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Project Title: IGF-1 activates AMPK to regulate mitochondrial fission/fusion proteins in adult rat sensory neurons

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Summary (250 words max single spaced):

Insulin-like growth factor (IGF-1) augments neuronal regeneration in the peripheral nervous system, and this signalling axis is disrupted in diabetic peripheral neuropathy (DPN). Recent work has implicated AMP-activated protein kinase (AMPK) and mitochondrial dysfunction in the etiology. Mitochondrial fission and fusion are processes that regulate the shape, size and movement of mitochondria, and have been implicated in disease. Using cultured rat dorsal root ganglia (DRGs) derived from age-matched control and streptozotocin(STZ)-induced rats, the role of IGF-1 in AMPK activation and mitochondrial dynamics was explored. We predicted that IGF-1 would increase AMPK activation and lead to changes in mitochondrial fission/fusion protein expression. IGF-1 increased AMPK activation in a dose-dependent manner. IGF-1-induced AMPK activation occurred after 1 hour, and remained activated for 24 hours. LKB1, a known upstream activator of AMPK, was also activated in a dose dependent manner. When treated for 24 hours, cultured DRGs elevated the mitochondrial fusion proteins, mitofusin 1 and 2 (Mfn1 and Mfn2). Treatments for 15, 30 and 60 minutes with IGF-1 showed a marked decrease in DRP-1, a protein involved in mitochondrial fission. In order to establish a link between AMPK activation and mitochondrial fusion, cultured DRGs were treated with an AMPK inhibitor, followed by IGF-1 treatment. Raised expression of Mfn1 triggered by IGF-1 was reduced to control levels with AMPK inhibitor treatment. Finally, DRG cultures from STZ-induced diabetic rats exhibited disrupted responses to IGF-1. These results provide evidence that IGF1 stimulates AMPK, influences mitochondrial dynamics through AMPK and that this pathway is disrupted in diabetes.

Student Signature 

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INTRODUCTION & BACKGROUND

Diabetes is one of the most common chronic health condition affecting Canadians today. According to the Public Health Agency of Canada, there are over 2.4 million Canadians with diabetes or prediabetes. This epidemic is also getting worse, with an increase in diagnoses of over 70% between 1999 and 2009 (Public Health Agency of Canada 2011). Diabetes comes with a host of complications including neuropathy of the peripheral and autonomic nervous systems. This neurodegenerative disease is one of the most common complications afflicting people with diabetes. Over 50% of patients with diabetes are expected to develop peripheral neuropathy during their lifetime (Boulton et al. 2005). Diabetic peripheral neuropathy (DPN) presents as a distal, symmetrical polyneuropathy, in what is referred to as a “stocking and glove” distribution. This reflects the propensity of DPN to begin in the most distal portions of the extremities and progress proximally (Edwards et al. 2008). The neuropathy is caused by a dying back of the nerve endings, and can involve both small and large fibres; as such, the symptoms between individuals can vary. Large fiber degradation leads to impaired proprioception and light touch sensation, while small fibre disease impairs pain and temperature perception, causing paresthesias, dysesthesias and neuropathic pain. Additionally, the severity of damage also contributes to symptom presentation, varying from diminished or absent deep-tendon reflexes, to distal weakness. Advanced DPN can lead to serious complications for patients, including ulceration, foot deformity such as “Mallet toes” and neuroarthropathy (Quattrini et al. 2007). The loss of proprioception, sensation and the accumulation of deformities often lead to sores and ulcers going unnoticed for long periods of time, resulting in soft tissue and bone infections, which ultimately end with amputation. Over 60% of non-traumatic amputations in North America are a result of DPN, numbering over 70,000 in 2004 alone (American Diabetes Association 2011). This causes substantial physical and psychological stress on people suffering with diabetes, as well as an immense cost on the healthcare system. For example, the Canadian Diabetes Association estimates that diabetic foot ulcers cost the Province of Ontario up to $400 million in direct health care costs, and up to $50 million in indirect healthcare costs per annum (Canadian Diabetes Association 2016).

The physiology underlying diabetic peripheral neuropathy remains unclear. Although there are multiple etiologies which have been reported to account for the DPN, hyperglycemia is central to all of them. As such, the majority of proposed biochemical pathophysiological mechanisms revolve around glucose metabolic pathways, such as the polyol pathway, the hexosamine pathway, the protein kinase C pathway, the poly ADP-ribose polymerase pathway and the advanced glycation endproducts (AGE) pathway. Broadly speaking, these mechanisms involve the accumulation of glucose leading to its conversion to various reduced states, which result in an increased oxidative stress burden on the cells through the accumulation of reactive oxygen species (ROS). Although each pathway plays a role independently, it was thought that collectively they cause an imbalance in the redox state of the cell. Excess formation of ROS leads to mitochondrial damage and impairment, which has been shown to play a major role in diabetic neuropathy. ROS such as superoxide and hydrogen peroxide are produced by mitochondria under normal conditions via the electron transport chain; however normal cellular detoxification agents such as superoxide dismutase, catalase and glutathione are sufficient in removing them. This model proposes that the hyperglycemia experienced by cells leads to increased mitochondrial activity, raising the overall production of ROS and thus overwhelming these cellular detoxification agents, causing damage to lipids, protein and DNA, finally compromising cell activity and functioning (Vincent et al. 2004).

There are, however, some pressing issues in the oxidative stress theory as it pertains to diabetic neuropathy. For one, the source of ROS is not as clear as once proposed. Although it was once

assumed that the increase in glucose led to an increase in the electron transport chain, and a subsequent increase in ROS generation by the mitochondria, this work was done in endothelial cells under acute conditions (Brownlee 2001). In fact, this mechanism did not extend to chronic hyperglycemia, nor to other cell types: mitochondrial respiration and enzymatic activities were found to be reduced in diabetic rat hearts, as well as in the skeletal muscle of patients with type 2 diabetes (Lashin et al. 2006; Patti et al. 2003; Bugger & Abel 2009). This work has been corroborated in the dorsal root ganglia of sensory neurons and nephrons in type 1 and type 2 diabetic rodent models (Dugan et al. 2013; Akude et al. 2011; Roy Chowdhury et al. 2010; Zhang et al. 2011). Not only do diabetic neurons experience decreased mitochondrial function, this can be corrected through the administration of neurotrophic hormones or insulin, independent of hyperglycemia (Huang et al. 2003). Moreover, a key feature of peripheral nerves is their ability to regenerate, which is lost under diabetic conditions (Polydefkis & Griffin 2017). The inability to regenerate nerve fibers is related to the degree of neuropathy, and does not appear to involve the oxidative stress pathway. Finally, it does not explain the selective nature and length dependent pattern of neuropathy found in diabetic individuals. Therefore, although oxidative stress may play a role in the metabolic syndrome experienced by cells under diabetic conditions, its role in DPN still remains controversial and it is clear that other models are required to explain the pathophysiology behind DPN.

