the dominant neurotrophin in the PNS, and has long been proven to induce axonal plasticity and regeneration [7].

Studies conducted on the hair follicle of murine dermis demonstrate increased GDNF mRNA expression in the active growth phase of the follicle, and a corresponding decrease in the regressive phase. This pattern of neurotrophic expression dependent on target tissue growth confirms the interaction between target cutaneous organs and nerve growth [6, 8]. It also suggests a potential regulatory link between tissue energy requirements and nerve growth, which is described in detail below.

Axon Regeneration-Cytokines

Cytokines have been the subject of much study in the nervous system. Early studies identified an increase in proinflammatory cytokine expression at the site of peripheral sensory neuron injury [9]. This has been associated with the temporary inflammation of Wallerian degeneration which involves the activation of cytokines, infiltration of macrophages, and regulation of neuropathic pain and growth via neurotrophins [9, 10]. In more recent studies, the relationship between cytokines and neurotrophic factors has been more closely examined. Their signaling pathways, and downstream effects are also being explored to identify potential roles in neuronal regeneration post nerve injury.

An increasingly well described family of cytokines is the interleukin-6-related cytokines (gp130-related cytokines). Interleukin-6 (IL-6) is a cytokine with a variety of functions including promoting differentiation of B and T cells of the immune system, and development of hepatocytes and neuronal cells [11]. Cytokines related to IL-6, share the necessity of binding a common accessory signal transducer to effect a signal. This common protein is known as gp130. It is ubiquitously expressed in tissues of the human body [11]. Along with IL-6, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) are other gp130-related cytokines. All three cytokines signal through a tyrosine-kinase receptor-linked pathway known as JAK/STAT3 [11]. The activation of signal transducer and activator of transcription 3 (STAT3) by neuronal injury has been implicated in peripheral nerve regeneration, particularly in DRG [12]. STAT3 is activated by phosphorylation of either its tyrosine705 residue (Tyr705), or serine727 residue (Ser727). The phosphorylated STAT3-Ser727 translocates to the mitochondria, and phosphorylated STAT3-Tyrosine705 translocates to the nucleus to regulate gene transcription [12, 13]. Evidence that CNTF and LIF induce phosphorylation of STAT3 at Tyr705 in murine DRG confirms their upstream role in peripheral nerve regeneration [12].

Proposed mechanisms of action to induce regeneration are specific to each cytokine. CNTF, for example, signals through the nuclear factor kappa B (NFκB) transcription factor [14]. NFκB forms a complex with other transcriptional components and upon degradation of its cytoplasmic inhibitor, translocates to the nucleus to regulate gene transcription. NFκB is associated with cytokine expression and cell survival [14]. This is clearly demonstrated in cultured adult sensory neurons, where CNTF enhances neurite outgrowth through an NFκB dependent pathway [14].

Besides the gp130-related cytokines, others have been examined for potential roles in peripheral nerve regeneration. As mentioned above, Wallerian degeneration is a part of nerve regeneration, as it clears the site of nerve injury. A review regarding Wallerian degeneration (WD) noted that TNFα, and IL-1β cytokines are of particular significance to WD because of their well-defined roles in inflammation [9]. Both could activate a network of cytokines in WD, similar to their action in inflammation; they could contribute to macrophage recruitment and function (e.g., myelin removal by phagocytosis); and they could regulate growth of peripheral neurons by regulating NGF production in PNS fibroblasts [9]. Several of these potential roles have been examined.

Tumor necrosis factor-alpha (TNFα) is known for activating the acute phase reaction of inflammation, and regulating immune cells [15]. It has both proliferative, and apoptotic regulatory abilities. Signaling mediated by TNFα has been well described. It activates NFκB, and MAPK pathways which lead to expression of proinflammatory cytokines, and the activation of the typically pro-apoptotic JNK transcription factor, respectively [15, 16]. Interestingly, TNFα has been shown to promote neurite outgrowth in both normal and diabetic adult rat DRG sensory neurons. This induction of growth is attributed to activation of NFκB [16].

Interleukin-1-beta (IL-1β) is known for its proinflammatory properties, and regulation of proliferation, differentiation, and apoptosis [17]. In neuronal injury, it signals through the JAK/STAT3 pathway to promote nerve regeneration. Recent studies have identified the translocation of pSTAT3-Ser727 to the mitochondria to regulate activity of electron transport Complex I [13, 18]. Evidence of this translocation was associated with NGF-promoted neurite outgrowth, thus confirming the significant role of mitochondria in nerve regeneration [13]. A proposed mechanism for diabetic neuropathy hypothesizes that neuropathy results from poor nerve regeneration, secondary to abnormal mitochondrial function [13]. This will be closely examined below.

The functions of the cytokines and their corresponding evidence above, are presented under normal physiological conditions. In diabetes, the cellular environment is highly altered. Hyperglycemia, and lack or resistance to insulin contribute to an aberrant environment.

Pathogenesis of Diabetic Neuropathy

Hyperglycemic stress

Sensory neurons are particularly vulnerable to hyperglycemia for several reasons. Firstly, they take up glucose independently of insulin [19]. Most cells in the body take up glucose through the GLUT4 transporter regulated by insulin. In liver, muscle and adipose cells, glucose transporter 4 (GLUT-4) is the most common [20]. CNS neurons have some protection via the blood-brain barrier. For peripheral nerves, however, without the regulatory effect of insulin, glucose entry is unrestricted [20]. Under hyperglycemic conditions, this allows excessive glucose into the cells. Secondly, energy requirements of neurons are high. The majority of energy expenditure is in maintaining the ion concentration gradients of the cell. The action potentials of neurons depend fully on these carefully balanced gradients. The intracellular bioavailable form of energy is ATP, usually produced from glucose. It is required for the function of ion channels along the axon. The typically long axons of sensory neurons necessitate distant transport of proteins and nutrients, and this, too, elevates energy requirements. Finally, sensory neurons do not respond well to disruptions in energy availability [20].

Polyol pathway

Among the many proposed mechanisms leading to diabetic neuropathy, the polyol pathway is a dominant one. In hyperglycemic states, excess glucose in neurons saturates the catabolic pathway known as glycolysis. Glycolysis is the first of three cellular respiratory pathways that ultimately produce ATP for the cell [19, 20]. If the first enzyme of glycolysis, hexokinase, cannot phosphorylate glucose, it is diverted to the polyol pathway. Its first enzyme, aldose reductase, has a lower affinity for glucose than hexokinase, and readily converts glucose into the intermediate, sorbitol. Sorbitol accumulates within the cell and adversely affects its function in three major ways. As an osmolyte, it draws excess water into the cell, causing tissue swelling and damage; and it downregulates endogenous osmolytes such as myoinositol, which have some neuroprotective action. The formation of sorbitol promotes oxidative stress, via consumption of the reducing agent NADPH. Inadequate NADPH prevents the function of glutathione reductase, an enzyme involved in reducing reactive oxygen species (ROS). Ultimately, the accumulation of ROS leads to oxidative stress [20].

