

**INTERMITTENT FASTING- DEPENDENT LEPTIN SIGNALLING
PROMOTES AXONAL REGENERATION IN THE PERIPHERAL AND
CENTRAL NERVOUS SYSTEM**

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1. ABSTRACT

Spinal cord injury affects thousands world- wide and yet clinical therapies remain elusive. This is partially because the central nervous system (CNS) is unable to regenerate. However, the peripheral nervous system (PNS) can mount a regenerative response, facilitating axonal growth and plasticity. Environmental factors such as exercise, diet and sleep have been shown to impact this regenerative capacity. Intermittent fasting (IF) for example, enhances axonal regeneration and functional recovery after sciatic nerve crush (SNC) and spinal cord injury. The endogenous molecular mechanisms for how it does so however, remain outstanding in the field. One of the key regulators of the fasting state within the body is leptin, the satiety hormone responsible for energy homeostasis. Many of leptin's downstream signalling pathways overlap with established regenerative mechanisms and previous work has characterised leptin's action in wound healing and regulating injury inflammation. It was hypothesised that leptin may drive the pro-regenerative effects seen with intermittent fasting following injury in the nervous system. RNA sequencing of dorsal root ganglia (DRG) neurons after IF revealed a number of metabolic changes, including significant upregulation of leptin signalling. Validation experiments with qPCR and immunohistochemistry confirmed this, showing neuronal leptin expression increases after SNC injury. To determine if leptin is central to this regenerative phenotype, deletion of the leptin receptor via Cre- injection in leptin receptor (LepR) FLOX mice ablated leptin's downstream signalling cascades and attenuated the regenerative response seen in IF. Independently of IF, overexpression experiments with AAV-Leptin-GFP viruses followed by either SNC or dorsal column axotomy (DCA) showed leptin enhanced axonal regeneration in both the PNS and CNS, displaying robust regeneration through the lesion site in the spinal cord. Subsequent investigations exploring the molecular mechanisms underlying leptin's regenerative phenotype found leptin upregulates transcription after spinal cord injury, modulating a dynamic euchromatin environment to facilitate regeneration-associated gene (RAG) expression. In summation, this data outlines a novel therapeutic role of leptin in nerve repair and functional recovery after injury.

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DECLARATION

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualifications.

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'It eluded us then, but that's no matter- tomorrow we will run faster, stretch out our arms farther... until one fine morning.

So we beat on, boats against the current, borne back ceaselessly into the past.'

~ Francis Scott Fitzgerald

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*'To see a world in a grain of sand,
And heaven in a wildflower,
Hold infinity in the palm of your hand,
And eternity in an hour'
~ William Blake*

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ABBREVIATIONS

Acetyl-CoA = acetyl coenzyme A

AD = Alzheimers disease

ADF = alternate day fasting

ADP = Adenosine diphosphate

AKT = phosphoinositide 3-kinases (PI3K)/serine/threonine protein kinase B

AL = Ad libitum

AMP = Adenosine monophosphate

ANOVA = analysis of variance

ATF3 = Activating Transcription Factor 3

ATP = Adenosine triphosphate

A β = amyloid beta

BBB = Basso-Beattie-Bresnahan

BBB = blood brain barrier

Bcl-2 = B-cell lymphoma 2

BDNF = Brain derived neurotrophic factor

bFGF = basic fibroblast growth factor

BMS = Basso mouse scale for locomotion

BP = Biological Process

BSA = bovine serum albumin

Ca²⁺ = Calcium

CaMK = Ca²⁺-calmodulin-dependent protein kinases

cAMP = 3',5'-cyclic adenosine monophosphate

Casp3 =Caspase 3

CBP = CREB-Binding Protein

CEBP = CCAAT-enhancer-binding proteins

ChAT = choline acetyl transferase

CMT1A = Charcot-Marie-Tooth type 1A

CNS = central nervous system

CR = caloric restriction

CREB = cAMP - response element binding protein
CSPGs = Chondroitin sulfate proteoglycan
CST = Corticospinal tract
CTB = Cholera toxin B
CXCL1 = chemokine (C-X-C motif) ligand 1
CXCR2 = C-X-C Motif Chemokine Receptor 2
DAVID = Database for Annotation, Visualization, and Integrated Discovery
db = diabetes gene
DCA = dorsal column axotomy
DLK1 = Delta Like Non-Canonical Notch Ligand 1
DMEM = Dulbecco's Modified Eagle Medium
DNA = Deoxyribonucleic acid
DRG = dorsal root ganglion
ECM = extracellular matrix
EDTA = Ethylenediaminetetraacetic acid
EE = enriched environment
EGFR = Epidermal growth factor receptor
ELISA = Enzyme-linked Immunosorbent Assay
EOD Fasting = every other day fasting
ERK = Extracellular-signal-regulated kinase
FBS = Foetal bovine serum
FGF2 = Fibroblast Growth Factor 2
GABA = gamma-Aminobutyric acid
GAP43 = Growth Associated Protein 43
GDNF = glial cell line-derived neurotrophic factor
GFAP = glial fibrillary acidic protein
GO = Gene Ontology
GPRs = G Protein-coupled receptors
HBSS = Hanks balanced salt solution
HDAC 3 = Histone deacetylase 3

HDAC 5 = histone deacetylase 5
Hif- 1 = hypoxia-inducible factor 1
HPA = hypothalamic-pituitary-adrenal axis
HSP70 = heat shock protein 70
IF = intermittent fasting
IFN γ = interferon gamma
IGF-1 = insulin like growth factor 1
IHC = immunohistochemistry
IL1 = Interleukin 1
IL10 = Interleukin 10
IL13 = Interleukin 13
IL17b = Interleukin 17B
IL18 = Interleukin 18
IL1 α = Interleukin 1 alpha
IL1 β = Interleukin 1 beta
IL6 = Interleukin 6
IP = intraperitoneal
IPA = indole-3-propionic acid
IRS = inflammatory response system
JAK = Janus kinase
JIP3 = JNK-interacting protein 3
JNK = JUN N-terminal kinase
JUN = Jun Proto-Oncogene
KAT2B = Lysine Acetyltransferase 2B
LDL = low density lipoprotein
LepR = leptin receptor
LepR- b = leptin receptor long isoform b
LIF = Leukemia inhibitory factor
logFC = log₂ Fold Change
MAG = Myelin associated glycoprotein

MAPK = Mitogen-activated protein kinase
MHC I = major histocompatibility complex I
mRNA = messenger RNA
NAD = nicotinamide adenine dinucleotide
NADPH = Reduced nicotinamide adenine dinucleotide phosphate
NF- κ B = Nuclear factor kappa B
NF200 = neurofilament 200
NGF = nerve growth factor
NGFb = Nerve Growth Factor (Beta Polypeptide)
NgR1 = Nogo receptor 1
NK cell = Natural killer cell
NKT cell = Natural killer T-cell
NogoA = Neurite outgrowth inhibitor A
NR = nuclear receptor
NPY = neuropeptide Y
NT-3 = Neurtrophin-3
NT-4 = Neurtrophin-4
ob = obesity gene
OB- Rb = long intracellular form of the leptin receptor
OMgp = oligodendrocyte myelin glycoprotein
PBS = Phosphate buffered saline
PDL = Poly-D-lysine
PI3K = Phosphoinositide 3-kinases
PKA = Protein kinase A
PKC = Protein kinase C
PLC = Phospholipase C
PNI = peripheral nerve injury
PNS = peripheral nervous system
POMC = proopiomelanocortin
PRKD1 = Protein Kinase D1

PTEN = Phosphatase and tensin homologue
RAGs = regeneration-associated genes
RNA = Ribonucleic acid
RNA-Seq = RNA sequencing
ROS = reactive oxygen species
SCFAs = short chain fatty acids
SCG-10 = Superior cervical ganglion-10
SCI = spinal cord injury
SEM = standard error of the mean
SHP2 = Src homology-2 domain-containing protein tyrosine phosphatase-2
SIRT3 = sirtuin 3
SNC = Sciatic nerve crush
SNPH = Syntrophin
SOX10 = SRY-Box Transcription Factor 10
SOX11 = SRY-Box Transcription Factor 11
STAT = signal transducers and activators of transcription
STAT3 = signal transducers and activators of transcription 3
TBI = traumatic brain injury
TET3 = tet methylcytosine dioxygenase 3
TF = transcription factor
Th1 cell = Type 1 helper T-cells
TLR = Toll like receptor
TLR4 = Toll like receptor 4
TNFR = tumor necrosis factor receptor
TNF- α = tumor necrosis factor alpha
Treg = regulatory T-cell
TrKA = receptor tyrosine kinase A
TrKB = receptor tyrosine kinase B
TrKC = receptor tyrosine kinase C
TX100 = Triton X 100

vGAT = synaptic vesicle GABAergic transporter

VGCC = voltage gated calcium channel

vGLUT = synaptic vesicle Glutamatergic transporter

β IIITub = β III Tubulin

2. INTRODUCTION

2.1 Nervous System Anatomy

The nervous system is a highly complex, integrated circuitry of multidirectional communication exchanged between the central and peripheral components. The central nervous system (CNS) consists of the brain and spinal cord, relaying descending and ascending communication to the remaining neurons throughout the body which form the peripheral nervous system (PNS) (Figure 1). The PNS is a collective term for all of the structures outside of the CNS, including cranial and spinal nerves, clustered ganglia, sensory receptors and the enteric plexus (Ramon Y Cajal, 1990, Kandel et al., 2000, Purves et al., 2019). The sensory division of the PNS acquires and processes information from the environment, linking sensory receptors within and on the body surface to sensory circuits in the CNS (Squire et al., 2012, Bear et al., 2020, Hans et al., 2020). For example, the visual and auditory systems. The motor division of the PNS responds to environmental stimuli by generating movements or other behaviours, classified into either the somatic or autonomic motor systems. The

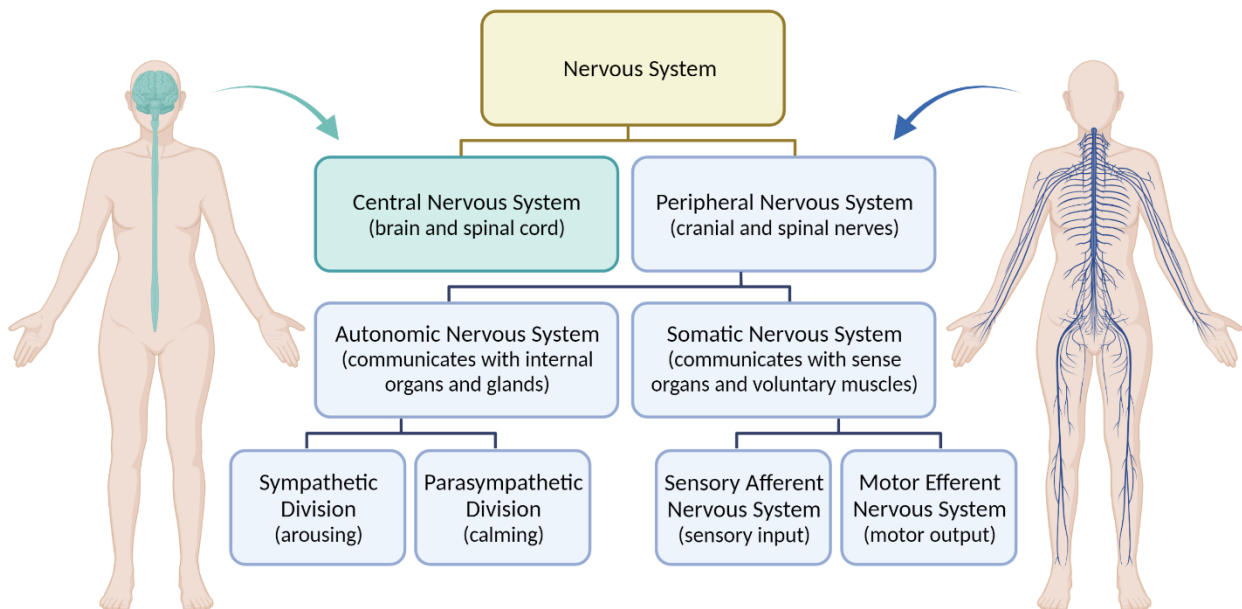


Figure 1: Divisions of the Central and Peripheral Nervous System

The central nervous system is comprised of the brain and spinal cord which connects to the peripheral cranial and spinal nerves to relay and receive both sensory and motor communication. Figure created in BioRender.

somatic motor division describes motor axons which connect the brain and spinal cord to skeletal muscle, whereas the autonomic division (also called the visceral system) contains the cells and axons which innervate smooth muscle, cardiac muscle and glands (Figure 1) (Lawrence and Kuypers, 1968, Appenzeller, 1990, Brodal, 2004, Graziano, 2006). The somatic division is responsible for receiving sensory information from the skin, muscles and joints regarding touch, pressure and proprioception to execute voluntary motor commands such as running or lifting (LaMotte and Srinivasan, 1987, Hans et al., 2020). These signals are relayed through either afferent sensory fibres or efferent motor fibres. The autonomic division mediates sensation and motor control of the viscera, vascular system and exocrine glands, controlling most involuntary (visceral) behaviour (Appenzeller, 1990). It is further sub-divided into the sympathetic, parasympathetic and enteric nervous systems (Figure 2). The sympathetic circuitry responds to stress, commonly known as the 'flight-or-fight' response. The sympathetic pre-ganglion cell column lies lateral to the spinal cord, extending between the cervical and lumbar sacral enlargement from T1 to L3, regulating many peripheral target organs from a substantial distance away (Purves et al., 2019, Bear et al., 2020). The parasympathetic neurons however, conserve body resources and restore homeostasis in a 'rest-and-digest' response. These ganglia lie in the cranial zone and are comprised of the cranial nerves and sacral segments 2 to 4, synapsing within or adjacent to their target organs (Appenzeller, 1990, Jansen et al., 1995). The last component of the visceral division is the enteric system, formulated from small ganglia and neurons within the wall of the gut, responsible for modulating smooth muscle function, gastric motility and secretion (Figure 3) (Costa et al., 2000, Furness, 2008, Furness, 2012). The enteric nervous system extends throughout the entire length of the gastrointestinal tract, containing over 100 million sensory and motor neurons to regulate the gut independently of the CNS (Kulkarni et al., 2018). Sensory neurons monitor mechanical and chemical changes within the gastrointestinal tract, whereas motor neurons control smooth muscle contraction to aid the passage of food (Furness, 2012, Kulkarni et al., 2018). Though the enteric system operates independently of the sympathetic and parasympathetic systems, it interacts synergistically to coordinate appropriate nervous system responses to environmental stimuli.

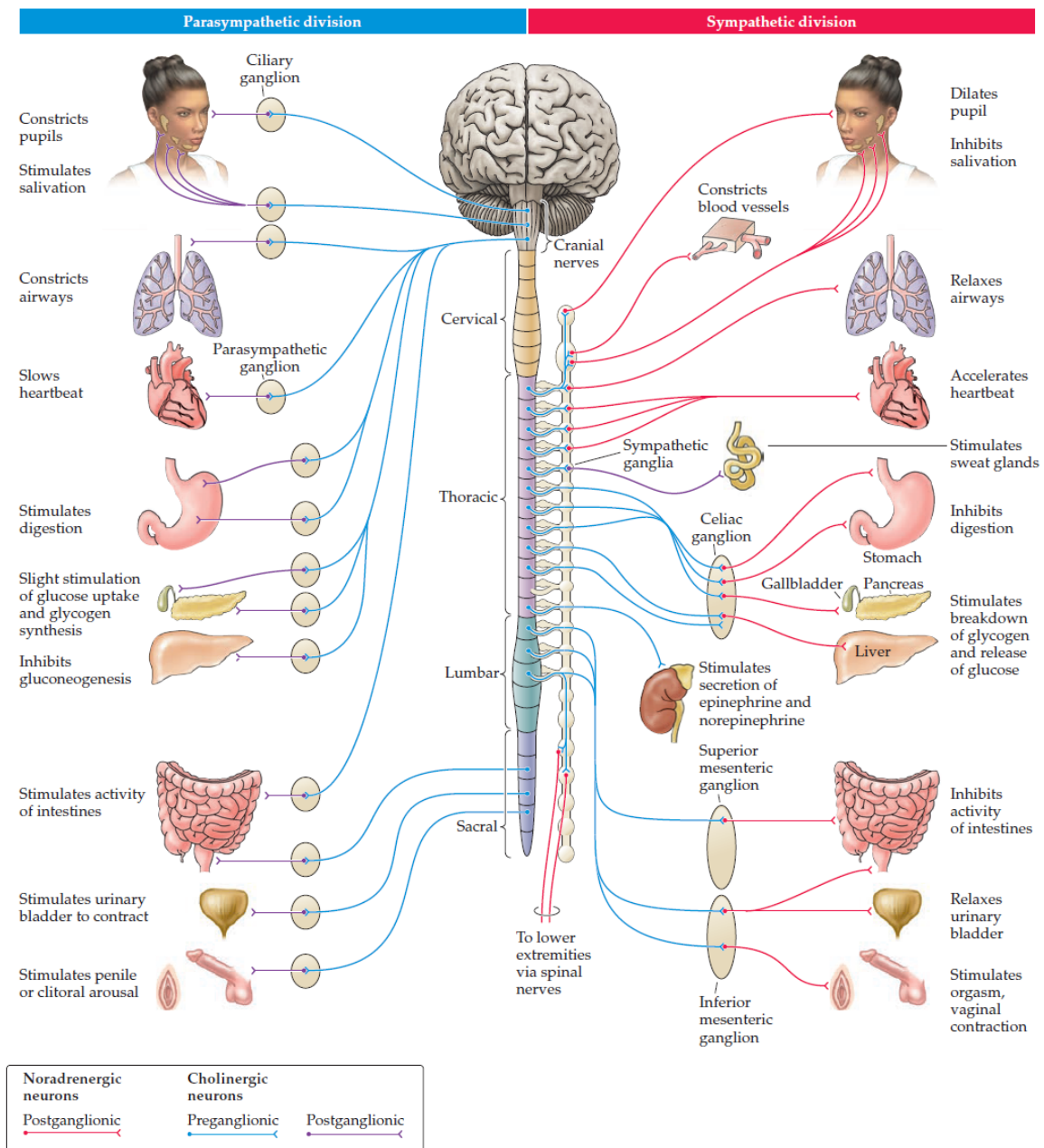


Figure 2: Sympathetic and Parasympathetic Nervous Systems

The autonomic nervous system regulates sensation and motor control of the viscera, vascular system and exocrine glands; controlling involuntary behaviour. The sympathetic circuitry mediates the 'fight-or-flight' response, stimulating arousal active mechanisms. The parasympathetic circuitry controls the 'rest-and-digest' physiology to restore energy homeostasis. (Purves et al., 2018).

In addition to anatomical and functional classifications of the nervous system, specific terminology distinguishes peripheral cell and axon locations from central. The nerve cell bodies which lie in the PNS are located in ganglia; pockets containing nerve cell bodies and local supporting cells (Kandel et al., 2000). Peripheral axons merge to form bundles called nerves, several of which are myelinated by the unique peripheral

glial cells, termed Schwann cells (Purves et al., 2019). However, CNS neurons within the brain are arranged as either nuclei or cortices, clusters of neurons with similar connections and functions; or a sheet-like arrangement of neurons such as in the cerebral hemispheres or cerebellum (Squire et al., 2012, Purves et al., 2019). Axons in the CNS gather into tracts, the equivalent of the peripheral 'nerve' and the terms 'Grey matter' and 'White matter' are used to distinguish between cell-body dense or axon tract- dense areas of the CNS (Squire et al., 2012). For example, within the PNS, the cell bodies of sensory neurons within the voluntary somatic division lie in the dorsal root ganglia (DRG) and cranial ganglia, which line the spinal cord (Figure 4, Figure 5) (Brodal, 2004). A combination of somatosensory projections from nociceptors and mechanoreceptors in the skin and proprioceptors in the muscle, enter into the DRG where their cell bodies lie. Due to their pseudounipolar morphology, the same cell body extends a branch beyond the DRG to relay these signals within the different dorsal

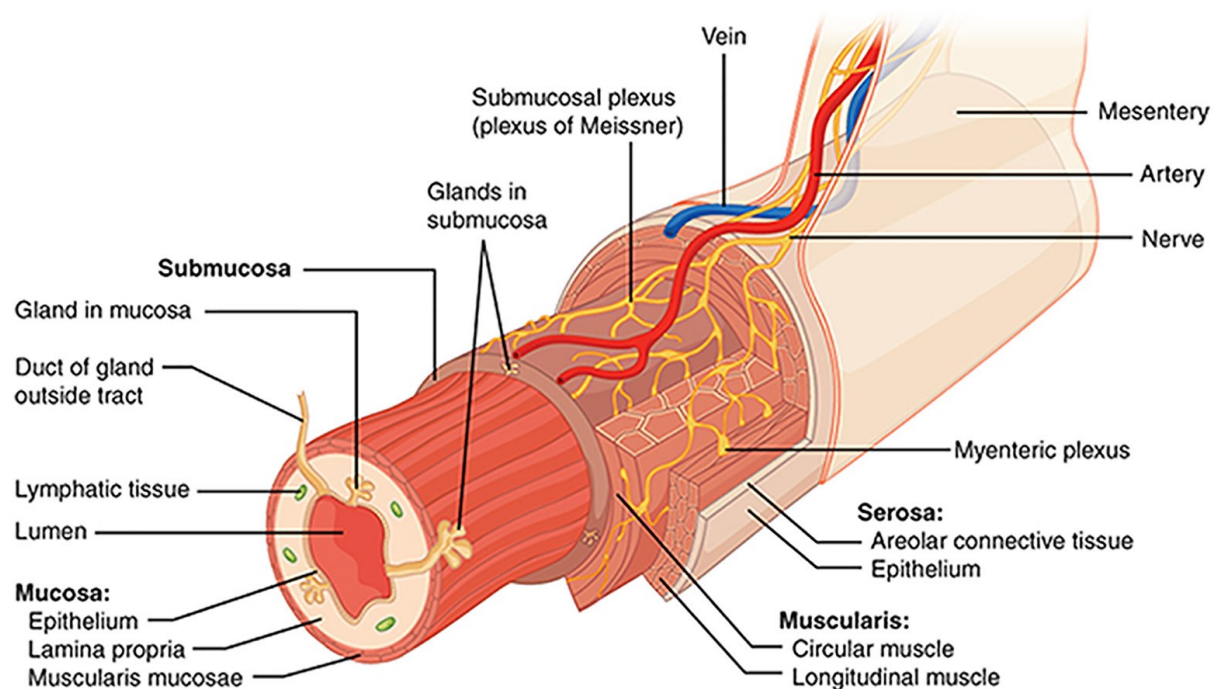
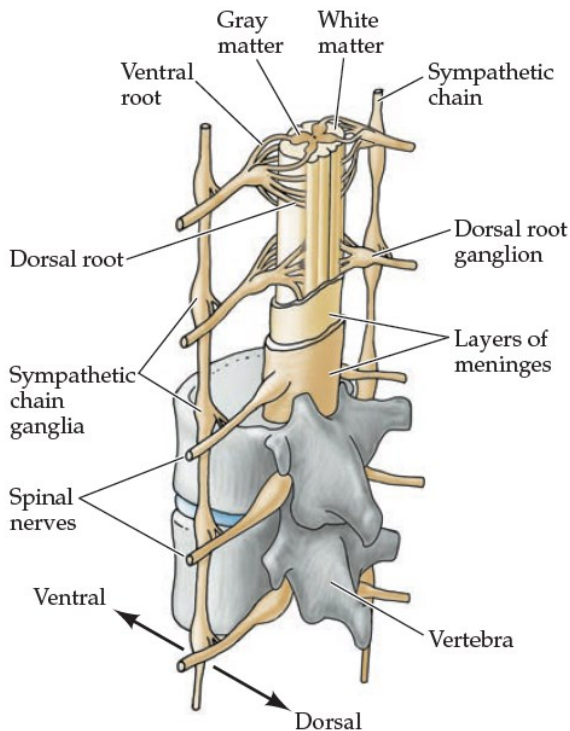


Figure 3: Anatomy of the Enteric Nervous System

Organization of the enteric nervous system within the gut wall of the small intestine, illustrating the laminar organization of the bowel in three dimensions from the mesentery to the lumen. The two major plexuses of the enteric system are the myenteric plexus, located between the circular and longitudinal muscle layers in the muscularis externa, and the submucosal plexus, located in the submucosa. Sensory monitoring and motor adjustments enable optimal functioning of the gut (Kulkarni et al., 2018).

A)



B)

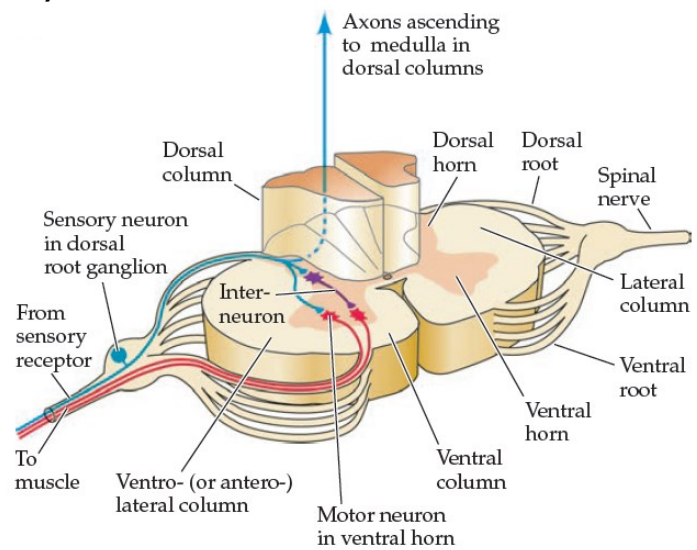


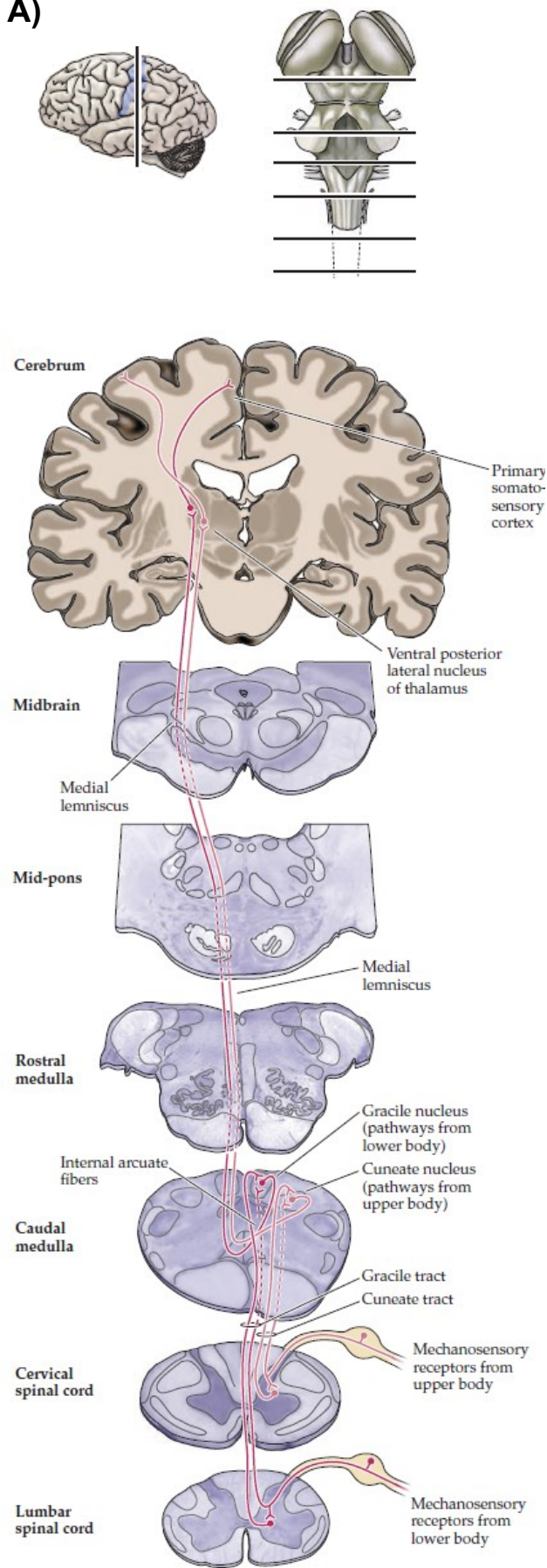
Figure 4: Spinal Cord and Dorsal Root Ganglia Anatomy

A: Relationship between the spinal cord and spinal nerves in the vertebral column. Sensory information carried by spinal nerves enters the cord via the dorsal root ganglia (DRG), whereas motor commands exit the cord via the ventral roots.

B: Internal structures of the spinal cord. Pseudounipolar sensory neurons from the periphery (blue) enter into the dorsal root ganglia where their cell bodies lie. The central branch of these neurons extend into the spinal cord and synapse within the ascending dorsal columns. Descending motor information from the corticospinal tracts synapse onto motor neurons (red) within the ventral horn of the spinal cord. Motor neurons exit into the periphery via the DRG to synapse onto their target effectors such as skeletal muscles. (Purves et al., 2018).

layers of the spinal cord, or directly synapse with interneurons which connect to the muscle as part of the reflex circuit (Hans et al., 2020). This somatosensory information then reaches the brain via the ascending dorsal column and spinothalamic tracts. Similarly, motor neurons also have pseudounipolar anatomy and house their cell bodies within the DRG (Figure 4, Figure 5) (Ramon Y Cajal, 1990, Lai et al., 2016). However, in contrast to the sensory system, motor efferent fibres relay voluntary commands from the brain out into the periphery via the descending lateral and anterior lateral corticospinal tracts, located in the ventral horn of the spinal cord (Lawrence and Kuypers, 1968, Bear et al., 2020). These neurons have a direct connection from the

A)



B)

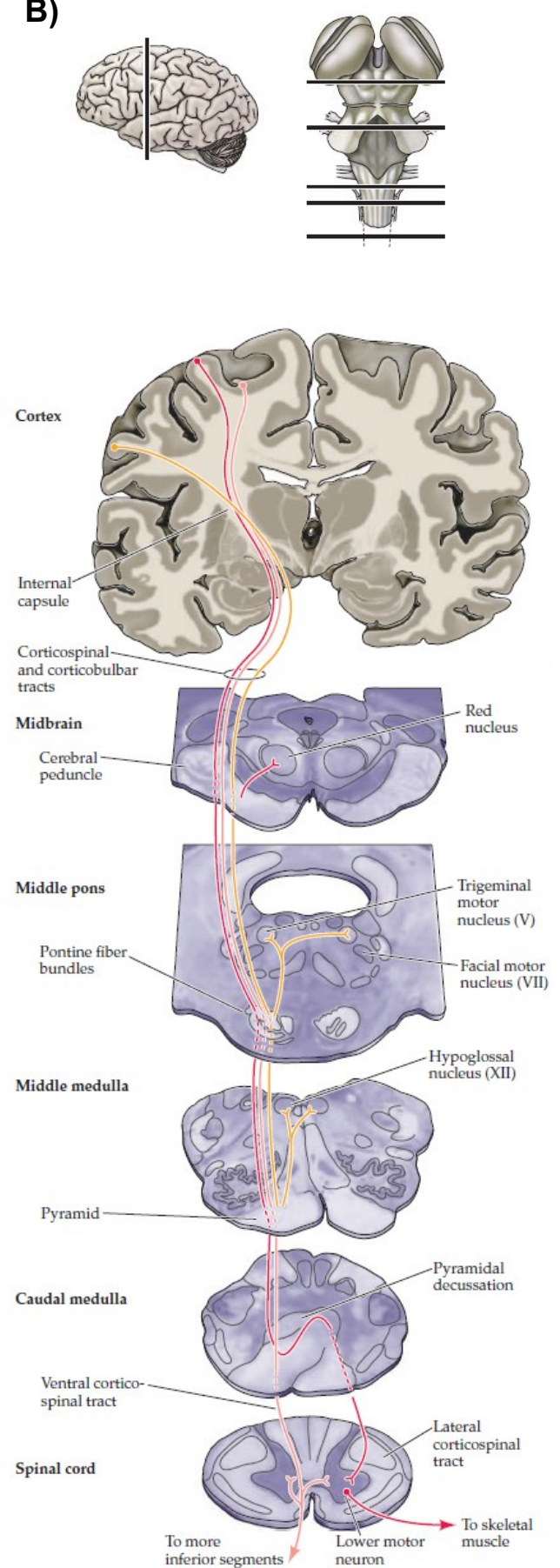


Figure 5: Anatomy of Sensory and Motor System Circuitry

A: Ascending dorsal column medial lemniscus pathway carries mechanosensory information from the posterior third of the head and the rest of the body up to the primary somatosensory cortex. The trigeminal portion of the mechanosensory system carries similar information from the face (not shown).

B: Descending corticospinal and corticobulbar tracts. Neurons in the motor cortex give rise to axons that travel through the internal capsule and coalesce on the ventral surface of the midbrain, within the cerebral peduncle. These axons continue through the pons and lie on the ventral surface of the medulla, forming the pyramids. Most pyramidal fibres cross in the caudal medulla to form the lateral corticospinal tract in the spinal cord. Axons which do not cross form the ventral corticospinal tract. These motor efferents synapse directly with their target effectors (Purves et al., 2018).

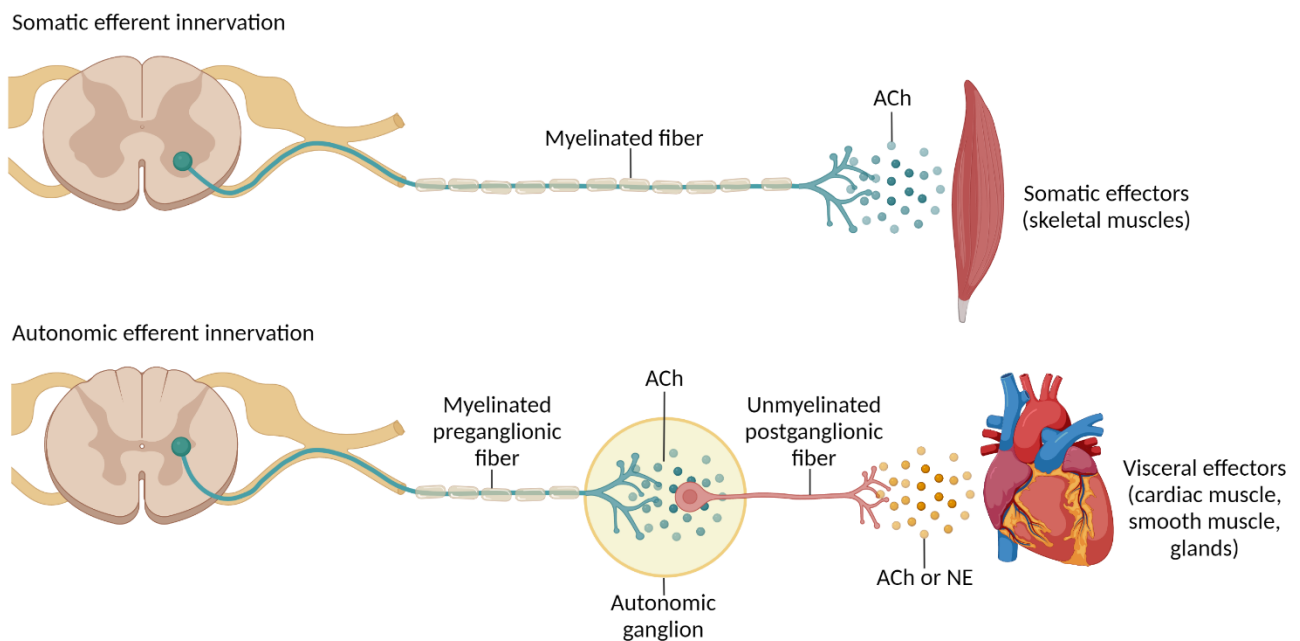


Figure 6: Somatic and Autonomic Neuronal Divisions

The somatic motor division connects the brain and spinal cord to skeletal muscle to control voluntary movement, whereas the autonomic division (visceral system) is responsible for involuntary behaviour and innervates smooth muscle, cardiac muscle and glands. Somatic efferents exit the spinal cord and synapse directly onto their target organs in the periphery, releasing neurotransmitters such as acetylcholine to initiate skeletal muscle contraction. Autonomic efferents synapse within autonomic ganglia onto postganglionic neurons, before these unmyelinated neurons synapse at their target visceral effectors and release neurotransmitters such as noradrenaline. Figure created in BioRender.

CNS to somatic effectors such as the skeletal muscles. Contrastingly, this is not the case in the involuntary autonomic system which contains pre and post-ganglionic neurons (Figure 6) (Watson, 1995). Autonomic pre-ganglionic neurons lie in the CNS and are fast-conducting myelinated neurons. They synapse onto post-ganglionic unmyelinated neurons within the autonomic ganglia, releasing neurotransmitters such as acetylcholine across the pre-synaptic space in order to transmit their signal (Watson, 1995, Bear et al., 2020). Unmyelinated post-ganglionic neurons lie in the PNS and synapse onto visceral targets such as cardiac muscle or smooth muscle, releasing neurotransmitters such as noradrenaline to regulate their function (Cooper et al., 2003, Squire et al., 2012, Bear et al., 2020). These neurons mark the junction between the CNS and PNS, as myelinated pre-ganglionic neurons originate from the neural tube (CNS) whereas unmyelinated post-ganglionic neurons arise from neural crest cells (PNS) (Lai et al., 2016).

Specialised fibre types ensure appropriate relay timing of nervous system stimuli. Motor neurons are classified as A α neurons, whereas sensory neurons can be further subclassified into A β touch and proprioception fibres; A γ muscle tone fibres; A δ pain, cold temperature and touch neurons; and C fibres responsible for pain, warm temperature and touch (Figure 7) (Seddighi et al., 2016). Diameter and myelination determine the speed of axon potential conduction, aligning with the properties of the central circuits and unique behavioural demands for each sensory afferent (Purves et al., 2019). Large diameter neurons supply sensory receptors in the muscles essential for proprioception. Smaller diameter afferents convey information regarding touch, whereas the smallest diameter C fibres are responsible for nociception, pain and temperature sensing (Ramon Y Cajal, 1990, Kandel et al., 2000, Purves et al., 2019). In descending order, sensory afferents A α , A β and A δ are myelinated, where A α fibres are wrapped in the thickest myelin sheath and consequently exert the fastest conduction velocities (Waxman, 1980, Etxeberria et al., 2016). The nociceptive C fibres are unmyelinated and have the slowest conduction velocity of all the sensory afferent types (Waxman, 1980, Etxeberria et al., 2016). PNS Schwann cells develop from neural crest tissue and either surround single axons to form the myelin sheath and enhance conduction speed; or develop into non-myelinating support cells (Fancy et al., 2011, Lai et al., 2016). In the CNS, oligodendrocytes are responsible for axon myelination and can cover multiple axons at a time, adjusting myelin thickness in

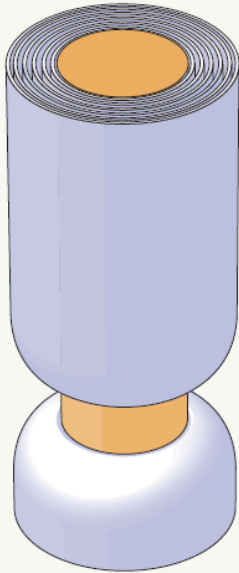
Axons from skin	$A\alpha$	$A\beta$	$A\delta$	C
Axons from muscles	Group I	II	III	IV
				
Diameter (μm)	13–20	6–12	1–5	0.2–1.5
Speed (m/sec)	80–120	35–75	5–30	0.5–2
Sensory receptors	Proprioceptors of skeletal muscle	Mechanoreceptors of skin	Pain, temperature	Temperature, pain, itch

Figure 7: Sensory Afferent Subtypes

Somatic sensory afferents are classified according to their conduction velocity. The three main categories are A, B, and C. A comprises the largest and fastest axons, C the smallest and slowest. Mechanoreceptor axons generally fall into category A. Additionally, the A group is subdivided into α (the fastest), β , and δ (the slowest). Muscle afferent axons are also usually classified into four additional groups—I (the fastest), II, III, and IV (the slowest). The diameter of an axon is correlated with its conduction velocity and with the type of sensory receptor to which it is connected. (Bear et al., 2001).

correlation with axon diameter (McTigue and Tripathi, 2008, Bradl and Lassmann, 2010). Structurally, peripheral sensory afferents are myelinated by Schwann cells, which are in turn coated by a basal laminae and a connective tissue compartment called the endoneurium (Seddighi et al., 2016). Grouped endoneurium compartments form a fascicle, insheathed in a perineurium, which when grouped together with blood vessels and surrounded by an epineurium; combine to form individual peripheral nerves (Figure 8) (Sunderland, 1990, Seddighi et al., 2016). Collectively, all fibre types and neural pathways work in synchronisation to optimise sensory input from the environment and coordinate motor outputs in response.