Recent work has shifted towards the mitochondria as a key player in understanding the underlying pathophysiology. The innervation of distal dermal and epidermal tissue is maintained through constant remodelling, sprouting and regeneration of dermal and epidermal nerve fibers (Diamond et al. 1992). In order to maintain this plasticity, neurons require high energy consumption, which can only be driven by the high ATP production capabilities of mitochondria (Fernyhough, 2015). Animal models suggest that mitochondrial function is not able to cope with energy demands in diabetes. Mitochondria in diabetic individuals have structural abnormalities. Schwann cells of myelinated and unmyelinated axons in the sural nerves of persons with diabetes showed degenerative and proliferative changes in mitochondrial number and size when exposed to hyperglycemic environments (Kalichman et al. 1998). Additionally, individuals with neuropathy have aberrant mitochondria in skin fibres (Casanova-Molla et al. 2012). In the prevertebral sympathetic ganglia of mice treated with streptozotocin (used to model type 1 diabetes), mitochondria appear to be significantly smaller and hyperchromatically denser than controls. Finally, intraepidermal axons, those responsible for innervating the epidermis within the skin, appear to accumulate mitochondria, vesicles and neurofilaments along their axons, contributing to axonal swelling (Lauria et al. 2003). These morphological differences appear to come with physiological differences as well. In both rat and mice models of both type 1 and type 2 diabetes, there is mitochondrial respiratory chain dysfunction recorded in their dorsal root ganglia (DRG) (Roy Chowdhury et al. 2010). Additionally, there is a consistent down regulation of proteins involved in the mitochondrial complexes (Roy Chowdhury, 2011) Although there is a clear down regulation of mitochondrial function in diabetic neurons, the link between this dysfunction and clinically relevant diabetic neuropathy still remains opaque.

Hereditary diseases of the peripheral nervous system may, however, provide some clarity. Specifically, Charcot-Marie-Tooth disease (CMT). CMT is a common inherited disorder of the peripheral nerves, whose symptoms include slowly progressive distal weakness, muscle atrophy and sensory loss that parallels that of diabetic neuropathy (Prior et al. 2017). There are several different subtypes of CMT, but CMT2A shares many similarities with DPN, from not only the nature of the neuropathy itself, but its length dependant distribution. The most common mutations identified in CMT2A are point mutations in the MFN2 gene, coding for mitofusin 2 (Misko, 2010). Mitofusin 2, along with its partner Mitofusin 1, are outer mitochondrial membrane proteins that

mediate the attachment of mitochondria to molecular motors. They play an integral role in mitochondrial fusion (Youle et al. 2012).

Because of their central role in cellular bioenergetics, mitochondria must be capable of responding to increased cellular energy demands, especially in neurons. Mitochondrial fusion and fission are essential to this process. Fusion is the process of joining two otherwise separate mitochondria together. This occurs when there is an increased cellular demand, and results in increased oxidative phosphorylation (Fang et al. 2016). Additionally, fusion events can occur between dysfunctional mitochondria and healthy mitochondria, enabling the later to compensate for the former. Although the mechanism and role of mitochondrial fusion is not completely known, interruption of mitochondrial fusion leads to a loss of mitochondrial potential and diminished oxidative phosphorylation (Olichon et al. 2003). Mitochondrial fission is mediated by a cystolic dynamin family protein, DRP1. DRP1 forms spirals around the mitochondria which constrict and cut both the inner and outer membrane. Fission is essential for growing and dividing cells – when cells divide, their mitochondria must be divided up between them. However, it has been unclear as to what role mitochondrial fission plays in non-proliferating cells, although it is evident that the mechanisms are active within them, as neurons cannot survive without mitochondrial fission (Youle et al. 2012). Rates of mitochondrial fission and fusion appear to depend on cellular metabolism. In situations with low glucose in which cells must rely on oxidative phosphorylation, mitochondria fusion is favoured (Rossignol et al. 2004). Additionally, fusion can be used as a protective mechanism to repair mitochondria damaged by ROS. Finally, autophagy, a mechanism utilized by cells to degrade cellular components such as protein aggregates, essential for the maintenance of a healthy mitochondrial network, utilizes mitochondrial fusion and fission process (Youle et al. 2012)

In order to elucidate the link between mitochondrial functioning and the hyperglycemia experienced by neurons under diabetic conditions, another player must be introduced. Adenosine monophosphate-activated protein kinase (AMPK) is an enzyme involved in the control of mitochondrial biogenesis and functioning, and is key to maintaining healthy mitochondria (Egan et al. 2011). AMPK is a Ser/Thr kinase, often referred to as a “master regulator” of cellular metabolism. AMPK is activated through sensing the AMP to ATP ratio within cells: when ATP levels are low (and thus AMP concentrations high), AMP binds to AMPK, facilitating its activation. Once activated, this protein phosphorylates enzymes necessary for catabolism, through the phosphorylation of PGC-1α, a coactivator of several transcription factors, influencing the energy expenditure in of the cell (Feige, 2007). AMPK is essential for the cellular balance between catabolism and anabolism (Hardie, 2008). AMPK has also been implicated directly in diabetes. AMPK signaling is depressed in DRG sensory neurons in both rats and mice with type 1 and type 2 diabetes. AMPK is also involved in physiological relevant ways: direct the blockade of the AMPK pathway with the use of dominant negative mutants results in impaired neurite outgrowth. Meanwhile, the activation of AMPK with resveratrol, a drug used to enhance AMPK phosphorylation, results in normalization of thermal sensitivity and in increase in intraepidermal nerve fiber density within the feet of diabetic mice (Roy Chowdhury et al. 2012).

Growth factors are essential in promoting growth & survival in neurons (Zochodne 2016). In vivo, Schwann cells and target tissues are responsible for providing these growth factors, however damage to Schwann cells and target delivery in diabetic neuropathy has lead to their implication in disease. In diabetic models, levels of nerve growth factor (NGF) were reduced under diabetic conditions, but returned to normal once normal glucose levels were restored, pointing to possible regulation via hyperglycemia or loss of insulin (Hellweg et al. 1994). Along with NGF, levels of insulin-like growth factor-1 (IGF-1) decrease with diabetes. IGF-1 is a polypeptide growth factor primarily produced and secreted by the liver. This hormone has many proposed physiological

functions, the best known being its role in growth during development. However, CNS tissue appears to retain widespread distribution of its receptors (IGF-1 receptors). IGF-1 has a distinct role in neurons, and is involved in neuronal survival, axonal outgrowth, differentiation and the maintenance of synaptic connections (Recio-Pinto, 1988). The IGF-1 receptor is a heterotetrameric protein that possesses the ability to autophosphorylate through its intracellular tyrosine kinase domain (De Meyts & Whittaker 2002). IGF-1 has been postulated as a potential treatment for various neuropathies. Subcutaneous administration of IGF-1 enhances functional recovery in motor neuropathies (Lewis et al. 1993). Additionally, IGF-1 acts directly on sensory neurons, stimulating their regeneration in a dose-dependant manner (Recio-Pinto, 1988). Although its receptor and effects have been well characterized, IGF-1's mechanism of action, particularly in neurons, is not well known.

We sought to investigate the role of AMPK and mitochondrial fission/fusion in the regulation of mitochondrial function and the possible role of IGF-1 in the modulation of these pathways. Based on work in other cell types, we hypothesised that IGF-1 would increase AMPK activation, as well as have a subsequent effect on mitochondrial fission/fusion. Dorsal root ganglia were cultured from adult control and streptozotocin-diabetic rats with/without IGF-1 in order to stimulate neurite outgrowth. Then, using quantitative Western blotting, we determined that IGF-1 stimulated sensory neurons through activation of the AMPK pathways. Furthermore, IGF-1 stimulated mitochondrial fusion through the recruitment of MFN1 and MFN2, while inhibiting DRP1. With the use of neurons derived from streptozotocin-diabetic rats and cultured under hyperglycemic conditions, we found evidence that IGF-1 was able to stimulate the mitochondrial fusion proteins, but at higher doses than in control. This work contributes to a growing body of data suggesting that aberrant mitochondrial fusion and fission are major components contributing to the pathophysiology of diabetic neuropathy, and suggests that IGF-1 may play a role in both its modulation and eventual therapeutic treatment.

