Interleukin-17A (IL-17A) Enhances Axonal Regeneration and Mitochondrial Function of Normal and Diabetic Sensory Neurons

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Abstract

Rationale and hypothesis: Diabetic neuropathy involves dying back of nerve endings that reflects impairment in axonal plasticity and regenerative nerve growth. Metabolic changes in diabetes can lead to a dysregulation of hormonal mediators, such as cytokines. Thus I studied the effect of interleukin-17A (IL-17A), a proinflammatory cytokine produced by T-cells, on the phenotype of sensory neurons derived from control or diabetic rats. I hypothesized that IL-17A induces neurite outgrowth in sensory neurons through signaling pathways that enhance mitochondrial function. IL-17A can also reverse impaired nerve regeneration associated with diabetes.

Objectives: Determine the ability of IL-17A to enhance neurite outgrowth in cultured sensory neurons. Investigate the signalling pathways activated by IL-17A and mechanistically link to neurite outgrowth. Study the ability of IL-17A to improve mitochondrial function of sensory neurons (since axon outgrowth consumes high levels of ATP).

Methodology: Cultured adult dorsal root ganglia (DRG) sensory neurons derived from age matched control or streptozotocin (STZ)-induced type 1 diabetic rats were fixed and stained for fluorescent imaging to determine total neurite outgrowth. Western blotting determined the levels of MAPK and PI-3K activation by IL-17A and for measuring levels of proteins of mitochondrial oxidative phosphorylation pathway. Mitochondrial bioenergetic function was tested in cultured DRG neurons using the Seahorse XF Analyzer.

Results: I found that IL-17A (10 ng/ml; $P<0.05$) significantly increased total neurite outgrowth in cultures derived from both control and STZ-diabetic rat models. This enhancement was mediated by IL-17A-dependent activation of MAPK and PI-3K pathways with maximal effect at

15 minutes ($P<0.05$). Pharmacological blockade of one of these activated pathways led to total inhibition of neurite outgrowth. IL-17A improved mitochondrial bioenergetic function of sensory neurons. Bioenergetics function was associated with augmented expression of proteins of mitochondrial oxidative phosphorylation.

Conclusion: IL-17A enhanced axonal plasticity through activation of MAPK and PI-3K pathways and was associated with augmented mitochondrial bioenergetics function in sensory neurons.

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30. IL-17A did not affect mitochondrial mass in cultured sensory neurons
31. IL-17A caused a small but significant increase in porin expression

Chapter 1:

Background and literature review

1.1. Introduction to nerve regeneration:

1.1.1. The peripheral nervous system (PNS):

The peripheral nervous system (PNS) is a complex network of neurons that connects different organs in the body to the central nervous system (CNS). PNS is divided into two main groups; the somatic nervous system (SNS) and the autonomic nervous system (ANS). The SNS is responsible for voluntary movement and sensation. It consists of two types of neurons: sensory which carries information from the periphery to the CNS, and motor which transmits signals from CNS to the skeletal muscles. On the other hand, the ANS controls visceral functions below the level of consciousness. This includes cardio regulation, respiration, digestion, sweating and erectile function. It is divided into the sympathetic system that is responsible for the "fight-or-flight" response, and the parasympathetic system which controls body function at rest.

Sensory neurons are divided into subgroups that include somatic, vision and auditory sensory neurons. The somatic sensory neurons are responsible for temperature, touch, pressure and pain sensation. These functions are carried out through mechanoreceptors that include proprioceptors which provide spatial information for the extremities and other parts of the body, and nociceptors that are responsible for processing pain and temperature changes [1]. On the other hand, motor neurons are classified according to their target to either somatic neurons, which innervate the muscles, or visceral neurons, that innervate different organs such as cardiac and smooth muscles.

1.1.2. Peripheral nerve structure and function:

In general, a neuron consists of a cell body, an axon and dendrites. In cell culture, dendrites and axons are termed neurites because it is hard to distinguish axons from dendrites before differentiation is complete. Neurons can be either bipolar, which have two processes extended from their cell bodies such as neurons responsible for smell, sight, taste and hearing, or they can be unipolar which have only one process such as sensory neurons found in the dorsal root ganglia. However, the most common type is multipolar where multiple dendrites arise from the cell body and branch through the system to form the dendritic tree (Fig.1), whereas only one axon arises from the cell body and extends for a long distance until it reaches its target. In synapses, signals are passed from the axon to be received by other neuron's dendrites [2].

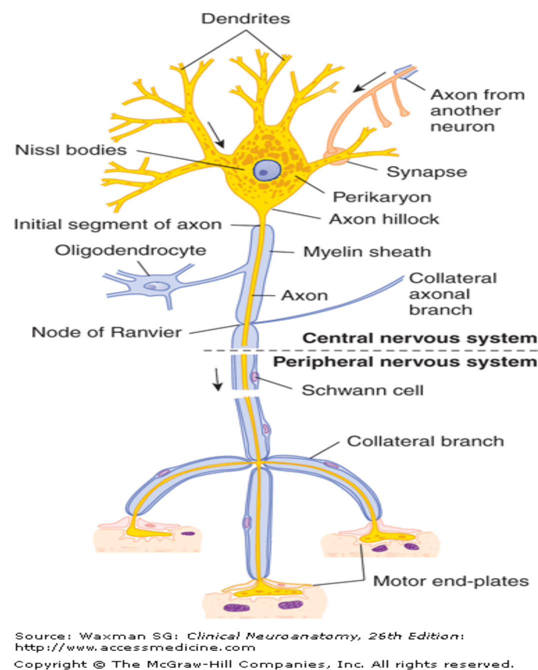

Fig.1: Schematic drawing of a Nissl-stained motor neuron. The myelin sheath is produced by oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system. Note the three motor end-plates, which transmit the nerve impulses to striated skeletal muscle fibers. Arrows show the direction of the nerve impulse [2].

Depending on their velocity of conduction, peripheral nerve fibers can be divided into three groups: A, B and C. A-fibers are myelinated fast-conducting fibers. A-fibers have subtypes such as alpha that is the motor and proprioceptive fibers of the A type, beta that carry sensory information such as touch, and delta which carry sensory signals related to pain, temperature and pressure. B-fibers are myelinated with a fast conduction velocity and detect fine touch, while C-fibres are unmyelinated slow-conducting fibers that are responsible for pain, touch and pressure.

In terms of structure, peripheral nerves are mainly composed of fascicles that are closely covered by perineurium and separated by interfascicular epineurium. Epineurium is a layer of connective tissues that surrounds multiple nerve fascicles. Axons inside the fascicle are surrounded by endoneurial fluid and covered by myelin which is made from successive wrappings of Schwann cells (Fig.2) [3].

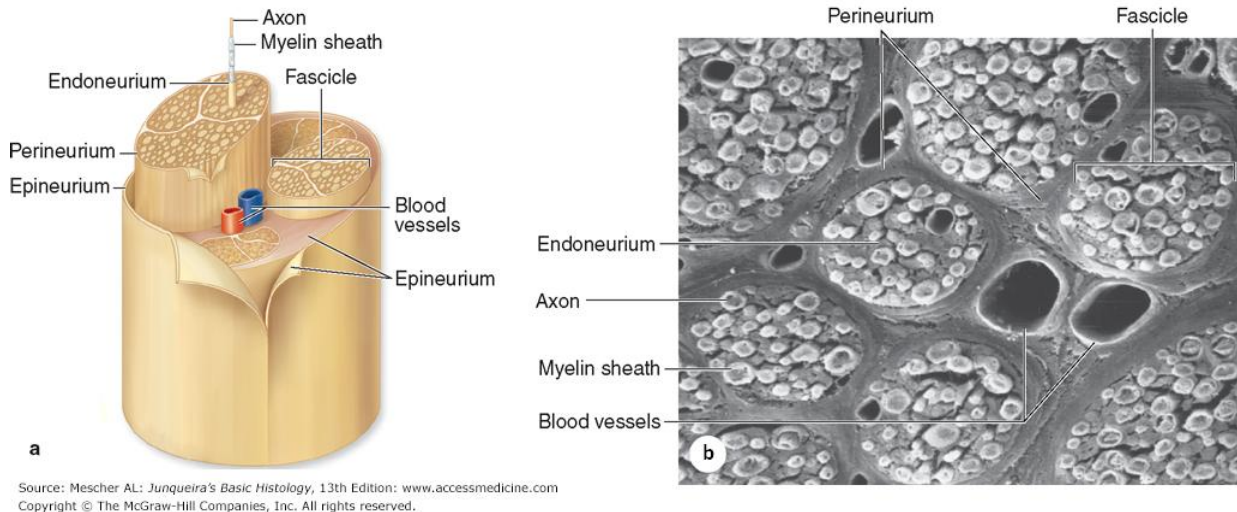

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Fig.2: (a) The diagram shows the relationship among the three connective tissue layers in large peripheral nerves. The epineurium consists of a dense superficial region and a looser deep region that contains the larger blood vessels. (b) SEM of transverse sections of a large peripheral nerve showing several fascicles, each surrounded by perineurium and packed with endoneurium around the individual myelin sheaths. Each fascicle contains at least one capillary. Endothelial cells of these capillaries are tightly joined as part of the blood-nerve barrier and regulate the

kinds of plasma substances released to the endoneurium. Larger blood vessels course through the deep epineurium that fills the space around the perineurium and fascicles. X450. (Junqueira's Basic Histology, 13e, Chapter 9. Nerve Tissue & the Nervous System)

Schwann cells (SCs) are the glia of the PNS. They contribute to the PNS by providing support for neurons, aiding in conduction of nerve impulses, and promoting nerve regeneration. Schwann cells form the myelin sheath in myelinated axons. The gaps in the myelinated axons, which are known as Nodes of Ranvier, increase the conduction velocity as signals jump faster from node to node to reach the other neuron's dendrites or the target in a process called saltatory conduction (Fig.1) [4].

1.1.3. Blood supply to nerve:

The nerve endings are supplied by blood vessels with a capillary plexus that penetrate the perineurium. The branches of the plexus run parallel with the fibres and are connected to each other by short transverse vessels. Endoneurial capillaries have atypically large diameters with big intercapillary distances. Two functionally independent vascular systems supply the peripheral nerves: an extrinsic system (regional nutritive vessels and epineurial vessels) and an intrinsic system (longitudinally running microvessels in the endoneurium). A cross connection between these two systems produces a huge overlap between the regions of the segmental arteries. Peripheral nerves have a high degree of resistance to ischemia due to the unique pattern of vessels and high basal nerve blood flow compared to metabolic requirements [5].

1.1.4. Challenges of axon survival:

Unlike the PNS, the CNS is well protected by bone structure that minimizes its injury. PNS is well exposed to all kinds of mechanical stresses. Due to their extreme length, axons face lots of

challenges for survival. Among these stresses is the need for axonal transport to and from the cell body. Any disruption of this process could lead to axonopathy or neurodegenerative disease. Physical vulnerability of the axon adds more stress to its function. Whether it is a full transection, a transection caused by inflammation, or long term pressure that damages the axons, nerve regeneration is an important process for axons to retain their function [6].

Adult neurons in the PNS respond to nerve damage by two types of growth, collateral sprouting and regeneration. Collateral sprouting is the growth of a new axon along the shaft of an intact axon, while regeneration is the re-growing of an injured axon. In collateral sprouting, uninjured axons expand their innervation area by branching to the denervated tissue. Collateral sprouting can restore sensation in the absence of regenerative growth in the injured nerve. It is sometimes referred to as a possible mode of sensory recovery [7]. However, unlike regeneration, collateral sprouting is fully dependent on nerve growth factor (NGF) and it can be totally abolished by blocking NGF function using NGF antibodies [8].

1.1.5. Process of Wallerian degeneration:

In the peripheral nervous system, nerves sustain their target's innervations in normal conditions. After nerve injury, their connection is lost, and thus neurons try to reinnervate their targets to retain its function by undergoing axonal regeneration. Following nerve injury, the nerve begins to degenerate by a process called Wallerian degeneration. Starting from the distal stump of the lesion, Schwann cells, with the help of macrophages, phagocytose the nerve's myelin sheath and axon debris (Fig.3) [9]. Schwann cells are very crucial in the regeneration process; besides their role in axon phagocytosis, the basal lamina contains neurite growth promoters such as laminin and fibronectin which help in growth induction.

At the other end of the nerve, and within a few hours of injury, the proximal stump starts to sprout toward the distal stump and gives arise to many axons. Some of these axons disappear, while the others enlarge and increase their diameter and that depends on them having a good connection to the target (Fig.3) [10]. The distal stump secretes neurotrophic factors that have a chemotactic effect to attract the growth direction [11]; however, the accuracy of the reconnection depends on whether the nerve is crushed or cut. If the nerve is crushed and no tissue is missing, nerves can grow through the old endoneurial tube which guides it to the target more accurately. On the other hand, if the nerve is cut, the chances of sprouting to a wrong target are higher [12].

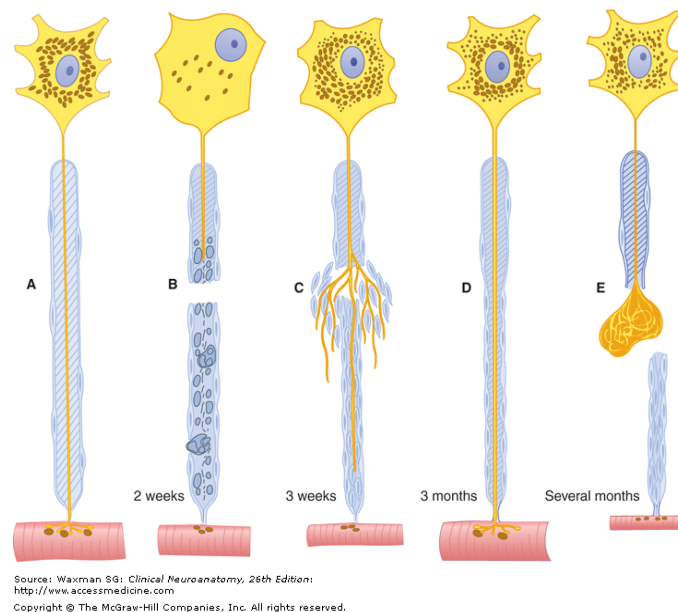


Fig.3: Main changes that take place in an injured nerve fiber. **A**: Normal nerve fiber, with its perikaryon and the effector cell (striated skeletal muscle). Notice the position of the neuron nucleus and the amount and distribution of Nissl bodies. **B**: When the fiber is injured, the neuronal nucleus moves to the cell periphery, Nissl bodies become greatly reduced in number (chromatolysis), and the nerve fiber distal to the injury degenerates along with its myelin sheath. Debris is phagocytized by macrophages. **C**: The muscle fiber shows pronounced disuse atrophy. Schwann cells proliferate, forming a compact cord that is penetrated by the growing axon. The axon grows at a rate of 0.5 to 3 mm/d. **D**: In this example, the nerve fiber regeneration was successful, and the muscle fiber was also regenerated

after receiving nerve stimuli. **E**: When the axon does not penetrate the cord of Schwann cells, its growth is not organized and successful regeneration does not occur [2].

The success of the regeneration depends on the presence of scar tissues or inflammation in the regenerated nerve. The regeneration process can be improved surgically by suturing the nerve epineurium; however, the disadvantages of this procedure are that it may increase scar formation, and the suturing material could cause fibrosis [13]. If a large portion of the nerve is missing after the injury, the gap can be filled by different materials such as an autologous cutaneous nerve, tube of silicon, collagen, evacuated muscle basal lamina or a regeneration chamber. This procedure could be improved by prefilling these tubes with different growth factors which help in enhancing the growth [14].

Regeneration requires formation of the axonal growth cone. The growth cone is a motile structure that is located on the very tips of nerve axons and dendrites. It produces molecules and signals that help in the regeneration process. It is formed as a result of disruption of the membrane and exposing axoplasm to the extracellular environment. Thus, calcium enters the axoplasm leading to degradation of the cytoskeleton structure. In addition, microtubules and neurofilament depolymerise and the terminals start to swell at the proximal stump. Along with the terminal swelling, microtubules and neurofilaments repolymerize to form the growth cone (Fig.4) [15].

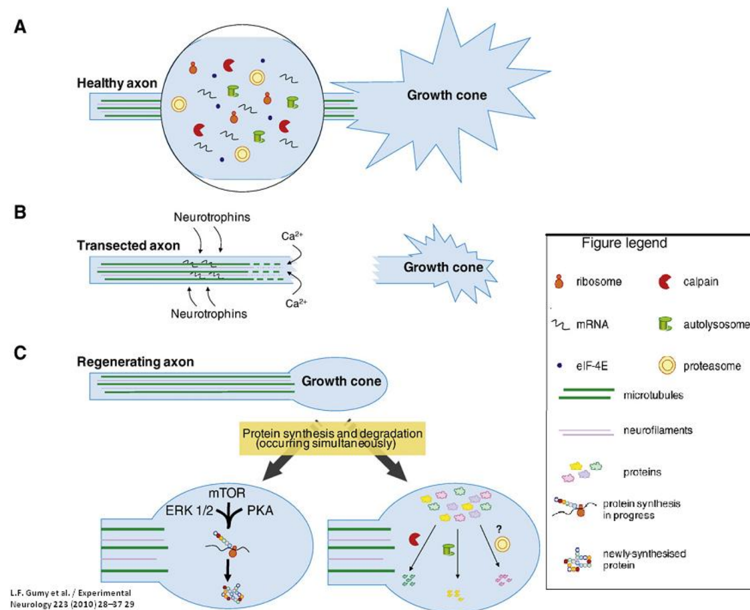


Fig.4.The formation of the growth cone. (A) Inside a healthy axon, a variety of protein synthesis and degradation machinery is present. (B) As a result of transection, the axon is divided into two parts. The distal portion undergoes Wallerian degeneration and will subsequently be degraded. In the proximal portion, calcium enters the axon due to the disruption to the plasma membrane, as well as via voltage-gated calcium channels. Cytoskeletal structures such as microtubules and neurofilament undergo depolymerisation and degradation. Neurotrophins from extracellular sources may also help the localisation of mRNAs. (C) Later on, a terminal swelling appears at the tip of the proximal stump, as regeneration ensues. Microtubules and neurofilaments undergo re-polymerisation. Protein synthesis and protein degradation occur simultaneously within the axon. Protein synthesis takes place via a mechanism dependent on mTOR, ERK 1/2 and PKA [15].

1.1.6. Wlds mouse model:

Despite the importance of Wallerian degeneration in the regeneration process, some mouse models undergoing delayed Wallerian degeneration (Wlds) were able to normally regenerate. The Wlds mice belong to the strain C57BL/Wlds. The Wlds mutation is an autosomal-dominant, naturally occurring mutation that affect chromosome 4 in the mouse. The mutated region contains two associated genes: nicotinamide mononucleotide adenlyl transferase 1 (Nmnat-1)

and ubiquitination factor e4b (Ube4b) and the protein formed by this mutation (Wlds protein) is only found in the nucleus[16]. In these mutant mice, nerves can still function up to 15 days after injury; on the other hand, nerves in normal mice die after 1 day of damage. The current reasoning behind this phenomenon is that these mice produce Wlds protein that substitute nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2). NMNAT2 is an enzyme that catalyzes an essential step in the NAD (NADP) biosynthetic pathway and serves as an axon survival factor. NMNAT2 is depleted after nerve injury, however, Wlds protein can substitute the axon maintenance function of NMNAT2 in primary cultures and also correct developmental defects associated with NMNAT2 deficiency [17]. Wlds neuroprotective activity mainly depends on two domains; N16 and NMNAT and the whole Wlds process could be abolished in their absence [18].

1.1.7. Changes in nerve cell body:

Following nerve damage, the cell body is affected by two kinds of signals: negative signals, which arise from the interruption of normal retrograde influence of neurotrophic factors; and positive signals that are not present in intact neurons but are increased after axotomy to stimulate the cell body response. Positive signals are mostly influenced by mediators such as cytokines [19]. These signals stimulate the cell body to produce a large amount of protein including growth associated proteins (GAPs), actin and tubulin. The stimulated proteins play a key role in neurite formation, regeneration, and plasticity. The cell body in itself is not a limiting factor in the regeneration process as the axon starts to grow a few hours after the injury, even before the cell body starts to respond. In addition, studies on axons that were completely disconnected from their cell body demonstrated the ability to regenerate and grow without their cell body's support.

This supported the notion of the nerve regeneration process being independent of the cell body during the initial response to damage [10].

Neurotrophic factors, such as nerve growth factor (NGF), play an important role in growth during embryonic development; they activate the Trk receptor which increases axonal growth by promoting both extracellular-signal-regulated kinases (ERK) and phosphatidylinositide 3-kinase (PI-3K) pathways (Fig.5). It has been proven that some of these neurotrophic factors are also increased after axotomy. This suggests an important role for these neurotrophic factors in mediating nerve regeneration after injury [20].

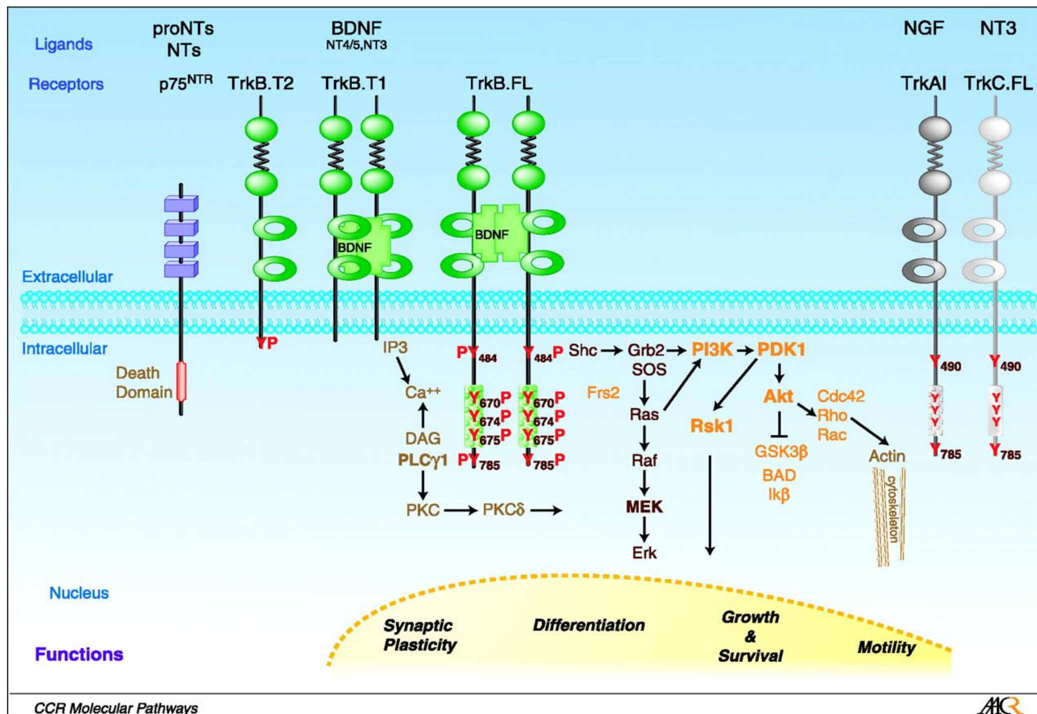


Fig.5: Schematic representation of Trk receptor tyrosine kinases and major signal transduction pathways. Activation of the TrkB-TK leads to phosphorylation (P) of a number of tyrosines (Y) in the TK domain, as well as the juxta membrane domain. These PY residues serve as docking sites for cytoplasmic proteins, such as Shc and PLCγ, whose recruitment in turn leads to activation of downstream mediators of the MAPK, PLCγ and PI-3 kinase pathways. Ultimately, these signals are transduced to the nucleus to mediate transcriptional programs that regulate cellular

functions, such as synaptic plasticity, differentiation, growth, survival, and motility. Other members of the Trk family, TrkA and TrkC, are depicted to illustrate the structural conservation of this gene family. P75 is a pan-neurotrophin (NT) receptor, which is structurally in the Death Receptor family of membrane-bound receptors. [21].

1.1.8. Regeneration in CNS:

In contrast to the PNS, the CNS has a low tendency for axonal regeneration. Current literature suggests that this is because the CNS lacks Schwann cells and has instead oligodendrocytes and astrocytes. Oligodendrocytes produce myelin associated protein and other potent axonal regeneration inhibitors such as neurite growth inhibitor (NI-35/250) [22]. On the other hand astrocytes proliferate after CNS damage to fill in the injured spaces and form a glial limiting membrane that has the capacity to wall larger spaces. The formed wall stops axonal growth and prevents it from penetrating this mechanical barrier [23]. In addition, astrocytes produce protease inhibitors, such as nexin-I and a2-macroglobulin [24], and other molecules such as chondroitin sulphate proteoglycan which have neurite inhibitory activity. Reticulon-4 or Nogo is another potent inhibitor of neurite outgrowth specific to the CNS system. It has three isoforms: Nogo A, B and C. Nogo-A has two inhibitory domains: amino-Nogo, a strong inhibitor of neurite outgrowth, and Nogo-66, which is involved in growth cone destruction [25]. The combinations of these factors stop most of the regeneration in CNS and prevent its success.

Contrary to the PNS, the CNS has no regeneration response after injury. However, peripheral axotomy occurring prior to a dorsal column injury forms a conditioning lesion which helps the axotomized central processes of DRG to grow inside the spinal cord overcoming neutralizing CNS inhibitory signals. This means that axons can grow back after spinal injury with the aid of PNS regeneration signalling [26].

Efficient regeneration in the CNS could be achieved by bridge grafting of a substrate such as embryonic astrocytes or dissociated Schwann cells which substitute for the CNS inhibitory environment. Regeneration in the CNS could also be triggered pharmacologically with an antibody against myelin-associated inhibitor of neurite growth which neutralizes the neurite growth inhibition properties of NI-35/250 proteins [27], or by injecting neurotrophic factors at the lesion site.

1.1.9. Neurodegenerative disorders and cytokines:

Neurodegenerative diseases are associated with progressive loss of structure or functions of neurons. They include Parkinson's, Alzheimer's, Huntington's and diabetic neuropathy. Diabetic neuropathy is a type of neurodegenerative disease associated with impaired peripheral nerve regeneration and collateral sprouting. It is thought to be caused by many factors such as failure to provide proper metabolic support for repair (e.g. excessive glucose flux via polyol pathway triggering oxidative stress), less support of axons by their Schwann cells, lack of trophic factors, attenuated inflammation and defects in the entry and actions of inflammatory cells within the injury [28].

Since they are involved in nerve lesions and repair in the PNS, cytokines have been linked to some neurodegenerative diseases. Proinflammatory cytokines, such as tumor necrosis factor (TNF), are thought to be important in the process of inflammatory demyelinating neuropathies as they increase vascular permeability and blood nerve barrier breakdown [29]. Nevertheless, it has been suggested that TNFα neurotoxic effects are important in Wallerian degeneration [30]. In addition, when TNFα was injected into a mouse sciatic nerve, it induced axonal degeneration of up to 80% of the nerve fibers [31]. Interleukin-1 may play several roles in the course of

Wallerian degeneration as well. It acts as a co-mitogen for Schwann cells [32], and it is involved in nerve regeneration and induces production of NGF and other neurotrophic factors, such as leukemia inhibitory factor (LIF) in nerve tissue [33].

1.2. Mechanism of cytokine signalling:

1.2.1. The cytokine family of growth factors:

Cytokines are hormonal mediator peptides that act as signalling molecules. They are also referred to as immunomodulating agents, and include interleukins and interferons. The term cytokine was originally used in immunology to describe peptides that are involved in inflammatory and immune reactions. This term is synonymous in neurobiology for peptides that have multifunctions and target multiple cells in the nervous system [34]. Cytokines are made by many cell populations; however, they are mainly produced by helper T cells (Th) and macrophages. Cytokines regulate host responses to infection, immune responses, inflammation and trauma. They can be either proinflammatory, such as IL-1, IL-6, IL-8, IL-17 and TNFα which initiate an inflammatory response necessary to recruit granulocytes and lymphocytes to fight disease; or anti-inflammatory such as IL-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13, which reduce inflammation and promote healing once the injury or infection is cleared [35]. If cytokines act on the cells that secrete them, this process is called an autocrine action; however, if they act on nearby cells or on distant cells, it is called a paracrine action or an endocrine action, respectively. Another criteria of some cytokines is that they might have a pleiotropic effect which means that a single cytokine is either acting on, or produced from, several different cell types [36].