Oxidative Stress and AGEs

Oxidative stress also leads to further detrimental effects in the cell. DNA strand breakage, for example, activates poly (ADP-ribose) polymerase (PARP). This enzyme lowers intracellular concentrations of NAD+, thereby decreasing the oxidation of substrates in mitochondrial respiration, which ultimately results in decreased ATP production. Insufficient energy results in neuron dysfunction and axonal degeneration [20].

Another source of peripheral neuropathy is known as protein glycation. Elevated extracellular and intracellular glucose levels result in the formation of advanced glycation end-products (AGEs). These are proteins bound by conformationally modified glucose, and by binding to the receptor for AGEs (RAGE), they activate pathways such as NFκB [20, 21]. Glycosylation of intracellular neuronal proteins such as tubulin causes impaired axonal transport [1]. AGEs affect various biological functions of the neuron resulting in neuronal damage.

Mitochondrial dysfunction

Finally, a prominent emerging pathogenic theory for diabetic neuropathy implicates mitochondrial dysfunction. Sensory neurons of streptozotocin-diabetic rats have been shown to display abnormal inner mitochondrial membrane depolarization [22]. Many transcription factors, pathways, and cytokines implicated in nerve regeneration have been associated with an abnormal mitochondrial phenotype in diabetes [13, 16]. These include IL-1β, the JAK/STAT3 pathway, and the AMPK/PGC1α pathway [13, 16, 23].

Cytokines and mitochondrial function

Studies of IL-1β-induced phosphorylation of STAT3 in DRG neurons of diabetic mice showed significantly diminished expression of both p-STAT3-Tyr705 and p-STAT3-Ser727 compared to control mice [13]. IL-1β significantly improved mitochondrial maximal respiration in these diabetic cells. Consequently, JAK/STAT pathway inhibitor AG490 significantly reduced maximal respiration in IL-1β treated cells [13]. In effect, IL-1β signaling through JAK/STAT3 is linked to mitochondrial respiratory function. In mitochondrial dysfunction, IL-1β signaling is impaired, thus its role in nerve regeneration is restricted.

The AMPK pathway

An additional signaling pathway associated with metabolic regulation is known as the AMP-activated protein kinase (AMPK) pathway. AMPK is a heterotrimeric complex that is activated by one of two factors [23]. An upstream kinase known as LKB1 phosphorylates the Threonine172 residue of the alpha subunit of AMPK, particularly during metabolic stress. LKB1 is activated by an increase in the AMP/ATP ratio in the cell. This means that AMPK is directly modulated by AMP levels. Therefore, any stress that disturbs energy balance such as hypoxia, or toxins to mitochondrial complexes will result in activation of this pathway [23].

The other kinase that activates AMPK, is triggered by elevated cytoplasmic Ca^{2+}. Calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ) also phosphorylates Thr172 on the alpha subunit of AMPK, but is not regulated by AMP/ATP levels. It is found only in certain cells including neurons [23].

AMPK has an important role in energy balance at both the cellular level and the whole-body level. Activation of AMPK switches on energy-producing pathways, and switches off energy-consuming pathways [24]. Specialized glucose-sensing cells (β cells of the pancreas and neurons in the hypothalamus), when stimulated with AMPK, result in the same effects that hypoglycemia produces: decreased insulin secretion, and increased feeding behaviour [23]. AMPK regulates energy balance at the whole-body level. This reveals a potential therapeutic role for AMPK in metabolic syndromes such as diabetes, and obesity [23].

Downstream of AMPK, a transcriptional coactivator known as peroxisome proliferator-activated receptor gamma coactivator-1-α (PGC-1α) has been characterised as a vast regulator of energy metabolism. It is phosphorylated by AMPK under conditions of energy depletion [24]. PGC-1α is mostly expressed in tissues with high energy oxidative capacity such as the heart, liver, and skeletal muscle. In Type 2 diabetic patients, skeletal muscle exhibits decreased levels of PGC-1α [24]. It is possible to conclude that PGC-1α is closely linked to energy balance requirements of this tissue.

The neuron, too, has been shown to express the AMPK/PGC-1α pathway. In diabetes, the nutrient excess may switch off AMPK signaling and PGC-1α activation in the neuron resulting in decreased mitochondrial function [19]. Evidence of down regulated expression of AMPK, PGC-1α, and mitochondrial proteins in DRG of streptozotocin-diabetic rats, suggests a link between impaired signalling and mitochondrial dysfunction and confirms that mitochondrial dysfunction is involved in diabetic neuropathy [19].

Thymic Stromal Lymphopoietin

Thymic stromal lymphopoietin (TSLP) is a cytokine well characterized in the field of immunology. It is an IL-7-like cytokine that has been implicated in a variety of inflammatory and allergic conditions including atopic dermatitis, asthma, allergic rhinitis, and rheumatoid arthritis [25]. Studies have shown that “TSLP is a critical factor sufficient and necessary to induce or maintain allergic airway inflammation” [26]. TSLP activates myeloid dendritic cells to promote CD4+ and CD8+ Th2 cell differentiation [25]. Besides the pro-allergic phenotype of TSLP, it has not been well characterized regarding signaling and mechanisms of expression [25].

The receptor for TSLP is part of the IL-2 receptor family. These receptors are composed of one specific ligand binding chain, and a non-specific common chain [27]. The TSLP receptor

contains a common IL-7Rα chain and its specific ligand binding chain known as TSLP-R or CRLF2 (cytokine receptor-like factor2) [28]. TSLP is known to signal via activation of STAT5 [28]. Interestingly, a more recent study demonstrated up regulation of TSLP in human airway smooth muscle cells by the influence of TNFα and IL-1β [26]. This was mediated by NFκB activation. Knowing that TNFα and IL-1β are associated with nerve regeneration, it is possible that TSLP, as a downstream cytokine, may also play a role in nerve regeneration.

Based on the previous knowledge that JAK/STAT3 is a common signaling pathway amongst cytokines involved in nerve regeneration, and that TSLP has been shown to signal via STAT5, I chose to examine TSLP signaling through JAK/STAT3.

HYPOTHESIS

TSLP signals through JAK/STAT3 and AMPK to modulate mitochondrial function and induce neurite outgrowth.

OBJECTIVES

To study the effect of cytokine TSLP on the phenotype of sensory neurons.
To investigate signaling pathways activated by TSLP.
To explore effect of TSLP on mitochondrial bioenergetics.

MATERIALS AND METHODS

The following are abridged methods previously described in Saleh et al, 2013 [13]; Gardiner et al, 2005 [29]; in an unpublished article-Smith et al, 2014 [30]; Roy Chowdhury et al, 2012 [31]; and discussed, in part, in the review by Brand and Nicholls, 2011 [32].