MATERIALS AND METHODS

Induction, treatment and confirmation of diabetes

Male Sprague-Dawley rats (275-325 g) were used as models of type 1 diabetes after delivery of a single intraperitoneal injection of 75-85 mg/kg of streptozotocin (Sigma). Age match control animals were injected with saline. Animals were euthanized and tissue collected after 30 weeks with diabetes**.** Before euthanasia, existence of neuropathy was confirmed using a thermal sensitivity detector (these animals exhibit sensory loss and so depression of thermal sensation in the hind pay). Non-fasting blood glucose concentrations were measured using a AlphaTRAK glucometer (Abbott) and all diabetic rats exhibited greater than 30 mM glucose in plasma. Animal procedures followed guidelines laid out by the University of Manitoba Animal Care Committee using the Canadian Council of Animal Care guidelines.

Adult rat dorsal root ganglia sensory neuron culture

Sensory neurons were isolated and dissociated from the DRG of adult male Sprague-Dawley rats. Cervical, thoracic and lumbar DRGs (45-50/ animal) were dissected aseptically and collected in F-12 growth medium. Ganglia were cleaned of roots and capsular connective tissue. Cleaned DRGs were enzymatically treated with collagenase and trypsin at 37°C, twice for 1 hour with 0.125% collagenase, washed, and once with 0.25% trypsin in F-12 growth media for 25 minutes. A single-cell suspension was then obtained through trituration of the enzymatically softened ganglia by 20-30 passes through the tip of a fire-polished, siliconized Pasteur pipette. Cells were run through a 70 μm cell strainer, then centrifuged at 500 rpm for 5 minutes, whereupon viable neurons were lightly pelleted leaving myelin debris, dead cells and small non-neuronal cells in

suspension. This was followed by a BSA column to further purify the neuronal population and a 900 rpm centrifugation for 10 minutes. Cells were then plated on culture dishes previously coated with polyornithine (500 ug/ml) and laminin (5 ug/ml). Cells were plated in F12:N2, a media used to promote growth in primary cell culture of both post-mitotic peripheral and central nervous system neurons. containing 10 mM of glucose, with the appropriate IGF-1 (Sigma Aldrich E. Coli derived human recombinant) or insulin treatments.

Western Blotting

Following treatment, F12:N2 media was removed, and cultured sensory neurons were harvested and homogenized in ice-cold stabilization buffer (neurofilament buffer) containing: 0.1 M PIPES, 5 mM $MgCl_2$, 5 mM EGTA, 0.5% Triton X-100, 20% glycerol, 10 mM NaF, 1 mM PMSF and protease inhibitor cocktail (Fernyhough et al. 1999).The protein content was measured using the Bradford method (1976).

DRG lysates, 5μg of total protein, from cell culture were resolved on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel and electroblotted onto nitrocellulose membrane. Blots were then blocked in 5% non-fat milk containing 0.05% Tween-20, and rinsed in tris-buffered saline with 0.05% Tween-20.

Blots were incubated with antibodies for the following proteins: phosphorylated AMPK (on Thr 172, P-AMPK; 1:500 Santa Cruz Biotechnology Inc), total AMPK (T-AMPK; 1:500, Cell Signaling Technology), phosphorylated LKB1 (p-LKBI,1:500 Santa Cruz Biotechnology Inc), phosphorylated Akt (p-AKT), Mfn1 (1:500, Santa Cruz Biotechnology Inc), Mfn2 (1:500, Santa Cruz Biotechnology Inc), DRP1 (1:500, Santa Cruz Biotechnology Inc) and phosphorylated DRP1 (1:500, Cell Signaling Technology). Total protein bands were captured by chemiluminescent imaging of the blot after gel activation (TGX Stain-Free™ FastCast Acrylamide Solutions, Bio-Rad, CA, USA), in addition to use of ERK for target protein normalization. The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (H+L) or donkey anit-mouse IgG (H+L) from Jackson ImmunoResearch Laboratories, PA, USA. The blots were incubated in ECL Advance (GE Healthcare) and imaged using a Bio-Rad ChemiDoc image analyzer (Bio-Rad)

Statistical Analysis

Data was analyzed using one-way ANOVA followed by Dunnett's or Tukey's post hoc tests, as appropriate and indicated (GraphPad Prism 4, GraphPad Softward). A P value <0.05 was considered to be significant.

RESULTS

IGF-1 Treatments Increase AMPK Phosphorylation

In order to evaluate AMPK activation, control rat DRGs were cultured in F12:N2 solutions containing IGF-1 varying from 1-100 nM; 1-10 nM interacts only with the IGF-1 type 1 receptors, whereas at 100nM, there may be crossover with the insulin receptors (Fernyhough et al. 1993). Following treatment, the cells were lysed, prepared for Western Blot and probed for phosphorylated AMPK Thr^{172}, the activation loop of its catalytic α subunit (Hardie et al. 1999). Protein levels of total AMPK were also measured, in order to ensure that any change in pAMPK could not be attributed to an increase in AMPK expression. All data was normalized to T-ERK, since this protein does not change under stimulatory conditions, and is thus used as a loading control. We found significant increases in control rat DRGs treated with 1, 10 and 100 nM concentrations of IGF-1 for 24 hours (Figure 1). There were no reciprocal significant differences in total AMPK levels (Figure 2).

IGF-1 Treatments Increase Phosphorylation of LKB1, Upstream AMPK activator

LKB1 has been implicated as an upstream kinase of AMPK, leading to its activation (Hong et al. 2003). Phosphorylated LKB-1 Ser^{428} has been linked to AMPK activation (Xie et al. 2008). Meanwhile, Akt, also an upstream kinase to AMPK, has been shown to be an AMPK inhibitor. Akt itself is phosphorylated on its S^{473} site, it becomes active (P. Liu et al. 2014). In order to evaluate the upstream influences of IGF-1 on its AMPK activity, both phosphorylated LBK1 and phosphorylated Akt were probed. Control rat DRGs were cultured in 1, 10 and 100 nM concentrations of IGF-1 for 24 hours, and their lysates were probed for Akt and phosphorylated LBK1. There was a significant increase in pLBK1 in the 100 nM treatment group, with the others trending up as well (Figure 3). Interestingly p-Akt was not shown to increase with treatments, with no significant differences noted between groups (Figure 4). Akt signaling through IGF-1 had previously been reported in human embryonic kidney cells, albeit with significantly shorter treatments (Hawley et al. 2014).

IGF-1 Elicits Quick and Sustained AMPK Activation

A time course treatment was done on cultured control rat DRGs. DRGs were treated with 10 nM solutions of IGF-1 for 30 minutes, 1 hour and 6 hours before being harvested lysed and probed for pAMPK Thr^{172}. A significant increase in pAMPK was found after 1 hour of treatment, and persisted up to 6 hours (Figure 5). This corresponds with the sustained increase found at 24 hours in previous blots (see above).

IGF-1 Influences Expression of Mitochondrial Fusion and Fission Proteins

Mitochondrial dysfunction plays an important role in diabetes, and recent work has pointed to mitochondrial fusion and fission as being important regulators of mitochondrial viability (Fang et al. 2016). Furthermore, AMPK activation has been shown to influence mitochondrial dysfunction in sensory neurons (Roy Chowdhury et al. 2012). We sought to investigate if IGF-1 affected expression of mitochondrial fission/fusion proteins. Mfn1 and 2 are mitochondrial fusion proteins implicated in a group of hereditary polyneuropathies known as Charcot-Marie-Tooth Disease, while DRP1 is a fusion protein implicated in apoptosis (Youle et al. 2012).

Cultured control rat DRGs were treated with 10 nm of IGF-1 for various time points up to 24 hours, and Mfn1, Mfn2 and DRP1 levels were quantified through Western Blotting. At 15, 30 and 60 minutes, there was no significant differences found in Mfn1 against control (Figure 6). However, Mfn2 saw a significant increase at 1 hour, but none at 15 minutes nor 30 minutes (Figure 7). There were significant differences found between untreated and 15, 30 and 60-minute treatment groups in DRP1, all of which showed much significant decreases in DRP1 protein levels (Figure 8).

