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NEURONS AND GLIA IN THE PERIPHERAL NERVOUS SYSTEM: INTERACTIONS IN HEALTH AND DISEASE

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Neurons and glia in the Peripheral Nervous System: interactions in health and disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Dedicated to my family

Dedicado à minha família

ABSTRACT

The peripheral nervous system (PNS) is composed of nerves and ganglia that connect the brain and the spinal cord (the central nervous system, CNS) to the outside world. The nerves contain the extensions (axons) of both sensory and motor neurons, which allow us to detect and respond to different stimuli. For example, nociceptive neurons can detect pain and alpha-motoneurons can control muscle contraction and movement. In addition to neurons, nerves also contain glial or support cells called Schwann cells (SC), which are responsible for the structural organization of nerves, myelination of large caliber axons and engulfment of small caliber nerve fibers. Myelin is crucial for the fast transmission of action potentials and acts as a protective layer around axons. Schwann cells are also able to provide trophic and metabolic support to neurons, and their dysfunction can drive axonal pathology in the absence of changes to myelin itself. Neurons and glia have been well studied in the CNS, but there has been an increased interest in the PNS at the level of development and long-term maintenance. Our contributions to this field are tri-partite:

In **Study I** we questioned if monocarboxylate transporters MCT1 and MCT4 expressed by SC contribute to metabolic support of peripheral neurons. We employed *Cre-Lox* technology to conditionally delete the genes *Slc16a1* and *Slc16a3*, coding respectively for MCT1 and MCT4, in SC. We found that both glial MCTs are largely dispensable for the development and myelination of nerves but MCT1 contributes to long term maintenance of neuromuscular innervation. Absence of MCT1 in SC induced transcriptional changes in spinal cord motoneurons, which may reflect reduced metabolic support and may be an early sign of neuronal pathology. These results suggest that MCT1 expressed by SC contributes to the support of motoneurons, but the relatively mild phenotype observed indicates that the remaining MCTs expressed by SC and neurons may play an important part as well.

In **Study II** we focused on the development of sensory neurons and investigated the function of PRDM12, an epigenetic regulator that can cause congenital insensitivity to pain when mutated in humans. We established constitutive knockout (KO) mice lacking the expression of *Prdm12* and discovered that absence of *Prdm12* results in a complete loss of the $TRKA^+$ subpopulation in the dorsal root ganglion (DRG), which gives rise to nociceptors. This is due in part to reduced proliferation of $SOX10^+$ precursor cells. Overexpression of *Prdm12* in chicken embryos was not sufficient to induce a nociceptor fate but it did prevent the differentiation of alternative sensory neuron subtypes. Finally, we found that while the number of $Ngn1^+$ and $Ngn2^+$ cells was unchanged in the *Prdm12* KO at embryonic day E10.5, at E12.5 the expression of *Ngn1* was greatly reduced, suggesting *Prdm12* is necessary to maintain its expression. We conclude that *Prdm12* is necessary to maintain the expression of *Ngn1* in the developing mouse DRG, it modulates the proliferation of $SOX0^+$ progenitors and restricts the differentiation potential of sensory neurons to the nociceptive TRKA-expressing fate.

In **Study III** we evaluated the effect of overexpressing a constitutively active form of NFATc4 in myelinating glia, *in vivo*. NFATc4 is transcription factor regulated by the

phospholipase Cγ/calcium/calcineurin signaling pathway. Inhibition of this pathway in neural crest cells leads to impaired SC development and reduced expression of *Krox20*, the master regulatory gene of myelin gene expression. We hypothesized that overexpression of an active (phosphorylation-resistant) form of NFATc4 in myelinating glia may lead to hypermyelination, but instead the transgenic mice developed a pronounced neuropathy phenotype. This was characterized by hind-limb clasping and reduced motor nerve conduction velocity. We found that at birth, the sciatic nerves of these mice were developmentally delayed, presenting a larger area occupied by unsorted bundles of axons and a complete absence of axons undergoing myelination. RNA sequencing of newborn sciatic nerves showed a dramatic alteration of the transcriptional landscape, allowing us to conclude that the timing and intensity of NFATc4 activation are crucial for proper development of peripheral nerves.

Additionally, **Paper IV** is a mini-review of the topic "Metabolic Interaction Between Schwann Cells and Axons Under Physiological and Disease Conditions". In it we review recent studies on the role of SC in the metabolic support of axons and compare them to the current knowledge of the same function in CNS glia. We propose that metabolic support of axons may be the primary function of axon-ensheathing glia, as can be seen in lampreys and fruit flies, with myelination becoming an evolutionarily advantageous feature of higher vertebrates. Then we present different mouse models of SC metabolic dysfunction, focusing on the myelin-independent features of the phenotypes, and finally, we suggest that SC-axon metabolic interactions may pose an interesting target to treat peripheral neuropathic disorders such as diabetic neuropathy and Charcot-Marie-Tooth disease.

RESUMO

O sistema nervoso periférico (SNP) é composto por nervos e gânglios que ligam o cérebro e a medula espinal (o sistema nervoso central, SNC) ao mundo externo. Os nervos contêm extensões (axónios) de neurónios sensoriais e motores que nos permitem detectar e responder a diferentes estímulos. Por exemplo, os neurónios nociceptivos detectam a sensação de dor e os neurónios motores alfa controlam a contracção muscular e os movimentos. Além dos neurónios, os nervos também contêm células gliais ou de suporte chamadas células de Schwann (CS), que são responsáveis pela organização estrutural dos nervos, mielinização de axónios de grande calibre e envolvimento de fibras nervosas de pequeno calibre. A mielina é crucial para a transmissão rápida dos potenciais de acção e actua como uma camada protectora ao redor dos axónios. As CS também são capazes de fornecer suporte trófico e metabólico aos neurónios, e a sua disfunção pode conduzir à patologia axonal mesmo na ausência de alterações na própria mielina. Neurónios e glia têm sido bem estudados no SNC, mas tem havido um aumento do interesse no SNP ao nível do seu desenvolvimento e manutenção a longo prazo. As nossas contribuições para este campo são tripartidas:

No **Estudo I** questionámos se os transportadores de monocarboxilatos MCT1 e MCT4 expressos por CS contribuem para o suporte metabólico dos neurónios periféricos. Empregámos a tecnologia *Cre-Lox* para excluir condicionalmente os genes *Slc16a1* e *Slc16a3*, que codificam respectivamente MCT1 e MCT4, em CS. Descobrimos que ambos os MCTs gliais são largamente dispensáveis para o desenvolvimento e mielinização dos nervos, mas o MCT1 em particular pode contribuir para a manutenção a longo prazo da inervação neuromuscular. A ausência de MCT1 em CS induziu alterações transcricionais nos neurónios motores da medula espinal, que podem reflectir a redução do suporte metabólico e podem ser um sinal precoce de patologia neuronal. Estes resultados sugerem que o MCT1 expresso por CS contribui para o suporte de neurónios motores, mas o fenótipo relativamente suave observado indica que os MCTs restantes expressos por CS e neurónios também podem desempenhar um papel importante.

No **Estudo II**, concentrámo-nos no desenvolvimento de neurónios sensoriais e investigámos a função de PRDM12, um regulador epigenético que pode causar insensibilidade congénita à dor quando mutado em humanos. Estabelecemos ratinhos knockout constitutivos (KO) sem a expressão de *Prdm12* e descobrimos que a ausência deste gene resulta numa perda completa da subpopulação $TRKA^+$ no gânglio da raiz dorsal (GRD), que dá origem a nociceptores. Isso deve-se em parte à proliferação reduzida de células precursoras $SOX10^+$. A sobreexpressão de *Prdm12* em embriões de galinha não foi suficiente para induzir a diferenciação de nociceptores, mas impediu a diferenciação de subtipos de neurónios sensoriais alternativos. Finalmente, descobrimos que, embora o número de células $Ngn1^+$ e $Ngn2^+$ permanecesse inalterado nos animais *Prdm12* KO no dia embrionário E10.5, a E12.5 a expressão de *Ngn1* estava reduzida, sugerindo que *Prdm12* é necessário para manter a sua expressão. Concluímos que *Prdm12* é necessário para sustentar a expressão de *Ngn1* no GRD

de ratinho em desenvolvimento, modula a proliferação de progenitores $SOX0^{+}$ e restringe o potencial de diferenciação de neurónios sensoriais ao destino nociceptivo que expressa TRKA.

No **Estudo III**, avaliamos o efeito da sobreexpressão de uma forma constitutivamente activa de NFATc4 na glia mielinizante, *in vivo*. NFATc4 é um fator de transcrição regulado pela via de sinalização fosfolipase Cγ/ cálcio/ calcineurina. A inibição desta via nas células da crista neural leva ao comprometimento do desenvolvimento de CS e à redução da expressão de *Krox20*, o gene regulador mestre da expressão de genes da mielina. A nossa hipótese era que a sobreexpressão de uma forma activa (resistente à fosforilação) de NFATc4 na glia mielinizante poderia levar à hipermielinização, mas em vez disso, os ratinhos transgénicos desenvolveram um fenótipo de neuropatia pronunciado, caracterizado por encolhimento dos membros posteriores e redução da velocidade de condução nervosa motora. Descobrimos que, aquando do nascimento, os nervos ciáticos desses ratinhos apresentavam um atraso no desenvolvimento, possuindo uma área maior ocupada por feixes de axónios desorganizados e uma completa ausência de axónios em mielinização. A sequenciação de ARN dos nervos ciáticos de ratinhos recém-nascidos mostrou uma alteração dramática da paisagem transcricional, permitindo-nos concluir que o momento e a intensidade da activação de NFATc4 são cruciais para o desenvolvimento adequado dos nervos periféricos.

Além disso, o **Artigo IV** é uma mini-revisão do tópico interacção metabólica entre células de Schwann e axónios em condições fisiológicas e de doença. Nele, revimos estudos recentes sobre o papel das CS no suporte metabólico de axónios e comparámo-los com o conhecimento actual da mesma função pela glia do SNC. Nós propomos que o suporte metabólico dos axónios pode ser a função primária da glia que embainha os axónios, como pode ser visto em lampreias e moscas-da-fruta, e que a mielinização se tornou uma vantagem evolutiva dos vertebrados superiores. Em seguida, apresentamos diferentes modelos de ratinhos com disfunção metabólica das CS, com foco nos fenótipos com características independentes da mielina e, finalmente, sugerimos que as interacções metabólicas axónio-CS podem representar um alvo interessante para tratar distúrbios neuropáticos periféricos, como a neuropatia diabética e a doença de Charcot-Marie-Tooth.