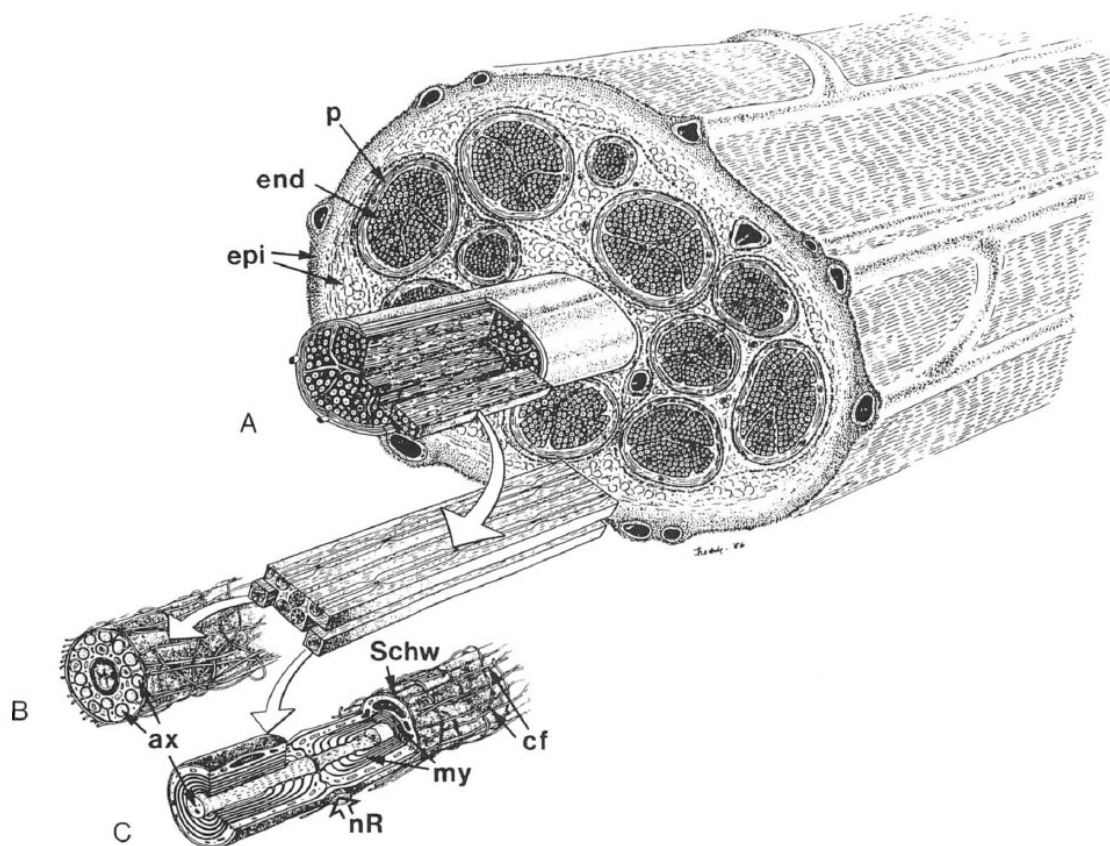


Figure 8: Peripheral Nerve Anatomy

The microanatomy of the peripheral nerve trunk and its various components. A: Bundles of nerve fibres are surrounded by the perineurium (p), creating fascicles that are embedded in loose connective tissue called the epineurium (epi) and are grouped together with blood vessels. The intrafascicular connective tissue is called the endoneurium (end) and sheaths the nerve fibres within fascicles. The appearance of nonmyelinated and myelinated nerve fibres are illustrated in B and C, respectively. Schw = Schwann cell; my = myelin sheath; ax = axon; nR = node of Ranvier. (Lundborg and Dahlin, 1996).

2.2 Spinal Cord Injury: Regenerative Failure

The central and peripheral nervous systems are highly intricate and finely balanced, optimised through years of conserved evolution to ensure organism survival. Spinal cord injury (SCI) is the result of disruption to descending and ascending white matter tracts, inhibiting communication between the brain and spinal cord. These injuries cause loss of autonomic, sensory and motor function, with devastating consequences for quality of life in patients. The global prevalence of SCI is 27 million people, with an annual incidence rate of 195 cases per million in European countries (Jazayeri et al., 2015, Collaborators, 2016, Badhiwala et al., 2019, Shiferaw et al., 2020), imposing significant financial constraints on health care systems, with average lifetime costs reaching up to \$2 million per person depending on location and severity of injury (McDonald and Sadowsky, 2002). Despite several decades of research, an effective therapy is yet to be developed and SCI imposes a large unmet clinical need. One difficulty levied by SCI is the heterogeneity of injury severity between patients. The most common causes of SCI include driving accidents, falls, gunshot wounds, motorbike crashes and surgical complications (McDonald and Sadowsky, 2002, Chiu et al., 2010, Chen et al., 2013, Center, 2015). Traffic accidents and car crashes are the leading cause of SCI and disproportionately affect young males, until approximately 45 years old, where falls become the leading factor (Chiu et al., 2010, Chen et al., 2013). Injured nerves experience mechanical trauma upon impact, compression forces, twisting and stretching from fractured or displaced bone fragments (McDonald and Sadowsky, 2002). Severed axons, broken neural cell-membranes and micro-haemorrhages precede ischemia, neural shock and an extensive inflammatory response (McDonald and Sadowsky, 2002), though the scale to which these events occur is constrained by initial injury severity. Injury trauma is assessed using the American Spinal Injury Association (ASIA) impairment scale, with the worst clinical impairment measured at an 'A' grade and normal motor and sensory functioning an 'E' grade. Concurrently with severity, injury location substantially affects ASIA scale assessment in patients. The spinal cord is divided into dermatomes, each segment of the cord innervating a specific neuronal subset in a hierarchical manner, from cervical and thoracic segments down to lumbar and sacral innervation (Kramer et al., 2010, Bear et al., 2020). Injury location, therefore, is key, as all systems below the injury site are in most part either entirely or partially lost. Injury at the thoracic level would impact walking and bladder function for example, but upper body systems such as arm

movements and breathing remain intact. Patients may have reasonable independence and quality of life with wheelchair aids. Whereas injury at the cervical level impacts all functionality below the neck. Consequently, SCI research requires a diverse range of injury models, from crush, to contusion, to axotomy, which makes translational of therapies into the clinic difficult. Nevertheless, characterising SCI with animal models remains invaluable, providing essential insight into the regenerative differences between the CNS and PNS.

Neuroplasticity is the process whereby axons and synapses adapt to continuously changing cellular environments within established and novel circuits, including collateral sprouting of spared and injured axons, structural synaptic remodelling and long- distance axon regeneration (Hutson and Di Giovanni, 2019). Axon regeneration however, is the re-growth of transected axons across a lesion site towards their original synaptic targets (Hutson and Di Giovanni, 2019). All processes, whether through circuit re-organisation or formation of new growth cones from transected nerves, can lead to restoration of function; though the biological mechanisms to execute this are very different. Central nervous system axons fail to regenerate after injury owing to poor intrinsic regenerative properties and the presence of an inhibitory glial environment (Kaplan et al., 2015). Whereas peripheral axons are able to mount a regenerative response, though limited and slow in the adult mammalian system (Schmidhammer et al., 2007, Seddighi et al., 2016). During development, neurons first establish polarity to distinguish between specialised dendrites for signal transduction and the axon, specialised for signal transmission and neurotransmitter secretion (Purves et al., 2019). Microtubule and actin components of the cytoskeleton distribute along a newly formed neurite to establish the axon, which then navigates to its synaptic partners via the highly motile growth cone and its lamellipodia extensions (Gordon-Weeks, 2004, Lowery and Vactor, 2009, Purves et al., 2019). Once arrived, the growth cone forms either a presynaptic axonal ending or the terminal domain of a dendrite (Gordon-Weeks, 2004, Lowery and Vactor, 2009, Purves et al., 2019). This growth and regenerative capacity declines with age as plastic neurons mature into established adult neurons with determined electrophysiological properties and polarised morphology (Weng et al., 2016, Purves et al., 2019, Fawcett, 2020). Observations from *in vivo* lesion experiments in embryonic chicken and newborn rodents, show the switch from a regeneration- permissive to a nonpermissive property of CNS tissue is correlated in time and space with myelin formation (Reh and Kalil, 1982, Keirstead et

al., 1992, Silver et al., 2015). Despite maturation, peripheral adult neurons retain regenerative capabilities, mounting both an intrinsic transcriptional program and extrinsic immune-inflammatory response at the injury site. Understanding the regenerative cascades in the PNS affords the opportunity to identify promising molecular targets to modulate within CNS injuries and characterise future therapeutics.

2.2.1 CNS Regenerative Failure: Extrinsic Factors

Following impact trauma, the unfolding pathophysiology of the spinal cord can be characterised into primary and secondary responses. In the primary response immediately following injury, ion homeostasis is dysregulated due to neuronal membrane rupture, along with haemorrhagic and ischaemic changes, which causes breakdown of the blood–CNS barrier and infiltration of early immune responders (Young and Koreh, 1986, Orr and Gensel, 2018, Hutson and Di Giovanni, 2019). In the secondary response, the injured spinal cord launches an extensive immuno-inflammatory reaction; inhibitory molecules of the myelin sheath and extracellular matrix are upregulated; and proliferation of astrocytes and microglia build a protective fibrotic scar supported by a glial border to contain the damage (Silver and Miller, 2004, Fawcett et al., 2012, Silver et al., 2015, Orr and Gensel, 2018). Central nervous system injury is typified by inflammation, progressive neurodegeneration, neuronal demyelination, apoptosis, reactive gliosis, macrophage infiltration and microglial activation. The major CNS inhibitory players within the extrinsic environment are either those associated with myelin or with the glial border surrounding the fibrotic scar. These pathways compound to inhibit axonal regeneration and ultimately, neurological disability from disrupted spinal connectivity. The following subchapter will outline the extracellular events following spinal cord injury and the role these cascades play in contributing to regenerative failure.

Within the adult mammalian CNS, axonal trauma inflicts tissue damage and transection of nerve fibres, the distal ends of which form dystrophic growth cones or ‘retraction bulbs’ (Cajal, 1928). Retraction bulbs are highly active structures stalled in a hostile injured environment (Tom et al., 2004), created by myelin associated inhibitors (MAIs) and inhibitory molecules of the glial border and fibrotic scar. For example, neurons grow successfully in peripheral nerve grafts but optic nerve explants from the CNS implanted into peripheral sciatic nerves fail to regenerate, indicating inhibitory environmental factors are involved in CNS regenerative failure (David and

Aguayo, 1981). In mixed optic nerve primary cultures, neurons adhered successfully with other neuronal cells and astrocytes but avoided mature oligodendrocytes, the myelinating cell type within the CNS (Schwab and Caroni, 1988). Isolated CNS rat myelin also possessed similar inhibitory properties compared to PNS myelin, demonstrating the inhibitory nature of CNS myelin in regeneration (Schwab and Caroni, 1988). Developmentally, inhibitory myelin components prevent aberrant neuronal sprouting and enable focused structural stabilisation of the highly complex CNS (Phokeo et al., 2002). However, once released into the neuronal environment post-injury, myelin associated inhibitors from oligodendrocytes and myelin debris can restrict axon growth via myelin-associated factors: neurite outgrowth inhibitor A (Nogo-A); myelin-associated glycoprotein (MAG); oligodendrocyte-myelin glycoprotein (OMgp); the transmembrane Semaphorins 4D, 5A and 3F; and ephrin B3 and A3 (Figure 9) (Schwab and Thoenen, 1985, McKerracher et al., 1994, Wang et al., 2002, Filbin, 2003, Moreau-Fauvarque et al., 2003, Benson et al., 2005, Giger et al., 2010). Nogo is a membrane protein expressed in CNS oligodendrocytes; inhibitory to neurite outgrowth (Oertle et al., 2003). Previous research has established Nogo-A inhibition enhances regeneration, sprouting and functional recovery following spinal cord injury in both rodent models and non-human primates (Chen et al., 2000, Schwab, 2004, Liebscher et al., 2005, Freund et al., 2006, Schwab and Strittmatter, 2014, Chen et al., 2017). MAG is a transmembrane protein essential for myelin formation and maintenance, promoting axon outgrowth during cell development but acting in an inhibitory capacity within the adult mammalian system (McKerracher et al., 1994, Tang et al., 2001, Kaplan et al., 2015). OMgp is enriched within cells surrounding the nodes of Ranvier to prevent collateral sprouting and once released into the extracellular environment following injury, inhibits neurite outgrowth (Kottis et al., 2002, Wang et al., 2002, Huang et al., 2005). Ephrin B3 and SemaD4/CD100 mediate axonal pathfinding during development but inhibit regeneration via disruptive guidance cues in adult CNS myelin (Moreau-Fauvarque et al., 2003, Benson et al., 2005, Yiu and He, 2006). Following injury, Nogo-A, MAG and OMgp all bind Nogo receptor 1 (NgR1) among others, to mediate these inhibitory effects and activate intracellular inhibitory cascades (Filbin, 2003, Yiu and He, 2006, Fawcett et al., 2012). However, whether combinatorial deletion of one or multiple inhibitory factors would aid regeneration after spinal cord injury remains under discussion. These treatments may risk impacting healthy inhibitory myelination to maintain synapse and axon guidance homeostasis, resulting

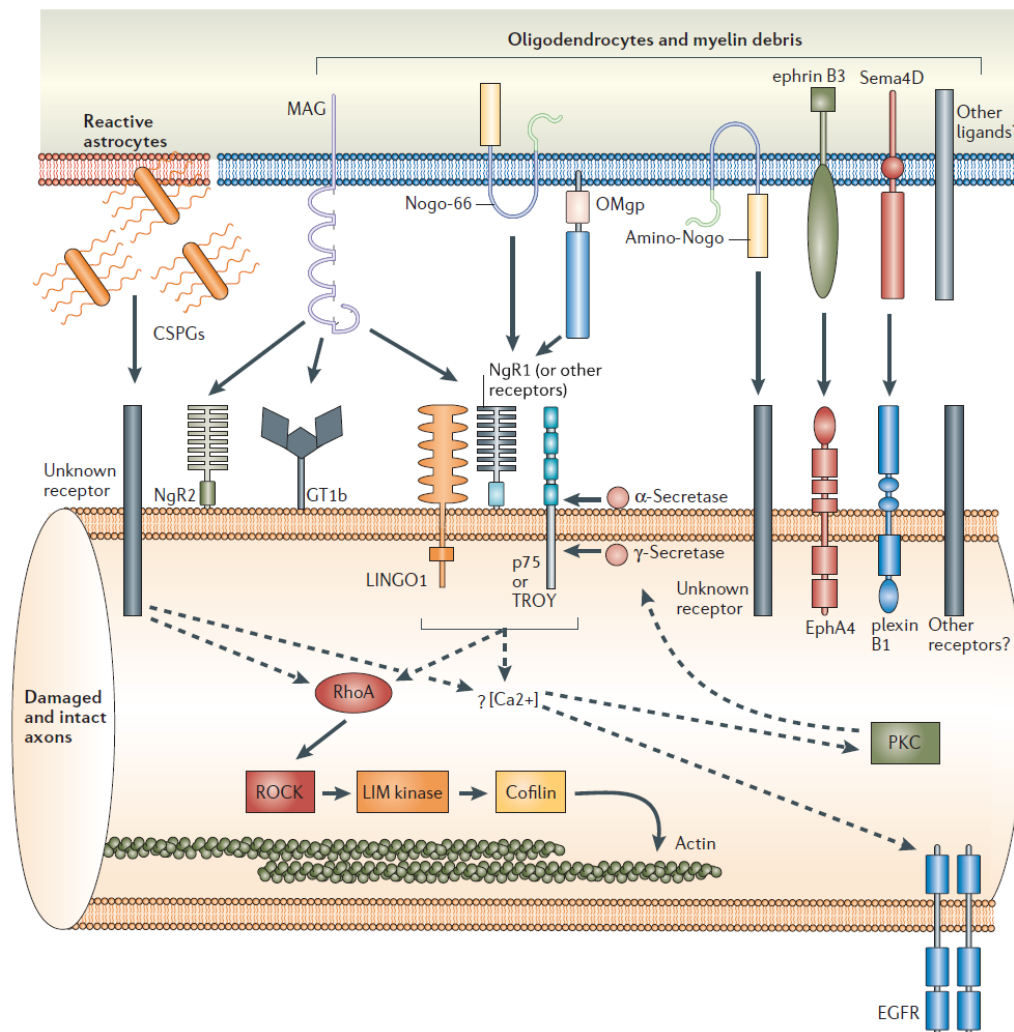


Figure 9: Inhibitory Spinal Cord Injury Signalling

The molecular inhibitors of the adult CNS environment include reactive astrocytes, chondroitin sulphate proteoglycans (CSPGs) and myelin-associated inhibitors, including myelin-associated glycoprotein (MAG), Nogo-A, oligodendrocyte myelin glycoprotein (OMgp), ephrin B3 and the transmembrane semaphorin 4D. Both the 66 amino acid loop (Nogo-66) and the amino-terminal domain (amino-Nogo) are inhibitory for axon outgrowth. Among the signalling components common to CSPGs and myelin is the activation of RhoA and a rise in intracellular calcium. Signalling pathways downstream of RhoA are well characterized (solid arrows), whereas the relationship between components upstream of RhoA and the role of calcium influx are still ambiguous (dashed arrows). RhoA- ROCK activation phosphorylates various target proteins, particularly those that regulate actin- myosin cytoskeleton contractility. One of the main ROCK substrates is myosin light chain (MLC), initiating stress-fibre formation and increased cellular contractility. ROCK also phosphorylates collapse in response mediator protein 2 (CRMP2), a neuronal protein involved in growth cone collapse. (Yiu and He, 2006).

in epilepsy or hyper-connectivity (Liu et al., 2011, Silver et al., 2015). Strong inhibition of one factor may also activate compensatory mechanisms, Nogo-A knock out mice for example show upregulation of several ephrins and semaphorins and their receptors (Lee et al., 2010, Kempf et al., 2013). However, pre-clinical studies of human AXER-204; a 'protein net' which traps myelin associated axonal inhibitors such as Nogo-A, MAG and OMgp; has shown improved functional recovery and enhanced axonal growth in rats (Wang et al., 2014). Human clinical trials of AXER-204 are ongoing (Sartori et al., 2020). A phase I trial of intrathecal delivery of an anti-Nogo-A antibody, ATI355, has been successfully completed and is well- tolerated in patients with acute SCI (Kucher et al., 2018, Hutson and Di Giovanni, 2019). Further trials will test efficacy after SCI. It is likely regenerative treatments are neuron-specific, utilising single cell methodologies and a series of systematic deletion studies would help us to further understand these networks and enable successful transfer to clinical trials.

In addition to degenerating myelin and astrogliosis, oligodendrocyte precursor cells, microglia, macrophages, fibroblasts, vascular endothelial cells and inflammatory cells all infiltrate the injury site to help form a fibrotic scar with a lesion cavity, surrounded by a glial border primarily composed of astrocytes (Figure 10) (Rudge and Silver, 1990, Silver and Miller, 2004, Fawcett, 2006). In the early stages of scar formation, inflammatory components from the blood stream activate surrounding microglia, followed by activation and proliferation of oligodendrocyte precursors (Fawcett et al., 2012, Silver et al., 2015, Orr and Gensel, 2018). Astrocyte hypertrophy and increased production of intermediate filament proteins Glial Fibrillary Acidic Protein (GFAP) and vimentin prevent axons growing past the glial scar (McKeon et al., 1991, Schwab and Strittmatter, 2014). Meningeal cells and endothelial cells invade the lesion cavity to form the collagenous lesion core and membrane; juxtaposed to the glial border (Silver et al., 2015). A chemical barrier is generated from up-regulation of inhibitory molecules within and surrounding the lesion site, including myelin inhibitory components, chondroitin sulphate proteoglycans (CSPGs), semaphorin III, fibrinogen and recruitment of immune cells, leading to production of free radicals and pro-inflammatory cytokines (Morgenstern et al., 2002, Schwab and Strittmatter, 2014). Together these inhibitors create a hostile CNS glial environment against axon repair. The extracellular matrix is a network of proteoglycans and glycoproteins responsible for cell attachment, movement, communication and growth in a healthy system (Bradbury et al., 2002, Laabs et al., 2005), but which inhibit sprouting and regeneration

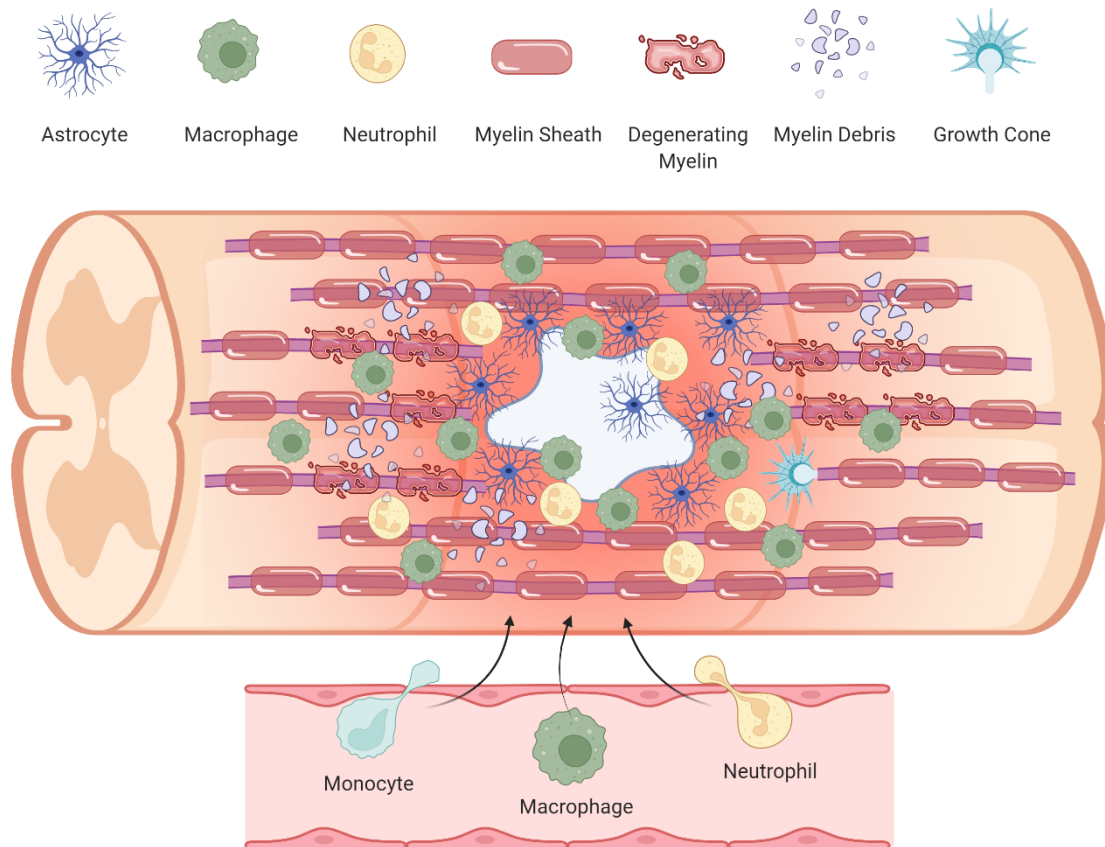


Figure 10: Spinal Cord Injury Lesion Site

Impact trauma to the spinal cord ruptures the blood-CNS barrier and causes widespread dispersion of inhibitory myelin components. Resident and infiltrating immune cells are activated to launch a chronic inflammatory-immune response which fails to clear obstructive molecules. At the injury centre, a cavity forms surrounded by a collagenous scar and astrocytic border. Damaged and demyelinated axons fail to formulate growth cones or initiate axonal regeneration, leading to regenerative failure and axonal die-back. Figure created in BioRender.

post-injury (Karimi-Abdolrezaee et al., 2010, Burnside et al., 2018, Rosenzweig et al., 2019). Key members are the CSPGs, produced by astrocytes, oligodendrocytes and meningeal cells (Galtrey and Fawcett, 2007). Chondroitin sulphate proteoglycans are protein anchors for glycan chains, functioning as barrier molecules as part of the perineuronal net in several biological events and include neurocan, phosphacan, versican and aggrecan, all of which are upregulated in CNS scar tissue and can inhibit axon regeneration (Bradbury et al., 2002, Morgenstern et al., 2002, Laabs et al., 2005, Galtrey and Fawcett, 2007). Inhibiting CSPGs and increasing chondroitinase in SCI has been shown to improve axonal sprouting, increase axonal regeneration and

improve functional recovery in rodent and primate SCI models (Bradbury et al., 2002, Burnside et al., 2018, Rosenzweig et al., 2019). One inhibitory effect of CSPGs is to inactivate integrins, surface receptor proteins essential for interacting with surrounding ECM molecules to facilitate axon growth and cell movement (Tan et al., 2011). Integrin activation following SCI can also improve regenerative outcomes following injury (Tan et al., 2011, Nieuwenhuis et al., 2018). The collagenous scar within the lesion core also creates a dense sheet- like network of ECM molecules interconnected with laminin, fibronectin and CSPGs among others (Hermanns et al., 2001, Schwab et al., 2001, Fawcett, 2006, Hermanns et al., 2006). This creates a complex physical barrier for regenerating axons to navigate.

The other major inhibitory component of the lesion core is the glial border surrounding the collagenous scar. Astrocytes rapidly migrate to the outer edges of the lesion core and proliferate at the lesion margin (Faulkner et al., 2004, Wanner et al., 2013). Intermediate filament proteins such as GFAP and nestin proliferate, leading to cellular hypertrophy, before the gliotic layer is restructured from a longitudinal alignment to one perpendicular to the length of the cord, obstructing any re-growth of new axonal projections (Wilhelmsson et al., 2004, Bardehle et al., 2013, Wanner et al., 2013). Targeted suppression of astrocyte signalling pathways reduces inhibitory scar formation and facilitates axon growth and SCI recovery, specifically, inhibition of TGF- β /Smad, TLR, JAK/STAT3, and JNK/c-Jun signalling (Shen et al., 2014). However, astrocytic scar formation is an essential component of the wound-healing process and astrocyte inhibition may interfere with ECM deposition of growth supportive substrates and neurotrophins (O'Shea et al., 2017, Orr and Gensel, 2018). Using genetic loss-of-function mutations in mice to prevent astrocyte scar formation; attenuate scar-forming astrocytes; or ablate chronic astrocytic scars; all approaches resulted in failure to produce spontaneous regrowth of transected corticospinal, sensory or serotonergic axons through severe spinal cord injury (SCI) lesions (Anderson et al., 2016). Further studies show astrocytes support axonal growth both during CNS development and following mature CNS injury (Mason et al., 1988, Kawaja and Gage, 1991, Lee et al., 2010, Lutz and Barres, 2014), facilitating axon regeneration across CNS lesions only when scar-forming astrocyte bridges are present (Liu et al., 2011, Zukor et al., 2013). Understanding how to modulate astrocytes of the glial border without disruption of essential wound-healing structural bridges is ongoing. For example, sustained delivery of axon-specific growth factors which are insufficiently expressed in SCI lesions,

combined with activation of neuron-intrinsic growth programs, can stimulate robust regrowth of transected axons along supportive matrix cues, despite the presence of inhibitory molecules (Anderson et al., 2016, Anderson et al., 2018). These combinatorial therapeutic approaches show promise.

The final contributor toward the inhibitory CNS environment is the sustained immuno-inflammatory response following injury. Resident microglia are activated and are among the first to infiltrate the lesion site to instigate formation of the glial-collagenous scar (Dibaj et al., 2010, Bellver-Landete et al., 2019). Injury trauma ruptures blood vessels and enables peripheral immune cells to infiltrate the lesion core. Within the adult rodent mammalian system, neutrophils arrive from the periphery within two hours via chemoattractant signalling released from activated astrocytes at the glial border, propagating early astrocyte activation and macrophage recruitment (Campbell et al., 2002, Silver and Miller, 2004, Pineau et al., 2010). Subsequently, peripheral macrophages and monocytes enter the lesion site three days post-injury and peak at seven days (Nguyen et al., 2011). Monocytes differentiate into tissue macrophages, implicated in both tissue repair and secondary tissue damage (Silver et al., 2015). Macrophages can be broadly categorised as either M1-type proinflammatory cells or M2-type anti-inflammatory cells. Following SCI, M1-type macrophages responsible for secondary tissue damage, neuronal loss and axon retraction persist in the lesion site, whereas M2-type macrophage numbers are depleted one-week post-injury (Kigerl et al., 2006, Martinez et al., 2009). These neurotoxic M1-type macrophages release reactive oxygen species and proinflammatory cytokines such as TNF α , IL1 β and IL6 (Horn et al., 2008, Kigerl et al., 2009). Additionally, macrophages and oligodendrocytes fail to remove myelin debris at the lesion site and compound axonal demyelination, accumulating as cell waste alongside apoptotic neutrophils (Totoiu and Keirstead, 2005, Wang et al., 2015). In a healthy wound-healing response, T-helper2 T cells expressing anti-inflammatory cytokines IL4, IL13 and IL10, convert M1-type macrophages into anti-inflammatory M2-type macrophages, creating a more permissible environment for axon repair (Kigerl et al., 2009, Martinez et al., 2009, Shechter et al., 2011). However, this does not happen following SCI due to myelin debris and cell waste build-up at the injury, contributing to chronic inflammation (Kigerl et al., 2009, Wang et al., 2015). Therapies targeting the inflammatory-immune response post-injury have shown macrophage depletion during the first 1–2 weeks post- SCI consistently reduces secondary or bystander tissue injury, leading to

improved recovery of sensory, motor, or autonomic functions (Giulian and Robertson, 1990, Blight, 1994, Popovich et al., 1999, Gris et al., 2004, Popovich and Longbrake, 2008, Zhu et al., 2015). Contradictory papers however, have demonstrated harmful impacts of macrophage depletion such as poorer neurological outcomes and reduced wound healing, owing to the many beneficial factors they can confer such as growth factor release and lipid recycling needed for membrane rebuilding (Heumann et al., 1987, Schwartz, 2003, Shechter et al., 2009). Establishing a nuanced understanding and finding the right balance in therapeutic immuno-modulation will take time. The immune response is highly dynamic after injury, the instigation and duration of macrophage depletion may fluctuate depending on the post-injury timeframe and the heterogeneity of macrophage subtypes makes blanket-treatments ineffective or detrimental. Selective depletion of macrophage subtypes may yield more consistent therapeutic outcomes (Gensel et al., 2017, Jeong et al., 2017, Orr and Gensel, 2018). In summary, inefficient debris clearance of myelin components, formation of a glial-collagenous scar and a chronic immune-inflammatory response all compound to restrict axon growth physically and chemically, resulting in a poor regenerative response in the CNS.

2.2.2 CNS Regenerative Failure: Intrinsic Signalling

Regenerative outcomes for individual neurons do not solely depend on extrinsic environmental factors, each cell possesses the required receptor machinery and internal signalling mechanisms to respond to surrounding environmental cues. This sub-chapter will explore the intrinsic qualities of a neuron following SCI; its response to injury and environmental signals; and subsequent internal physiological changes which fail to facilitate robust regeneration. Upon impact, tissues, blood vessels and axonal membranes are ruptured, releasing debris and inhibitory myelin components into the lesion site which trigger intracellular signalling communication within and between cells. Rho kinases are serine/threonine kinases important in fundamental processes of cell migration, proliferation and survival (Mueller et al., 2005). Receptor complexes for myelin inhibitory factors MAG and Nogo-A (NgR1, Nogo-66, p75, TROY, LINGO1) stimulate RhoA and its effector, RhoA-associated kinase (ROCK) (Mueller et al., 2005), in addition to neuronal receptors for the semaphorin and ephrin families (Shamah et al., 2001, Borisoff et al., 2003). Activation of RhoA post-injury can inhibit neurite outgrowth and sprouting, correlated with axon guidance repulsion (Hall, 1998,

Kaplan et al., 2015). ROCK has several downstream targets (Figure 9) such as myosin light chain, LIM kinase and cofilin, inducing growth cone collapse and decreased axonal outgrowth (Mueller et al., 2005, Hsieh et al., 2006). ROCK also inhibits protein synthesis and cell growth via downregulating mammalian target of rapamycin (mTOR) signalling (Liu et al., 2011). Consequentially, inhibition of ROCK pathways to enhance axonal regeneration is a developing treatment. Inhibiting RhoA using C3 transferase promotes axon outgrowth on inhibitory substrates, as can pharmacological inhibition of ROCK (Dergham et al., 2002, Niederöst et al., 2002, Fournier et al., 2003). Many of these receptors and signalling pathways are also activated by CSPGs. For example, outgrowth inhibition mediated by CSPGs can be ameliorated by blocking RhoA- ROCK signalling (Davies et al., 1999, Dergham et al., 2002). Inhibitory myelin components and CSPGs also stimulate protein kinase C (PKC) and the epidermal growth factor receptor (EGFR) (Yiu and He, 2006). Pharmacological block of PKC for example, minimises outgrowth inhibition and RhoA activation (Hasegawa et al., 2004, Sivasankaran et al., 2004).

Additionally, neurotrophins and neurotrophic factors critically regulate cell survival and differentiation (Ferguson and Son, 2011); and exogenous neurotrophin application to injured CNS neurons increases axon growth (Hendriks et al., 2004). Following peripheral nerve injury, neurotrophic factors such as nerve growth factor (NGF), insulin-like growth factor 1 (IGF-1) and brain- derived neurotrophic factor (BDNF), support regenerative mechanisms (Kiryu-Seo and Kiyama, 2011, Keefe et al., 2017). However, this does not occur in the CNS and contributes to regenerative failure. Topical application of BDNF or IGF-1 within 30 minutes of a focal trauma to the rat spinal cord attenuated breakdown of the blood–spinal cord barrier; oedema; motor dysfunction; and cell injury (Sharma, 2007). Combinations of select neurotrophins BDNF, glial cell line–derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3) and NGF further enhanced the neuroprotective efficacy of growth factors in SCI (Sharma, 2007). Neurotrophins predominantly bind and signal through the p75 neurotrophin receptor or the tyrosine kinase receptor family, enhancing cell signalling via nuclear factor κ B (NF- κ B), Akt, and c-Jun N-terminal kinase (JNK) (Mendell et al., 2001, Sharma, 2007). Many neurotrophin downstream signalling cascades activate regeneration associated genes (RAGs) such as Akt and c-Jun. Capitalising on the intrinsic capacity of a damaged cell in addition to facilitating a more permissive environment is essential for axonal regeneration.

Within the busy injury site, many of the cells present, including glia and immune cells, directly influence and regulate the growth programmes of neurons at the transcriptional and post-transcriptional level. Thus, 'extrinsic' growth regulatory cues interact with and codetermine the 'intrinsic' ability of injured CNS neurons to form regenerating sprouts and elongate over long distances (Silver et al., 2015). This is a major area of therapeutic focus, using animal models and pharmacology to attenuate inhibitory growth signalling and boost intrinsic growth programmes (Cafferty et al., 2008, Zörner and Schwab, 2010, Hollis and Tuszynski, 2011, Liu et al., 2011). Injured peripheral neurons launch a transcriptional regenerative program to enhance expression of RAGs, triggered by calcium signalling and receptor-mediated cues from components within the lesion. Peripheral injury is characterised by an increase in chromatin accessibility at the enhancers and promoters of RAGs, linked to upregulation of growth-promoting transcription factors and an increase in neuroregeneration (Palmisano et al., 2019). This programme fails to launch following CNS injury, maintaining heterochromatic chromatin states and repressive epigenomic markers such as H3K27me3 (Palmisano et al., 2019), contributing to regenerative failure. Collectively, CNS regenerative outcomes are constrained by downstream signalling of inhibitory molecules at the CNS lesion site in conjunction with physical and chemical barriers presented by the neuronal environment. In contrast, the time-course of events and subsequent outcome after peripheral nerve injury is very different.

2.3 Peripheral Nerve Injury: Regenerative Success

2.3.1 PNS Regenerative Success: Extrinsic Factors

In contrast to the CNS, injured peripheral axons trigger an intrinsic regenerative cascade, supported by a local permissive environment encouraging growth cone development (Weng et al., 2016). Following axonal trauma and membrane rupture, the axons and their surrounding myelin fragment and swell, inhibiting signal conduction (Siddique and Thakor, 2014). The distal axon segment undergoes Wallerian degeneration; a series of cellular events initiating anterograde degeneration of axons and the myelin sheath post-lesion (Reier and Lane, 2008, Siddique and Thakor, 2014). Growth-promoting neurotrophic signalling and a swift immune response to injury

successfully clears injury site debris to create a permissive growth- promoting environment for regenerating axons. This subchapter will explore the extracellular cascades following injury in the peripheral system and subsequent axonal regeneration and wound repair.

Wallerian degeneration was first described by Augustus Volney Waller using sectioned frog glossopharyngeal and hypoglossal nerves (Waller, 1850, Stoll et al., 2002, Gaudet et al., 2011). After PNS injury, detached axon segments remain intact for several days, continuing to transmit action potentials when stimulated (Luttges et al., 1976, Tsao et al., 1999). This transmission lag persists for 24-48 hours in rodents and up to several days in the primate mammalian system, including humans, prior to axonal degeneration (Gilliat and Hjorth, 1972, Lubińska, 1977, Chaudhry and Cornblath, 1992). Once this lag period concludes, axons swell and granular disintegration of the cytoskeleton into fine debris is triggered (Figure 11) (George et al., 1995, Kerschensteiner et al., 2005). Neuronal membrane rupture alters ionic homeostasis, preceding a large calcium influx into the axoplasm which activates calpain, a protease essential for cytoskeletal degradation and axon regeneration (George et al., 1995, Wang et al., 2004, Touma et al., 2007, Stirling and Stys, 2010). This degenerative process is essential to make way for other cells in the extracellular environment to execute their reparative function.

The first cells to respond to this degenerative process are the Schwann cells, the ensheathing glial cells of the PNS critical for normal nerve function. Neurons of the CNS are myelinated by oligodendrocytes, whereas PNS neurons are myelinated via individual Schwann cells surrounded by a basal lamina (Siddique and Thakor, 2014). Schwann cells and the basal lamina provide access to growth promoting extracellular matrix molecules and protection from inhibitory molecules, a contributing factor to regenerative failure in the CNS, as the lamina is absent (Höke, 2006, Gaudet et al., 2011). Schwann cells are sensitised to their environment, expressing a number of Toll-like receptors able to recognise nearby tissue damage through binding foreign endogenous ligands released in injury, such as heat shock proteins, mRNA and degraded ECM (Vabulas et al., 2001, Karikó et al., 2004, Goethals et al., 2010, Lee et al., 2016). These Schwann cells act as sentinels in the PNS; rapidly responding to adverse change. In the first four days following injury, Schwann cells in the distal segment of the injured nerve dedifferentiate, cease myelin protein production and upregulate RAG expression (Pellegrino et al., 1986, Trapp et al., 1988, Liu et al., 1995).

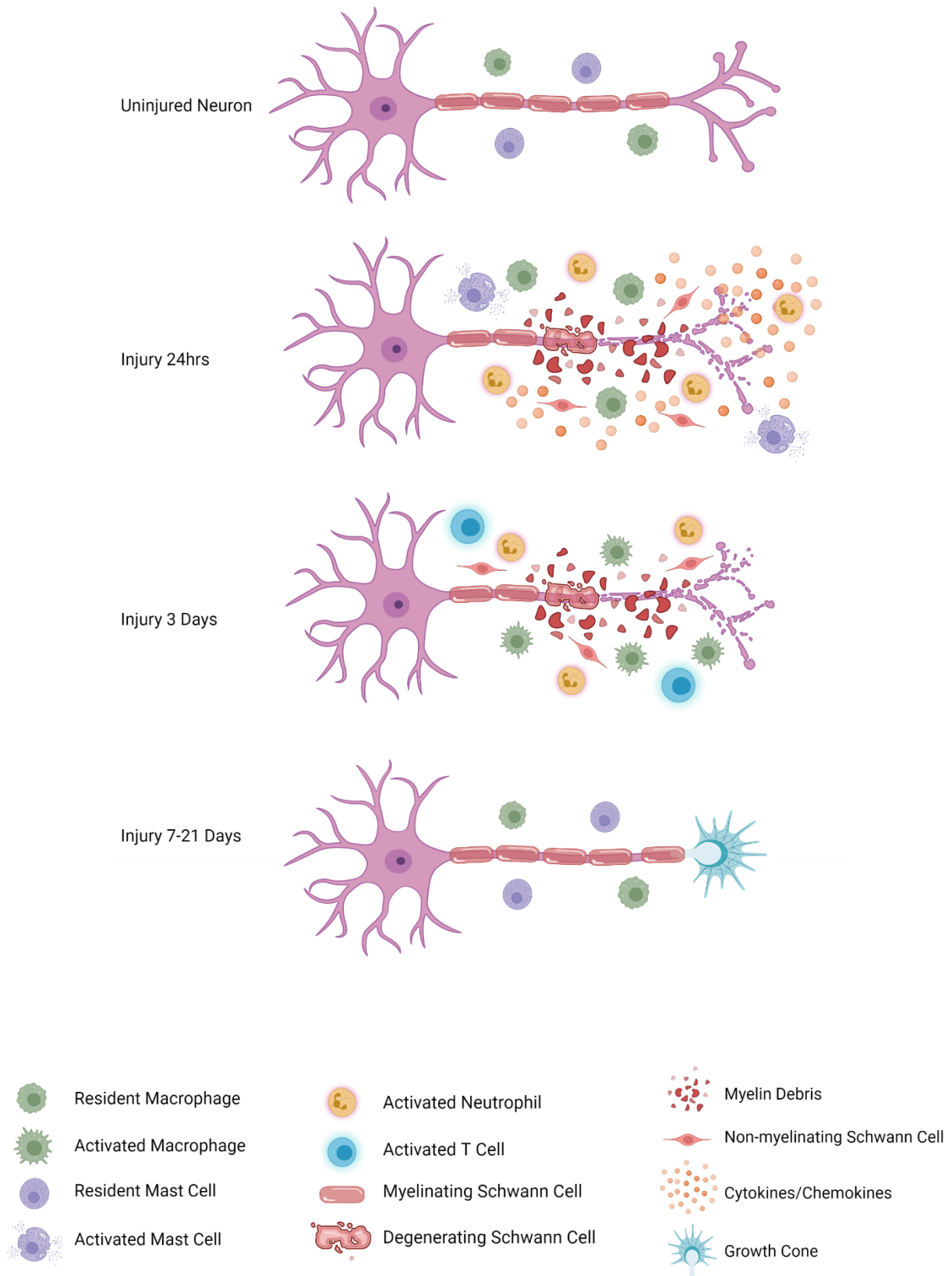


Figure 11: Peripheral Nervous System Wallerian Degeneration

Resident mast cells, macrophages and myelinating Schwann cells maintain a healthy neuronal environment in the uninjured neuron. Within a day of injury, Schwann cells de-differentiate to form non-myelinating Schwann cells responsible for neuronal support and phagocytosing debris. Schwann cells release cytokine, chemokines and neurotrophic factors to activate and recruit immune cells to the injury site. The distal portion of the axon begins to degenerate. Activated neutrophils infiltrate from the circulating blood to release reactive oxygen species and phagocytose inhibitory myelin factors. This clearance response is subsequently enhanced by activated macrophages and T-cells entering the injury site, removing disintegrated axon components and further debris. Cytokine signalling stimulates intracellular cascades promoting growth cone formation and axon outgrowth. In the following two- week period post-injury, anti-inflammatory signalling converts activated M1 macrophages into M2 macrophages, prior to mass efflux of infiltrating immune responders out of the injury site. In the newly created growth- permissive environment, growth cones develop at severed distal axon stumps and begin to extend along the bands of Büngner formed by migrating Schwann cells, eventually guiding axons back to their peripheral targets. Figure created in BioRender.