1.2.2. Cytokine receptors:

Cytokines act on their targets by binding to their specific cell receptors. Based on their structure, cytokine receptors are divided into: a) type I cytokine receptors, whose members have certain conserved motifs in their extracellular amino-acid domain including those that have glycoprotein 130 (gp130) subunits in their intercellular region such as interleukin receptors IL-6RA, IL-11RA, LIFR and CNTFR; b) type II cytokine receptors, whose members are receptors mainly for interferons; c) immunoglobulin (Ig) superfamily, which is ubiquitously present throughout several cells and tissues of the vertebrate body and include interleukin -1 receptor; and d) tumor necrosis factor receptor family TNFR which includes receptors that have a cysteine-rich common extracellular binding domain (Fig.6) [37].

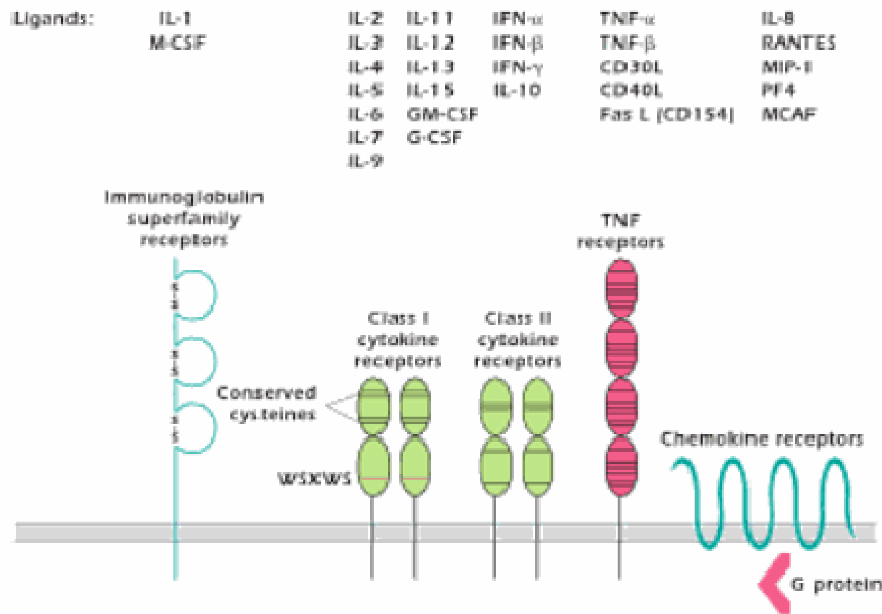


Fig.6 Types of cytokines receptors and their ligands. Based on their three-dimensional structure, cytokine receptors are divided in to: immunoglobulin superfamily receptors, class I cytokine receptors, class II cytokine receptors TNF receptors and chemokine receptors. [37]

1.2.2.1. Glycoprotein 130 cytokine family receptors:

Glycoprotein 130 (gp130) cytokines, also known as interleukin 6 (IL-6) family, include leukemia inhibitory factor (LIF), interleukin 11 (IL-11), ciliary neurotrophic factor (CNTF), oncostatin-M, and cardiotrophin-1. All gp130 cytokines use the same receptor signaling subunit and form homodimers or heterodimers of gp130 with additional α or β receptor subunits. IL-6 and IL-11 form a homodimer complex through dimerization of two gp130 subunits; on the other hand, CNTF, LIF and OSM make a heterodimeric complex of gp130 plus β subunit leukemia inhibitory factor receptor (LIFR). To form a high affinity complex, IL-6, IL-11 and CNTF first bind to the α receptor subunit (IL-6Rα, IL-11 Rα , CNTF Rα respectively) (Fig.7). gp130 and LIFR do not contain tyrosine kinase activity; but after receptor activation, Janus kinases (JAKs) phosphorylate tyrosine residues in the cytoplasmic region of gp130 [38].

	LIFR containing		**Non-LIFR containing**		
Cytokine	**CNTF**	**LIF**	**IL-6**	**IL-11**	**NP**
Transmembrane receptor	LIFR gp130	LIFR gp130	gp130 gp130	gp130 gp130	? gp130
α Receptor	CNTFRα	-	IL-6Rα	IL-11Rα	CNTFRα

Fig.7: Components of gp130 cytokine receptors. Edited figure [39].

Sensory neuron sensitivity to IL-6 cytokines is determined by the expression level of α and β subunits. The vast majority of sensory neurons have gp130 and LIFR. Gp130 have homogenous distribution across somatic sensory neuron subpopulations. This wide spread of IL-6 family-signaling subunit (gp130) indicates a potential response pathway for these cytokines in sensory neurons [38].

After axotomy, neither the levels of gp130 protein nor the sensory neuron response to gp130 ligands change. However, a significant increase in the expression of cytoplasmic LIFR was observed; yet this induction was not accompanied with an increase in the level of LIFR protein at any time point after axotomy. Thus the increase in the cytoplasmic LIFR did not necessarily mean an increase in receptor synthesis. The cytoplasmic LIFR was co-localized in small diameter neurons which were mostly nociceptive in function [38].

1.2.2.2. Toll/interleukin-1 receptor (TIR) family:

The TIR is a large receptor family that is involved in immune and inflammatory responses. Based on its extracellular domain, it is divided into the immunoglobulin (Ig) domain family and the leucine-rich repeat (LRR) motif family.All members of the TIR family share the same intracellular TIR domain which is important in the initiation of signalling (Fig.8). Adapter proteins such as myeloid differentiation primary response gene 88 (MyD88), TIR-domain containing adapter protein (TIRAP), and TIR-domain-containing adapter-inducing interferon-β (TRIF) are recruited to the TIR domain to aid in signalling and add specificity [40].

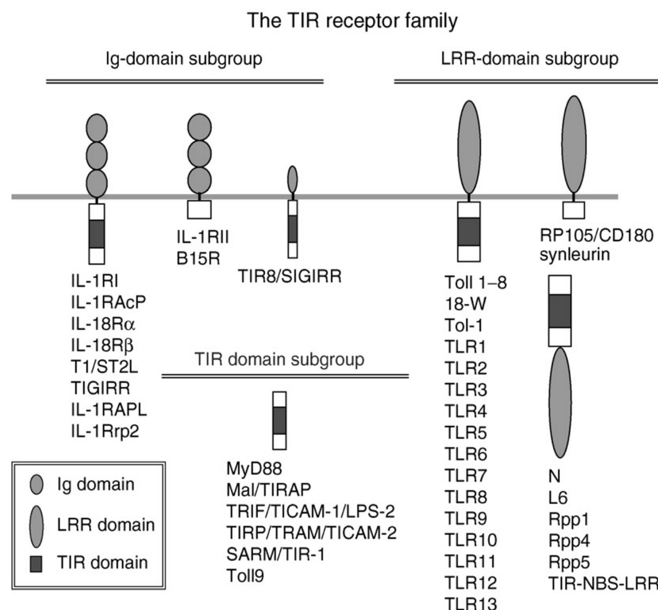


Fig.8. TIR receptor family subgroups. The TIR receptor family can be divided into two main subgroups based on the extracellular domains, those containing an immunoglobulin (Ig)-like domain such as IL-1R1 and IL-1R18, and those characterized by a leucine-rich repeat motif (LRR) which include TLR [41]

The Ig family include IL-1 receptor type I (IL-1RI), its accessory protein (IL-1RAcP), IL-18 receptors (IL-18Ra and IL-18Rb), regulatory receptors (T1/ST2) and IL-1R-like receptors. It also includes some orphan receptors whose endogenous ligands have not yet been identified such as Interleukin 1 receptor accessory protein-like (IL-1RAPL), three immunoglobulin domain-containing IL-1 receptor-related (TIGIRR) and, single immunoglobulin and toll-interleukin (SIGIRR) [40].

1.2.2.3. Interleukin-1 receptors (IL-1R) and their ligands:

IL-1R is composed of two chains: IL-1R type I (IL-1RI) and IL-1R accessory protein (IL-1RAcP). The main ligand for IL-1RI is the IL-1 family which is composed of 11 members that show resemblance in amino acid sequence and gene structure. Among the IL-1 family is IL-1-like molecules (IL-1α, IL-1β, and the antagonist IL-1Ra) and IL-18. IL-1R can be activated by either IL-1α or IL-1β and deactivated by IL-1 receptor antagonist (IL-1Ra) (Fig.9). IL-1 connects with IL-1RI in two places, a large area in the groove between the first and second domain, and a smaller area next to the third domain. However, IL-1Ra binds only to the large area and fails to interact with the smaller one. On the other hand, IL-1RAcP which is a homolog of IL-1RI doesn't bind to any of IL-1R ligands. Upon activation of IL-1RI, IL-1RAcP forms a high affinity receptor complex which is important for signal initiation [41].

IL-1 receptor type II (IL-1RII) is another receptor for IL-1. Its extracellular domain can bind to IL-1 but its intercellular domain is too short and doesn't contain TIR which makes it incapable of

initiating signals. IL-1RII has higher affinity to IL-1β and lower affinity to IL-1α and it acts as an inhibitor to IL-1 action since it guides the receptor to a non-signalling complex (Fig.9) [41].

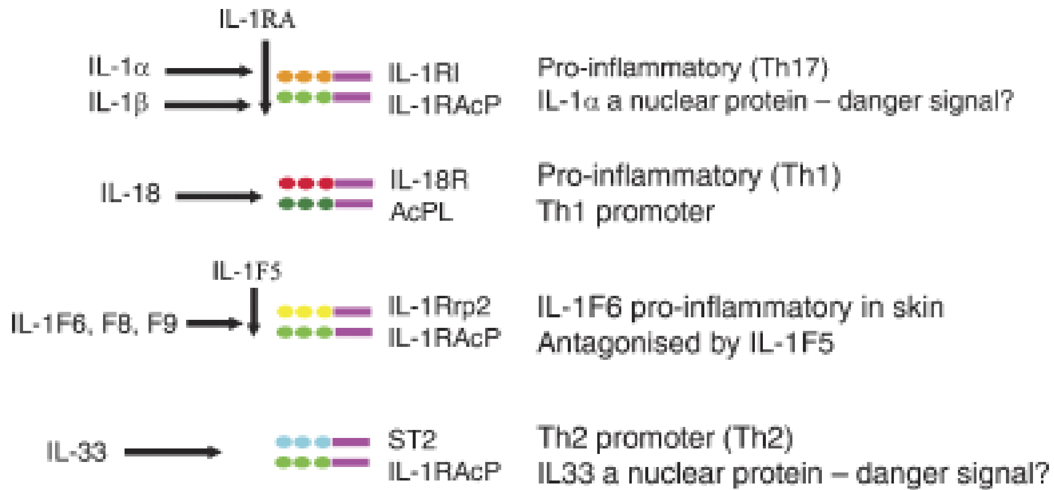


Fig.9: The IL-1 family of ligands and their receptors. The receptor complexes for IL-1a and IL-1b (and IL-1RA), IL-18, IL-1F6-F9 (and the antagonist IL-1F5), and IL-33 are shown. Each IL-1 family member appears to activate different T-cell subsets. IL-1 is likely to be pro-inflammatory due to its capacity to activate Th17 cells. IL-18 is pro-inflammatory because of its effects on Th1 cells. IL-33 activates Th2 cells. Finally IL-1F6, F8, and F9 are pro-inflammatory in skin, where their effects are antagonized by IL-1F5 [40]

1.2.3. Cytokine signalling to NF-κB:

NF-κB is a family of transcription factors that get activated by different stimulants such as cytokines, injuries or infectious agents. NF-κB has important functions in humans. It regulates gene expression of inflammatory response and promotes cell survival, proliferation and differentiation. The NF-κB family consists of five proteins: c-Rel, RelB, p65, p105 and p100. NF-κB is kept inactive in the cytoplasm by IκB [42]. Cytokines such as IL-1β and TNFα activate NF-κB signalling pathway through IL-1 receptor (IL-1R) and TNF receptor 1 (TNFR1), respectively.

1.2.4. Transactivation between PI-3K and STAT:

Activated signalling pathways might enter a transduction loop that leads to a continuous activation. Excessive apoptotic cell death is involved in the pathogenesis of neurodegenerative disorders; however, cytokines might affect cell apoptosis through their activated signalling pathways. [43]. Rat ventricular myocytes have anti-apoptotic properties, probably due to high levels of AKT and STAT3 [44]. These anti-apoptotic properties could be mediated by a positive transduction loop between AKT/NF-κB and JAK/STAT3 as inhibition of AKT by PI-3K inhibitor decreased STAT3 expression, and activation of AKT through constitutively activated AKT (caAKT) increased STAT3 levels. On the other hand, inhibition of STAT3 by JAK2 inhibitor decreased AKT expression, and activation of NF-κB by an activator increased STAT3 levels (Fig.10). This crosstalk between these two signalling pathways protects the cell from apoptosis [44]. Also PI-3K/AKT plays a role in tyrosine phosphorylation of STAT via members of Src tyrosine kinase.

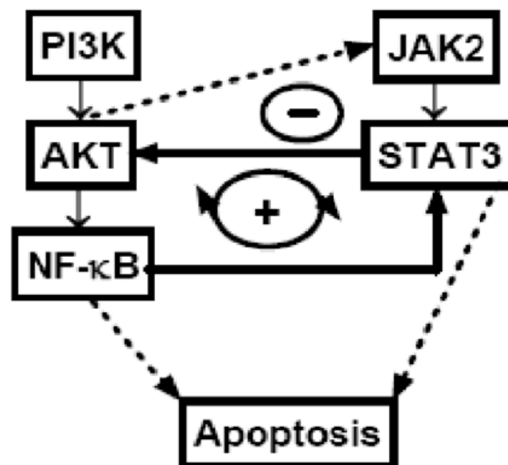


Fig.10: Schematic depiction of dual crosstalks between PI3K/AKT/NF-κB and JAK2/STAT3 pathways: the positive transactivation loop that maintains higher levels of AKT and STAT3 and the negative feedback mechanism that

keeps balanced AKT and STAT3 activities. Solid lines indicate positive actions and dashed lines indicate negative actions [44].

1.3. Cytokine modulation of nerve regeneration:

Cytokines are produced in the peripheral nerve tissue during normal and pathological conditions. They are produced by resident and recruited macrophages, mast cells, endothelial cells, and Schwann cells. Following peripheral nerve injury, macrophages and Schwann cells that are recruited to the site of injury facilitate regeneration by secreting cytokines and specific growth factors required for nerve regeneration [36]. Cytokines may be transported retrogradely from the periphery through axonal transport to the DRG where they can have profound effects on neuronal activity. After nerve injury, the localized inflammatory irritation of DRG increases the pro-inflammatory cytokines and decreases the anti-inflammatory ones [45]. Some inflammatory cytokines are linked to contralateral hyperalgesia /allodynia and are implicated in nerve-injury and inflammation-induced central sensitization [36].

1.3.1. Interleukin-1β (IL-1β):

IL-1β is a proinflammatory cytokine that is produced in glial cells to regulate immune cell activation. Besides its function in the immune system, IL-1β has an important role in the nervous system. It has been strongly linked to nerve regeneration for many reasons, particularly its involvement in Schwann cell proliferation and induction of neurotrophic factors [33, 46]. An increase in IL-1β starts after one day of nerve injury and continues for seven days. This induction leads to enhancement of axon outgrowth through deactivation of RhoA which is responsible for myelin-associated glycoprotein (MAG) synthesis, and thus holding back MAG's inhibition of growth and retraction of axon outgrowth [47]. Over 70% of DRG neurons express IL-1 β mRNA

and its expression is increased in neurodegenerative diseases such as multiple sclerosis and Alzheimer's. IL-1β is considered as an important mediator in nociception as it regulates nociception in peripheral inflammatory processes. Administration of IL-1β in low doses could cause hyperalgesia, while it has an analgesic effect at high doses [48].

IL-1β is synthesised after activation of toll like receptor-4 (TLR4) by bacterial lipopolysaccharides (LPS). This activation largely depends on NF-κB and MAPK signalling pathways which in turn stimulate macrophages and microglia to synthesise pro-IL-1β. Purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7) is a receptor that is expressed solely by microglia and has a key role in converting pro-IL-1β to its active form. P2RX7 needs high concentrations of ATP to be activated; once it is activated it stimulates caspase-1, which is also known as IL-1 converting enzyme (ICE), to convert pro-IL-1β to its mature form (Fig.11) [49].

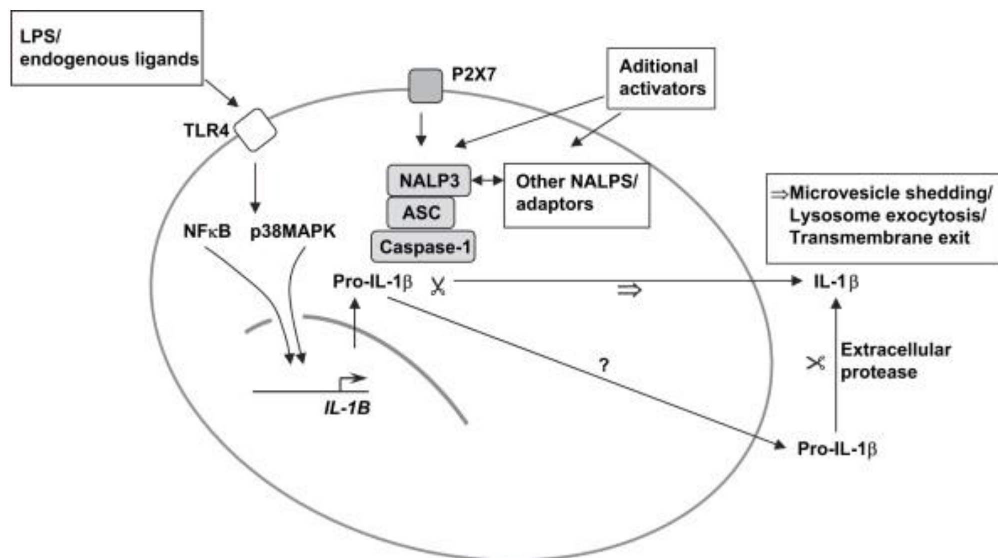


Fig.11: Mechanism of IL-1 β synthesis and release. TLR4 is activated by bacterial LPS leading to IL-1β synthesis through NF-κB and MAPK. P2RX7 plays important role in activating pro- IL-1β and converting it to its mature form through caspase-1 [49]

It was suggested that IL-1 β has an autocrine and paracrine signaling function in sensory neurons since both IL-1RI and IL-1 β were found in sensory neuron and glial cells. IL-1β has an unusual secretory pathway as it has never been found in the Golgi apparatus or associated with the cell membrane. It was later found to be located in the cytoplasm but it was noticed that there is an increase in lactate dehydrogenase (LDH) levels accompanied by IL-1 β increase; since IL-1 β needs caspase I for its activation, it was suggested that cell lysis could be the mechanism of IL-1 β secretion [49].

1.3.2. Glycoprotein 130 (gp130) cytokines:

In the PNS, cytokines have an important role in injury response. Many cytokines are up regulated in the distal stump after axotomy. This increase is accompanied by induction of neurotrophic factors and enhancement of axon outgrowth. The glycoprotein 130 (gp130) cytokine family includes IL-6, LIF, IL-11, CNTF, oncostatin-M and cardiotrophin-1. They have pleiotropic effects and act through activation of the gp130 subunit in their receptor. Gp130 cytokines enhance gene expression and induce growth through activation of JAK/STAT and MAPK signalling pathways. Gp130 cytokines are mainly produced by satellite and Schwann cells; they have a paracrine action as they get transported retrogradely to the cell body after axotomy [50]. In the cell body, gp130 cytokines induce production of new neuropeptides that were not produced before the nerve damage, such as galanin, substance P, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). These neuropeptides are known as regeneration associated genes (RAG) and they have important roles in neuronal survival and regeneration [50]. Gp130 cytokines are considered a positive signal, and when they were applied to uninjured neurons in the presence of anti-NGF, as a negative signal, they produced a huge synergistic enhancement of RAG production [50]. On the other hand, IL-6

phosphorylates Na^+, K^+, Cl^- co-transporter NKCC1 in injured sensory neurons leading to intercellular chloride accumulation. This process was shown to promote growth in DRG neuronal culture [51].

Gp130 cytokines activate STAT3 which serves as a transcription factor in the nucleus and enhances expression of target genes involved in cell proliferation, differentiation and apoptosis. Each cytokine has a distinct role in different target cells, and this cell-specific response depends, in part, on the expression level of gp130 cytokine receptor [52]. For instance, IL-6R is highly expressed in Schwann cells but not in DRG neurons and thus IL-6, and in contrast to LIF and CNTF, activates STAT3 in Schwann cells but not in DRG neurons.

1.3.2.1. Interleukin 6 (IL-6):

IL-6 rapidly increases in neurons 1 to 4 days after nerve transection. It is not detected in uninjured neurons but is found in abundance during development. IL-6 is synthesised in abundance with astrocytes and microglia as the main source in the CNS. IL-6 mRNA was detected within a subpopulation of medium and large size DRG neurons [53]. This observation suggests some cell specific gene regulation similar to that which restricts the distribution of neurofilament and neurotrophin receptors in the sensory neurons [54]. IL-6 acts on both neuronal and non-neuronal cells and its autocrine action include changes in the synthesis of peptides, growth associated molecules and possibly the synthesis of trophic molecules.

1.3.2.2. Ciliary neurotrophic factor (CNTF):

CNTF was first identified as a growth factor that promotes chick ciliary ganglion neuron survival. Later it was found to have an effect on a wide range of peripheral and central neurons [55]. It was noticed that CNTF levels were downregulated in the nerve distal stumps after axonal

injury. These recovered to the original levels after axon regeneration [56]. CNTF acts on neurons via its cell surface receptor CNTF receptor α (CNTFRα), gp130 and LIF receptor (LIFR). When CNTF binds to CNTFRα, it forms an active trimeric receptor complex at the cell membrane due to heterodimerization of gp130 and LIFR. This complex activates the family proteins (JAK1/JAK2) leading to tyrosine phosphorylation and nuclear translocation of STAT3. Phosphorylated STAT3 dimers then activate target gene transcription (Fig.12). CNTF activates PI-3K and MAPK as well and all three pathways have an important role in mediating CNTF neuropoietic function [56]. CNTF has neuroprotective properties. It promotes survival and axonal regeneration in injured neurons. Its efficacy is more noticeable in conditions that lack anti-oxidants which prove its high activity in an oxidative environment. In support of this finding, CNTF was able to reverse nerve dysfunction in neurodegenerative disorders associated with oxidative stress such as diabetes [57].

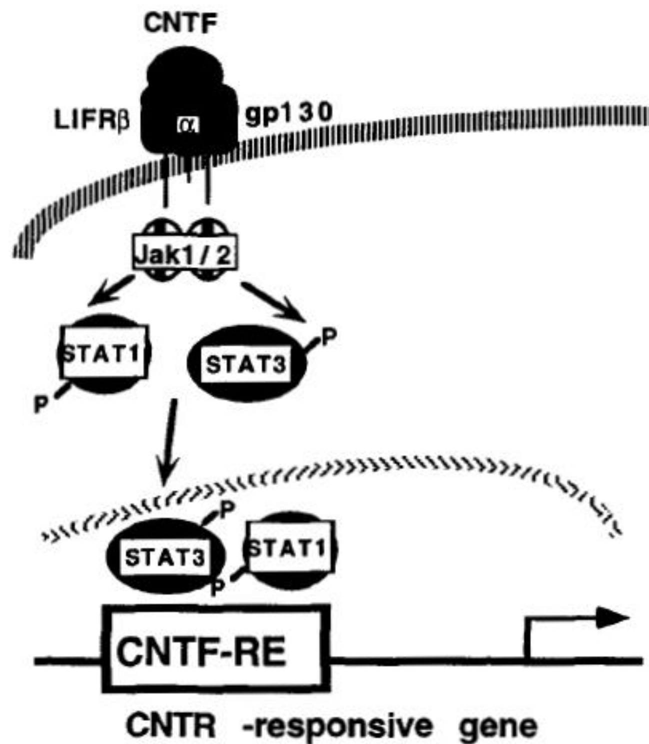


Fig.12: CNTF signaling involves the Jak-STAT pathway. CNTF binds to the CNTF-specific a receptor and to the leukemia inhibitory factor (LIF)-receptor subunit and the related gpl30 forming a trimeric receptor. This activates the associated Janus tyrosine kinase (Jak 1 or Jala), which phosphorylates STAT-1 and/or STAT3. The phosphorylated proteins translocate to the nucleus, where they bind to CNTF-responsive elements (CNTF-RE) on CNTF-responsive genes and thereby initiate transcription [58]

CNTF increased the number of viable neurons and neurite-bearing cells in culture of adult rat DRG. NGF and GDNF only promoted neurite elongation with no significant enhancement for neuronal survival. CNTF stimulated a wider subpopulation of DRG and was able to enhance the survival of both small and large diameter DRG. The NGF and GDNF effect was only seen in small diameter neurons. The abundant expression of the CNTF receptor in nearly all neurons could explain its effect on broad subpopulations of neurons [56]. MAPK, PI-3K and JAK/STAT are involved in CNTF induction of neurite outgrowth. Also its survival promoting effect is mediated by the same pathways with the exception of MAPK [56].

As with other CNS neurons, retinal ganglion cells (RGC) fail to regenerate after optic nerve axotomy. This is a consequence of the inhibitory effect of MAG and inadequate intrinsic ability of CNS neurons to regrow in the CNS milieu. On the other hand, intravitreal application of CNTF protected injured RGCs from cell death and promoted axon regeneration in the optic nerve [59]. This could be due to CNTF direct and rapid activation of both JAK/STAT3 and PI-3K signaling pathway specifically in RGCs. An indirect effect may also be mediated by induction of CNTF expression in the retinal astrocytes which is mediated by MAPK/ERK. This endogenous CNTF leads to further activation of JAK/STAT3 and enhancement of axonal growth, so the axon growth enhancement by CNTF is partially mediated indirectly by a mechanism that depends on endogenous CNTF expression [60]. Another *in vivo* study suggests

that the role of CNTF in mediating a conditioning effect is due, at least in part, to induction of the transcription factor c-jun and three effector molecules Reg-2, SPRR1A and GAP-43 [61].

cAMP:

cAMP has an important role in neuronal survival, modulation of axonal guidance and enhancement of neurite outgrowth [62]. The basal levels of cAMP are usually low but they increase 1 day after injury. Direct injection of cAMP mimics the effect of peripheral lesion and induces dorsal column regeneration through the PKA signaling pathway [63]. Increasing intracellular levels of cAMP through administration of forskolin (a drug that activates adenylyl cyclase and increases intracellular levels of cAMP) to cultured RGC enhanced the growth promoting effect of CNTF, while forskolin alone modestly increased the growth. This observation suggests that the increase in cAMP was additive to the effect of CNTF on RGC, yet cAMP is not crucial for CNTF action [60]. On the other hand, intraocular administration of cAMP analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) was not able to induce growth in axotomized adult RGCs. However it was able to potentiate CNTF induced axonal regeneration in RGCs [64]. This enhancement is essentially a result of the PKA signaling pathway with the involvement of other pathways such as PI-3K, JAK/STAT3 and MAPK. Activation of PI-3K and MAPK lead to inactivation of the pro-apoptotic Bcl-2-associated death promoter (BAD) and activation of anti-apoptotic bcl-2 proteins [65] causing enhancement of RGC survival. In addition, cAMP potentiation of CNTF growth promoting effect is a result of upregulation of CNTFR α in the retina [62]. The induction of growth accompanied by application of cAMP is mediated, in part, through promoting the synthesis of neuropoietic cytokines such as LIF and IL-6 within the DRG as well as induction of cAMP regulated transcription factor response element binding protein (CREB) which acts within the neuron to promote its survival [61].