Induction of type I diabetes in rats

Male Sprague Dawley rats were made diabetic with a single intraperitoneal injection of 75mg/kg streptozotocin (STZ; Sigma, USA). Sensory neuropathy was confirmed in rodents at 2 months using development of thermal hypoalgesia in the hind paw as a marker (this is indicative of loss of thermal sensitivity as a result of distal fiber loss in the skin). Tissue was collected from rodents after 1-7 months of type 1 diabetes. Animal procedures followed guidelines of University of Manitoba Animal Care Committee using Canadian Council of Animal Care rules [13].

Sensory neuron cultures and treatments

Rats were killed by decapitation. DRG were removed, cleaned of connective tissue and chemically dissociated in 0.125% collagenase in Ham's F12 (Sigma) in two 45 minute treatments. DRG were then transferred to 0.25% trypsin for 25 min. Trypsin was then inactivated using 30% fetal bovine serum (Sigma) [29]. Ganglia were washed twice with dilute fetal bovine serum, and once in Ham's F12 before being mechanically dissociated by gentle trituration using a borosilicate glass pipette [29]. Dissociated neurons were passed through 70μm mesh (NUNC, USA) to remove undissociated cells and myelin debris, and centifugated at 3000rpm for 5 min. Cells were resuspended in Ham's F12 and centrifuged through 15% bovine serum albumin at 9000rpm for 10 min, to remove non-neuronal cells [29]. Neurons were resuspended in modified Ham's F12 in the presence of modified Bottenstein's N2 supplement without insulin (see [13] for constituents of N2). Cells were seeded onto NUNC plates precoated with 2μg/ml laminin-1 (Sigma) [29]. In some experiments the media was also supplemented with a low dose cocktail of neurotrophic factors (0.1ng/ml NGF, 1.0 ng/ml GDNF, 1.0ng/ml NT-3, and 0.1nM insulin—all from Promega, USA) [13]. Neurons were incubated for 18 h in 5% CO_2 at

37ºC [29]. In some experiments, TSLP (R&D systems; Lot: IDK0513121) was added to the cells before incubation overnight; in others, it was added at specified times prior to cell harvesting.

Immunocytochemistry and quantification of neurite outgrowth

Immunostaining-based fluorescent images were captured and the mean pixel area determined using ImageJ software (adjusted for the cell body signal) [13]. All values were normalized for neuronal number.

Western blotting

Cultured DRG neurons were harvested after 24 h in ice-cold stabilization buffer containing: 0.1M Pipes, 5mM $MgCl_2$, 5mM EGTA, 0.5% Triton X-100, 20% glycerol, 10mM NaF, 1mM PMSF, and protease inhibitor cocktail. Proteins were assayed using DC protein assay (BioRad, CA, USA) and Western blot analysis performed as previously described [31] with the addition of stain-free technology (BioRad). The antibodies were used at the following concentrations: p-STAT3 and T-STAT3 at 1:1000, pAMPK at 1:500; and T-ERK at 1:5000.

Measurement of mitochondrial respiration in cultured DRG neurons

An XF24 Analyzer (Seahorse Biosciences, USA) was used to measure neuronal bioenergetics function. The XF24 creates a transient 7µl chamber in specialized 24-well microplates that allows for oxygen consumption rate (OCR) to be monitored in real time [13]. Mitochondrial respiration analysis was performed as previously described [13, 29]. The step-wise addition of specific mitochondrial protein toxins allowed determination of the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production, the level of non-ATP-linked oxygen consumption (proton leak), the maximal respiration capacity and the non-mitochondrial oxygen consumption [13]. Following OCR measurement, the cells were harvested and a DC protein assay was conducted for normalization to total protein per well. Data are expressed as OCR in pmoles/min for 1000 cells.

RESULTS

Experiments were conducted under defined conditions on dissociated cultures of DRG sensory neurons derived from adult male Sprague Dawley rats. The rats were between 200-400g.

TSLP induces neurite outgrowth in DRG neurons derived from normal and diabetic rats in a dose-dependent manner

To study the effect of TSLP on the phenotype of sensory neurons, adult rat DRG neurons were cultured for 24 hours in the presence of a low dose growth factor cocktail (LDGF), and treated with three different concentrations of TSLP (5, 10, 25ng/ml) (Figure 1). The culture was fixed, stained for neuron specific β-tubulin III (Figure 2), and the levels of total neurite outgrowth were quantified. TSLP induced neurite outgrowth in a dose-dependent manner. Neurites stemmed from both large and small sized cells. The growth was significantly higher between the control cells and those treated with 10ng/ml TSLP (*$p<0.05$). This appeared to be the concentration to induce peak growth, as the cells at 25ng/ml expressed less neurite outgrowth.

A similar culture was performed on DRG neurons derived from a streptozotocin-induced (STZ) diabetic rat, a model of type I diabetes. In the absence of LDGF and treated with 5, 10, and 25ng/ml TSLP for 24 hours, the diabetic neurons exhibited an enhanced phenotype of neurites (Figure 3). Both large and small sized cells grew neurites. Again, the dose-dependent effect of

TSLP seemed to peak at 10ng/ml at which stage growth was significant compared to the control (*p<0.05).

TSLP mediates enhanced expression of p-STAT3-Ser727 in normal DRG neurons

The ability of TSLP to signal through the JAK/STAT3 pathway was investigated. Adult rat DRG neurons were cultured in the presence of LDGF for 24 hours. Prior to collection for Western blot, the cells were treated for 30 minutes with TSLP at 5, 10, and 25ng/ml. Expression of phosphorylated STAT3 at residues Tyrosine705 and Serine727 was investigated via Western blot. Expression of p-STAT3-Tyr705 in cells treated with TSLP remained equal to control levels (data not shown). Expression of p-STAT3-Ser727, however, increased in a dose-dependent manner, when normalized to housekeeping protein ERK (Figure 4a), with significant expression at 5 and 10ng/ml TSLP compared to control. Based on previous work in our lab, total ERK expression remains stable in both normal and diabetic DRG neurons. For this reason, it is preferred as a loading control for Western blots. When normalizing p-STAT3-Ser727 levels to total STAT3, a slight elevation in expression at 5 and 10ng/ml was noted, but was not statistically significant (Figure 4b). Similar in trend to the previous neurite outgrowth experiments, the 25ng/ml treatment group had a reduced expression of p-STAT3-Ser727 when normalized to either total ERK or total STAT3.

JAK/STAT inhibitor AG490 inhibits TSLP-mediated expression of p-STAT3-Ser727

To assess the potential link between TSLP-induced neurite outgrowth and TSLP signaling through the JAK/STAT3 pathway, an inhibitor experiment was conducted. Adult rat DRG neurons were cultured for 24 hours in the presence of LDGF, and treated in three groups of 5ng/ml TSLP, 10uM AG490—a common JAK/STAT inhibitor—and a combination of both 5ng/ml TSLP and 10uM AG490 (Figure 5). Tyrphostin AG490 (AG490) is a JAK2 inhibitor [33]. In a review of the JAK2 gene, at least 6 references to studies involving AG490 demonstrated inhibition of the JAK/STAT pathway [33]. JAK2 is immediately upstream of STAT3, therefore inhibition of JAK2 will prevent the phosphorylation of STAT3 and any downstream effects. In this case, the downstream effect of JAK/STAT3 inhibition may be reduced neurite outgrowth, if the two processes are associated. The culture was fixed, stained for β-tubulin III and the levels of total neurite outgrowth were quantified. As seen previously, the 5ng/ml TSLP treatment induced significant neurite outgrowth compared to all other groups (one-way ANOVA with Tukey's test). The AG490-treated cells expressed neurite outgrowth similar to control levels. Finally, the TSLP + AG490–treated cells expressed inhibited neurite outgrowth, similar to control levels.