Control rat DRGs were cultured in 10 nM IGF-1 solutions for 3, 6 and 24 hours. We found no significant differences between control and 3 or 6 hour treatments in Mfn1, however a significant difference at 24 hours (Figure 9). A similar trend was observed with Mfn2 (Figure 10). Of note, a significant difference was not found at the 3-hour treatment, however one was noted at 1 hour in a previous blot (see above). DRP1 showed no significant differences between the 3, 6 and 24-hour treatment groups (Figure 11).

Link Between IGF-1 AMPK activation and IGF-1 Mitofusin Increase

In order to investigate the link between IGF-1 activation of AMPK and the increase in Mfn1 and Mfn2, an AMPK inhibitor was utilized. Compound C (6-[4-(2-Piperidin-1-ylenthoxy) phenyl]-3-pyridin-4-ylpyrazole [1,5-a]pyrimidine) is a potent cell-permeable AMPK inhibitor (X. Liu et al. 2014). We incubated cells with Compound C, followed by treatment with 10 nM of IGF-1 for 24 hours. We found a significant difference between control and 10 nM IGF-1 treated DRGs in Mfn1,

replicating an earlier finding. There was no significant difference between the control and Compound C only group, although there was an upward trend noted in the Compound C only group. There was a significant increase in the IGF-1 treatment group and the IGF-1 + Compound C treatment group. There was no difference between the Compound C group and the IGF-1 + Compound C group (Figure 12). There were no significant differences between Mfn2 in any of the groups (not shown). Blots were normalized to total lane protein rather than ERK-1, due to disruption in the ERK-1 pathway with Compound C.

Diabetes Disrupts IGF-1 Induced Mitofusin Increase

Streptozotocin is utilized to induce diabetes in rats, and is a prevalent model utilized in the study of diabetic neuropathy (Yorek 2016). A dose of streptozotocin it administered to adult rats, destroying their β islet cells and inducing insulin-deficient diabetes (Rees & Alcolado 2005). Streptozotocin-diabetic rats DRGs were cultured in 10 and 100 nM concentrations of IGF-1 for 24 hours. When blotted for Mfn1, no significant differences were found between untreated and treated groups (Figure 13). However, there was a significant differences noted between the 10 nM and 100 nM treatment groups, with 10 nM having a higher signal. There was a significant difference between untreated and the 100 nM treatment group in Mfn2 (Figure 14). Streptozotocin-diabetic rats DRGs were also cultured in 10 and 100 nM insulin for 24 hours, in order to compare the results to those found in the IGF-1 treatment groups. No significant differences were found between treatment groups and controls, in either Mfn1 or Mfn2 (Figure 15 and 16). Blots were normalized to total lane protein rather than ERK-1, due to disruption in the ERK-1 pathway in diabetic animals.

DISCUSSION

The findings in this study indicate that IGF-1 activates AMPK in a dose dependent manner in rat dorsal root ganglia, providing evidence that AMPK is likely involved in downstream growth pathways in neurons. Evidence for a link between IGF-1 and mitochondrial dynamics was also provided, specifically in relation to fission and fusion. Although the results are preliminary, they help shed light on how IGF-1 works in sensory neurons, as well as provides a connection between AMPK activation and mitochondrial dynamics, helping elucidate a possible pathway from IGF-1 receptor to structural and functional changes in sensory neurons.

There was a significant increase in phosphorylated AMPK at the Thr172 site after 24-hour treatments with 1nm, 10 nM and 100 nM concentrations of IGF1. Additionally, an effect was seen as early as 1 hour after treatment. These results suggest a potent and sustained increase in activated AMPK. Previous work has shown that similar concentrations of IGF-1 lead to enhanced neurite outgrowth in cultured DRGs (Fernyhough, 1993). This work provides evidence that IGF-1's effect on neurite outgrowth may be mediated through the stimulation of AMPK.

AMPK is regulated in several different ways. AMPK is a heterotrimer, consisting of three different subunits: one catalytic α subunit, a regulatory β subunit and a γ subunits. Binding of AMP to the γ subunit activates AMPK through the promotion of phosphorylation at its Thr^{172} site. Some cells have the additional ability to activate AMPK through direct phosphorylation via regulatory kinases (Hardie 2008). The mechanism through which IGF-1 stimulates AMPK was investigated through the probing of two of its known regulators, LKB1 and Akt. LKB1 is a well known activator of AMPK (Shackelford, 2009). In fact, LKB1's activation through phosphorylation at Ser^{429} has been linked to the activity of metformin, a drug commonly used to combat diabetes (Xie, 2008). Akt, on the other hand, has been shown to inhibit AMPK activity through the phosphorylation of Ser^{487}, also referred to as the "ST loop". Akt itself is activated through mTOR by phosphorylation at its S^{473}

site (Liu, 2014). The data revealed a steady increase in phosphorylated LKB1 Ser^{429}, however it was only significant in 100 nM IGF-1 treatment group, despite the general upward trend towards significance. A comparison between total LKB-1 and pLKB-1 may be more sensitive in detecting a change at 1 or 10 nM of IGF-1. pAkt levels remained stable throughout all treatment groups, suggesting that Akt does not play a role in IGF-1 signaling in sensory neurons at these time points. This is contrary to other work that found IGF-1 signals through Akt, and decreased AMPK Thr^{172} activation in human embryonic kidney cells, although this work was done using short term treatments of IGF-2 (Hawley et al. 2014). This discrepancy may be due to the conditions and cell types used: neuronal cells may simply utilize unique IGF-1 signaling pathways. One consequence of AMPK inhibition in non-neuronal cells is the increased usage of the mTOR pathway, shifting the cell to anabolic actions such as lipogenesis and glycogen synthesis, pathways not highly represented in neuronal cells. Moreover, this pathway had been studied primarily in a human embryonic kidney cell line (HEK-293), whilst under serum starvation. This experiment was done on cultured rat DRG, with glucose enriched media. We contend that this medium is much more conducive and realist to the study of diabetes, particularly in terms of AMPK, as it is immensely sensitive to cell starvation.

We sought to link IGF-1 to mitochondrial dynamics in cultured rat DRGs by probing for changes in mitochondrial fission and fusion proteins after treatment with 10 nM IGF-1 solutions at various time points. A time course experiment of less than 1 hour found no differences in mitochondrial fusion proteins levels, Mfn1, although a significant difference in Mfn2 at 1 hour was noted, although that effect was not noted at 3 hours. This may be due to a false positive, or possibly fluctuations in Mfn2 levels due to some unknown mechanism. There was however a significant decrease in DRP1 with 10 nM treatments of IGF-1 for 15, 30 and 60 minutes. This suggests that IGF-1 may play a role in modulating DRP1. DRP1 is involved in mitochondrial fission, essentially breaking up large mitochondria into smaller functional mitochondria. Reduction in DRP1 increases mitochondrial connectivity and fusion (Kageyama et al. 2012). Moreover, mitochondrial length can be significantly increased through knockdown or interference of DRP1 in neuronal cell (Uo et al. 2010). The decrease found in DRP1 may then indicate a trend towards mitochondrial fusion, which has been proposed to help mitochondria in times of increased cellular demand of oxidative phosphorylation (Fang et al. 2016). This all suggests that IGF-1 causes a decrease in mitochondrial fission, discouraging the production of smaller, fragmented mitochondria. The mechanism through which this is facilitated is not clear. The quick timing suggest that this change is not at the transcriptional level. DRP1 has several different post-translational regulators, that act through phosphorylation, SUMOylation and ubiquitination (Singh & Sharma 2017). One plausible explanation for the precipitous decrease in DRP1 by IGF-1 stimulation would be the later. Ubiquitination is a post-translational process utilized by cells to degrade proteins, and plays a regulatory role in mitochondrial integrity (Iglewski et al. 2011). The details of posttranslational modifications of DRP1 and its effects on fission are not yet well understood, and pursuit of how this process works in neurons and its impact on disease would be an obvious next step.