1.3.2.3. Leukemia inhibitory factor (LIF):

LIF is induced after nerve injury. LIF mRNA starts to increase 6 hours after the injury at the site of the lesion but not further distally. Twenty four hours later, LIF mRNA was detected in the entire distal stump and remained high for 3 days. This biphasic pattern of LIF induction is similar to what is seen for NGF. This observation suggests that these two factors might be regulated by the same cellular and molecular mechanism in injured nerves [66]. Schwann cells synthesise LIF under normal conditions, and despite the ability of IL-1β to stimulate LIF production in synovial fibroblasts [67], it doesn't cause any further induction of Schwann cells to produce LIF [66].

Following nerve injury, neurons undergo a two phase regenerative responses. The first phase within the first 48 hrs is characterized by short and highly arborized sprouting with limited linear extension. In the second phase, growth is characterized by enhanced neurite elongation and reduction in neurite branching [68]. Although LIF had been shown to promote neuronal survival in developing systems [69], there was no significant change in the amount of adult neurons surviving after LIF treatment [70]. LIF induces transcription factors such as galanin and Reg-2 which might be responsible its ability to promote axonal growth. LIF is thought to be responsible for the elongating phase of neurite extension since its role in regeneration is mostly to mediate neurite elongation rather than arborizing. Its effect is mainly noticed in the small diameter neurons. LIF is a key component of the conditioning response. The conditioning effect was significantly depleted in the sensory neurons of LIF -/- mice. The effect was completely enhanced after addition of exogenous LIF. It has been suggested that the intrinsic growth state of sensory neurons is closely regulated by LIF [70].

1.3.3. STAT3 and the conditioning effect:

Injuries to peripheral and central axons lead to different molecular changes in DRG neurons, but only the peripheral changes cause induction of growth. In the central axon, regeneration can happen if we overcome the inhibitory environment by increasing the intrinsic neuronal growth ability [71]. Studies have suggested involvement of gp130 in mediating the growth in dorsal column axons through the STAT3 pathway. Conditioning effects on neurons after axotomy is blocked in mice with null mutation of genes for LIF or IL-6 [72]. STAT3 is phosphorylated in DRG neurons after peripheral but not dorsal column injury. Blocking STAT3 activation caused inhibition of neuronal response to sciatic nerve transection. As a consequence, dorsal column axons fail to regenerate after conditioning injury. This observation suggests that the injury induced signaling pathway is crucial for increasing neuronal growth after a conditioning lesion [71]. Neuropoietic cytokines are necessary for a full conditioning response and STAT3 alone is sufficient to stimulate neurite outgrowth [73].

1.3.4. Endogenous inhibition of STAT3 signaling:

Suppressor of cytokine signaling (SOCS3) is a protein that provides feedback inhibition of STAT3 signaling through binding to tyrosine residue 759 of gp130. It has a very important function in various types of cells including macrophages and neurons. Since STAT3 signaling is involved in promoting axonal growth in neurons, blocking STAT3 signaling by SOCS3 partially stopped neurite outgrowth. SOCS3 inhibited growth through blocking nuclear translocation of endogenous and exogenous STAT3. The SOCS3 partial blocking could be due to activation of SOCS3 in only a selected amount of neurons, or because it is partially activated in all neurons [73]. Besides its inhibition of STAT3 signaling, SOCS3 inhibits neurite outgrowth through

interfering with gp130/JAK/STAT3 signaling and interacting with other signaling molecules such as growth hormone receptor, insulin receptor and gp120 rasGAP [73].

1.3.5. Cytokines in pain:

Similar to IL-1β, TNFα levels are increased after nerve injury. At the same time TNFα promotes IL-1β production, and this results in further production of IL-1β. Acute application of TNFα topically increases action potential firing in sciatic nerve and activation of C-fibres in the sural nerve [74]**.** When TNFα or Il-1β were applied for a longer time, they increased excitability and sensitivity of sensory neurons through increasing the production of COX II in both neuronal and non-neuronal cells. This in turn induced prostaglandin synthesis (PGE2 and PGI2) and altered the expression of prostanoid receptors [75].

TNFα and IL-1β modulate nociception directly by activating neurons, and indirectly by infiltration of immune cells and activating non-neuronal cells. Immune cells are involved in pain after nerve injury. Both neutrophils and T-lymphocytes have been shown to influence pain sensitivity after nerve trauma [76]. After nerve injury, TNFα and IL-1β induce infiltration of neutrophils and M1 monocytes/macrophages into the distal stump. This is found to be directly involved in neuropathic pain since IL-1R1 -/- and TNFR1 -/- mice had reduced nociceptive hypersensitivity and were less sensitive to mechanical pain after injury [77]. Regeneration in these mice was severely affected due to blocking of cytokine signalling. On the other hand, reducing neutrophils decreased neuropathic pain after injury without interfering with nerve recovery or growth. This observation suggests that blocking neutrophil entry after peripheral nerve injury is better than neutralizing proinflammatory cytokines in neuropathic pain treatment [77].

IL-1β is involved in mediating pain and hyperalgesia. It increases mechanosensitivity of the peripheral receptive field in DRG. Many other mediators such as substance P, bradykinin, chemokines and prostaglandins are involved in nociception and signal via G protein coupled receptors (GPCRs). G protein coupled receptor kinase 2 (GRK2) regulates the responsiveness of GPCRs. It phosphorylates agonist-occupied GPCRs resulting in arrestin binding and receptor internalization. Thus, GRK2 activity could affect nociception [78].

Bradykinin (BK) activates bradykinin receptor 2 (B2R) which undergoes desensitization after ligand binding. IL-1β increased the responsiveness of B2R and weakened its internalization in DRG; however, it didn't increase the proportion of neurons expressing B2R. On the other hand, IL-1β was found to decrease GRK2 levels in DRG, besides an extensive co-localization of B2R-like IR, IL-1R1-like IR, and GRK2-like IR in many DRG neurons. This observation suggests that IL-1β reduction of B2R internalization was mediated by IL-1β down regulation of GRK2 [78].

1.4. Diabetes:

Diabetes is a metabolic disorder characterized by increased levels of blood sugar. Nearly 347 million people worldwide have diabetes [79]. In North America 19 million people currently have diabetes which has an incidence of 6%. Diabetes is caused by lack of insulin synthesis due to destruction of pancreatic β cells. This type is known as insulin dependent diabetes mellitus (IDDM) or type 1 diabetes. Type 1 diabetes accounts for around 10% of the diabetic patients. Many risk factors can be attributed to type 1 diabetes such as genetic and environmental factors. The other major type of diabetes is non-insulin dependent diabetes mellitus (NIDDM) also known as type 2 diabetes. It is characterized by lack of insulin sensitivity and accounts for

around 90% of diabetic patients. Early signs of diabetes are polyuria, polydipsia and polyphagia, however in the long term, if diabetes is not well controlled it leads to serious complications such as retinopathy, nephropathy, cardiomyopathy and neuropathy.

1.4.1 Diabetic neuropathy:

Half of the diabetic patients are at risk of developing neuropathy. Patients with clinical diabetic neuropathy mostly exhibit a distal symmetrical form of the disorder. Signs and symptoms affect more-distal parts of the lower limbs and eventually the proximal parts of the upper limbs, indicating that the longest nerve fibers are affected first [80]. Diabetic neuropathy is manifested by deficits in motor and sensory nerve conduction velocity. It is also associated with structural changes in peripheral nerve including endoneurial microangiopathy, abnormal Schwann cell pathology, axonal degeneration, paranodal demyelination and loss of myelinated and unmyelinated fibers [81]. The neuropathy could occurs in sympathetic, motor and sensory neurons. The autonomic neuropathy arises from damage to small myelinated and unmyelinated nerve fibres which results in autonomic failure together with reduced thermal and pain sensation [82]. Autonomic neuropathy could cause cardiovascular disturbances, postural hypotension, gastroparesis and bladder atony. Sensory neuropathy starts with progressive unpleasant sensory sensations in the feet including tingling, burning pain and contact pain. The loss of sensation progresses to ulceration and in severe cases leads to amputation.

1.4.2. Pathogenesis of diabetic neuropathy:

Beside the hyperglycemic state and the metabolic reactions which contribute to the development of neuropathy, the dysregulation of insulin action and signal transduction also play an important role in the progress of structural degeneration and fiber loss in diabetes [83]. Lack of insulin

action affects the expression of neurotrophic factors and their receptors in neurons leading to sever axonal atrophy and loss. This is more profound in type 1 diabetic neuropathy due to lack of insulin secretion [83]. Different hypotheses have been proposed to understand the etiology underlying diabetic neuropathy.

1.4.2.1. Polyol pathway activation:

The entry of glucose into neuronal cells doesn't require the action of insulin, thus glucose enters freely to neurons where it is used in energy production. In normal conditions, the unused glucose is converted to glucose-6-phosphate by the hexokinase pathway and only a few molecules go through the polyol pathway where they are converted to sorbitol by aldose reductase (AR) and sorbitol dehydrogenase. However, due to the hyperglycemic state in diabetes, the hexokinase pathway gets saturated; thus, more glucose flux occurs to the AR pathway and increases the production of sorbitol. The accumulated sorbitol increases osmotic stress and leads to nerve damage. The consumption of NADPH by the polyol pathway compromises the glutathione (GSH) cycle resulting in increased production of superoxide and hydroxyl radicals and leading to oxidative stress [84] (Fig.13).

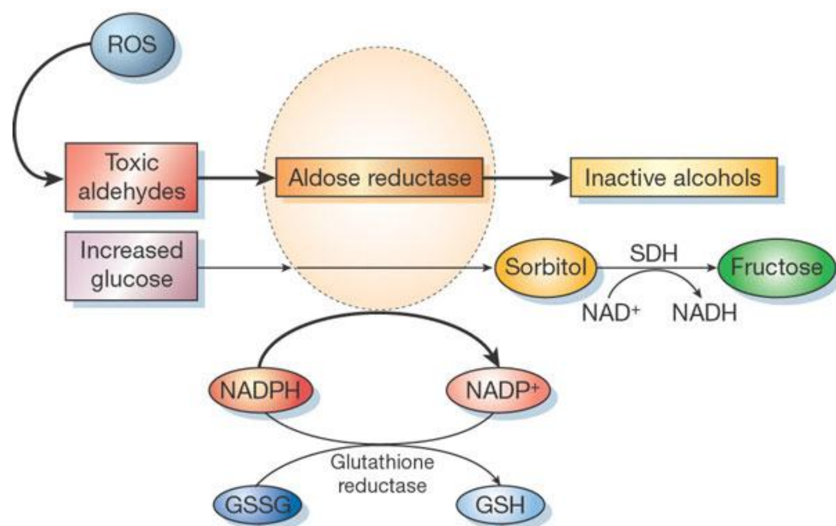


Fig.13: Aldose reductase and the polyol pathway. Aldose reductase reduces aldehydes generated by reactive oxygen species (ROS) to inactive alcohols, and glucose to sorbitol, using NADPH as a co-factor. In cells where aldose reductase activity is sufficient to deplete reduced glutathione (GSH), oxidative stress is augmented. Sorbitol dehydrogenase (SDH) oxidizes sorbitol to fructose using NAD+ as a co-factor [84].

1.4.2.2. Glycated proteins:

Hyperglycemia causes non-enzymatic glycation of proteins. An alteration to the protein structure happens through addition of open chain glucose to lysine groups on proteins which leads to abnormal protein function and activation of the receptor for advanced glycation end products (RAGE). Some of these glycosylated proteins such as N(epsilon)-carboxymethyllysine, have been implicated in the pathology of diabetic neuropathy as they were detected in sural, peroneal and saphenous nerves of human diabetics [85].

1.4.2.3. Neurotrophic factors

NGF and other neurotrophins have a crucial role in expression of the adult neuronal phenotype. Neurotrophin levels are reduced in diabetic patients which impairs maintenance of the normal phenotype of adult sensory neurons. In rodent models of diabetes, there is decreased retrograde axonal transport of NGF and decreased support of NGF-dependent sensory neurons along with downregulation of some neuropeptides, substance P and calcitonin gene-related peptide (CGRP). These incidents were caused by deficits in NGF expression and downregulation of its high-affinity receptor trkA. Also a decrease in the response to NT-3 as well as an NT-3 deficiency could have a major effect on the loss of protective sensation in diabetic neuropathy [86].

1.4.2.4. Oxidative stress

When free radicals overcome antioxidants, it results in a state called oxidative stress. Free radicals bind to proteins and nucleic acids leading to impairment of their function such as cell signaling and transportation. Excessive production of reactive oxygen species (ROS) in oxidative stress induces neurodegeneration [87]. Oxidative stress arises, in part, from vascular abnormalities and coupled with microangiopathy in the nerve is considered one of the main causes of nerve damage in diabetes in humans. A decrease in antioxidants, plasma GSH and GSH metabolizing enzymes was found in both type 1 and 2 diabetes which indicates an oxidative stress in diabetes [88]. Oxidative stress in nerve tissue happens in diabetes due to impaired neurotrophic factors function, decreased nerve blood flow and increased polyol pathway flux [89].

1.4.2.5. Mitochondrial dysfunction

Neurons have wide variations in ATP demands, but diabetes decreases their mitochondrial function and lowers their spare respiratory capacity, which limits the neurons ability to meet the increase in energetic demands. For this reason, mitochondrial dysfunction is considered as a central mediator in development of many neurodegenerative diseases including diabetic neuropathy [90]. Sensory neurons of diabetic rodents exhibit an abnormal mitochondrial phenotype that contributes to the etiology of diabetic neuropathy [90]. Lumbar DRG isolated from STZ-induced diabetic rat exhibit a reduced rate of oxygen consumption and depression of respiratory complex activity that correlated with suppression of expression of proteins of the respiratory chain complexes [91]. Changes in mitochondria number and size have been observed in the Schwann cells of diabetic sensory neuropathy in human and animal models [92].

Dysfunction in mitochondria has been also noticed in pre and post synaptic elements in prevertebral sympathetic ganglia of diabetic mice [93].

Neuronal death:

Pathological cellular insults or physiological death signals can activate apoptotic pathways. Any dysfunction or abnormalities in mitochondrial membrane potential, ROS generation or activation of the permeability transition pore (PTP) could lead to activation of apoptotic pathways and cell death. Although apoptosis of neurons in the DRG of STZ-induced diabetic rat has been reported in sensory diabetic neuropathy [94], there is no evidence of cell death triggered by apoptosis in both experimental and human forms of diabetic neuropathy [95].

1.4.3. Cytokines as a treatment for diabetic neuropathy:

Some cytokines have positive effects on neurodegenerative disorder models such as diabetic neuropathy. Many cytokines have been studied and found to reverse neuronal damage caused by diabetes through different mechanisms.

CNTF had reduced bioactivity in the streptozotocin (STZ)-induced diabetic rat a model of type 1 diabetes [96], and treatment with CNTF reversed the diabetes-induced deficit in nerve regeneration after systemic application [57]. CNTF was able to enhance neurite outgrowth in sensory neurons derived from 2-5 month STZ-induced diabetic rat models through activation of the NF-κB signaling pathway. Furthermore, CNTF reversed the deficit in mitochondrial function of diabetic neurons via NF-κB [97]. This enhancement of mitochondrial function allowed neurons to increase ATP synthesis under stress conditions to meet the high energy demand by the growth cone. In terms of the basal levels of neurite outgrowth, diabetic neurons had lower levels than the age matched control. However, upon application of CNTF, the response was

similar in the diabetic and control cells. Diabetic neurons had no lack of sensitivity to CNTF since no decrease in CNTFRα was found. Nevertheless, factors downstream of NF-κB such as a decrease in the expression of structural proteins could be behind the lower levels of basal neurite outgrowth in diabetic neurons [97].

TNFα was shown to be downregulated in lumbar DRG of 2-5 month STZ-induced diabetic rats [98]. NF-κB activity was impaired in diabetic neurons as well. TNFα activated NF-κB and enhanced neurite outgrowth mainly in medium to large diameter neurons, and this enhancement was abolished by an NF-κB blocker. This observation proved that TNFα enhancement of neurite outgrowth in diabetic neurons was mediated by NF-κB [98]. Thus, it has been suggested that reduced expression of TNFα in lumbar DRG in diabetes is contributing to the impairment of axonal growth seen in diabetic sensory neurons.

Similar to TNFα and CNTF, IL-1β expression was reduced in sensory neurons and peripheral nerves in DRG isolated from STZ-induced diabetic rats [99]. It was found that IL-1β application to cultures derived from diabetic rats augmented neurite outgrowth partially via the STAT3 pathway. IL-1β was able to reverse mitochondrial dysfunction in cultured diabetic neurons. This effect was mediated by the JAK/STAT3 pathway since it was noticed that STAT3 localized in mitochondria and is known to interact with complex I components of the electron transport system [99]. Cultured sensory neurons expressed IL-1β and exhibited endogenous transcriptional activity for the IL-1β promoter [99]. A reduction in neurite outgrowth was observed after blocking endogenous IL-1β. This suggests that IL-1β has an autocrine pathway where endogenous IL-1β promotes neurite outgrowth. As a result, IL-1β activates STAT3 which targets multiple sites in the axons such as the mitochondria and microtubule networks to promote neurite outgrowth and enhance axonal plasticity [99].

Local administration of IL-1β on transected and sutured rat sciatic nerves for 2 weeks enhanced sensory functional recovery, as evaluated by toe pinch test [100]. It also increased the number and the area of neurofilament-positive axons. This data proved that IL-1 may contribute to sensory nerve regeneration following sciatic nerve injury by promoting axonal extension *in vivo* [100].

Moreover, IL-1 contributes to neuropathic pain through activation of a cascade of events, leading to ectopic afferent discharges that trigger and maintain central sensitization [101]. However, chronic pharmacological blockade of IL-1 signaling by IL-1ra markedly reduced neuropathic pain symptoms, as reflected by attenuated mechanical allodynia, autotomy, and spontaneous ectopic activity. Thus, blockade of IL-1 signaling may provide effective pain relief for patients suffering from neuropathic pain. [102].

1.5. Interleukin-17 (IL-17):

IL-17 is a proinflammatory cytokine that is exclusively produced by T-helper cells (TH) and induced by IL-23. It contributes to the pathogenesis of inflammatory diseases by amplifying the effector immune response that increases tissue damage [103]. The members of IL-17 family are six cytokines that share 20-50% homology to IL-17 namely IL-17 A, B, C, D, E and F. The receptor of IL-17A is IL-17R which is a single transmembrane protein that is expressed in all tissues [104]. IL-17 promotes the production of other cytokines and chemokines such as IL-6 and IL-8 and has been shown to augment many of the destructive effects of IL-1 and TNFα in cartilage, synovium, and meniscus [105]. IL-17 and its receptors bear no resemblance to any other known families of protein which gives them a very distinct and potent signaling system.

IL-17R activates ERK , JNK and p38 MAPK pathways (Fig.14) [106] which results in upregulation of IL-6, IL-1 and NF-κB [107].

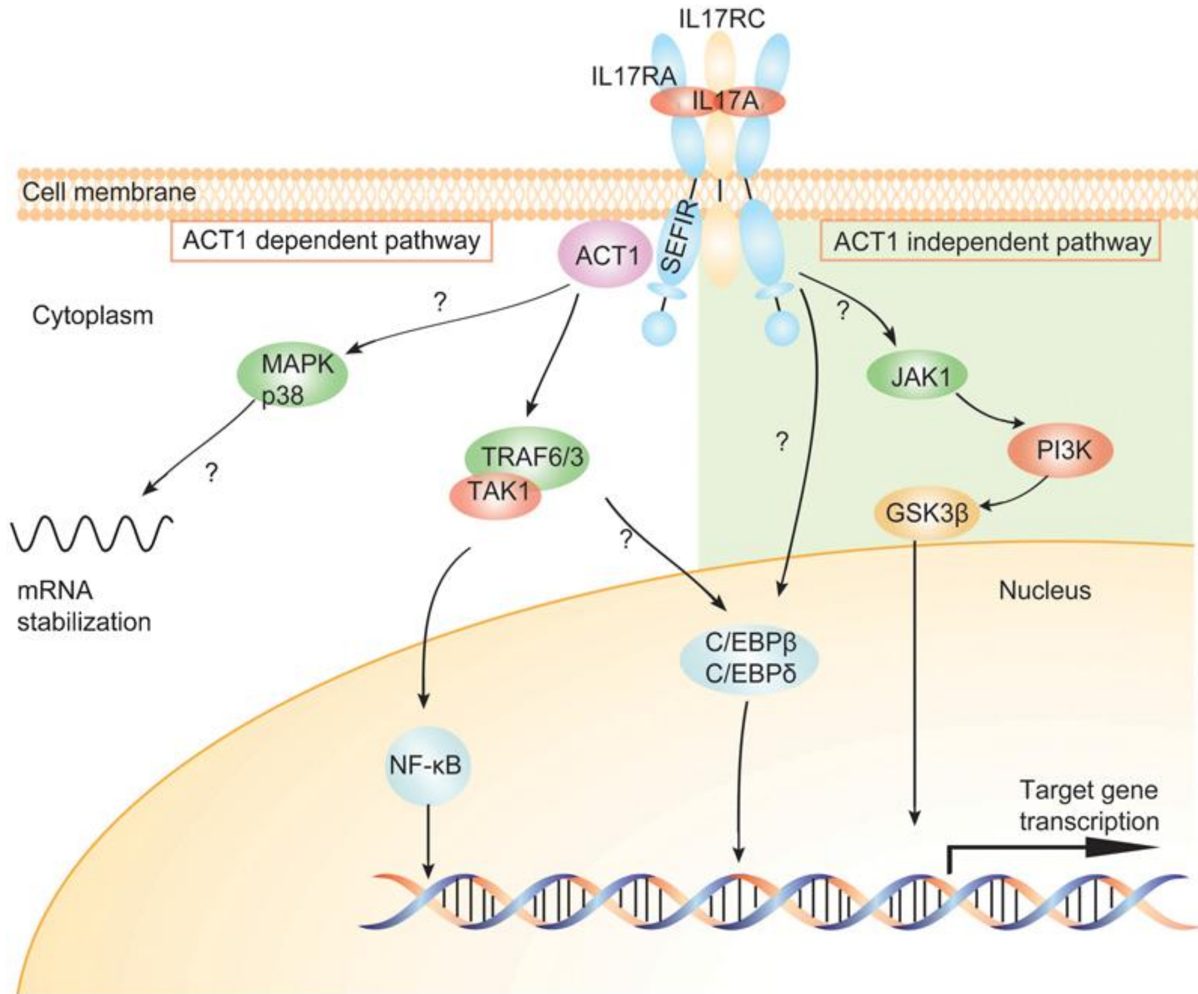


Fig.14: Signal pathways of IL-17A. The IL-17R complex is composed of two IL-17RA and one IL-17RC; both subunits encode SEFIR domains. After activation, the intracellular IL-17A signaling includes ACT1-dependent and -independent downstream pathways. Left: the ACT1-dependent pathway: IL-17RA engages its SEFIR domain to recruit the adaptor protein ACT1. ACT1 contains a TRAF6-binding motif and can bind TRAF6, TRAF3 and TAK1, which subsequently leads to activation of the canonical NF-κB pathway. ACT1 is also required for the activation of MAPK p38, and this pathway leads to the stabilization of mRNAs, particularly those encoding chemokines and cytokines. Right: the ACT1-independent pathway involves JAK1 and PI3K, followed by subsequent inactivation of GSK-3β. Both ACT1-dependent and -independent pathways contribute to the activation of transcription factors

C/EBP-β and C/EBP-δ. ACT1, nuclear factor-κB activator 1; C/EBP, CCAAT/enhancer-binding protein; GSK-3β; JAK1; MAPK,; NF-κB,; PI3K,; SEFIR, SEF/IL-17R; TAK1, TGF-β-activated kinase 1; TRAF6 [108].

1.5.1. IL-17 in neurons:

IL-17 plays an important role in autoimmune disorders that are accompanied by neuronal dysfunction such as multiple sclerosis (MS) and irritable bowel disease (IBD). Despite the general consensus that IL-17 is a damaging proinflammatory cytokine, there is emerging evidence that it plays a role in homeostasis and tissue repair [109]. In a recent study, systemic treatment with an IL-17 function-blocking antibody attenuated restoration of sub-basal nerve plexus density after corneal injury. The arrival of trophic factor-secreting neutrophils were behind the IL-17 neurotrophic effect in cornea [110]. In contrast to the indirect effect of IL-17 on the cornea, another study found a direct effect of IL-17A on the superior mesenteric ganglion (SMG). IL-17A increased neurite outgrowth in adult sympathetic postganglionic neurons by activation of NF-κB and suppression of Ca^{+2} influx [111]. An increase in Ca^{+2} can abolish neurite sprouting [112]. Depolarization in neurons can inhibit neurite outgrowth due to inward current through N-type voltage-gated calcium channels (VGCCs) [113]. IL-17 inhibition of Ca^{+2} current in SMG neurons through inhibition of VGCCs in cell body and growth cone promoted neurite outgrowth. This process was mediated by NF-κB. Blocking NF-κB prevented IL-17 induction of growth and alteration of Ca^{+2}.

IL-17A can act as a pain mediator and play a role in mechanonociception. Its application on isolated rat sensory neurons activated signaling cascades and enhanced neuronal excitability [114]. IL-17A induced a long term sensitization of rat joint nociceptor to mechanical stimuli and this effect was not prevented by either IL-6 or TNFα neutralization. It was also found that IL-17A is mainly a sensitizer for C fibers since Aδ fibers were only sensitized at a dose that was

toxic for C fibers [114]. IL-17RA is highly expressed in the majority of rat DRG neurons. However, the amount of IL-17R in neurons is higher than TNFR and IL-1R which are only localized in 30-50% of DRG neurons [78, 115]. IL-17A was able to activate PKB/Akt and ERK. This pathway is usually phosphorylated once neurons are hyperexcited [116]. Similar to prostaglandins and TNFα, IL-17 regulates the activation of voltage gated ion channels because it generates hyperexcitability of small to medium sized DRG neurons within a few minutes of application. The previous findings proved that IL-17A action on mechanosensitivity is coming from a direct action on neurons. IL-17 is involved in neuropathic pain since it is highly expressed in satellite cells of DRGs which plays an important role in pathologic destruction and repair following neuronal injury [117].

1.6. Conclusion:

Cytokines are known for their function in the immune system and their part in mediating inflammation. However, their effect is not limited to immune cells. Some cytokines have been shown to have a neurotrophic capacity. They have an important role in mediating nerve response to injury and promoting nerve regeneration in the peripheral nervous system. Thus, in view of the fact that cytokines are part of the wound healing process after nerve injury, they are becoming a major interest in drug targeting in the treatment of neurodegenerative disorders. In addition, these findings encourage further exploration of other cytokines that might be associated with the neuronal regeneration process.

Giving the previous facts, I studied the effect of IL-17A on the phenotype of sensory neurons derived from control or diabetic rats. I **hypothesized** that IL-17A induces neurite outgrowth in

sensory neurons through signaling pathways that enhance mitochondrial function. IL-17A can also reverse impaired nerve regeneration associated with diabetes.

Our **objectives** were to determine the ability of IL-17A to enhance neurite outgrowth in cultured sensory neurons and to investigate the signalling pathways activated by IL-17A and mechanistically linked to neurite outgrowth. Furthermore, since mitochondrial dysfunction is a feature of the pathogenesis of diabetic neuropathy, and since axon outgrowth consumes high levels of ATP, I investigated the ability of IL-17A to improve mitochondrial function of sensory neurons.

Chapter 2:

Research design and methods

The methods of this project were adapted and copied from protocols and papers published from Dr. Paul Fernyhough's lab [97-99] [118, 119].

2.1. Adult rat DRG sensory neuron culture

DRG sensory neurons from adult male Sprague-Dawley rats were isolated and dissociated using previously described methods [120]. Rats were either control or 3-5 month STZ-diabetic rats. Rats were made diabetic with a single i.p. injection of 75 mg/kg STZ (Sigma) which causes degeneration of Langerhans islet beta cells and induces experimental type 1 diabetes mellitus. Within weeks of induction, STZ-diabetic rats undergo deficits in nerve conduction velocity, abnormalities in nerve blood flow and change in pain perception threshold [121]. All animal protocols carefully followed the Canadian Committee on Animal Care (CCAC) guidelines. DRG neurons were plated onto poly-d-L-ornithine hydrobromide and laminin-coated multi-well plates for neuronal survival and axon outgrowth studies and 25 mm glass cover slips for immunocytochemistry. Neurons were grown in defined Hams F-12 medium with modified Bottenstein and Sato's N2 medium (with no insulin) containing 0.1 mg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite, 1 mg/ml BSA, and supplemented with neurotrophic factors (NTFs): 0.1 nM insulin, 0.1 ng/ml nerve growth factor (NGF), 0.1 ng/ml neurotrophin-3 (NT-3), 1 ng/ml glial cell line derived neurotrophic factor (GDNF). Neurons derived from control animals were exposed to 10 mM glucose and 0.1 nM insulin. STZ-induced

diabetic neurons were cultured in the presence of 25 mM glucose without adding insulin to mimic physiological conditions.