THESIS CONSTITUENT PAPERS

I. **Bouçanova, F.**, Pollmeier, G., Sandor, K., Morado Urbina, C., Nijssen, J., Médard, J. J., Bartesaghi, L., Pellerin, L., Svensson, C. I., Hedlund, E., Chrast, R. (2020). Disrupted function of lactate transporter MCT1, but not MCT4, in Schwann cells affects the maintenance of motor end-plate innervation.
Glia. 2020; 1– 13

II. Bartesaghi, L.*, Wang, Y.*, Fontanet, P., Wanderoy, S., Berger, F., Wu, H., Akkuratova, N., **Bouçanova, F.**, Médard, J. J., Petitpré, C., Landy, M. A., Zhang, M. D., Harrer, P., Stendel, C., Stucka, R., Dusl, M., Kastriti, M. E., Croci, L., Lai, H. C., Consalez, G. G., Pattyn, A., Ernfors, P., Senderek, J., Adameyko, I., Lallemend, F., Hadjab, S., Chrast, R. (2019). PRDM12 Is Required for Initiation of the Nociceptive Neuron Lineage during Neurogenesis.
Cell reports, 26(13), 3484–3492.e4
* Authors contributed equally

III. Domènech-Estévez, E., Smith, R., **Bouçanova, F.**, Médard, J. J., Pollmeier, G., Nijssen, J., Svaren, J., Einheber, S., Hedlund, E., Chrast, R., Salzer, J. L.*, and Baloui, H.*. Sustained NFAT-mediated signaling in myelinating glia induces neuropathy
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* Authors contributed equally

REVIEW ARTICLE INCLUDED IN THIS THESIS

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OTHER ARTICLES NOT INCLUDED IN THIS THESIS

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Under revision in *Brain*

* Authors contributed equally

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CONTENTS

LIST OF ABBREVIATIONS

2-DG	2-deoxyglucose
AAV	Adeno-associated virus
ADAM17	A disintegrin and metalloproteinase 17
AKT/PKB	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine 5’-monophosphate
AMPK	AMP-dependent kinase
ANLS	Astrocyte-neuron lactate shuttle
ATP	Adenosine 5’-triphosphate
ATPase	Adenosine triphosphatase
BACE	Beta-site APP cleaving enzyme or Beta-secretase
BDNF	Bone-derived neurotrophic factor
BMP	Bone morphogenic protein
C9orf72	Chromosome 9, open reading frame 72
cAMP	Cyclic adenosine-monophosphate
CDC42	Cell division control protein 42
CIP	Congenital insensitivity to pain
cKO	Conditional knockout
CMT	Charcot-Marie-Tooth disease
CnB	Calcineurin B, regulatory subunit
CNP/CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CoA	Coenzyme-A
COX10	Cytochrome C oxidase assembly factor heme A:farnesyltransferase
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
dbcAMP	Dibutyryl-cAMP
dCKO	Double conditional knockout
DN	Diabetic neuropathy

DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
E day	Embryonic day
EGFP	Enhanced green fluorescent protein
EGR2/KROX20	Early growth response 2
EHMT	Euchromatic histone lysine methyltransferase 2
ER	Endoplasmic reticulum
ErbB2/3	Erb-B2/3 receptor tyrosine kinase
ERK	Extracellular regulated kinase
FAK	Focal adhesion kinase
FUS	RNA-binding protein FUS (Fused in Sarcoma)
GDP	Guanosine 5'-diphosphate
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GJB1	Gap junction protein beta 1
GLI	Glioma-associated zinc finger protein
GP	Glycogen phosphorylase
GPR/GPCR	G-protein coupled receptor
Grb2	Growth factor receptor-bound protein 2
GS	Glycogen synthase
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine 5'-triphosphate
GTPase	Guanosine triphosphatase
H3K9	Histone 3 lysine 9
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HK	Hexokinase
IP_3	Inositol 1,4,5-trisphosphate
iPSC	Induced pluripotent stem cells
iSC	Immature Schwann cell
ISL1	Islet 1

LCM-Seq	Laser capture microdissection and RNA sequencing
LDH	Lactate dehydrogenase
LKB1/Stk11	Liver kinase B1/ Serine/threonine kinase 11
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCT	Monocarboxylate transporter
MFN2	Mitofusin 2
mGluR	Metabotropic glutamate receptors
MN	Motoneuron
MNCV	Motor nerve conduction velocity
MNX1/HB9	Motor neuron and pancreas homeobox 1/ homeobox gene HB9
MPZ	Myelin protein zero
mRNA	Messenger ribonucleic acid
mSC	Myelinating Schwann cell
mTORC	Mechanistic or mammalian target of rapamycin complex
MYRF	Myelin regulatory factor
NAD^+/NADH	Nicotinamide adenine dinucleotide, oxidised form/reduced form
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate, oxidised form/reduced form
NCC	Neural crest cell
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
Ngfr/P75ntr	Nerve growth factor receptor/ low affinity neurotrophin receptor
NGN	Neurogenin
NKX2.2	NK2 homeobox 2
NKX6.1	NK6 homeobox 1
Ntrk1/2/3	Neurotrophic receptor tyrosine kinase 1/2/3
NMDAR	N-methyl-D-aspartate receptor
NMJ	Neuromuscular junction
nmSC	Non-myelinating Schwann cell

NRG	Neuregulin
NT	Neurotrophin
OL	Oligodendrocyte
OLIG2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte precursor cell
OXPHOS	Oxidative phosphorylation
P day	Post-natal day
Par	Partitioning defective
PAX	Paired box
pCMBS	p-Chloromercuribenzene sulfonate
PDC	Pyruvate dehydrogenase complex
PERIPH	Peripherin
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase A
PLCγ	Phospholipase Cγ
PLP	Proteolipid protein
PMP22	Peripheral myelin protein 22
PN	Peripheral neuropathy
PNS	Peripheral nervous system
POU3F1/OCT6/SCIP	POU Domain, Class 3, Transcription Factor 1/ Octamer-Binding Transcription Factor 6/Suppressed cyclic AMP-inducible POU
PPP	Pentose phosphate pathway
PRDM12	PRDI-BF1 and RIZ homology domain-containing protein 12
PTEN	Phosphatase and tensin homologue
qPCR	Quantitative polimerase chain reaction
RAC1	RAS-related C3 botulinum toxin substrate 1
RET	Rearranged during transfection tyrosine kinase receptor
Rheb	RAS-homolog enriched in brain
Rho	RAS homolog family member A
RNA	Ribonucleic acid

RNS/ROS	Reactive nitrogen/oxygen species
SC	Schwann cell
SCN	Sodium voltage-gated channel family
SCP	Schwann cell precursor
SH3TC2	SH3 Domain And Tetratricopeptide Repeats 2
Shc	Src homology domain-containing transforming protein
SHH	Sonic hedgehog
shRNA	Small hairpin ribonucleic acid
SLC	Solute carrier family
SN	Sciatic nerve
SNCV	Sensory nerve conduction velocity
SOD1	Superoxide dismutase 1
SOX	SRY-Box Transcription Factor
TCA	Tricarboxylic acid cycle or Krebs cycle
TDP-43	TAR DNA-binding protein 43
TFAM	Transcription factor A, mitochondrial
TFEB	Transcription factor EB
TG	Trigeminal ganglia
Tg	Transgenic
TRK	Tyrosine receptor kinase
TSC2	Tuberous sclerosis complex 2
WNT	Wingless-type MMTV integration site

1 INTRODUCTION

Imagine yourself ambling through a forest. Trees rise from the ground and branch towards the sky, the air is filled with sounds of birds and other unidentified animals, and you can sense the odor of decomposing vegetation under your feet. You see a bush heavy with ripe berries and you reach out to pick one. Alas! A sudden, sharp unpleasantness drives your hand back and is followed by a dull, prolonged, disagreeable sensation, as a bright red drop begins to form on your fingertip.

The situation I just described will be familiar to most people, but how does it work? It is the result of a sophisticated system that allows us to detect stimuli from our external (and internal) environment and respond accordingly. In a dangerous situation, such as getting pricked by a bush of wild berries, we hardly even acknowledge the trigger before our body spontaneously reacts to protect itself. In fast sequence, peripheral pain receptors on our skin detect the presence of a noxious stimulus and send an urgent message to our center of command. From there, an order is instantly sent towards our muscles to quickly change the position of the limb and move it out of harm's way. Only then do we consciously understand what just happened and acknowledge the throbbing sensation coursing through our injured finger.

The main components of this peripheral system are: fast, myelinated sensory neurons, which detect a stimulus and send a quick warning signal; motor neurons, that control our movements; and slow, unmyelinated sensory neurons, which carry long lasting pain, perhaps as a reminder to be more cautious next time. Myelin, the accelerator of impulse transmission, is also an important part of this system, as are the glial cells that produce it - Schwann cells. In addition to the sensory detectors and the motor effectors are spinal interneurons, which belong to the central nervous system and complete the circuit.

In the following sections of this thesis I will introduce in further detail these different components, as well as a few disorders that may affect their proper function. I will describe some of the knowledge we currently have on the interactions between neurons and glia and explain the steps my collaborators and I have taken to address some of the gaps in that knowledge.

2 THE PERIPHERAL NERVOUS SYSTEM

The peripheral nervous system, or PNS, is composed of sensory and motor nerves that radiate from our central neural organs (the brain and spinal cord) and innervate our peripheral organs, such as the skin, muscles, and glands. It can be further subdivided into somatic PNS, responsible for voluntary movement and reflexes, and autonomic PNS, which controls the function of our internal organs, such as digestion (Purves, Augustine et al. 2018).

The PNS is composed of two main types of neurons, motor and sensory, as well as one main type of glia, Schwann cells (SC) (Figure 2-1). Spinal cord interneurons, an essential connection to the central nervous system (CNS), will not be covered in this thesis.