An extended time course treatment with 10 nM of IGF-1 solution of control rat DRGs for 3, 6 and 24 hours yielded no significant differences in DRP1 expression. There were however, significant increases in both Mfn1 and Mfn2 at 24 hours. The length of time suggests a transcriptional up regulation of Mfn1/2 proteins due to IGF-1 treatment. However, in order to link AMPK and the mitofusin proteins, AMPK was inhibited with the use of Compound C. The data suggested that when AMPK was inhibited, the rise in Mfn1 did not occur. This is a promising sign that Mfn1 may be regulated by AMPK. However, this result must be tempered with some possible caveats. First, there was no difference found in Mfn2, despite the previously reported increase in Mfn2 at 24 hours. This discrepancy would best be solved through replication of both experiments, preferably with larger groups. Second, although Compound C has been used extensively in the literature as

a AMPK inhibitor, its mechanism is not well understood. It is not believed to inhibit AMPK directly, but rather effect several pathways simultaneously; as such it is a difficult inhibitor to work with, and results should be embraced with caution (Bai et al. 2016). Although no other AMPK inhibitor exists, AMPK stimulators such as resveratrol may help bolster the link between Mfn1 and AMPK (Dasgupta & Milbrandt 2007). However, on its face, this data does suggest a link between activated AMPK and Mfn1, and the possibility that Mfn2 works through a separate mechanism than Mfn1. Mfn1 and Mfn2 are highly homologous proteins: however there is evidence that the two may diverge in function: Mfn1 has been shown to be more effective in tethering mitochondrial membranes and may function alone to fuse mitochondrial membranes with Opa1; additionally, Mfn2 alone may play a role in mitochondrial transport as well as fusion (Ishihara, 2004; Misko et al. 2010).

Streptozotocin-induced diabetic rat DRGs were treated with 10 and 100 nM of IGF-1 and insulin respectively. Streptozotocin is utilized to induce diabetes in rodents, and is a prevalent model utilized in the study of diabetic neuropathy (Yorek 2016). A dose of streptozotocin is administered to adult rodents, destroying their β islet cells and inducing insulin-deficient diabetes (Rees, 2005). There were no differences in Mfn1 or Mfn2 with insulin treatments. With IGF-1 treatments, there was no significant difference in untreated vs treated groups, but a significant difference between the 10 nM IGF-1 group and the 100 nM IGF-1 group. This suggests that IGF-1 does stimulate Mfn1 in diabetic neurons, however more replications would be required to make a definitive conclusion. Additionally, there was a significant difference found in Mfn2 between control and 100 nM groups. Together, these results suggest that the pathways involving mitochondrial dynamics are still present in diabetes, but altered. Moreover, insulin was unable to stimulate either mitofusin, suggesting that IGF-1 plays a unique role in mitochondrial dynamics in diabetes. Higher concentrations of IGF-1 were required to stimulate the mitofusins. The cause and exact nature of the discrepancy between control and diabetic rats is currently unknown. It is possible that IGF-1 receptors are down regulated under diabetic conditions, resulting in a blunted response, or that the response was temporally different to the control animals, and thus could not be captured at 24 hours. It is clear however that diabetes does alter cellular response to IGF-1 with regard to mitochondrial dynamics. The discrepancy between the Mfn1 and Mfn2 results in the diabetic DRG cultures underscores the possibility that these proteins are regulated differently, but that IGF-1 affects both. There are however, some issues that must be pointed out. The first is the normalization method used in this analysis: ERK-1, generally known as a housekeeping protein, had been used loading control for all other analysis in this study, except for the diabetic animals and those treated with Compound C. The reason for this discrepancy stems from a significant, systematic change noted in ERK-1 levels in these animals. The blots were normalized to total protein as an alternative (Aldridge et al. 2008). It is possible that ERK-1 is involved in the IGF-1 or AMPK pathways, and thus future experiments might benefit with the use of other loading controls, such as β-actin. Overall, however, this data does provide evidence that IGF-1 modulates mitochondrial dynamics in diabetes.

This work fits into a larger growing body of research that highlights the importance of AMPK in diabetes. AMPK functions to balance catabolism and anabolism within cells: when there are sufficient nutrients, AMPK is inhibited, and anabolic processes such as protein, glycogen and lipid synthesis are activated, primarily through up regulation of the mTOR pathway. Conversely, under starvation conditions, the AMPK pathway is activated, promoting catabolism, leading to increased mitochondrial oxidative phosphorylation, in order to optimize ATP production (Hardie et al. 1999). The picture, however, becomes more complicated under a different form of nutrient stress, in the form of excess nutrients. Glucose is the primary energy source for sensory neurons; mitochondria provide the bulk of its ATP, with glycolysis providing a small amount. OXPHOS and glycolysis are minor contributors, while there is no oxidation of fatty acids present in neurons (Belanger et al.

2011). Glucose uptake in neurons is independent of insulin, utilizing the GLUT 1/3 transporters (Simpson et al. 2007). It is postulated that high intracellular glucose suppresses AMPK, and particularly in chronic hyperglycemic conditions such as in diabetes, cells begin to rely primarily on glycolysis for energy production, and down regulate mitochondrial proteins, thus mitochondrial functioning. This phenomenon has been documented in rat DRGs: both mitochondrial proteins and functioning were down regulated in streptozotocin-diabetic rat DRGs under hyperglycemic conditions. There was also a corresponding functional consequence: neurite outgrowth was reduced, as well as thermal hypoalgesia and skin intraepidermal nerve fibre loss in *in vivo* mice models (Roy Chowdhury et al. 2012)

Remodeling is essential in the terminal ends of peripheral neurons because of their demand to undergo constant growth. IGF-1 and insulin play a trophic role in neurons outside of metabolism. IGF-1 has been shown to increase neurite outgrowth in cultured adult rat sensory neurons (Recio-Pinto & Ichii 1988). Phosphorylated AMPK has been linked to mitochondrial function, and subsequently an increase in neurite outgrowth. However, a definitive link between AMPK activation and IGF-1 has not been documented in neurons. What this work suggests is the possibility that IGF-1 may reverse the effects of AMPK inhibition in diabetes, or conversely, that the lack of IGF-1 signalling under diabetic conditions may also contribute to AMPK down regulation. It also speaks to a possible mechanism that may help elucidate the pattern of neuropathy inherent to diabetes and the similarity it shares with hereditary diseases of the peripheral system. Hyperglycemia, lack of trophic factors, or both lead to dysfunction in AMPK, which results in mitochondrial dysfunction, quite possibly through disruption of fusion and fission processes. This effect is two fold – neurons are unable to generate sufficient levels of ATP to complete ATP reliant tasks, such as the constant growth and remodelling necessary for maintenance of dermal and epidermal innervation. Additionally, mitochondria are unable to properly fragment and reassemble, making shuttling mitochondria down to the free nerve ending at the periphery, where they are required, more difficult. Approximately half of ATP consumption in neurons occurs at the tip of the axon, an area known as the growth cone, in order to maintain plasticity and motility (Bernstein, 2003). If mitochondria are unable to translocate there, neurons would not be able to maintain the growth cone. Mitofusins may also play a direct role in this process: Mfn2 has been shown to have direct influence over mitochondrial shuttling, as well as its role in fusion (Misko et al. 2010). This shuttling and metabolic dysfunction may then contribute to length dependent axonal degeneration. Moreover, mitochondrial fission/fusion fits nicely with oxidative stress models of diabetes as well, as mitochondria fragmentation has been associated with excessive generation of reactive oxygen species and mitochondrial death (Brooks et al. 2009). Although speculative, elucidating the link between AMPK and mitochondrial dynamics is crucial to developing a robust understanding of the aberrant pathway at work in diabetes.