2.2. Assessment of total neurite outgrowth

Rat neurons grown on glass cover slips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 15 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 5 min. Cells were then incubated in Blocking Buffer (Roche, Indianapolis, IN, USA) diluted with FBS and 1.0 mM PBS (1:1:3) for 1 h then rinsed three times with PBS. The primary antibody used was a β -tubulin isotype III (1:1000) neuron-specific from (Sigma Aldrich, Canada). The antibody was added to all wells and plates were incubated at 4 C overnight. The following day, the coverslips were incubated with CY3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature, and then mounted and imaged using a Carl Zeiss Axioscope-2 fluorescence microscope equipped with an AxioCam camera. Images were captured using Axio-Vision4.8 software [97]. Quantification of total neurite outgrowth in rat cultures was done through measuring the mean pixel area of the captured images using ImageJ software, which was adjusted for the cell body signal. All values were adjusted for neuronal number [118]. In this culture system the level of total neurite outgrowth has been previously validated to be directly related to an arborizing form of axonal plasticity and homologous to collateral sprouting *in vivo* [68].

2.3 Western blotting for P-ERK, P-AKT, OXYPHOS and Porin

Rat DRG neurons were harvested after either 3 hr or 24 hr of culture in ice-cold stabilization 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 [118]. Proteins assay was performed DC protein assay (BioRad; Hercules, CA, USA) and Western blot analysis was conducted. The samples (5 µg total protein/lane) were resolved on a 10% SDS-PAGE gel, and electroblotted (100 V, 1 h) onto a nitrocellulose membrane. Blots were then blocked in 5% nonfat milk (containing 0.05% Tween) overnight at 4-C, rinsed in TBS-T, and then incubated with the primary antibody for P-ERK, P-AKT (1:1000; Santa Cruz Biotechnologies, CA), OXYPHOS (1:500; MitoScience), or Porin (1:1000; MitoScience). Total ERK (1:1500; Santa Cruz Biotechnologies, CA) was used as a loading control (usually its expression does not change in DRG under diabetic conditions) [118]. Secondary antibody was applied for 1 h at room temperature after 5 - 6 washes of 10 min in PBS-T. The blots were rinsed, incubated in Western Blotting Luminol Reagent (Santa Cruz Biotechnologies, CA, USA), and imaged using the BioRad Fluor-S image analyzer [97].

2.4. Measurement of mitochondrial respiration in cultured DRG neurons

An XF24 Analyzer (Seahorse Biosciences, Billerica, MA, USA) was used to measure the neuronal bioenergetic function of mouse and rat cultures. The XF24 creates a transient 7 µl chamber in specialized 24-well microplates that allows for the oxygen consumption rate (OCR) to be monitored in real time [122]. Culture medium was changed 1 h before the assay to unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) supplemented with 1 mM pyruvate, and 10 mM D-glucose. Neuron density in the range of 2000-4000 cells per well gave linear OCR. Oligomycin (1 µM), carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP; 1.0 uM) and rotenone (1 µM) + antimycin A (1 µM) were injected sequentially through ports in the Seahorse Flux Pak cartridges. Each loop was started by mixing for 3 min, and then delayed for 2 min and OCR measured for 3 min [97]. This 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 respiratory capacity and nonmitochondrial oxygen consumption [122]. Oligomycin inhibits the ATP synthase leading to a build-up of the proton gradient that inhibits electron flux and determines the coupling efficiency. Injecting FCCP to the culture determines the maximal capacity to reduce oxygen. Finally, rotenone (complex I inhibitor) + antimycin A (complex III inhibitor) were injected to inhibit the flux of electrons, and stop the oxygen consumption at cytochrome c oxidase. The remaining OCR reveled after this intervention is primarily non-mitochondrial [97]. After finishing the OCR measurement, cells were either immediately fixed and stained for β tubulin III to count the cells per well, or harvested for a protein assay. The stained plates were then inserted into a Cellomics Arrayscan-VTI HCS Reader (Thermo Scientific, Pittsburgh, PA, USA) equipped with Cellomics Arrayscan-VTI software to determine total neuronal number in each well. Data were either expressed as OCR in pmol/min for 1000 cells or in pmol/min for 10 μg protein [97].

2.5. Measuring mitochondrial mass

MitoTracker Deep Red FM (Invitrogen) stain was used to measure the mitochondrial mass since it detects mitochondria irrespective of mitochondrial potential across the inner mitochondrial membrane. MitoTracker Deep Red is well retained after fixation allowing for immunostaining afterward. DRG neurons from control rat were plated on 35 mm high μ-Dish (Ibidi, CA) for 24 hr. Cells were incubated with 100 nM MitoTracker Deep Red FM for 30 min at 37 C. Cells were then washed and fixed as described above and stained for β -tubulin isotype III (1:500) as a primary antibody and Alexa fluor 488 (1:1000) as a secondary antibody. Fluorescence was detected with a Carl Zeiss LSM510 confocal inverted microscope and fluorescent intensities for neurons stained with MitoTracker Deep Red were measured using ZEN software.

2.6. Spectrophotometric measurements for COX-IV enzymatic activity

Enzymatic activity of cytochrome *c* oxidase (COX-IV) in cultured DRGs was measured spectrophotometrically using a temperature-controlled Ultrospec 2100 ultraviolet-visible spectrophotometer (Biopharmacia Biotech, Uppsala, Sweden). COX activity was measured at 25 C for 2 min by following the rate of oxidation of reduced cytochrome *c* at 550 nm. Cytochrome *c* was reduced by sodium dithionite and excess dithionite was removed by filtration through a Sephadex G-25 column. The efficacy of reduction and the auto-oxidation rate were controlled. COX-IV activity was measured by monitoring the absorbance decrease of reduced cytochrome *c* at 550 nm. The reaction was started by addition of 30 µM reduced cytochrome *c* into 20 mMol/l phosphate buffer containing cultured DRG (6.3 µg protein) solubilized with 0.12% lauryl maltoside to facilitate the access of substrate to the mitochondria [91].

2.7. Statistical analysis

Data were expressed as mean +/- SEM and were subjected to one-way ANOVA with post-hoc comparison using Dunnett's test for dose response experiments and when a comparison of treatment groups to a single control was needed. Tukey’s test was also used to find means that are significantly different from each other between different treatment groups. In addition, two-tailed student’s t test was performed on some data. GraphPad Prism software was used to perform the statistical analysis. For each experiment, one rat was used with cultures performed in replicate (n=3 or 4). Some experiments were independently repeated for 1 or 2 times. Data presented is for representative experiment from each study.

Chapter 3:

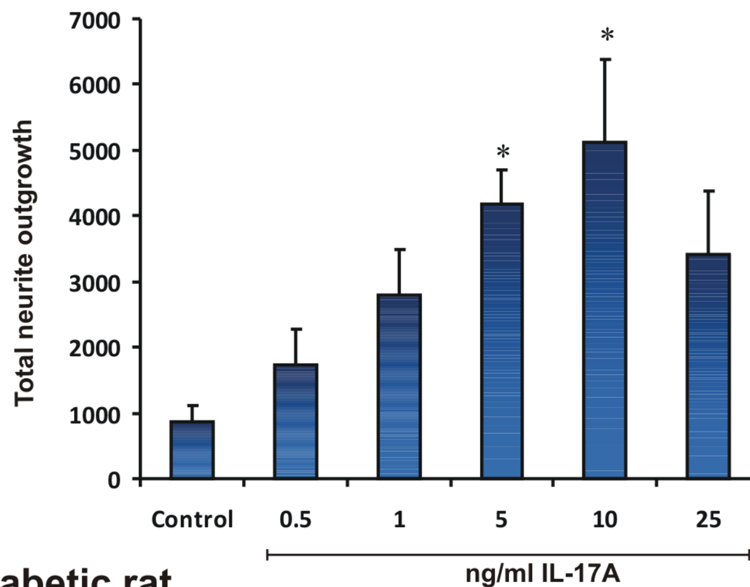
IL-17A enhanced neurite outgrowth in normal and diabetic sensory neurons through activation of MAPK and PI-3K signaling pathways.

The effect of IL-17A on the neurite outgrowth of normal and STZ-diabetic sensory neurons was tested as well as the pathways activated by IL-17A treatment and mechanistically linked to neurite outgrowth. The following results were found:

3.1 IL-17A enhances neurite outgrowth in adult rat sensory neurons:

The ability of IL-17A to enhance adult sensory neuron axon regeneration was investigated. DRG sensory neurons were collected from adult control or 3-5 months STZ-induced diabetic rats. Cells were cultured and left to grow under defined conditions and treated with different concentrations of IL-17A for 24 h in the presence of a low dose cocktail of neurotrophic factors that help in maintaining the viability of the cells without interfering with the growth. Cells were stained for neuron specific β-tubulin III after fixation. The cultures had diverse cells populations and different sizes and classes of neurons, which is representative to what is normally seen in DRGs *in vivo* [123]. The range of cell size was maintained *in vitro* with no loss of range in the small cells. Small neurons account for about 75% of the total population of neurons in the culture. The neurite outgrowth was mainly derived from medium to large diameter sensory neurons. The branching pattern and axon networking were similar in most neurons (Fig. 16). Minimal non-neuronal cells were found in the culture (detected by 4',6-diamidino-2-phenylindole (DAPI)). Fluorescent images were taken from the cultures to quantify the levels of

total neurite outgrowth. The total neurite outgrowth is related to an arborizing form of axonal plasticity and collateral sprouting *in vivo* [68]. IL-17A enhanced neurite outgrowth in a dose-dependent manner in control neurons (Fig. 15A). A 3 to 4 fold increase was seen at both 5 and 10 ng/ml (*$p<0.05$ vs control) concentrations. Also, IL-17A was able to enhance neurite outgrowth in neurons derived from STZ-induced diabetic rats (Fig. 15B). The enhancement was 3 to 4 fold at both 10 and 25 ng/ml IL-17A (*$p<0.05$ vs control) concentrations.



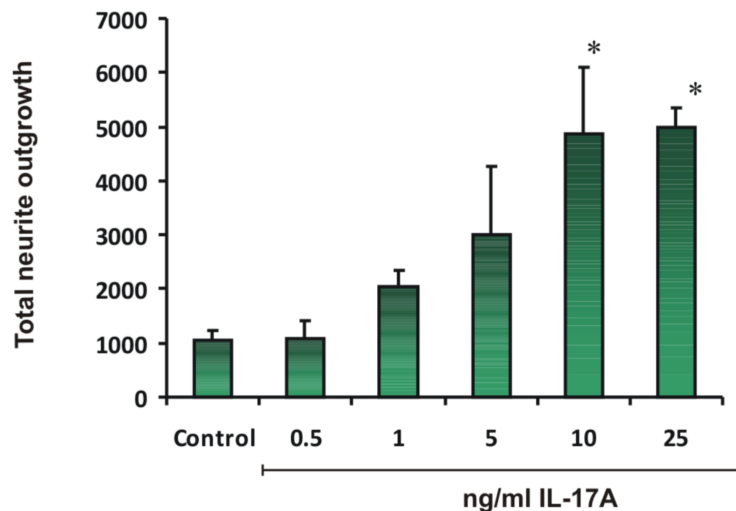
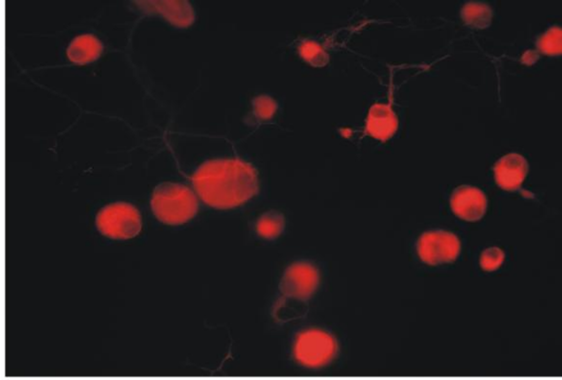
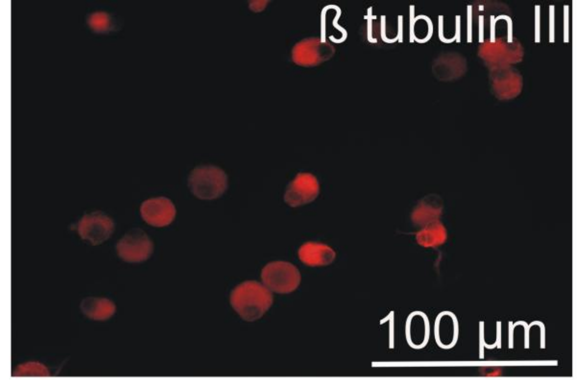
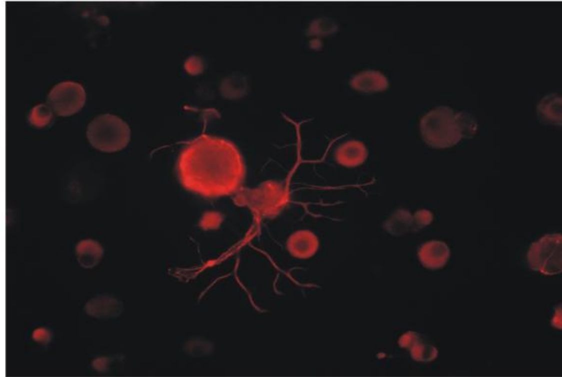
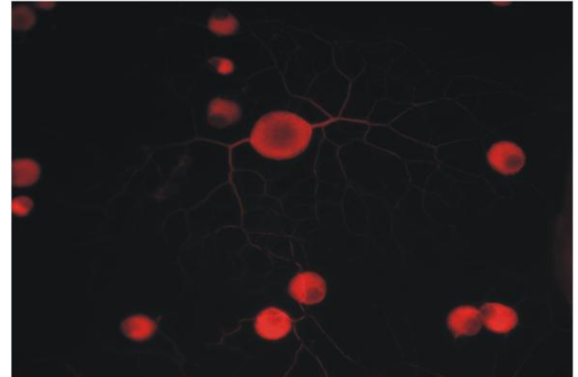


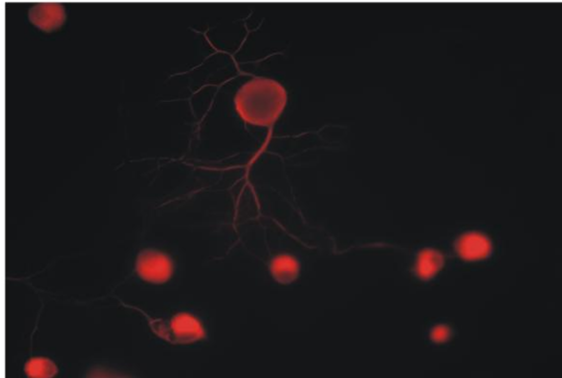
Fig.15 IL-17A enhanced neurite outgrowth in normal and diabetic sensory neurons. In (A) total neurite outgrowth of adult DRG sensory neurons derived from normal rats in response to a different concentrations of IL-17A grown for 24 h in the presence of low dose cocktail neurotrophic growth factors (GF).. The levels of neurite outgrowth were adjusted to cell number. Values are means ± SEM (n = 4 replicates); *p < 0.05 vs control (oneway ANOVA with Dunnett's *posthoc* test). In (B) total neurite outgrowth of sensory neurons, isolated from an STZ-induced diabetic rat, in response to a different concentrations of IL-17A in the presence of a low dose cocktail of neurotrophic growth factors. After fixation, cultures were stained for neuronal specific β-tubulin III. Values are means ± SEM (n = 4 replicates); *p < 0.05 vs control (oneway ANOVA with Dunnett's *posthoc* test).

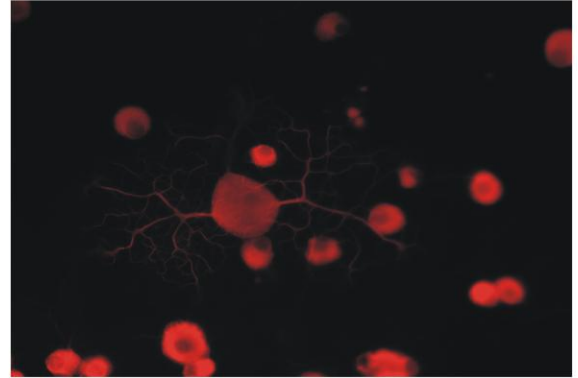

A- Control

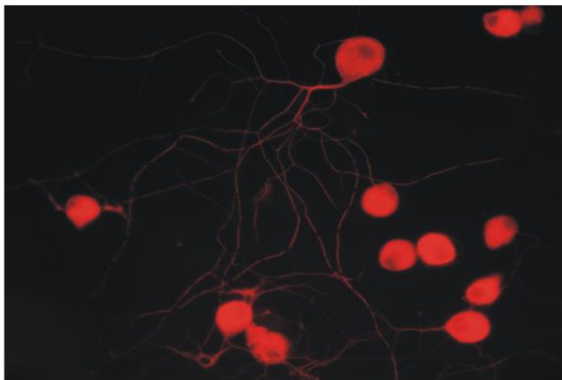

ß tubulin III
100 µm


B- IL- 17A 1 ng/ml




C- IL-17A 5 ng/ml




D- IL-17A 10 ng/ml

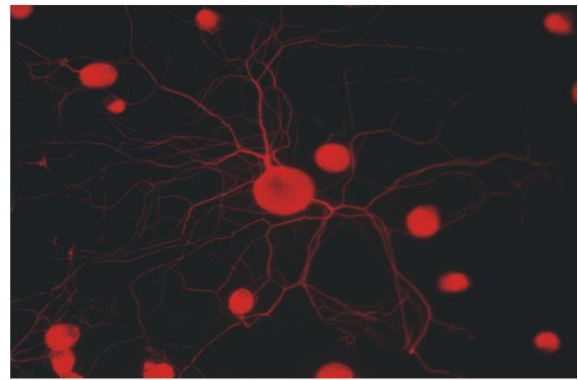


Fig.16 Images of cultures stained for neuron-specific β-tubulin III. (A) Untreated control neuronal cells, (B) treated for 24 h with IL-17A (1 ng/ml), (C) treated with IL-17 (5 ng/ml) and (D) treated with IL-17A (10 ng/ml). Size marker indicates 100 μm.

3.2 IL-17A activates MAPK and PI-3K signaling pathways in normal and STZ-induced diabetic sensory neuron culture.

After determining the optimal concentration of IL-17A that drives the neurite outgrowth in sensory neurons, I investigated the ability of IL-17A to activate MAPK and PI-3K pathways in cultured adult sensory neurons. Neurons were cultured and incubated for 3 hr in absence of neurotrophic factors (to prevent overlapping activation of these pathways by the neurotrophins) and then treated with 10 ng/ml IL-17A for either 15 min or 30 min. After lysing the cells, I determined the levels of phosphorylated ERK (P-ERK) and phosphorylated AKT (P-AKT). I found increases in both P-ERK and P-AKT levels in control sensory neurons. IL-17A activated MAPK through increasing ERK1/2 phosphorylation on the Thr 202/Tyr 204 epitopes (44 and 42 kDa) with peak activation after 15 mins. IL-17A also activated PI-3K through enhancing AKT1/2/3 phosphorylation on Ser 473 epitope (65 kDa) with peak value after 15 min (Fig. 17). In STZ-induced diabetic rats, IL-17A was able to enhance P-ERK and P-AKT levels and activate both MAPK and PI-3K in sensory neurons when normalized to T-ERK (usually the expression of T-ERK is not changed by the diabetic state in DRG) [99]. Significant activation was observed after 15 min of treatment. In opposition to the normal sensory neurons which reverted to basal level after 30 min, STZ-induced diabetic neurons had a significant elevation after 30 min (Fig. 18).

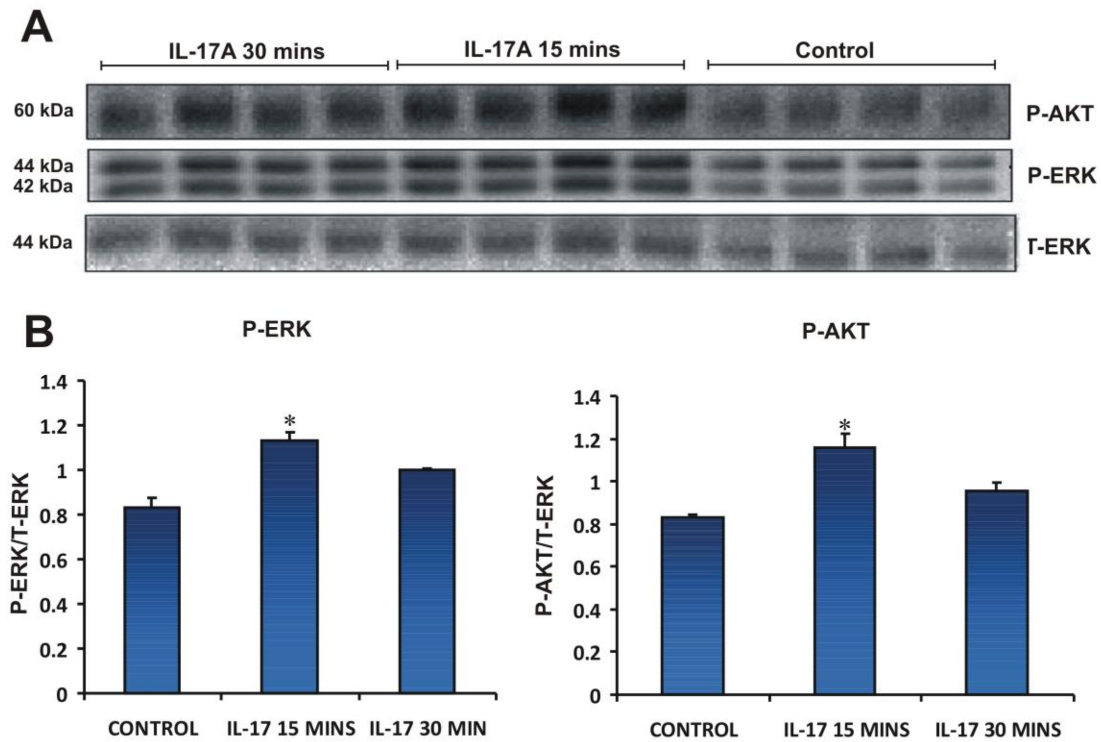


Fig.17 IL-17A activates MAPK and PI-3K signaling pathway in cultured normal sensory neurons. Neurons isolated from a control rat were treated for 15 min or 30 min with 10 ng/ml IL-17A in the absence of neurotrophic factors. Western blots for phosphorylated-ERK (P-ERK), phosphorylated-AKT (P-AKT) and total ERK (T-ERK) are shown in (A). Data for P-ERK and P-AKT activation normalized against T- ERK is shown in (B Values are means ± SEM (n = 4 replicates), 2 separate experiments. *p < 0.05 vs control (oneway ANOVA with Tukey's *posthoc* test).

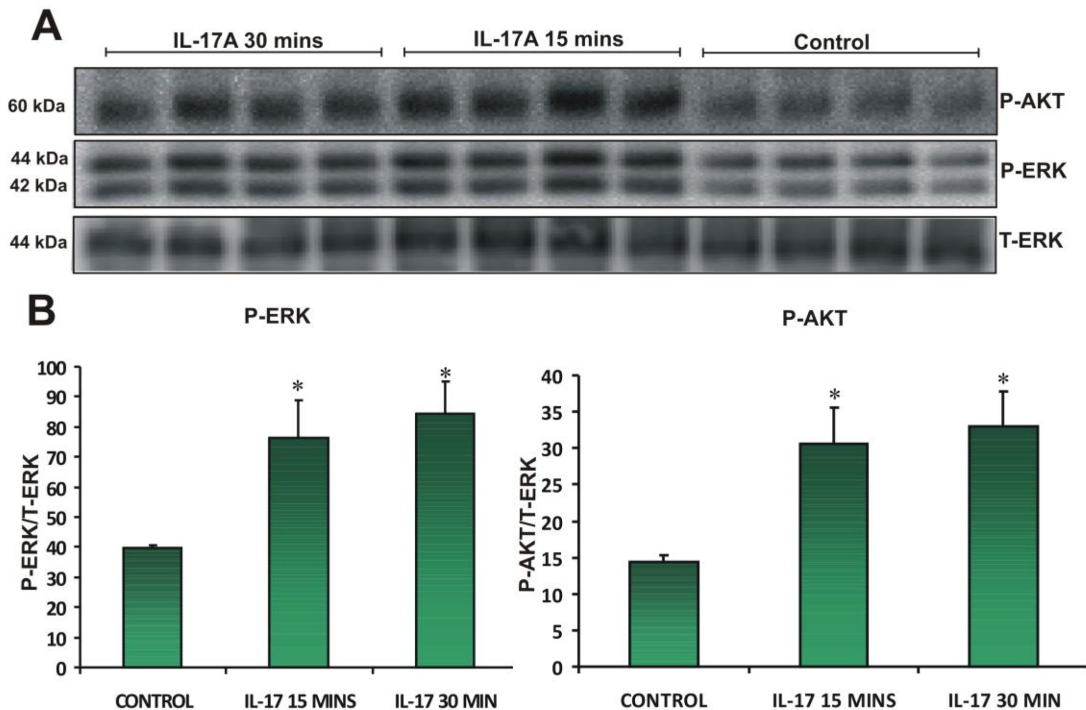


Fig.18 IL-17A activates MAPK and PI-3K signaling pathway in cultured diabetic sensory neurons. Neurons isolated from an STZ-induced diabetic rat were treated for 15 min and 30 mins with 10 ng/ml IL-17A in the absence of neurotrophic factors. Western blots for P-ERK, P-AKT and T- ERK are shown in (A). Data for P-ERK and P-AKT activation normalized against T- ERK is shown in (B). Values are means ± SEM (n = 4 replicates). *$p < 0.05$ vs control (oneway ANOVA with Tukey's *posthoc* test).

3.3 IL-17A-induction of neurite outgrowth is mediated by PI-3K pathway in normal sensory neurons.

I tested the association of the PI-3K signal transduction pathway in IL-17A-directed neurite outgrowth by treating the cells with different concentrations of the PI-3K inhibitor, Ly294002. Ly294002 is a potent competitive reversible inhibitor of class I phosphoinositide 3-kinases (PI3Ks) that acts on the ATP binding site of the catalytic subunit of the enzyme [124]. Cultures were treated with either 1, 3 or 10 μM Ly294002 +/- IL-17A in the presence of neurotrophins.

The impact of the inhibitor on IL-17A dependent neurite outgrowth was assessed. In absence of IL-17A, Ly294002 treatment had no effect on neurite outgrowth (Fig. 19A). IL-17A treatment caused 3 fold increase in total neurite outgrowth that was completely blocked by 3 and 10 μM Ly294002. This suggests the involvement of PI-3K in mediating IL-17A induction of neurite outgrowth (Fig. 19B).