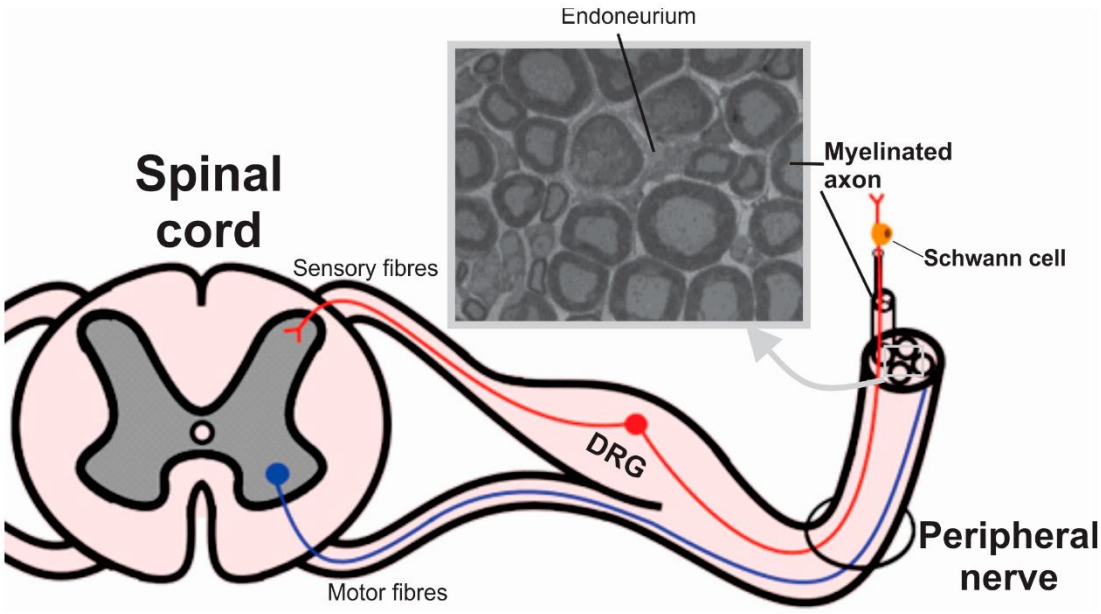

Figure 2-1 Schematic representation of the structure of the PNS. Schematic cross-section of the spinal cord (left) showing a central region composed of grey matter surrounded by white matter. The cell bodies of motor neurons can be found in the ventral horns of the spinal cord (represented by a blue circle) and their axons extend through the ventral root to innervate skeletal muscles. The dorsal root ganglion (DRG) contains the cell bodies of sensory neurons (red circle) and they extend their axon towards the periphery to innervate sensory structures (as those in the skin), and into the spinal cord through the dorsal root to transmit sensory input to the CNS. Axons are wrapped by Schwann cells (SC) and together they make up the endoneurium (inset: electron microscopy image showing dark myelin rings produced by myelinating SCs). Image reproduced from Barton et al. (2017).

2.1 MOTOR NEURONS

Spinal cord motor neurons (also spelled motoneurons, MN) are multipolar cholinergic neurons that can be found in the ventral horns of the spinal cord and which control bodily movements (Purves, Augustine et al. 2018). They extend their axons through a motor exit point in the ventral spinal cord, forming a ventral root adjacent to a dorsal root ganglion (DRG), before merging with sensory axons into a spinal nerve. The axons of MNs form efferent fibers which send impulses from the CNS towards effector organs like muscles. Histologically speaking, the ventral root is easily distinguished from the dorsal as its axons are generally myelinated (as opposed to the presence of bundles of unmyelinated axons in the dorsal root).

During embryonic development, a concentration gradient of growth factors is established after the closure of the neural tube (neurulation), with high levels of WNT and BMP secreted by the roof plate (dorsal) and SHH released by the floor plate (ventral) (Stifani 2014). On the lateral axis, structures called somites (that will originate bones and muscles) express retinoic acid and, together, these three signals will induce different cellular fates in the developing neurons in a dorsal-to-ventral pattern. Higher levels of SHH in the ventral spinal cord will promote the activity of GLI proteins in progenitor cells (Niewiadomski, Kong et al. 2014, Stifani 2014), which will, in turn, promote or repress different transcription factors, further reinforcing the dorsal-to-ventral signal gradient. Cross-repression of these third level regulators will ensure the formation of mutually exclusive domains where the progenitors of different neuronal types can form. In a dorsal-ventral order those domains will be p0, p1, p2, pMN and p3 and they will give rise, respectively, to V0, V1 and V2 interneurons, motoneurons and finally V3 interneurons. The pMN domain will express a unique combination of transcription factors including NKX6.1, PAX6, and OLIG2, followed by (among others) NGN2 and MNX1/HB9 (Stifani 2014). This will drive the post-mitotic differentiation of MNs. Later, these early MNs will further organize along the rostral-caudal axis forming columns and specify into target-specific pools.

Within the pools there are 3 subtypes of motoneurons: alpha, beta and gamma (Stifani 2014). Alpha MNs innervate extrafusal muscle fibers (the main constituents of skeletal muscle) and drive their contraction. They are morphologically larger than the other types and end at a neuromuscular junction or motor endplate. The neuromuscular junction is the synapse between the MN terminals and the muscle, a “pretzel”-shaped structure on the muscle fiber rich in nicotinic acetylcholine receptors (Purves, Augustine et al. 2018). Binding of the neurotransmitter to its receptors triggers sodium ion influx into the muscle fiber and leads to muscle contraction. Alpha MNs can be directly innervated by sensory neurons, which enables fast reflex responses, or by interneurons, which modulate motoneuron activity and transmit sensory and motor information between the CNS and PNS (Zavvarian, Hong et al. 2020). Gamma MNs innervate intrafusal muscle fibers (fibers that make up the muscle spindle), respond to indirect sensory input and regulate the sensitivity of spindles, by increasing the tension of intrafusal muscle fibers (Eccles, Eccles et al. 1960, Stifani 2014). Beta MNs are the least abundant and less is known about them. Their interaction with both intra- and extrafusal muscle fibers allows them to modulate both muscle spindle tension and muscle contraction (Bessou, Emonet-Denand et al. 1965, Stifani 2014).

Among the diseases affecting spinal cord MNs, amyotrophic lateral sclerosis (ALS) is one of the most common, affecting between 4.1 and 8.4 people per 100000 (Longinetti and Fang 2019). It is a degenerative disorder that leads to the denervation of muscles and death of motoneurons (Siddique and Siddique 2001 [updated 2019]). Muscle atrophy, weakness and cramps, as well as difficulty swallowing, are some symptoms that may be present. ALS is a progressive and fatal disorder, often caused by paralysis of respiratory muscles. 10-15% of patients have a known genetic mutation causing ALS (in genes such as *SOD1*, *c9orf72*, *FUS* and *TDP-43*), whereas most cases are of unknown origin. The disease mechanisms can involve

protein aggregation and ER stress, altered RNA metabolism, mitochondrial dysfunction and oxidative stress, as well as excitotoxicity (Le Gall, Anakor et al. 2020). Due to the different mechanisms involved and the variable disease presentation, no disease-modifying treatments have yet reached clinical practice.

2.2 SENSORY NEURONS

Sensory neurons can be found in sensory ganglia outside of the brain and spinal cord, such as trigeminal ganglia (TG, which relay sensory information from the face/head) and dorsal root ganglia (DRG, carry sensory inputs from the body) (Purves, Augustine et al. 2018). Pairs of DRGs can be found along the spinal cord, to which they are connected by dorsal (sensory) roots. Inside the DRG lie the cell bodies of sensory neurons derived from neural crest precursors. These neurons possess a pseudo-unipolar morphology with a bifurcated axon that extends towards the spinal cord (through the dorsal root) on one side and the body's periphery (through the spinal nerve) on the other. Sensory axons constitute afferent fibers, bringing information from peripheral sensory structures into the CNS. Different types of sensory neurons will innervate different sensory structures or organs. The terminals of nociceptors (neurons that sense pain, heat, and itch) lie inside the epidermis of the skin as free nerve endings, while those of mechanoreceptors (neurons that sense touch) can be found in encapsulated organs such as Meissner's corpuscles and Pacini's corpuscles, and proprioceptors (neurons that sense body position) innervate muscle spindles.

In development, a structure called the neural crest forms after the process of neurulation. Neural crest cells (NCC) are multipotent precursors that can originate not only sensory neurons but also glia and non-neural cells such as melanocytes (Le Douarin, Calloni et al. 2008). In mice, NCCs can be directed to differentiate into sensory neurons through two waves of neurogenesis (Lallemend and Ernfors 2012). This process begins between embryonic days E9.5 and E11.5, with a first wave mediated by neurogenin 2 (NGN2), a proneuronal transcription factor. This wave originates large, myelinated mechanoreceptors and proprioceptors, as well as a subgroup of small, thinly myelinated nociceptors. Mechanoreceptors can be further characterized by the expression of tyrosine receptor kinase (TRK) B, the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin NT4/5, whereas proprioceptors express TRKC, the receptor for NT3. Between days E10.5 and E13.5, a second, NGN1-dependent wave originates most small, unmyelinated nociceptors, which express TRKA, the receptor for nerve growth factor (NGF) (Lallemend and Ernfors 2012).

Sensory neurons, especially nociceptors, can be particularly affected by peripheral neuropathies (disorders that affect the PNS) such as diabetic neuropathy. Their small caliber, long axons and thin or absent myelin may increase their susceptibility to oxidative stress, mechanical injury and metabolic insults. Degeneration of these axons can lead to reduced sensation and numbness, while their regeneration can cause positive symptoms like pain and sensitivity to non-noxious stimuli (Said, Slama et al. 1983, Malik, Tesfaye et al. 2005, Zenker,

Ziegler et al. 2013, Feldman, Nave et al. 2017). In animal models of peripheral neuropathy, small caliber C-fibers are sometimes the first to be lost. Skin innervation can be assessed as part of the diagnostic procedure and the extent of loss of free nerve endings increases with disease severity (Ebenezer, McArthur et al. 2007).