This work is preliminary and supplements a larger body of work surrounding IGF-1 and mitochondrial functioning; however, it does suggest that IGF-1 stimulates AMPK activation in sensory neurons, the first line victims of neuropathy in diabetes. Furthermore, it implicates mitochondrial dynamics in IGF-1's mechanism of action, specifically through the regulation of mitochondrial fusion and fission pathways. Future work would be aimed at visualizing the mitochondria after IGF-1 treatment using confocal microscopy: this would provide real time data on mitochondrial morphology and localization. The applications of this work are potentially wide reaching: IGF-1 receptors may become useful targets in future drug design, and targeting the IGF-1 pathway may lead to development of new pharmaceutical options for those suffering from diabetic neuropathy.

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Type II i

TNF & JNK insulin receptor: type II diabetes (skeletal muscle) high TNF, activated JAK IRS phos non responsive insulin -> insulin insensitivity in TYPE II. Evidence of

inflammation in type I, JNK activated (fernhough 1999) in neurons, stress activated neurons. Insensitivty pathway.

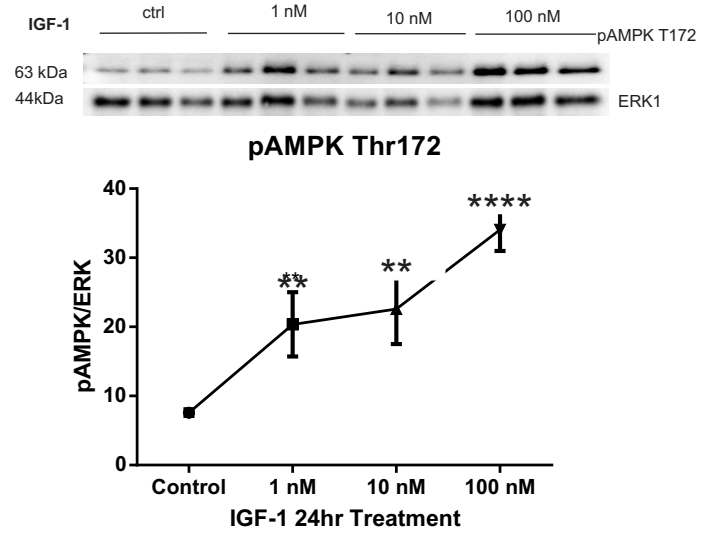

Figure 1: IGF-1 (1, 10 and 100 nM) increased the expression of pAMPK Thr172 in control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM, *P<0.05 ; **P<0.01 ; ***P<0.001 ; ****P<0.0001).

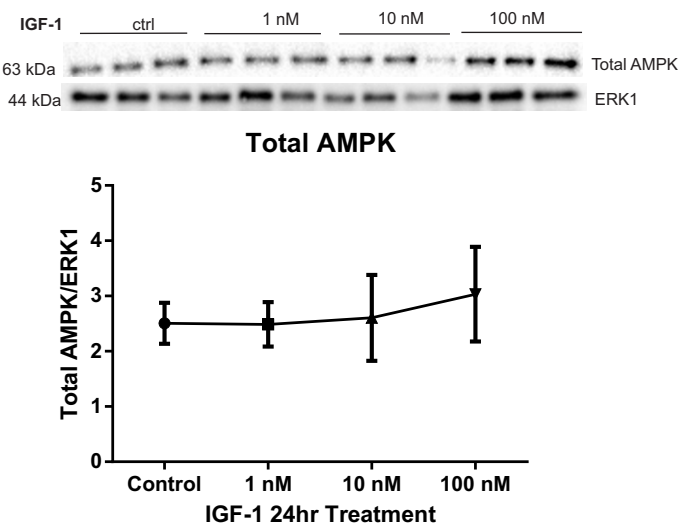

Figure 2: No significant difference in expression of Total AMPK with the 24 hr IGF-1 treatment (1, 10 and 100 nM) of control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM).

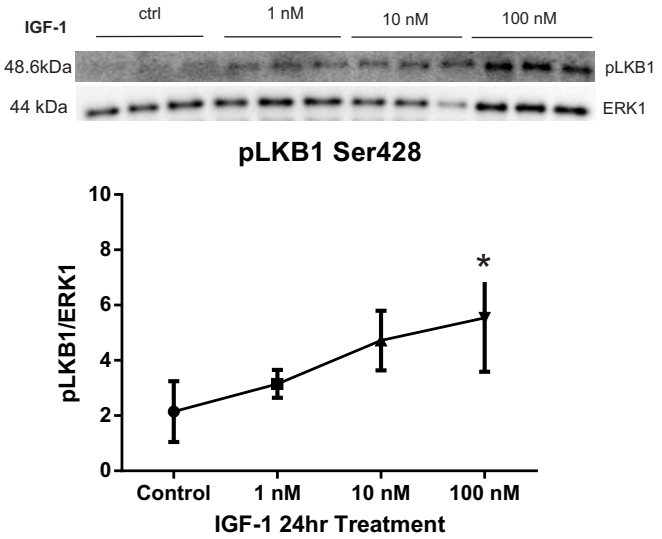

Figure 3: IGF-1 (1, 10 and 100 nM) increased the expression of pLKB1 in control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM, *P<0.05).

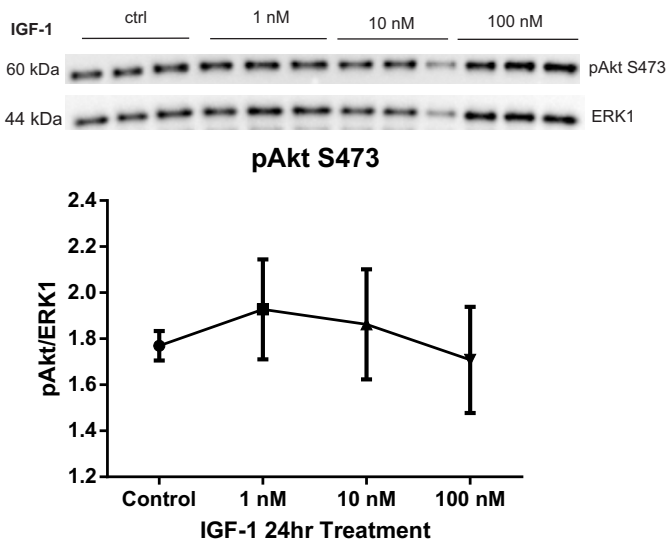

Figure 4: No significant difference in expression of pAkt S473 with the 24 hr IGF-1 treatment (1, 10 and 100 nM) of control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM).