Consequentially, the proximal axon produces multiple sprouts containing growth cones that initiate regeneration (Burnett and Zager, 2004). Schwann cells divide and proliferate, aligning in columns along the intact basement membrane to form the Bands of Bünger to guide sprouting axons and release nerve growth factors and guidance cues (Stoll et al., 1989, Burnett and Zager, 2004). As Schwann cells re-establish themselves along the axon and the process of re-myelination begins, the protective basal lamina also reforms and contributes once again to supporting healthy functioning of the neuron. Additionally, Schwann cells act as the primary phagocytotic cell type present within the injury site for the first five days, removing myelin debris acting as a physical barrier to regrowing axons (Perry et al., 1995). They also secrete trophic factors and ECM molecules (laminin 2, laminin 8, NGF) to promote axon growth, along with cytokines and chemokines (TNF- α , IL-1 α , IL-1 β , LIF, IL-6) to recruit immune cells to the injured distal nerve stump (Gaudet et al., 2011). However, the supportive functionality of a denervated Schwann cell declines within eight weeks, undergoing apoptosis one to two months after injury (Sulaiman and Gordon, 2000). This eventual time-dependent loss of support in the distal nerve is one of the contributing factors which limits successful long-distance peripheral regeneration (Gaudet et al., 2011).

Within a week of injury, peripheral and tissue resident immune cells are recruited to the injury site via Schwann cell chemotaxis, where they assume the primary role in

debris removal and growth factor production (Tofaris et al., 2002). Phagocytosing neutrophils are the first inflammatory leukocytes to enter the injury site from the circulation, releasing reactive oxygen species and actively recruiting monocytes during this early stage of Wallerian degeneration (Nathan, 2006). Neutrophil density at the injury site dramatically increases within eight hours and peaks at twenty-four hours (Perkins and Tracey, 2000). Neutrophils have a high turnover rate, briefly phagocytosing debris before undergoing apoptosis and giving way to a macrophage-dominated response (Kennedy and DeLeo, 2009). Resident macrophages present in the local tissue express MHC and complement receptor 3, which endow macrophages with both antigen presenting and surveillance capacities, key for responding to inflammatory signals, proliferation and phagocytosing myelin debris (Brück and Friede, 1991, Shen et al., 2000, Mueller et al., 2003). Within two days, infiltrating macrophages from the blood arrive, accumulating within the injured nerve alongside blood-derived monocytes recruited by Schwann cell and macrophage-derived cytokine-chemokine signalling (Siebert et al., 2000, Mueller et al., 2003). These hematogenous macrophages also produce trophic factors such as NGF to aid regeneration (Heumann et al., 1987, Hikawa and Takenaka, 1996). Conditional depletion of CD11-b positive macrophages 12 hours prior to a sciatic nerve crush in mice reduced myelin debris clearance; caused a loss of neurotrophin synthesis; and reduced regenerative and functional recovery outcomes (Barrette et al., 2008). Whereas neurites from DRG explants grew more robustly when treated with macrophage-conditioned media, supporting the beneficial role of a rapid and efficient macrophage response in PNS axon regrowth (Fleur et al., 1996). In contrast, inflammation in the CNS is significantly more cytotoxic, partially as a result of a protracted inflammatory macrophage response (Gaudet et al., 2011). In addition to their well characterised role mediating degeneration at the peripheral nerve distal stump, macrophages also interact directly with the proximal axon to facilitate regeneration. For instance, exosomes release from macrophages are retrogradely transported to the neuronal cell soma to mediate NOX2-dependent PTEN-oxidisation, promoting axonal regeneration after sciatic nerve crush injury (Hervera et al., 2018). Finally, T lymphocytes arrive three days post-injury and peak at day 28 (Moalem et al., 2004). T lymphocytes produce both pro and anti-inflammatory cytokines to support a diverse cellular response as needed. Type 1 helper T cells (Th1) release pro-inflammatory cytokines such as TNF- α and IFN- γ to activate macrophages, neutrophils and natural killer cells in a coordinated 'clean-up'

response (Mosmann et al., 1986, London et al., 1998). Whereas Type 2 helper T cells (Th2) release anti-inflammatory signals (IL-4, IL-10) to inhibit macrophage functioning and regulate the pro-inflammatory cascades (London et al., 1998, Xin et al., 2011). Successful peripheral axon regeneration is associated with a rapid, efficient inflammatory response, which concludes once the injury site is clear. Robust removal of inhibitory myelin components and clearance of physical obstacles creates a permissive environment for regenerating axons and improved prospects for functional recovery.

Another major difference between the PNS injury environment and CNS injury is the presence of neurotrophins and their associated signalling cascades. Many cells within the peripheral injury site such as Schwann cells and macrophages, release or instigate release of neurotrophic factors including: NGF, neurotrophin-3 (NT-3), BDNF, ciliary neurotrophic factor (CNTF) and glial cell-derived neurotrophic factor (GDNF) among others. Nerve growth factor, NT-3 and BDNF all induce axon growth via localised activation of PI3K at the growth cone and regulation of cytoskeleton protein-binding proteins (Zhou et al., 2004, Zhou et al., 2006, Chen et al., 2007). Neurotrophic factors also play important roles in regulating Schwann cell differentiation and axon remyelination, key processes in the end stages of Wallerian degeneration (Cosgaya et al., 2002, Chen et al., 2007). The majority of neurotrophin signalling is mediated via two key receptor types, the tyrosine kinase receptors and p75 (Chao, 2003). For example, p75^{NTR}–/– mice exhibit reduced myelination, fewer myelinated axons and thinner myelin sheaths compared to wild type mice (Cosgaya et al., 2002). Brain derived neurotrophic factor binds the tyrosine kinase receptor TrkB, though the specifics of its therapeutic action post-injury remain to be clarified. Application of low doses of BDNF at the injury site promote regeneration in chronic injury, but higher doses inhibit regeneration (Boyd and Gordon, 2002). Several examples of therapeutic uses of neurotrophins post-injury demonstrate beneficial effects on nerve outgrowth, axon regeneration, remyelination and sustaining longer-term regenerative processes (Ye and Houle, 1997, Terenghi, 1999, Jones et al., 2001, Boyd and Gordon, 2003, Cui, 2006, Gordon, 2009).

Collectively, the events following PNS injury formulate a locally permissive environment. Successful clearance of axonal and myelin debris via Schwann cells and macrophages, in addition to support from axon guidance cues, neurotrophins, cytokines and growth factors are a stark contrast to the molecular cascades following

CNS injury (Chen et al., 2007). The enabling PNS environment may be so influential that it was hypothesised that the regenerative potentiality of central neurons may only be expressed when the CNS neuroglial environment is altered to resemble that of the PNS (David and Aguayo, 1981).

2.3.2 PNS Regenerative Success: Intrinsic Programme

Beside the environmental challenges, injured peripheral neurons initiate a programme of gene expression that provides the cytoskeletal, axoplasmic, transcriptional and epigenetic elements necessary to support neurite outgrowth and regeneration (Woolf, 2001). This constitutes the intrinsic growth capacity of the axon, encompassing changes at the nuclear level, in addition to modulation of local protein translation; retrograde and anterograde axonal transport; and microtubule dynamics (Mar et al., 2014). Following peripheral axonal injury (Figure 12), calcium influx into the axoplasm from ruptured cell membranes elicits intracellular signalling required for resealing the axonal membrane, local protein synthesis and assembly of a competent growth cone

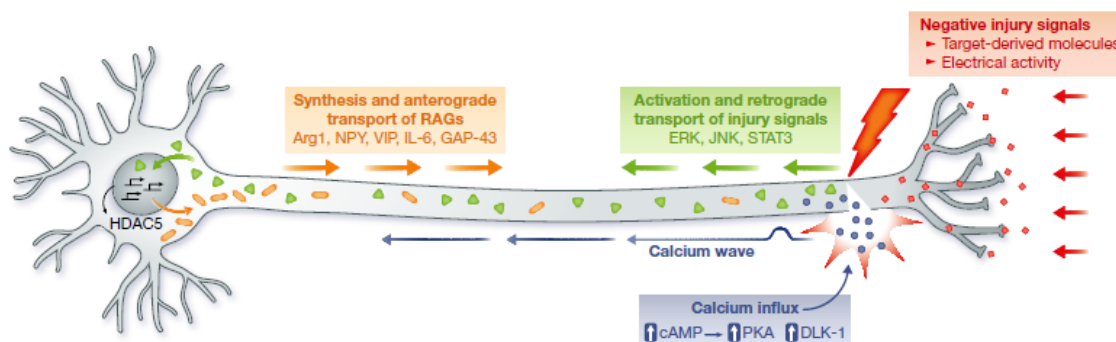


Figure 12: Peripheral Nerve Injury Signalling Cascades

Injury at the peripheral nerve disrupts neuronal membranes and ionic homeostasis. Calcium influx into the axoplasm activates cAMP and PKA signalling to DLK-1, promoting growth cone formation, local protein synthesis and resealing of the axonal membrane. This calcium wave back-propagates to the cell body, triggering HDAC5 nuclear export and initiation of the intrinsic regenerative transcription programme. Within the cell body, RAGs necessary to mount a regenerative response are expressed (Arg1, NPY, IL-6, GAP-43 and others). Retrograde transport of injury signals including ERK, JNK and STAT3 is facilitated by local protein synthesis at the injury site. Vimentin and Importin β 1 are translated to create an Importin- pERK- Dynein complex, which transports injury signals to the cell body via microtubule networks. Increased anterograde transport of mitochondria to the lesion via Kinesin- microtubule interaction facilitates this local protein synthesis. (Mar 2014).

(Krause et al., 1994, Chierzi et al., 2005, Kamber et al., 2009). Adenylate cyclase is activated, increasing cAMP levels to activate dual leucine zipper kinase (DLK-1) and initiate cytoskeleton transformation required for growth cone assembly and retrograde transport of injury signalling molecules (Ghosh-Roy et al., 2010). This calcium wave continues to travel to the soma to activate protein kinase C μ and instigate the nuclear export of histone deacetylase 5 (HDAC5), increasing histone acetylation and activation of the regenerative transcription programme (Cho et al., 2013). Calcium- stimulated retrograde transport of locally activated injury signals to the cell body increases RAG expression, including extracellular signal- regulated kinase (ERK), c-Jun N- terminal kinases (JNK) and signal transducer and activator of transcription 3 (STAT3) (Ben-Yaakov et al., 2012, Mar et al., 2014). Simultaneously, anterograde axonal transport of injury signals to the distal axon tip ensures structural components, vesicles and organelles are supplied locally within the neuron to support regeneration (Mar et al., 2014). Consequently, injury activates an intrinsic growth capacity in the cell body and the proximal stump formulates a growth cone for axon extension, indicated via increased cell volume from production of RNA and growth proteins (Maggi et al., 2003, Abe and Cavalli, 2008).

The effector output of intrinsic RAG programmes activated in peripheral injury are mediated by chromatin and epigenetic processes within the cell nucleus. Chromatin is formed from a repeat unit called a nucleosome, consisting of negatively charged DNA wrapped around a positively charged histone H3/H4 tetramer and two pairs of H2 dimers (Luger et al., 1997). Chromatin compactness and accessibility is mediated by multiple biochemical interactions which influence gene expression and phenotype without altering the primary DNA sequence, a phenomenon known as epigenetics (Margueron and Reinberg, 2010, Bannister and Kouzarides, 2011). Modifications include DNA chemical changes and histone post- translational alterations such as acetylation, methylation and phosphorylation on histone N-terminus tails (Weng et al., 2016). For example, histone acetylation neutralises the positive histone charge, decreasing the strength of binding to the negative DNA backbone, associated with an open euchromatin conformation and transcriptionally active states (Shahbazian and Grunstein, 2007, Trakhtenberg and Goldberg, 2012). Whereas deacetylation and methylation of histones is often a repressive mark associated with gene silencing and heterochromatin (Kouzarides, 2007, Lindner et al., 2013). Heterochromatin states of tightly packaged nucleosomes render DNA inaccessible to transcription factors, a

mechanism used to silence genes (Sterner and Berger, 2000). The balance between acetylation and deacetylation is mediated by enzymes, histone acetyltransferases (HATs) which facilitate acetylation and histone deacetylases (HDACs) which remove acetylation (Jacob et al., 2011). These epigenetic interactions have proven key to intrinsic neuronal regenerative mechanisms. Inhibition of histone deacetylation via HDAC inhibitor trichostatin A (TSA) induces histone H3K9/14 hyperacetylation and axonal outgrowth (Gaub et al., 2010). Following peripheral injury, increased histone acetylation and RAG transcription initiates a regenerative programme. However, central injury of retinal ganglion cells fails to export nuclear HDAC5, triggering histone deacetylation and suppressed axonal regeneration (Cho et al., 2013). Comparative examination of the epigenetic landscape following PNS and CNS injury revealed PNS regeneration in DRG neurons is associated with a more accessible euchromatin conformation, characterised by high levels of H3K9ac and H3K27ac at the promoters of injury responsive genes (Palmisano et al., 2019). Whereas following a central lesion, chromatin remains in a “silent” non-accessible heterochromatic state (Palmisano et al., 2019). Neurons are also not the only cell type to adapt their epigenetic characteristics following peripheral injury. Proliferating Schwann cells present within the PNS injury site alter their transcriptional programme to support damaged neurons. Schwann cells increase active markers such as H3K27ac and H3K4me3 at enhancers and promoters for key growth hormone and neurotrophic factor genes, including GDNF and sonic hedgehog (Shh) (Ma et al., 2016). Peripheral injury also instigates reversion of polycomb repression within Schwann cells, complexes responsible for catalysing trimethylation at lysine 27 of histone H3 (H3K27me3) which produces a transcriptionally repressive chromatin environment (Ma et al., 2016).

The intrinsic transcriptional program activated by PNS injury upregulates expression of RAGs and epigenetic markers associated with gene transcription. Regeneration associated genes are defined as any molecule which plays a role in neurite and axon outgrowth following injury, consisting of transcription factors, neuropeptides, neurotrophins, adhesion molecules and structural proteins (Giovanni, 2009). Transcription factors are hubs (highly connected genes) in the RAG network, controlling the coordinate expression of several RAGs contributing to post-injury axon outgrowth, including c-Jun, activating transcription factor 3 (ATF3) (activated by JNK), Sox11, Smad1, CREB, arginase-1, EGR1, Fos, SMAD1, STAT3 and BDNF (Smith and Skene, 1997, Xiao et al., 2002, Lindwall and Kanje, 2005, Deng et al., 2009, Van

Kesteren et al., 2011, Chandran et al., 2016). A principle goal has been to identify master regulators that coordinate the regenerative program to understand the global gene regulation process underlying intrinsic growth ability (Kiryu-Seo and Kiyama, 2011). Therefore, several RAG signalling pathways, HATs and HDACs have been characterised and modulated therapeutically following CNS injury, to increase RAG transcription and central axon regeneration. For example, the HAT complex CBP/p300 acetylates histone 3 at K9-14 and the transcription factor p53, activating a transcriptional program which regulates RAG expression in rodents (Tedeschi et al., 2009, Gaub et al., 2010, Gaub et al., 2011). The HAT p300 directly binds and acetylates histones at the promoter of growth-associated protein 43 (GAP-43), coronin 1 b, and Sprr1a, driving RAG expression (Gaub et al., 2011). This system was recreated in an optic nerve crush model where neuronal p300 overexpression promoted axon growth in the CNS (Gaub et al., 2011). In addition to its effects on cell survival, development and plasticity, CREB- dependent transcription is activated by phosphorylation activity from protein kinase A, stimulated by calcium influx and cAMP following nerve injury (Gao et al., 2004, Hannila and Filbin, 2008, Tedeschi, 2012). Overexpression of CREB increases regeneration in ascending dorsal column axons after spinal cord injury, upregulating Arginase I which promotes polyamine synthesis and cytoskeleton tubulin regulation (Gao et al., 2004, Ma et al., 2010). Another RAG activated by growth factor, cytokine and inflammatory signalling following PNS injury is c-Jun, a component of the heterodimeric AP-1 transcription factor (Herdegen et al., 1997, Jochum et al., 2001, Tedeschi, 2012). C-Jun activity is controlled by Jun N-terminal kinases (JNK) to enable the JNK/c-Jun transcriptional pathway to act as a barometer in response to nerve injury, rapidly retrogradely transporting JNKs to the cell body following nerve injury to activate c-Jun (Herdegen et al., 1997, Raivich et al., 2004, Makwana and Raivich, 2005). Upregulation of c-Jun improves regenerative responses in several injury models (Herdegen et al., 1991, Jenkins and Hunt, 1991, Lindwall and Kanje, 2005) and absence of c-Jun impairs RAG expression of CD44, Galanin and $\alpha 7 \beta 1$ integrin, affecting target reinnervation and functional recovery (Herdegen et al., 1997, Raivich et al., 2004, Teng and Tang, 2006). Alternatively, inhibitors of RAG pathways can be targeted to promote regeneration. For example, phosphatase and tensin homolog (PTEN) acts as an antagonist to PI3K, inactivating protein kinase B and mTOR signalling beneficial for regeneration (Mar et al., 2014). In CNS retinal ganglion cells, PTEN deletion promotes robust axon regeneration after

optic nerve injury (Park et al., 2008). In the PNS following nerve axotomy, PTEN-depleted adult sensory neurons also showed increased axon regeneration (Christie et al., 2010).

The research exploring selective RAG activation, combinatorial RAG activation, overexpression or inhibition of factors to modulate transcription and epigenetic markers in regeneration is extensive (Giovanni, 2009, Kiryu-Seo and Kiyama, 2011, Tedeschi, 2012, Lindner et al., 2013, He and Jin, 2016, Fawcett, 2020). Yet collective efforts to outline the clinical applications for SCI patients are ongoing, as many of these networks are broad with diffuse downstream targets. Time, new technologies and robust *in vivo* models which sufficiently recapitulate human injury conditions will improve our understanding and translational success in the future.

2.4 Regenerative Models

The spinal cord injury field first established its understanding of development and growth before the concept of regeneration became distinct. It was originally thought that regeneration simply equated to recapitulating an earlier growth state, but non-mammalian and invertebrate studies dispelled this notion and provided injury-specific insight. These organisms also showed a more permissive regenerative capacity, in vertebrates such as fish, newts and salamanders for example, the CNS is able to repair itself once damaged and regenerate entire limbs, tails and body parts (Gaze, 1970, Brockes and Kumar, 2008). This impressive capacity to establish full functional recovery in the nervous system provided valuable opportunity to investigate the physiological mechanisms responsible for successful regeneration. The giant axons in *Aplysia californica* clarified the molecular events in growth cone formation and many of the essential proteins retrogradely transported to the cell soma following injury (Schmied and Ambron, 1997, Zhang and Ambron, 2000, Sung et al., 2004, Bradke et al., 2012). *Drosophila melanogaster*, *C.elegans* and zebra fish have also contributed greatly to understanding of regenerative cascades, particularly useful for their short life cycle, transparent physiology and simple nervous systems (Xiong et al., 2010, Ghosh-Roy et al., 2012, Song et al., 2015). Transparent *C.elegans* for example, have been used to study precise single axon axotomy using lasers, to characterise the variable responses initiated in axon re-growth in different neuronal subtypes and provide insight into intrinsic regenerative cascades in particular, as laser lesions exert minimal tissue damage (Yanik et al., 2004, Wu et al., 2007, Canty et al., 2013). However, progressing

from primitive vertebrates such as newts to higher mammals such as frogs and rodents, new limb growth and regenerative ability is lost within the evolutionary chain (Lang et al., 1995, Fawcett et al., 2012). Limb damage in adult *Xenopus* regenerates a spike of tissue without a paw, whereas newts and salamanders can generate a fully patterned limb (Fawcett et al., 2012). Context and translation are, therefore, essential. If regenerative studies are hoped to facilitate therapeutic recovery for human patients, research using mammals and non-human primates are required to recreate the complexity of the human nervous system.

The overarching goal in spinal cord injury research is to restore axonal regeneration and functional recovery within the CNS. Understanding the intrinsic regenerative differences between the CNS and PNS within the adult mammalian system is key to achieve this. The sciatic dorsal root ganglia provide an elegant system to study these differential signalling mechanisms (Figure 13), comprised of pseudo-unipolar primary sensory neurons extending one axonal branch into the peripheral sciatic nerve and the other into the dorsal column of the spinal cord (Giovanni, 2009). This model enables direct comparison within the same neuronal cell body between regenerative mechanisms following peripheral sciatic nerve axotomy (SNA) or regenerative-inhibitory pathways after central dorsal column axotomy (DCA). As described previously, injury at the peripheral branch launches an intrinsic regenerative program as the distal axon undergoes Wallerian degeneration prior to growth cone formation, axon extension and target re-innervation. Injury to the central branch of DRG neurons fails to instigate successful extracellular clean-up of inhibitory myelin debris, maintains a chronic inflammatory environment and fails to instigate an intrinsic transcriptional programme, resulting in retraction bulb formation and axonal die-back. The DRG model helped to highlight the importance of the intrinsic cascade in the regenerative processes. Previous injury at the peripheral branch, termed a conditioning lesion, facilitates regeneration of central sensory axons toward re-innervation of brain and spinal targets, following a subsequent injury at the central branch (Woolf, 2001, Hoffman, 2010). The conditioning effect activates regenerative machinery prior to the CNS lesion, mediated by increased cAMP levels to enable neurons to overcome myelin inhibition and sustain RAG expression via central epigenetic mechanisms (Smith and Skene, 1997, Qiu et al., 2002, Ylera et al., 2009, Mar et al., 2014). Identification and manipulation of essential regenerative propagators in SNA can focus target selection to stimulate central axonal growth following DCA. As such, these *in*

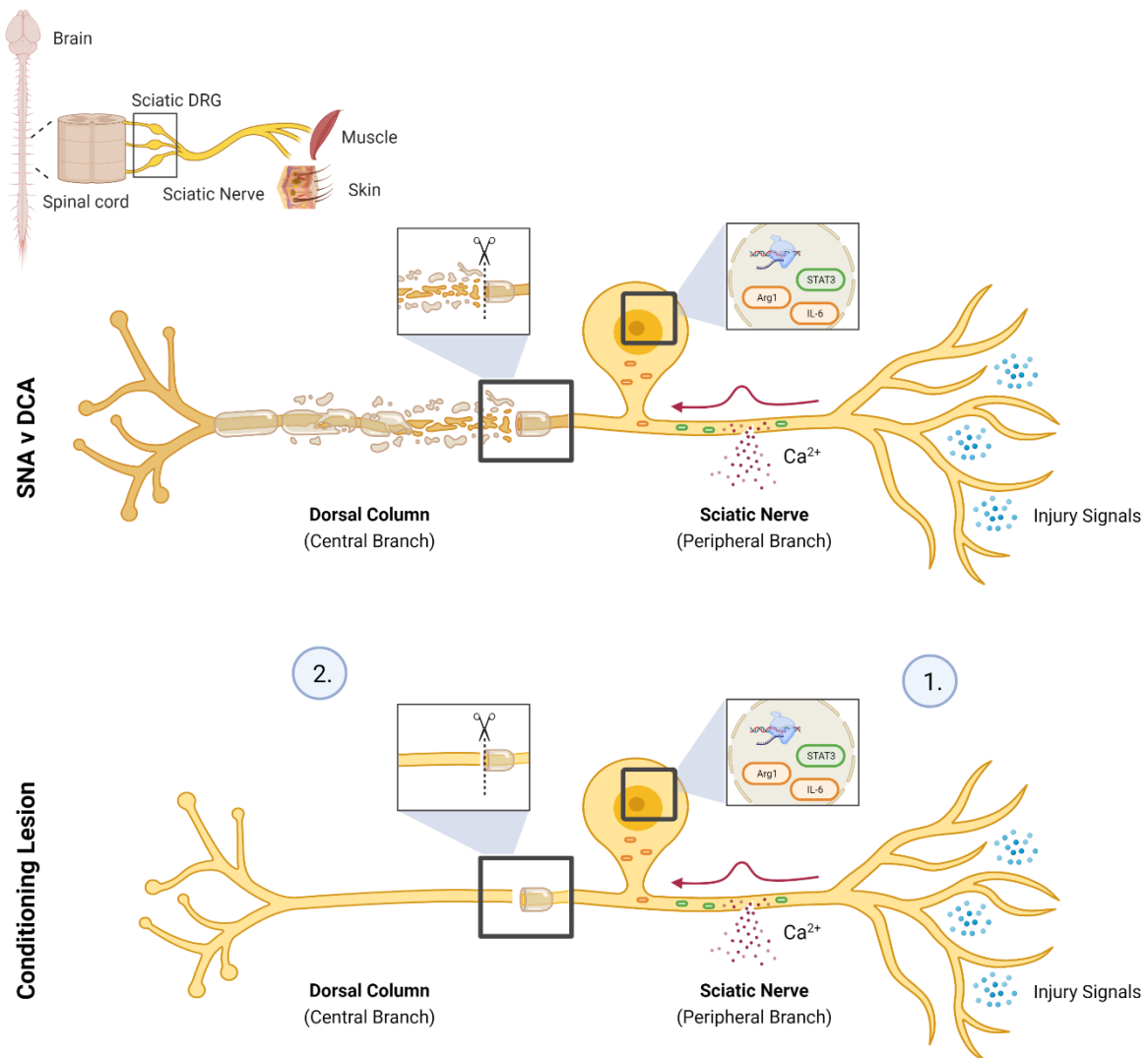


Figure 13: The Dorsal Root Ganglia Model

The pseudounipolar neurons of the dorsal root ganglia extend one central branch into the dorsal columns of the spinal cord and one peripheral branch into the sciatic nerve of the leg. This provides an elegant opportunity to study the regenerative difference between the peripheral system and the central system within the same cell body. Sciatic nerve axotomy (SNA) and peripheral injury triggers Wallerian degeneration and an intrinsic regenerative cascade to enhance axonal outgrowth. Dorsal column axotomy (DCA) and central injury results in axonal degeneration and retraction bulb formation. Injury to the sciatic nerve, synthesis of necessary regenerative proteins and axonal repair, prior to injury at the central branch (conditioning lesion) enhances regeneration and repair of that same central branch. Figure created in BioRender.

vivo injury models are essential for exploring regenerative hypotheses and potential therapies.

The spinal cord injury field has made good progress characterising post-injury cascades with regenerative models such as DRG neurons and the conditioning lesion. Recently, injury-independent physiological mediators such as circadian rhythms, exercise and diet have also been explored within the context of axonal regeneration. Conserved physiological signalling pathways key to organism survival may offer opportunities to discover new pathways to modulate regeneration both independently of and in the context of, axonal injury. For example, an enriched environment with more exercise opportunities conferred neuroprotective properties on mouse DRG sensory neurons prior to injury, which gave rise to a more robust regenerative output following nerve injury (Hutson et al., 2019). Many of the signalling pathways activated within neurons during exercise overlap with those modulated through dietary regimens (Mattson et al., 2018). It's possible that broad metabolic regulators fundamental to basic cell processes may exact diverse physiological changes which could be manipulated in the context of axonal regeneration.

2.5 Intermittent Fasting

2.5.1 The Evolution of Energy Homeostasis

The human body is intrinsically connected to the environment surrounding it, processing external stimuli and regulating the needs of the organism for optimal survival. Energy homeostasis and feeding behaviour are highly conserved mechanisms of health regulation that date back to hunter-gatherer societies and their predecessors. The thrifty gene hypothesis posits that in the 'wild' where calories are limited or supply inconsistent, deposition in fat stores provides protection against future starvation when food is scarce (Friedman, 2019). This would have benefitted early humans who foraged for food and ate only what the environment provided, often going for several days without food before eating again (Mattson, 2012). This feeding behaviour is termed intermittent fasting (IF). Modern day famines significant enough to exert a selection pressure on humans with low adipose levels are infrequent (Speakman, 2007, Speakman, 2018), though this would have impacted past hunter-gatherer societies and there is likely a minimum adipose level required to promote

survival during lean times (Speakman, 2007, Prentice et al., 2008, Dhurandhar, 2016). Conversely, the predator- release hypothesis predicted higher adipose tissue mass was disadvantageous to survival by limiting an animal's mobility and its capacity to escape danger (Speakman, 2007). Over time, a balance in adipose levels was determined via natural selection, formulating sensitive and efficient energy homeostasis mechanisms within humans. However, it is theorised that the absence of predators among most modern human populations, coupled with food excess in developed countries, has led to an increased incidence of obesity as a result of genetic drift from accumulation of predisposing alleles that are no longer selected against (Friedman, 2019). The extent to which genetics plays a role in obesity and other metabolic disorders is controversial (Diamond, 2003) but understanding how IF and past feeding behaviours impact energy homeostasis is crucial for the current understanding of health and longevity mechanisms in the modern human.

Adaptation towards IF feeding behaviour does not just strengthen survival of the organism but is also shown to increase physical and mental performance. Goodrick *et al.* compared an ad libitum (AL) diet to every-other-day (EOD) fasting in male rats and found the mean lifespan of EOD rats was increased by 83% compared to AL rats (Goodrick et al., 1982). This was one of the first papers to suggest a relationship between IF and longevity. Several studies have built on this early work to shape the existing field, exploring the many systemic and physiological impacts IF has on tissues throughout the body (Anson et al., 2003, Gómez Acuña et al., 2013, Li et al., 2013, Longo and Mattson, 2014, Longo and Panda, 2016, Mattson et al., 2017, Patterson and Sears, 2017, de Cabo and Mattson, 2019). Intermittent fasting can be defined as 'alternate- day fasting' consisting of 24-hour cycles of ad libitum food consumption followed by 24 hours of fasting (no food or energy- containing beverages) (Longo and Mattson, 2014, Longo and Panda, 2016). Cycles can last for a few days or up to several weeks and modified fasting regimens may alter the frequency or time period of the fast, such as the 5:2 diet based on severe energy restriction for 2 non-consecutive days and 5 days of ad libitum eating (Patterson and Sears, 2017). Intermittent fasting is a form of dietary restriction centred around complete fast days. The other dietary restriction proven effective in increasing life span and disease resistance is caloric restriction (CR) (Anson et al., 2003). The caloric restriction paradigm reduces food intake to a level below that which would be consumed voluntarily, to around 20-40% of the usual daily intake (Longo and Mattson, 2014). A large body of data has been built comparing

CR to IF. Both interventions have a similar effect on reduction of visceral fat, adiponectin, insulin levels and insulin resistance (Barnosky et al., 2014, Patterson and Sears, 2017). Caloric restriction is more successful in decreasing body weight but IF is more effective at decreasing glucose and insulin concentrations and increasing IGF-1 and β -Hydroxybutyrate levels (Anson et al., 2003). Intermittent fasting stimulates ketone production and activates neuroprotective mechanisms via upregulation of stress protein chaperones and neurotrophic factors, improving neuronal survival after injury (Anson et al., 2003). Crucially, intermittent fasting alters fasting levels of key stress resistance and neuroprotective molecules independently of overall caloric intake and body weight, enabling clear elucidation of the importance of these signalling cascades in disease or injury states.

Our diet and the way humans eat is intrinsically linked to our survival and conserved mechanisms of energy homeostasis. The IF lifestyle of hunter gatherer societies whilst no longer prevalent in the age of food abundance in many developed nations, it is still reflected in religious practices such as Ramadan, instances of war such as the Dutch Hunger Winter and developing countries who regularly experience famine. These cohorts and research studies provide a wealth of information about how IF impacts health and disease.

2.5.2 The Physiology of Intermittent Fasting

Intermittent fasting influences numerous cellular and metabolic cascades throughout the body, impacting key energy homeostasis processes. Energy homeostasis can be defined as the balance between energy input and output. The second law of thermodynamics states energy within a system must be conserved and any change must result from either a change in the energy input to, or output from, that system (Bray, 1990, Schneider and Kay, 1994). In animals, changes in fat mass derive from changes in energy input (food intake) or expenditure (Friedman, 2019). Energy homeostasis is the biological mechanism indexing this process to maintain the stability of weight and adipose tissue mass (Adolph, 1947, Kennedy, 1953). Eating behaviour is regulated via the interaction of the homeostatic and hedonic systems. The homeostatic system controls energy balance, increasing the motivation to eat following depletion of energy stores (Lutter and Nestler, 2009). It is comprised of peripheral hormonal regulators of hunger, satiety and adiposity levels, such as leptin, ghrelin, and insulin (Hommel et al., 2006). These neuropeptides act on central hypothalamic and

brainstem circuits to stimulate or inhibit feeding, to maintain appropriate levels of energy balance (Figure 14) (Kenny, 2011). Vagal nerve innervation to the midbrain, projecting to the hypothalamus, also signals nutritional state, depending on whether a person has recently eaten (Morton et al., 2006). Following food consumption, plasma glucose and amino acid levels rise, accompanied by enhanced parasympathetic activity (Geary, 2004). Beta cells of the pancreas secrete insulin in response, promoting glycolysis and synthesis of energy storage molecules such as fatty acids and triglycerides (Schulinkamp et al., 2000). The orexigenic hormone ghrelin decreases, whereas the satiety hormone leptin increases, targeting receptors within the hypothalamus to inhibit feeding (Geary, 2004). Leptin stimulates pro-opiomelanocortin (POMC) neurons and inhibits neuropeptide Y and Agouti-related protein (NPY/AGRP) neurons in the arcuate nucleus of the hypothalamus (Stephens et al., 1995, Friedman and Halaas, 1998, Elias et al., 1999, Cowley et al., 2001). Intermittent fasting however, incorporates fast days into feeding behaviour, alternating energy production between catabolic and anabolic states. Catabolism can be defined as a 'breakdown' process such as the oxidation of glucose from energy stores following a fast day (Wolfe, 2006, Judelson et al., 2008). Whereas anabolism is a synthesis process, creating proteins from amino acids metabolised from recently ingested food (Wolfe, 2006, Judelson et al., 2008). The body interchanges between the two depending on energy availability. During a fast day systemic leptin levels decrease drastically, increasing NPY and AGRP to stimulate eating behaviour, instigating the switch from carbohydrate to fat-based metabolism during lipolysis and gluconeogenesis (Ahima and Flier, 2000). This fall in leptin mediates the adaptation to a fasting state. As such, adipose tissue is a highly dynamic organ playing a crucial role in the systemic control of energy balance; communicating nutritional changes to CNS centres that control appetite and linking changes in the levels of energy stores to adaptive changes in the neuroendocrine axis, immune function and the function of arguably all other physiological systems (Friedman, 2019).

Obesity studies were seminal for identifying the components responsible for energy balance. In particular, the identification of the recessive (*ob*) mutation and the diabetes (*db*) mutation, both of which cause mice to present with an obese phenotype (Hummel et al., 1966, Coleman, 1978). A similar phenotype also develops in genetically obese rats carrying the fatty (*fa*) mutation (Zucker and Zucker, 1961). These phenotypes cause extreme obesity and insulin-resistant diabetes as part of a complex syndrome

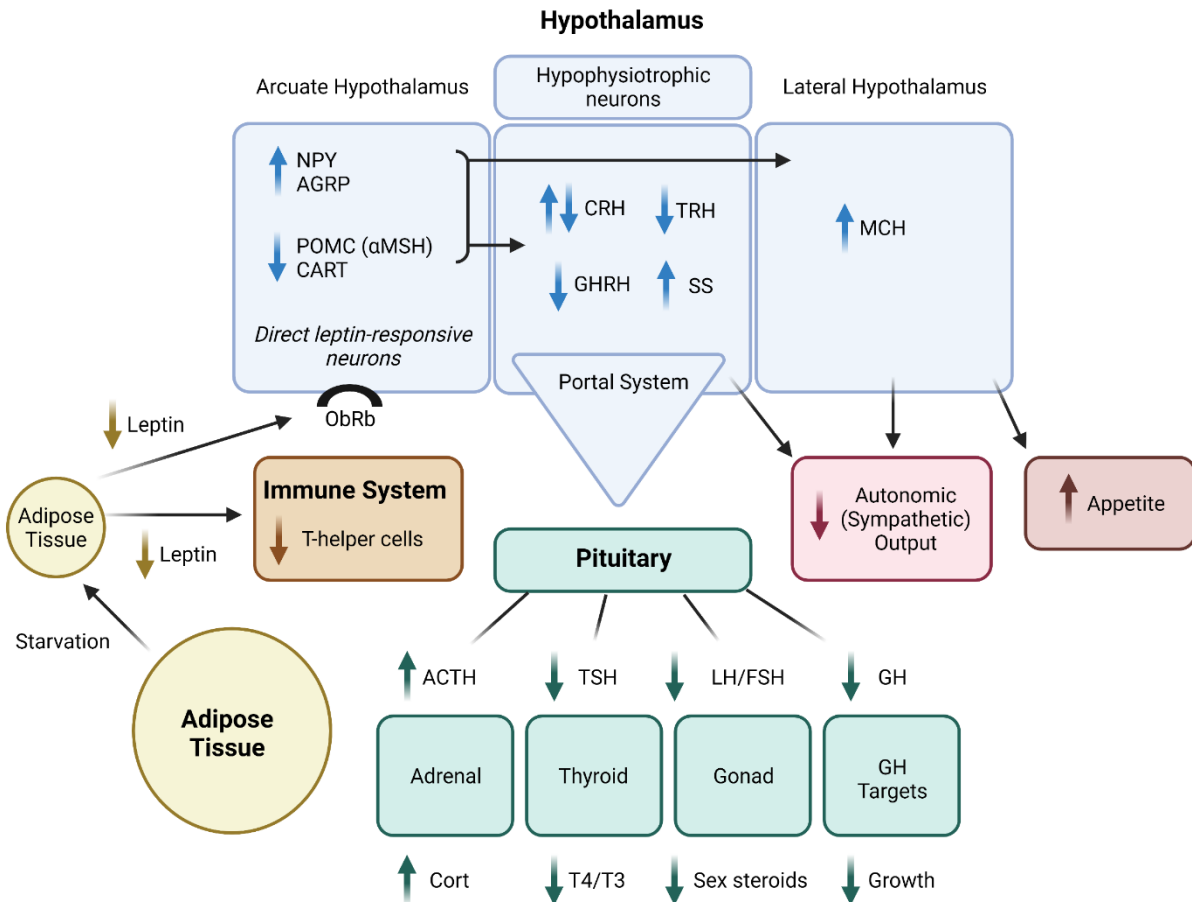


Figure 14: Leptin Targets in the Brain

Circulating leptin levels released by adipose tissue fall during intermittent fasting, increasing neuropeptide Y (NPY) and agouti-related peptide (AGRP) levels, and decreasing proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) levels in the arcuate hypothalamic nucleus. NPY, POMC, AGRP, and CART neurons all bind and respond directly to leptin. NPY and AGRP stimulate feeding (orexigenic), whereas α -melanocyte stimulating hormone (a product of POMC) and CART inhibit feeding (anorexigenic). These neurons project to the lateral hypothalamus and regulate the expression of melanin-concentrating hormone (MCH), a major stimulator of feeding. In addition, leptin targets in the arcuate hypothalamic nucleus respond to low leptin levels by regulating the neuroendocrine axis and decreasing sympathetic nervous output. Falling leptin levels also suppress immune function. Figure created in BioRender, adapted from Ahima and Flier 2000. CRH (corticotropin-releasing hormone), TRH (thyrotropin-releasing hormone), GHRH (growth hormone–releasing hormone), SS (somatostatin), GH (growth hormone), ACTH (adrenocorticotropin), TSH (thyroid-stimulating hormone), LH (luteinizing hormone), FSH (follicle-stimulating hormone), Cort (cortisol), T4/T3 (thyroxine/ triiodothyronine).

including infertility, immune dysregulation and hypothermia (Friedman and Halaas, 1998). Early parabiosis studies (the surgical union of two animals, resulting in chronic blood exchange) determined a blood-borne humoral factor caused weight loss (Hervey, 1959). Wild type mice parabiosed to *db* mice and wild type rats parabiosed *fa/fa* rats lost large amounts of weight, similarly to *ob* mice parabiosed to *db* mice (Coleman and Hummel, 1969, Coleman, 1973, Coleman, 1978, Harris et al., 1987). This suggested *ob* mice carried a mutation in the humoral factor regulating weight loss (Coleman, 1973). In aggregate, *ob* mice are obese because they do not produce a hormone that regulates food intake and body weight, whereas *db* mice and *fa* rats are obese because they do not express the receptor for that factor (Friedman, 2019). It was not until 1994 and the discovery of the *ob* gene (Zhang et al., 1994), that this humoral factor was identified as leptin. Leptin is encoded for by the obesity gene (*ob*) located on chromosome 7q31.3 in humans and circulates in the blood as a 16KDa satiety hormone (Isse et al., 1995). Leptin is synthesised by white adipose tissue but is expressed in multiple peripheral locations including bone marrow, ovary, placenta, stomach and lymphoid tissue (Masuzaki et al., 1997, Bado et al., 1998). Leptin targets leptin receptors on hypothalamic nuclei and brainstem neuronal circuits implicated in feeding regulation (Kwon et al., 2016). During fasting, systemic leptin decreases, NPY increases to stimulate feeding and melanin-concentrating hormone (MCH) increases to initiate food intake (Ahima and Flier, 2000). Leptin's role as a homeostatic regulator of food intake and energy stores is well established through disease models. Several studies link leptin resistance to obesity and mutations in the *ob* gene trigger early onset obesity. For example, *ob/ob* mice are leptin deficient, hyperphagic and morbidly obese, with multiple metabolic and neuroendocrine abnormalities (Zhang et al., 1994). Leptin administration significantly decreases body weight and adipose tissue mass in *ob* mice and wild type animals but has no discernible effect in *db* animals (Campfield et al., 1995, Halaas et al., 1995, Pelleymounter et al., 1995). Leptin levels increase when fat mass increases, suppressing food intake, whereas weight loss leads to a decrease in leptin levels and a consequent increase in food intake, maintaining homeostatic control of adipose tissue mass with no effect on decreasing lean mass (Friedman, 2019).