2.3 SCHWANN CELLS

Schwann cells (SC) are the myelinating cells of the PNS. Like sensory neurons, they also arise from migratory NCCs, and populate the developing nerve around day E12 in mouse (Jessen and Mirsky 2005). There are two main types of SC - myelinating (mSC) and non-myelinating (nmSC) (Figure 2-2). Individual mSC interact with segments of larger caliber axons and wrap them in a lipid and protein-rich membranous structure termed a myelin sheath. On the other hand, nmSC engulf multiple small caliber axons forming a so-called Remak bundle and can be also called Remak cells (Griffin and Thompson 2008). The main role of myelin is to provide electrical insulation to the axonal membrane, allowing the flow of ions in and out of the axon only at discrete points called nodes of Ranvier. This creates a "saltatory" conduction of action potentials, increasing the speed and efficiency of impulse transmission (Nave 2010). SCs also provide axons with physical protection and trophic and metabolic support, produce growth factors and aid their regeneration after injury (Jessen and Mirsky 2016).

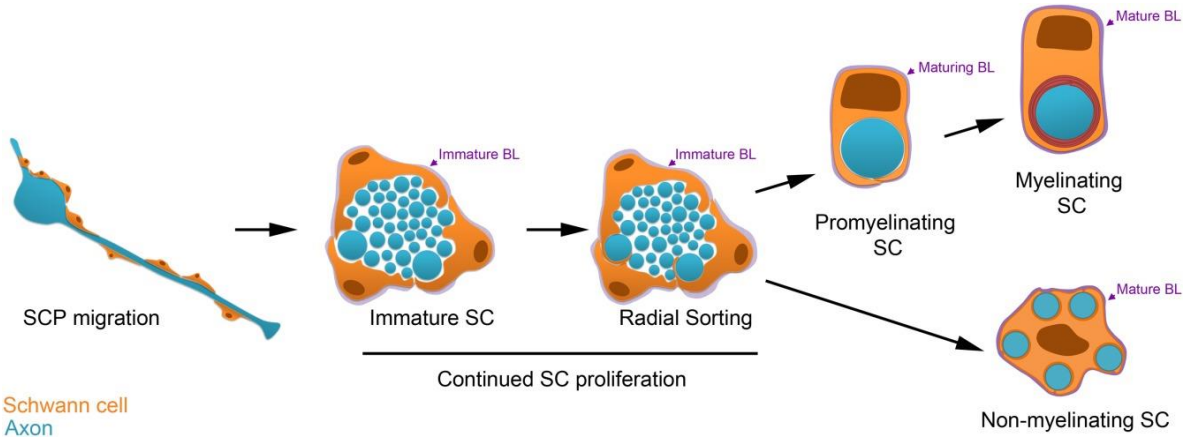

Figure 2-2 Development and maturation process of Schwann cells (BL - basal lamina). Reproduced from Monk et al. (2015)

The relationship between axons and SCs is bi-directional, as neurons instruct the maturation of SCs and vice-versa. The process of myelination induces changes in the axonal membrane, restricting voltage-gated sodium channels to the node of Ranvier and voltage-gated potassium channels to the juxtaparanodal region (Salzer, Brophy et al. 2008). Inside the axon, neurofilaments may become phosphorylated, which increases the space between filaments and drives the radial growth of the axon (Garcia, Lobsiger et al. 2003). Subcellular organelles like mitochondria may also be relocated to the proximity of the node of Ranvier, possibly to provide

energy for membrane repolarization (Perkins and Ellisman 2011). Unmyelinated small caliber axons have long been considered to possess uniformly distributed ion channels in their membranes (Black, Foster et al. 1981), but this idea has recently been challenged by the observation that sodium channels may be enriched in lipid rafts of nociceptors (Pristera, Baker et al. 2012). This implies the possibility of micro-saltatory conduction in unmyelinated axons (Neishabouri and Faisal 2014).

The CNS counterpart of mSCs are oligodendrocytes (OL). Unlike mSCs, OLs can myelinate multiple neurons simultaneously and their origin lies in the neural tube. There are two waves of oligodendrocyte development in the brain and spinal cord (Richardson, Kessaris et al. 2006). One occurs in the ventral ventricular zone of the spinal cord, with OL precursor cells (OPC) arising from the same domain as MNs, and is followed by a second wave derived from dorsal precursor cells. In the brain, a similar pattern occurs as first wave OLs arise from the medial ganglionic eminence (ventral part of the brain) and migrate through the brain, followed by cells from the lateral/caudal ganglionic eminence. In post-natal development, OLs originate from cortical OPCs and replace the embryonic cells. Despite their similar functions, OLs and mSCs differ in their expression of transcription factors and other proteins. For example, OLs express NKX2.2 and MYRF transcription factors (Qi, Cai et al. 2001, Hornig, Frob et al. 2013), while mSCs express transcription factor KROX20 (Topilko, Schneider-Maunoury et al. 1994) and major peripheral myelin constituent MPZ (Lemke, Lamar et al. 1988). Another difference is OLs' ability to myelinate in a cell-autonomous fashion, as they can wrap artificial fibers (Lee, Leach et al. 2012), while mSCs require axonal and extracellular matrix cues for myelination.

As it will be mentioned in later sections of this thesis, there are often comparisons and parallels between the biology of SCs and OLs. Both express SOX10, CNP and myelin proteins such as MBP, MAG and PLP. nmSCs also have some commonalities with astrocytes, as they express GFAP and can accumulate glycogen, for example. An additional feature of all three types of glial cells is their ability to provide metabolic support to neurons in the form of energy-rich substrates, such as lactate and pyruvate. The metabolic interactions between SC and neurons have been reviewed in Paper IV of this thesis (Boucanova and Chrast 2020) and our contributions to the study of SC-mediated metabolic support of neurons will be presented in Paper I (Boucanova, Pollmeier et al. 2020).

2.4 PERIPHERAL NEUROPATHIES AND THERAPEUTIC APPROACHES

A peripheral neuropathy (PN) is a disorder that affects the PNS. Symptoms of PN can be positive (pain, tingling, allodynia) or negative (diminished sensation, numbness, muscle atrophy, paralysis). It may be hereditary or acquired, and it can target primarily the neuronal or the glial compartment. PNs can be a consequence of damage to a peripheral nerve (such as carpal tunnel syndrome), exposure to chemical insults (heavy metals, alcohol abuse,

chemotherapy), infectious disease or its treatment (HIV), diabetes and autoimmunity (Guillain-Barré syndrome).

Among the hereditary PNs, the most common is a group of disorders termed Charco-Marie-Tooth diseases (CMT), affecting 1:2500 people. Symptoms include foot and leg deformities, difficulty walking and abnormal gait. CMT can be divided in two main groups: demyelinating or axonal. Demyelinating CMTs are caused by mutations in myelin genes, such as CMT1A, caused by a duplication of *PMP22*, and CMT1B, caused by a mutation in *MPZ* (Weis, Claeys et al. 2017). These mutations can cause thinner or less compact myelin, leading to reduced nerve conduction velocity. Axonal CMTs can be caused by mutations in genes related to axonal transport of different cargoes, namely mitochondria (for example *MFN2*, *GDAP1*, neurofilaments and kinesins (Barneo-Munoz, Juarez et al. 2015, Beijer, Sisto et al. 2019, Bernard-Marissal, van Hameren et al. 2019)) and the main consequence is degeneration of axons, detectable by reduced amplitude of action potentials with preserved or minor alterations to conduction velocity.

A few therapeutic approaches have been proposed for the treatment of demyelinating PNs. For example, in a rat model of CMT1A, a diet supplement containing phosphatidylcholine and phosphatidylethanolamine improved myelination, nerve conduction velocity and grip strength (Fledrich, Abdelaal et al. 2018). Also, a combination of gene therapy and diet supplementation (expression of neurotrophin 3 together with pyruvate treatment) in Trembler-J mice let to benefits in myelination and nerve conduction (Sahenk, Yalvac et al. 2018). However, the most promising treatment is a cocktail of baclofen, sorbitol and naltrexone called PXT3003. In animal studies, this treatment led to reduced levels of *Pmp22* mRNA which in turn ameliorated the molecular signaling that drives the disease (Prukop, Stenzel et al. 2019). After completing a phase II and a phase III clinical trials, this treatment is undergoing a second phase III trial (identifier NCT03023540) for up to two years, to study the safety of long-term exposure to the drugs and disease-modifying effects. Gene therapy approaches have also been tested in mouse models of demyelinating CMT1X, caused by loss of function of GJB1/connexin 32, and CMT4C, caused by mutation of SH3TC2 (Sargiannidou, Kagiava et al. 2020). In these studies, the administration of lentiviral vectors carrying wild-type GJB1 (Kagiava, Sargiannidou et al. 2016, Kagiava, Karaiskos et al. 2018) or SH3TC2 (Schiza, Georgiou et al. 2019) was performed intrathecally (into the spine so as to reach the cerebral-spinal fluid). This approach avoids systemic distribution and allows the dispersal of substances to the PNS. The genetic constructs included the promoter for MPZ to limit the expression to mSCs. In both studies there was an improvement at the level of myelinated fibers and impulse conduction, however caution should be exercised particularly when different mutations lead to different disease mechanisms. Connexin32 mutations that lead to ER or Golgi accumulation did not benefit from increased load of wild-type connexin32 as the two forms of the protein would interact physically during the assembly of gap-junctions. Translation into human trials is yet to be tested, as multiple parameters need to be optimized: first, the use of lentiviral *versus* adeno-associated virus (AAV) vectors; second, the route of administration, intrathecal *versus* intramuscular or intravenous. Lentiviral vectors allow the use of larger constructs with full

length promoters, but AAVs are safer as they do not integrate into the genome. Different routes of administration will require different viral loads (greater for intravenous route), different levels of invasiveness and procedure risk, as well as different efficiency of transduction of the target cells. A few clinical trials for related diseases are ongoing such as giant axonal neuropathy (ClinicalTrials.gov Identifier: NCT02362438) and Batten disease (NCT02725580) (intrathecal administration), as well as spinal muscular atrophy (NCT02122952) (intravenous route). The results of these clinical trials may provide a foundation for the design of future studies in patients with PNs.