TSLP mediates enhanced expression of pAMPK in normal DRG neurons

Adult rat DRG neurons were cultured without LDGF for 24 hours. Prior to collection for Western blot, the cells were treated with 10ng/ml TSLP for 15, 30, or 60 minutes (Figure 6). Expression of phosphorylated AMPK was investigated via Western blot, and was found to increase in a time-dependent manner, when normalized to ERK, with significant expression at 60min compared to control. The 30min treatment group, however, expressed reduced levels of pAMPK. When normalizing pAMPK expression to total membrane protein, an identical trend was demonstrated.

A similar Western blot was performed in STZ-diabetic rat DRG neurons. The diabetic neurons were cultured for 24h, in the absence of LDGF, and were treated with 10ng/ml TSLP for 15, 30, or 60 minutes. They exhibited a time-dependent increase in pAMPK expression when normalized with either ERK or total membrane protein (Figure 7a,b). Significant expression was

noted at 60min compared to control, when normalized to total protein. No statistically significant expression was noted when data normalized to ERK.

TSLP mediates enhanced mitochondrial bioenergetics in DRG neurons

To explore effect of TSLP on mitochondrial bioenergetics, adult rat DRG neurons were cultured for 24 hours in the presence of LDGF, and treated with 10ng/ml TSLP (Figure 8). A real-time test of oxygen consumption rate, or mitochondrial bioenergetics under stress was performed. Compared to control basal respiration, maximal respiration, and spare respiratory capacity of cells treated with TSLP were significantly elevated (Figure 9a,b,c).

DISCUSSION

Effect of TSLP on sensory neuron phenotype

The effect of TSLP on the phenotype of sensory neurons was explored with DRG neuron cultures. A cocktail of low dose growth factors (LDGF) was used in the normal DRG culture. This cocktail is used to improve cultured cells' viability, but does not influence in any significant way, the neurite outgrowth. In the diabetic DRG culture, LDGFs were not included. This was to reduce any possible effect of the growth factors on neurite outgrowth, in order to see a clear effect between the treatment groups. Diabetic rats have obvious neuropathy, demonstrated by thermal hypoalgesia [34]. Neurite outgrowth, presumably, is more difficult to induce in diabetic cultured neurons. For this reason, I chose to remove the growth factors, in order to view the clearest representation of neurite outgrowth of diabetic DRG neurons. The resulting dose-dependent effect of TSLP on normal and diabetic sensory neurons demonstrated a peak of induction of growth at 10ng/ml, followed by a slight decrease at 25ng/ml (Figure 1a,b). This reduction in neurite outgrowth may be due to an inhibitory pathway triggered by higher concentrations of TSLP. It is also possible that at a certain concentration, the TSLP receptor becomes internalized by the cell, and prevents further stimulation-in effect, a negative feedback system.

TSLP signals through the JAK/STAT3 pathway

Interestingly, the study of TSLP-mediated phosphorylation of STAT3-Ser727 demonstrated an elevated expression at the same concentrations of TSLP that had previously induced neurite outgrowth (Figure 4a,b). If TSLP can induce neurite outgrowth in both normal and diabetic DRG cultures at 5 and 10ng/ml, and also enhance expression of p-STAT3-Ser727 at those concentrations, the two phenomena may be related. TSLP may signal through JAK/STAT3 via phosphorylation of STAT3-Ser727 to induce neurite outgrowth.

The normalization of p-STAT3-Ser727 levels to total STAT3 was conducted to account for any basal level of STAT3 activation. As a mediator of cell growth, differentiation and survival signals [35] STAT3 is an active transcription factor involved in many functions. Normalization theoretically ensures that basal activation of STAT3 for other functions does not influence the levels activated by TSLP. In the case of my culture treated with TSLP, however, normalization to total STAT3 produced different results compared to data normalized to housekeeping protein ERK. As mentioned previously, ERK is used as a loading control, ensuring probed levels of protein are normalized to the quantity of protein loaded into each well. The discrepancy between the two sets of results (Figure 4a,b) may be accounted for by the independent activation of T-STAT3 by other pathways. TSLP may have an indirect effect on T-STAT3 that affects the resulting normalized expression of p-STAT3.

TSLP mediates neurite outgrowth through JAK/STAT3

The potential link between TSLP-induced neurite outgrowth and TSLP signaling through the JAK/STAT3 pathway was investigated by blocking TSLP signaling through JAK/STAT3. This was achieved by the JAK2 inhibitor, AG490. The neurite outgrowth induced by TSLP was inhibited by AG490 (Figure 5). This clearly demonstrates that TSLP signals through JAK/STAT3 to induce neurite outgrowth. It may then be possible that down regulation of TSLP signaling through JAK/STAT3 contributes to deficit in neurite outgrowth. This is certainly a concept seen with the IL-1β cytokine. IL-1β signaling through JAK/STAT3 is linked to mitochondrial respiratory function. In diabetes, reduced levels of IL-1β prevent JAK/STAT3 modulation of mitochondrial function, and poor nerve regeneration (ie. neuropathy) results. This is a plausible mechanism in TSLP-induced neurite outgrowth.

TSLP signals through the AMPK pathway

The role of AMPK in energy balance at both the cellular and whole-body level implicates it in mitochondrial regulation. Because p-STAT3-Ser727 translocates to the mitochondria [13], and because TSLP is able to mediate its phosphorylation, a link between TSLP and the mitochondria can be proposed. The AMPK pathway offers a complementary approach to investigate the potential relationship between TSLP and regulation of mitochondrial function. A time course treatment at 10ng/ml TSLP was conducted. This concentration was selected because it induced maximal outgrowth in previous dose response experiments. The resulting significant increase in pAMPK expression at 60min in both normal and diabetic neurons (Figure 6,7) indicates that optimal TSLP-triggered phosphorylation of AMPK occurs at that time point. A proposed mechanism of action involves TSLP binding to its receptor, perhaps activating CaMKKβ which, in turn, activates AMPK via Thr172 residue phosphorylation. AMPK mediates up regulation of mitochondrial proteins to produce energy necessary for neurite outgrowth [23]. Further work is necessary in determining the precise mechanism by which TSLP activates AMPK.