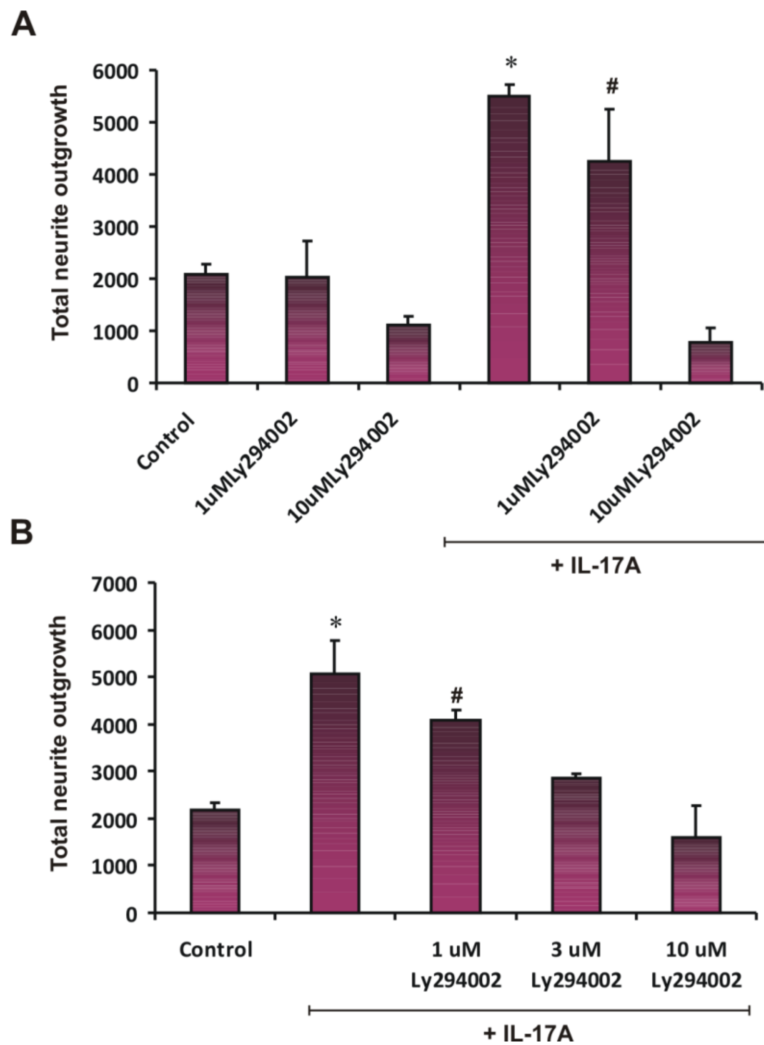


Fig.19 Blockade of PI-3K signaling inhibits IL-17A-induced neurite outgrowth in normal sensory neurons. DRG sensory neurons from control rats were cultured for 1 day in the presence of neurotrophic factors and were exposed to PI-3K inhibitor, Ly294002 in (A) at1 and10 μM concentrations ± IL-17A (10 ng/ml) and in (B) at 1, 3 and 10 μM concentrations + IL-17A. After fixation, neurons were stained for neuron-specific β-tubulin III. In A and B the

impact on total neurite outgrowth is shown. Data are mean ± SEM (*n* = 4 replicates). In (A) *P < 0.05 vs 1 μM Ly294002, 10 μM Ly294002, 10 μM Ly294002+IL-17A and control. #P < 0.05 vs 10 μM Ly294002+IL-17A. In (B) *P < 0.05 vs 3 μM Ly294002+IL-17A, 10 μM Ly294002+IL-17A and control. #P < 0.05 vs 10 μM Ly294002+IL-17A. All statistical comparisons were performed by oneway ANOVA with Tukey's *posthoc* test.

3.4 IL-17A-induction of neurite outgrowth is mediated by MAPK pathway in normal sensory neurons.

I tested the role of the MAPK signal transduction pathway in IL-17A-directed neurite outgrowth by treating the cells with different concentrations of U0126 (MAPK inhibitor). U0126 blocks the activation of MAPK (ERK 1/2) by inhibiting the kinase activity of MEK 1/2. MEK phosphorylates the threonine and tyrosine (T183 and Y185) residues on ERKs 1 and 2 resulting in their activation [125]. U0126 inhibition of MEK is noncompetitive with respect to ERK and ATP, suggesting that it binds in a unique site in MEK and this may underlie its selectivity against MEK [126]. Cultures were treated with either 1, 3 or 10 μM U0126 +/- IL-17A in the presence of neurotrophins. I studied the effect of MAPK inhibitor on IL-17A-neurite outgrowth induction. In absence of IL-17A, U0126 treatment had no effect on neurite outgrowth (Fig. 20A). The 3 fold enhancement of neurite outgrowth that was carried out by IL-17A was completely blocked by 3 or 10 μM U0126. This supports the fact that IL-17A-enhancement of neurite outgrowth is mediated by the MAPK pathway (Fig. 20B).

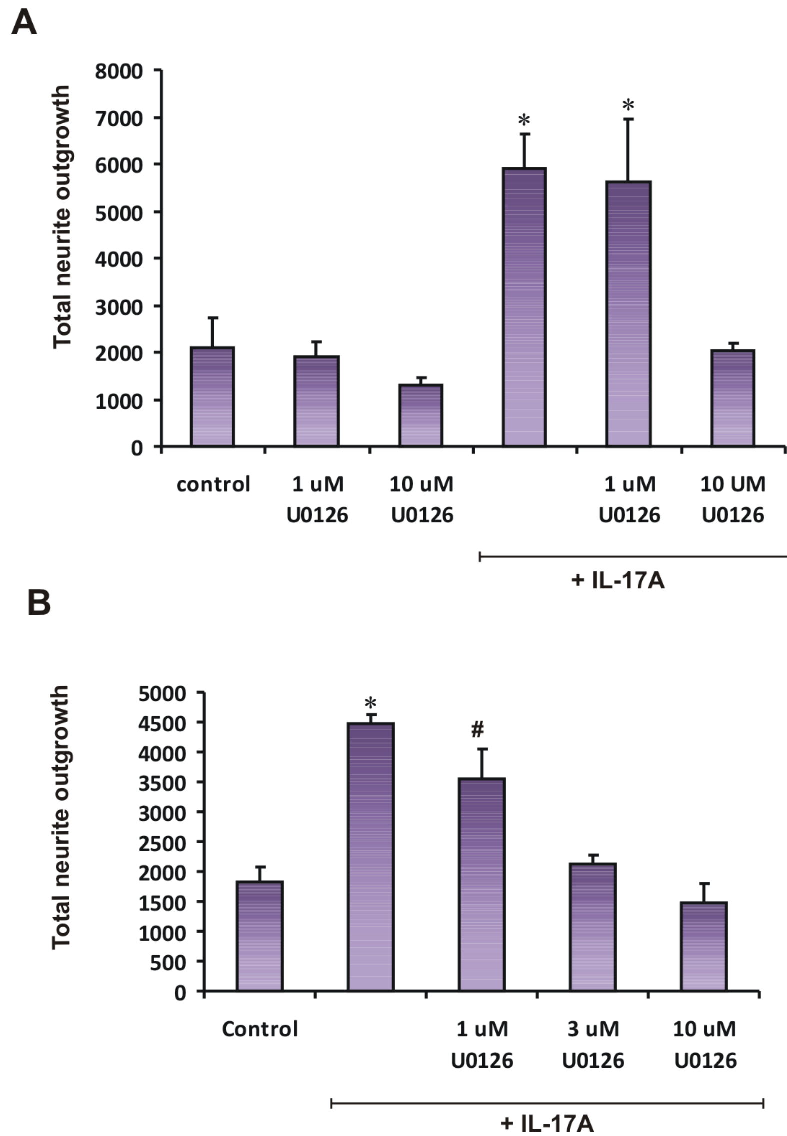


Fig.20 Blockade of MAPK signaling inhibits IL-17A-induced neurite outgrowth in normal sensory neurons. DRG sensory neurons from control rats were cultured for 1 day in the presence of neurotrophic factors and were exposed to the pharmacological inhibitor of MAPK, U0126 in (A) at 1 and10 µM concentrations ± IL-17A (10 ng/ml) and in (B) at 1, 3 and 10 µM concentrations + IL-17A. Neurons were fixed and stained for neuron-specific β-tubulin III. In A & B is shown the impact on total neurite outgrowth. Data are mean ± SEM (*n* = 4 replicates). In (A) *P < 0.05 vs 1 µM U0126, 10 µM U0126, 10 µM U0126+IL-17A and control. In (B) *P < 0.05 vs 3 µM U0126+IL-17A, 10 µM U0126+IL-17A and control. #P < 0.05 vs 10 µM U0126+IL-17A and control. All statistical comparisons by oneway ANOVA with Tukey's *posthoc* test.

3.5 IL-17A-induction of neurite outgrowth in diabetic sensory neurons is mediated by MAPK and PI-3K

The role of MAPK and PI-3K in mediating IL-17A-induction of neurite outgrowth in diabetic sensory neurons was investigated. Neurons were treated with either 3 μM Ly294002 or 3 μM U0126 in the presence or absence of IL-17A and the presence of neurotrophins. The impact of the inhibitors on IL-17A dependent neurite outgrowth was assessed. In absence of IL-17A, neither Ly294002, nor U0126 treatment had a significant impact on the basal neurite outgrowth (Fig. 21). However, IL-17A treatment caused 3 fold increase in total neurite outgrowth that was completely blocked by 3 μM Ly294002 or 3 μM U0126. This suggests the involvement of PI-3K and MAPK in mediating IL-17A induction of neurite outgrowth in diabetic sensory neurons (Fig. 21).

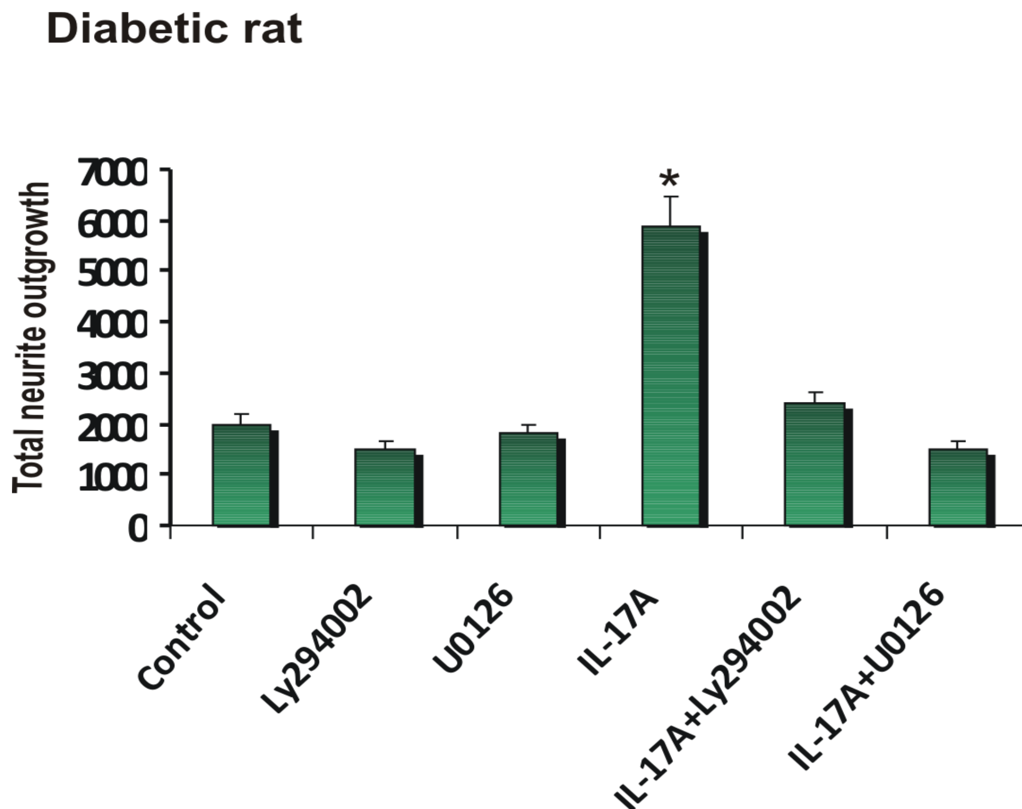


Fig.21 Blockade of MAPK and PI-3K signaling inhibits IL-17A-induced neurite outgrowth in diabetic sensory neurons. DRG sensory neurons from diabetic rats were cultured for 1 day in the presence of neurotrophic factors and were exposed to the pharmacological inhibitor of MAPK, 3 μM U0126 ± IL-17A (10 ng/ml) and inhibitor of PI-3K, 3 μM Ly294002 ± IL-17A (10 ng/ml). After fixation, neurons were stained for neuron-specific β-tubulin III. Data are mean ± SEM ($n = 4$ replicates). *$P < 0.05$ vs 3 μM U0126, 3 μM Ly294002, 3 μM U0126+IL-17A, 3 μM Ly294002+IL-17A and control. All statistical comparisons by oneway ANOVA with Tukey's *posthoc* test.

3.6 Ly294002 and U0126 inhibited IL-17A-activation of P-AKT and P-ERK respectively, in cultured sensory neurons.

I tested the ability of Ly294002 to inhibit P-AKT activation, and U0126 to inhibit P-ERK activation that was carried out in the presence of IL-17A treatment in sensory neurons. Neurons were incubated for 3 hrs prior to applying either 3 μM Ly294002 or 3 μM U0126 for 1 hr. Cells were treated with 10 ng/ml IL-17A for 15 min in absence of neurotrophic factors. After lysing the cells, Western blotting was performed and I determined the levels of P-ERK and P-AKT (Fig. 22A). I found that IL-17A increased P-AKT activation; however, the level of P-AKT relative to T-ERK was reduced in Ly294002 treated neurons. Also IL-17A increased P-ERK activation, but the level of P-ERK relative to T-ERK was reduced in U0126 treated neurons which reflects U0126 inhibition of P-ERK activation by IL-17A (Fig. 22B).

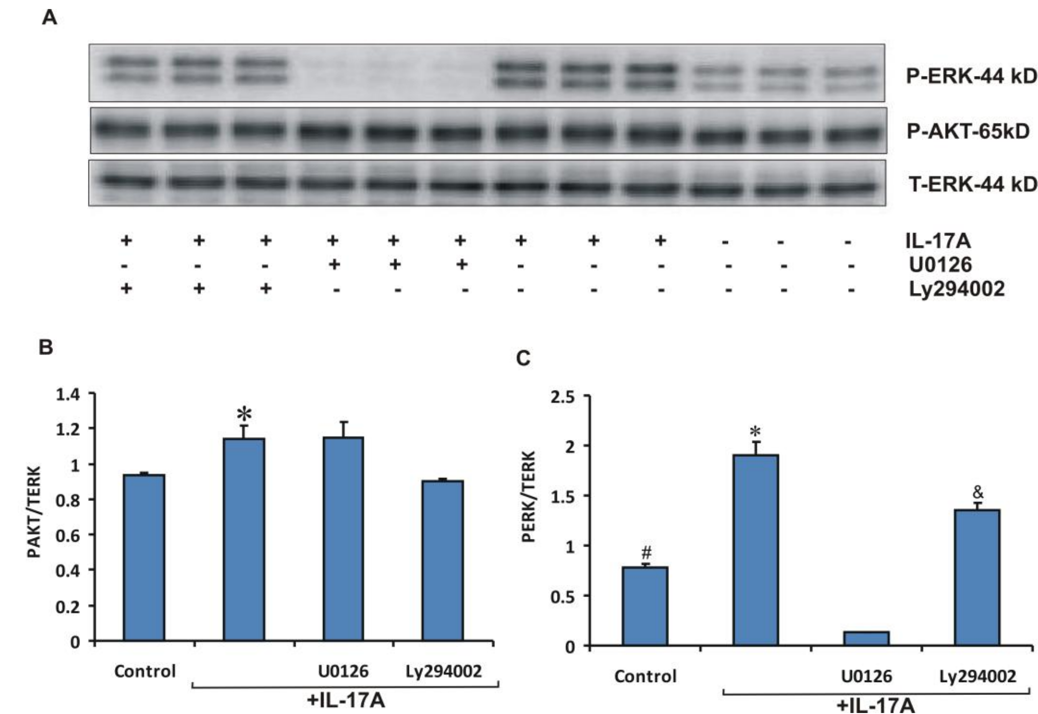


Fig.22 U0126 (MAPK inhibitor) blocked IL-17A-activation of P-ERK and Ly294002 (PI-3K inhibitor) blocked IL-17A-activation of P-AKT. Sensory neurons were isolated from a control rat, plated and treated for 1 hr with either 3 μM U0126 or 3 μM Ly294002. Then cells were treated with 10 ng/ml IL-17A for 15 mins in the absence of neurotrophic factors. Panel (A) shows western blots for P-ERK, P-AKT and T-ERK. (B) Graphed data for P-AKT activation normalized against T- ERK. Ly294002 was able to block IL-17A- activation of P-AKT. Values are means ± SEM (n = 3 replicates) *$p < 0.05$ vs control and Ly294002 (oneway ANOVA with Tukey's *posthoc* test). (C) Graphed data for P-ERK activation normalized against T- ERK. U0126 was able to block IL-17A-activation of P-ERK. Values are means ± SEM (n = 3 replicates) *$p < 0.05$ vs control, U0126 and Ly294002. #$p < 0.05$ vs IL-17A, U0126 and Ly294002. &$p < 0.05$ vs control, U0126 and IL-17A (oneway ANOVA with Tukey's *posthoc* test).

Chapter 4:

IL-17A improved mitochondrial bioenergetic function of sensory neurons and was associated with augmented expression of proteins of mitochondrial oxidative phosphorylation.

Mitochondrial dysfunction has been identified as a key component of the pathogenesis of diabetic neuropathy and considered as a central mediator in triggering nerve degeneration. This is due to mitochondria crucial role in commanding cells bioenergetic. This is important in neurons which have high demand of energy and any impaired in bioenergetic status may induce oxidative stress [127]. Giving the role of mitochondria in providing the ATP for high energy requiring axonal functions and growth cone motile, studying mitochondrial function was proposed to provide an explanation for IL-17A-induction of neurite outgrowth. I investigated the mitochondrial bioenergetic function, oxidative phosphorylation pathway, enzymatic activity and mitochondrial mass and I had the following results:

4.1 IL-17A augmented mitochondrial bioenergetic function in cultured adult sensory neurons.

Functional mitochondria and sufficient ATP are needed by the growth cone to drive neurite outgrowth. DRG neurons derived from a control rat were cultured for 24 hr in the presence of neurotrophic factors and then treated with IL-17A for either 3 or 6 hr. Oxygen consumption rate (OCR) was measured to evaluate the mitochondrial bioenergetics profile using the Seahorse Biosciences XF24 analyzer. This machine allows studying the different behavior of mitochondria through injecting different substances during respiration measurement. After measuring the basal

respiration, oligomycin is injected which inhibits the ATP synthase leading to a build-up of the proton gradient. This also leads to a dearth of ATP that inhibits electron flux and determines the coupling efficiency. Injecting FCCP into the culture determines the maximal capacity to reduce oxygen. Finally, rotenone (complex I inhibitor) + antimycin A (complex III inhibitor) were injected to inhibit the flux of electrons, and stop the oxygen consumption at cytochrome c oxidase [97]. This final step revealed the non-mitochondrial OCR. Data are expressed as OCR in pmol/min for 1000 cells (usually we have 2000-4000 cells per well) [119]. The OCR of neurons that were cultured for 3 or 6 h with IL-17A, and induced by the uncoupler FCCP (1 μM) was significantly enhanced (Fig.23A). Maximal respiration, coupling efficiency, and spare respiratory capacity are presented for control (blue), 3 hr IL-17A (green) and 6 hr IL-17A (pink). The nonmitochondrial respiration (e) was subtracted from all the parameters. All the parameters were calculated according to a previously described method [119]. Graphed data showed significant improvement in basal respiration, maximal OCR and spare respiratory capacity in both 3 and 6 hr IL-17A treated neurons. No significant impact was seen on coupling efficiency by IL-17A treatment (Fig. 23B).

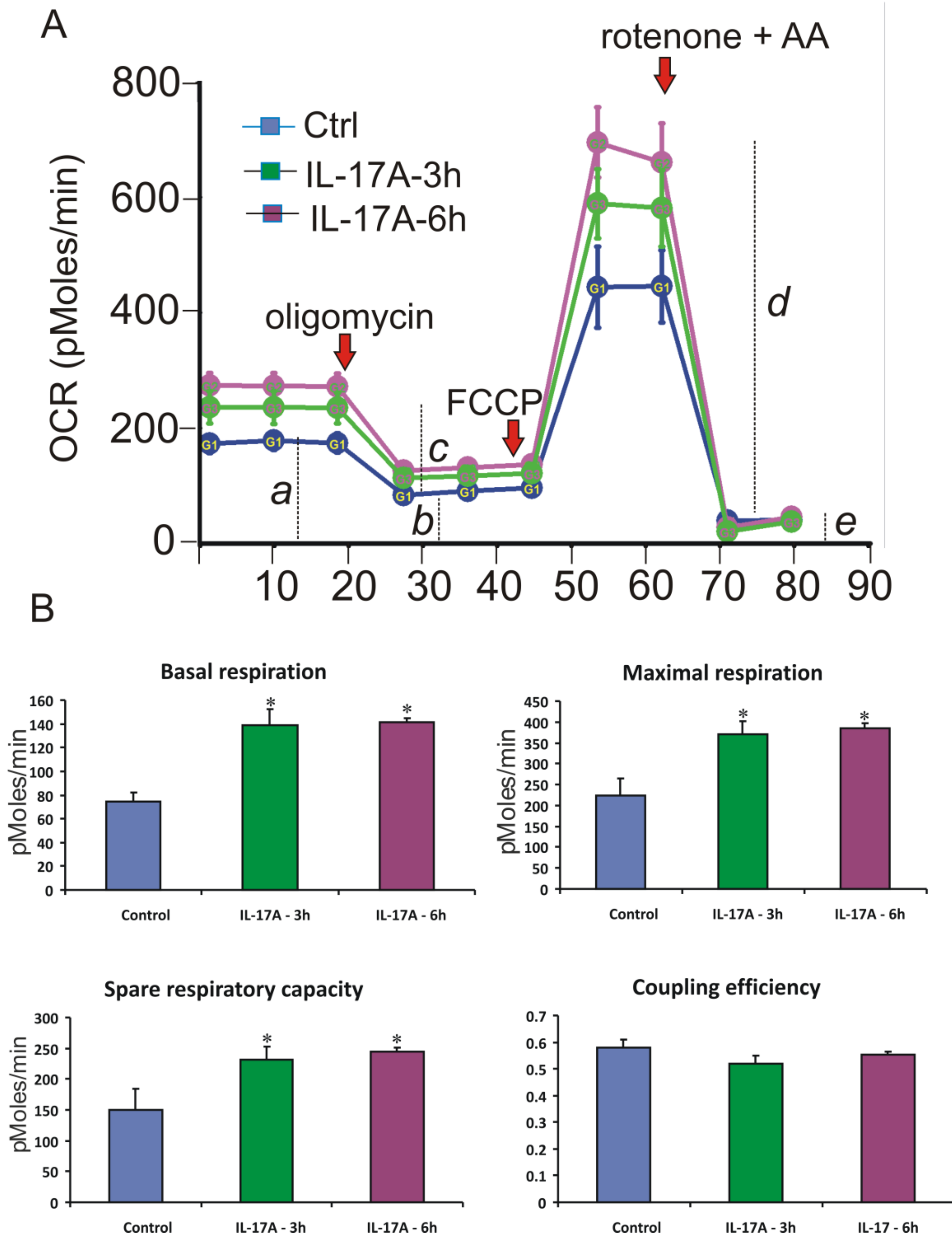


Fig.23 IL-17A enhanced mitochondrial bioenergetic function**.** DRG sensory neurons from control rats were cultured for 1 day in the presence of neurotrophic factors and were treated after that with 10 ng/ml IL-17A for either 3 or 6 hrs**.** Oxygen consumption rate (OCR) was measured at basal level, then different compounds were sequentially

added: oligomycin (1 μM), FCCP (1 μM), and rotenone (1 μM) + antimycin A (AA; 1 μM) to the DRGs. Dotted lines, a- e in have been used later for quantification of bioenergetics parameters [97]. Data are expressed as OCR in pmol/min for 1000 cells. (B) Graphed data showing the mitochondrial parameters. All the parameters were calculated according to a previously described method, basal respiration (a-e), maximal respiration (d-e), coupling efficiency (c-e/a-e) and spare respiratory capacity (d-a) [119],. *$p < 0.05$ vs control, $n = 5$ replicate. (oneway ANOVA with Tukey's *posthoc* test).

4.2 IL-17A augmentation of mitochondrial bioenergetics function in normal and diabetic sensory neurons was mediated by PI-3K and MAPK.

I tested whether promoting MAPK and PI-3K signaling contributes to the ability of IL-17A to induce mitochondrial function in sensory neurons. DRG neurons derived from normal or diabetic rat were cultured and treated with IL-17A and co-treated with either 3 μM Ly294002 or 3 μM U0126 for 24 hr in presence of neurotrophic factors. OCR was measured through Seahorse Biosciences XF24. Twenty four hr IL-17A treatment enhanced mitochondrial bioenergetics function in diabetic (Fig. 26) and normal (Fig. 24) sensory neurons. That was demonstrated by the significant increase in basal respiration, maximal respiration and spare respiratory capacity. However, the basal respiration was significantly suppressed by PI-3K inhibitor, Ly294002, in normal but not in diabetic sensory neurons. Maximal OCR and spare respiratory capacity were also inhibited by MAPK inhibitor U0126 and PI-3K inhibitor Ly294002 in normal (Fig.25) and diabetic neurons (Fig. 26). Coupling efficiency was not impacted by IL-17A treatment, or the inhibitors. Thus, IL-17A augmentation of mitochondrial bioenergetics function was mediated by MAPK and PI-3K pathways in normal and diabetic sensory neurons.

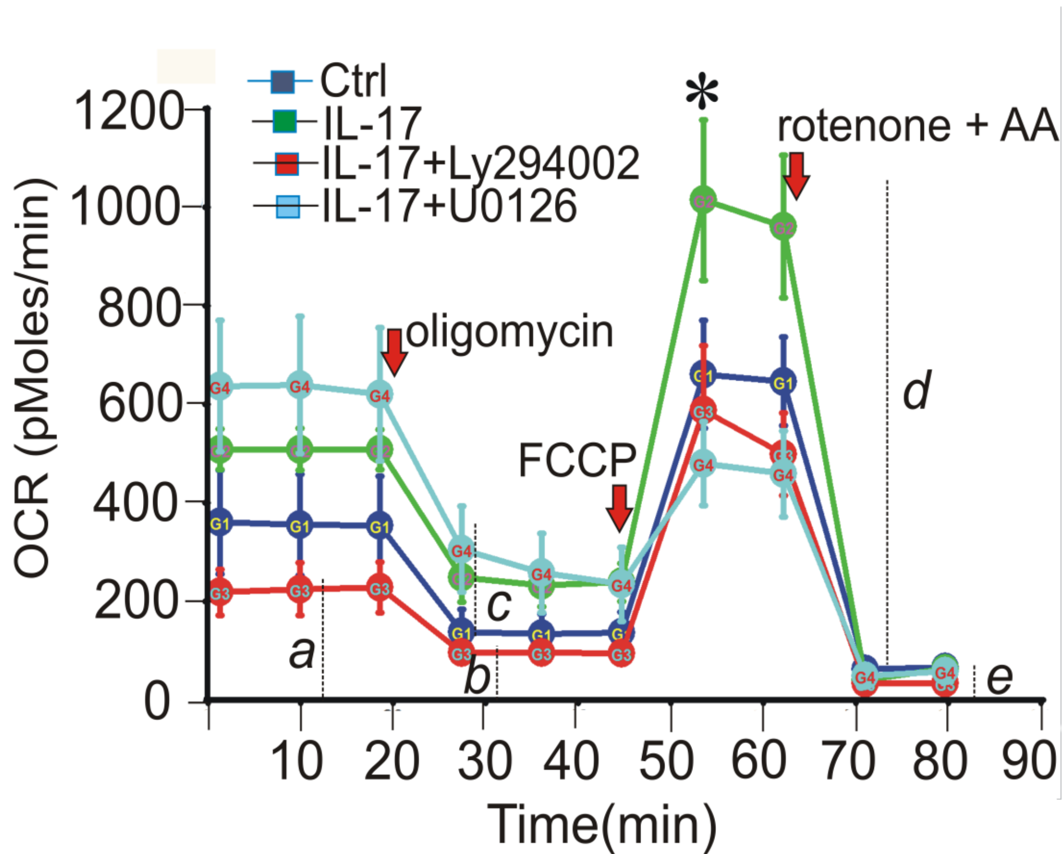


Fig.24 Mitochondrial bioenergetics are enhanced by IL-17A through MAPK and PI-3K in normal sensory neurons. DRG sensory neurons from control rats were cultured for 1 day in the presence of neurotrophic factors and were exposed to the pharmacological inhibitor of MAPK (U0126) and PI-3K inhibitor (Ly294002). 10 ng/ml IL-17A enhanced mitochondrial bioenergetics function and this enhancement was blocked by 3 μM Ly294002 and 3 μM U0126. Data are expressed as OCR in pmol/min for 1000 cells. *$p < 0.05$ vs control, IL-17+Ly294002 and IL-17+U0126. (oneway ANOVA with Tukey's *posthoc* test).