The most common form of PN, however, is diabetic neuropathy (DN), affecting up to 50% of diabetes patients (Hicks and Selvin 2019). According to the World Health Organization, in 2014, 8.5% of adults had diabetes, representing 422 million people (www.who.int/news-room/fact-sheets/detail/diabetes). As previously mentioned, DN can lead to degeneration of small sensory axons, such as unmyelinated nociceptive C fibers and small myelinated axons. Diabetes is characterized by a sustained metabolic imbalance derived from high levels of blood glucose, which can be due to an inability to produce insulin (diabetes type I) or a metabolic syndrome (diabetes type II). Hyperglycemia can lead to the activation of the polyol pathway, high levels of ROS, oxidative stress and mitochondrial dysfunction, activation of downstream pathways leading to inflammation and decreased intracellular energy levels, as well as production of advanced glycation end products that can modify proteins and impair their function or trigger inflammation (Feldman, Nave et al. 2017). Not only can diabetes directly affect peripheral neuron function and survival, by the aforementioned mechanisms, but it might also affect SC-axon interactions. For example, the high levels of dietary lipids that contribute to type II diabetes, could also lead to altered lipid metabolism in SCs. In a mouse model of disrupted mitochondrial metabolism (Viader, Sasaki et al. 2013), SCs produce high levels of acylcarnitines and transport them to axons, where they exert a toxic effect and lead to degeneration by increasing intracellular calcium. A similar mechanism could be at play in diabetes leading to neuropathy. Alternatively, under diabetic conditions SCs could provide inappropriate metabolic support to neurons, either by utilizing glucose-derived products for their own metabolism or by overloading the axon with monocarboxylic acids and causing acidosis (Feldman, Nave et al. 2017). Thus, the involvement of SC in diabetic neuropathy is a subject that requires more investigation.

In the realm of speculation, it would be interesting to test the use of a ketogenic diet to ameliorate diabetic neuropathy and axonal forms of CMT. A ketogenic diet eliminates carbohydrates and replaces them with lipids and proteins. This leads the body to drive gluconeogenesis from non-carbohydrate precursors and increases the production of ketone bodies. Ketone bodies are small high-energy molecules that can be directly used by mitochondria and can cross the blood-brain barrier. Not to be confused with a high fat diet containing, for example, 54% fat and 24% carbohydrates in addition to proteins, and which can be used experimentally to induce diabetes in mice (Cooper, Menta et al. 2018). In this study, Copper *et al.* took mice that had previously been on a high fat diet and treated them with a ketogenic diet. The diet change did not lead to weight loss or reverse the animals' diabetic state,

but it improved skin innervation and symptoms such as mechanical allodynia, which are signs of neuropathy. A ketogenic diet can also improve mitochondrial function and reduce the production of reactive oxygen species in the sciatic nerve (Cooper, McCoin et al. 2018) as well as improve myelination and axonal abnormalities in the CNS in a model of Pelizaeus-Merzbacher disease (Stumpf, Berghoff et al. 2019). In humans, ketogenic diets have been proposed for the treatment of diseases such as Alzheimer's and Parkinson's (Wlodarek 2019), but it is still unclear if they would be beneficial for PNs.

3 CONGENITAL INSENSITIVITY TO PAIN

Mutations in genes that code for specific voltage-gated sodium channels (*SCN9A* and *SCN11A)* or the NGF/TRKA (*NTRK1*) pathway can cause congenital insensitivity to pain (CIP) in humans (Nahorski, Chen et al. 2015) due to absence or dysfunction of nociceptive neurons. These mutations are rare and only a few patients have been identified/studied. Patients with CIP often show mutilation of the mouth, tongue, hands and feet, bruises and deformities caused by poorly healed fractures (Leipold, Liebmann et al. 2013). Some forms of CIP may be accompanied by anhidrosis (inability to sweat) and mental retardation (Indo, Tsuruta et al. 1996).

The molecular mechanisms that regulate the development of nociceptors are still poorly understood. However, recent developments in this field by our group and others (Chen, Auer-Grumbach et al. 2015, Bartesaghi, Wang et al. 2019, Desiderio, Vermeiren et al. 2019) have identified the epigenetic regulator PRDM12 as an important factor for the initiation of the nociceptor lineage. Our contributions to the study of PRDM12 in nociceptor development can be found in Paper II of this thesis (Bartesaghi, Wang et al. 2019). Patients with *PRDM12* mutations manifest clinically with frequent injuries to the hands, feet, and mouth/tongue. In these patients, the sensation of heat and cold is also affected, but the sensation of touch, vibration and proprioception are not, indicating that the development of mechanoreceptors and proprioceptors is independent of nociceptors and of PRDM12 (Chen, Auer-Grumbach et al. 2015).

PRDM12 (PRDI-BF1 and RIZ homology domain-containing protein 12) is an epigenetic regulator of gene expression. Although it does not possess intrinsic enzymatic activity, it can recruit a methyltransferase (G9a, encoded by *EHMT2* (Yang and Shinkai 2013)) to modify histone H3 at lysine 9 by adding two methyl groups (H3K9me2), which can act as a repressor of gene transcription. In adult humans, *PRDM12* has been described as expressed exclusively in the DRG (Chen, Auer-Grumbach et al. 2015), but in mice it is also found in some regions of the brain, such as the hypothalamus (Hael, Rojo et al. 2020). Constitutive deletion of *Prdm12* in mice is lethal (Bartesaghi, Wang et al. 2019, Desiderio, Vermeiren et al. 2019) while the conditional deletion (under *Advillin-Cre* driver, which targets peripheral sensory neurons) leads to injuries similar to those of human patients (Desiderio, Vermeiren et al. 2019). The constitutive ablation of *Prdm12* leads to a complete absence of $TRKA^+$ neurons, without major changes to $TRKB^+$ and $TRKC^+$ subpopulations. This is due to reduced proliferation of $SOX10^+$ precursors at E11.5-E12.5 and interrupted expression of NGN1 (while the transcript of *Ngn1* can be detected at E10.5, it is no longer present at E12.5) (Bartesaghi, Wang et al. 2019). Overexpression of *Prdm12* in chicken embryos is not sufficient to induce the differentiation of $TRKA^+$ neurons, but it is able to repress an alternative fate as proprioceptors or mechanoreceptors.

In a recent preprint submitted to bioRxiv server (Landy, Goyal et al. 2020), Landy and colleagues evaluate the role of *Prdm12* in the developing and adult DRG using a conditional

Advillin-Cre driver and an inducible *Advillin-CreERT2* driver. In agreement with the previous publications mentioned, deletion of *Prdm12* in sensory neurons during embryogenesis leads to reduced numbers of nociceptors and a marked sensory phenotype with reduced response to mechanical, cold and itchy stimuli. Non-painful touch sensation was unaffected as expected, and neither was heat sensitivity (which may be due to the timing of gene inactivation at E12.5, while *Prdm12* starts to be expressed at E9.5). Interestingly, the deletion of *Prdm12* in the adult stage did not affect nociceptor function or numbers, suggesting it is necessary for nociceptor development but not for their maintenance. RNA sequencing of DRGs from adult control and induced *Prdm12* cKO mice showed a majority of differentially expressed genes were decreased, which suggests PRDM12 may act as a transcriptional activator in the adult, instead of a repressor as previously indicated. The consequence of these transcriptional changes in adult nociceptor function is so far unknown.

4 FACTORS THAT REGULATE SCHWANN CELL DEVELOPMENT

During development, NCCs delaminate from the dorsal part of the neural tube and migrate outwards to reach their final destinations, starting around embryonic day E8.5-E10 (Marmigere and Ernfors 2007, Jacob 2015). During this journey, they begin to express transcription factors and other molecules that will determine their differentiation. One such transcription factor is SOX10. SOX10 expression can be found in precursor cells that will originate peripheral glia, sensory DRG neurons and melanocytes, for example. In the developing DRG, its expression is downregulated in cells that will become neurons, but it is maintained at high levels in progenitors of SC by a regulatory network involving chromatin remodelers HDAC1/2 and transcription factor PAX3. SOX10 also induces the expression of ErbB3, a receptor of neuregulin 1 type III (NRG1III), in SC precursors (SCP). NRG1III is expressed at the axonal surface and its signaling in the SC lineage promotes SC proliferation, survival and myelination (Birchmeier and Nave 2008, Salzer 2012, Salzer 2015).

SCPs accompany developing axons as they extend towards their target tissues, possibly protecting the axons' growth cones from inhibitory molecules in the extracellular milieu (Wanner, Mahoney et al. 2006). During this process they proliferate in response to Notch signaling, via ERK and JNK signaling pathways, and they transition to immature SCs (iSC) (Woodhoo, Alonso et al. 2009, Fledrich, Kungl et al. 2019). iSCs stop migrating and form families of 3-8 cells around bundles of axons. At this stage, iSCs start to deposit a basal lamina and initiate the process of radial sorting: an iSC will extend a process into the bundle and establish a one-to-one relationship with an axon if its diameter is greater than 1µm. This decision is based on the expression level of NRG1III at the axonal surface and will instruct the promyelinating SC to initiate the myelination program between the late embryonic stages and early post-natal, as well as the thickness of the myelin sheath (Michailov, Sereda et al. 2004, Taveggia, Zanazzi et al. 2005). The remaining smaller-caliber axons will instead be engulfed by a single mature non-myelinating SC in a Remak bundle, as previously mentioned.

4.1 EXTRACELLULAR SIGNALS CONVERGE AT THE TRANSCRIPTIONAL LEVEL

Different signaling pathways become activated after NRG1III binding to ErbB2/3 receptors: autophosphorylation of ErbB2/3 on tyrosine residues recruits adaptor molecules including Grb2, Shc, PLCγ and PI3K, which will activate different pathways such as MAPK/ERK, FAK, calcium/calcineurin/NFAT and AKT (Newbern and Birchmeier 2010) (Figure 4-1). In parallel, the extracellular matrix also signals to myelinating SCs via integrins, G-protein coupled receptors (such as GPR126) and others (Monk, Feltri et al. 2015). These signals can increase cAMP levels and activate PKA, as well as activate small Rho GTPases RAC1 and CDC42 that control the cytoskeleton. All these different signals can converge to finetune the timing and expression of transcription factors, enabling the correct myelination of

peripheral axons. SOX10 together with cAMP-response element binding protein (CREB) and NF-κB can promote the expression of OCT6 in pre-myelinating SC and then SOX10 and OCT6 cooperatively drive the expression of KROX20, the master regulator of myelin gene expression (Topilko, Schneider-Maunoury et al. 1994). At a later stage, OCT6 is downregulated to allow myelination to progress. The post translational processing of axonal NRG1III by secretases can also modulate the timing and intensity of the signaling network (Monk, Feltri et al. 2015, Fledrich, Kungl et al. 2019). Extracellular cleavage by β-secretase BACE-1 creates a fragment that can activate PI3K signaling to promote myelination, while cleavage by α-secretase ADAM17 prevents it. The intracellular domain of NRG1III can also be processed by the γ-secretase complex and induce the neuron to express the enzyme prostaglandin D2 synthase. The product, prostaglandin D2, can bind GPR44 at the SC surface, which can lead to activation of transcription factor NFATc4 by a mechanism independent of PLCγ/ calcineurin (Trimarco, Forese et al. 2014).