TSLP enhances mitochondrial bioenergetics

Mitochondrial function is necessary in providing energy for nerve regeneration. Neurons have a high and constant demand for energy—not only in regular physiological function, but in particular when growing neurites. The mitochondrial bioenergetics influenced by TSLP demonstrate an increased spare respiratory capacity (Figure 9c). The spare respiratory capacity is the difference between maximal respiration and basal. It is the reserve capacity of the mitochondria for work. This means that TSLP enhances the ability of mitochondria to work under high demand for energy. Thus, it may be possible that TSLP induces neurite outgrowth through enhancement of mitochondrial function.

CONCLUSION

The effect of TSLP on normal and diabetic sensory neurons resulted in a dose-dependent induction of neurite outgrowth. TSLP was shown to signal through the AMPK pathway. TSLP was shown to also signal through JAK/STAT3 to induce neurite outgrowth. It may be possible that down regulation of TSLP signaling through JAK/STAT3 contributes to a deficit in neurite outgrowth. The mitochondrial bioenergetics of sensory neurons influenced by TSLP, mediated in part via JAK/STAT3 and AMPK pathways, demonstrated an increased spare respiratory capacity meaning that TSLP enhances the ability of mitochondria to work under high demand for energy. Therefore, the role of TSLP in enhancing mitochondrial efficiency may be a mechanism worth targeting therapeutically, in the treatment of diabetic neuropathy and possibly other diseases of the peripheral nervous system.

REFERENCES

1. Belchetz PE, Hammond P, *Mosby's Color Atlas and Text of Diabetes and Endocrinology.* London: Mosby; 2003.
2. Kandel ER, Schwartz J, Jessell T, *Principles of Neural Science*. 2000.
3. Waxman S, *Clinical Neuroanatomy.* 27th ed. Lange; 2013.
4. Crossman AR, N.D., *Neuroanatomy: an Illustrated Colour Text, 4th Edition.* Elsevier; 2010.
5. Weissmiller, A.M. and C. Wu, *Current advances in using neurotrophic factors to treat neurodegenerative disorders.* Transl Neurodegener, 2012. **1**(1): p. 14.
6. Hendrix, S. and E.M. Peters, *Neuronal plasticity and neuroregeneration in the skin -- the role of inflammation.* J Neuroimmunol, 2007. **184**(1-2): p. 113-26.
7. Lindsay, R.M., *Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons.* J Neurosci, 1988. **8**(7): p. 2394-405.
8. Botchkareva, N.V., et al., *New roles for glial cell line-derived neurotrophic factor and neurturin: involvement in hair cycle control.* Am J Pathol, 2000. **156**(3): p. 1041-53.
9. Shamash, S., F. Reichert, and S. Rotshenker, *The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta.* J Neurosci, 2002. **22**(8): p. 3052-60.
10. Golz, G., et al., *The cytokine/neurotrophin axis in peripheral axon outgrowth.* Eur J Neurosci, 2006. **24**(10): p. 2721-30.
11. Kishimoto, T., et al., *Interleukin-6 family of cytokines and gp130.* Blood, 1995. **86**(4): p. 1243-54.
12. Wang, L., et al., *Cell type-specific STAT3 activation by gp130-related cytokines in the peripheral nerves.* Neuroreport, 2009. **20**(7): p. 663-8.
13. Saleh, A., et al., *Diabetes impairs an interleukin-1beta-dependent pathway that enhances neurite outgrowth through JAK/STAT3 modulation of mitochondrial bioenergetics in adult sensory neurons.* Mol Brain, 2013. **6**: p. 45.
14. Saleh, A., et al., *Ciliary neurotrophic factor activates NF-kappaB to enhance mitochondrial bioenergetics and prevent neuropathy in sensory neurons of streptozotocin-induced diabetic rodents.* Neuropharmacology, 2013. **65**: p. 65-73.
15. Gaur, U. and B.B. Aggarwal, *Regulation of proliferation, survival and apoptosis by members of the TNF superfamily.* Biochem Pharmacol, 2003. **66**(8): p. 1403-8.
16. Saleh, A., et al., *Tumor necrosis factor-alpha elevates neurite outgrowth through an NF-kappaB-dependent pathway in cultured adult sensory neurons: Diminished expression in diabetes may contribute to sensory neuropathy.* Brain Res, 2011. **1423**: p. 87-95.
17. 3553, Gene ID., *IL1B interleukin 1, beta [Homo sapiens (human)].* <http://www.ncbi.nlm.nih.gov/gene/3553> Updated 3-Aug-2014
18. Wegrzyn, J., et al., *Function of mitochondrial Stat3 in cellular respiration.* Science, 2009. **323**(5915): p. 793-7.
19. Chowdhury, S.K., R.T. Dobrowsky, and P. Fernyhough, *Nutrient excess and altered mitochondrial proteome and function contribute to neurodegeneration in diabetes.* Mitochondrion, 2011. **11**(6): p. 845-54.
20. Tomlinson, D.R. and N.J. Gardiner, *Glucose neurotoxicity.* Nat Rev Neurosci, 2008. **9**(1): p. 36-45.
21. Goldin, A., et al., *Advanced glycation end products: sparking the development of diabetic vascular injury.* Circulation, 2006. **114**(6): p. 597-605.
22. Chowdhury, S.K., D.R. Smith, and P. Fernyhough, *The role of aberrant mitochondrial bioenergetics in diabetic neuropathy.* Neurobiol Dis, 2013. **51**: p. 56-65.
23. Hardie, D.G., *AMPK: a key regulator of energy balance in the single cell and the whole organism.* Int J Obes (Lond), 2008. **32 Suppl 4**: p. S7-12.
24. Canto, C. and J. Auwerx, *PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure.* Curr Opin Lipidol, 2009. **20**(2): p. 98-105.
25. Takai, T., *TSLP expression: cellular sources, triggers, and regulatory mechanisms.* Allergol Int, 2012. **61**(1): p. 3-17.
26. Redhu, N.S., et al., *Essential role of NF-kappaB and AP-1 transcription factors in TNF-alpha-induced TSLP expression in human airway smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2011. **300**(3): p. L479-85.
27. Kisseleva, T., et al., *Signaling through the JAK/STAT pathway, recent advances and future challenges.* Gene, 2002. **285**(1-2): p. 1-24.
28. Isaksen, D.E., et al., *Requirement for stat5 in thymic stromal lymphopoietin-mediated signal transduction.* J Immunol, 1999. **163**(11): p. 5971-7.
29. Gardiner, N.J., et al., *Alpha7 integrin mediates neurite outgrowth of distinct populations of adult sensory neurons.* Mol Cell Neurosci, 2005. **28**(2): p. 229-40.
30. Smith, D.R., and S.R. Chowdhury, A. Saleh, P. Fernyhough, *Analysis of cellular bioenergetics in cultured adult sensory neurons.* unpublished, 2014.
31. Roy Chowdhury, S.K., et al., *Impaired adenosine monophosphate-activated protein kinase signalling in dorsal root ganglia neurons is linked to mitochondrial dysfunction and peripheral neuropathy in diabetes.* Brain, 2012. **135**(Pt 6): p. 1751-66.
32. Brand, M.D. and D.G. Nicholls, *Assessing mitochondrial dysfunction in cells.* Biochem J, 2011. **435**(2): p. 297-312.
33. al., W.K.e., *Gene Review Jak2- Janus kinase 2.*
34. Calcutt, N.A., J.D. Freshwater, and A.P. Mizisin, *Prevention of sensory disorders in diabetic Sprague-Dawley rats by aldose reductase inhibition or treatment with ciliary neurotrophic factor.* Diabetologia, 2004. **47**(4): p. 718-24.
35. Hirano, T., K. Ishihara, and M. Hibi, *Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors.* Oncogene, 2000. **19**(21): p. 2548-56.