Leptin resistance and increasing obesity rates in the modern age may reflect the body's inability to store energy efficiently at times of abundance (evolutionary disadvantageous) (Ahima and Flier, 2000). The dominant role of leptin in energy homeostasis is therefore likely as an adaptor to fasting. Starvation initiates neural,

metabolic, hormonal and behavioural modifications to maintain energy supply to the brain and promote survival, processes triggered by falling leptin levels (Ahima and Flier, 2000). Leptin can therefore be described as a signal between fasted and fed states.

2.5.3 Intermittent Fasting in Health and Disease

The presence of fasting adaptations is not unique to humans but is seen throughout the animal kingdom, from single- cell organisms, to *C. elegans*, to mammals (Longo and Mattson, 2014). *E. coli* switched from a nutrient-rich broth to a calorie-free medium survived four times longer than controls (Gonidakis et al., 2010) and yeast *S. cerevisiae* moved from standard growth medium to water also underwent a consistent 2-fold chronological lifespan extension, reversible with the addition of various nutrients (Longo et al., 1997, Longo et al., 2012). Lifespan extension and increased stress resistance is also observed after IF in the nematode worm *C. elegans* and in *Drosophila* flies (Lee et al., 2006, Piper and Partridge, 2007), in addition to the many fasting studies performed in mice and rats. The establishment and maintenance of fasting mechanisms within single cell organisms, conserved and adapted throughout evolution, underscores the impact and diffuse reach IF exerts on longevity and survival. Intermittent fasting instigates several metabolic changes within the mammalian system including decreased glucose, insulin and leptin concentrations; a change in energy synthesis to ketogenesis to boost energy efficiency; increased insulin sensitivity; and increased fatty acid concentrations (Mattson, 2012, Hatchwell et al., 2020). Potent changes in metabolic and cellular pathways promotes stress resistance, lipolysis and autophagy (Longo and Mattson, 2014). Further to this, IF enhances parasympathetic activity in autonomic neurons targeting the gut, heart and arteries via acetylcholine signalling to improve gut motility and decrease heart rate and blood pressure (Longo and Mattson, 2014). Cycles of fast- feeding behaviour have also been implicated in gut microbiome changes, the vast, diverse microbial community which resides in the intestinal tract. The gut microbiome impacts metabolic health by regulating inflammatory responses, fermenting fibre and synthesising essential amino acids (Kohl et al., 2014). Cyclical changes in feeding behaviour can instigate similar changes in gut microbiota, contributing to the diversity of gut microflora and beneficial metabolic consequences (Patterson and Sears, 2017). For example, IF was shown to confer protection in a multiple sclerosis animal model via altering the composition and

metabolic cascades of gut microbiota (Cignarella et al., 2018). In parallel with metabolic changes, IF impacts the nervous system, modifying brain neurochemistry and neural network activity to optimise brain function and peripheral energy metabolism (Longo and Mattson, 2014). Studies have shown IF improves performance of sensory and motor behavioural tests (Singh et al., 2012) and learning and memory outcomes via enhancing synaptic plasticity in the mouse hippocampus (Dasgupta et al., 2018). Fasting also enriches neuronal network activity in brain regions involved in cognition, boosting synthesis of BDNF and signalling cascades controlling upregulation of plasticity and neurogenesis, and suppression of oxidative stress (Rothman et al., 2012, Li et al., 2013, Baik et al., 2020). However, the extent and mechanisms by which IF mediates neuronal function and plasticity is still not fully understood.

Collectively, mechanisms of IF converge to create a robust neuroprotective and stress- resistant physiological response to ensure optimal adaptation to external environmental stimuli and survival of the organism. This is clearly reflected in the health benefits IF exerts in a diverse series of injury and disease cases (Figure 15). For example, IF attenuates the inflammatory reaction to ischemic stroke (Fann et al., 2014) and decreases cell death after injury (Fann et al., 2017). Multiple neuroprotective proteins are upregulated in the brain following IF, including BDNF, stress response proteins and antioxidant enzymes (Lee et al., 2002), providing a protective microenvironment in neurons. Intermittent fasting has also been shown to combat cancer, reducing lymphoma incidence and delaying spontaneous tumorigenesis (Descamps, 2005). Multiple cycles of IF were shown to be as effective as chemo therapeutic agents in delaying tumour progression in several cancer varieties in mice (Lee et al., 2012). The efficacy and format of IF- treatment cycles for cancer are variable, reviewed in more detail in Longo *et al.* (2014).

Intermittent fasting has a strong impact on metabolic processes and related pathologies such as diabetes, obesity and cardiovascular disease. Common aspects of metabolic diseases include abnormal adiposity, insulin resistance, elevated triglycerides and hypertension (Mattson, 2012, Mattson et al., 2017). Intermittent fasting combats all of these elements by decreasing abdominal fat, inflammation and blood pressure and increasing insulin sensitivity, to improve the functional capacity of nervous and cardiovascular systems within the body (Wan et al., 2003a, Castello et al., 2010, Harvie et al., 2011, Gotthardt et al., 2016, Joslin et al., 2017). Obesity cohorts which have undergone an IF regimen show decreased cholesterol, blood glucose,

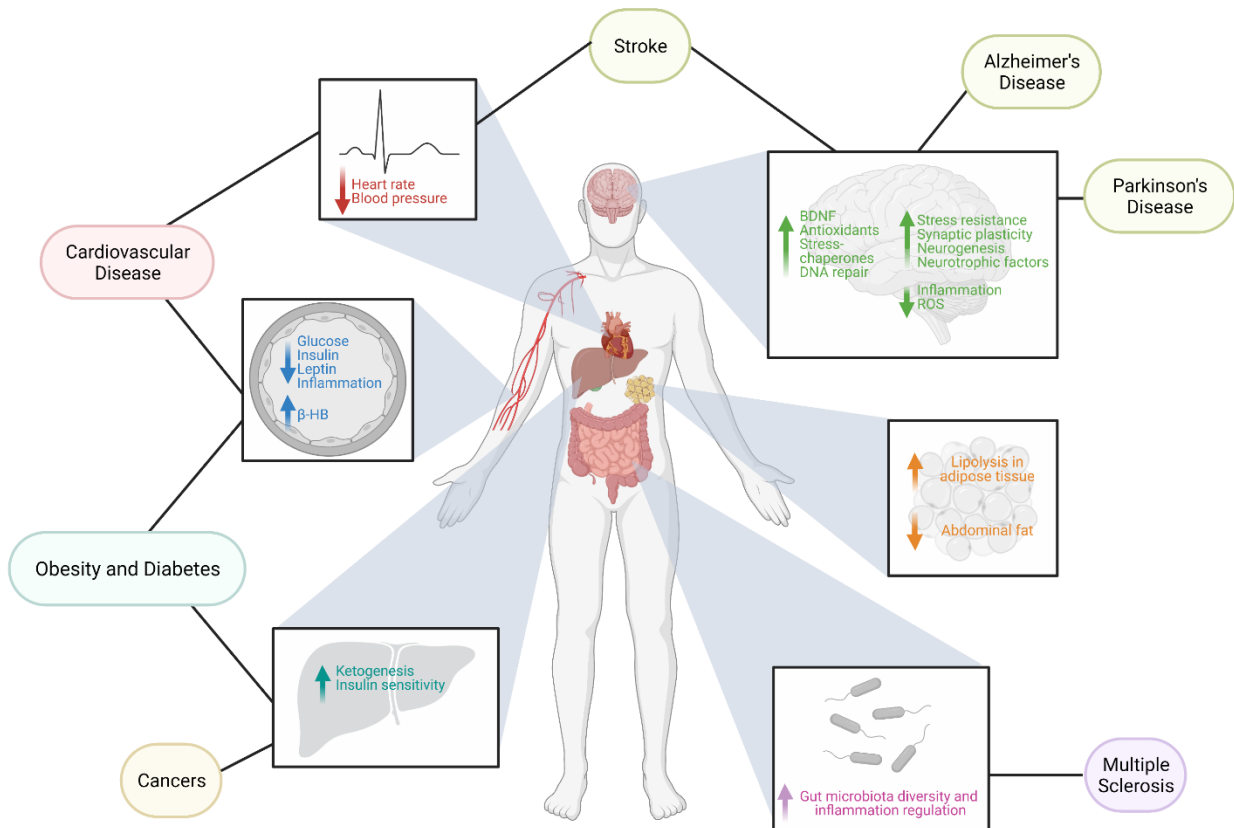


Figure 15: Physiological Effects of Intermittent Fasting in Health and Disease

Intermittent fasting (IF) has diverse physiological impacts on numerous organs and tissues in the body. Fasting initiates several metabolic changes within the adult mammalian system including decreased glucose, insulin and leptin concentrations within circulating blood; upregulation of insulin sensitivity and ketogenesis; and increased lipolysis within adipose fat tissue. These all exert beneficial changes in numerous diseases such as obesity, diabetes, cancer and cardiovascular disease. Reduced blood pressure and heart rate during IF also protects against stroke. Intermittent fasting diversifies the gut microbiome population, impacting metabolic health and regulation of inflammatory responses. A broad range of effects have also been characterised within the nervous system, enhanced antioxidants, stress-response components, synaptic plasticity and neurogenesis, whilst downregulating inflammation and reactive oxygen species following IF. Beneficial effects of IF in neurodegenerative diseases such as Alzheimer's, Parkinson's and memory have been described. Figure created in BioRender.

systemic inflammation and oxidative stress levels (Varady et al., 2009, Klempel et al., 2013). Similar outcomes are reflected in healthy subjects too, with IF reducing the risk of cardiovascular disease in non-obese humans (Tinsley and La Bounty, 2015, Longo and Panda, 2016). Additional links have been established between IF and longevity cascades, prompting the hypothesis that IF may counteract disease processes associated with ageing, a major risk factor for neurodegenerative diseases. In neurodegenerative disease pathologies, multiple brain regions exhibit progressive degeneration and neuronal death, impairment of adaptive cellular stress responses, accumulation of extracellular A β and intracellular Tau aggregates, mitochondrial dysfunction and cellular energy deficits (Kapogiannis and Mattson, 2011, Mattson, 2012). Alternating between fast and fed states is known to boost antioxidant defences, increase neurotrophic factors, such as BDNF and FGF2, and decrease pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) (Arumugam et al., 2010). Multiple neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's disease, exhibit fewer clinical symptoms and show decreased neuronal dysfunction as a result of IF effects (Mattson et al., 2017). Patients with mild cognitive impairment maintained on a low glycemic diet for a month improved delayed visual memory, cerebrospinal fluid biomarkers of A β metabolism and brain bioenergetics (Bayer-Carter et al., 2011). The shift to ketogenesis instigated by IF may also play a role in cytoprotective effects, as it is reported that rats fed a ketogenic diet exhibit increased resistance to seizures (Bough et al., 1999) and β -Hydroxybutyrate protects neurons in rodent models of Alzheimer's and Parkinson's disease (Kashiwaya et al., 2000). Intermittent fasting also promotes restoration of damaged nerve cell circuits by stimulating synapse formation and production of new neurons from neural stem cells (Lee et al., 2002).

The impacts of an IF regimen are not constrained to the protein level but influence transcriptomics, genomics and gene expression. Fasting increases neuronal activity, enhances stress response cascades and upregulates neurotrophic factors. Several transcriptional regulators responsive to these neurotrophic factors and reactive oxygen species (ROS) are consequentially activated, such as CREB, NF- κ B and Hif-1 (Mattson, 2012). These in turn modulate expression of neuroplasticity and cell survival genes, including antioxidant enzymes, anti-apoptotic Bcl-2 family members, glutamate receptor subunits, calcium binding proteins and cell adhesion proteins (Mattson, 2012). Some of the first indicators that IF influences genomics were demonstrated in large

famine cohorts, for example, survivors of the Dutch Hunger Winter of 1944. Pregnant women who suffered malnutrition during only the first three months of pregnancy gave birth to babies of a standard healthy weight, but once these babies had reached adulthood they were at higher risk of obesity and other disorders (Lumey et al., 1995a, Lumey et al., 1995b). When women from this set of babies became mothers themselves, their first- born child was heavier than control groups, despite the baby never being exposed to a period of malnutrition (Lumey et al., 1995a, Lumey, 1998). Conversely, pregnant women malnourished in their third trimester gave birth to smaller babies (Stein et al., 1995), emphasising how the timing of food restriction during development can instigate drastically different outcomes. Whether these effects were due to epigenetics (altered DNA methylation or histone modifications) or an altered intra-uterine environment, is difficult to determine. Cases from the male line however strengthen the support for the epigenetic hypothesis. Famine records from Sweden in the early 20th century describe periods of severe food shortages interspersed with periods of plenty. If food was scarce during a male's pre-pubescent growth period, his son was at decreased risk of dying from cardiovascular disease, but if the reverse was true, access to a surfeit of food led to increased risk of dying due to diabetic illness (Kaati et al., 2002). Collectively, data supports the hypothesis that poor nutrition during pregnancy affects the offspring and that there are transgenerational consequences of food availability in the grandparental generation, mediated via epigenetics (Heijmans et al., 2008). These conclusions are not unique to extrapolation from human cohorts but are reflected in controlled animal studies too. Male rats fed a high fat diet were overweight, had a high percentage of fat to muscle and exhibited type- 2 diabetes symptoms (Ng et al., 2010). When mated with standard diet females, the offspring were a normal weight but showed diabetes- type abnormalities and mis-regulation of metabolic genes (Ng et al., 2010). Similar results were mirrored in another study, where male mice fed a diet abnormally low in protein and high in sugar were bred with females on a standard diet (Carone et al., 2010). Transcriptomic data revealed abnormal metabolic gene regulation and changes in epigenetic modifications in the livers of three- week- old pups born of the males on the modified diet (Carone et al., 2010). Together, data from famine and obesity studies indicate that a parent's diet can directly influence epigenetic modifications and re-program metabolic gene expression, impacting the health of the offspring.

Intermittent fasting was a fundamental challenge during the evolution of animals; the brain and multiple organ systems forced to respond adaptively to optimise the individual's resistance to injury and disease (Mattson et al., 2017). These effects are evident in almost every cell type within the body, extending through to epigenetic signatures and transgenerational inheritance in offspring. Whilst predominantly neuroprotective, IF is also capable of stimulating active regeneration and growth.

2.5.4 Intermittent Fasting and The Nervous System

Developments in the spinal cord injury field suggest injury- independent signals within the adult mammalian system trigger regenerative signalling pathways post- injury, such as signalling cascades regulated by environmental changes. For example, an enriched environment (EE), exercise and intermittent fasting (IF) have all been shown to enhance synaptic plasticity and reduce neuronal apoptosis, propagating neurogenesis and increasing the likelihood of survival following injury (Fontán-Lozano et al., 2007, Li et al., 2013, Bayat et al., 2015). Environmental enrichment exerted therapeutic effects in a model of traumatic brain injury (TBI), attenuating cognitive deficits and preserving tissue integrity after injury (Passineau et al., 2001). Placing mice in EE, with increased exercise and play, has a pre-conditioning protective effect on proprioceptive neurons, improving functional recovery after sciatic nerve injury (Schmidhammer et al., 2007). Positive enhancements of the central nervous system have also been demonstrated, where application of EE improved sensory and motor recovery following spinal cord injury in rats (Berrocal et al., 2007, Hutson et al., 2019) and enhanced spinal cord plasticity in a rat model of cerebral palsy (Marques et al., 2014). Many of the molecular pathways activated during exercise and EE overlap significantly with IF cascades (Mattson et al., 2018). Both EE and IF were shown to alleviate pain in a model of peripheral nerve neuropathic pain (Madorsky et al., 2009, Vachon et al., 2013). Charcot-Marie-Tooth type 1A (CMT1A) neuropathies are progressive demyelinating disorders of the PNS, characterised by misexpression of peripheral myelin protein 22 (PMP22) (Madorsky et al., 2009). Dietary IF treatment alleviated CMTA1 phenotypes by decreasing PMP22 aggregates and enhancing cytosolic chaperones, resulting in improved locomotor performance (Madorsky et al., 2009). Additional findings on nervous system health and disease resistance after IF are mirrored in Jeong et al. (2011). Ad libitum feeding was compared to a three week pre- and post- injury IF regimen and found IF improved functional recovery after a moderate spinal cord

contusion injury (Jeong et al., 2011). Intermittent fasting improved motor and sensory function measured via behavioural tests such as the Basso–Beattie–Bresnahan (BBB) locomotor open field, the CatWalk and GridWalk assessments (Jeong et al., 2011). This complements previous work demonstrating IF improved gait- pattern, forelimb function in the ladder and cylinder test, decreased lesion size and enhanced corticospinal axonal sprouting (Plunet et al., 2008, Plunet et al., 2010, Sayadi et al., 2021).

2.6 Experimental Rationale

Intermittent fasting mediates several neuroprotective signalling cascades enhancing synaptic plasticity and stress- resistance, both pre- and post-injury. Recent efforts to characterise IF's mechanism of action in the context of axonal regeneration has shown IF promotes axonal regeneration post- sciatic nerve crush injury, facilitating re-innervation of sciatic nerve fibres into large diameter sensory neurons of the leg muscle (Serger et al., 2022). Metabolomics performed after IF found the gut metabolite indole-3-propionic acid was upregulated and mediated an extrinsic regenerative pathway via neutrophil chemotaxis into the DRG microenvironment (Serger et al., 2022). This demonstrates IF primes neurons for axonal regeneration via local and systemic modifications in metabolic and cellular signalling pathways. Despite IF's regenerative signalling capacity following axonal injury, the endogenous intrinsic mechanism through which it mediates these effects remains unknown. Greater understanding of intracellular metabolic signalling cascades initiated after IF and nerve injury may offer solutions. I hypothesise IF will increase intrinsic neuronal signalling and facilitate regenerative epigenetic modifications that formulate a euchromatic transcriptional state. This thesis will establish the answer to this hypothesis with the following aims:

1. Investigate IF- dependent regenerative signalling cascades via transcriptional molecular studies.
2. Explore novel applications of intrinsic IF signalling in peripheral and central neuronal injury.

3. METHODS

3.1 In Vivo Methods

3.1.1 Animal Preparation

Animal work was conducted in accordance with UK Home Office licence legislation, the Animals Scientific Procedures Act of 1986 and approved by Imperial College Ethics Committee. Animal work was conducted with male and female C57B16/J (Harlan) mice (6-8 weeks) between 20-30g. Prior to surgery, mice were anaesthetised (1% Oxygen and 4% Isoflurane (2% for maintenance)) until the pedal reflex was no longer present and analgesics were administered via subcutaneous injection (carprophen 5mg/kg and buprenorphine 0.1mg/kg). Animal body temperature was maintained at 37°C throughout surgical procedures with the use of a heat mat.

3.1.2 Intermittent Fasting Regimen

Male C57B16/J mice (6-8 weeks) were maintained on intermittent fasting regimens for 10 days. Mice were housed on a 12-hour light/dark cycle with water available *ad libitum*, randomly assigned to intermittent fasting (IF) or *ad libitum* (AL) experimental groups. The IF group did not have access to food (fasting) during the first 24 hours and were subsequently fasted every second day (e.g. fasting during 0–24 h, 48–72 h, 96–120 h) with *ad libitum* access to food on the alternating days (24–48 h, 72–96 h, 120–144 h). Food was weighed and added to the food hopper of the animal's cage at 9.30am (aside from the fast days for the IF groups) and remaining food was weighed 24 hours later the following morning. Food provided was RM1 standard chow for rodents (RM1 (E) Special Diet Services, 801002: crude fat, crude protein, crude fibre, crude ash, lysine, methionine, calcium, phosphorus, sodium, magnesium, copper). Tissue and biological material were either collected on day 10 of the regimen or were collected every day during the 10 day IF regimen. Sciatic DRGs were dissected and either processed for RNA extraction or protein extraction; dissociated and cultured for 12 hours; or fixed in PFA and processed for immunofluorescence. Blood was collected from AL and IF mice and the serum extracted.

3.1.3 Sciatic Nerve Crush Surgery

Sciatic nerve surgeries (Figure 16) were conducted with male C57B16/J (Harlan) mice (6-8 weeks). Mice were anesthetised, analgesics administered and lower limbs prepared for surgery. The legs and surface area over the hip were shaved and Iodine solution applied to sterilise the surgery area. The skin below the hip bone was opened with a small scissor incision. Blunt dissection of biceps femoris and gluteus maximus exposed the sciatic nerve between the femur bone and tendon. The sciatic nerve was lifted using a surgical nerve hook. The proximal side of exposed nerve where the three fascicles are sequentially aligned was placed on the bottom jaw of a pair of super-fine hemostatic forceps (Fine Science Tools, 13020-12), 1.5 mm from the forceps tip. The nerve was crushed orthogonally for 30 seconds (45 seconds for a functional recovery experiment) on the tightest setting of the forceps with no nerve stretch. The crush was performed approximately 20mm distally from the L4-L6 DRGs. The skin was closed with suture clips. Animals recovered in 30°C incubator and were euthanized 24 hours or 72 hours after surgery.

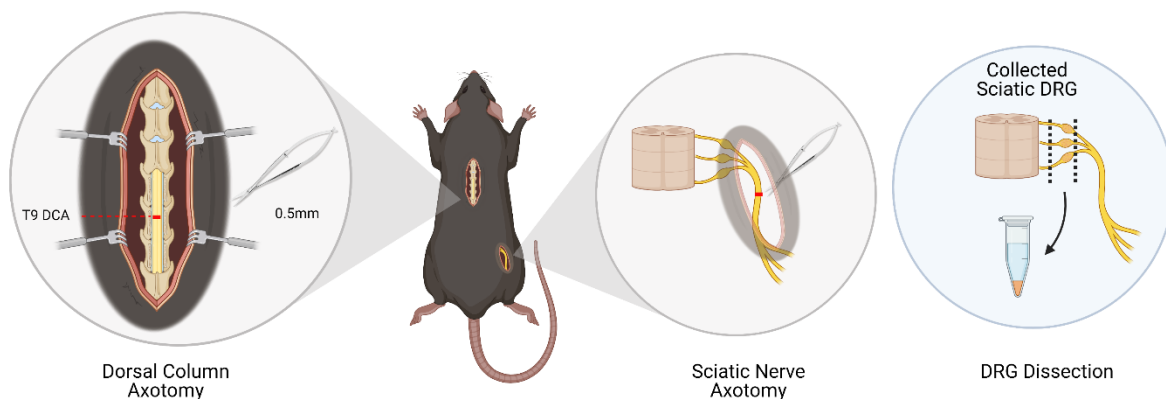


Figure 16: Dorsal Colum Axotomy and Sciatic Nerve Crush

Schematic diagram of peripheral and central nerve injuries.

3.1.4 Dorsal Column Axotomy

Mice were anesthetised as described in 3.1.1 'animal preparation'. The fur over the thoracic spinal cord was removed and swabbed with iodine. A mid-line incision was performed on the skin over the vertebrae and the superficial fat moved aside to enable muscle tissue dissection to expose laminae T9-11. A T10 laminectomy was performed and the dura matter carefully punctured above T10 (Figure 16). A dorsal column axotomy was performed at T10 to a depth of 0.5 mm with micro-scissors. Muscle tissue and skin were sutured individually before mice recovered in a 37°C incubation box and were monitored for adverse effects post- surgery. Post-surgery carprophen (5mg/kg) was administered via the drinking water for three days and mice underwent daily checks for general health, motility and autophagy of the toes for the remainder of the experiment.

3.1.5 Sciatic Nerve Injection and Axonal Tracing

Animals for sciatic nerve injection were prepared in the same manner as for sciatic nerve crush surgery. The skin and muscle at the distal thigh was opened with a small scissor incision to expose the distal extreme of the sciatic nerve. The sciatic nerve was lifted away from the muscle using curved forceps and a drop of saline solution added to prevent drying. Approximately 3µL of injectable is loaded into a Hamilton syringe and needle (NDL small RN ga34/15mm/pst45°) (Hamilton) and injected bilaterally. The needle tip is inserted into the distal end of the sciatic nerve and the contents injected over a 5-minute period. The skin is closed with suture clips and animals recovered in a 30°C incubator. All adeno-associated viruses (AAVs) were injected and a 4-week time period lapsed to ensure robust virus expression within sciatic DRG neurons.

To study leptin overexpression in sensory neurons of the DRG, either AAV5-GFP or AAV5- Leptin- GFP (Duke University, Viral Vector Core) was injected (Figure 17), prior to either a SNC injury or a DCA injury. For deletion experiments examining the loss of leptin signalling, either AAV1- GFP or AAV1- Cre- GFP (tebu-bio) were injected into Leptin-receptor FLOX mice to delete the leptin receptor. To assess spinal cord regeneration, six weeks post- spinal cord injury the sensory axons in the dorsal columns were traced with 15% Dextran Alexa Fluor 555 (D34679, ThermoFisher Scientific). Dextran was injected following distal sciatic nerve ligation and crush using 5 mm surgery forceps (91150-20 Inox-Electronic). Five days post- dextran tracing,

mice were terminally anaesthetised and transcardially perfused with 10 mL of PBS (pH 7.4, Sigma), followed by 20 mL of 4% paraformaldehyde in PBS (Sigma).

<i>Name</i>	<i>Serotype</i>	<i>Titre</i>	<i>Company</i>
AAV- GFP	5	1.54X10 ¹² VG/mL	Duke University, Viral Vector Core
AAV- Leptin- GFP	5	1.23X10 ¹² VG/mL	Duke University, Viral Vector Core
AAV- GFP	1	1.17X10 ¹³ VG/mL	Tebu-bio
AAV- Cre- GFP	1	1.12X10 ¹³ VG/mL	Tebu-bio

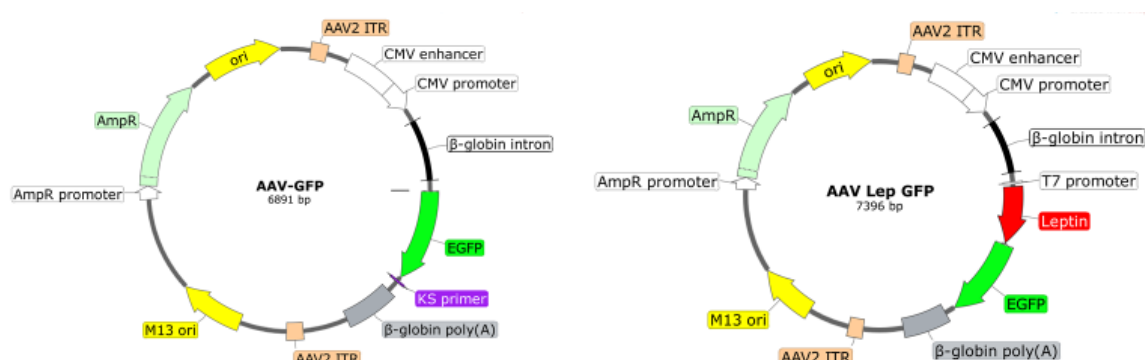


Figure 17: Plasmid Gene Maps

Gene maps of control and overexpression plasmids used directly for electroporation and the creation of AAV-GFP and AAV-Leptin-GFP viral vectors. AAV-GFP contains standard AAV components and an Enhanced Green Fluorescent Protein (EGFP; green) gene. AAV-Lep-GFP contains standard AAV components, EGFP and Leptin (red) genes. Images visualised using Snapgene.

3.1.6 Intramuscular Injection

Following sciatic nerve crush injury and 21 days of regeneration, 5µL of 1% Cholera toxin B (CTB; List Biological Laboratories) was injected into the tibialis anterior and medial gastrocnemius muscles using a Hamilton syringe (NDL small RN ga34/15mm/pst45°). Five days later the animals were sacrificed and the DRGs and epidermis of the skin dissected.

3.1.7 Systemic Leptin Injection Treatment Regimen

Male and female LepR FLOX mice were distributed evenly between four groups to ensure equal balance of gender and age. Two groups received AAV1-GFP sciatic

nerve injections and the remaining groups AAV1-Cre-GFP. To assess the impact of leptin delivery post-injury, all mice underwent SNC injury prior to intraperitoneal injections of either saline vehicle or 20µg/day recombinant mouse leptin (L3772, Sigma Aldrich). Mice receiving leptin were dosed once per day for four days and their weight monitored.

3.1.8 Tissue Dissection

Sciatic nerve and DRG: Mice are culled from live via cervical neck dislocation. The head is cut off (Student Scissors Straight, Fine Science Tools) and the skin removed. The cadaver is placed on its back ventral- side facing upward, pressure applied to the ribs to push the internal organs down and open scissors placed horizontally over the legs to cut beneath the stomach to remove internal organs. The cadaver is pinned to a Styrofoam board ventral- side facing down. Muscle is re-opened at the surgery site and cleared away from the sciatic nerve along with any connective tissue. Nerve is severed at the ankle and the hip and placed in 4% PFA for 4 hours on ice. Mouse is rotated ventral- side facing upwards. Using a dissection microscope (Stemi 2000-C Stereo Microscope, Carl Zeiss) the muscle over the vertebrae is pulled away using forceps (Student Dumont #5 Forceps, Fine Science Tools). The vertebrae is lifted and cut away (Student Vannas Spring Scissors, Fine Science Tools) to reveal the spinal cord and dorsal root ganglia (DRG). Lifting the peripheral branch of the DRG, careful not to touch the DRG itself, the central axonal branch is cut with microscissors (Vannas Spring Scissors Straight), followed by the peripheral branch just below the forceps. The DRG are placed directly into 4% PFA for 4 hours on ice. Nerves and DRG are transferred to 30% sucrose for 5 days before they are embedded in Tissue-Tek OCT. Tissue is sectioned on the cryostat at 10 µm thickness.

Spinal Cord: Mice are overdosed with an i.p injection containing Ketamine (1:10) and Xylazine (1:20) until the pedal reflex is absent. Once unconscious, the thoracic cavity is opened to expose the heart and a perfusion needle inserted directly into the lower ventricle. The mouse is first perfused with 10 mL of 1X PBS (pH 7.4, Sigma) followed by 20 mL of 4% PFA (Sigma). Once perfused with fixative the organs are removed and the vertebrae lifted to expose the spinal cord as described above. The dorsal roots are cut and the spinal cord is carefully dissected and transferred to 4% PFA at 4°C

overnight. Cords are transferred to 30% sucrose for 5 days before they are embedded in Tissue-Tek OCT. Tissue is sectioned on the cryostat at 20 µm thickness.

3.2 Ex Vivo and In Vitro Methods

3.2.1 Blood Serum Preparation

Blood was collected via cardiac puncture and transferred directly into a vacutainer (Becton Dickinson). Blood was left to clot undisturbed at room temperature for 30 minutes before Eppendorf's were centrifuged at 2000g for 10 minutes at 4°C. Erythrocytes were pulled to the bottom and the clear serum was immediately transferred into a new Eppendorf and stored at -20°C until use.

3.2.2 Leptin ELISA

Leptin protein concentrations of extracted serum from IF and AL mice were analysed with a leptin ELISA kit (ab100718, Abcam). Briefly, leptin standards and serum samples are pipetted into a pre-coated 96 well plate and left to incubate overnight. Leptin present in the samples bind to immobilised leptin antibodies coated on the bottom of the plate. Wells are washed and biotinylated anti- mouse leptin antibody is added. Unbound biotinylated antibody is washed away and HRP- conjugated streptavidin is added. Wells are washed once again and a TMB substrate solution added to create a colour change. The colour develops in proportion to the amount of leptin bound. A stop solution is added and the colour intensity (optical density) is recorded at 450 nm. A standard curve and sample concentrations are calculated.

3.2.3 Protein Extraction

Lysis buffer is prepared on ice with RIPA buffer, phosphatase inhibitors (PhosSTOP, Roche) and protease inhibitors (cOmplete Protease Inhibitor cocktail, Roche). A 50 µL volume of lysis buffer is added to each cell/tissue sample on ice and homogenised with a protease-free pestle. Samples are rotated at 4°C for 30 minutes before centrifuging at 14,000 rpm at 4°C for 20 minutes. The supernatant is removed and immediately transferred to a new Eppendorf. Samples are either frozen at -20 or undergo BCA assay quantification. Protein concentration was calculated using the Pierce bicinchoninic (BCA) protein assay (Thermo Scientific). Briefly, 1 µL of protein lysate is

combined with 140 μL of reagent mix containing bicinchoninic acid. Proteins reduce Cu^{2+} to Cu^{1+} in an alkaline solution, producing a purple colour change after a 30 minute incubation at 37°C . The higher the protein content, the darker the colour change. This is quantified using a colourimeter measuring optical density and compared against a standard curve of known protein values.

3.2.4 Western Blot

A 15% separating gel (H_2O , 30% Acrylamide bisulphate, 1.5M tris-HCL pH8.8, 10% SDS, 10% APS, TEMED) is created with a stacking gel (H_2O , 30% Acrylamide bisulphate, 1.0M tris-HCL pH6.8, 10% SDS, 10% APS, TEMED) set on top at a 1.5mm thickness. The chamber is filled with running buffer (900mL dH_2O , 100mL SGS 10X stock solution) and the gel submerged. Protein samples are thawed on ice and combined with loading dye (7 μL), water and reducing agent buffer (2.8 μL) at a 28 μL volume. Samples are heated to 70°C for 10 minutes and placed on ice for a further 5 minutes. Samples are loaded into each well of the gel along with one well for a spectra multicolour broad-range protein ladder (10 μL of ladder + 18 μL of loading dye). The lid of the chamber is secured and the gel run at 80V for 40 minutes until samples have passed through to the separating gel, following which the gel is run at 100V for 1 hour. Once the samples have run, the gel is removed from the chamber and transferred onto an iBlot NC mini stack according to manufacturer's instructions. The P3 transfer protocol (20V, 7 minutes) is run to transfer protein samples from the gel to the membrane using the iBlot 2 Dry Blotting System. The membrane is blocked for 1 hour in a tray of 5% milk (5g milk powder in 100mL of 1X TBST) with gentle rocking. Primary antibodies are diluted in 5% milk and added to 50mL falcon tubes. The membrane is cut using a scalpel and added to the falcons containing primary antibody. Membranes are incubated in antibody overnight at 4°C whilst rotating. The membranes are washed in 1X TBST (3 times, 5 minutes) and incubated in secondary antibodies diluted in 5% milk for 1hr at room temperature. The membranes are washed again in 1X TBST (3 times, 5 minutes), stained with an ECL plus kit and imaged using a Syngene GeneGnome Western Blot Imager and GeneSys software.

3.2.5 Immunofluorescence

Immunohistochemistry: Sciatic nerves and DRGs were cut to 10µm sections on a CryoJane (Leica CM1850) cryostat before samples were mounted and dried. Tissue was rehydrated in 1X phosphate buffer solution (PBS) (3X 5 minutes) before blocking buffer (PBS + 8% BSA + 0.3% Triton X100) was added for 1 hour at room temperature (RT) to prevent non-specific binding. Blocking solution was removed and tissue incubated in primary antibody solution (PBS + 2% BSA + 0.1% Triton X100 + primary antibodies) at 4°C overnight. Repeated 1X PBS wash prior to secondary antibody incubation for 1 hour at room temperature (PBS + 2% BSA + 0.1% Triton X100 + secondary antibodies in 1:1000 dilution). Tissue was stained with DAPI (Hoechst 1:100000) for 10 minutes. Tissue was washed in PBS and coverslips mounted with Mowiol and left to dry overnight. Slides were stored at 4°C until imaged.

Immunocytochemistry: Plated cells were fixed in 4% PFA for 15 minutes and washed three times with 1X PBS. Cells were permeabilised and blocked for 1 hour at room temperature with blocking solution (PBS + 2% BSA + 0.3% Triton X100). Blocking solution was removed and cells incubated in primary antibody solution (PBS + 2% BSA + 0.1% Triton X100 + primary antibodies) at room temperature overnight. Cells were washed with PBS and incubated for 1 hour at room temperature (PBS + 2% BSA + 0.1% Triton X100 + secondary antibodies in 1:1000 dilution). Cells were stained with DAPI (Hoechst 1:100000) for 10 minutes and stored covered at 4°C until imaged.

<i>Name</i>	<i>Species</i>	<i>Dilution</i>	<i>Company</i>
<i>Leptin</i>	Rabbit	1:300	Abcam, UK
<i>Leptin Receptor</i>	Mouse	1:100	R&D Systems, USA
<i>STMN2 (SCG10)</i>	Rabbit	1:1000	Novus Biologicals, USA
<i>βIII-Tubulin</i>	Chicken	1:1000	Novus Biologicals, USA
<i>pSTAT3</i>	Rabbit	1:100	Cell Signalling Technology, USA
<i>NeuN</i>	Chicken	1:1000	Sigma-Aldrich, Germany
<i>Alexa Flour 488</i>	Goat anti-rabbit	1:1000	Thermo Fisher, US
<i>Alexa Flour 488</i>	Goat anti-chicken	1:1000	Thermo Fisher, US
<i>Alexa Flour 568</i>	Goat Anti-rabbit	1:1000	Thermo Fisher, US
<i>Alexa Flour 568</i>	Mouse	1:1000	Thermo Fisher, US

<i>Alexa Flour 594</i>	Anti-mouse	1:1000	Thermo Fisher, US
<i>Alexa Flour 594</i>	Anti-rabbit	1:1000	Thermo Fisher, US

3.2.6 RNA Extraction

RNA was extracted using the RNeasy Mini Kit (Qiagen) according to protocol guidelines. Briefly, tissue was stored in RNAlater until RNA extraction, before homogenisation in RLT lysis buffer, β -mecaptoethanol and 70% ethanol. Solution was loaded onto a spin column and centrifuged at 8000g for 1 minute. The column was washed with buffer RW1 followed by buffer RPE and eluted in RNase- free water.

3.2.7 cDNA Synthesis

RNA underwent DNase treatment to remove contaminants. A reaction mixture of 1 μ g of RNA, water, 2 μ L DNase (50 μ g/ μ L) and 1 μ L 10X buffer is treated for 30 minutes at 37°C. Enzyme reaction is terminated via addition of 1.1 μ L of EDTA (50mM) per sample and incubated at 65°C for 10 minutes. cDNA is created in accordance with Invitrogen's SuperScript First- strand synthesis system for RT-PCR. Briefly, 10 μ L of RNA is combined with 1 μ L of dNTP mix (10mM), 1 μ L of random hexamers (50ng/ μ L) and 1 μ L of OligodT mix (0.5 μ g/ μ L) and incubated at 65°C for 5 minutes. On ice, 9 μ L of buffer mix (2 μ L 10X Reverse Transcriptase Buffer, 4 μ L 25mM MgCl₂, 2 μ L 0.1M DTT and 1 μ L 40U/ μ L RNaseOUT) is added to each sample and incubated at RT for 2 minutes. Per sample, 1 μ L of SuperScript II Reverse Transcriptase is added, incubated at RT for 10 minutes and 42°C for 50 minutes. Reaction is terminated at 70°C for 15 minutes and chilled on ice before addition of 1 μ L of RNase H incubated at 37°C for 20 minutes. DNA stored at -20°C and used for qPCR.

3.2.8 Quantitative Polymerase Chain Reaction (qPCR)

Primers (mus musculus, mm10) were designed in NCBI Primer Blast for Leptin and the leptin receptor.

Leptin Forward Primer: CAGCTGCAAGGTGCAAGAAG

Leptin Reverse Primer: GATACCGACTGCGTGTGTGA

Leptin Receptor Forward Primer: TATGTGGTCCAGGTTCTGCTG

Leptin Receptor Reverse Primer: GCCATGAGAAGGTAACGTCC

Real-time polymerase chain reaction was performed as previously reported by Thermo Fisher Scientific. In summary, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, UK) was added to a master mix of purified water, forward and reverse primers (Eurofins Genomics, UK) before addition of DNA sample. A qPCR-7900HT real-time PCR system machine (Applied Biosystems) and the SDS 2.4 programme was used to run and analyse samples. Cycle conditions were as follows: 95° for 5 minutes hot start, 45 cycles of 95° for 20 seconds denaturation, 56° for 20 seconds annealing, 72° for 30 seconds elongation and a final dissociation stage. Ct values were used for quantitative analysis using the $2^{-\Delta\Delta Ct}$ formula and appropriate statistical analysis performed.