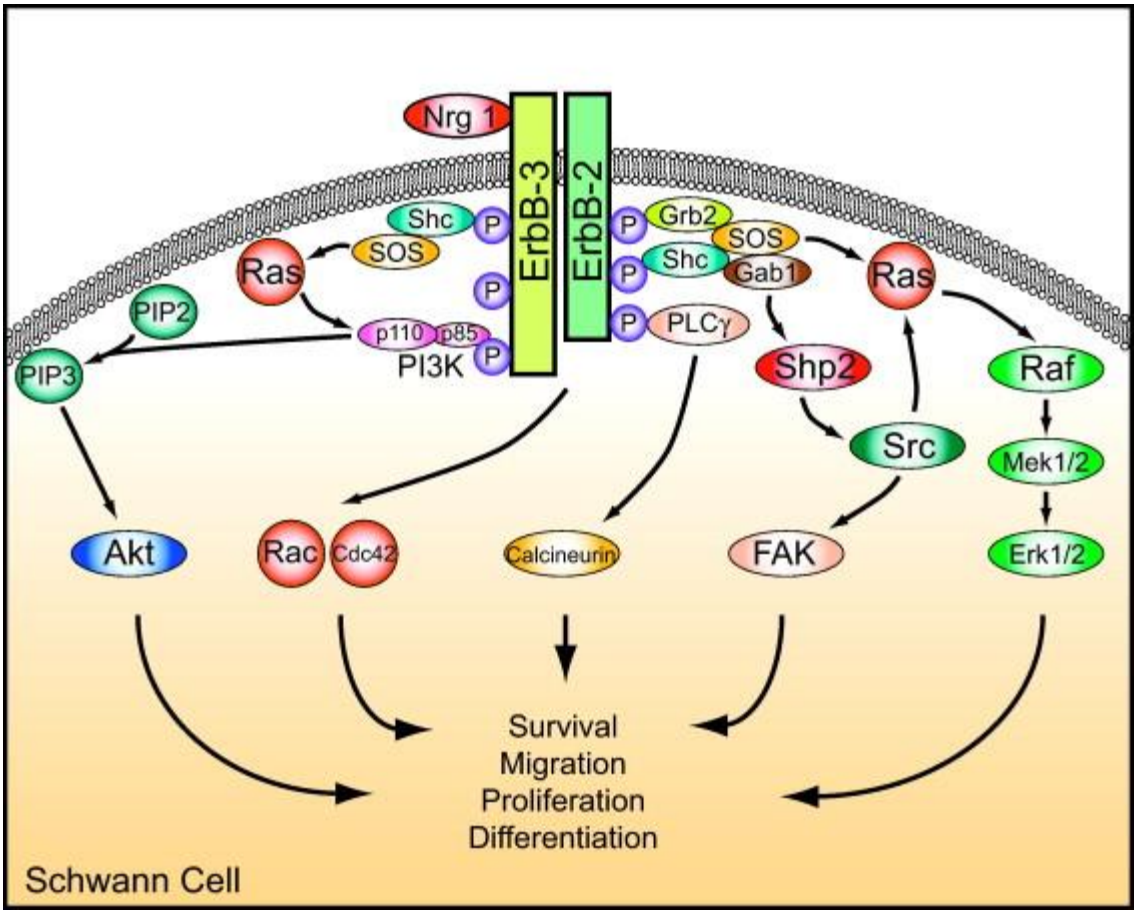

Figure 4-1 Schematic representation of pathways downstream of NRG1/ErbB signaling in SCs. Reproduced from Newbern and Birchmeier (2010).

4.2 PI3K/AKT/mTOR

The PI3K/AKT pathway is an important regulator of SC biology, acting downstream of NGR1III/ErbB. The constitutive activation of AKT or elimination of its inhibitor, PTEN, drive excessive production of myelin (hypermyelination), with formation of outfoldings and

tomacula that can impair normal PNS function (Goebbels, Oltrogge et al. 2012, Domenech-Estevez, Baloui et al. 2016). Treatment of these animals with mTOR inhibitors like rapamycin can rescue the phenotype while the genetic deletion of mTOR causes hypomyelination. AKT can activate the mTOR pathway by phosphorylating TSC2, an important inhibitor of mTORC1. TSC2 is a GTPase activating protein that maintains Rheb (a small GTPase activator of mTORC1) in the GDP-bound state, inhibiting its function (Beirowski 2019).

The mTOR pathway can regulate metabolism by promoting protein and lipid synthesis necessary for cell growth in response to growth factor signaling and nutrient availability (Figlia, Gerber et al. 2018). Interestingly, mTOR needs to be tightly regulated before and after the onset of myelination. Prior to onset, high levels of mTOR inhibit KROX20 expression, likely to ensure completion of axonal sorting before progressing to a myelinating state. Then, mTOR activity is reduced, KROX20 levels rise and myelin genes begin to be highly expressed. At this stage, inducing the activation of mTOR (by elimination of its regulator TSC2, for example) will lead to excessive myelin production. Several direct downstream effectors, feedback loops and alternative pathway branches modulate the effects of mTOR activity.

4.3 LKB1

The LKB1/AMPK pathway can also regulate SC biology in a variety of ways (Beirowski 2019). Canonically, LKB1 (liver kinase B1) can sense changes in the energy status of the cell and phosphorylate AMPK (AMP-dependent kinase), activating it. Binding of AMP to AMPK further boosts its activity and promotes catabolic processes such as glycolysis and fatty acid oxidation, the tricarboxylic acid cycle, autophagy and oxidative phosphorylation, increasing the production of ATP. This pathway can antagonize the mTOR pathway, which promotes anabolic, or biosynthetic, pathways instead, spending ATP to do so. However, LKB1 in SC also plays important roles in cell polarity and SC-mediated axonal support in AMPK-independent ways.

Conditional ablation of LKB1 in myelinating glia (using the *Cnp-Cre* driver, active from E12) prevents SCs to switch from glycolytic to oxidative metabolism during their transition from a proliferative state to a differentiating state (Pooya, Liu et al. 2014). This is characterized by reduced mitochondrial membrane potential, reduced oxygen consumption and increased lactate/glucose ratio. Mutant mice present a pronounced neuropathic phenotype, with hypomyelination and reduced compound muscle action potentials, but mRNA and protein levels of myelin-related genes were generally preserved. Instead, myelin lipids were greatly reduced and this was linked to lower levels of citrate, an important intermediate metabolite of the tricarboxylic acid cycle and lipid synthesis. The overexpression of citrate synthase in LKB1-deficient SCs was able to promote myelination in SC-DRG neuron co-cultures, partially rescuing the deficit observed. A different study focused instead on the role of LKB1 as a regulator of cellular polarity (Shen, Chen et al. 2014). LKB1 is the mammalian ortholog of Par4, a polarity regulator in *C. elegans*. The subcellular localization of LKB1 can be regulated

by phosphorylation in SC-DRG cultures. When phosphorylated, it can be found at the SC-axon interface where it colocalizes with Par3, another polarity-related protein. Viral induction of LKB1 depletion leads to mislocalization of Par3, suggesting LKB1 is necessary for this effect. The researchers demonstrated that LKB1 can be regulated by cAMP/PKA signaling by treating cultured SCs with forskolin, a stimulant of adenylate cyclase, to increase cAMP levels or by expressing a dominant negative form of PKA, a kinase known to phosphorylate LKB1 in other species.

These two studies show the complexity and interconnectedness of the network regulating SC differentiation and initiation of myelination. A third study evaluated instead the role of LKB1 on long-term axonal maintenance by SC. LKB1 was knocked out in SC specifically using the *Mpz-Cre* driver (active between E13.5 and E14.5), leading to a delay in sorting and onset of myelination (Beirowski, Babetto et al. 2014). This delay was resolved by post-natal day P30, although myelin thickness remained reduced. The sciatic nerve showed a similar metabolic dysregulation, with reduced lipids as seen by Pooya and colleagues, as well as increased AMP/ATP ratio, reduced NAD^+/NADH and increased lactate. At P90 some animals began showing signs of peripheral neuropathy, which became more pronounced with aging, and there were signs of axonal degeneration of both myelinated and unmyelinated fibers, with overt absence of small unmyelinated fibers at 1 year of age. The loss of sensory axons was more pronounced and led to functional deficits, whereas the motor function was relatively preserved. Interestingly, axonal loss was not preceded by demyelination or inflammation, suggesting it was due to the metabolic imbalance in SCs, and there were also no major changes to SC polarity. The authors found increased activity of AMPK, despite absent LKB1, and mitochondrial dysfunction. Finally, the researchers inhibited glycolysis using 2-deoxyglucose (2-DG) to effectively reduce lactate production, but this further exacerbated axonal loss. The authors suggest SC-derived lactate could be a compensatory way of supporting axonal metabolism, however, the 2-DG treatment may have starved LKB1-deficient SCs, leading to additional toxic effects.

5 THE ROLE OF NFAT IN MYELINATING GLIA

As previously mentioned, NRG1III/ErbB signaling can activate multiple downstream pathways that control sorting and myelination. One such pathway begins with the activation of PLCγ (Hogan, Chen et al. 2003). This enzyme catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG can further activate PKC and IP_3 binds to receptors on the endoplasmic reticulum (ER) to trigger the release of calcium from ER storage. Increased levels of Ca^{2+}, together with calmodulin, can activate the phosphatase calcineurin which will dephosphorylate different targets.