FIGURES

All data are mean ± SEM unless otherwise stated. Statistical analyses were conducted on GraphPadPrism software.

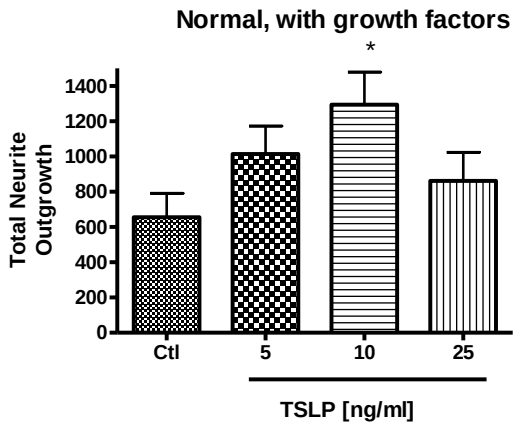

Figure 1 Normal adult rat DRG sensory neurons treated with 5, 10, and 25ng/ml doses of TSLP in the presence of low dose neurotrophic growth factor cocktail (LDGF) for 24 hours. (n=5 replicates) *p<0.05 for Ctl vs 10ng/ml TSLP via one-way ANOVA with Dunnett's test

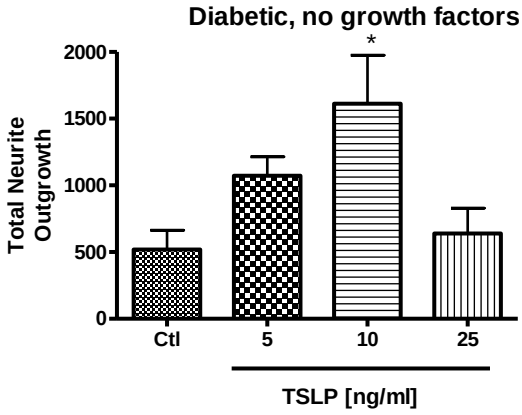

Figure 3 Diabetic adult rat DRG sensory neurons treated with 5, 10, 25ng/ml doses of TSLP without LDGF for 24 hours. (n=5 replicates) *p<0.05 for Ctl vs 10ng/ml TSLP via one-way ANOVA with Dunnett's test

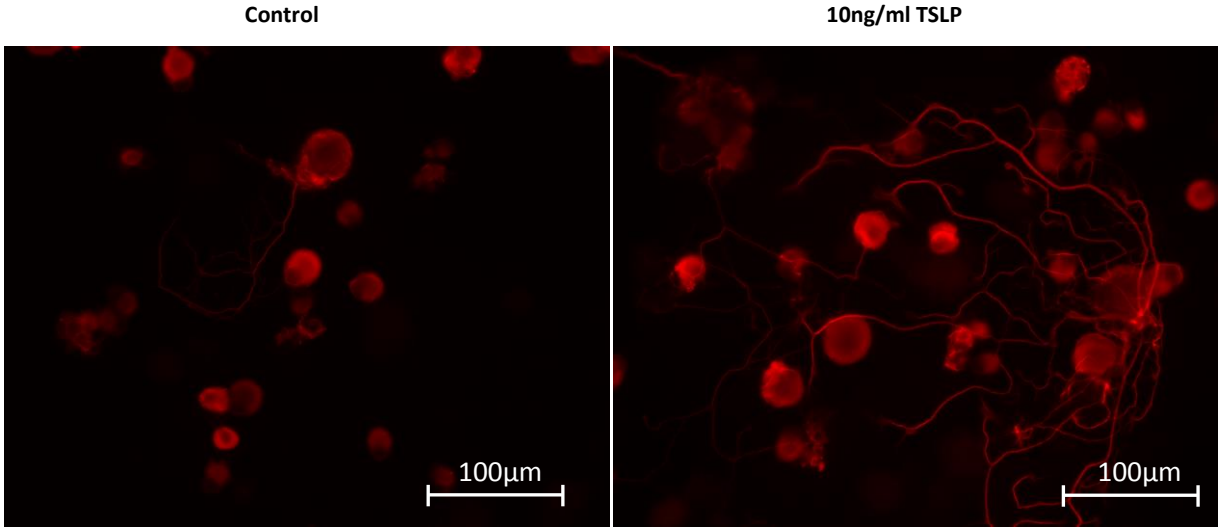

Figure 2 Immuncytochemistry-DRG sensory neurons stained with β tubulin III. Comparison of neurite outgrowth between control and 10ng/ml TSLP.