3.2.9 RNA Sequencing

Mice which had undergone either 10 days IF or AL feeding were sacrificed and L4-L6 DRG dissected. RNA was extracted as described in 3.2.6. Residual DNA contamination was removed by treating spin columns with 40 units of RNase-free DNase I (Qiagen) for 15 min at 23°C prior to RNA elution. RNA concentration and purity were verified using the Agilent 2100 Bioanalyzer (Agilent) and RNA with a RIN factor above 8 was used for library preparation. Imperial College BRC Genomic Facility generated cDNA libraries for each sample via the TruSeq stranded mRNA Sample Preparation Kit A (Illumina, San Diego CA) and sequenced using Illumina HiSeq 4000 (PE 2x75 bp) sequencing.

Mice received either AAV-GFP or AAV-Leptin-GFP sciatic nerve injections and a T9 DCA 4 weeks later. Twenty-four hours after DCA injury, L4-6 DRG were dissected and underwent a neuronal gradient enrichment protocol, described in 3.2.12. RNA was extracted as described in 3.2.6 and RNA sequencing preparation executed as described above.

3.2.10 RNA Sequencing Analysis

Sanger Illumina 1.9 paired end sequencing was performed using 75 base pair reads. Quality control checks of raw Fastq files were conducted to ensure sufficient overlap for copied DNA fragments. Bad quality reads were removed (Phred score below 20) and TrimGalore software used to remove the adapter sequences from the gene reads.

The bioinformatic programmes Salmon and Deseq2 were used to map RNA reads to the reference library to determine which genes are differentially expressed within the dataset. Various statistical stringency points can be applied at this level depending on what is appropriate to the dataset (IF v AL RNAseq: $P < 0.05$, AAV-GFP DCA v AAV-Leptin-GFP DCA: $FDR < 0.05$). Differentially expressed genes were classified as any value greater than (upregulated) or less than (downregulated) a Log fold change of zero. Downstream gene enrichment analysis was carried out using DAVID (Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>)), which used either Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to classify gene functions and biological pathways. Similarly, multiple statistical stringencies determined the significance of the genes for each GO/KEGG term. For both RNAseq datasets genes were grouped with a p value less than 0.01 and were ranked via categories with the largest gene count (number of genes) to the smallest gene count.

3.2.11 DRG Cell Culture

Prior to dissection, 24-well or 96-well plates were coated with PDL (Poly-D-Lysine) and laminin for 1-2 hours. DRGs were dissected (either total or sciatic only), stored in 1x HBSS and digested in 500 μ L Digest Solution (2% Collagenase Type II, 1% Dispase, DMEM) at 37°C for 45 minutes in a water bath. Cells were centrifuged at 800rpm for 2 minutes and subsequently washed in 1mL of DRG media (1000 μ L: 100 μ L FBS (HIA), 20 μ L B27 (50X), 440 μ L DMEM, 440 μ L F12). The media was replaced and DRGs were dissociated 20 times with gentle pipetting. Cells were quantified with a haemocytometer and centrifuged at 1000 rpm for 5 minutes. DRG media was removed and cells resuspended in DRG Culture Media (10 μ L Pen/Strep, 20 μ L B27 (50X), 485 μ L DMEM, 485 μ L F12). 400 μ L of cell solution was plated at a confluency of 4000 cells/well in a 24 well plate. 40 μ L of cell solution was plated at a confluency of 1600 cells/well in a 96 well plate. This ensures sparse density to prevent cross-over of arborised neurites. Cells were cultured for 12 hours for standard outgrowth experiments, before being fixed in 4% PFA for 15 minutes, washed with PBS and stained for various antibodies.

Ex vivo leptin treatment (recombinant mouse leptin, L3772, Sigma Aldrich) of neuronal cultures also incorporated various leptin doses (50ng/mL, 100ng/mL, 1µg/mL) into the media of cultured neurons prior to the 12-hour incubation.

3.2.12 DRG Electroporation

Sciatic DRGs were dissected as described in 3.1.8 and placed into HBSS. The DRGs were dissociated with collagenase and dispase for 45 minutes as described above in 3.2.11. Cells from each biological replicate were resuspended and transferred into one falcon tube. Cells were washed three times with PBS and resuspended in buffer 'R'. Resuspended cells are combined with 5 µL of vector DNA (2µg/µL) and drawn up into the electroporation pipette. Neurons are either combined with a GFP control plasmid or a leptin-GFP plasmid in which the leptin gene is overexpressed under the CMV promoter (Figure 17). The electroporation pipette is slotted into the machine chamber containing E₂ buffer and a 1500V current run for 10ms in three pulses. Once the electroporation process is completed, cells are added directly into warm culture media and cultured for 48 hours in plates coated with poly-D-lysine. Cells are fixed with 4% PFA and stained for β III-tubulin to measure outgrowth in GFP⁺ cells.

3.2.12 DRG Neuronal Gradient

Prior to dissection, 24-well or 96-well plates were coated with PDL (Poly-D-Lysine) and laminin for 1-2 hours. DRGs were dissected (either total or sciatic only), stored in 1x HBSS on ice and digested in 500µL of Digest Solution (2.5mg/mL Collagenase Type II, 1mg/mL Dispase, DMEM/F12, Papain) at 37°C for 30 minutes in a water bath. Cells were centrifuged at 800rpm for 2 minutes and subsequently washed in 1mL of DRG media (Volume for 1 biological replicate: 500µL FBS (HIA), 100µL B27 (50X), 4400µL DMEM/F12, Hepes 1:50, Rnase inhibitor 20U/µL). The media was replaced at a lower 700µL volume and DRG dissociated 10 times with slow pipetting using a sterile glass Pasteur pipette. Dissociation was repeated using a P1000 pipette 15 times. A 50mL falcon tube with a 70µm cell strainer placed on top is prepared for each mouse. Dissociated DRG were left to settle for 1 minute and the cell suspension added to the filter. An additional 700µL of DRG media was added to remaining undissociated tissue and pipetting repeated until all tissue was filtered. For each mouse, a 15% BSA cushion (1mL 30% BSA to 1mL of DMEM) is created in a 15mL falcon tube. The filtered cell

suspension for each mouse is added on top of a BSA gradient on ice. Gradients are centrifuged at 80g for 8 minutes at 4°C. The gradient is removed and the neuronal pellet is either resuspended in RNAlater for RNA extraction or lysis buffer for protein extraction. RNAseq samples pooled 3 mice per replicate for sufficient RNA material.

3.2.14 Ex Vivo Virus Infection

The DRG are dissected fresh as described in 3.1.8 and plated in a 24 well plate at 4000 cell/well as described in 3.2.11. 1µL of virus (AAV-GFP or AAV-Leptin-GFP) is diluted in 2mL of culture media. Dissociated and washed cells are centrifuged into a pellet and resuspended in the 'virus media'. 300µL of cells are plated per well. After three days GFP expression is checked and cells are infected with fresh virus media. Cells are cultured for a total of 5 days to establish sufficient virus infection within neuronal cells. Cells are subsequently fixed in 4% PFA and washed in 1X PBS prior to immunostaining.

3.3. Analysis and Statistics

3.3.1 Microscopy

Photomicrographs of DRG outgrowth and tissue immunofluorescence were taken with a Nikon Eclipse TE2000 microscope and an optiMOS scMOS camera at either 10x or 20x magnification using micro-manager 1.4 software. Photomicrographs of axonal regeneration and tissue immunofluorescence were imaged with a Zeiss Axio Observer and a Hamamatsu Flash 4.0 fast camera at either 10x or 20x magnification. Confocal images were taken with a Leica TCS SP5 II confocal microscope at 20X magnification and processed with the LAS-AM Leica software (Leica).

3.3.2 Image Analysis

DRG Outgrowth Quantification: 15 neurons/well were imaged, with an average of three wells per biological replicate. A minimum of three biological replicates were used per experimental condition. Subsequent outgrowth measurements were conducted in ImageJ (Fiji) using the NeuronJ plugin. The total distance of combined neurite outgrowth was measured per neuron and the average distance per mouse calculated for each experimental group.

Ex Vivo Virus Validation: 15 neurons/well were imaged, with an average of six wells per biological replicate. The ratio of GFP⁺ neurons was calculated for each well to determine the infection efficiency for both the AAV-GFP and AAV-Leptin-GFP virus.

DRG Protein Expression Quantification:

For each biological replicate, all six DRGs were imaged in triplicate, averaging 18-20 images per mouse. A minimum of three biological replicates were used per experimental condition. Intensity measurements were conducted in ImageJ (Fiji). Cytoplasmic stains were quantified by outlining each neuron and nuclear stain by outlining each nucleus. An average of 30 neurons were quantified for each DRG.

PNS Axonal Regeneration Quantification: Sciatic nerves were imaged following nerve crush injury conditions. Both sciatic nerves from one mouse were analysed individually and an average created for each biological replicate. A minimum of three biological replicates were used per experimental condition. The injury site was identified from sudden loss of axonal tracts; a large increase in STMN2 intensity; and clusters of nuclear cell bodies preceding sparse nuclear staining, indicative of cell death and accumulating immune cells. Intensity measurements were conducted in ImageJ (Fiji) (Figure 18). The staining intensity of regenerating fibres was measured at 100µm intervals extending distally from the crush site and normalised to the intensity of axons pre-lesion. The regeneration index is the point at which 50% of axonal intensity is reached. The distance of the furthest regenerating axon beyond the crush site was also plotted.

CNS Axonal Regeneration Quantification: Spinal cords were imaged following a dorsal column axotomy. A minimum of eight biological replicates were used per experimental condition. Intensity measurements were conducted in ImageJ (Fiji). The staining intensity of dextran⁺ fibres was measured at 100µm intervals extending from -700µm before the injury site to 700µm beyond the injury site. Intensity measurements were normalised to the intensity of axons pre-lesion (-700µm distance) and plotted against distance beyond the injury site. The distance of the furthest regenerating axon beyond the injury site was measured from the distal lesion border to the axon tip. For each

spinal cord, GFAP lesion area and intensity was measured by tracing the border of GFAP⁺ cells.

3.3.3 Statistical Analysis

All data analysis was performed blind to the experimental group. Statistical analysis was conducted in GraphPad Prism 7 and data is presented as mean $\pm$ SEM. A significance threshold was determined as $p < 0.05$ and significance levels are displayed as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Normally distributed data containing two groups was analysed using an unpaired two-tailed Student's t-test. Normally distributed data containing over two groups was analysed using a one-way ANOVA. A Two-way ANOVA with Tukey's or Sidak's post-hoc test was applied when examining two independent variables within one dependent variable. Where multiple images or nerves were taken from one biological replicate, these were averaged to avoid pseudo-replication and maintain independence. The assumptions of normality and homogeneity of variance were assessed by visual inspection of residual plots.

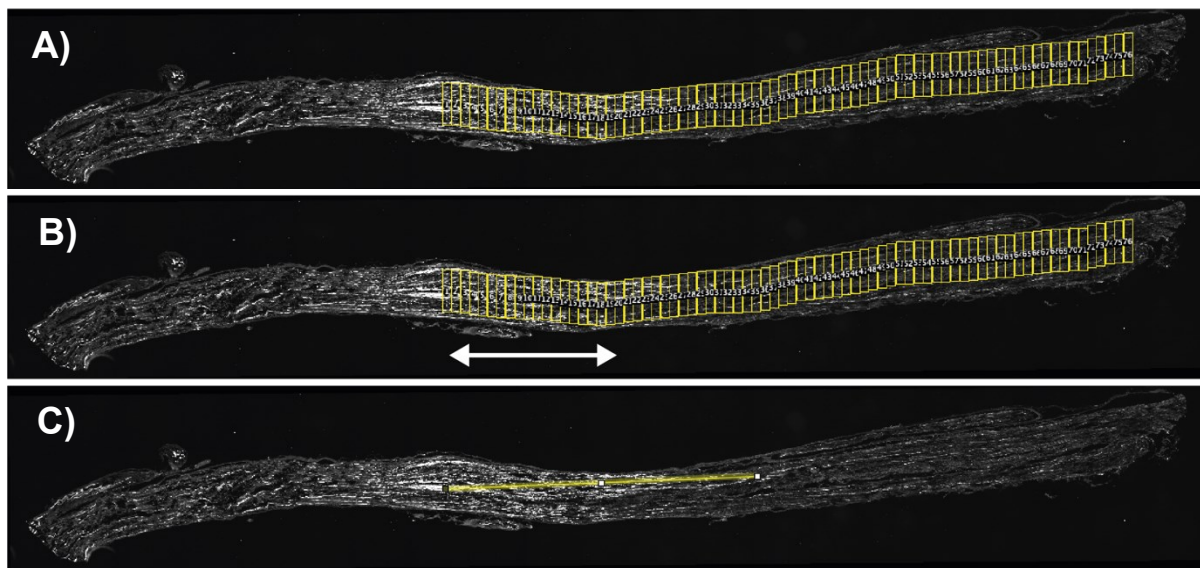


Figure 18: Axonal Regeneration Measures

A. Percentage Intensity: quantified using a FIJI macro plugin where the fluorescence intensity was normalised to background nerve staining at 100µm intervals.

B. Regeneration Index: the distance (µm) from the crush site to 50% fluorescence intensity.

C. Longest Axon: the distance (µm) from the crush site to the longest axon tip.

Figure created in Fiji and Adobe Illustrator by Phoebe Liddell.

4. RESULTS

4.1 Intermittent Fasting Promotes Leptin Expression and Signalling in DRG Neurons Preceding and After a Sciatic Nerve Injury

Differential gene expression changes were investigated following IF to determine the endogenous molecular pathways underpinning the IF-dependent regenerative phenotype. RNA sequencing (RNAseq) analysis from L4-L6 sciatic DRG RNA was conducted after 10 days of IF or AL diet (Figure 19A-H), which was recently shown to prime axonal regeneration of sensory DRG neurons (Sergey et al., 2022). Analysis of the RNAseq data ($p < 0.05$) revealed a large number of genes are differentially expressed following IF compared to AL, with 639 genes upregulated and 868 genes downregulated (Figure 19B). Analysis of downregulated Gene ontology (GO) transcripts showed an enrichment ($p < 0.01$) in pathways related to the 'immune response', 'inflammatory response' and 'chemotaxis' (Figure 19C). Many of these immune pathways are present within disease processes, reflected in the KEGG categories displayed (Figure 19D). Intermittent fasting also downregulated cell adhesion genes such as Col18a1, Fn1, Itga3, Pdpn and Lama5, in addition to many factors essential for a broad spectrum of tissue growth and development, including angiogenesis, heart development, lung development and extracellular matrix reorganisation. Downregulated GO categories suppressed in IF reflect redirection away from anabolic processes toward pathways associated with stress resistance and organism survival.

Among upregulated differentially expressed transcripts following IF, the most represented GO categories ($p < 0.01$) predominantly included 'metabolic pathways' such as 'steroid biosynthesis processes', 'lipid metabolic process' and 'cellular oxidant detoxification' (Figure 19E-G). Other key pathways included 'nervous system development', 'ion transport' and 'chemical synaptic transmission', suggesting IF widely impacts cell communication, neurotransmitter transport and possibly affects neuronal growth and development within DRG neurons. These themes were also present within the KEGG analysis ($p < 0.01$), revealing enhanced cAMP signalling, steroid biosynthesis and fatty acid metabolism (Figure 19F). The most upregulated KEGG category 'Biosynthesis of antibiotics' contained several key metabolic enzymes such as ATP-dependent 6-phosphofructokinase (Pfkfb), Gamma- enolase (Eno2), and Dihydrolipoyl dehydrogenase (Dld), all important for processes such as glycolysis,

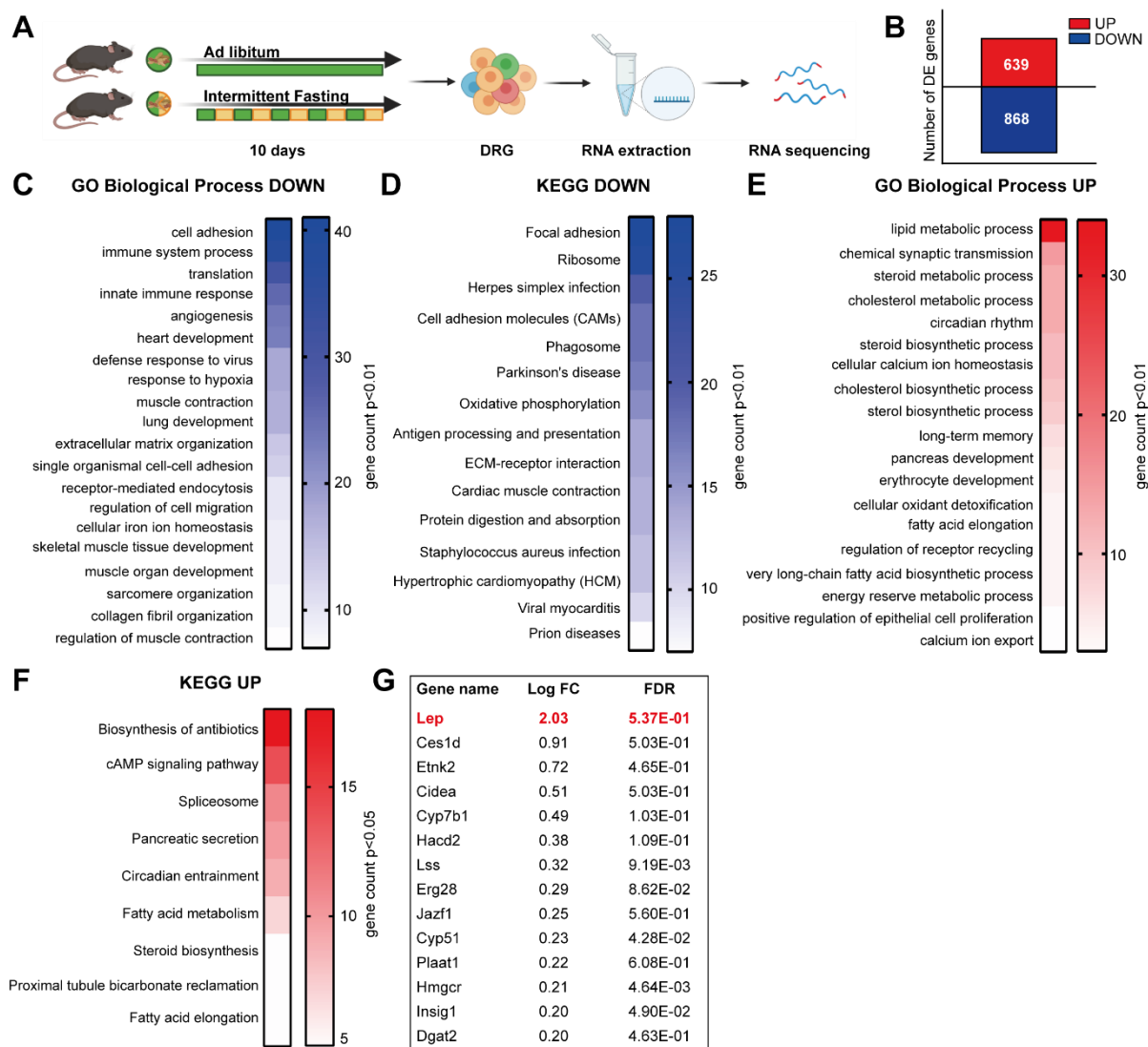


Figure 19: Intermittent Fasting Upregulated Lipid Metabolic Processes and Leptin Signalling within DRG Neurons.

A: Schematic of experimental procedure. RNA sequencing analysis from DRG bulk tissue following 10 days of intermittent fasting compared to ad libitum controls (n= 3 mice per group).

B: Number of differentially expressed up and down regulated genes in IF v AL dataset where $p < 0.05$. 639 genes were upregulated following IF and 868 genes were downregulated.

C: Heatmap visualising DAVID Gene Ontology (GO) analysis of the top 25 gene categories most significantly downregulated. Shown are pathways graphed via gene count at $p < 0.01$. Blue indicates the most significantly downregulated genes.

D: Heatmap visualising DAVID KEGG analysis of the top 25 gene categories most significantly downregulated. Shown are pathways graphed via gene count at $p < 0.01$. Blue indicates the most significantly downregulated genes.

E: Heatmap visualising GO analysis of the top 25 gene categories most significantly upregulated. Shown are pathways graphed via gene count at $p < 0.01$. Red indicates the most significantly upregulated genes.

F: Heatmap visualising DAVID KEGG analysis of the top 25 gene categories most significantly upregulated. Shown are pathways graphed via gene count at $p < 0.05$. Red indicates the most significantly upregulated pathways.

G: Table listing the logarithmic expression fold change (LogFC) and FDR (< 0.05) of the most significantly upregulated genes within the 'lipid metabolic process' GO term.

glycine cleavage systems, mitochondrial complexes or exerting neuroprotective properties in CNS neurons. Strikingly, detailed exploration of genes within the most upregulated GO transcripts found the leptin gene had the highest differential fold change (LogFC 2.03) and a significant FDR value (FDR <0.05; a stringent analysis cut-off of the most significant gene expression changes) (Figure 19G). Leptin expression is strongly dependent upon dietary changes (Ahima and Flier, 2000) and was differentially upregulated in numerous metabolic GO terms, supporting its known physiological impact. This suggests IF-dependent leptin-signalling may underlie metabolic and neuronal communication changes observed after fasting within DRG neurons. Understanding leptin's expression profile within neurons during IF before and after injury will provide clearer understanding of the hormone's potential role in regeneration.

Initially, RT-PCR from DRG tissue, including from a neuronal enriched preparation, showed that leptin is present within the DRG and enriched within DRG neurons (Figure 20A). To characterise the cellular localisation and expression of leptin and the leptin receptor within the DRG, immunofluorescence and RT-PCR for leptin and the LepR was performed on sections of DRG following 10 days of IF vs AL diet. Indeed, leptin expression was upregulated at both the mRNA (1.6 fold) and protein level (1.3 fold) within DRG neurons after IF (Figure 20B-J). The LepR was also selectively expressed in neurons, but expression was unchanged at either the mRNA or protein level during IF (Figure 20F-G). However, it's possible leptin signalling mediated by the leptin receptor increased following IF as indicated by increased pSTAT3, a well-known leptin-LepR phosphorylation signalling target (Figure 20H). These results are supported by time-course data mapping leptin expression at the protein level across the early, middle and late stages of the IF regimen (Figure 20I-J). Leptin protein expression on the first fast day of the regimen, whilst increased, did take until the second refed day to reach an approximate average 1.4-fold increase observed for the remaining days of the regimen. Collectively, this data indicates that even early stages of the IF regimen can activate neuronal leptin signalling. However, the inverse relationship is observed within the systemic system. Circulating serum leptin concentrations show a decrease after one day of fasting (Figure 20K), in keeping with previous reports. This indicates that whilst leptin's action as a satiety hormone is active systemically and downregulated following fasting, it's expression and physiological role within neurons is different and may play a role in the neuronal injury response.

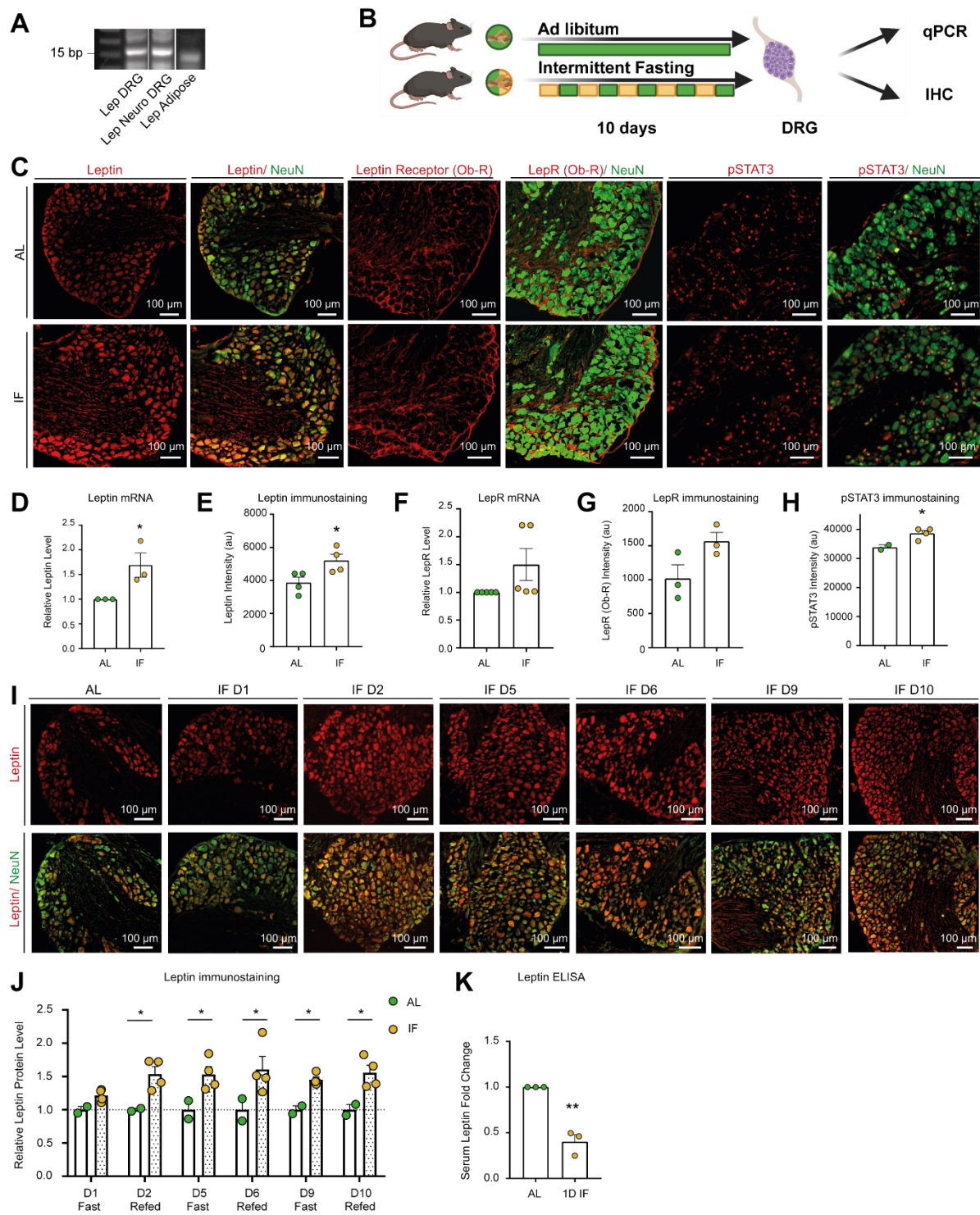


Figure 20: Leptin DRG Expression Increases after 10 days of Intermittent Fasting.

A: Agarose gel visualisation of sample DNA from bulk DRG, neuronal-enriched DRG tissue, cortical tissue and adipose tissue for leptin expression.

B: Schematic of experimental procedure. 10- day IF regimen, DRG tissue dissected on a re-fed day and processed for qPCR and immunohistochemistry.

C: Representative immunofluorescent images for graphs D-H. (Scale bar: 100 μ m).

D-E: (D) Total DRG leptin mRNA expression 10 day IF samples v AL controls, quantified via qPCR normalised to GAPDH. (* $p < 0.05$, $n = 3$ mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM). **(E)** DRG leptin protein expression 10 day IF samples v AL controls, immunofluorescence analysis. (* $p < 0.05$, $n = 4$ mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).

F-G: (F) Total DRG leptin receptor mRNA expression 10 day IF v AL controls, quantified via qPCR normalised to GAPDH. ($p = 0.08$, $n = 5$ mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM). **(G)** DRG leptin receptor membrane protein (Ob-R) expression 10 day IF samples v AL controls, immunofluorescence analysis. ($p = 0.08$, $n = 3$ mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).

H: DRG pSTAT3 protein expression 10 day IF samples v AL controls, immunofluorescence analysis. (* $p < 0.05$, $n = 4$ mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).

I: Representative DRG images of leptin protein expression over the 10 day IF regimen.

J: DRG leptin protein expression day 1-10 IF fast and re-red samples v AL controls. (* $p < 0.05$, $n = 4$ mice per group, Two-way ANOVA with Sidak's multiple comparison's test, mean $\pm$ SEM).

K: Leptin serum ELISA of circulating leptin concentrations in AL or IF mice. (** $p < 0.01$, $n = 3$ mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).

Leptin expression and pSTAT3 also show an IF-dependent increase in DRG neurons following sciatic nerve crush injury compared to animals on an AL diet as shown by RT-PCR, immunoblotting and immunofluorescence (Figure 21A-N). To determine whether IF-dependent leptin signalling enhances axonal regeneration, neuronal DRG leptin was measured after 1 day of fasting compared to AL control following sciatic nerve crush injury and 72 hours of regeneration. One day of fasting significantly enhanced leptin expression within DRG neurons, accompanied by a corresponding increase in axonal regeneration past the crush site (Figure 21A-E). Data shows one day of fasting significantly impacts neuronal leptin expression and regenerative output. For example, leptin expression following a 10-day IF regimen without injury effects a 1.34-fold change or 34% increase in leptin expression within DRG neurons (Figure 20E). Following 1 day of fasting, a sciatic nerve crush injury and 72 hours of regeneration, leptin expression within DRG neurons increases by 1.59-fold or 59% (Figure 21E). A similar relationship is observed with pSTAT3 before (1.14-fold) and after injury (2.19-fold) (Figure 20H and 21N). Introduction of an injury condition enhances leptin expression and downstream signalling. To ascertain the importance of an injury condition in boosting DRG leptin signalling and clarify the relationship between leptin and the IF regenerative phenotype, DRG leptin expression after a 10d IF regimen and sciatic nerve crush was measured. Neuronal DRG leptin showed a 6.0-fold upregulation at the mRNA level (Figure 21I) and a 2.9-fold increase at the protein level (Figure 21J, K). Leptin expression was increased substantially more after IF and

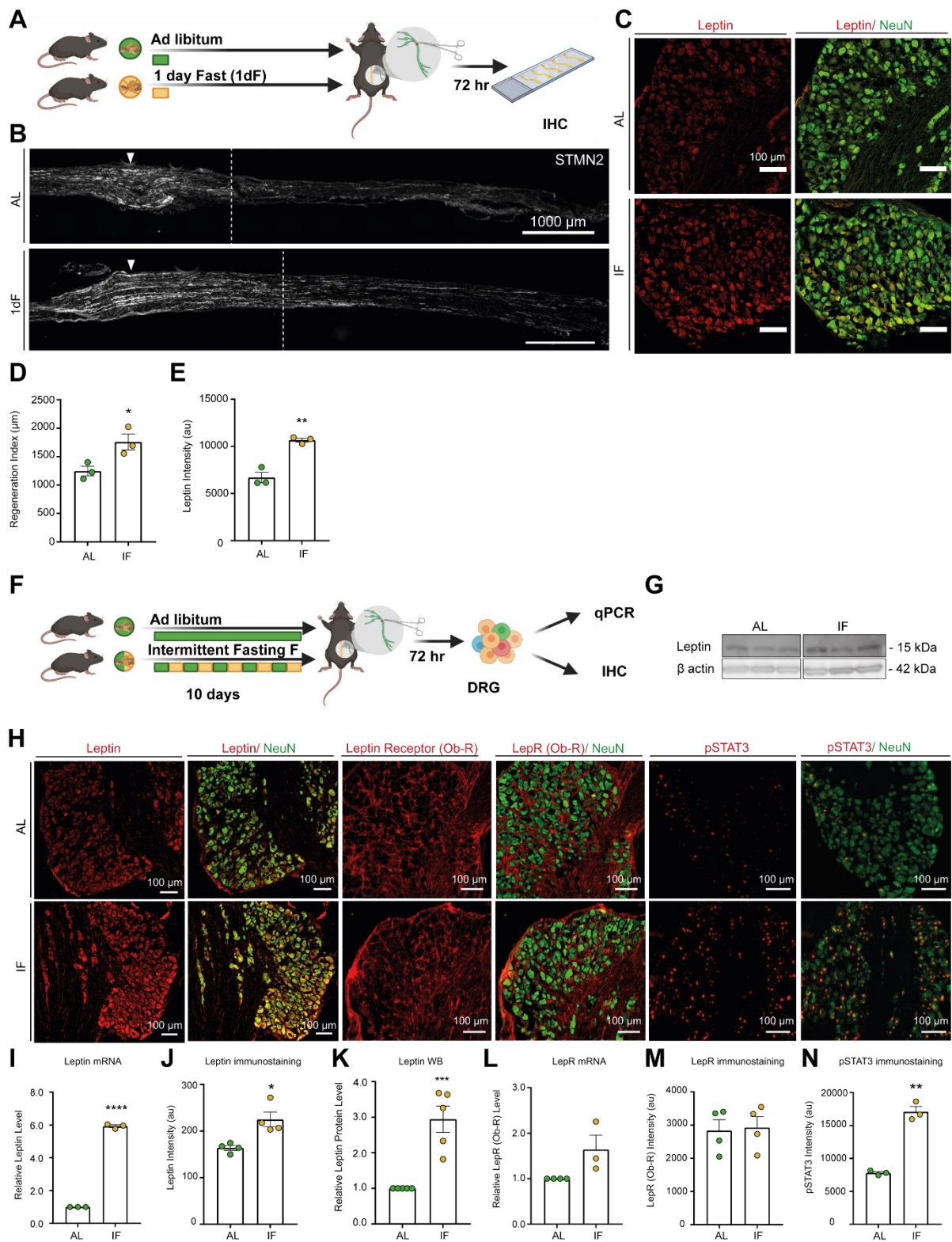


Figure 21: DRG Leptin Expression Increases after 10 days of Intermittent Fasting and Enhances Axonal Regeneration post- Sciatic Nerve Crush Injury.

- A:** Schematic of 1d fasting followed by SNC and 72hr regeneration.
- B:** Representative micrographs of longitudinal sections of 1dF sciatic nerves 72h after SNC, immuno-stained for SCG-10 (STMN2) (Scale bar: 1000 μ m). The white arrow indicates the crush site and the dashed line marks the regeneration index; the point at which SCG-10 intensity reaches 50%.
- C:** Representative DRG images of leptin intensity for one day fasting (1dF) samples compared to ad libitum (AL) controls. (Scale bar: 100 μ m). DRG following 1 day of fasting show a 1.5-fold increase in leptin intensity.
- D:** Quantification of regeneration index, the point at which SCG-10 intensity reaches 50%, normalised to crush site. (n=6 nerves from 3 mice per group with a bilateral sciatic nerve crush; * $p < 0.05$, unpaired two-tailed student's T-test, mean $\pm$ SEM).
- E:** Quantification of leptin intensity, presented in (C). (** $p < 0.01$, n=3 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).
- F:** 10 day IF regimen, followed by sciatic nerve crush and 72 hr regeneration. DRG tissue dissected on a fast day and processed for qPCR, western blot and immunofluorescence imaging.
- G:** Immunoblot shows leptin expression in sciatic DRG neurons following 10 days of IF or AL diet, a sciatic nerve crush injury and three days of regeneration. Normalised to β - actin.
- H:** Representative immunofluorescence images of leptin, the leptin receptor and downstream pSTAT3 protein expression within sciatic DRG neurons following 10 days IF or AL diet, sciatic nerve crush injury and a 72-hr regeneration period. Co-stained with neuronal marker NeuN to demonstrate neuronal localisation.
- I:** DRG leptin (**I**) and leptin receptor (**L**) mRNA expression from 10 day IF samples v AL controls, followed by sciatic nerve crush injury and three days regeneration. Quantified via qPCR and normalised to neurofilament. (**I**) (**** $p < 0.0001$, n=3 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM). (**L**) $p = 0.058$, n=3 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).
- J:** Immunofluorescence quantification of DRG leptin (**J**), the leptin receptor (**M**) and pSTAT3 (**N**) protein expression represented in panel H. (**J**) (* $p < 0.0108$, n=4 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM). (**M**) ($p = 0.852$, n=4 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM). (**N**) (** $p < 0.01$, n=3 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).
- K:** Immunoblot quantification of leptin protein expression levels in (G). The relative leptin protein levels were quantified following normalisation to β - actin. (*** $p < 0.001$, n= 5 mice per group, unpaired two-tailed student's T-test, mean $\pm$ SEM).

injury, compared to IF alone. Neuronal DRG leptin climbed from a 1.59-fold increase after 1 fast day with injury to a 2.9-fold increase following 10 days of IF with injury, indicating leptin accumulation is still increasing during the early stage of the IF regimen. Similarly, in line with the non-injury data, LepR expression did not change following IF and injury (Figure 21L, M) but LepR downstream signalling marker pSTAT3 expression did increase (Figure 21N). Collectively, data suggests that leptin expression and signalling in DRG sensory neurons is induced by IF and further amplified during nerve injury. However, IF activates many processes and signalling pathways within the body independently of leptin. Therefore, to determine the centrality of IF-dependent leptin signalling in axonal regeneration, a series of leptin receptor deletion experiments were performed.

4.2 Leptin Receptor Deletion Significantly Impairs Intermittent Fasting-Dependent Nerve Regeneration

To ascertain whether the IF-dependent regenerative phenotype requires leptin signalling, a conditional deletion of the LepR in DRG neurons was carried out. Either

an AAV-Cre-GFP or AAV-GFP virus was injected directly into the sciatic nerve of LepR FLOX mice to delete the LepR in DRG neurons. Virus expression for four weeks ensured optimal expression of the transgene, which was followed by 10d IF or AL diet and sciatic nerve crush injury (Figure 22A-E). Analysis of axonal regeneration performed 72 hours after SNC, showed that LepR deletion attenuated IF-dependent peripheral axonal regeneration equivalent to AL control levels (Figure 22B-C). Measurement of the regeneration index (the distance between the crush site and the point at which SCG-10 intensity is 50% of that at the crush site) showed a significant increase in regeneration following IF in the AAV-GFP control group (AL: $1183 \pm 56.4 \mu\text{m}$, IF: $1674 \pm 323.7 \mu\text{m}$). This regenerative advantage was lost however in the AAV-Cre-GFP group in which the LepR was deleted (AL: $1008 \pm 373.2 \mu\text{m}$, IF: $1104 \pm 167.8 \mu\text{m}$). Attenuation of the IF regenerative phenotype to AL control levels upon LepR deletion demonstrates leptin signalling via its receptor is required for IF-dependent axonal regeneration. Quantification of DRG neurons from these groups confirm successful membrane LepR deletion in Cre-GFP LepR FLOX mice (Figure 22D-E).

These results indicate leptin is a key player in IF-dependent axonal regeneration. To ascertain the effects of direct leptin activation, we examined whether leptin administration would enhance neuronal outgrowth via LepR signalling. Increasing concentrations of leptin (50 ng/mL, 100 ng/mL and 1 $\mu\text{g/mL}$) were added to DRG cultures from AL mice and outgrowth measured after 12 hours (Figure 23A, C). Leptin treatment significantly increased outgrowth compared to vehicle controls. Additionally, the effect of leptin treatment on neurite outgrowth was measured in 10d IF and AL states (Figure 23B, D). As expected, IF increased neuronal outgrowth compared to AL control (AL: $721 \pm 188.7 \mu\text{m}$, IF: $1241 \pm 288.6 \mu\text{m}$) and leptin treatment boosted outgrowth in AL cultures. Neurite outgrowth increased equivalently in IF and IF + leptin conditions compared to AL controls, supporting the hypothesis that leptin drives the growth- promoting regenerative phenotype produced by IF within DRG neurons. Similar results were observed *in vivo*. LepR FLOX mice were injected with either AAV-GFP or AAV- Cre- GFP and the virus expressed for 4 weeks before mice underwent sciatic nerve crush injury. Following injury, mice were treated with intraperitoneal injections of 20 $\mu\text{g/ day}$ of leptin for 4 days vs saline control (Figure 24A). Strikingly, within the AAV-GFP control system and intact leptin signalling cascade, leptin treatment post- injury significantly increased axonal regeneration compared to saline

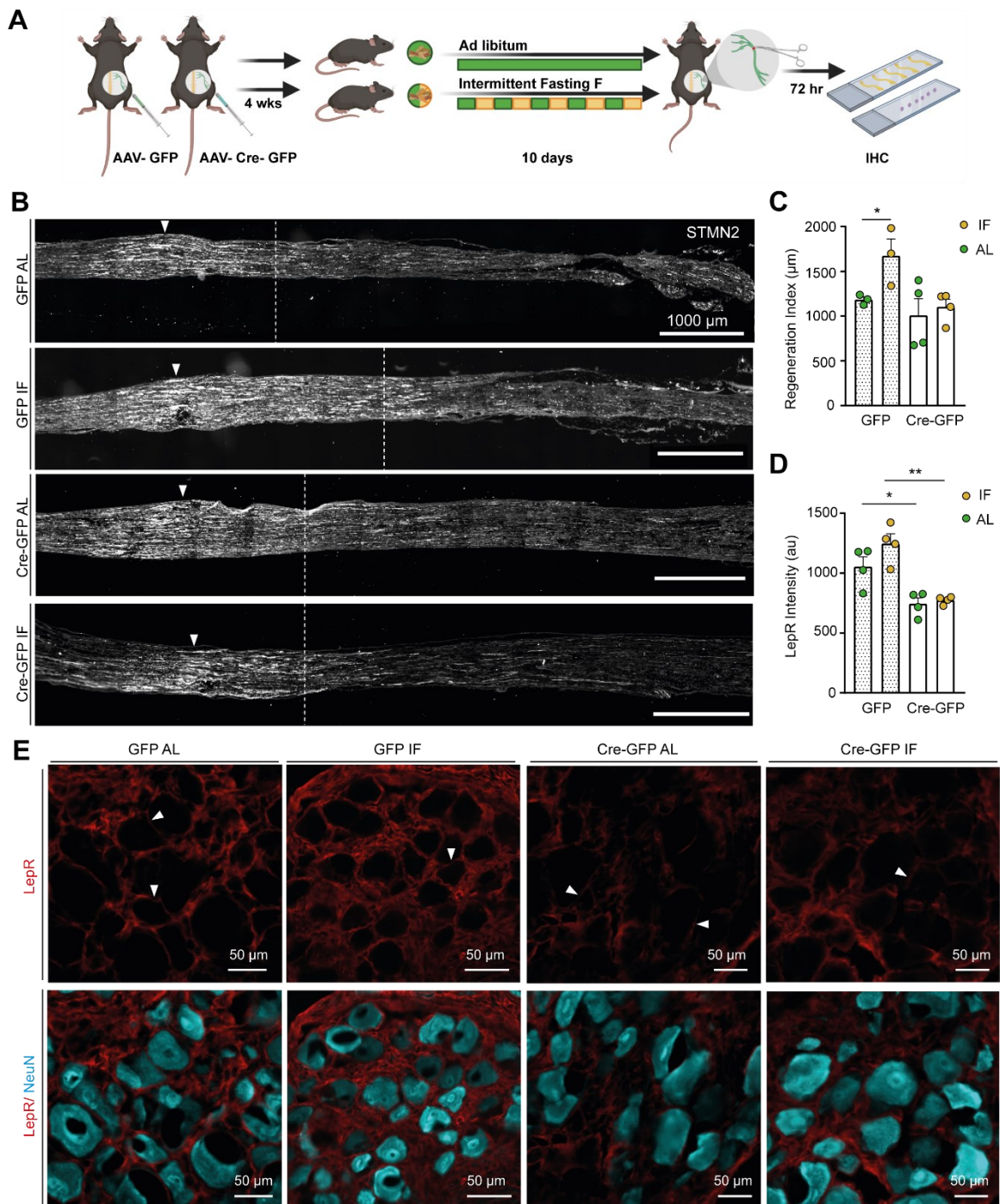


Figure 22: Leptin Receptor Deletion Attenuated Intermittent Fasting- Dependent Axonal Regeneration.