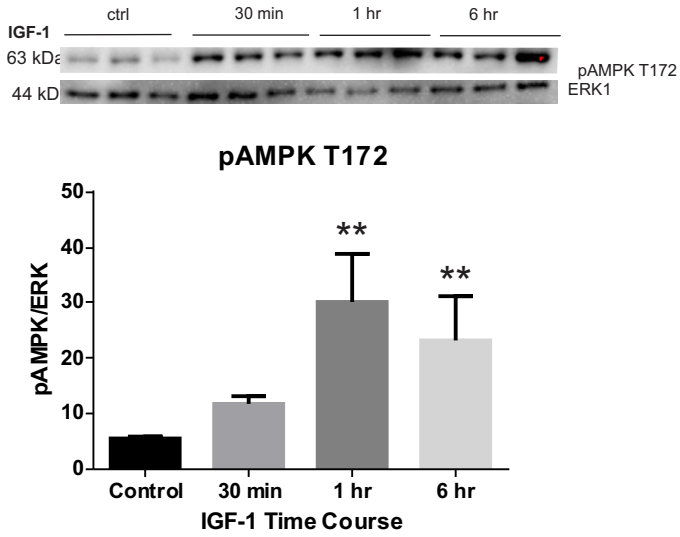

Figure 5: IGF-1 (10 nM) increased the expression of pLKB1 in control rat DRGs at 1hr and 6 hrs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM, *P<0.05 ; **P<0.01).

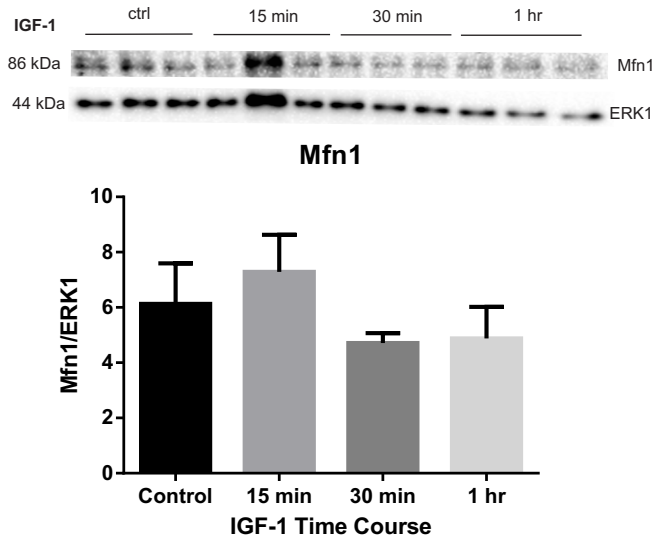

Figure 6: No significant difference in expression of Mfn1 with 15, 30 or 60 minutes treatments with IGF-1 (10 nM) of control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM).

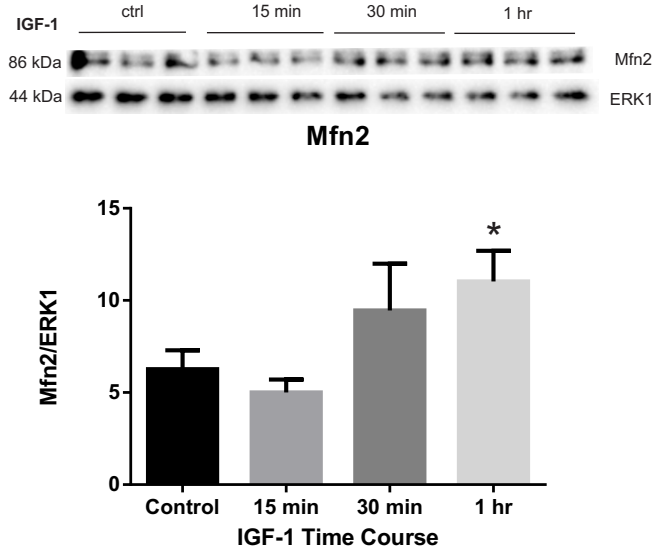

Figure 7: 1hr treatment with IGF-1 (10 nM) increased the expression of Mfn2, with no significant difference in expression with 15, 30 minute treatments in control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM, *P<0.05).

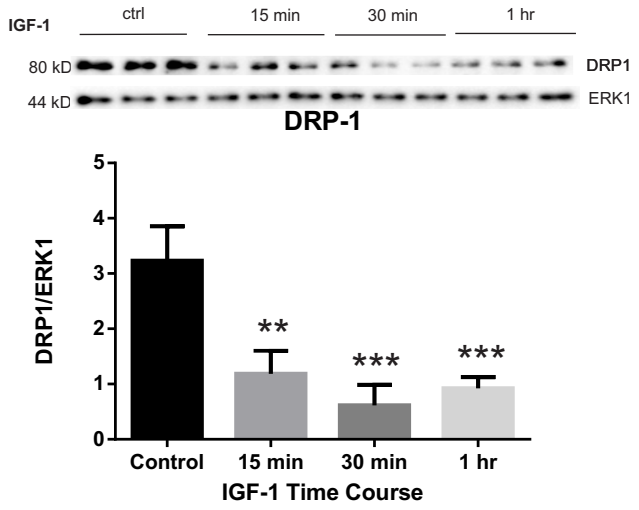

Figure 8: IGF-1 (10 nm) decreases the expression of DRP1 with 15, 30 and 60 minute treatments in control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM, *P<0.05 ; **P<0.01 ; ***P<0.001).

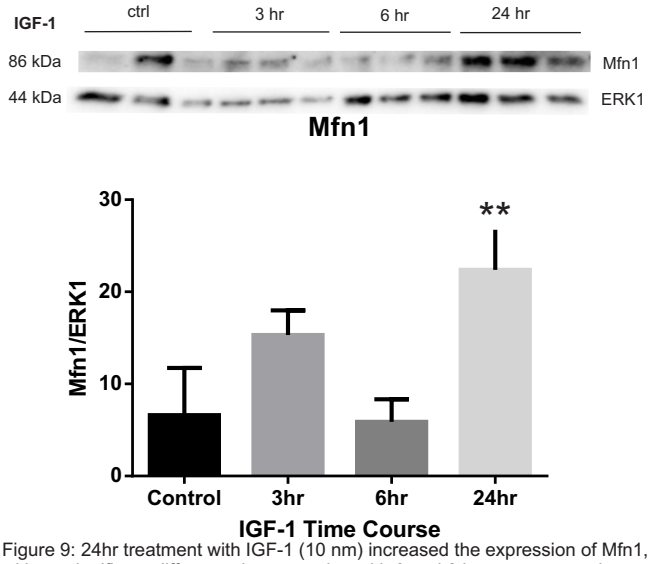

Figure 9: 24hr treatment with IGF-1 (10 nm) increased the expression of Mfn1, with no significant difference in expression with 3 and 6 hour treatments in control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM,

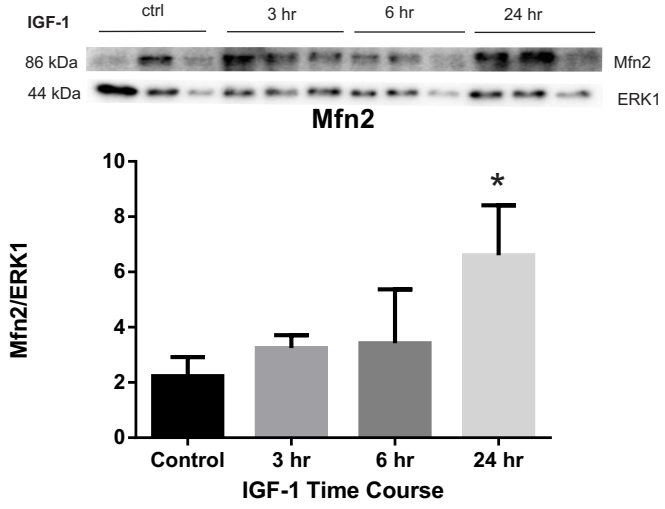

Figure 10: 24hr treatment with IGF-1 (10 nm) increased the expression of Mfn2, with no significant difference in expression with 3 and 6 hour treatments in control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM,

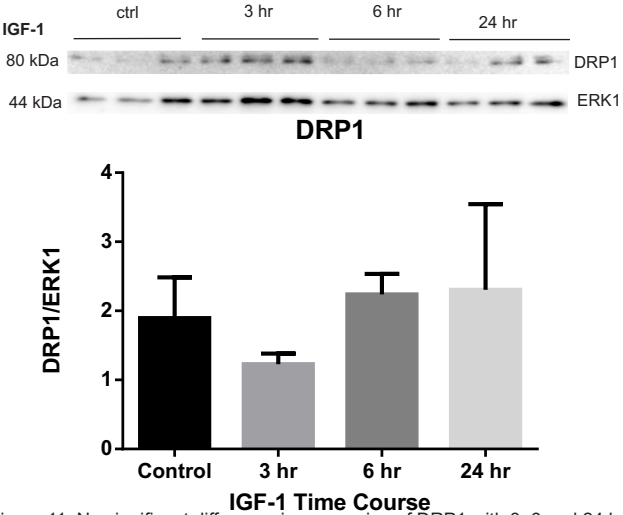

Figure 11: No significant difference in expression of DRP1 with 3, 6 and 24 hour treatments with IGF-1 (10 nM) of control rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=3, M +/- SEM).