One of calcineurin's targets is NFATc4, a member of a family of transcription factors named Nuclear Factor of Activated T cells (the other members are NFATc1, c2, c3 and NFAT5 (Muller and Rao 2010)). NFATc1-c4 are regulated by phosphorylation by kinases such as PKA and GSK3β, becoming retained in the cytoplasm, and then activated by phosphatases, such as calcineurin. After dephosphorylation, cytosolic NFATs translocate to the nucleus where they bind a nuclear partner and induce transcription of target genes. In SCs, NFATc4 can cooperate with SOX10 to activate the transcription of KROX20 and MPZ (Figure 5-1) (Kao, Wu et al. 2009), and this can be further potentiated by cAMP (Kipanyula, Woodhoo et al. 2013). In the CNS, NFATs also mediate calcium signaling in OL, by interacting with SOX10 to regulate the expression of transcription factors NKX2.2 and OLIG2 (Weider, Starost et al. 2018).

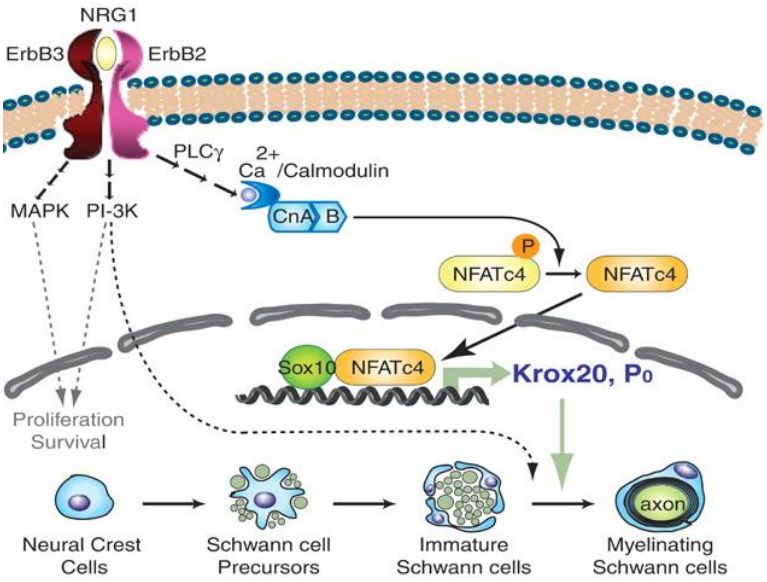

Figure 5-1 Schematic representation of a signaling pathway downstream of NRG1/ErbB, involving NFATc4 dephosphorylation by calcineurin (CnA/B) and promotion of transcription of myelin-related genes Krox20 and MPZ (P0). Reproduced from Kao et al. (2009).

Kao and colleagues (2009) studied a mouse model in which the regulatory subunit of calcineurin (CnB) was conditionally deleted in NCCs, by using the *Wnt1* promoter (active from embryonic day E8 (Hari, Miescher et al. 2012)). These animals died shortly after birth and showed increased phosphorylation of NFATc3 and NFATc4 in cultured DRGs, indicating that

the activation of these proteins depends primarily on calcineurin. In the sciatic nerve (SN), transgenic mice presented a delay in sorting and myelination, reduced levels of KROX20 and myelin proteins, whereas *in vitro* SCs lacking CnB were unable to activate NFAT dephosphorylation in response to stimulation with NRG1. Using mass spectrometry, the researchers identified SOX proteins as partners of NFATs and further validated that NFATc4 binds to SOX10 in cultured SCs. Using luciferase reporter assays, Kao and colleagues demonstrated that NFATc4 cooperates with SOX10 to activate the *Krox20* myelin-specific enhancer and the *Mpz* promoter, leading them to conclude that calcineurin/NFATc4 signaling is an important driver of SC maturation.

The expression of KROX20 is known to be regulated by cAMP, a co-activator of PKA and of CREB transcription factor. *In vitro,* this is achieved by culturing SCs in the presence of forskolin, an activator of adenylate cyclase. Kipanyula and colleagues (2013) found that treatment of cultured rat SCs with dbcAMP, an analog of cAMP, induced expression of KROX20 and it was further increased by adding a calcium ionophore, while the ionophore on its own did not have an effect (Kipanyula, Woodhoo et al. 2013). The synergistic effect of calcium could be prevented by co-treatment with cyclosporin A, an inhibitor of calcineurin, or by transfection of SCs with PVIVIT, a sequence that blocks the calcineurin-NFAT interaction. These results suggest that the effect of Ca^{2+}/calcineurin/NFAT as a promoter of KROX20 expression depends on cAMP signaling. The authors also found that nuclear translocation of a GFP-tagged NFATc2 construct depended on treatment with dbcAMP, not calcium, and it could be blocked by inhibition of calcineurin. These results suggest that co-activation of calcineurin/NFAT and cAMP/PKA pathways downstream of NRG1III/ErbB signalling is important for the optimal expression of master regulator gene *Krox20.*

The perinatal lethality of the CnB conditional knockout mouse developed by Kao *et al.* left open the question of what role does calcineurin play in SC during post-natal development. To address this question, Reed and colleagues (2020) developed a SC-specific CnB knockout by using the *Mpz-Cre* driver. The mutant mice presented a delay in radial sorting that was maintained into adulthood (some fibers were never myelinated, and some were inclusively demyelinated), but the myelin thickness was normal. The expression of transcription factors KROX20 and OCT6 was normal, as was the phosphorylation of AKT and ERK, suggesting calcineurin is dispensable in PNS post-natal development. However, these mice presented impaired injury response, due to reduced myelin clearance and autophagy, and this was related to reduced activation of TFEB transcription factor (an essential gene for lysosome production) (Reed, Frick et al. 2020).

These results paint a complex picture of the role of calcineurin/NFAT signaling in SC biology. It is possible that its role depends on the timing and intensity of activation/inhibition and may be modulated by other signaling pathways. Our contributions to the study of the role of NFATc4 in SCs will be presented in Paper III of this thesis (Domènech-Estévez et al., manuscript).

6 THE ROLE OF GLIA IN THE METABOLIC SUPPORT OF AXONS

As mentioned in previous sections, axons in the PNS are always in close contact with glial cells, whether in the form of myelinating SCs or Remak cells. The same can be said of the CNS, where axons can be wrapped by OL processes in myelin sheaths or they can be directly contacted by astrocytes. Astrocytes cover a large portion of the neuropil, interacting with axons, dendrites, synapses and blood vessels. They contribute to the recycling of neurotransmitters such as glutamate and mediate the flux of blood-borne substances into the brain parenchyma, contributing to the blood-brain barrier. Astrocytes are also known to store glucose in the form of glycogen to be used as an energy source, a function identified in SCs as well (Evans, Brown et al. 2013).

These types of cells that closely interact with and support neuronal function can be found in vertebrates and invertebrates alike, suggesting an ancient evolutionary history. *Drosophila* present several types of glia, some similar to astrocytes and others called wrapping glia, reminiscent of Remak cells and that enwrap axons of motor and sensory neurons (Freeman 2015). Jawless fish like lampreys (*Petromyzon marinus*) also present glia similar to SCs that express *SoxE* genes (orthologues to mammalian *Sox9* and *Sox10*) and sort axons according to their caliber: those greater than 3µm become enwrapped by individual cells while smaller ones remain in bundles (Weil, Heibeck et al. 2018). The absence of structured myelin in these animals suggests an important function of axon-ensheathing cells unrelated to fast saltatory conduction. Some of the topics in this section have also been discussed in Paper IV of this thesis (Boucanova and Chrast 2020).

6.1 BIOENERGETICS OF THE NERVOUS SYSTEM

The generation and propagation of action potentials by neurons is an energy-intensive process. Na/K ATPases work against the flow of sodium and potassium ions to restore the membrane potential, using an ATP molecule for each cycle that imports two K^+ ions and exports three Na^+. Estimates of oxygen consumption allow us to evaluate the extent of oxidative phosphorylation (OXPHOS). Estimates for the human brain suggest it uses 20% of the body's energy, despite representing 2% of its mass (Rolfe and Brown 1997). OXPHOS occurs in the mitochondria and relies on reducing molecules such as NADH to produce ATP. NADH can be produced in small amounts by glycolysis (the conversion of glucose to pyruvate) and in greater amounts by the tricarboxylic acid cycle (TCA cycle), which is fueled by acetyl-CoA produced from glycolysis-derived pyruvate, oxidized lipids and amino-acids. NADH and other molecules donate electrons to the respiratory chain of OXPHOS and drive a proton gradient across the mitochondrial membrane that is used to generate ATP by ATP synthase. Molecular oxygen is the final electron acceptor and it binds protons producing water molecules. However, some electrons escape the respiratory chain and generate reactive oxygen and nitrogen species (ROS and RNS), which can damage cellular proteins and lipids. Due to their

high oxidative metabolism, neurons are especially at risk for this type of damage (Chen, Guo et al. 2012) and to combat it, they must possess a large store of antioxidants (Bolanos and Almeida 2010). Glutathione is one of the main antioxidant molecules in cells and it is regenerated by NADPH, a reducing molecule produced by the pentose phosphate pathway (PPP). The PPP uses glucose to generate 5-carbon cyclical molecules and NADPH. In proliferative cells, this is important for production of nucleotides and the by-product NADPH is necessary for lipid synthesis of dividing plasma membranes, but this is obviously not the case of post-mitotic neurons. Figure 6-1 summarizes the different ways that glucose can be processed in cells.