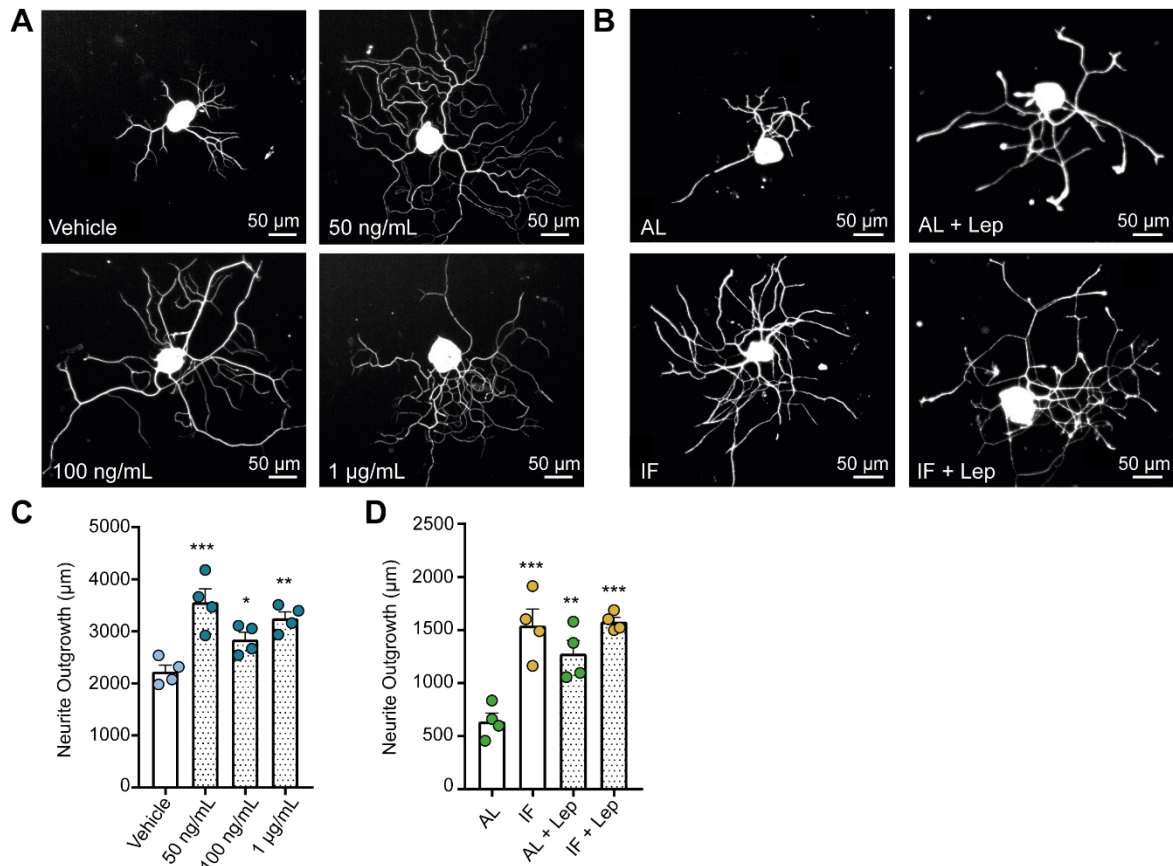
A: Schematic of neuronal LepR deletion, presented in B-C. AAV-GFP or AAV-Cre-GFP viruses were injected directly into the sciatic nerve of LepR FLOX mice to delete the receptor from neuronal cells within the DRG. Mice underwent a 10 day IF regimen or AL diet prior to sciatic nerve crush and 72hr regeneration.

B: Micrographs showing representative longitudinal sections of sciatic nerves 72h after SNC, immune-stained with SCG-10 (STMN2) (Scale bar: 1000 μ m). The white arrow indicates the crush site and the dashed line marks the regeneration index- the point at which SCG-10 intensity reaches 50%. Nerves are AAV- GFP control samples or AAV-Cre-GFP experimental samples in which the LepR has been deleted.

C: Quantification of regeneration index, the point at which SCG-10 intensity reaches 50%, normalised to crush site. (n=4 mice per group with a bilateral sciatic nerve crush, * $p < 0.05$, one-way-ANOVA with Tukey post-hoc test, mean $\pm$ SEM).

D: Quantification of leptin receptor DRG intensity within the AAV-GFP control system and AAV-Cre-GFP LepR FLOX system (n=4 mice per group, * $p < 0.05$, ** $p < 0.01$, one-way-ANOVA with Tukey post-hoc test, mean $\pm$ SEM).

E: Representative DRG images of Leptin receptor DRG expression quantified in D.



control (Saline: $981 \pm 50.2 \mu\text{m}$, Leptin: $1554 \pm 132.3 \mu\text{m}$) (Figure 24B-D). However, the regenerative capacity of post-injury leptin treatment was attenuated within the Cre-FLOX LepR deletion system. Deletion of the LepR within DRG neurons ablated nerve regeneration in the leptin treatment group, supporting previously discussed data determining the essential role of the LepR in regenerative leptin signalling. Quantification within DRG neurons confirmed leptin protein expression increased following post-injury leptin treatment; an effect which is lost following deletion of the LepR in the Cre-FLOX system (Figure 24E-F). Neuronal pSTAT3 expression also increased in leptin-treated intact mice, suggesting activation of LepR downstream

signalling cascades (Figure 24E, G). In summary, these results show that independently from IF, leptin promotes nerve regeneration when delivered following a nerve injury and that leptin-dependent axonal regeneration requires the LepR.

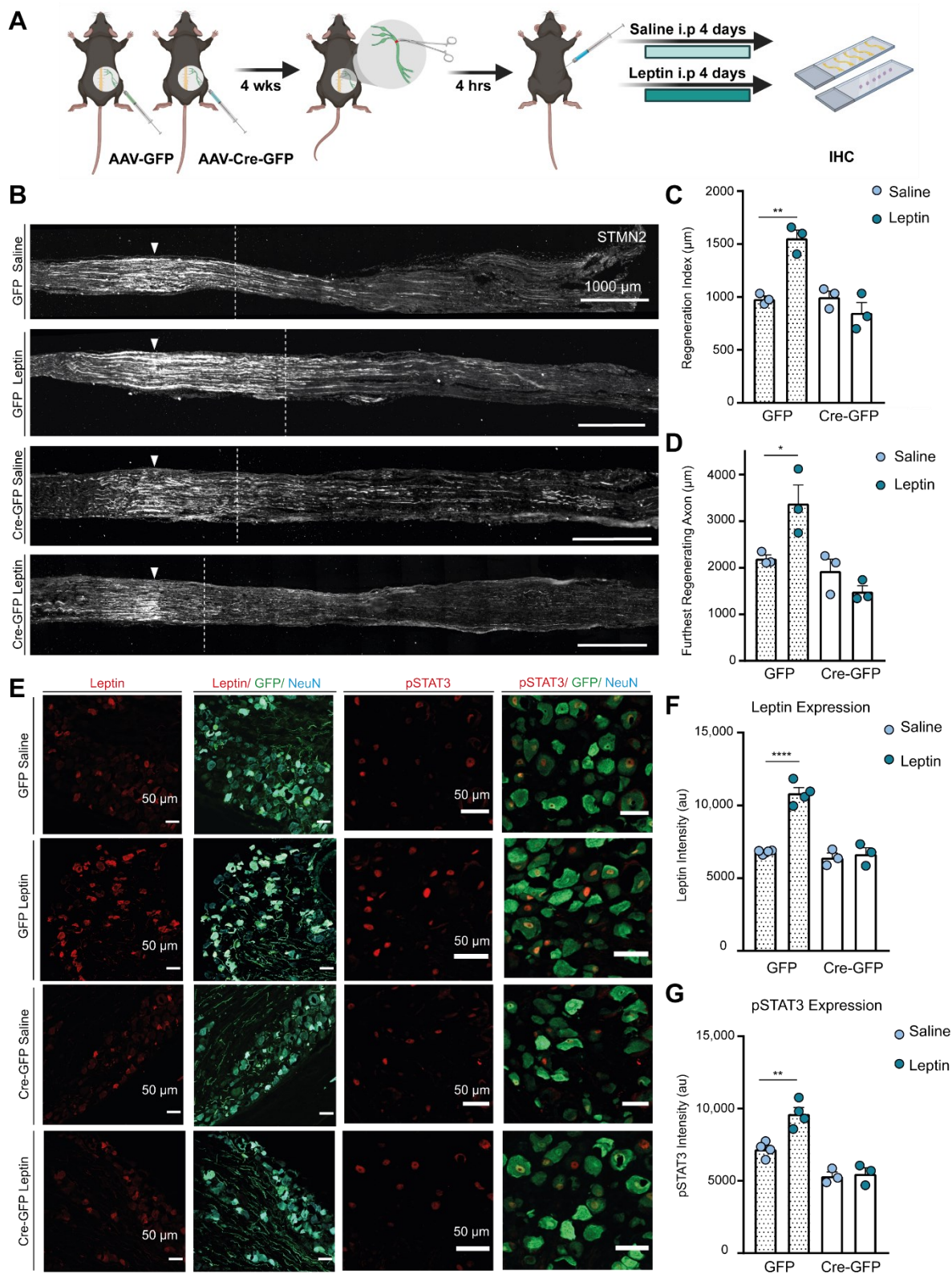


Figure 24: Leptin Treatment Enhanced Axonal Regeneration post- Sciatic Nerve Crush Injury.

A: Experimental schematic. LepR FLOX mice received either AAV-GFP or AAV-Cre-GFP sciatic nerve injections 4 weeks prior to a sciatic nerve crush injury. Mice recovered for 4 hours before receiving the first intra- peritoneal (i.p) dose injection of either 20 µg of leptin or saline control. Treatment injections were delivered every morning for 4 days before mice were culled. Nerves, DRG and serum were collected.

B: Micrographs showing representative longitudinal sections of sciatic nerves 72h after SNC immune-stained with SCG-10 (STMN2) (Scale bar: 1000 µm). The white arrow indicates the crush site and the dashed line marks the regeneration index- the point at which SCG-10 intensity reaches 50%.

C: Quantification of regeneration index, the point at which SCG-10 intensity reaches 50%, normalised to crush site. (n= 3 mice per group with a bilateral sciatic nerve crush, ** p<0.01, ordinary one-way ANOVA with Tukey's post-hoc test, mean±SEM).

D: Quantification of furthest regenerating axon beyond the crush site. (n= 3 mice per group with a bilateral sciatic nerve crush, * p<0.05, ordinary one-way ANOVA with Tukey's post-hoc test, mean±SEM).

E: Representative DRG images of leptin and pSTAT3 expression.

F: Quantification of leptin DRG intensity within the AAV-GFP control system and AAV-Cre-GFP LepR FLOX system. (n= 3-4 mice per group with a bilateral sciatic nerve crush, **** p<0.0001, ordinary one-way ANOVA with Tukey's post-hoc test, mean±SEM).

G: Quantification of pSTAT3 DRG intensity within the AAV-GFP control system and AAV-Cre-GFP LepR FLOX system. (n= 3-4 mice per group with a bilateral sciatic nerve crush, ** p<0.01, ordinary one-way ANOVA with Tukey's post-hoc test, mean±SEM).

4.3 Leptin Overexpression Enhances Axonal Regeneration after Peripheral and Central Nervous System Injury

Having ascertained IF- dependent and independent pro-regenerative leptin signalling acts via the LepR and its downstream signalling cascades, further experiments aimed to establish whether neuronal leptin overexpression would enhance both regenerative growth of sensory neurons in culture and following an injury condition in the peripheral or central nervous system environments. DRG neuronal cultures dissected from AL fed mice and electroporated with a leptin-GFP plasmid showed significantly enhanced neurite outgrowth ($2118 \pm 257.4 \mu\text{m}$) compared to GFP controls ($1467 \pm 154.7 \mu\text{m}$) (Figure 25A-C). Next, overexpression experiments comparing AAV-GFP and AAV-Leptin-GFP delivery into the sciatic nerve and sensory neurons of the DRG, showed that leptin overexpression significantly increased regeneration beyond the crush site 72 hours following sciatic nerve crush injury (GFP: $1233 \pm 142.0 \mu\text{m}$, Leptin-GFP: $1835 \pm 219.6 \mu\text{m}$) (Figure 26A-G). This supports previous conclusions determining leptin's regenerative effect independently of IF. Quantification of infection efficiency also determined that DRG neurons had a good AAV infection rate of 60% within *ex vivo* cultures (Figure 27A-B). However, the peripheral nervous system is regeneration-competent and able to mount a regenerative response post- injury, whereas central nervous system axons fail to regenerate (Kaplan et al., 2015). Therefore, we challenged the regenerative ability of leptin overexpression further in the context of spinal cord injury. AAV-Leptin-GFP or AAV-GFP viruses were injected into the sciatic nerves and a 4- week time period lapsed to enable robust overexpression prior to a T9

dorsal column axotomy (DCA). Axonal regeneration of DRG sensory fibres was measured 6 weeks later via dextran labelling (Figure 28A). Excitingly, leptin overexpression increased axonal regeneration within the spinal cord, mounting a robust regenerative response through the spinal cord lesion site, astrocytic border (visualised with an anti- GFAP antibody) and collagenous scar (Figure 28B-D). Leptin overexpression increased the distance and density of regenerating fibres within the spinal cord, showing significantly longer far- reaching regenerating fibres compared to AAV GFP controls (Figure 28D). This was also reflected in caudal axon retraction measures. The distance from the lesion epicentre to healthy tract fibres caudal to the injury site was much larger in AAV- GFP controls, where injured axons exhibited 'die back' and retraction from the injury centre (Figure 28D). In comparison, fibres from leptin overexpression cords were maintained at the lesion epicentre, extending through the astrocytic-collagenous scar to the rostral spinal cord. Spinal cord cross sections 5mm above the injury site confirmed the absence of spared fibres within rostral sections while dextran tracing of lumbar neurons was observed in caudal sections (Figure 29A). Additionally, the lesion area and lesion GFAP intensity between groups remained equal, confirming injury severity was consistent between the control and leptin overexpression groups (Figure 29B-D). Together, data shows that neuronal leptin overexpression significantly promotes axonal regeneration of sensory neurons in both the peripheral and central nervous system.

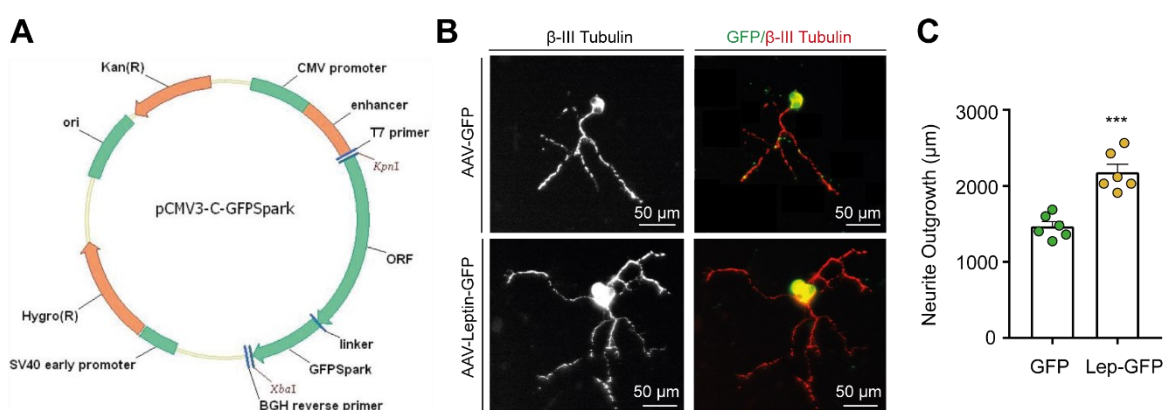


Figure 25: Leptin Overexpression Enhanced Neurite Outgrowth.

A: Schematic of Leptin-GFP plasmid electroporated into DRG neuronal cultures, presented in B-C.

B: Representative images of GFP⁺ DRG neurons immune-stained with βIII-Tubulin. (Scale bar: 50 μm).

C: Quantification of neurite outgrowth immune-stained for GFP and βIII-Tubulin. (n=6 wells from 3 mice per group, *** p<0.001, unpaired two-tailed student's T-test, mean±SEM).

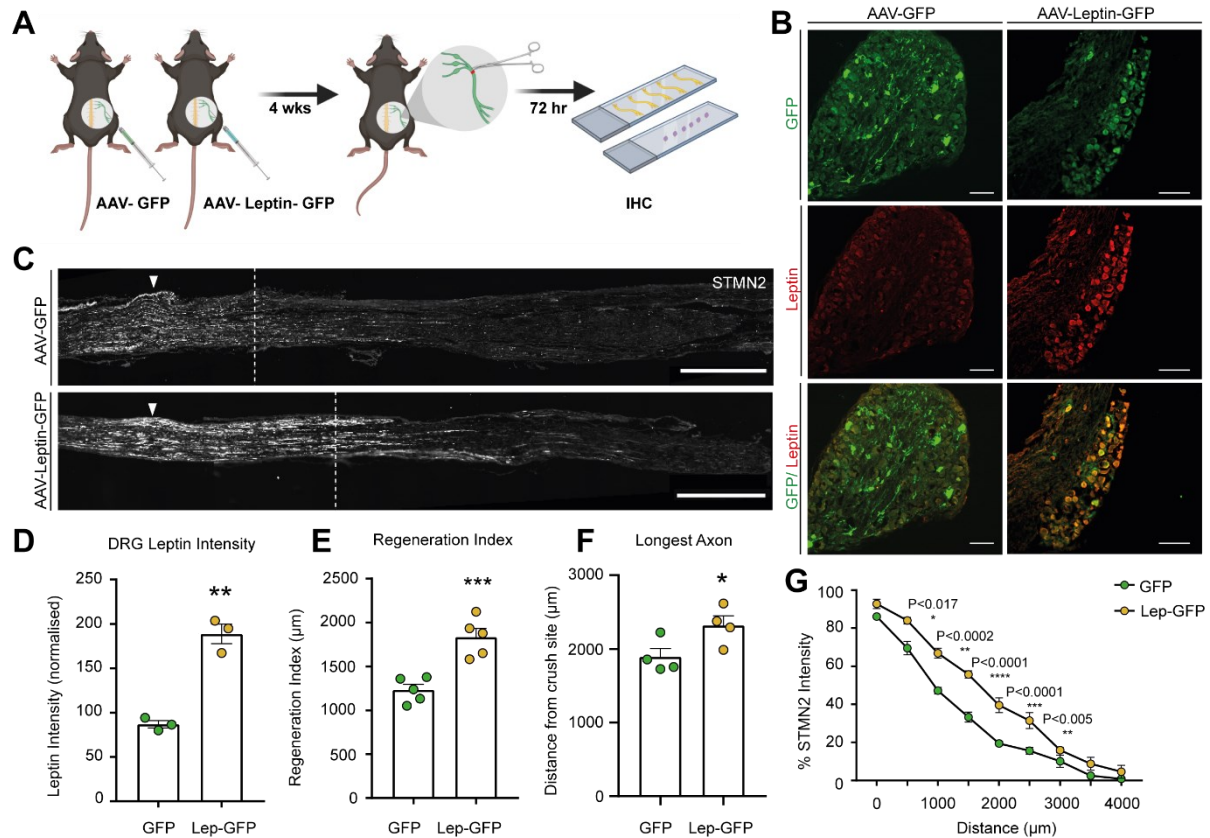


Figure 26: Leptin Overexpression Enhanced Axonal Regeneration following Sciatic Nerve Crush Injury.

A: Schematic of experimental leptin overexpression procedure.

B: Representative DRG images immune-stained for leptin and GFP following overexpression or control virus injections.

C: Micrographs showing representative longitudinal sections of sciatic nerves 72h after SNC immune-stained with SCG-10 (STMN2) (Scale bar: 1000 μ m). The white arrow indicates the crush site and the dashed line marks the regeneration index- the point at which SCG-10 intensity reaches 50%. Nerves are AAV- GFP control samples or AAV-Leptin-GFP experimental samples in which leptin is overexpressed.

D: Quantification of DRG leptin intensity, presented in **(B)**. (** p<0.01, n=3 mice per group, unpaired two-tailed student's T-test, mean±SEM).

E: Quantification of regeneration index, the point at which SCG-10 intensity reaches 50%, normalised to crush site. (n=10 nerves from 5 mice per group with a bilateral sciatic nerve crush, *** p<0.001, unpaired two-tailed student's T-test, mean±SEM).

F: Quantification of longest regenerating axon beyond the crush site. (n=8 nerves from 4 mice per group with a bilateral sciatic nerve crush, * p<0.05, unpaired two-tailed student's T-test, mean±SEM).

G: Quantification of percentage STMN2 intensity beyond the crush site. (n=4 mice per group, **** p<0.0001, Two-way ANOVA with Tukey's post-hoc test, mean±SEM).

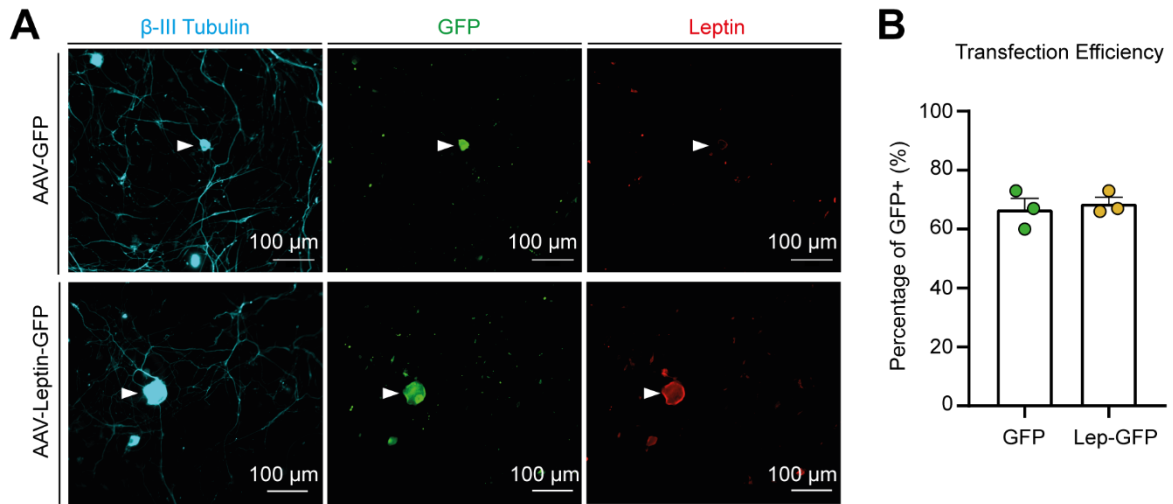


Figure 27: Leptin Overexpression Infection Efficiency.

A: Representative images of AAV- GFP and AAV- Leptin-GFP virus validation in neuronal cell culture.

B: Quantification of AAV- GFP and AAV-Leptin-GFP virus infection efficiency, summarised as percentage of GFP⁺ cells per DRG.

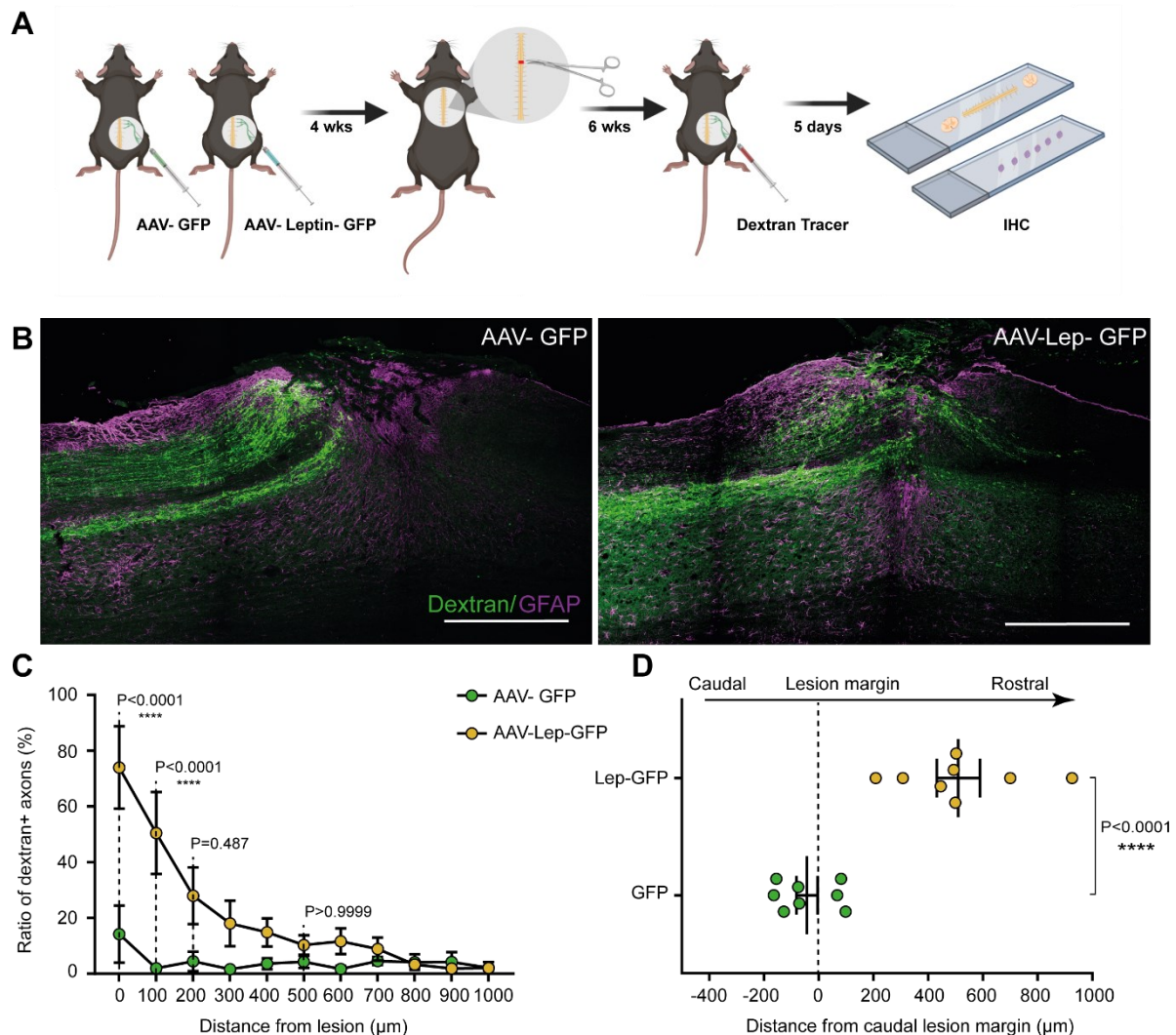


Figure 28: Leptin Overexpression Enhanced Spinal Cord Regeneration post- Dorsal Column Axotomy.

A: Schematic of experimental leptin overexpression procedure, presented in B-C. AAV-GFP or AAV-Leptin-GFP viruses were injected directly into the sciatic nerve of C57BL/6 mice until fully expressed after 4 weeks. Mice underwent a T9 dorsal column axotomy and a 6-week regeneration window prior to dextran tracer injections into the sciatic nerve to visualise regenerating fibres.

B: Micrographs showing representative longitudinal sections of the spinal cord after DCA, immune-stained with GFAP to visualise the astrocytic scar (Scale bar: 500 μ m). Cords are AAV-GFP control samples or AAV-Leptin-GFP experimental samples in which leptin has been overexpressed.

C: Quantification of dextran intensity past the lesion epicentre, corresponding to the number of regenerating fibres present in the rostral spinal cord. (n=8 mice per group, **** $p < 0.0001$, Two-way ANOVA with Tukey's post-hoc test, mean $\pm$ SEM).

D: Quantification of the furthest regenerating fibre beyond the lesion epicentre following DCA and quantification of axon retraction from the lesion epicentre to the nearest caudal bundle of dorsal column axons as a measure of 'die-back'. (n=8 mice per group, **** $p < 0.0001$, unpaired two-tailed student's T-test, mean $\pm$ SEM).

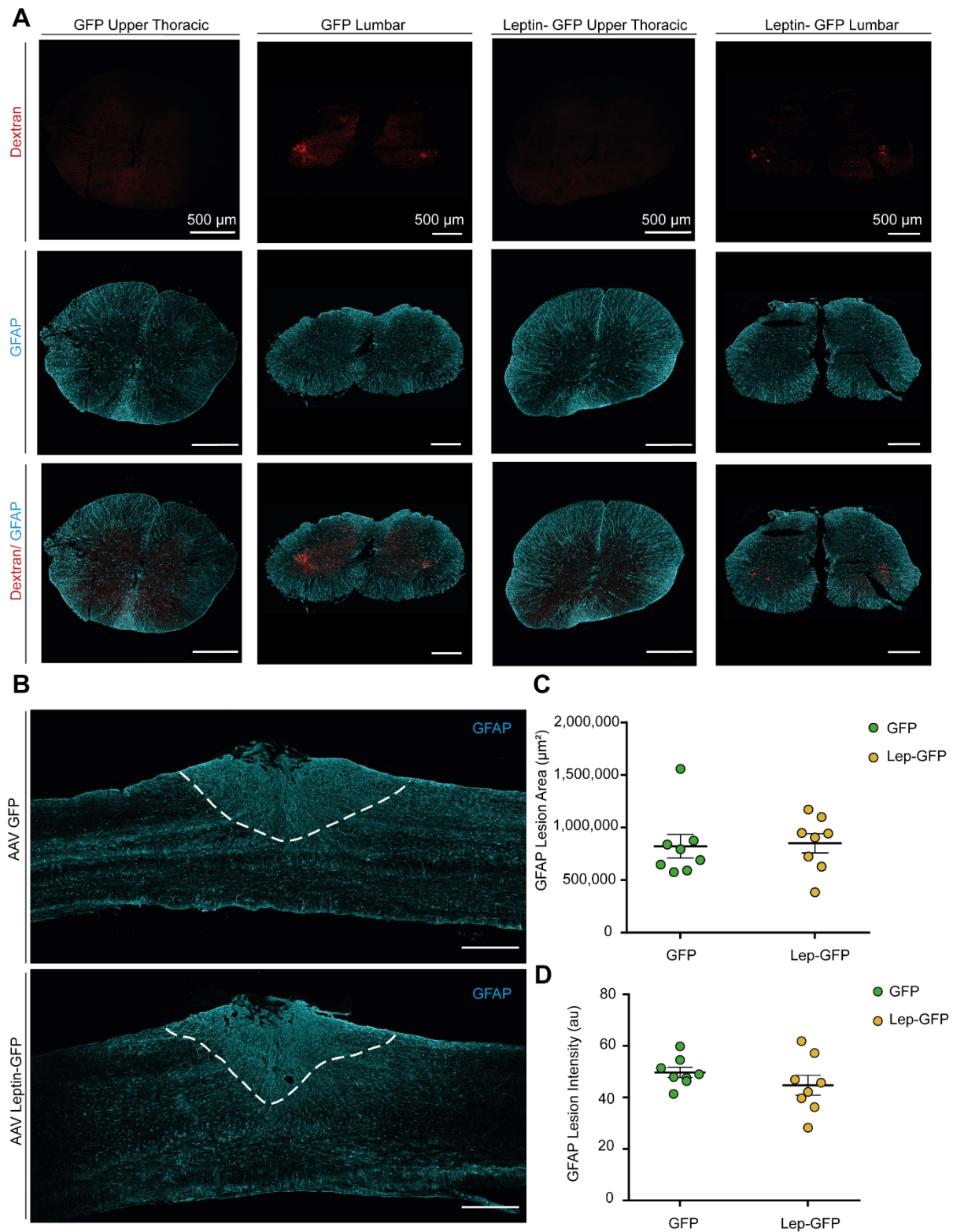


Figure 29: Dorsal Column Axotomy Injury Controls.

A: Representative spinal cord images of upper thoracic and lumbar sections above and below the T9 dorsal column injury site, co-immuno-stained with antibodies against GFAP and Dextran. Scale bar 500 μm .

B: Micrographs showing representative longitudinal sections of the spinal cord after DCA, immune-stained with GFAP to visualise the astrocytic scar (Scale bar: 500 μm).

C: Quantification of GFAP lesion area, corresponding to the size of the lesion core and indicative of injury consistency between animals. ($n=8$ mice per group, $p=0.846$, unpaired two-tailed student's T-test, mean $\pm$ SEM).

D: Quantification of GFAP intensity, corresponding to the number astrocytes present within the lesion core. ($n=8$ mice per group, $p=0.268$, unpaired two-tailed student's T-test, mean $\pm$ SEM).

4.4 Leptin Overexpression in Sensory DRG Neurons Significantly Promotes Gene Transcription

Leptin overexpression exerts a significant regenerative response in both the peripheral and central nervous system that requires the leptin receptor. However, the molecular mechanisms underpinning this phenotype remain unknown. To understand the regenerative molecular pathways stimulated following leptin treatment and spinal cord injury, RNA sequencing was conducted on a neuronal- enriched L4-L6 sciatic DRG neuronal population following AAV-Leptin-GFP or AAV-GFP overexpression and T9 spinal dorsal column axotomy (Figure 30A). Stringent differential gene expression analysis (FDR<0.05) revealed 938 upregulated and 373 downregulated transcripts following leptin overexpression and DCA compared to GFP (Figure 30B). Analysis of the most significantly downregulated GO pathways ($p<0.01$) following leptin overexpression and spinal cord injury reveal a number of categories, including 'oxidation reduction process', 'translation', 'apoptosis' and 'catabolic proteolysis' (Figure 30C-D). For example, genes in the oxidation-reduction pathway included *Ndufb3,5,11*; NADH dehydrogenase subunit complexes forming the mitochondrial respiratory chain and *Gfer*, a sulfhydryl oxidase essential for disulphide bond formation and protein folding. Many ribosomal and mitochondrial proteins are also downregulated in the 'translation' category (*Mpl15*, *Rpl19*, *Rps2*), along with translational elongation factors essential for the early stages of protein synthesis (*Eef1a1*, *Eif2s2*, *Eif5a*). Downregulated processes impacted by leptin overexpression are varied and reflect cell homeostasis mechanisms favouring energy-conservative pathways. In particular, downregulation of translation and formation of new proteins at the 24-hour time- point post-injury.

Strikingly, the almost three-fold increase in upregulated versus downregulated transcripts (Figure 30B) suggest that leptin overexpression drives gene transcription. In line with this prediction, upregulated genes displayed a strong enrichment for several broad biological themes such as 'transcription', 'growth and development', 'phosphorylation' and 'chromatin remodelling' (Figure 30E-G), revealing a novel role of leptin in regulating transcription and chromatin remodelling following spinal cord injury. Upregulated genes ($p<0.01$) in the GO analysis are enriched for terms including 'regulation of transcription', 'RNA polymerase II activity', 'protein phosphorylation', 'mRNA processing' and 'RNA splicing' (Figure 30F). For example: the histone

acetyltransferase *p300*; the transcription factor *Sp1* involved in cell growth, neurite outgrowth and synaptogenesis; *Med25* the mediator complex which regulates transcription of RNA polymerase II- dependent genes; in addition to other activators, repressors and broad transcriptional regulators. KEGG pathways list pro-regenerative signalling cascades including 'MAP kinase', 'PI3K-AKT' ($p < 0.05$) and strong cell-receptor communication such as 'ECM- receptor interaction' (Figure 30E), indicating receptor- driven regenerative signalling is activated upon leptin overexpression and spinal cord injury. The KEGG analysis, similar to GO categories, highlights the importance of mRNA with pathways such as 'Spliceosome' and 'mRNA surveillance pathway' upregulated after injury, suggesting an active transcriptional environment. Detailed exploration of differentially expressed genes essential to transcriptional regulation revealed a high percentage of genes involved in either RNA processing or chromatin architecture and transcription (Figure 30G). Genes such as *Klf4*, *Jun* and *Sox2* were included within the 'transcriptional regulators' group, modulating transcription factor expression and gene activity in development and growth factor signalling. Chromatin architecture genes and remodeller examples included *Brd2* (Bromodomain containing protein 2; binds hyperacetylated chromatin to regulate transcription), *Smarcc2* (SWI/SNF complex subunit which alters DNA-nucleosome topology) and *Trim24* (Transcriptional coactivator that regulates cell proliferation and apoptosis). A range of epigenetic modifiers were also upregulated, encompassing both repressors (HDAC3,5,6,7 and polycomb complexes) and activators (*p300*~ HAT; *Yeats2*~ component of ATAC acetyltransferase complex; and *Sirt6*~ NAD-dependent sirtuin important for telomere maintenance and DNA damage). Several RNA polymerase II subunit components key for transcriptional elongation and termination were also significantly enriched. Collectively, data suggests leptin signalling activates dynamic epigenetic modifications to stimulate an open euchromatin conformation which facilitates an increase in RNA polymerase II activity, enhancing transcriptional elongation and subsequent downstream activation of regeneration associated genes. This offers exciting insight into how neuronal leptin either stimulated endogenously by IF or delivered therapeutically, can regulate the transcriptional landscape to enhance axonal regeneration in nerve injuries.

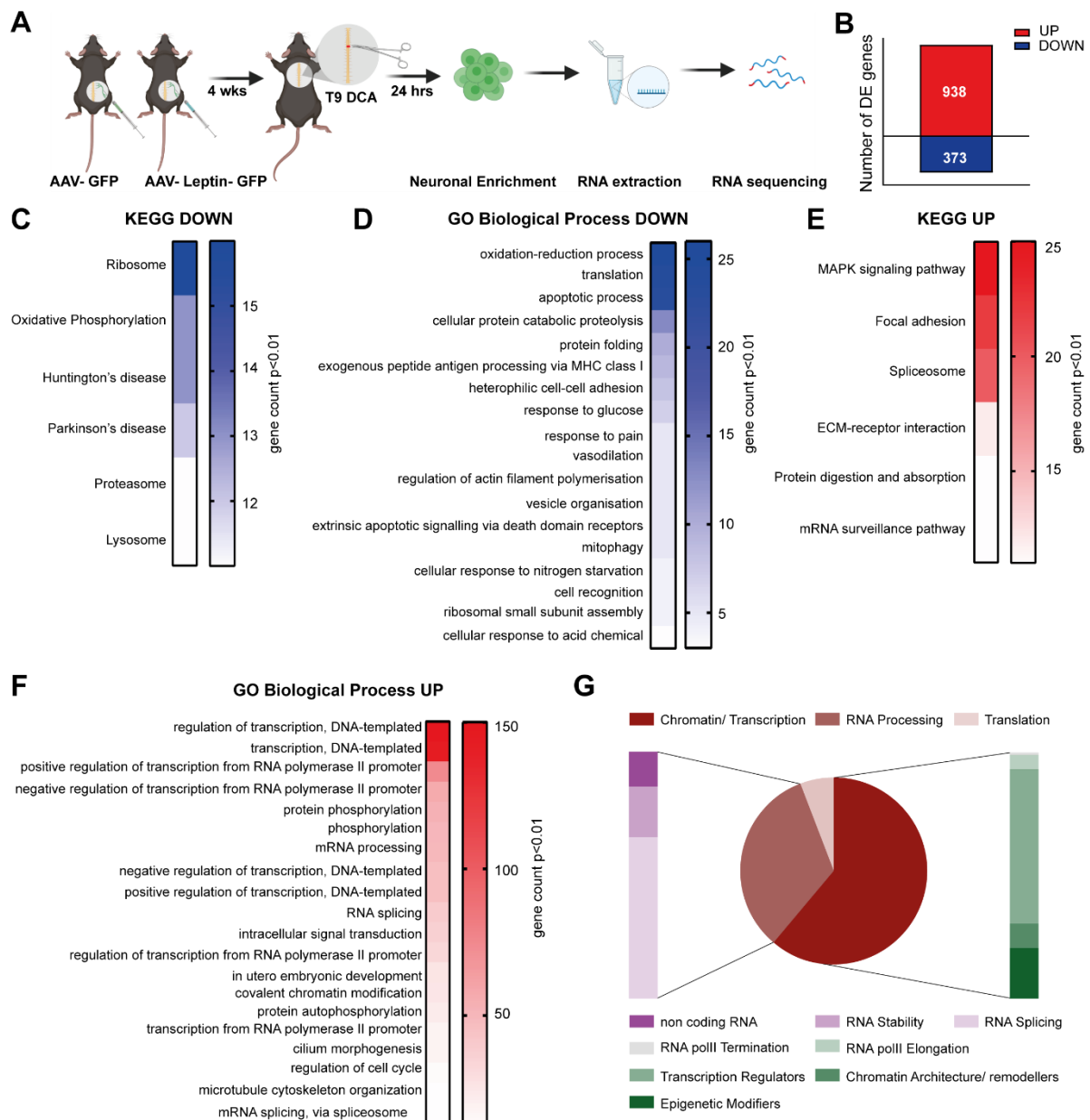


Figure 30: Leptin Signalling Modulates Transcription and Chromatin Remodelling.