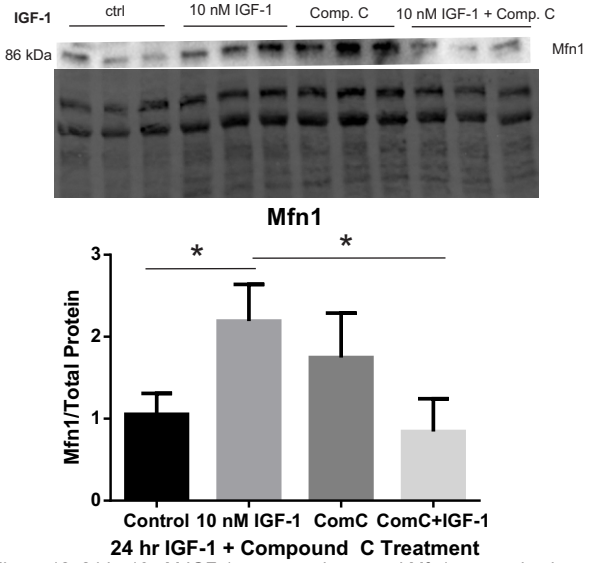

Figure 12: 24 hr 10 nM IGF-1 treatment increased Mfn1 expression in control rat DRGs. The addition of Compound C, an AMPK inhibitor, negated that effect, with a significant difference between IGF-1 treated DRGs and IGF-1 + Compound C treated DRGs. (One -way ANOVA-Tukey's post hoc test, n=3, M +/- SEM, *P<0.05).

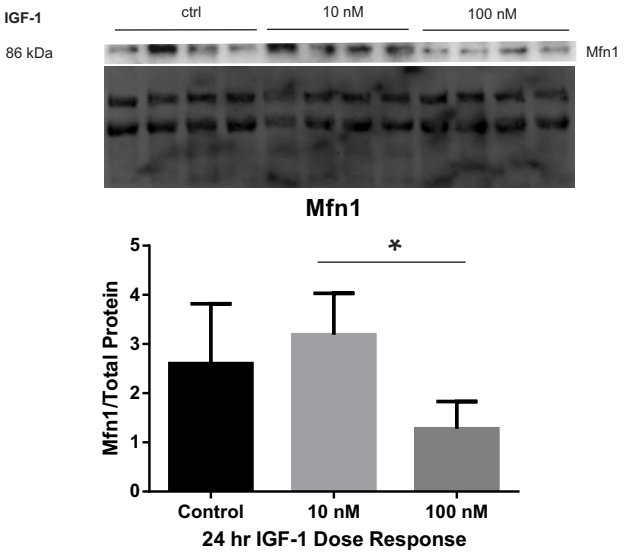

Figure 13: No significant difference in expression of Mfn1 with 24hr IGF-1treatments (10 and 100 nM) of streptozotocin-diabetic rat DRGs. However, a significant difference between 10 nM and 100 nM treatments was noted. (One -way ANOVA-Tukey's post hoc test, n=4, M +/- SEM, *P<0.05).

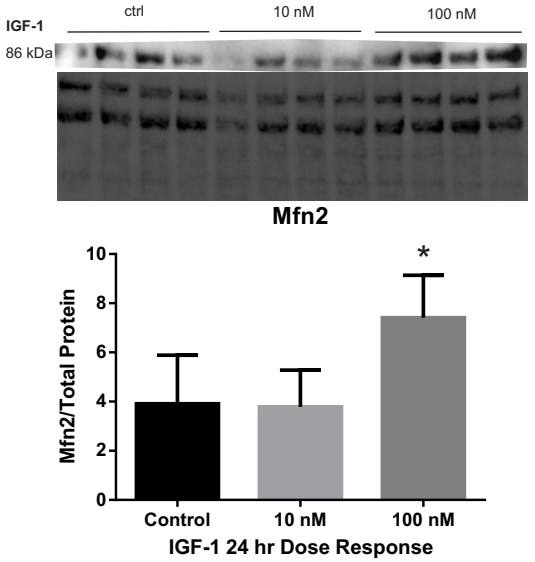

Figure 14: 24 hr 100 nM treatment with IGF-1 increases MFN2 expression, with no difference at 10 nM in streptozotocin-diabetic rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=4, M +/- SEM, *P<0.05).

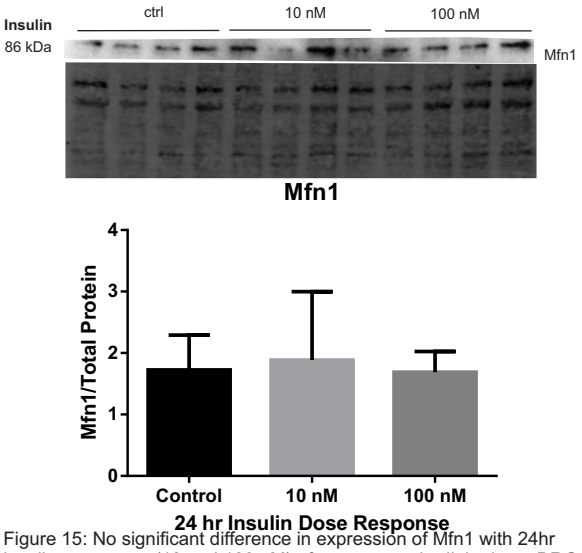

Figure 15: No significant difference in expression of Mfn1 with 24hr insulin treatments (10 and 100 nM) of streptozotocin-diabetic rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=4, M +/- SEM).

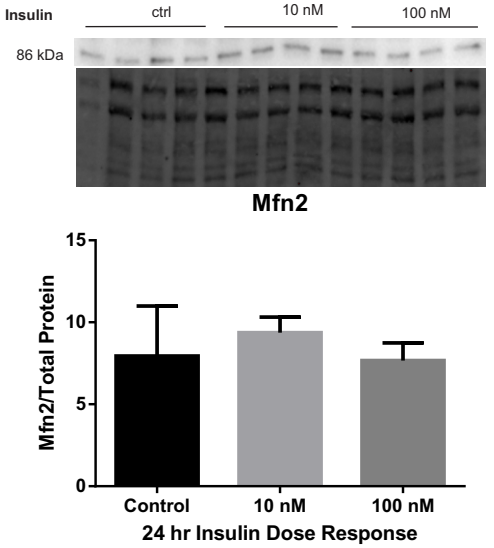

Figure 16: No significant difference in expression of Mfn1 with 24 hr insulin treatments (10 and 100 nM) of streptozotocin-diabetic rat DRGs (One -way ANOVA-Dunnett's post hoc test, n=4, M +/- SEM)