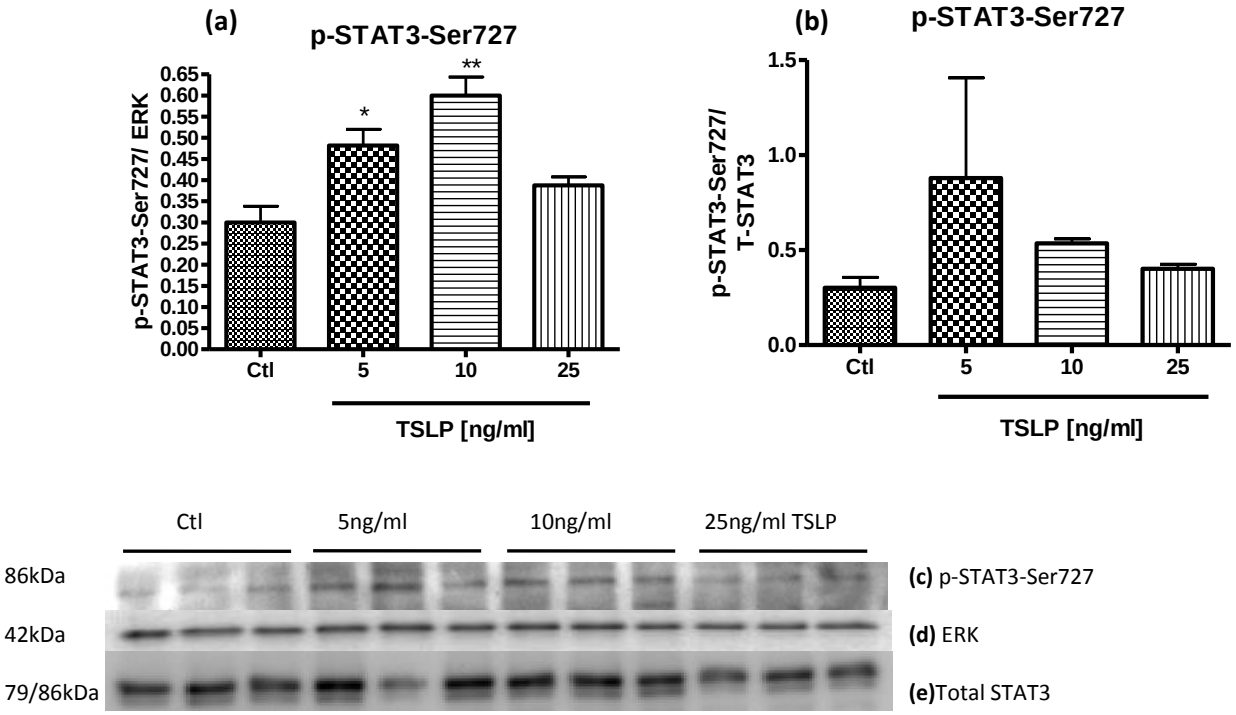

Figure 4 Western blot of adult rat DRG cultured for 24 hours with LDGF and treated for 30 minutes with TSLP at 5, 10 and 25ng/ml. (a) Levels of phospho-STAT3-Ser727 were normalized against housekeeping protein ERK. (b) Phospho-STAT3-Ser727 levels were normalized against total STAT3. (c) Western blot probed for phospho-STAT3-Serine727. (d) Blot reprobed for ERK. (e) Blot reprobed for total STAT3. (n=3 replicates) *p< 0.05 for Control vs 5ng/ml TSLP and **p<0.01 for Control vs. 10 ng/ml TSLP via one-way ANOVA with Dunnett's test

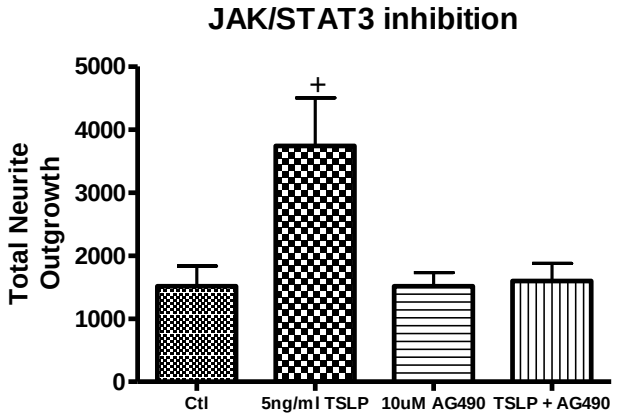

Figure 5 Adult rat DRG sensory neurons treated with TSLP, JAK/STAT signaling pathway inhibitor AG490, and both 5ng/ml TSLP and 10uM AG490 combined, in the presence of LDGF for 24 hours. (n=5 replicates) +p<0.05 for 5ng/ml TSLP compared to all other groups via one-way ANOVA with Tukey's test

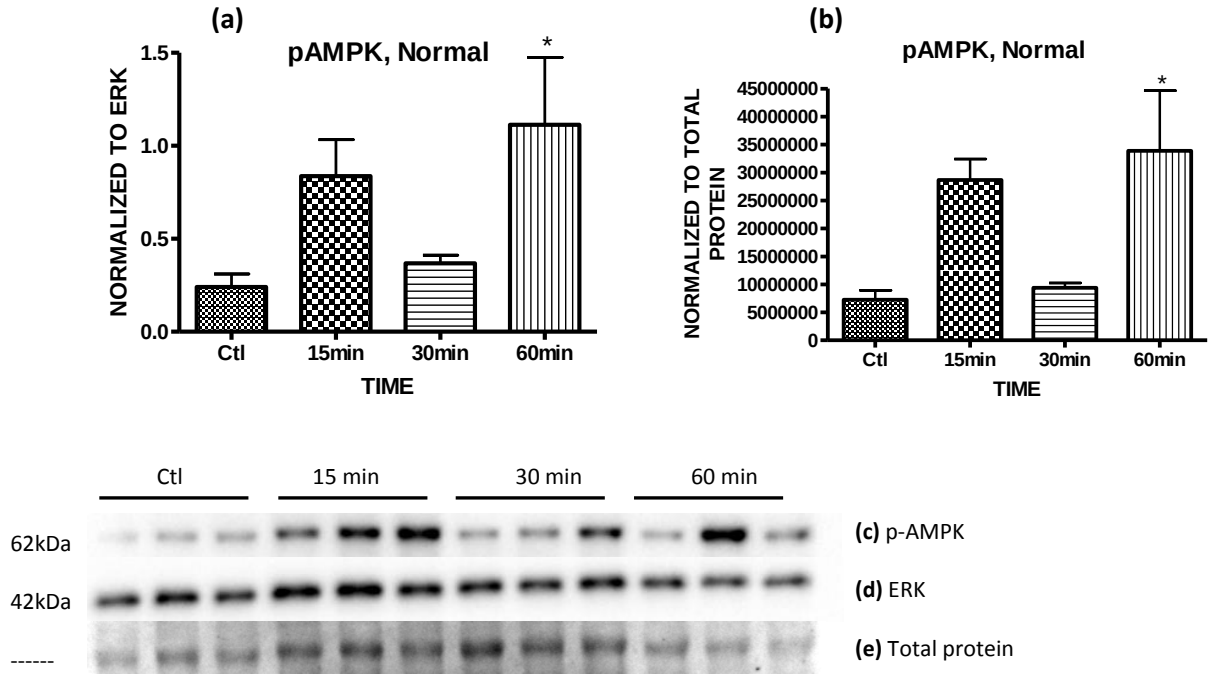

Figure 6 Western blot of adult rat DRG cultured for 24 hours without neurotrophic growth factors; treated for 15, 30, and 60 minutes with 10ng/ml TSLP. (a) phospho-AMPK levels were normalized against housekeeping protein T-ERK (b) Levels of phospho-AMPK were normalized against total protein (c) Western blot probed for phospho-AMPK (d) Blot reprobed for T-ERK. (e) Blot probed for total protein (n=3 replicates) *p< 0.05 for Control vs 60min via one-way ANOVA with Dunnett's test