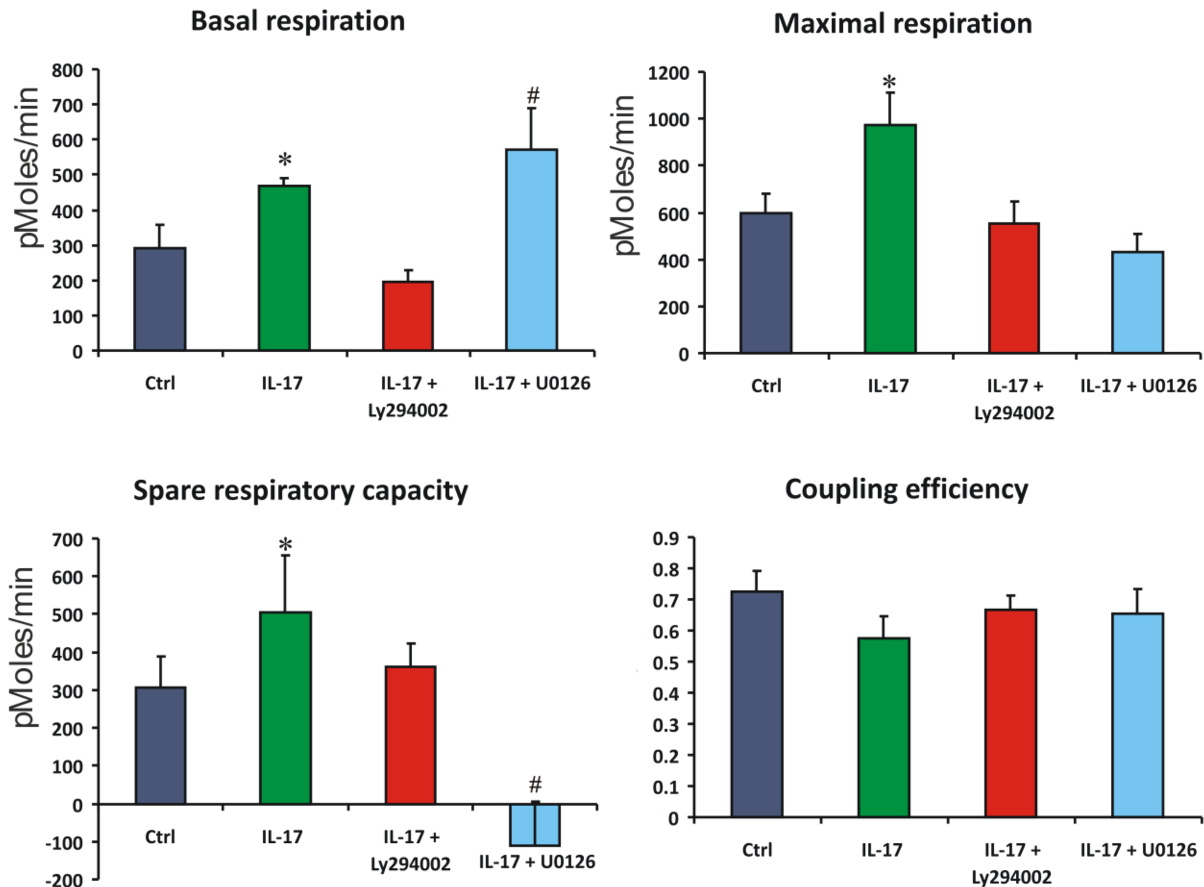
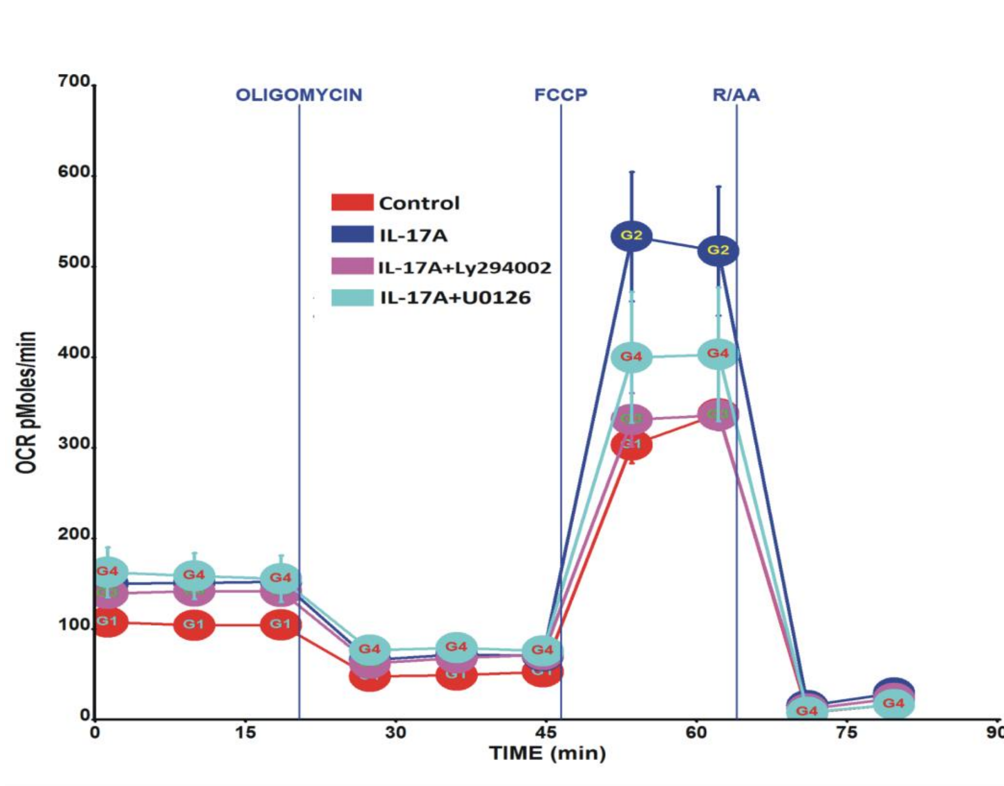
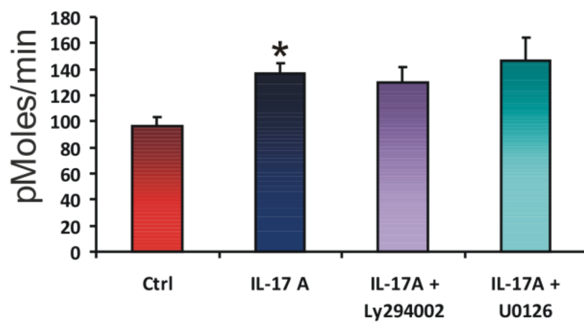
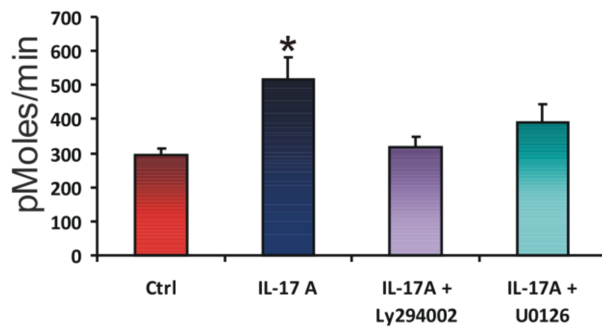


Fig.25 IL-17A enhanced mitochondrial maximal respiration through MAPK and PI-3K pathways in normal sensory neurons. The OCR measurements of control (violet) and cells treated with IL-17A (green), and cells treated with 10 ng/ml IL-17A + 3 μM Ly294002 (red) or 10 ng/ml IL-17A + 3 μM U0126 (blue) at the 1 μM concentration of FCCP were plotted. Data are expressed as OCR in pmol/min for 1000 cells, values are mean +/- SEM of n = 4 replicate. In basal respiration *p < 0.05 vs control and IL-17A+Ly294002, #p < 0.05 vs control and IL-17A+ Ly294002. In maximal respiration *p < 0.05 vs control, IL-17A+Ly294002 and IL-17A+U0126. In spare respiratory capacity *p < 0.05 vs control, IL-17A+Ly294002 and IL-17A+U0126, #p < 0.05 vs control, IL-17A and IL-17A+Ly294002. All statistical comparisons by oneway ANOVA with Tukey's *posthoc* test.


OLIGOMYCIN
FCCP
R/AA
Control
IL-17A
IL-17A+Ly294002
IL-17A+U0126
OCR pMoles/min
TIME (min)


Basal respiration
pMoles/min
*
Ctrl
IL-17 A
IL-17A + Ly294002
IL-17A + U0126


Maximal respiration
pMoles/min
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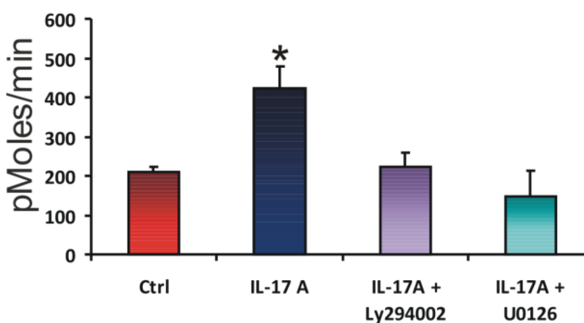
IL-17 A
IL-17A + Ly294002
IL-17A + U0126


Spare respiratory capacity
pMoles/min
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IL-17 A
IL-17A + Ly294002
IL-17A + U0126

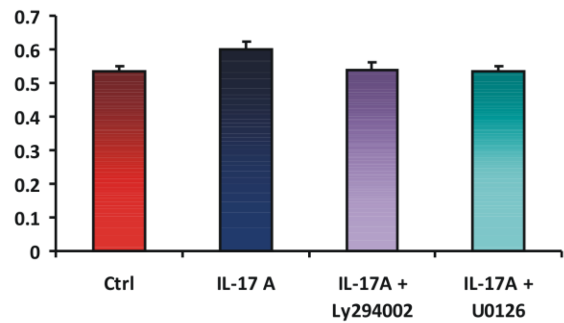

Coupling efficiency
Ctrl
IL-17 A
IL-17A + Ly294002
IL-17A + U0126

Fig.26 IL-17A enhanced mitochondrial maximal respiration through MAPK and PI-3K pathways in diabetic sensory neurons. The OCR measurements of control (red) and cells treated with IL-17A (deep blue), and cells treated with 10 ng/ml IL-17A + 3 μM Ly294002 (purple) or 10 ng/ml IL-17A + 3 μM U0126 (blue) at the 1 μM concentration of FCCP were plotted. Data are expressed as OCR in pmol/min for 10 μg protein, values are mean +/- SEM of n = 4 replicate. In basal respiration *$p < 0.05$ vs control. In maximal respiration and spare respiratory capacity *$p < 0.05$ vs control, IL-17A+Ly294002 and IL-17A+U0126. All statistical comparisons by oneway ANOVA with Tukey's *posthoc* test.

4.3 IL-17A enhanced the enzymatic activity of mitochondrial COX-IV

To investigate the mechanism behind IL-17A enhancement of mitochondrial bioenergetics, I tested the enzymatic activity of complex IV cytochrome *c* oxidase (COX-IV) which is the last enzyme complex in the respiratory electron transport chain of mitochondria. DRG neurons derived from control rat were cultured and treated with IL-17A in presence of neurotrophic factors for 24 hr. Cells were harvested and protein assay was performed and the activity of COX IV was measured by Ultrospec 2100 ultraviolet-visible spectrophotometer. Cells treated with IL-17A had a higher value for the slope (Fig. 27A). This reflects a decrease in the absorbance of reduced cytochrome *c* which means that IL-17A was able to enhance the rate of oxidation of reduced cytochrome *c*. Thus, IL-17A treated neurons exhibited significantly higher COX-IV enzymatic activity (in nmol/min/mg total protein) compared to untreated neurons (Fig. 27B).

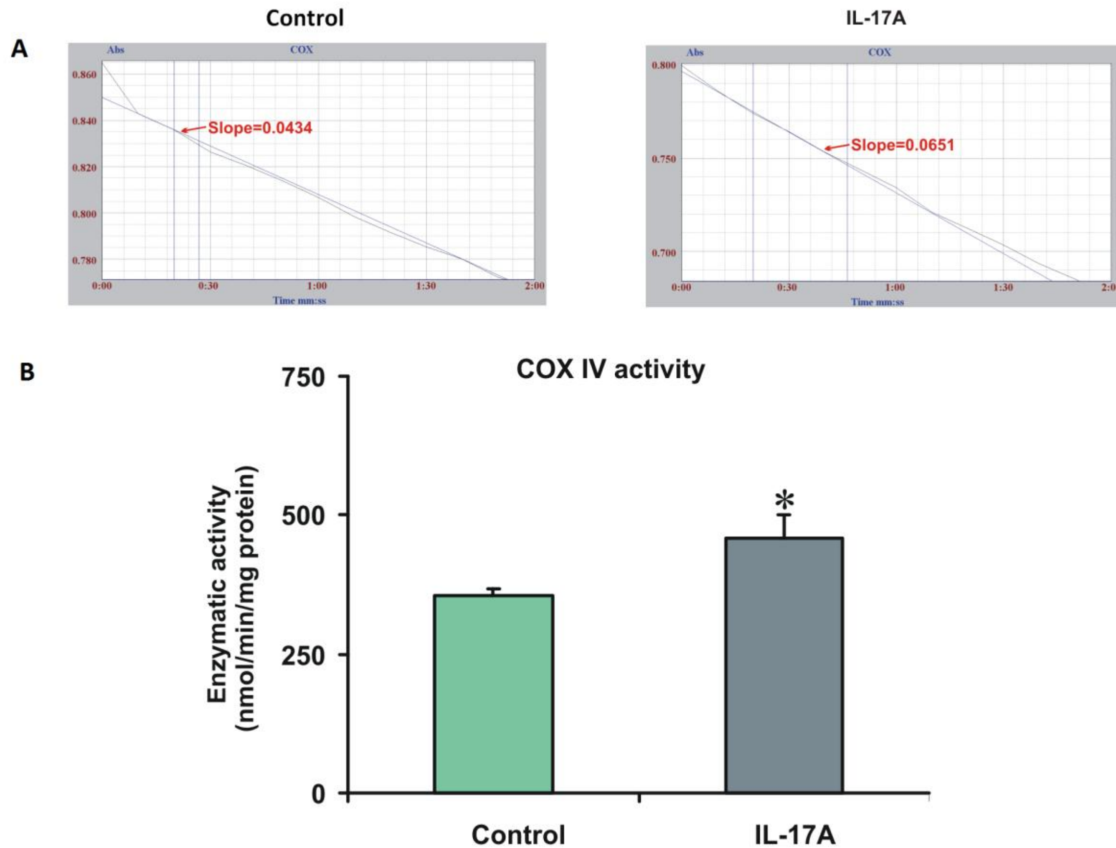


Fig.27 IL-17A enhanced the enzymatic activity of mitochondrial COX-IV. Neurons isolated from a control rat were cultured and treated for 24 hrs with 10 ng/ml IL-17A in the presence of low dose neurotrophic factors. (A) in the spectrophotometric assay of COX-IV activity, the slope for the IL-17A treated neurons shows a higher value than in the control neurons. (B) Neurons treated with IL-17A show significantly higher COX-IV enzymatic activity (in nmol/min/mg total protein). Values are mean +/- SEM of n = 3 replicate, *$p < 0.05$ by Student's t-Test.

4.4 The expression of proteins of the mitochondrial oxidative phosphorylation pathway was enhanced by IL-17A and this enhancement was mediated by PI-3K in cultured normal sensory neurons.

I investigated the ability of IL-17A to enhance mitochondrial protein expression. Sensory neurons were isolated from a control rat and cultured in the presence of neurotrophic factors and IL-17A for 24 or 48 hr. Cells were harvested and Western blot was performed. Blots were

incubated with a primary anti-body cocktail detecting proteins of mitochondrial oxidative phosphorylation that included proteins belonging to Complex II subunit succinate dehydrogenase B (SDHB), Complex III subunit ubiquinol-cytochrome *c* reductase core protein II (UQCRC2) , Complex IV subunit cytochrome oxidase II (COX-II), and Complex V subunit ATP5A (Fig.28 A). IL-17A significantly increased the expression levels of specific components of complex II, III and IV after 24 hr of treatment. Similar results were found after 48 hr of IL-17A treatment but no significant impact of IL-17A after 24 hr or 48 hr was seen on complex V ATP5A expression (Fig.28 B).

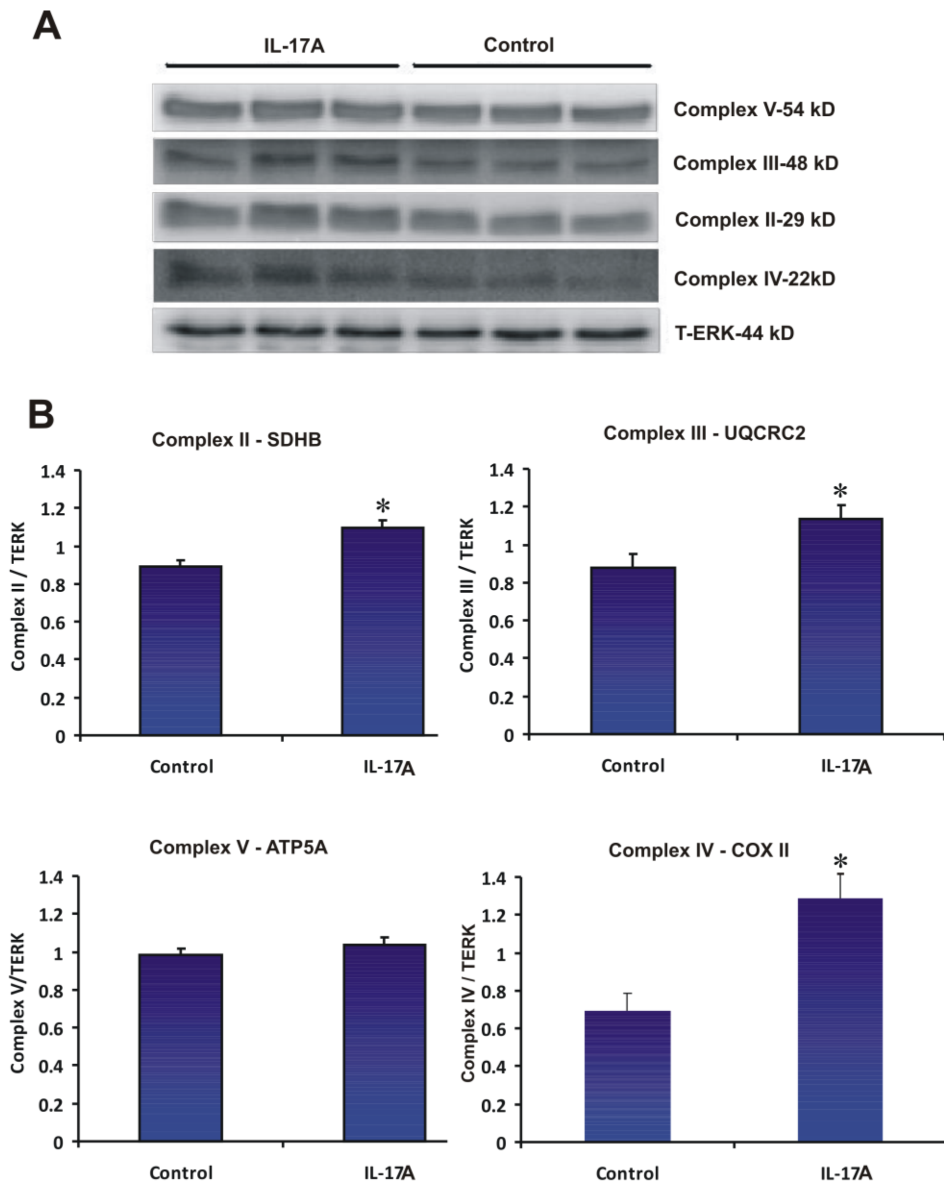


Fig.28 IL-17A enhanced the expression of proteins of the mitochondrial oxidative phosphorylation pathway in cultured normal sensory neurons. Neurons isolated from a control rat were cultured and treated for 24 hrs with 10 ng/ml IL-17A in the presence of neurotrophic factors. (A) Western blots for proteins belonging to Complex II subunit (SDHB), Complex III subunit (UQCRC2), Complex IV subunit (COX-II), and Complex V subunit ATP5A are shown. (B) Data from the different complexes indicate that IL-17A significantly increased the levels of specific components of complex II, III and IV and did not affect complex V ATP5A expression. Same results were found after 48 hr IL-17A treatment. Values are mean +/- SEM (n = 3 replicate); *$p < 0.05$ by Student's t-Test.

When neurons were co-treated with Ly294002 or U0126 in the presence of IL-17A for 24 hr, the expression level of specific components of complex II, III and IV was significantly suppressed by Ly294002 treatment. This means that IL-17A expression enhancement of these complexes was mediated by the PI-3K pathway (Fig.29). U0126 did not inhibit the expression of the complexes significantly.

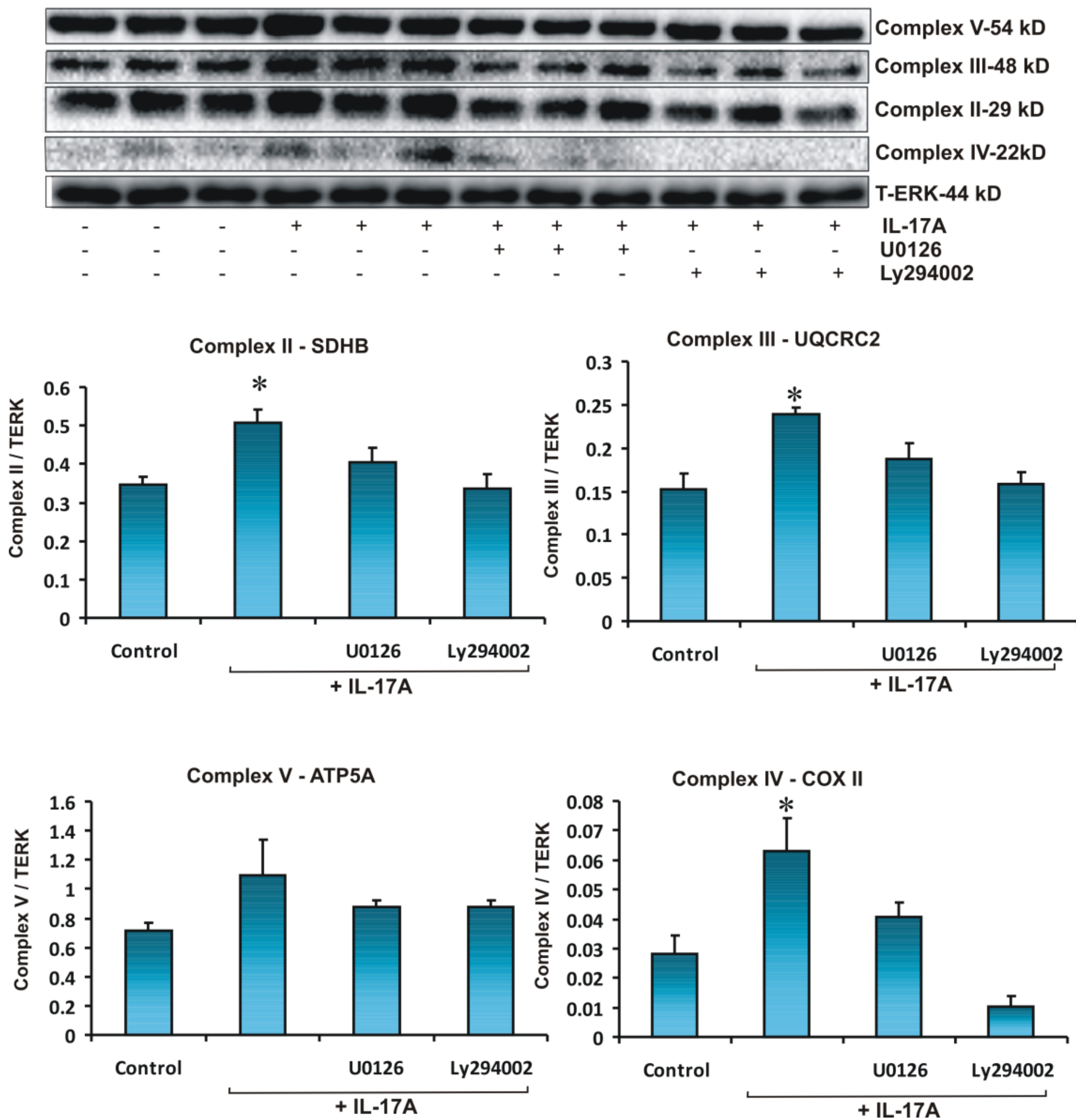
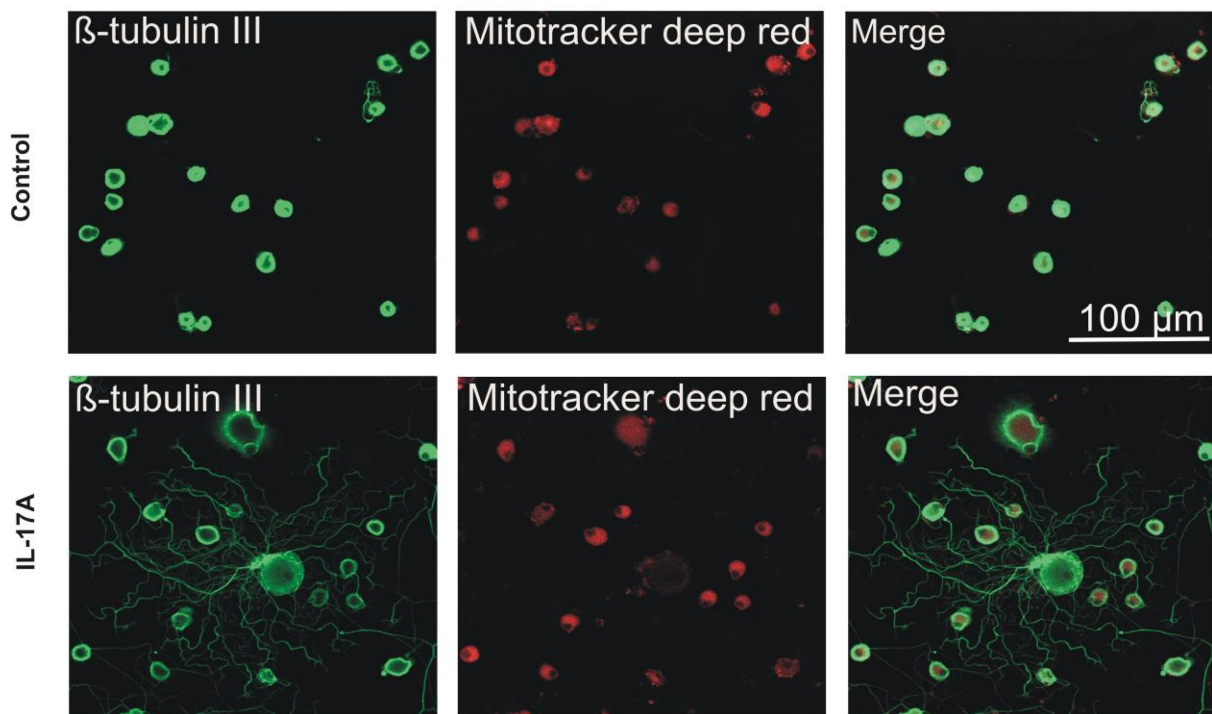


Fig.29 IL-17A enhanced expression of proteins of mitochondrial oxidative phosphorylation was mediated by PI-3K. Neurons isolated from a control rat were treated for 24 hrs with 10 ng/ml IL-17A in the presence of neurotrophic

factors and 3 µM U0126 (MAPK inhibitor) or 3 µM Ly294002 (PI-3K inhibitor). IL-17A induction of expression of proteins belonging to complex II, complex III and complex IV was significantly inhibited by PI-3K inhibitor. Data are mean ± SEM ($n = 3$ replicates); *$p < 0.05$ vs control and Ly294002+IL-17 by oneway ANOVA with Tukey's *posthoc* test.