A: Schematic of experimental procedure. RNA sequencing analysis from DRG neuronal enriched tissue following leptin overexpression and dorsal column axotomy compared to GFP controls (n = 4 mice per group).

B: Number of differentially expressed up and downregulated genes in AAV-Leptin-GFP + DCA dataset. 938 genes were upregulated following leptin overexpression and spinal cord injury and 373 genes were downregulated.

C: DAVID KEGG analysis sorted all significant genes (FDR<0.05). Heatmap visualising KEGG analysis of the most significantly downregulated genes. Shown are pathways graphed via gene count ($p < 0.01$). Blue indicates the most significantly downregulated pathways.

D: DAVID Bioinformatics Database GO analysis sorted all significant genes (FDR<0.05), encompassing three categories: biological process, molecular function and cellular component. Heatmap visualising GO analysis of downregulated genes categorised via gene count ($p < 0.01$). Blue indicates the most significantly downregulated genes.

E: Heatmap visualising KEGG analysis (FDR<0.05) of the most significantly upregulated genes. Shown are pathways graphed via gene count ($p < 0.01$). Red indicates the most significantly upregulated pathways.

F: Heatmap visualising GO analysis (FDR<0.05) of upregulated genes categorised via significance. Shown are pathways graphed via gene count ($p < 0.01$). Red indicates the most significantly upregulated genes.

G: Pie chart representation of upregulated genes from 'regulation of transcription, DNA templated' category. Genes fall into three main processes: chromatin/ transcription, RNA processing and translation. Further division of the RNA processing genes (purple) and the chromatin/ transcription genes (green) reveal the distribution and variety of functions these genes regulate.

5. DISCUSSION

5.1 Overview

Adult mammalian nerves possess limited regenerative capacity, with slow, insufficient fibre numbers that fail to reinnervate tissue targets and promote functional recovery. Injury independent environmental regulators of cell physiology such as exercise, environmental enrichment and diet can impact neuronal health and promote re-equilibrium following injury. Intermittent fasting regulates a wide range of metabolic cascades which influence longevity and upregulates neuroprotective stress protein chaperones and neurotrophic factors to improve neuronal survival after injury. Data has shown IF improves clinical outcomes in several neurodegenerative and cardiovascular diseases, including CMT1A neuropathies and spinal cord injury. Collectively, results indicated IF may exert not just a neuroprotective function but a regenerative one too. Mechanisms predominantly characterised activity within the extracellular space and intrinsic endogenous communications remain unanswered. The aim of this research was to investigate intrinsic IF- dependent regenerative signalling and explore novel application in peripheral and central neuronal injury.

In summary, 10 days of IF upregulated neuronal leptin expression within the DRG, maintained throughout the length of the IF regimen. Axonal injury amplified this expression further and activated downstream LepR signalling cascades. Deletion of the LepR inhibited regenerative outcomes mediated by IF, highlighting the importance of leptin signalling in the IF regenerative phenotype. Leptin delivery in neuronal culture and post-SNC leptin treatment both increased neurite outgrowth and regeneration, supporting the role of leptin as a promising therapeutic agent in the treatment of nerve injuries. Targeted overexpression of leptin within DRG neurons also enhanced regenerative outcomes in both the peripheral and central nervous system, facilitating fibre extension through the lesion core in the injured spinal cord. Investigation of leptin's impact on gene expression revealed leptin upregulated gene expression and epigenetic modifications after spinal cord injury, boosting RAG expression and regenerative pathways. Together, this data outlines a novel regenerative role of leptin following both peripheral nerve and central spinal cord injury.

5.2 Intermittent Fasting Promotes DRG Leptin Expression and Signalling

Intermittent fasting impacts multiple cell types and tissues within the body. The effect of IF on longevity, metabolic adaptation to energy deficient environments and immune-inflammatory inhibition are well characterised (Goodrick et al., 1982, Varady et al., 2010, Longo and Mattson, 2014, Patterson and Sears, 2017, de Cabo and Mattson, 2019, Hatchwell et al., 2020, Vasim et al., 2022). However, the relationship between diet, metabolism, intermittent fasting and nervous system injury is a developing area. The majority of experiments conducted in this field focus on functional recovery after spinal cord injury without further exploration into the regulatory cascades responsible for the IF- regenerative phenotype (Plunet et al., 2008, Plunet et al., 2010, Jeong et al., 2011). Recently, IF was shown to enhance axonal regeneration following sciatic nerve crush injury, increasing several gut microbial metabolites including indole-propionate acid, which in turn promoted nerve regeneration (Sergey et al., 2022). Intermittent fasting dynamically regulated the gut microbiome and subsequent synthesised metabolites to modify the nerve's response to injury. Indole-propionate acid actively recruited neutrophils into the DRG microenvironment to facilitate regeneration and epidermal reinnervation (Sergey et al., 2022). This exemplifies one mechanism of action within the extracellular injury space and recruitment of external cell types. However, the effect of IF on endogenous metabolic signalling pathways within DRG neurons was not investigated. Therefore, metabolic and signalling pathways needed for IF-dependent axonal regeneration remained unexplored. To understand the impact of IF upon gene expression, RNAseq after IF was performed (Figure 19A-H). As expected, IF downregulates cell adhesion, immune pathways, translation, tissue development and extracellular matrix reorganisation (Figure 19C-D). In line with previously published work (Johnson et al., 2007, Vasconcelos et al., 2014), IF regulates inflammatory- immune cascades, including cell adhesion proteins involved in these pathways. In response to IF, adipocytes secrete adiponectin which exerts anti-atherosclerotic and anti-inflammatory effects by preventing monocyte adhesion to endothelial cells (Malinowski et al., 2019, Vasim et al., 2022). Adiponectin also inhibits the release of vascular cell adhesion molecule 1 (VCAM-1), endothelial-leukocyte adhesion molecule 1 (ELAM-1), and intracellular adhesive molecule 1 (ICAM-1) to reduce local and systemic inflammation (Malinowski et al., 2019). Intermittent fasted states are typified by low levels of amino acids, glucose and insulin. Downregulation of the insulin–insulin-like growth factor 1 (IGF-1) signalling pathway

and reduction of circulating amino acids represses the activity of mTOR, resulting in inhibition of protein synthesis and stimulation of autophagy (de Cabo and Mattson, 2019). To sustain survival, the organism minimises energy-consuming processes (protein synthesis, growth and reproduction), to prioritise maintenance and repair systems, enhancing stress resistance and mitochondrial biogenesis (de Cabo and Mattson, 2019).

Unsurprisingly, upregulated gene ontology terms were predominantly metabolic processes such as lipid, steroid and cholesterol metabolism; all pathways which are modulated during IF (Varady et al., 2009, Mattson, 2012, Klempel et al., 2013, Tinsley and La Bounty, 2015, Longo and Panda, 2016). Upregulated terms such as 'chemical synaptic transmission' and 'cAMP signalling' also indicate cell-cell communication within and between neurons increases during IF, including a number of interesting genes involved in sensory neuron metabolic signalling such as NMDA, AMPA, cannabinoid, opioid, serotonin and dopamine receptor subunits. This corroborates previous data detailing that IF increases gene expression of key regulators of neuronal activity (Fontán-Lozano et al., 2007, Dasgupta et al., 2018, Kim et al., 2018) and metabolic regulation (Wan et al., 2003b, Qiu et al., 2012, Santos and Macedo, 2018). The extent to which neuronal signalling and synaptic formation contributes to axonal regeneration is unclear, conflicting data shows facilitation and inhibition of regeneration following induced neuronal and synaptic activity (Li et al., 2016, Tedeschi et al., 2016). Despite this, the link between metabolism and neuronal activity does indicate how IF might drive sensory neuron regeneration, supported by recently published work (Serger et al., 2022). This relationship is also reflected consistently throughout numerous gene ontology and KEGG categories, such as upregulation of metabolic genes (Eno2) which confer neuroprotective properties within CNS neurons (Figure 19F). Detailed exploration of the most upregulated genes identified the leptin gene with the largest differential fold change and significant FDR value (Figure 19G), present within multiple upregulated GO terms. Leptin is a known regulator of fasting cascades during IF (Ahima and Flier, 2000, Schulingkamp et al., 2000, Geary, 2004, Hommel et al., 2006), of which many of its downstream signalling mechanisms intersect with regenerative pathways to boost neuronal signalling (Elsaeidi et al., 2014, Wada et al., 2014, Ryu et al., 2019). It was possible leptin signalling mediated by IF enhanced endogenous neuronal metabolism and intracellular communication, however, the specific characterisation of leptin's role in repair and regeneration remained

unanswered. Consequently, subsequent experiments aimed to establish the role of IF-dependent leptin in regenerative signalling cascades and determine a novel role of leptin treatment in the context of nerve and spinal cord injuries.

Firstly, leptin's expression profile was characterised in both naïve and injury conditions during IF. Leptin is endogenously expressed within neurons and this expression increases following IF (Figure 20A-K). There are multiple endogenous sites of peripheral leptin production such as the stomach, placenta and bone marrow, including potentially within DRG tissue, though leptin's role at these various sites is unknown (Masuzaki et al., 1997, Bado et al., 1998, Chang et al., 2017b). In contrast, LepR expression did not change after IF. Only a small percentage (5- 25%) of LepR isoforms are present at the plasma membrane, ready to initiate leptin's downstream signalling cascades once bound (Gorska et al., 2010, Zhou and Rui, 2013). The majority of LepR subunits are localised in the golgi apparatus and endosomes, though the function of the intracellular LepR is unclear (Zhou and Rui, 2013). After ligand binding, the receptors are internalized into early endosomes via clathrin- coated vesicles before the receptor is degraded and recycled to the cell surface membrane (Gorska et al., 2010). It is possible most of the LepR-b remains on the cell surface membrane, internalised and replaced as needed to propagate cell-cell signalling. This may account for why neuronal LepR expression does not change following IF within the DRG.

Systemically, leptin levels within the body fall during IF as it is a satiety hormone (Figure 20K) (Boden et al., 1996, Weigle et al., 1997, Burnett et al., 2017). However, the inverse relationship is true within the DRG neurons, indicating leptin function within the DRG microenvironment is distinct from its role as a homeostatic regulator. Many of the downstream cascades activated by the leptin receptor are known regeneration associated genes, such as pSTAT3 (Schwaiger et al., 2000, Zhou and Too, 2011, Pellegrino and Habecker, 2013, Wada et al., 2014). Despite no change in LepR expression following IF, an increase in pSTAT3 was observed within neurons (Figure 20F-H), suggesting active leptin-LepR signalling. I hypothesised leptin signalling stimulated by IF functions as a mediator of regenerative signalling within neurons. This hypothesis is supported by data demonstrating IF prior to an injury condition increased DRG leptin expression and axonal regeneration following SNC (Figure 21A-N) (Serger et al., 2022). Both a day of fasting and 10 days of IF increase axonal regeneration. Previously established fasting regimens within the literature fluctuate between 30 days

up to a year, reflecting the dietary regimens commonly undertaken by humans (Moro et al., 2016, Anton et al., 2018, Malinowski et al., 2019, Tinsley et al., 2019, Nowosad and Sujka, 2021, Vasim et al., 2022). The regenerative difference between 10 days and 30 days of IF prior to SNC found no change in axonal regeneration (Serger et al., 2022) and subsequent experiments were undertaken with a 10 day IF regimen length.

The rapidity of a regenerative outcome even with a short fasting regimen of one day only suggests a cell signalling mechanism which generates effector outcomes in the cell within a matter of hours. G-protein coupled receptors with an internal signalling domain are specialised in rapid multi-pathway cascades once their external subunit is bound, co-ordinating a sensitive response to external stimuli (Rosenbaum et al., 2009, Amisten et al., 2015). The long isoform of the leptin receptor is comprised of both an extracellular subunit and an intracellular signalling domain which activates rapid downstream signalling cascades in response to leptin binding (Zhou and Rui, 2013, Wada et al., 2014). Interestingly, one day of fasting alone was not sufficient to significantly increase leptin expression within neurons (Figure 20I-J), but addition of an injury condition was (Figure 21C-E). It is possible that calcium influx from neuronal membrane rupture and retrograde transport of injury signalling molecules facilitates anterograde transport of mitochondria to the injury site which may aid local neuronal leptin synthesis and secretion (Lee and Fried, 2009, Tao et al., 2014, Wang et al., 2021). Mechanisms activated in injury repair may help to propagate leptin signalling. Neurons also express the LepR on their surface membranes. Active synthesis and secretion of neuronal leptin instigated by IF and boosted upon nerve injury may signal in an autocrine and paracrine manner to enable a rapid leptin signalling response between multiple neuronal subtypes within the DRG. For example, leptin stimulates immune cells required for atherosclerosis to release proinflammatory cytokines, which in turn form a feedback loop by promoting leptin synthesis *in vivo*, suggesting leptin exerts an autocrine/ paracrine mode of action (Sarraf et al., 1997, Schneiderman et al., 2012). Additional studies have also shown leptin modulates adventitial pericytes and reproductive processes via autocrine/paracrine signalling (González et al., 2000, Riu et al., 2017). The precise mechanisms as to how IF regulates leptin signalling remain of interest.

In summary, IF regulates metabolic pathways and leptin signalling, enhancing axonal regeneration after SNC injury. However, intermittent fasting modulates numerous factors which may influence axonal regeneration in isolation of leptin, such

as neurotrophic factors, metabolites and insulin. These factors may also stimulate pSTAT3 expression in addition to leptin. To establish the centrality of leptin signalling within this regenerative phenotype, endogenous leptin signalling was inhibited.

5.3 Leptin Receptor Deletion Attenuates Pro-Regenerative Leptin Signalling

Leptin exerts its effects via its receptor (LepR), encoded for by the LepR gene (*db*, diabetes gene) on chromosome 1p31.3 in humans (Zhou and Rui, 2013). The LepR belongs to the class I cytokine receptor family expressed in six isoforms due to alternate RNA splicing of the *db* gene (Gorska et al., 2010). The isoforms are divided into three classes, long, short and secretory. The short isoforms (OB- Ra, Rc, Rd) bind JAK kinases and activate signal transduction cascades associated with leptin internalisation and degradation (Uotani et al., 1999, Frühbeck, 2006). The soluble isoform (OB- Re), regulates serum leptin concentrations and serves as a carrier protein delivering leptin to its membrane receptors (Tartaglia et al., 1995, Cioffi et al., 1996, Barr et al., 1999, Chan et al., 2002), or binds leptin to inhibit signal transduction pathways (Gorska et al., 2010). All isoforms contain identical extracellular domains but only the long isoform (LepR-b or OB-Rb) contains an intracellular domain capable of propagating downstream cell signalling (Figure 31) (Wada et al., 2014). Activation of the LepR-b phosphorylates Janus kinase 2 (JAK2), initiating multiple signalling pathways such as janus- kinase- signal transducer activators of transcription (JAK2-STAT3/5), the protein tyrosine phosphatase 2- extracellular signal- related kinase pathway (SHP2-ERK) and the insulin receptor substrate- phosphoinositide 3- kinase signal transduction pathway (IRS-PI3K) (Zhou and Rui, 2013). Leptin binds with high affinity to the LepR homodimer, activating JAK2 which phosphorylates tyrosine residues (Tyr 985, 1077, 1138 and 1185) on the intracellular domain of the LepR (Wada et al., 2014). These phosphorylated tyrosine's serve as binding sites for signalling molecules containing a Src homology 2 (SH2) domain, enabling JAK2 to phosphorylate and activate them once bound (Zhou and Rui, 2013). For example, the SH2 domain of STAT3 binds at tyrosine residue 1138 where it is phosphorylated by JAK2, before pSTAT3 then dimerises and is translocated to the nucleus, where it regulates expression of target genes as a transcription factor (Vaisse et al., 1996, White et al., 1997, Zhou and Rui, 2013). Disruption of the STAT3 binding site via mutation of Tyr¹¹³⁸ triggers hyperphagia and obesity similar to *db/db* mice (Bates et al., 2003, Jiang et al., 2008), demonstrating the JAK2-STAT3 pathway is necessary for

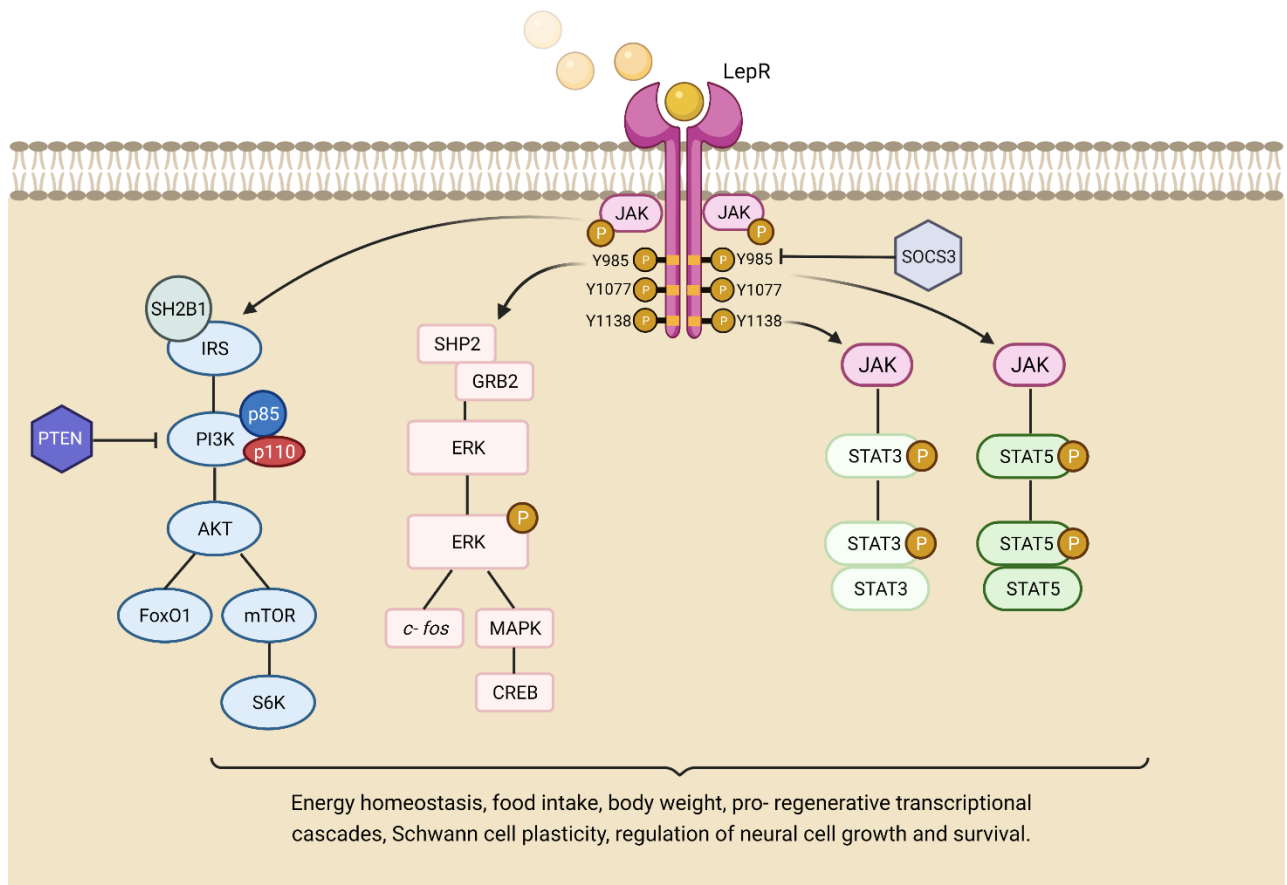


Figure 31: Intracellular Leptin Signalling

Leptin binds to the long isoform of the leptin receptor (LepR-b) and activates Janus kinase 2 (JAK2), which phosphorylates the LepR at multiple tyrosine sites: Tyr985, Tyr1077 and Tyr1138. Phospho-Tyr985, -Tyr1077 and -Tyr1138 bind intracellular signalling molecules and activate the JAK2/STAT3, JAK2/STAT5, PI3K/IRS/AKT, and SHP2/ERK pathways. These pathways regulate energy homeostasis, body weight, cell growth and regenerative mechanisms. Negative feedback regulation of LepR signalling by SOCS3 and PTEN prevents aberrant leptin signalling. Figure created in BioRender.

leptin's anti-obesity effects. Several signalling cascades are activated at these tyrosine sites; Tyr¹⁰⁷⁷ activates STAT5 (Gong et al., 2007, Mütze et al., 2007), which if activated in hypothalamic neurons suppresses food intake in mice (Lee et al., 2008); and Tyr⁹⁸⁵ binds the SH2 domain of SHP2 (Bjørnbæk et al., 2001, Rahmouni et al., 2009), stimulating the ERK pathway important for leptin's anorexigenic effects and initiation of brown adipose tissue thermogenesis (Rahmouni et al., 2009). Stimulation of the SHP2-ERK signalling pathway in turn activates mitogen-activated protein kinase (MAPK), a pathway which initiates apoptosis (Kim et al., 2003). Phosphorylation of tyrosine 985 also activates suppressor of cytokine signalling family 3 (SOCS3), which

suppresses LepR-b/ JAK2 pathways in a negative feedback loop to downregulate leptin action (Bjørnbæk et al., 2001). Equally, the IRS- PI3K pathway is required for leptin action (Jiang et al., 2008); pharmacological inhibition of hypothalamic PI3K prevented leptin- induced anorexia in mice (Niswender et al., 2001). Downstream effectors of the PI3K pathway are also regulators of eating behaviour, including Akt stimulation of forkhead box O1 (FoxO1) and the mammalian target of rapamycin (mTOR)- ribosomal S6 kinase (S6K) pathway (Morris and Rui, 2009). FoxO1 is a key transcription factor for gluconeogenesis (Kim et al., 2006), inhibited via Akt phosphorylation to prevent increased food intake upon leptin binding its receptor (Taniguchi et al., 2006). In contrast, leptin stimulates the mTOR complex 1, to activate S6K in hypothalamic neurons to protect against obesity (Cota et al., 2006, Blouet et al., 2008, Maya-Monteiro and Bozza, 2008). Collectively, these cascades all contribute to energy regulation and the reduction of feeding behaviour following leptin signalling. Only deletion of the LepR-b isoform gives rise to an *ob/ob* phenotype, suggesting this isoform and its downstream intracellular cascades are key in mediating the action of leptin (Wada et al., 2014).

Intermittent fasting alters synthesis and circulation of anorexigenic hormones such as leptin within the body, influencing leptin signalling and potentially, regenerative LepR signalling pathways. Deletion of the LepR prior to IF inhibited axonal regeneration (Figure 22A-E), indicating that leptin and the downstream cascades activated by the LepR are essential for IF-dependent regenerative impact. Many of the LepR- b signalling cascades are established contributors in regenerative mechanisms. The JAK- STAT pathway is a key mediator of both peripheral and central axonal regeneration. Following axotomy in rat facial and hypoglossal neurons, activated STAT3 protein was identified within three hours in neurons and after one day in astrocytes, remaining activated until the time of reinnervation (Schwaiger et al., 2000). These pSTAT3 levels were detectable for more than three months and consequentially thought to be involved in the switch from a physiological gene expression program to an alternative 'regenerative' program following injury (Schwaiger et al., 2000). This is supported by data demonstrating axonal regeneration in the spinal cord following a conditioning lesion requires activation of the JAK-STAT cascade, as regeneration was significantly attenuated in the adult dorsal column following infusion of the JAK2 inhibitor AG490, suppressing STAT3 phosphorylation (Qiu et al., 2005). Leptin also regulates expression of other STAT3 target genes in the hypothalamus, including c-

fos and c-jun, subunits of the AP-1 transcription factor required for successful axonal regeneration (Raivich et al., 2004, Arthur-Farraj et al., 2012, Ruff et al., 2012). Following injury, upregulation of STAT3 and subsequent transcriptional programs may sensitise the neuron to various cytokines and growth factors, to modulate the immuno-inflammatory response at the lesion site and aid regeneration (Schwaiger et al., 2000, Pellegrino and Habecker, 2013, Elsaiedi et al., 2014). STAT3 also integrates cytokine and neurotrophin signals to promote sympathetic axonal regeneration via regulation of microtubule dynamics and mitochondrial ROS production (Zhou and Too, 2011, Selvaraj et al., 2012).

Several other LepR signalling pathways also contribute to regenerative outputs. The SHP2- ERK pathway regulates synaptic plasticity and memory; ROS production in macrophages; phosphorylation of the Grainy head transcription factor essential for wound healing; and controls Schwann cell plasticity and peripheral nerve regeneration *in vivo* (Kim and McGinnis, 2011, Napoli et al., 2012, Li et al., 2015, Ryu et al., 2019). Downstream stimulants of this pathway, such as MAPK, also coordinate regenerative responses (Agthong et al., 2009, Nix et al., 2011, Gerdtts et al., 2016). The PI3K- Akt pathway is anti- apoptotic, shown to enhance the regenerative properties of the cytokine erythropoietin and stimulate neurite outgrowth *in vitro* (Kretz et al., 2005, Park et al., 2015). In a model of rat facial nerve axotomy, PI3K- Akt signalling improved neuronal survival, axonal growth and axonal branching (Huang et al., 2017). Subsequent Akt stimulation of mTOR propagates these pro-regenerative effects, regulating neuronal cell growth, proliferation, metabolism, motility and survival (Ma and Blenis, 2009, Laplante and Sabatini, 2012, Yang et al., 2014). Collectively, activated LepR pathways key to systemic regulation of energy homeostasis, overlap substantially with regenerative repair mechanisms. Despite this, the impact of leptin treatment in nerve injury is poorly understood.

The addition of leptin to AL neuronal DRG cultures increased outgrowth but did not have an additive effect in IF conditions, indicating leptin drives the outgrowth response observed following fasting (Figure 23A-D). This is supported by previous literature which has also shown leptin stimulates axonal growth of both adult and neonatal sympathetic neurons *in vitro* via STAT3, suggesting leptin enhances sympathetic nerve innervation (Pellegrino et al., 2014). Addition of leptin to *in vitro* neuronal cultures enhanced neurite outgrowth via STAT3 activation in control and gp130^{-/-} cultures, acting as a cytokine in gp130^{-/-} deficient neurons (Quarta et al., 2014). This shows

leptin acts as a repair agent, enhancing outgrowth and the number of neurite-bearing cells (Quarta et al., 2014). These results were recapitulated *in vivo*, where post-injury leptin treatment increased axonal regeneration compared to saline controls (Figure 24A-G). A detailed expanse of literature has trialled several therapeutic leptin regimens. Physiological serum leptin concentrations in the adult mouse peak at 4ngmL^{-1} following feeding and drop to 1.5ngmL^{-1} during fasting (Ahima et al., 1996). The majority of experiments test leptin delivery at $20\text{ }\mu\text{g/day}$ (Ahima et al., 1996), $12\text{ }\mu\text{g/day}$ (Halaas et al., 1997) and $10\text{ }\mu\text{g/day}$ (Harris et al., 1998), though therapeutic studies can range from $3\text{-}250\text{ }\mu\text{g/day}$ (Campfield et al., 1995, Halaas et al., 1995, Pellemounter et al., 1995, Weigle et al., 1997, Burnett et al., 2017). Dose-response studies show very high doses cause excessive intermittent elevation of serum leptin concentration, but found that $20\mu\text{g/day}$ exerts a significant physiological outcome without side-effects (Harris et al., 1997, Harris et al., 1998). Though the choice of $20\text{ }\mu\text{g/day}$ of leptin is far above the natural circulating level, it was sufficient to garner a rapid physiological outcome within a short regenerative timeframe for this proof-of-principle experiment, without adverse side effects and in keeping with previous dose regimens cited in the literature. We found that $20\text{ }\mu\text{g/day}$ of leptin delivered via intraperitoneal injection significantly enhanced axonal regeneration after injury. The leptin-regeneration phenotype was again lost following LepR knock-down, underscoring the importance of the receptor in mediating the regenerative response (Figure 24A-G). These results suggest that circulating serum levels of leptin need to operate through its receptor in neurons to enhance sensory nerve regeneration. It is interesting to speculate on the relationship between serum leptin and neuronal leptin and a potential autocrine/paracrine mechanism of activation (Sarraf et al., 1997, González et al., 2000, Schneiderman et al., 2012, Riu et al., 2017). Gene therapy to enable targeted neuronal treatment without impacting the systemic leptin levels may be the best approach to avoid unwanted side effects such as extreme weight loss. For example, obese rats with high circulating leptin levels were found to have increased axon growth and sympathetic hyperinnervation of the heart compared to rats fed normal chow (McCully et al., 2013).

These results demonstrate IF initiates regenerative leptin signalling mediated via the LepR. These mechanisms are also stimulated following systemic leptin treatment post-injury, suggesting leptin may offer translational therapeutic opportunities in nerve

injury. Outstanding questions regarding targeted nervous system leptin delivery were addressed in subsequent experiments.

5.4 Leptin Overexpression Enhances Axonal Regeneration in the PNS and CNS

The expression of leptin and its receptor is predominantly contextualised within leptin's role as a regulator of energy homeostasis. The LepR is expressed extensively throughout the brain, including the somatosensory cortex, hippocampus, substantia nigra pars compacta and hypothalamus (dorsomedial, lateral, ventromedial, arcuate nucleus and ventral pre-mammillary nucleus), where leptin mediates regulatory control over POMC neurons and eating behaviour (Huang et al., 1996, Schwartz et al., 1996, Elmquist et al., 1998, Burguera et al., 2000, Zhou and Rui, 2013). However, leptin is also expressed in peripheral tissues including the stomach, mammary glands, placenta, skeletal muscle, heart and kidney (Masuzaki et al., 1997, Bado et al., 1998, Smith-Kirwin et al., 1998, Morash et al., 1999, Wilkinson et al., 2000, Tessier et al., 2013, Feijóo-Bandín et al., 2015), though the function of endogenous peripheral leptin at different synthesis sites remains unclear. It is possible leptin exerts a unique function within peripheral tissues, juxtaposed to its role as an energy homeostasis regulator. The data previously described points toward a novel role of leptin in axonal outgrowth and regeneration.

The relationship between leptin and the nervous system is a developing field. For instance, leptin signalling is implicated in several diseases; its neuroprotective effects in CNS diseases such as Alzheimer's and Parkinson's are reviewed extensively elsewhere (Signore et al., 2008, Tang, 2008, Davis et al., 2014, Li et al., 2016, Fujita and Yamashita, 2019). Leptin also regulates neurogenesis in the developing CNS, synaptogenesis and neuronal excitability (Bouret, 2010, Paz-Filho et al., 2010, Arnoldussen et al., 2014, McGregor and Harvey, 2018); and is implicated in enhancing neuronal plasticity. Neural plasticity refers to the ability of a neuron or the brain to adapt to external or internal stimuli, via cellular events such as cell migration, cell survival and modification of mature synapses (Pilar-Cuéllar et al., 2013, Liu et al., 2017, Ge et al., 2018). Leptin was reported to modulate hippocampal transmission synaptic efficacy, long- term potentiation, excitatory synaptic strength and cell proliferation (Harvey, 2013). Peripheral leptin injections in mice also decreased stress- evoked glutamate release in a tail- suspension test (Wang et al., 2015). Together, these studies demonstrate leptin has broad effects on aspects of neural plasticity,

proliferation and protection, reflected in its abundant expression locations. However, the interaction between systemic circulating leptin and endogenous neuronal leptin remained unclear. Leptin overexpression in neuronal cultures increased axonal outgrowth and when overexpressed specifically within DRG neurons, increased axonal regeneration after both a peripheral sciatic and central spinal injury (Figure 25-29), demonstrating that intrinsic neuronal leptin propagates regenerative outcomes. Supporting literature has shown leptin is an important component in the wound healing processes (Murad et al., 2003). Leptin expression was steadily upregulated in skin tissue, blood vessel walls and fibroblasts following incisional wounds, serving as an autocrine/paracrine regulator of normal wound healing (Murad et al., 2003). Treatment with neutralising anti-leptin antibodies slowed healing progression, preventing wound closure and contraction (Murad et al., 2003).

Besides leptin's neuroprotective and wound healing effects, studies have also identified endogenous leptin expression within un-lesioned spinal cords (Fernández-Martos et al., 2012) and pseudo-unipolar neurons of the dorsal root ganglia (Chang et al., 2017b). Following preganglionic cervical root avulsion in C57BL6 mice, a large increase in leptin and LepR expression was observed in DRG neurons (Chang et al., 2017a). The significance of this *de novo* production of neuronal leptin was not investigated at the time but establishing the purpose of intraneuronal leptin will positively impact the outcome of future CNS diseases and injuries. I found that neuronal leptin overexpression enhances axonal regeneration in the dorsal column of the spinal cord (Figure 28), indicating that local production of leptin in neurons is key to neuronal repair after injury.

Interestingly, leptin and the LepR are also expressed in the non-neuronal glial cells of the CNS, including astrocytes, microglia and oligodendrocytes (Fujita and Yamashita, 2019), possibly contributing to modification of the inhibitory growth environment surrounding the neuron after injury. Leptin is reported to affect cytokine production in microglia and macrophages, stimulating the LepR- b isoform to induce interleukin 1 β production via STAT3 activation (Pinteaux et al., 2007, Tang et al., 2007, Lafrance et al., 2010). In a partial sciatic nerve ligation model (Seltzer et al., 1990), there was an increase in leptin expression in adipocytes and in the epineurium of the injured sciatic nerve (Maeda et al., 2009). This study found macrophages were recruited to the perineurium of the sciatic nerve, expressing the LepR-b; leptin then stimulated these macrophages via the JAK-STAT pathway to aid debris clearance at

the lesion site (Maeda et al., 2009). In contrast, another study reports leptin signalling reduced microglial and macrophage activation, contributing to functional recovery in a rat SCI (Fernández-Martos et al., 2012). Following a moderate contusion SCI, leptin and its receptor was upregulated in neuronal and glial cells (Fernández-Martos et al., 2012). An intraparenchymal leptin injection enhanced expression of neuroprotective genes, reduced inflammation and significantly improved sensory and motor functional outcomes after SCI, decreasing the area of Iba-1 positive microglia and macrophages (Fernández-Martos et al., 2012). However, some studies show high leptin concentrations facilitate neuropathic pain development following injury (Seltzer et al., 1990, Lim et al., 2009, Chang et al., 2017a, Inoue and Tsuda, 2018); its therapeutic role in injury repair therefore requires further exploration. For example, leptin-controlled inflammatory modulation in microglia has diverse effects which will be more clearly established with future cell- type specific leptin deletion studies. Staining of spinal cord tissue also demonstrated LepR-b protein expression in oligodendrocyte precursor cells (Matoba et al., 2017), of which expression increased following SCI (Fernández-Martos et al., 2012). Leptin treatment following SCI also enhanced myelin preservation caudal to the lesion site, indicating leptin signalling in oligodendrocyte lineage cells may contribute to cell and tissue repair after injury (Fernández-Martos et al., 2012, Fujita and Yamashita, 2019).

Altogether, current literature has demonstrated leptin enhances repair mechanisms after spinal cord injury and exerts neuroprotective effects following peripheral nerve injury, focusing heavily on neuroimmunology and extrinsic wound-healing responses. Recent data now clarifies the impact of intracellular leptin signalling in facilitating axonal regeneration and downstream effector mechanisms in both peripheral and central nervous system injury. One of the remaining outstanding questions is *how* leptin drives these regenerative outcomes within the neuron.

5.5 Regenerative Leptin Signalling Modulates Transcription and Chromatin

Remodelling

To ascertain the intrinsic molecular mechanism underpinning the regenerative response following leptin overexpression and spinal cord injury, the neuronal transcriptome was investigated 24-hours after injury (Figure 30). Downregulated transcripts were varied, encompassing biological processes such as redox reactions, translation and apoptosis. Oxidation- reduction processes, or redox reactions, occur

concurrently with the loss or gain of electrons. Many biological processes within the cell including enzymes and organelles produce reactive oxygen species (ROS), small volatile molecules which are formed by the partial reduction of oxygen (Kim et al., 2015, Hervera et al., 2018). Mitochondria for instance, are a large contributor of ROS via the electron transport chain (Ray et al., 2012). Classically characterised as triggers for oxidative stress and tissue damage in many disease processes such as Huntington's and Parkinson's, NOX-dependent ROS production also function as signalling molecules in the immune-inflammatory response which proves beneficial for axonal regeneration (Bouloumié et al., 1999, Chetboun et al., 2012, Hervera et al., 2018). The biological context, location and timing of ROS response can be either beneficial or detrimental in injury and disease cases. In this instance, leptin downregulates oxidation- reduction processes 24-hours after injury (Figure 30D), potentially reducing tissue damage in the early injury phase. Leptin has also been shown to reduce apoptosis and modulate immune components during the immuno-inflammatory response, downregulating cytokine and chemokine release by certain cells of the innate and adaptive immune system (Mancuso et al., 2001, Zarkesh-Esfahani et al., 2004, Johnson et al., 2007, Pérez-Pérez et al., 2017). Many of these immune pathways are present within disease processes, reflected in the KEGG categories displayed. Most strikingly however, downregulation of translation, ribosomal activity and protein processes occurs immediately after injury, reflecting biological re-prioritisation away from energy-intensive processes to energy-conservative pathways (Bisby and Tetzlaff, 1992, Gummy et al., 2010). Leptin was also shown to downregulate genes involved in protein catabolic proteolysis, the breakdown of proteins into smaller peptides during cellular maintenance, injury conditions and apoptosis. Successful regeneration-permissive environments promote local protein production after nerve injury (Gummy et al., 2010, Jung et al., 2012). It is possible that directly after injury leptin prepares an active transcriptional landscape by promoting gene expression and euchromatin, pausing protein activity whilst the cell focuses on mRNA production. Later timepoints may show an increase in protein synthesis as these transcripts are subsequently translated into proteins used to facilitate the repair processes.

The majority of gene transcripts were upregulated following leptin overexpression, showing a strong enrichment for GO pathways such as 'transcription', 'growth and development', 'phosphorylation' and 'chromatin remodelling'. Leptin overexpression

in the context of spinal cord injury upregulated many epigenetic modifiers to encourage euchromatin and enhance the activity of RNA polymerase II, transcriptional elongation and RAG expression. Epigenetic modifications alter gene expression without changes to the DNA code, encompassing changes in DNA accessibility (de/methylation), chromatin dynamics (posttranslational histone modifications) and mRNA regulation (microRNAs) (Loscalzo and Handy, 2014, Aragonès et al., 2016). The majority of epigenetic modifications observed after leptin treatment are dynamic posttranslational histone modifications (Figure 30), though leptin is also known to influence global DNA methylation levels in studies using *ob/ob* mice (Kamei et al., 2010, Sonne et al., 2017, You et al., 2017). Histone acetylation is associated with gene activation. Histone H3 acetylation at lysine residues 9, 14, 18, and 27 (H3-K9/14/18/27 ac) and H4 acetylation for example, are all hallmarks of transcriptional activation and are often found in active promoter regions around the transcription start site (Lindner et al., 2013). Whereas histone methylation alters gene-outcomes based on the degree of methylation (mono, di or tri) and location of the methyl lysine residues (Greer and Shi, 2012). For instance, H3K4me2/3 and H3K27me2/3 are active transcriptional marks but H3K9me3, H3K27me3 and H4K20me3 repress gene expression (Wang et al., 2008, Bannister and Kouzarides, 2011, Greer and Shi, 2012, Farooq et al., 2016). Epigenetic modifications upregulated in the dataset contained a range of active gene markers (*Ep300*, *Rnf111*, *Sat1*, *Yeats2*) and repressive markers (*HDAC5-7*, *Kdm6b*, *Pcgf1*, *Sirt6-7*) (Figure 30). Research has shown leptin treatment increases HDAC5 expression in the hypothalamus of leptin-deficient *ob/ob* mice and knockout of HDAC5 caused mice to be more prone to diet-induced obesity (Kabra et al., 2016). However, neuronal HDAC5 nuclear export is required to enable transcriptional activation and launch of the intrinsic regenerative program in regenerating neurons (Cho et al., 2013). Leptin-resistant *ob/ob* and *db/db* mice also exhibited reduced expression of the HDAC Sirtuin 1 (SIRT1), which was upregulated upon leptin-induced Nrf2- dependent signalling (Song et al., 2018). However, it is difficult to extrapolate whether leptin treatment in clinically deficient models would exert similar effects in healthy control mice or different neuronal types. The context of different cell types is essential for understanding the epigenetic regulatory balance. The field of leptin-induced epigenetic modifications as a whole is heavily under-researched and a substantial area for future investigation in the context of injury conditions and metabolic disorders.