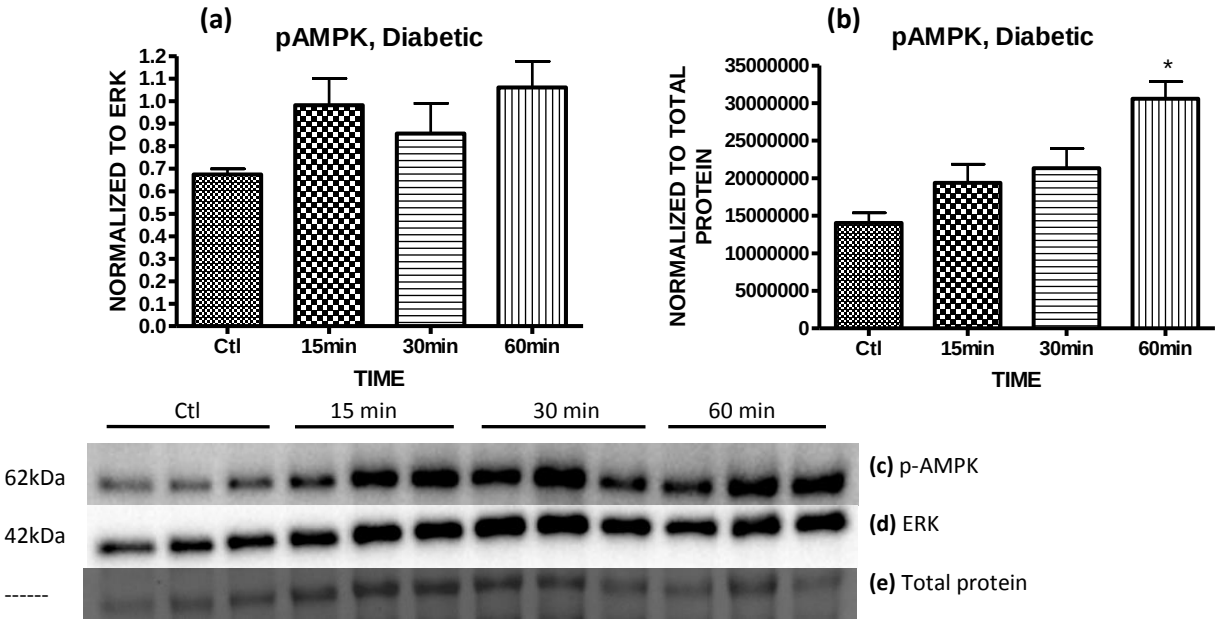

Figure 7 Western blot of diabetic adult rat DRG cultured for 24 hours without neurotrophic growth factors; treated for 15, 30, and 60 minutes with 10ng/ml TSLP. (a) phospho-AMPK levels were normalized against housekeeping protein ERK. (b) Levels of phospho-AMPK were normalized against total protein (c) Western blot probed for phospho-AMPK (d) Blot reprobed for ERK (e) Blot probed for total protein (n=3 replicates) *p< 0.05 for Control vs 60min via one-way ANOVA with Dunnett's test

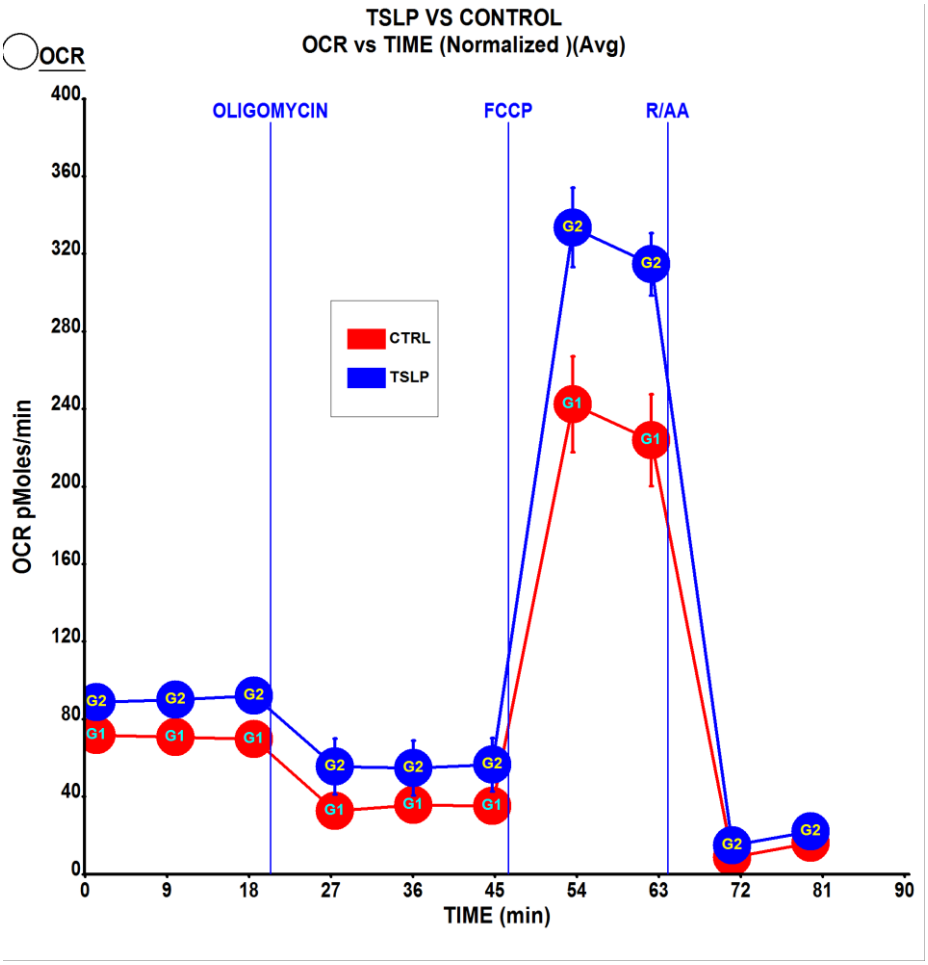

Figure 8 Adult rat DRG sensory neurons cultured overnight in LDGF; control vs 10ng/ml TSLP. Mitochondrial bioenergetics demonstrated under stress. Inhibitors added at staggered timepoints as indicated. (n=4 replicates)

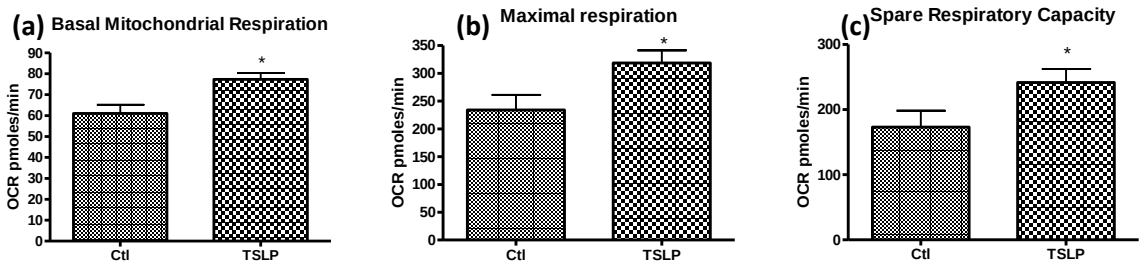

Figure 9 Adult rat DRG sensory neurons cultured overnight in LDGF; control vs 10ng/ml TSLP. (a) Basal respiration corrected for non-mitochondrial respiration (b) Maximal respiration corrected for non-mitochondrial respiration. (c) Spare respiratory capacity = Corrected Maximal respiration – Corrected Basal respiration. (n=4 replicates) *p< 0.05 for Control vs 10ng/ml TSLP via Student's t-test