4.5 Mitochondrial mass was not affected by IL-17A treatment in cultured sensory neurons.

The ability of IL-17A to increase mitochondrial mass was tested. DRG neurons from control rats were cultured and treated with IL-17A in the presence of neurotrophic factor for 24 hr. Cells were loaded with MitoTracker deep red stain which was used to measure the mitochondrial mass since it detects mitochondria irrespective of potential across the inner mitochondrial membrane. Cells then were fixed and stained for neuron-specific β-tubulin III. Although IL-17A enhanced neurite outgrowth in the culture, it did not change the fluorescence intensity of Mitotracker stained cells. Thus, no difference was found in mitochondrial mass between the control and IL-17A treated neurons (Fig.30).



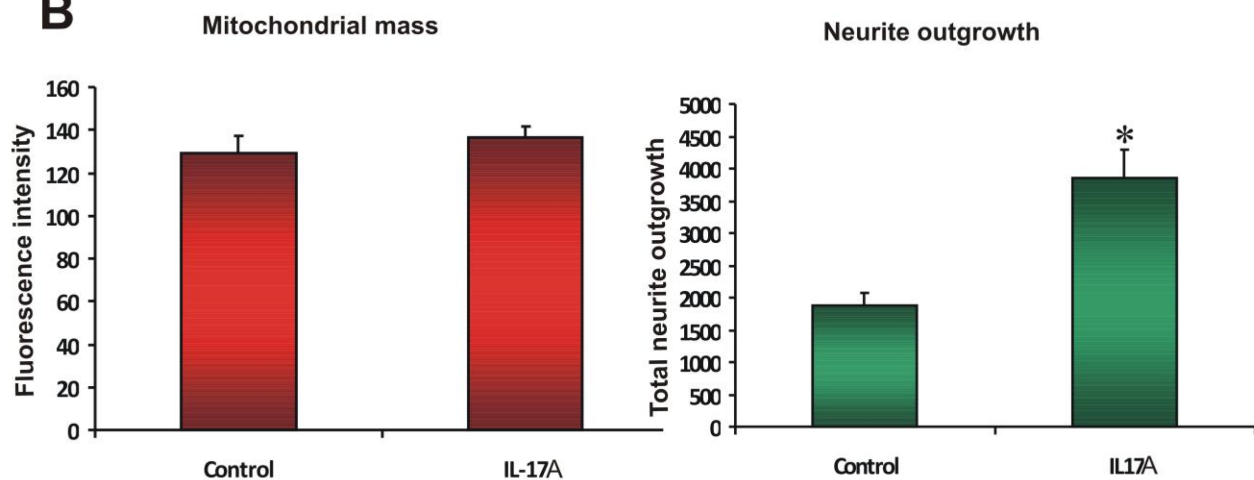


Fig.30 IL-17A did not affect mitochondrial mass in cultured sensory neurons. Sensory neurons were isolated from a control rat and cultured with 10 ng/ml IL-17A for 24 hrs in the presence of low dose neurotrophic factors. Cells were then loaded with Mitotracker deep red (100 nM) for 30 mins. After fixation, neurons were stained for neuron-specific β-tubulin III. (A) Fluorescent images reveal that IL-17A increased neurite outgrowth but did not change the fluorescence intensity of Mitotracker stained cells. Size marker indicates 100 μm. (B) IL-17A significantly enhanced

neurite outgrowth in the culture, however, the mitochondrial mass did not change in the treated neurons in comparison to the control. Values are mean +/- SEM, n = 3 replicate, *p < 0.05 by Student's t-Test.

4.6 IL-17A caused a small but significant increase in porin expression.

I further tested the affect of IL-17A on mitochondrial mass. Neurons were isolated from a control rat and cultured for 24 or 48 hr in the presence of neurotrophic factors and IL-17A. After harvesting the cells, I measured porin expression (mitochondrial membrane protein) by Western blotting. I found that IL-17A slightly increased porin expression in a statistically significant manner after 24 and 48 hr of IL-17A treatment (Fig. 31).

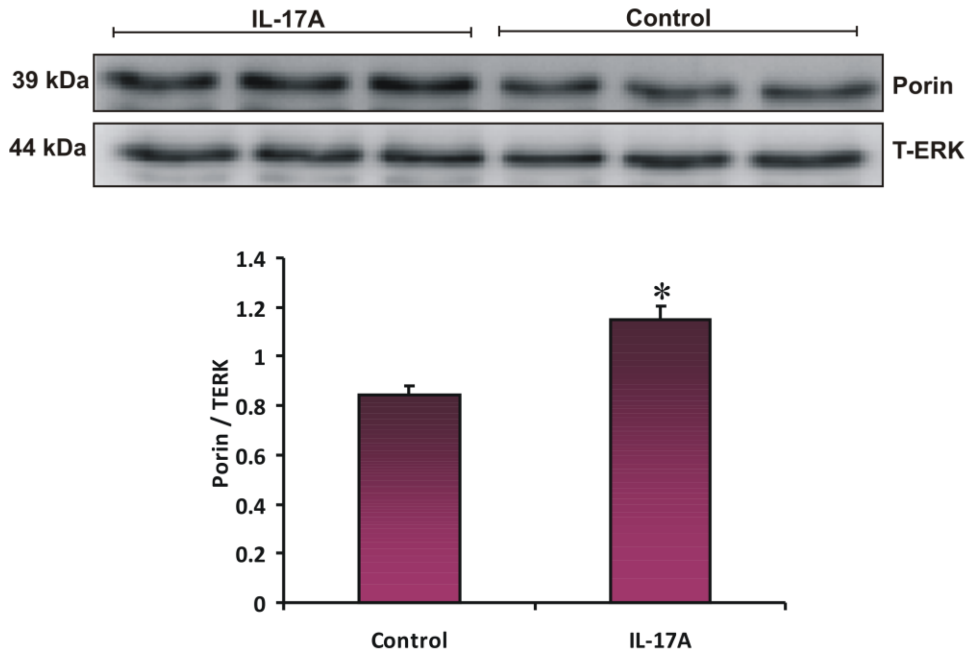


Fig.31 IL-17A caused a small but significant increase in porin expression. Neurons isolated from a control rat were cultured and treated for 24 hrs with 10 ng/ml IL-17A in the presence of low dose neurotrophic factors. IL-17A treated neurons showed a higher level of porin expression compared to non-treated neurons. Same results were found after 48 hr IL-17A treatment. Values are mean +/- SEM, n = 3 replicate, *p < 0.05 by Student's t-Test.

Chapter 5:

Discussion

The aim of this project was to study the signal transduction pathways activated by IL-17A and linked to neurite outgrowth in adult sensory neurons. The findings of this study should help in identifying new drug treatments for diabetic neuropathy and other neurodegenerative diseases. I found that IL-17A enhanced total neurite outgrowth in cultures derived from both control and STZ-induced diabetic rat models through IL-17A-dependent activation of MAPK and PI-3K pathways. In addition, IL-17A improved mitochondrial bioenergetics function of sensory neurons via MAPK and PI-3K and this improvement was associated with augmented expression of proteins of mitochondrial oxidative phosphorylation.

5.1 Cytokine levels and the inflammatory environment in diabetic sensory neurons

Metabolic changes associated with diabetes can eventually lead to a dysregulation of hormonal mediators, such as cytokines. Hyperglycemia has been demonstrated to impact on cytokine levels in different ways in various tissues. One negative impact could be due to lack of activation of important signaling pathways that are involved in inducing cytokine production such as NF-κB [128], or due to down-regulation of receptors responsible for specific cytokine production such as TLR4 [129]. Nevertheless, there is some evidence of a proinflammatory environment in longer term experimental diabetes in DRG and peripheral nerves from patients with minimal neuropathy [130]. Pathogenesis of diabetic neuropathy such as oxidative stress, AGE formation, and lipid peroxidation triggers inflammatory processes and NF-κB activation. This results in raising levels of inflammatory mediators and tissue injury at various sites [131]. Inflammation is

considered an aggravating factor for many neurodegenerative conditions including Alzheimer's disease, Parkinson's disease and diabetic neuropathy [132]. COX-2 and TNF-alpha activity have also been correlated with inflammatory damage in the pathophysiology of diabetic neuropathy [131]. Some markers of inflammation, such as C-reactive protein (CRP), IL-6 and TNF-α are shown to be high in diabetes which suggests a low-grade inflammation in this disease [133]. Increased migration of macrophages or oxidative-stress induced DNA damage appeared to be characteristic in peripheral nerve of STZ-diabetic rats [134]. In a long-term model of experimental diabetes that mimics human diabetic peripheral neuropathy, elevation in RAGE expression was found in the peripheral nervous system and this elevation was connected to the developed pathological changes [135]. However, there is little evidence of neuroinflammation in human sural nerve biopsies and post-mortem DRG samples from diabetic patients with symmetrical polyneuropathy that might contribute to the pathology of diabetes. No signs of macrophage invasion or activation of resident immune cells was observed in the lumbar DRG during the early stages of diabetes [95, 136]. A reduction in NF-κB activation was seen in the DRG of short-term STZ-induced diabetic mice and rats [128]. Deficits in protein expression of NF-κB p50 subunits and RNA expression were also found in DRG of STZ-induced diabetic rats [98]. Thus, there is no direct evidence of inflammation in DRG or nerves in the early stages of the disease in STZ-induced diabetic rats [98]. However, since diabetic neuropathy is associated with progressive neuronal degeneration, and since these neurons try to regenerate and undergo Wallerian degeneration, this leads to continuous recruitment of inflammatory components which eventually build up and enhance the inflammation in the long term. Thus, neuroinflammation could be seen in the end stage of diabetes that is accompanied by major fiber loss, and this inflammation might contribute to the etiology of neuropathy. The diabetic animals that were

used in the former study were 2-5 months STZ-induced diabetic with a moderate level of severity of type 1 diabetes but were neuropathic at the same time [98]. Emerging evidence suggests that pro-inflammatory and neuropoietic cytokines undergo a down-regulation in level of protein expression in neuronal tissues in animal models of type 1 diabetes. These studies found generalized and diminished levels of cytokine signaling that was demonstrated by low levels of some proinflammatory cytokines such as TNFα and IL-1β [98, 99] and CNTF [57]. Our preliminary data, not shown, showed reduced expression of IL-17A in the DRG of 3-5 month STZ-induced diabetic mice (in collaboration with Drs. Soussi-Gounni and Ali Saleh). Thus, I hypothesized that lower IL-17A expression might be involved in the early symptoms of sensory neuropathy.

5.2 IL-17A induction of neurite outgrowth

In the sympathetic nervous system, another group found increases in neurite outgrowth of adult postganglionic sympathetic neurons driven by IL-17A treatment [111]. Our results show for the first time the positive impact of IL-17A on adult sensory neurons. IL-17A was able to enhance neurite outgrowth in adult sensory neurons derived from control and STZ-induced diabetic rat models. This enhancement was mediated by MAPK and PI-3K dependent pathways. IL-17A augmented the growth in control neurons at the concentration of 5 and 10 ng/ml, while the growth was only significant at 10 and 25 ng/ml in STZ-induced diabetic neurons. Nevertheless, IL-17A doubled the neurite outgrowth at a concentration of 1 ng /ml in control, but only a small change was observed in STZ-induced diabetic neurons at this dose (Fig. 15A, 15B). These observations in diabetic models could be due to lack of sensitivity to IL-17A in diabetic sensory neurons, or due to downregulation of IL-17A receptor in the DRG of the diabetic rats. Dysregulation of factors upstream or downstream of MAPK and/or PI-3K signaling is another

possible cause as well. In addition, the induction of neurite outgrowth in the STZ-induced diabetic sensory neurons reaches a plateau after the concentration of 10 ng/ml (Fig. 15B) which could be due to saturation of IL-17A receptor signaling pathway.

IL-17A receptor was found in most neurons in DRG sections as well as in cultured DRG neurons [137]. However, IL-17A receptor was also expressed in the satellite cells of DRGs [114]. Therefore, and despite the fact that minimal non-neuronal cells were found in our cultures (detected by DAPI), there is still a possibility that part of the IL-17A signaling is mediated through satellite glial cells which produce factors that then support the neurons and thus indirectly increase neurite outgrowth. The results from the immunostaining studies suggest that IL-17A mainly modulated the neurite outgrowth in the medium to large diameter neurons as the neurites detected in the cultures were derived from this population of DRG neurons. In addition, blocking IL-17A-activated pathways (MAPK or PI-3K) totally inhibited IL-17A enhancement of neurite outgrowth in normal and diabetic sensory neurons. Thus, we propose that reduced IL-17A signaling may contribute to the deficit in axonal plasticity and regeneration that was observed in diabetic sensory neurons.

IL-17A promoted MAPK and PI-3K activation in sensory neuron culture. These pathways are of importance in neurons as MAPK regulates proliferation, gene expression, differentiation and cell survival [138], while PI-3K is involved in many cell functions such as cell growth, proliferation, differentiation, survival and intracellular trafficking [139]. MAPK and PI-3K can be activated rapidly by IL-17A. The level of phosphorylation for both P-ERK and P-AKT was enhanced after IL-17A treatment. The peak of activation was seen at 15 min in control neurons. In STZ-induced diabetic neurons treatment with IL-17A had a significant effect at both 15 and 30 min. No neurotrophins were added to these cultures which ensured no overlapping activation of pathways

between IL-17A and the neurotrophins since some of these growth factors, such as NGF, activate MAPK and PI-3K [140].

Pharmacological inhibition of MAPK and PI-3K signaling blocked IL-17A-dependent neurite outgrowth. Concentrations of 3 or 10 μM of both inhibitors totally blocked neurite outgrowth driven by IL-17A. However, adding different concentrations of the inhibitors to the control or diabetic neurons did not have a significant impact on the basal neurite outgrowth (Fig. 19A, 20A, 21). This confirms that the inhibition was acting only for IL-17A action rather than affecting the regeneration of basal neurite outgrowth.

5.3 The crosstalk between IL-17A activated pathways

Signaling pathways crosstalk with each other in which one pathway signals to either enhance or suppress signaling by another [141]. MAPK pathway stimulates the release of growth factors which can feed back onto cells to potentiate signaling pathways [142]. ERK and PI-3K were thought to be linear independent signaling pathways activated by different stimuli. However many studies suggested that they might intersect to regulate each other and co-regulate downstream functions [143]. Depending on cell type, PI-3K-AKT and MEK1/2-ERK1/2 signaling pathways work together to maintain cell viability [144]. It is known now that MAPK and PI-3K cross-talk extensively and regulate each other both positively and negatively [143].

MAPK inhibitor (U0126) blocked P-ERK activation by IL-17A, but it did not alter P-AKT activation. On the other hand PI-3K inhibitor (Ly294002) significantly inhibited IL-17A activation of P-AKT and P-ERK (Fig. 22). This informs us that MAPK and PI-3K pathways do not run in parallel in sensory neurons and somehow P-ERK activation depends on PI-3K or it could be that PI-3K in some steps works upstream from MAPK in sensory neurons. Inhibition of

ERK activation by pharmacological inhibitors of PI-3K has been reported in many studies. Wortmannin blocked the activation of ERK by insulin or insulin-like growth factor 1 (IGF 1) in rat skeletal-muscle cell line L6 cells [145]. Another group reported that MAPK activation by lysophosphatidic acid (LPA) was attenuated by the PI-3K inhibitors wortmannin and LY294002. They also suggested that PI-3K activity is required in MAPK signaling pathway at a point upstream of Sos and Ras activation [146]. In addition to cell type, signal strength plays an important role in ERK inhibition by PI-3K blockers. It was found that weak stimulation of ERK depends on PI-3K but strong stimulation is independent of PI-3K [147]. Evidence for the involvement of PI-3K has been found at a number of different positions in the pathway, however the mechanism involved in the ability of PI-3K inhibitors to block ERK activation remains unclear [148].

5.4 IL-17A induction of mitochondrial function

Diabetic sensory neurons have a limited ability to meet a wide variation in ATP demands due to mitochondrial dysfunction in diabetes. This limits the ability of neurons to regenerate. Thus mitochondrial dysfunction is considered as a central mediator in development of diabetic neuropathy and many neurodegenerative diseases [90]. Efficient mitochondrial function with enough supply of ATP to the motile growth cone is required by neurons to drive neurite outgrowth [149]. Although mitochondria of diabetic sensory neurons were found to be energetically stressed with reduced spare respiratory capacity, no evidence of mitochondrial loss in sensory neurons was seen [150]. Dysfunction in mitochondria has been also noticed in pre and post synaptic elements in prevertebral sympathetic ganglia of diabetic mice [93]. Impairment in respiratory chain activity was associated with downregulation of respiratory chain components of the mitochondrial proteome in diabetic sensory neurons [150].

The Seahorse XF analyzer was used to measure mitochondrial bioenergetic functions and cell respiration. Many parameters are given by this instrument such as ATP production rate, maximal respiration rate and spare respiratory capacity which gives us information about different behaviors of mitochondria in different conditions. Mitochondrial bioenergetic function was enhanced after 3 and 6 hr of IL-17A treatment in cultured control sensory neurons. Similar results were found after longer term treatment (24 hrs). IL-17A significantly increased maximal respiration and spare respiratory capacity in normal and diabetic sensory neurons via MAPK and PI-3K (Fig. 25, 26). Spare respiratory capacity determines neurons ability to meet energetic needs [119]. Thus, IL-17A allows the mitochondria to increase ATP synthesis under stress conditions, and since the ATP demand for growth cone motility is high, this increase in mitochondrial oxidative capacity could increase axonal plasticity [149]. Thus, we propose that IL-17A signals through MAPK and PI-3K signalling pathways to modulate mitochondrial function and this modulation may underlie the ability of IL-17A to induce neurite outgrowth and protect the neurons from pathogenesis of diabetic neuropathy.

MAPK and PI-3K mediation of IL-17A-induction of mitochondrial bioenergetics is not unusual. PI-3K regulates mitochondrial homeostasis in part through PGC-1β. In addition, blocking PI-3K induces ROS to arrest cell growth and reduces mitochondrial mass in human lung adenocarcinoma A549 cells [151]. In follicular and anaplastic carcinoma cell lines, acute PI-3K inhibition significantly decreased active mitochondria and was accompanied by a significant shift in oxygen consumption [152]. PI-3K provides mitochondrial protection through preserving mitochondrial membrane potential in oxidative stress and this effect happens upon poly ADP-ribose polymerase (PARP) inhibition in human liver cells [153]. Significant levels of MEK and ERK were localized to the mitochondria in alveolar macrophages [154], cardiomyocytes [155]

and in the mitochondria of the brain at the outer membrane/intermembrane space [156]. In addition, active ERK was found associated with mitochondrial proteins in midbrain tissues [157]. On top of that, inhibition of ERK activity induced a profound reduction in cellular ATP and was accompanied with a loss of mitochondrial transmembrane potential in alveolar macrophages [154]. ERK inhibition led to cytosolic release of mitochondrial proteins, caspase activation and cell death. The cell death induced by ERK inhibition had distinctive features of both apoptotic (caspase activation) and necrotic (ATP loss) cell death [154].

In addition to IL-17A, other cytokines such as TNFα, IL-1β and CNTF showed the ability to modulate mitochondrial function in sensory neurons [97-99] and chondrocyte cells [158]. Growth factors as well play roles in controlling mitochondrial function. BDNF, the activity-dependent neurotrophin, was shown to increase the respiratory coupling index (RCI) of mitochondria derived from rat brain through a MEK kinase mechanism via complex I [159]. Also BDNF enhances PGC-1α-mediated mitochondrial biogenesis in hippocampal neurons. This enhancement was demonstrated by elevated ATP production and increased mitochondrial mass indicated by a greater amount of MitoTracker-green fluorescence. In addition, levels of two mitochondrial proteins, cytochrome oxidase 1 and cyt-c were also elevated considerably in response to BDNF [160]. Furthermore, NGF takes part in modulating mitochondrial function. After re-exposure of NGF-deprived sympathetic neurons to NGF, mitochondria recovered their normal size and their cytochrome *c* content, by a process requiring de novo protein synthesis [161].

An interesting finding in our bioenergetics data was the increase in the basal respiration upon treatment with U0126 in normal sensory neurons. The mitochondria were working at their maximum capacity in that group as the uncoupler (FCCP) was not able to further enhance the

respiration. That was demonstrated in the spare respiratory capacity which had a value around zero in this treatment group. There is no clear explanation for this phenomenon, but it seems that MAPK is acting as a brake on basal respiration and blocking this brake increased the oxygen consumption at the basal level to its maximum.

5.5 The basis of enhanced mitochondrial function by IL-17A

To find an explanation for the enhanced mitochondrial bioenergetics function, I tested complex IV cytochrome *c* oxidase (COX) activity. COX IV is the final protein complex in the electron transport chain; it transfers electrons to oxygen and pumps protons through the membrane to form water by reducing oxygen. I found that the rate of oxidation of COX IV was promoted after IL-17A treatment and is best explained by an enhancement in the enzymatic activity of this complex. That encouraged us to test the expression of specific protein components of the different complexes that are involved in the ATP production and mitochondrial electron transport chain. Upon 24 hr IL-17A treatment, I found an increase in the expression of some mitochondrial oxidative phosphorylation proteins including subunit succinate dehydrogenase B (SDHB, part of Complex II), subunit ubiquinol-cytochrome *c* reductase core protein II (UQCRC2, part of Complex III) and subunit cytochrome oxidase II (COX-II, part of Complex IV). This data suggests that IL-17A is enriching protein levels for these complexes and increasing the activity of the mitochondrial electron transport chain. This augmentation of Complex activity might account for the induction of mitochondrial bioenergetics function. I could not find a change in the expression of subunit ATP5A, a subunit of Complex V. However, this does not necessarily mean that complex V expression is not altered after IL-17A treatment; it could be the expression of this particular subunit is not affected by IL-17A.

It is unlikely that the acute induction of mitochondrial bioenergetics observed after 3 and 6 hr could be due entirely to changes in gene expression. That's because any outcome of gene expression or protein synthesis requires more than 6 hrs to be visible. However the enhancement of the activity and expression of mitochondrial oxidative phosphorylation proteins that I noticed after 24 hr and 48 hr treatment suggests that the changes in gene expression come after this acute effect to reinforce the initial IL-17A-induced enhancement of mitochondrial function. On the other hand, the acute effect could be mediated by a rapid signaling pathway, or IL-17A could be somehow providing the electron transport chain with more substrates through inducing the enzymatic activity of the TCA cycle.

5.6 The role of mitochondrial mass in promoting bioenergetics function

Mitochondrial mass was investigated to check if an elevation in total mitochondrial number and or mass contributed to the observed promotion in bioenergetics function. Porin is a membrane protein that acts as a pore for molecules to diffuse into the organelle; it is mainly present in the mitochondrial membrane and is routinely used as a marker for mitochondrial identification by staining and of mass. After 24 hours of IL-17A treatment, there was a small, but significant, increase in porin expression which might indicate an increase in mitochondrial mass (Fig. 31). These results were confirmed using an alternative method. Cultured sensory neurons were loaded with MitoTracker deep red, a stain that binds to mitochondria regardless of the membrane potential and used as a marker for mitochondrial mass. Although IL-17A enhanced total neurite outgrowth in the culture, it did not change the fluorescence intensity of MitoTracker in the cell bodies of the neurons (Fig. 30). So in opposition to the porin results, this data suggests that total amount of mitochondria per cell did not change after IL-17A treatment. Therefore, an enhancement of mitochondrial mass does not account for the changes in bioenergetics function I

observe in mitochondria. The small increase in porin expression could be due to a specific effect of IL-17A on porin expression and not necessarily linked to increased mitochondrial mass. It was also noticed that the smaller sensory neurons, which are mostly thinly myelinated or unmyelinated neurons, had a higher mitochondrial content. This observation mimics what is usually seen *in vivo* as unmyelinated neurons have higher demand for energy to transduce the signal along the whole axon (there is no salutatory nerve conduction between Nodes of Ranvier) [162]. On the other hand, the medium to large neurons which are myelinated, had less mitochondrial mass. This is also understandable since myelinated neurons need less energy to transducer signals as the action potential jumps from one Node of Ranvier to another along the axon. Thus, mitochondrial content of medium to large neurons is found concentrated at these nodes, and inter-nodal areas have relatively sparse mitochondrial numbers [163]. Another explanation for the lower mitochondrial content in larger neurons which exhibited higher levels of neurite outgrowth is that the mitochondria migrate to areas of need. Axonal transport distributes mitochondria to regions of the neuron where their functions are required [164]. In addition, in cultured neurons, mitochondrial transport responds to growth cone activity [165]. Thus the organelle translocates to the end of the axon (to the growth cone) to produce the energy needed for growth cone motility.

5.7 Indirect mechanism for IL-17A action in neurons

Increasing the production of other cytokines that have an important role in the neuroregeneration process, such as TNFα, IL-1β and IL-6 by IL-17A [166] could also contribute to IL-17A action in sensory neurons. IL-1β has been strongly linked to nerve regeneration for many reasons, particularly its involvement in Schwann cell proliferation and induction of neurotrophic factors [33]. IL-6 (member of gp130 cytokines) enhances gene expression and induces growth through

activation of JAK/STAT and MAPK signaling pathways. It also induces the production of neuropeptides, known as regeneration associated genes (RAG), which have important roles in neuronal survival and regeneration [50]. In addition, TNFα induced neurite outgrowth in diabetic sensory neurons through NF-κB [98]. Thus, I think that IL-17A possibly mediates the previous actions in neurons indirectly through induction of such other cytokine production.

5.8 Conclusion:

This project demonstrates novel work that identifies, for the first time, the role of IL-17A in adult sensory neurons derived from normal or diabetic rats. The findings of this study should help in identifying new drug treatments for diabetic neuropathy and other neurodegenerative diseases. The results show that IL-17A treatment enhanced axonal plasticity in cultured sensory neurons from normal or diabetic rats. This enhancement was mediated by MAPK and PI-3K pathways, as a result, it is suggested that reduced IL-17A signaling may contribute to the deficit in axonal plasticity and regeneration within the target field of diabetic sensory neurons. IL-17A-induction of neurite outgrowth was mediated, in part, via enhancement of mitochondrial bioenergetics through the MAPK and PI-3K pathways. Thus, it is proposed that IL-17A signals through MAPK and PI-3K to modulate mitochondrial function and this modulation may underlie the ability of IL-17A to induce neurite outgrowth and protect the neurons from pathogenesis of diabetic neuropathy. It is also believed that IL-17A enriches the protein levels of mitochondrial oxidative phosphorylation and increases the activity of mitochondrial electron transport chain and this may lie beneath its ability to modulate mitochondrial function.