Many epigenetic modifications on histone tails are modulated by enzymes found coupled with other chromatin modifiers in large multiprotein complexes, where they can also operate as transcriptional co-activators (Esau et al., 2004, Milagro et al., 2009). A common pairing is KAT2B (lysine (K) acetyltransferase 2b), also known as CBP/P300-associated factor or PCAF (Lindner et al., 2013). Neurotrophic signalling in pro-regenerative conditions induces phosphorylation and nuclear translocation of PCAF with H3-K9 acetylation, in neuronal-like PC-12 cells during neurite outgrowth (Wong et al., 2004). Both the CBP and p300 mRNA transcripts were upregulated following leptin overexpression and DCA, in addition to several chromatin modifiers such as bromodomain proteins, condensing complex subunits, DNA helicase and SWI-SNF complex subunits (Figure 30). Interestingly, the PCAF–CBP/P300 complex interacts with transcriptional activators, such as FOS, JUN, MYB, HIF1A, TRP53, TRP53 and KLF13, to enhance promoter accessibility of RAGs essential for axonal regeneration including RAB13, CORO1B, and GAP43 (Sternier and Berger, 2000, Di Giovanni et al., 2006, Tedeschi et al., 2009, Gaub et al., 2010). Research has also shown that energy- regulation hormones such as insulin and leptin, recruit chromatin remodelling complexes important for neuronal mammalian development to promote target gene expression (Lee et al., 2007, Son and Crabtree, 2014, Sokpor et al., 2017). This is reflected in the dataset showing upregulation of the SWI-SNF complex following leptin overexpression. Other examples also include the BRG1/ BRM-associated factor (BAF) chromatin remodelling complexes required for neurogenesis and differentiation recruited by energy homeostasis hormones (Wang et al., 2013, Hota et al., 2019). Mutations in these complexes cause neurodevelopmental deficits, epilepsy and lead to loss of dendrites in human neurons (Bell et al., 2019), underscoring the importance of chromatin remodelling in neuronal functioning. Epigenetic and chromatin modifications enhance neuronal RAG expression, many of which were upregulated within the RNAseq dataset following leptin overexpression. For example, classical RAGs such as KLF4, c-JUN, ERK and FOS were all upregulated. Similarly, downstream RAG derivatives or RAG complex cofactors were also upregulated such as BIN1 and MYCBPAP, both of which are interacting and association proteins of the well characterised RAG MyC. Additional immunofluorescence staining for key RAGs within DRG tissue following LepGFP overexpression and DCA would help to validate some of the RAG targets identified with RNAseq. Collectively, leptin overexpression in the context of spinal cord injury activates a diverse range of epigenetic modifiers and

chromatin remodelling complexes to facilitate RAG expression and boost axonal regeneration.

An increasing body of literature has also shown that microRNA (miRNA) interference, short non-coding RNAs, are capable of regulating the expression of hundreds of mRNAs, widely impacting the transcriptome and proteome (Catalanotto et al., 2016, Wróblewski et al., 2019). MicroRNAs influence alternate splicing and are abundant in the nervous system, suggesting they may be particularly important in neuronal cells (Kiryu-Seo and Kiyama, 2011, Wróblewski et al., 2019). Research in the fields of neurogenesis, plasticity, and neurodegeneration have all identified miRNA interactions (Eacker et al., 2009, Siegel et al., 2011). Complex miRNA networks synergistically control axon regrowth, inflammation, apoptosis and myelination, altering their expression in DRG neurons after sciatic nerve and spinal cord injury (Zhang et al., 2011a, Ghibaudi et al., 2017, Van Battum et al., 2018). Leptin overexpression in DRG neurons significantly upregulated miRNA transcripts and genes involved in miRNA regulation such as: *Dalrd3*, *Kcnq1ot1*, *Neat1*, *Tnrc6a*, *Ddx17* and *Pan2* (Figure 30). The impact of leptin on miRNAs has been studied in several sites throughout the body; a potent regulator of miRNA expression in tissues affected by obesity, energy homeostasis and adipogenesis (Derghal et al., 2015, Zhang et al., 2017). For example, leptin controls miRNAs which regulate POMC neurons in the hypothalamus during metabolic imbalance (Derghal et al., 2015). Studies have also shown leptin administration in adult male rats during fasting or AL feeding differentially expressed several hypothalamic miRNAs (miR-132, miR-145, miR-30e, miR-132) and stimulated hippocampal synaptogenesis via leptin induced CREB phosphorylation (Dhar et al., 2014, Sangiao-Alvarellos et al., 2014). This data supports the role of leptin miRNA regulation in synaptic plasticity and enhancing neuronal regeneration, though it has wide-ranging impacts in multiple metabolic disorders, cancer and cardiovascular diseases (Dattaroy et al., 2015, Meerson and Yehuda, 2016). A strong interplay exists between epigenetic-chromatin modifications and miRNA regulatory mechanisms, forming a constantly evolving, sensitive feedback loop to regulate gene expression.

In combination, epigenetic, miRNA and chromatin conformation modifications converge to regulate gene expression and gene accessibility. Leptin in turn, also directly regulates transcriptional elongation and RNAPol2 activity (Figure 30). Messenger ribonucleoproteins (mRNPs) are created via a three-step process: first, the

transcription of a DNA template by RNA polymerase II (RNAPol2) to make pre-mRNA; second, the maturation of the pre-mRNA by processing factors; and third, packaging of the mature mRNA with proteins to make a particle competent for export to and translation in the cytoplasm (Bentley, 2014). The stages of transcription and processing occur simultaneously in living cells to enhance efficiency and accuracy of pre-mRNA maturation (Bird et al., 2004). This enables novel interactions with regulatory implications, including communication between splicing and chromatin modifications (Kolasinska-Zwierz et al., 2009, Bieberstein et al., 2012, Gómez Acuña et al., 2013). GO and KEGG analysis show a large portion of upregulated genes involved in the spliceosome and alternate splicing following spinal cord regeneration induced by leptin overexpression (Figure 30). This indicates leptin exerts modulatory effects on the spliceosome, diversifying mRNA transcripts and subsequent protein products required for axonal regeneration. The co-transcriptional nature of cells has shown that transcription initiation and elongation factors influence capping (Chiu et al., 2002), splicing (Monsalve et al., 2000, Benson et al., 2012, Huang et al., 2012) and 3' end formation (Rosonina et al., 2003). Conversely, spliceosome processing factors are also implicated as effectors of transcriptional initiation and elongation (Lenasi et al., 2011, Ji et al., 2013). Detailed exploration of upregulated transcription genes following neuronal leptin administration revealed a large increase in transcriptional elongation and RNAPol2 activity, suggesting a novel role of leptin in transcriptional regulation. The transcription elongation rate determines the delay between the appearances of upstream and downstream elements in the nascent pre-mRNA, which might compete with one another for RNA-binding and processing factors (Bentley, 2014). Should leptin enhance the elongation rate, this would enable a consistent increase in mRNA editing and production without increased competition for these factors.

In summation, at the level of the nucleus leptin impacts transcription factors, epigenetic mechanisms (posttranslational histone modifications, miRNA, DNA methylation) and transcriptional regulation via control of splicing and RNAPol2 activity. Intracellular LepR cytoplasmic signalling also activates regenerative molecules MAPK, PI3K and AMPK to initiate the intrinsic regenerative program (Moore and Goldberg, 2011, Saito and Cavalli, 2016, Kong et al., 2020). Many of these mechanisms regulate leptin expression in turn; discerning the differences between target and mediator will uncover many more exciting applications of leptin within the nervous system.

5.6 The Diverse Impacts of Leptin

This research has outlined how leptin signalling has enhanced axonal regeneration in the peripheral and central nervous system via modulation of epigenetic modifications, transcriptional control and activation of the intrinsic regenerative programme. However, leptin and its receptor are expressed at numerous sites and tissues throughout the body, activating a range of cellular cascades and additional extrinsic mechanisms which influence regenerative outcomes. Leptin is known to regulate the injury- site immune response following spinal cord injury for example, aiding growth and tissue repair (Maeda et al., 2009, Fernández-Martos et al., 2012, Fujita and Yamashita, 2019). This is in addition to broader physiological processes such as metabolic interaction with other energy homeostasis hormones; impact on the gut microbiome and synthesised metabolites; and modulation of feeding behaviour. In combination, the activation of intrinsic regenerative pathways together with extracellular contributors gives rise to a robust regenerative outcome (Figure 32). A summary of these processes are outlined below.

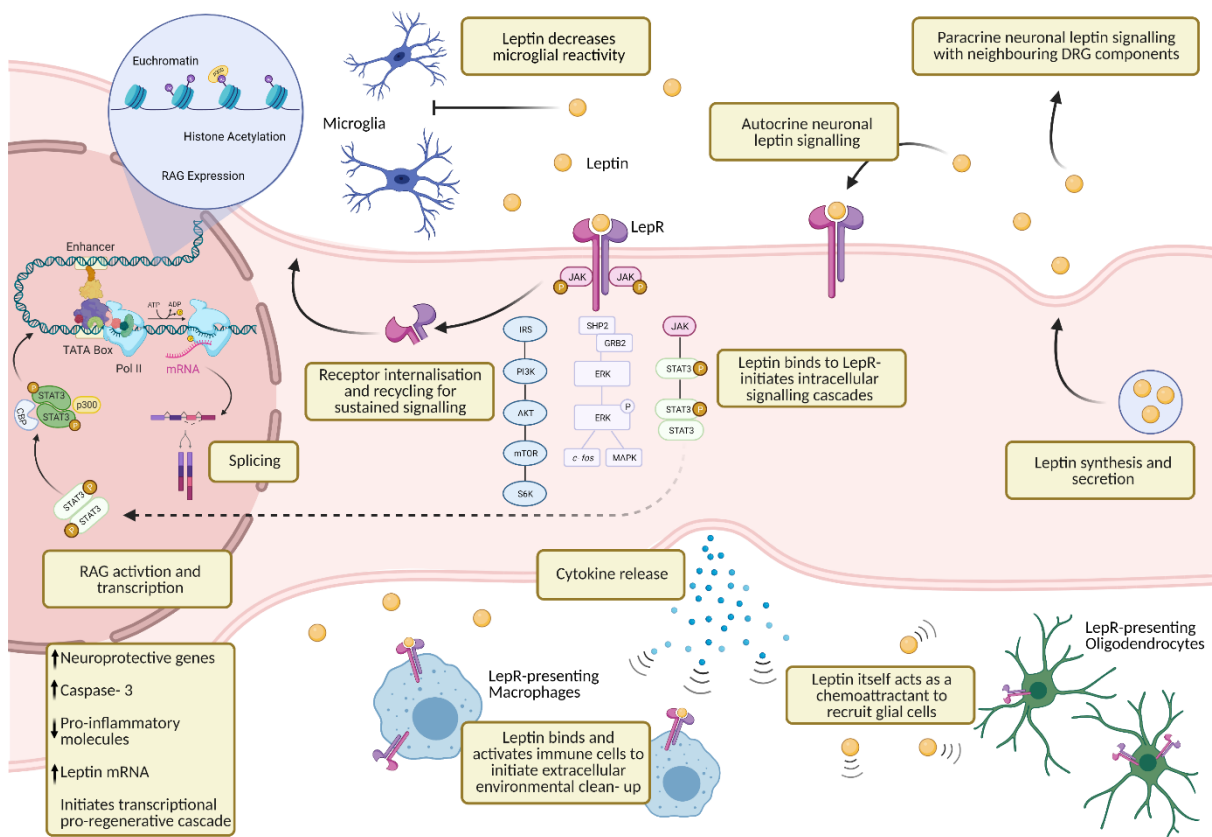


Figure 32: Neuronal Leptin Signalling Following Nerve Injury

Summary schematic of intracellular leptin-mediated action after axonal injury. Leptin synthesis and secretion is upregulated within neurons, binding to its receptor on the cell surface and those of adjacent neurons in an autocrine/paracrine propagation cascade. Downstream receptor signalling activates transcription, RAG expression and mRNA splicing. Extracellular immune-mediated events such as cytokine release and macrophage recruitment aid injury site debris clearance and eventual downregulation of inflammatory processes. In combination, leptin's neuronal actions foster a permissive regenerative environment for axon repair. Figure created in BioRender.

5.6.1 Metabolism:

Leptin is a homeostatic regulator released from adipose tissue, it circulates in the blood to reach its receptor targets within hypothalamic neurons of the brain, stimulating anorexigenic feeding behaviour. It acts in tandem with orexigenic hormones to maintain energy balance. Following eating, plasma glucose and amino acid levels increase, stimulating insulin secretion from β cells of the pancreas to activate glycolysis and leptin release from adipocytes. The LepR is abundantly expressed throughout the body, influencing multiple metabolic processes. For example, the homeostatic control of leptin signalling within the CNS can be summarised into 5 main signalling circuits: homeostatic feeding, anorexia, hedonic feeding, energy expenditure and glucose homeostasis circuits (Münzberg and Morrison, 2015). Consequently, leptin signalling directly regulates and is regulated by various homeostasis factors: insulin, ghrelin, glucocorticoids and cytokines (Park and Ahima, 2015). Leptin is known to regulate insulin sensitivity in the pancreas and via PI3K signalling in mediobasal hypothalamic neurons, acting as an important determinant of glucose metabolism (Poitout et al., 1998, Morton et al., 2005, Amitani et al., 2013). Both the LepR and the insulin receptor activate PI3K, either via JAK2 or activation of insulin receptor substrate 2 (IRS-2), respectively (Porte Jr et al., 2002). Cross-talk between downstream intracellular signalling via the LepR and insulin receptor occurs at the level of PI3K, though each receptor also exerts its own independent mechanisms (Cohen et al., 1996, Porte Jr et al., 2002). Leptin's regulation of metabolic processes such as insulin signalling, impact axonal regeneration. For example, active leptin signalling decreases food intake and increases energy expenditure by enhancing glycolysis, the breakdown of glucose for energy (Frederich et al., 1995, Douros et al., 2018). Metabolically reprogrammed glia promoted morphological and functional regeneration after CNS injury in *Drosophila* via

an increase in glycolysis (Li et al., 2020). Leptin is also an essential regulator of lipid metabolism, circulating leptin concentrations correlate directly with total body lipid content in mice (Frederich et al., 1995, Reidy and Weber, 2000). Lipids serve as signalling molecules and essential components of phospholipid membranes required for regenerating axons (Roy and Tedeschi, 2021). An increase in leptin and lipid molecules provide necessary components in axon growth and CNS repair. Similar to glucagon and catecholamines, leptin stimulates lipolysis by increasing cAMP concentrations (Reidy and Weber, 2000). It is well known cAMP elevation promotes axon growth and regeneration, sensitising neuronal responsiveness to growth factors, strengthening neurons against inhibitory myelin-associated molecules, in addition to cAMP-mediated activation of CREB and RAG transcription (Cui and So, 2004, Hannila and Filbin, 2008, Kurimoto et al., 2010). In summary, many of the metabolic pathways regulated by leptin underlie several regenerative processes within the cell which likely act simultaneously with leptin's intrinsic transcriptional response.

5.6.2 Microbiome:

Many of the metabolic pathways activated by IF and leptin are established within the neuroendocrine gut-brain-axis (Heiss and Olofsson, 2018). Gut-brain signalling is transduced via the Hypothalamic-pituitary-adrenal (HPA) axis or Vagus nerve, facilitating bidirectional exchange of hormones, neurotransmitters, gut peptides and cytokines in feedback loops (Mayer et al., 2015, Martin et al., 2018). Intermittent fasting alters the metabolites synthesised by the gut microbiome, such as short-chain fatty acids, which in turn influence energy uptake and regulation (Kasubuchi et al., 2015). This directly impacts nervous system functioning. For example, gut-brain signalling alters CNS development and neuronal plasticity, implicated in a number of neurodegenerative conditions including Parkinson's (Ghaisas et al., 2016, Sandhu et al., 2017). It is known that many of the substrates produced by the gut microbiome are also important signalling molecules in axonal regeneration. Most recently described, the gut metabolite indole-3-propionate (IPA) upregulated by IF and released from gut epithelia, stimulated neutrophil chemotaxis into the DRG microenvironment to enhance axonal regeneration (Sergey et al., 2022). Many of the satiety and energy homeostasis hormones such as leptin, insulin and ghrelin, regulate the microbiome and gut-brain axis as a collective (Dockray, 2014). This makes it difficult to distinguish the contribution of a single effector hormone. Currently the role of leptin within microbiome-

mediated axonal regeneration is unknown, though it likely contributes indirectly via control of energy metabolism.

Recent research has described the emergence of microbial endocrinology, the interactions between hormones and the gut microbiome and the two-way regulatory relationship where each impacts the other. The number of microbial cells present can be 10 times greater than the total number of cells in the human body, essential for breaking down dietary components, host immunity, degrading toxins and modulation of the endocrine system and hormonal secretion (Flint et al., 2012, Clarke et al., 2013, Elahi et al., 2013, Neuman et al., 2015). For example, research has shown Vancomycin antibiotic treatment in rats decreases circulating leptin levels by 38% (Lam et al., 2012). Vancomycin depletes gram positive bacteria such as *Clostridium sporogenes*, the bacteria responsible for synthesising indole metabolites such as IPA from tryptophan (Sergey et al., 2022). Indole-3-propionic acid was upregulated after IF and is central to the IF regenerative phenotype (Sergey et al., 2022). Depletion of gram-positive bacteria that metabolise tryptophan to IPA via vancomycin antibiotics abolishes the IF-dependent regenerative response (Sergey et al., 2022) and decreases circulating leptin levels (Lam et al., 2012). This indicates that gram-positive bacteria are required for IF-dependent regeneration and potentially influence leptin synthesis and circulation. Similar studies also show positive correlations between leptin synthesis and other bacterial types including *Bifidobacterium* and *Lactobacillus* (Queipo-Ortuño et al., 2013), while other bacteria (*Allobaculum*, *Prevotella*, *Bacteroides*) display a negative correlation with leptin levels (Ravussin et al., 2012, Queipo-Ortuño et al., 2013). It is thought that one mechanism through which bacteria control neuropeptides is via the release of somatostatin, which suppresses release of GI and pancreatic hormones (LeRoith et al., 1985, Neuman et al., 2015). Fetissov et al. also found autoantibodies against peptide hormones involved in appetite control within humans and rats which are altered in germ-free conditions (Fetissov et al., 2008). How leptin signalling is specifically impacted by IPA, indole metabolites and the gut microbiome would require systematic studies depleting alternating bacterial populations in turn. Leptin itself is also thought to regulate the microbiome and release of indole metabolites such as IPA. The absence of leptin signalling in leptin-deficient *ob/ob* or LepR-null *db/db* mice promotes hyperphagia and decreased energy expenditure and these mice display alterations in their gut microbiome (increased *Firmicutes* relative to *Bacteroidetes*), similar to those of diet-induced obese animals (Ley et al., 2005, Turnbaugh et al., 2008,

Geurts et al., 2011). Rajala et al. hypothesised that leptin action may modulate gut bacterial populations independently of feeding by controlling the expression of gut antimicrobial peptides. Leptin receptor signalling extrinsic to the intestinal epithelium and independently of food intake was found to control the gut microbiome (Rajala et al., 2014). Phylum level changes in faecal microbial content and antimicrobial peptide gene expression persisted in pair-fed *db/db* mice, suggesting that these differences did not stem from hyperphagia alone (Rajala et al., 2014). Additionally, serum from obese patients undergoing a gastric bypass analysed before surgery and three months after surgery found that of all the monitored feeding hormones (Adiponectin, Insulin, Ghrelin, Leptin, Resistin, GIP, Glucagon, GLP1, and PYY), only leptin was associated with alteration in gut metabolites (Seridi et al., 2018). Moreover, leptin was associated with tryptophan in support of a possible role of leptin in the regulation of serotonin synthesis pathways in the gut, in addition to the known synthesis of indole metabolites such as IPA (Seridi et al., 2018). It is interesting to speculate on the interdependence between IPA and leptin in IF-dependent regeneration and the wider impact of gut microbiome-mediated regulation of intracellular neuronal signalling.

5.6.3 Immune Response:

Energy metabolism underlies the basic physiological functioning of every cell in the body, including immune cells. Leptin regulates metabolic, immune and endocrine functions concurrently in a multi-layered manner (Schneiderman et al., 2012). As a class I cytokine and chemoattractant, leptin binds to its receptor expressed on several immune cell types (mast cells, neutrophil, macrophages, NK cells, B-cells, T-cells), participating in both innate and adaptive immune responses (Lord et al., 1998, Gorska et al., 2010, Reis et al., 2015, Pérez-Pérez et al., 2017). Leptin mediates a rapid innate immune response, facilitating debris clearance from the injury site (Naylor and Petri Jr, 2016), later enhancing adaptive immune coordination to drive the pro-inflammatory response towards an anti-inflammatory state (Mancuso et al., 2001). Leptin promotes proliferation of macrophages and mast cells, increasing phagocytosis and reducing apoptosis, participating as a trophic factor critical for cell function (Najib and Sánchez-Margalet, 2002). Additionally, leptin increases survival rate and migration of granulocytes, boosting neutrophil chemotaxis and cellular ROS production, both shown to enhance axonal regeneration (Ottonello et al., 2004, Matarese et al., 2005, Hervera et al., 2018, Serger et al., 2022). Several papers have also illustrated leptin's

involvement in leukocyte, T-cell and B-cell signalling, increasing T-cell cytokine, IFN- γ and TNF- α secretion; and B-cell IgG2 α release to drive inflammatory signalling in early injury stages (Martín-Romero and Sánchez-Margalet, 2001, Reis et al., 2015). However, mapping the neuro-immune communication mediated by leptin signalling before, during and after axonal injury remain unexplored. This would be an essential component in the consideration of neuro-immune modulation via therapeutic leptin delivery.

It has also been reported leptin synthesis is itself stimulated by endotoxins and cytokines (TNF, LIF, IL- 1) (Ahima and Flier, 2000). Therefore, injury and subsequent systemic inflammation stimulates leptin synthesis. Intermittent fasting- dependent CNS leptin upregulation may compound leptin- driven immune upregulation triggered by axonal injury, propagating a rapid neuroprotective and pro-regenerative response. Additionally, leptin may utilise other inflammatory mechanisms to clear debris from the injury site. Leptin treatment was shown to induce autophagy, both in cultured cells and in animals (Malik et al., 2011, Goldstein et al., 2019). Autophagy is a 'self- eating' detox process the body undergoes to clean out damaged cells and regenerate new ones (Malik et al., 2011). If leptin signalling initiates this detox process in hypothalamic neuronal cells (Meng and Cai, 2011, Quan et al., 2012), it is possible IF- dependent leptin signalling stimulates autophagy clearing processes following axonal injury, supported by neuro-immune interactions at the injury site. This dual mechanism of action, targeting both the intrinsic and extrinsic inhibitory environments following axonal injury, make leptin a very powerful moderator of regeneration.

5.6.4 Local Protein Translation

An essential necessity in axonal regeneration is the process of local translation, the ability to locally synthesise new proteins and degrade others at the injury site autonomously from the cell body (Gumy et al., 2010). As previously described, after PNS injury the distal portion of the axon undergoes Wallerian degeneration whilst the proximal axon connected to the cell body formulates a growth cone to facilitate regeneration. The processes of retraction, terminal enlargement formation, growth cone development and axon extension, all indicate a dynamic protein environment which regulates the balance between protein synthesis and degradation (Gumy et al., 2010). Severed axons of DRG neuronal cultures showed many neurons already form new growth-cones within 20 minutes of axotomy (Verma et al., 2005). This rapid

response supports the concept of local synthesis, as the majority of molecules synthesised by the cell body are not transported quickly enough to arrive within this timeframe (Gumy et al., 2010). Several studies in developing and regenerating sensory or motor axons show evidence of local translation and the presence of ribosomal proteins, translation initiation factors and cytoskeletal proteins within the axoplasmic compartment (Piper and Holt, 2004, Kun et al., 2007, Court FA et al., 2008). The composition of axonal mRNA also alters after injury and during regeneration, favouring an increase in transcripts essential for outgrowth, axon targeting and synapse formation (Yoo et al., 2010). Interestingly, localisation of ribosomes in the adult sensory axon showed endogenous ribosomes were detected in the mouse sciatic nerve and were supplemented with Schwann cell ribosomes after peripheral injury, as the cells appeared to transfer their own ribosomes into the axon following nerve damage (Court FA et al., 2008). However, the distribution of translational machinery and mRNAs differs between mature PNS and CNS axons and an explanation into this difference is ongoing (Verma et al., 2005).

Evidence shows extracellular stimuli can differentially localise specific mRNAs and translational machinery in the axon (Willis et al., 2007, Gumy et al., 2010) and it is likely both IF and neuronal leptin signalling regulate local protein synthesis in a manner which impacts axonal regeneration. Proteomics analysis from axonal lysates of rats which had undergone sciatic nerve crush injury identified a number of cytoskeleton proteins including β -actin, vimentin, peripherin and cofilin-1, in addition to heat shock proteins, anti-oxidant proteins and metabolic proteins (Willis et al., 2005). This accumulation of newly generated cytoskeletal proteins likely acts as a structural protein source for the formation of growth cones and regulation of cytoskeletal dynamics (Ghosh et al., 2004, Willis and Twiss, 2006, Gumy et al., 2010). Leptin is known to activate the RhoA/ROCK pathway within obese patients and induce cytoskeletal remodelling within nucleus pulposus cells of the intervertebral disc, inducing F-actin reorganisation and stress fibre formation (Li et al., 2014). Activation of RhoA-ROCK occurs after CNS injury and induces growth cone collapse. It is possible leptin may exert a dose effect whereby lower concentrations regulate cytoskeleton dynamics in a regenerative- permissive manner but chronic upregulation of high circulating leptin levels as observed in obese patients instigates a negative outcome. Subcutaneous mini pump delivery of leptin to ob/ob mice enhanced fat loss and showed cytoskeleton protein expression (α -actin, desmin, α tubulin) was altered by leptin within these mice,

suggesting interactions between mitochondria and the cytoskeleton are essential for normal mitochondrial motility and lipolysis (Zhang et al., 2011b).

Exploratory attempts to understand the molecular mechanism behind local protein synthesis identified mTOR and protein kinase p38, both of which regulate local protein synthesis in axon guidance. In DRG neuronal cultures, inhibitors against these molecules significantly reduced the incorporation of [3H]-leucine into axonal proteins and the regeneration of new growth cones after axotomy, highlighting their importance in protein synthesis (Verma et al., 2005, Gummy et al., 2010). Extracellular signal-regulated kinase 1,2 (ERK 1/2) and protein kinase A (PKA) are also essential for successful growth cone regeneration of axotomized DRG neurons, thought to mediate synthesis of growth-related proteins from local axonal mRNAs (Campbell and Holt, 2003, Chierzi et al., 2005). Leptin receptor signalling is known to increase both mTOR and ERK expression and may regulate local protein synthesis via these molecular routes (Wada et al., 2014). For example, placentas from gestational diabetes patients are challenged by structural differences and overgrowth (Perez-Perez et al., 2013). Leptin and LepR expression are increased in placentas from gestational diabetes, in addition to translation machinery activity and [3H]-leucine incorporation into proteins, compared to placentas from control pregnancies (Perez-Perez et al., 2013). Leptin signalling was associated with translational signalling and activation of signalling proteins regulating protein synthesis, such as mTOR, S6 Kinase, EIF4E-BP1, EIF4E, and eEF2 within the context of diabetes (Perez-Perez et al., 2013). Research has also shown that leptin stimulates BDNF translation in dendrites of hypothalamic neurons, essential in leptin's role in the regulation of body weight (Liao et al., 2012). This implicates leptin as a regulator of dendritic protein synthesis, mechanisms which may also be stimulated after injury within regenerative cascades.

In summary, leptin signalling cascades likely regulate cytoskeleton proteins and key molecular pathways involved in local protein synthesis to aid growth cone formation and regeneration of sensory axons after injury. Additional immunofluorescence staining of mTOR or pSTAT3 within DRG neurons in culture following leptin treatment would ascertain whether local protein translation was present within the neuronal dendrites.

5.7 Limitations and Future work

Interest in IF and the impact of metabolism in health and disease has grown rapidly with the increasing prevalence of obesity and cardiovascular diseases in modern populations. Many studies have outlined cellular and system- level processes mediated by IF which exert regenerative and wound healing responses. The research described here has characterised a novel neuronal mechanism via IF- dependent leptin signalling which facilitates axonal regeneration by modulating neuronal transcription. However, some areas remain underdeveloped with opportunity for further work.

Most notably, IF has divergent effects on almost every tissue type within the body, stimulating a vast array of overlapping and complementary cascades in addition to leptin signalling. It is conceivable multiple mechanisms mediate the improvements seen in functional recovery after IF, which when measured in isolation may not reach significance. Recently described were the effects of the gut metabolite IPA which utilised neutrophil chemotaxis within the DRG microenvironment to enhance regenerative outcomes (Serger et al., 2022). Similarly, the effect of IF on circadian rhythms and on neuroimmune modulatory mechanisms has been characterised (Vasconcelos et al., 2014, Longo and Panda, 2016, Mattson et al., 2017). How these pathways intersect with leptin-mediated actions within the neuron and whether they have an additive effect on regeneration remains unknown. Additionally, the physiological processes which regulate leptin signalling during IF are not fully characterised. I hypothesise an autocrine/paracrine mechanism of action between serum leptin concentrations and neuronal leptin synthesis, though the relationship and interdependence between the two is difficult to mechanistically establish. The metabolic "switch" that occurs in mice within 24 hours of fasting (depletion of glycogen and elevation of ketones), typically takes longer in humans and is likely dependent on diet and levels of physical activity (Cignarella et al., 2018). Metabolic and neuroendocrine adaptations to fasting mediated by leptin are also likely to be of greater survival value in rodents, as short-term starvation has more severe consequences in this species (Ahima and Flier, 2000). How the observed effects of leptin in sciatic nerve injury and SCI could be scaled for humans would require clinical studies. In this context, the centrality of leptin signalling in IF- dependent mechanisms would also need to be established further within patients. Intermittent fasting though beneficial for many tissues could nevertheless prove detrimental in patients recovering from SCI due

to weight loss and muscle atrophy concerns. Leptin however offers a strong translational opportunity in terms of an isolated neuronal delivery via gene therapy. It would also be of interest to determine the impact of therapeutic leptin delivery within the nervous system of healthy patients and whether this would exert 'IF-like' neuroprotective properties or trigger unwanted side-effects such as hyperinnervation and neuropathic pain.

Similarly, additional clinical factors which need to be considered are age and sex. The majority of IF studies have been performed in young organisms or have not compared their effects in young and old. Understanding the age-specific effects of these interventions is important considering the major changes in weight, growth hormones and steroid hormones that occur at different ages (Longo and Panda, 2016). For example, severe protein restriction causes weight loss in old but not young mice, and low protein intake is associated with protection from mortality in 65 and younger, but not 66 and older, individuals (Levine et al., 2014). Intermittent fasting initiated at 10 months in aged animals did not extend mean lifespan and in some cases even reduced lifespan (Goodrick et al., 1990, Arum et al., 2009). However, when initiated at 1.5 months in young animals, IF increased longevity (Goodrick et al., 1990). A possible reason may be that fasting is consistently protective in young and middle-aged laboratory rodents that are either gaining or maintaining body weight, but detrimental in older animals that, similarly to humans, begin to lose weight at advanced ages (Longo and Mattson, 2014). This may be particularly pertinent to leptin's mechanism of action as it is an anorexigenic hormone which can cause weight loss. Regenerative differences between young and aged mice have also demonstrated that DRG neurons of older mice expressed higher levels of CXCL13, attracting T-cell accumulation and triggering regenerative failure (Zhou et al., 2022). Both metabolic and regenerative differences advance with age. Metabolic differences in energy sensing satiety hormones such as leptin also fluctuate depending on the sex of the animal. Females contain higher circulating leptin levels within the serum and secrete leptin from adipose tissue deposits at a faster rate, despite adjusting for body fat content (Hickey et al., 1996, Hellström et al., 2000, Karastergiou et al., 2012). Following IF, differences in body weight gain and combinations of pro-survival gene expression can be observed with different sexes in the liver of mice (Piotrowska et al., 2016), in addition to differences in IF-dependent effect on lifespan (Li and Zhang, 2019). Data included in the present body of work used an equal combination of male and female mice for

experiments; leptin treatment or overexpression was shown to enhance axonal regeneration in both sexes. Future studies would benefit from specific sex-disaggregated data to distinguish any changes which may influence regenerative outcomes.

The body is able to exact specific physiological outcomes in response to stimuli, in part due to cell fate. Each cell type within the body is specialised for a particular function, including neurons. Neuronal subtypes therefore have divergent responses to injury; some more resistant than others to sprout or regenerate axons following injury (Lindner et al., 2013). The corticospinal tracts are the most refractory in comparison to the rubrospinal and raphe spinal axons to regenerate, only expressing regenerative gene activation in layer V neurons after intracortical but not intraspinal axotomy, suggesting retrograde transport which stimulates regenerative signalling is weaker within the corticospinal system (Fernandes et al., 1999, Mason et al., 2003, Giovanni, 2009). Transcriptional profiling of the DRG at single cell and population levels reveal a large molecular diversity of somatosensory neurons (Chiu et al., 2014), including previously unclassified subtypes (Usoskin et al., 2015). This is in addition to other DRG cell types, such as satellite cells, endothelial cells and immune cells. Transcriptomic RNAseq data exploring differences between IF and AL were inferred from bulk DRG tissue (Figure 19), diluting the specificity of the response described. Subsequent RNAseq analysis of leptin overexpression and DCA was collected from a neuronal-enriched population which had applied a gradient to remove other cell types within the DRG (Figure 30). This gave a clearer 'neuronal' response to leptin administration but did not distinguish between neuronal subtypes. Single cell sequencing would facilitate distinction of leptin's action on neuronal subpopulations as recent studies have highlighted unique injury responses in different types of DRG neurons, suggesting different gene regulatory networks are involved in nerve regeneration (Hu et al., 2016). Additionally, the neuronal gradient whilst effective at pooling the neurons and removing other cell types, satellite cells are notoriously 'sticky' and some likely remain within the sample. However, it is unlikely satellite-dependent leptin signalling contributes to the regenerative phenotype. Deletion of the LepR in LepR FLOX mice neurons ablated the regenerative phenotype observed with IF, supporting the conclusion that regenerative leptin signalling is neuronally driven. Leptin was also shown to exert a dose-effect within the nervous system. Lower doses of leptin corrected neuroendocrine abnormalities such as hypercorticism, whereas higher leptin doses above the normal

fed level inhibited feeding and prevented weight gain (Ioffe et al., 1998). This would suggest different leptin thresholds exact different physiological functions within neurons, potentially mediated by various neuronal subtypes (Ahima and Flier, 2000). Both metabolic function and injury response vary between individual neurons and between individuals themselves, though very little is known about the causes accounting for these differential abilities (Hawthorne et al., 2011, Canty et al., 2013, He and Jin, 2016). Future research mapping the contributions of each subtype would strengthen our understanding of potential therapeutic targets and provide a basis from which personalised medicine can develop.

Additional work characterising the mechanistic link between leptin signalling and transcriptional regulation may also offer confirmatory insight into neuronal responses after injury. Further exploration via ATAC sequencing would confirm hypothesised euchromatin conformation structures at the promoters and enhancers of RAGs, in addition to ChIP sequencing, immunofluorescent staining for epigenetic markers and transcriptional inhibition-rescue studies within DRG *ex vivo* cultures. However, the RNAseq dataset investigating the effect of leptin overexpression on central axonal regeneration is very strong, with clear enrichment of transcriptional categories at the highest bioinformatic stringency level.

5.8 Conclusion

Evolution has gone to considerable lengths to develop multiple inhibitors of regeneration and plasticity within the adult mammalian CNS, conferring what must be evolutionary advantageous outcomes. It is possible a hard-wired nervous system is more efficient; or that energetics and potential for mutation in maintaining regeneration and plasticity are unfavourable; or there is difficulty in controlling inflammation (Fawcett, 2020). Yet biological paradigms distinct from injury conditions such as exercise, sleep and diet can all impact neuronal physiology and alleviate some of these restrictive inhibitors upon injury. This thesis has explored the endogenous intrinsic signalling mechanisms initiated by IF within neurons and found leptin signalling was pervasive among many of the activated pathways. Neuronal leptin expression within DRGs was essential to the IF-dependent regenerative phenotype, as removal of the receptor and downstream signalling attenuated the regenerative response. Leptin overexpression within neurons and systemic leptin treatment both increased regenerative outcomes in PNS and CNS injury models, suggesting promising

applications of leptin delivery in the treatment of nerves and spinal injuries. Exploration of the leptin-dependent mechanism of action showed leptin regulated transcriptional output and chromatin conformation to formulate a permissive regenerative environment within DRG neurons, replicating the landscape signatures usually observed within the PNS, in the inhibitory CNS.

Due to the necessity of multidisciplinary research, the field of neurometabolism is developing, particularly in the context of injury and disease. The role of leptin as a satiety hormone is well established within the healthy system as well as its clinical connotations in obesity. A considered investigation into the summative contributions of neuronal subtypes and the relationship between systemic and DRG leptin concentrations will be necessary to understand how to therapeutically manipulate leptin *in vivo* without side effects and homeostasis disruption. The impact of leptin on different neuronal types such as corticospinal (CST) and rubrospinal tracts, in addition to the sensory neurons of the dorsal column, is unknown. Correlations have been established, looking at the effect of maternal undernutrition on neonatal development in rodents, results established a leptin surge at postnatal day 11 which stunted body weight, body length and altered pituitary hormone levels including growth hormones (Miles et al., 2022). These early leptin surges will have impacted nervous system development as a whole, but the specifics of discreet neuronal types were not investigated. Though CST motor fibres were not investigated in the research outlined in this thesis, leptin and LepR expression in the spinal cord will likely signal to these neurons. Neuron-centric treatments such as targeted gene therapy may therefore facilitate the transferal of leptin's beneficial effects without heavily impacting systemic circulating leptin and harmful side effects including weight loss, hyperinnervation, neuropathic pain or hyper-angiogenesis which disrupts the wound healing process (McCully et al., 2013, Kratofil et al., 2022). However, the nervous system and the vascular system are intrinsically linked and overexpression of leptin within neurons will likely raise circulating leptin levels. The timing and duration of leptin treatment will therefore be key. Intermittent leptin delivery either via an osmotic mini pump or skin patch may offer greater control and safety compared to a more protracted treatment such as gene therapy.

Additionally, the patient's injury profile must be taken into consideration. Patients with a cervical or thoracic SCI will experience hindered sympathetic innervation, including information to the stomach, pancreas, liver and adrenal glands which will

influence release of key endocrine hormones such as insulin, ghrelin and glucagon among others (Miscio et al., 2005). If a patient is already experiencing dysregulated energy homeostasis, the levels of circulating leptin may reach pathological levels associated with disease risk. However, patients with a lumbar injury may maintain relatively intact sympathetic signalling and are therefore better equipped to respond to therapeutic neuronal leptin delivery. Similarly, the length of time the patient has lived with a SCI will impact treatment. Chronic injuries will undergo axonal sprouting via spared fibres below the injury site, potentially remodelling the location and function of many axons in an effort to diversify the capacity of the neurons which remain. This can lead to unpredicted responses to treatment as many of the original neuronal target innervations have changed. It's also possible that over time with restricted movement, patient adipose levels increase and the patient is diagnosed with obesity (Latifi et al., 2013). The influence of co-morbidities such as SCI and obesity make treatments very challenging to practically implement. The majority of SCI patients are obese, have high circulating leptin levels due to fat redistribution and experience worse neurological regenerative outcomes compared to controls (Colak et al., 2000, Gezici et al., 2009, Latifi et al., 2013, Bekar et al., 2014). For example, obesity was shown to negatively impact peripheral regeneration after sciatic nerve crush in rats, reducing nerve diameter and displaying irregular myelin sheath morphology (Bekar et al., 2014). Obese mice and obese patients present clinically with high leptin resistance, however the cause of this resistance within patients and an effort to successfully understand leptin transfer across the blood brain barrier is ongoing (Rahmouni et al., 2002, Münzberg and Morrison, 2015, Pan et al., 2014, Izquierdo et al., 2019). Until considerable progress is made in this capacity, it's probable that leptin treatment for nerve injuries would not be beneficial for all SCI patients due to the difficulty in overcoming leptin resistance in those with high adipose deposits. The key goal in translating the regenerative effects of IF is identifying molecular mechanisms which will enable metabolic manipulation of a cell to encourage metabolic switching between glycolysis and ketogenesis. Whether the full effects of IF can be recapitulated by driving singular pathways is unlikely. However, both continuous and discrete leptin delivery either pre or post injury is pro-regenerative and it offers a clinical possibility in patients for whom fasting is not advisable. Further exploration into dosing concentrations and regimens will offer additional understanding into a potentially exciting new therapy for spinal cord injury.

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