6. Bibliography:

1. Lee, Y., C.H. Lee, and U. Oh, *Painful channels in sensory neurons.* Mol Cells, 2005. **20**(3): p. 315-24.
2. Waxman, S.G., *Clinical neuroanatomy*. 26th ed. A Lange medical book. 2010, New York: McGraw-Hill Medical. xi, 371 p.
3. Topp, K.S. and B.S. Boyd, *Structure and biomechanics of peripheral nerves: nerve responses to physical stresses and implications for physical therapist practice.* Phys Ther, 2006. **86**(1): p. 92-109.
4. Kalat, J.W., *Biological psychology*. 9th ed. 2007, Belmont, CA: Thomson/Wadsworth. xx, 582 p.
5. Elliott L. Mancall, D.G.B., *Gray's Clinical Neuroanatomy: The Anatomic Basis for Clinical Neuroscience*. 2011.
6. Coleman, M.P., *The challenges of axon survival: introduction to the special issue on axonal degeneration.* Exp Neurol, 2013. **246**: p. 1-5.
7. Inbal, R., et al., *Collateral sprouting in skin and sensory recovery after nerve injury in man.* Pain, 1987. **28**(2): p. 141-54.
8. Diamond, J., et al., *Evidence that endogenous beta nerve growth factor is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in adult rats.* Proc Natl Acad Sci U S A, 1987. **84**(18): p. 6596-600.
9. Cajal, S.R.Y., May, R.M., *Degeneration and Regeneration of the Nervous System. .* Am J Psychiatry, 1929. **86**: p. 212-218.
10. Fawcett, J.W. and R.J. Keynes, *Peripheral nerve regeneration.* Annu Rev Neurosci, 1990. **13**: p. 43-60.
11. Ochi, M., et al., *Further experimental evidence of selective nerve regeneration in aortic Y-chambers.* Scand J Plast Reconstr Surg Hand Surg, 1994. **28**(2): p. 137-41.
12. Haftek, J. and P.K. Thomas, *Electron-Microscope Observations on Effects of Localized Crush Injuries on Connective Tissues of Peripheral Nerve.* Journal of Anatomy, 1968. **103**: p. 233-&.
13. D.Tonge. and J. GOLDING., *Regeneration and repair of the peripheral nervous system.* Seminars in Neuroscience, 1993. **5**(6): p. 385-390.
14. Brecknell, J.E. and J.W. Fawcett, *Axonal regeneration.* Biol Rev Camb Philos Soc, 1996. **71**(2): p. 227-55.
15. Gumy, L.F., C.L. Tan, and J.W. Fawcett, *The role of local protein synthesis and degradation in axon regeneration.* Exp Neurol, 2010. **223**(1): p. 28-37.
16. Coleman, M.P., et al., *An 85-kb tandem triplication in the slow Wallerian degeneration (Wlds) mouse.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 9985-90.
17. Gilley, J., et al., *Rescue of peripheral and CNS axon defects in mice lacking NMNAT2.* J Neurosci. **33**(33): p. 13410-24.
18. Coleman, M.P. and M.R. Freeman, *Wallerian degeneration, wld(s), and nmnat.* Annu Rev Neurosci, 2010. **33**: p. 245-67.
19. Richardson, P.M., et al., *Responses of the nerve cell body to axotomy.* Neurosurgery, 2009. **65**(4 Suppl): p. A74-9.
20. Liu, R.Y. and W.D. Snider, *Different signaling pathways mediate regenerative versus developmental sensory axon growth.* J Neurosci, 2001. **21**(17): p. RC164.
21. Thiele, C.J., Z. Li, and A.E. McKee, *On Trk--the TrkB signal transduction pathway is an increasingly important target in cancer biology.* Clin Cancer Res, 2009. **15**(19): p. 5962-7.

22. Caroni, P. and M.E. Schwab, *Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading.* J Cell Biol, 1988. **106**(4): p. 1281-8.
23. Reier, P.J. and J.D. Houle, *The glial scar: its bearing on axonal elongation and transplantation approaches to CNS repair.* Adv Neurol, 1988. **47**: p. 87-138.
24. Bauer, J., et al., *Astrocytes synthesize and secrete alpha 2-macroglobulin: differences between the regulation of alpha 2-macroglobulin synthesis in rat liver and brain.* Adv Exp Med Biol, 1988. **240**: p. 199-205.
25. GrandPre, T., et al., *Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein.* Nature, 2000. **403**(6768): p. 439-44.
26. Snider, W.D., et al., *Signaling the pathway to regeneration.* Neuron, 2002. **35**(1): p. 13-6.
27. Caroni, P. and M.E. Schwab, *Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter.* Neuron, 1988. **1**(1): p. 85-96.
28. Kennedy, J.M. and D.W. Zochodne, *Impaired peripheral nerve regeneration in diabetes mellitus.* J Peripher Nerv Syst, 2005. **10**(2): p. 144-57.
29. Creange, A., G. Barlovatz-Meimon, and R.K. Gherardi, *Cytokines and peripheral nerve disorders.* Eur Cytokine Netw, 1997. **8**(2): p. 145-51.
30. Stoll, G., et al., *Tumor necrosis factor-alpha in immune-mediated demyelination and Wallerian degeneration of the rat peripheral nervous system.* J Neuroimmunol, 1993. **45**(1-2): p. 175-82.
31. Redford, E.J., S.M. Hall, and K.J. Smith, *Vascular changes and demyelination induced by the intraneural injection of tumour necrosis factor.* Brain, 1995. **118 (Pt 4)**: p. 869-78.
32. Lisak, R.P. and B. Bealmear, *Antibodies to interleukin-1 inhibit cytokine-induced proliferation of neonatal rat Schwann cells in vitro.* J Neuroimmunol, 1991. **31**(2): p. 123-32.
33. Lindholm, D., et al., *Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve.* Nature, 1987. **330**(6149): p. 658-9.
34. Unsicker, K., et al., *Cytokines in neural regeneration.* Curr Opin Neurobiol, 1992. **2**(5): p. 671-8.
35. Dinarello, C.A., *Proinflammatory cytokines.* Chest, 2000. **118**(2): p. 503-8.
36. Zhang, J.M. and J. An, *Cytokines, inflammation, and pain.* Int Anesthesiol Clin, 2007. **45**(2): p. 27-37.
37. Richard Coico, G.S., *Immunology: A Short Course, 6th Edition*. 2008: Wiley-Blackwell.
38. Gardiner, N.J., et al., *Expression of gp130 and leukaemia inhibitory factor receptor subunits in adult rat sensory neurones: regulation by nerve injury.* J Neurochem, 2002. **83**(1): p. 100-9.
39. White, U.A. and J.M. Stephens, *The gp130 receptor cytokine family: regulators of adipocyte development and function.* Curr Pharm Des, 2011. **17**(4): p. 340-6.
40. O'Neill, L.A., *The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress.* Immunol Rev, 2008. **226**: p. 10-8.
41. Boraschi, D. and A. Tagliabue, *The interleukin-1 receptor family.* Vitam Horm, 2006. **74**: p. 229-54.
42. Verstrepen, L., et al., *TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme.* Cell Mol Life Sci, 2008. **65**(19): p. 2964-78.
43. Casetta, I., V. Govoni, and E. Granieri, *Oxidative stress, antioxidants and neurodegenerative diseases.* Curr Pharm Des, 2005. **11**(16): p. 2033-52.
44. Lu, Y., et al., *JAK/STAT and PI3K/AKT pathways form a mutual transactivation loop and afford resistance to oxidative stress-induced apoptosis in cardiomyocytes.* Cell Physiol Biochem, 2008. **21**(4): p. 305-14.

45. Xie, W.R., et al., *Robust increase of cutaneous sensitivity, cytokine production and sympathetic sprouting in rats with localized inflammatory irritation of the spinal ganglia.* Neuroscience, 2006. **142**(3): p. 809-22.
46. Conti, G., et al., *Interleukin-1 beta and interferon-gamma induce proliferation and apoptosis in cultured Schwann cells.* J Neuroimmunol, 2002. **124**(1-2): p. 29-35.
47. Temporin, K., et al., *IL-1beta promotes neurite outgrowth by deactivating RhoA via p38 MAPK pathway.* Biochem Biophys Res Commun, 2008. **365**(2): p. 375-80.
48. Copray, J.C., et al., *Expression of interleukin-1 beta in rat dorsal root ganglia.* J Neuroimmunol, 2001. **118**(2): p. 203-11.
49. Simi, A., et al., *Mechanisms of regulation for interleukin-1beta in neurodegenerative disease.* Neuropharmacology, 2007. **52**(8): p. 1563-9.
50. Zigmond, R.E., *gp130 cytokines are positive signals triggering changes in gene expression and axon outgrowth in peripheral neurons following injury.* Front Mol Neurosci, 2011. **4**: p. 62.
51. Pieraut, S., et al., *NKCC1 phosphorylation stimulates neurite growth of injured adult sensory neurons.* J Neurosci, 2007. **27**(25): p. 6751-9.
52. Wang, L., et al., *Cell type-specific STAT3 activation by gp130-related cytokines in the peripheral nerves.* Neuroreport, 2009. **20**(7): p. 663-8.
53. Murphy, P.G., et al., *Induction of interleukin-6 in axotomized sensory neurons.* J Neurosci, 1995. **15**(7 Pt 2): p. 5130-8.
54. Verge, V.M., et al., *Influence of nerve growth factor on neurofilament gene expression in mature primary sensory neurons.* J Neurosci, 1990. **10**(6): p. 2018-25.
55. Siegel, S.G., B. Patton, and A.W. English, *Ciliary neurotrophic factor is required for motoneuron sprouting.* Exp Neurol, 2000. **166**(2): p. 205-12.
56. Sango, K., et al., *Neuroprotective properties of ciliary neurotrophic factor for cultured adult rat dorsal root ganglion neurons.* Histochem Cell Biol, 2008. **130**(4): p. 669-79.
57. Mizisin, A.P., et al., *Ciliary neurotrophic factor improves nerve conduction and ameliorates regeneration deficits in diabetic rats.* Diabetes, 2004. **53**(7): p. 1807-12.
58. Segal, R.A. and M.E. Greenberg, *Intracellular signaling pathways activated by neurotrophic factors.* Annu Rev Neurosci, 1996. **19**: p. 463-89.
59. Cui, Q. and A.R. Harvey, *CNTF promotes the regrowth of retinal ganglion cell axons into murine peripheral nerve grafts.* Neuroreport, 2000. **11**(18): p. 3999-4002.
60. Muller, A., et al., *Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF.* Mol Cell Neurosci, 2009. **41**(2): p. 233-46.
61. Wu, D., et al., *Actions of neuropoietic cytokines and cyclic AMP in regenerative conditioning of rat primary sensory neurons.* Exp Neurol, 2007. **204**(1): p. 66-76.
62. Park, K., et al., *Cellular mechanisms associated with spontaneous and ciliary neurotrophic factor-cAMP-induced survival and axonal regeneration of adult retinal ganglion cells.* J Neurosci, 2004. **24**(48): p. 10806-15.
63. Neumann, S., et al., *Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation.* Neuron, 2002. **34**(6): p. 885-93.
64. Cui, Q., et al., *Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons.* Mol Cell Neurosci, 2003. **22**(1): p. 49-61.
65. Alonzi, T., et al., *Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons.* Mol Cell Neurosci, 2001. **18**(3): p. 270-82.
66. Subang, M.C. and P.M. Richardson, *Synthesis of leukemia inhibitory factor in injured peripheral nerves and their cells.* Brain Res, 2001. **900**(2): p. 329-31.

67. Hamilton, J.A., P.M. Waring, and E.L. Filonzi, *Induction of leukemia inhibitory factor in human synovial fibroblasts by IL-1 and tumor necrosis factor-alpha.* J Immunol, 1993. **150**(4): p. 1496-502.
68. Smith, D.S. and J.H. Skene, *A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth.* J Neurosci, 1997. **17**(2): p. 646-58.
69. Murphy, M., et al., *Cytokines which signal through the LIF receptor and their actions in the nervous system.* Prog Neurobiol, 1997. **52**(5): p. 355-78.
70. Cafferty, W.B., et al., *Leukemia inhibitory factor determines the growth status of injured adult sensory neurons.* J Neurosci, 2001. **21**(18): p. 7161-70.
71. Qiu, J., et al., *Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation.* J Neurosci, 2005. **25**(7): p. 1645-53.
72. Cafferty, W.B., et al., *Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice.* J Neurosci, 2004. **24**(18): p. 4432-43.
73. Miao, T., et al., *Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons.* J Neurosci, 2006. **26**(37): p. 9512-9.
74. Junger, H. and L.S. Sorkin, *Nociceptive and inflammatory effects of subcutaneous TNFalpha.* Pain, 2000. **85**(1-2): p. 145-51.
75. Fehrenbacher, J.C., et al., *Tumor necrosis factor alpha and interleukin-1beta stimulate the expression of cyclooxygenase II but do not alter prostaglandin E2 receptor mRNA levels in cultured dorsal root ganglia cells.* Pain, 2005. **113**(1-2): p. 113-22.
76. Moalem, G., K. Xu, and L. Yu, *T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats.* Neuroscience, 2004. **129**(3): p. 767-77.
77. Nadeau, S., et al., *Functional recovery after peripheral nerve injury is dependent on the pro-inflammatory cytokines IL-1beta and TNF: implications for neuropathic pain.* J Neurosci, 2011. **31**(35): p. 12533-42.
78. von Banchet, G.S., et al., *Molecular effects of interleukin-1beta on dorsal root ganglion neurons: prevention of ligand-induced internalization of the bradykinin 2 receptor and downregulation of G protein-coupled receptor kinase 2.* Mol Cell Neurosci, 2011. **46**(1): p. 262-71.
79. Maruthur, N.M., *The growing prevalence of type 2 diabetes: increased incidence or improved survival?* Curr Diab Rep, 2013. **13**(6): p. 786-94.
80. Said, G., *Diabetic neuropathy--a review.* Nat Clin Pract Neurol, 2007. **3**(6): p. 331-40.
81. Toth, C., et al., *Diabetes mellitus and the sensory neuron.* J Neuropathol Exp Neurol, 2004. **63**(6): p. 561-73.
82. Watkins, P.J. and P.K. Thomas, *Diabetes mellitus and the nervous system.* J Neurol Neurosurg Psychiatry, 1998. **65**(5): p. 620-32.
83. Sima, A.A. and H. Kamiya, *Diabetic neuropathy differs in type 1 and type 2 diabetes.* Ann N Y Acad Sci, 2006. **1084**: p. 235-49.
84. Brownlee, M., *Biochemistry and molecular cell biology of diabetic complications.* Nature, 2001. **414**(6865): p. 813-20.
85. Sugimoto, K., et al., *Localization in human diabetic peripheral nerve of N(epsilon)-carboxymethyllysine-protein adducts, an advanced glycation endproduct.* Diabetologia, 1997. **40**(12): p. 1380-7.
86. Tomlinson, D.R., P. Fernyhough, and L.T. Diemel, *Role of neurotrophins in diabetic neuropathy and treatment with nerve growth factors.* Diabetes, 1997. **46 Suppl 2**: p. S43-9.
87. Obrosova, I.G., *How does glucose generate oxidative stress in peripheral nerve?* Int Rev Neurobiol, 2002. **50**: p. 3-35.

88. Vincent, A.M., et al., *Oxidative stress in the pathogenesis of diabetic neuropathy.* Endocr Rev, 2004. **25**(4): p. 612-28.
89. Yorek, M.A., *The role of oxidative stress in diabetic vascular and neural disease.* Free Radic Res, 2003. **37**(5): p. 471-80.
90. 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.
91. Chowdhury, S.K., et al., *Mitochondrial respiratory chain dysfunction in dorsal root ganglia of streptozotocin-induced diabetic rats and its correction by insulin treatment.* Diabetes, 2010. **59**(4): p. 1082-91.
92. Kalichman, M.W., H.C. Powell, and A.P. Mizisin, *Reactive, degenerative, and proliferative Schwann cell responses in experimental galactose and human diabetic neuropathy.* Acta Neuropathol, 1998. **95**(1): p. 47-56.
93. Schmidt, R.E., C.A. Parvin, and K.G. Green, *Synaptic ultrastructural alterations anticipate the development of neuroaxonal dystrophy in sympathetic ganglia of aged and diabetic mice.* J Neuropathol Exp Neurol, 2008. **67**(12): p. 1166-86.
94. Russell, J.W., et al., *Neurons undergo apoptosis in animal and cell culture models of diabetes.* Neurobiol Dis, 1999. **6**(5): p. 347-63.
95. Schmidt, R.E., et al., *Dystrophic axonal swellings develop as a function of age and diabetes in human dorsal root ganglia.* J Neuropathol Exp Neurol, 1997. **56**(9): p. 1028-43.
96. Calcutt, N.A., et al., *Reduced ciliary neuronotrophic factor-like activity in nerves from diabetic or galactose-fed rats.* Brain Res, 1992. **575**(2): p. 320-4.
97. 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.
98. 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. **1423**: p. 87-95.
99. 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**(1): p. 45.
100. Temporin, K., et al., *Interleukin-1 beta promotes sensory nerve regeneration after sciatic nerve injury.* Neurosci Lett, 2008. **440**(2): p. 130-3.
101. Ozaktay, A.C., et al., *Effects of interleukin-1 beta, interleukin-6, and tumor necrosis factor on sensitivity of dorsal root ganglion and peripheral receptive fields in rats.* Eur Spine J, 2006. **15**(10): p. 1529-37.
102. Gabay, E., et al., *Chronic blockade of interleukin-1 (IL-1) prevents and attenuates neuropathic pain behavior and spontaneous ectopic neuronal activity following nerve injury.* Eur J Pain, 2011. **15**(3): p. 242-8.
103. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* J Exp Med, 2005. **201**(2): p. 233-40.
104. Moseley, T.A., et al., *Interleukin-17 family and IL-17 receptors.* Cytokine Growth Factor Rev, 2003. **14**(2): p. 155-74.
105. Chabaud, M., et al., *Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines.* J Immunol, 1998. **161**(1): p. 409-14.

106. Subramaniam, S.V., L.L. Pearson, and S.E. Adunyah, *Interleukin-17 induces rapid tyrosine phosphorylation and activation of raf-1 kinase in human monocytic progenitor cell line U937.* Biochem Biophys Res Commun, 1999. **259**(1): p. 172-7.
107. Broxmeyer, H.E., *Is interleukin 17, an inducible cytokine that stimulates production of other cytokines, merely a redundant player in a sea of other biomolecules?* J Exp Med, 1996. **183**(6): p. 2411-5.
108. Xu, S. and X. Cao, *Interleukin-17 and its expanding biological functions.* Cell Mol Immunol, 2010. **7**(3): p. 164-74.
109. Littman, D.R. and A.Y. Rudensky, *Th17 and regulatory T cells in mediating and restraining inflammation.* Cell. **140**(6): p. 845-58.
110. Li, Z., et al., *IL-17 and VEGF are necessary for efficient corneal nerve regeneration.* Am J Pathol. **178**(3): p. 1106-16.
111. Chisholm, S.P., et al., *Interleukin-17A increases neurite outgrowth from adult postganglionic sympathetic neurons.* J Neurosci. **32**(4): p. 1146-55.
112. Bolsover, S.R., *Calcium signalling in growth cone migration.* Cell Calcium, 2005. **37**(5): p. 395-402.
113. Ryan, S.K., et al., *Glutamate regulates neurite outgrowth of cultured descending brain neurons from larval lamprey.* Dev Neurobiol, 2007. **67**(2): p. 173-88.
114. Richter, F., et al., *Interleukin-17 sensitizes joint nociceptors to mechanical stimuli and contributes to arthritic pain through neuronal interleukin-17 receptors in rodents.* Arthritis Rheum, 2012. **64**(12): p. 4125-34.
115. Boettger, M.K., et al., *Antinociceptive effects of tumor necrosis factor alpha neutralization in a rat model of antigen-induced arthritis: evidence of a neuronal target.* Arthritis Rheum, 2008. **58**(8): p. 2368-78.
116. Aley, K.O., et al., *Nociceptor sensitization by extracellular signal-regulated kinases.* J Neurosci, 2001. **21**(17): p. 6933-9.
117. Kim, C.F. and G. Moalem-Taylor, *Interleukin-17 contributes to neuroinflammation and neuropathic pain following peripheral nerve injury in mice.* J Pain, 2011. **12**(3): p. 370-83.
118. Fernyhough, P., et al., *Aberrant neurofilament phosphorylation in sensory neurons of rats with diabetic neuropathy.* Diabetes, 1999. **48**(4): p. 881-9.
119. Brand, M.D. and D.G. Nicholls, *Assessing mitochondrial dysfunction in cells.* Biochem J. **435**(2): p. 297-312.
120. 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.
121. Calcutt, N.A., et al., *Tactile allodynia and formalin hyperalgesia in streptozotocin-diabetic rats: effects of insulin, aldose reductase inhibition and lidocaine.* Pain, 1996. **68**(2-3): p. 293-9.
122. Hill, B.G., et al., *Importance of the bioenergetic reserve capacity in response to cardiomyocyte stress induced by 4-hydroxynonenal.* Biochem J, 2009. **424**(1): p. 99-107.
123. Devor, M., *Unexplained peculiarities of the dorsal root ganglion.* Pain, 1999. **Suppl 6**: p. S27-35.
124. Vlahos, C.J., et al., *A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002).* J Biol Chem, 1994. **269**(7): p. 5241-8.
125. Zheng, C.F. and K.L. Guan, *Cytoplasmic localization of the mitogen-activated protein kinase activator MEK.* J Biol Chem, 1994. **269**(31): p. 19947-52.
126. Favata, M.F., et al., *Identification of a novel inhibitor of mitogen-activated protein kinase kinase.* J Biol Chem, 1998. **273**(29): p. 18623-32.
127. Fernyhough, P., S.K. Roy Chowdhury, and R.E. Schmidt, *Mitochondrial stress and the pathogenesis of diabetic neuropathy.* Expert Rev Endocrinol Metab, 2010. **5**(1): p. 39-49.

128. Purves, T.D. and D.R. Tomlinson, *Diminished transcription factor survival signals in dorsal root ganglia in rats with streptozotocin-induced diabetes.* Ann N Y Acad Sci, 2002. **973**: p. 472-6.
129. Kanhaiya, A.N., Gupta SK, Kiran Singh, *Differential Expression of Toll like Receptor 4 in Type 2 Diabetic Patients with Impaired Wound Healing.* Diabetes & Metabolism, 2013. **4**: p. 260.
130. Drel, V.R., et al., *New therapeutic and biomarker discovery for peripheral diabetic neuropathy: PARP inhibitor, nitrotyrosine, and tumor necrosis factor-{alpha}.* Endocrinology. **151**(6): p. 2547-55.
131. Kumar, A. and S.S. Sharma, *NF-kappaB inhibitory action of resveratrol: a probable mechanism of neuroprotection in experimental diabetic neuropathy.* Biochem Biophys Res Commun, 2010. **394**(2): p. 360-5.
132. Cameron, N.E. and M.A. Cotter, *Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway.* Curr Drug Targets, 2008. **9**(1): p. 60-7.
133. Ho, E. and T.M. Bray, *Antioxidants, NFkappaB activation, and diabetogenesis.* Proc Soc Exp Biol Med, 1999. **222**(3): p. 205-13.
134. Yamagishi, S., et al., *Correction of protein kinase C activity and macrophage migration in peripheral nerve by pioglitazone, peroxisome proliferator activated-gamma-ligand, in insulin-deficient diabetic rats.* J Neurochem, 2008. **104**(2): p. 491-9.
135. Toth, C., et al., *Receptor for advanced glycation end products (RAGEs) and experimental diabetic neuropathy.* Diabetes, 2008. **57**(4): p. 1002-17.
136. Mizisin, A.P., et al., *Comparable myelinated nerve pathology in feline and human diabetes mellitus.* Acta Neuropathol, 2007. **113**(4): p. 431-42.
137. Segond von Banchet, G., et al., *Neuronal IL-17 receptor upregulates TRPV4 but not TRPV1 receptors in DRG neurons and mediates mechanical but not thermal hyperalgesia.* Mol Cell Neurosci, 2013. **52**: p. 152-60.
138. Cargnello, M. and P.P. Roux, *Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases.* Microbiol Mol Biol Rev, 2011. **75**(1): p. 50-83.
139. Arcaro, A. and A.S. Guerreiro, *The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications.* Curr Genomics, 2007. **8**(5): p. 271-306.
140. Hong, J., et al., *NGF promotes cell cycle progression by regulating D-type cyclins via PI3K/Akt and MAPK/Erk activation in human corneal epithelial cells.* Mol Vis, 2012. **18**: p. 758-64.
141. Reardon, D.B., et al., *Dominant negative EGFR-CD533 and inhibition of MAPK modify JNK1 activation and enhance radiation toxicity of human mammary carcinoma cells.* Oncogene, 1999. **18**(33): p. 4756-66.
142. Dent, P., et al., *Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death.* Mol Biol Cell, 1999. **10**(8): p. 2493-506.
143. Mendoza, M.C., E.E. Er, and J. Blenis, *The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation.* Trends Biochem Sci, 2011. **36**(6): p. 320-8.
144. Jarvis, W.D., et al., *Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine.* Mol Pharmacol, 1997. **52**(6): p. 935-47.
145. Cross, D.A., et al., *The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf.* Biochem J, 1994. **303 (Pt 1)**: p. 21-6.
146. Hawes, B.E., et al., *Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway.* J Biol Chem, 1996. **271**(21): p. 12133-6.

147. Duckworth, B.C. and L.C. Cantley, *Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength.* J Biol Chem, 1997. **272**(44): p. 27665-70.
148. Wennstrom, S. and J. Downward, *Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor.* Mol Cell Biol, 1999. **19**(6): p. 4279-88.
149. Bernstein, B.W. and J.R. Bamburg, *Actin-ATP hydrolysis is a major energy drain for neurons.* J Neurosci, 2003. **23**(1): p. 1-6.
150. Akude, E., et al., *Diminished superoxide generation is associated with respiratory chain dysfunction and changes in the mitochondrial proteome of sensory neurons from diabetic rats.* Diabetes, 2011. **60**(1): p. 288-97.
151. Gao, M., et al., *Phosphatidylinositol 3-kinase affects mitochondrial function in part through inducing peroxisome proliferator-activated receptor gamma coactivator-1beta expression.* Br J Pharmacol, 2011. **162**(4): p. 1000-8.
152. K Alexander Iwen, E.S., Julia Resch, Ulrich Lindner, Peter König, Hendrik Lehnert, Nina Perwitz, Saleh Ibrahim & Georg Brabant, *Mitochondrial mass and function is regulated by PI3K signaling in thyroid cancer cells.* Endocrine Abstracts, 2013.
153. Tapodi, A., et al., *Pivotal role of Akt activation in mitochondrial protection and cell survival by poly(ADP-ribose)polymerase-1 inhibition in oxidative stress.* J Biol Chem, 2005. **280**(42): p. 35767-75.
154. Monick, M.M., et al., *Constitutive ERK MAPK activity regulates macrophage ATP production and mitochondrial integrity.* J Immunol, 2008. **180**(11): p. 7485-96.
155. Baines, C.P., et al., *Mitochondrial PKCepsilon and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCepsilon-MAPK interactions and differential MAPK activation in PKCepsilon-induced cardioprotection.* Circ Res, 2002. **90**(4): p. 390-7.
156. Alonso, M., et al., *Mitochondrial extracellular signal-regulated kinases 1/2 (ERK1/2) are modulated during brain development.* J Neurochem, 2004. **89**(1): p. 248-56.
157. Zhu, J.H., et al., *Localization of phosphorylated ERK/MAP kinases to mitochondria and autophagosomes in Lewy body diseases.* Brain Pathol, 2003. **13**(4): p. 473-81.
158. Lopez-Armada, M.J., et al., *Mitochondrial activity is modulated by TNFalpha and IL-1beta in normal human chondrocyte cells.* Osteoarthritis Cartilage, 2006. **14**(10): p. 1011-22.
159. Markham, A., et al., *Brain-derived neurotrophic factor-mediated effects on mitochondrial respiratory coupling and neuroprotection share the same molecular signalling pathways.* Eur J Neurosci, 2012. **35**(3): p. 366-74.
160. Cheng, A., et al., *Involvement of PGC-1alpha in the formation and maintenance of neuronal dendritic spines.* Nat Commun, 2012. **3**: p. 1250.
161. Martinou, I., et al., *The release of cytochrome c from mitochondria during apoptosis of NGF-deprived sympathetic neurons is a reversible event.* J Cell Biol, 1999. **144**(5): p. 883-9.
162. Chowdhury, S.K., D.R. Smith, and P. Fernyhough, *The role of aberrant mitochondrial bioenergetics in diabetic neuropathy.* Neurobiol Dis. **51**: p. 56-65.
163. Ohno, N., et al., *Myelination and axonal electrical activity modulate the distribution and motility of mitochondria at CNS nodes of Ranvier.* J Neurosci, 2011. **31**(20): p. 7249-58.
164. Hollenbeck, P.J., *The pattern and mechanism of mitochondrial transport in axons.* Front Biosci, 1996. **1**: p. d91-102.
165. Morris, R.L. and P.J. Hollenbeck, *The regulation of bidirectional mitochondrial transport is coordinated with axonal outgrowth.* J Cell Sci, 1993. **104 (Pt 3)**: p. 917-27.
166. Aggarwal, S. and A.L. Gurney, *IL-17: prototype member of an emerging cytokine family.* J Leukoc Biol, 2002. **71**(1): p. 1-8.