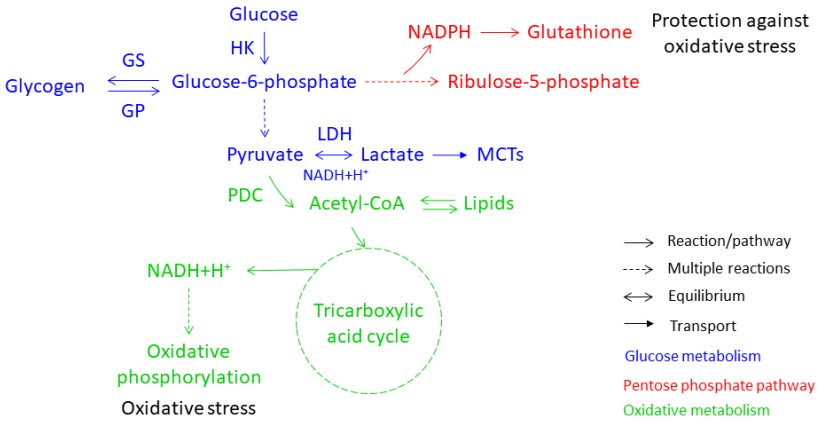

Figure 6-1 Summary of glucose metabolism in eukaryotic cells. HK – hexokinase; GS – glycogen synthase; GP – glycogen phosphorylase; LDH – lactate dehydrogenase; MCTs – monocarboxylate transporters; PDC – pyruvate dehydrogenase complex; NADH – nicotinamide adenine dinucleotide, reduced form; NADPH – nicotinamide adenine dinucleotide phosphate, reduced form.

At rest, the brain's usage of glucose and oxygen are well balanced, close to the expected stoichiometry of 6 O_2 to 1 glucose molecule (McKenna, Gruetter et al. 2005), but stimulation leads to a greater increase of glucose consumption than oxygen (Fox, Raichle et al. 1988). This has led scientists to propose that the brain can resort to aerobic glycolysis (known in cancer cells as Warburg effect) for energy production. However, glycolysis is a much less efficient process of ATP generation (2 ATP, 2 NADH and 2 pyruvate molecules per glucose molecule oxidized), and it can be quickly inhibited by its product NADH. In order to prevent NADH accumulation, pyruvate can be converted to lactate by lactate dehydrogenase, in a reversible reaction using NADH as a cofactor. The buildup of lactic acid in cells has additional detrimental effects such as acidosis, and so it must be exported by monocarboxylate transporters (MCTs) or other channels (for example, astrocytes express a lactate-permeable ion channel with a calculated conductance of 37 pS (picosiemens) (Sotelo-Hitschfeld, Niemeyer et al. 2015)). So, what could be the advantage of aerobic glycolysis in the brain, and which cells are responsible for this metabolic switch?

Evidence from *in vivo* and *in vitro* studies suggest that astrocytes are likely the main culprit for this phenomenon (Barros, Ruminot et al. 2020). Astrocytic stimulation by neuron-

released K^+ leads to inhibition of mitochondrial respiration and increased glycolysis. This causes an increase in lactate release, which can be taken up by neurons for energy production, (Sotelo-Hitschfeld, Niemeyer et al. 2015) and it also makes oxygen more available to neurons for use in OXPHOS. It is possible that outsourcing glycolysis to astrocytes may allow neuronal glucose to be used by the PPP instead. Additional signals such as ammonium ions from neurons and nitric oxide from the endothelium further regulate the rates of glycolysis and respiration in astrocytes and mediate the local, activity-dependent increases in lactate detected in certain brain regions (Barros, Ruminot et al. 2020). Nevertheless, neurons are not exclusively dependent on lactate for their metabolic needs and glucose may still play an important role in different physiological and pathological conditions (Ivanov, Malkov et al. 2014, Diaz-Garcia, Mongeon et al. 2017).

6.2 A GLIA-TO-NEURON LACTATE SHUTTLE

In the 1990s, Pellerin and Magistretti observed that cultured astrocytes respond to glutamate stimulation by increasing glycolysis and releasing lactate (Pellerin and Magistretti 1994). They called this phenomenon the astrocyte-neuron lactate shuttle (ANLS) and proposed that, upon neuronal activation, astrocytes can be stimulated to increase their glycolytic output and produce large amounts of lactate to be taken up by neurons and sustain their oxidative metabolism (Pellerin, Pellegri et al. 1998). To this day, this topic is the source of debate and many publications have provided evidence both for and against it (Chuquet, Quilichini et al. 2010, Wyss, Jolivet et al. 2011, Hall, Klein-Flugge et al. 2012, Machler, Wyss et al. 2016, Diaz-Garcia, Mongeon et al. 2017, Yellen 2018, Chamberlain and Sheng 2019). This hypothesis relies on the following observations: 1) astrocytes present a highly glycolytic metabolism, they are able to store glucose as glycogen, express LDH5 (an enzyme that predominantly converts pyruvate to lactate (Bittar, Charnay et al. 1996)), MCT1 (a medium affinity monocarboxylate transporter (Tekkok, Brown et al. 2005)) and MCT4 (low affinity MCT); these factors make astrocytes a net lactate producer. 2) Neurons present a more oxidative metabolism, express LDH1 (predominantly converts lactate to pyruvate) and MCT2 (a high affinity monocarboxylate transporter); these factors make neurons a net lactate consumer.

Inhibition of glycogenolysis in the hippocampus, or the ablation of MCTs, leads to amnesia in rats (Suzuki, Stern et al. 2011). This can be rescued by administration of L-lactate if the astrocytic MCTs are ablated, but not if the neuronal MCT2 is, suggesting the uptake of lactate into neurons is crucial for memory formation. In *Drosophila*, glycolytic metabolism and lactate transfer from glia supports neuronal function and survival (Volkenhoff, Weiler et al. 2015, Delgado, Oliva et al. 2018), and the ablation of a lactate transporter, *Chaski*, in glia leads to impaired locomotion and increased sensitivity to nutrient withdrawal. The lactate shuttle in *Drosophila* can act not only as a source of energy but also as a protection against oxidative stress: under ROS stress, photoreceptors in the eye can use lactate provided by pigment cells to synthesize lipids (Liu, MacKenzie et al. 2017). Then, they ship them back to the glial cells

where they accumulate as lipid droplets. If this process is blocked by ablation of neuronal MCTs, lactate import and lipid production are reduced and neurodegeneration delayed, but elimination of apolipoproteins blocks lipid transfer to glia and accelerates neuronal death. It is possible that the accumulation of lipid droplets and glycogen in glial cells could be an evolutionarily advantageous way for neurons to outsource metabolic support, while specializing in impulse transmission (Nave, Tzvetanova et al. 2017). This may have been one of the steps that enabled the evolution of more complex behaviors and cognitive functions in animals. But what about myelinating glia, are they also implicated in energy support of neurons?

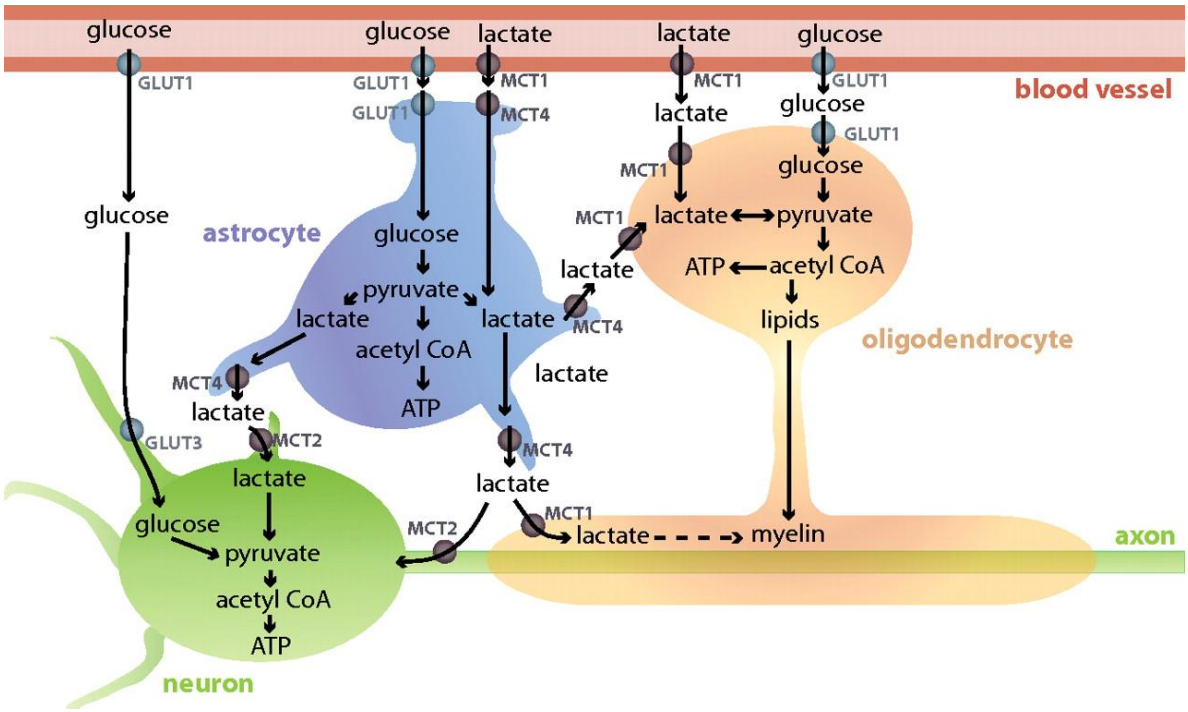

Figure 6-2 Schematic representation of glucose and lactate utilization by neurons, astrocytes and oligodendrocytes (OL). Blood-borne glucose and lactate can be taken up directly by cells of the CNS, to be utilized in energy production and lipid synthesis, for example. Alternatively, glucose can be converted to lactate in glial cells, astrocytes and OLs, and shuttled to neurons via monocarboxylate transporters (MCTs). Not represented: lactate transport between OLs and neurons. Reproduced from Rinholm et al. (2011).

Different studies have provided evidence that OLs can support axons in a myelin-independent manner. Animals that lack certain myelin-related genes, such as *Cnp* (Lappe-Siefke, Goebbels et al. 2003) and *Plp* (Griffiths, Klugmann et al. 1998) develop relatively normal myelin but later show signs of axonal degeneration. This is due, in part, to impaired interactions between the two cell types, namely at the level of the node of Ranvier and paranodal loops in animals lacking CNP (Rasband, Tayler et al. 2005, Edgar, McLaughlin et al. 2009), and the effect is exacerbated by absence of PLP, which leads to less-compact myelin (Rosenbluth, Nave et al. 2006). These observations suggest that axonal integrity depends greatly on proper contact with myelinating glia, or that abnormal contacts may have toxic consequences. OL metabolism is another important factor that supports axonal maintenance. During development, OLs can use both glucose and lactate for their metabolism (Rinholm,

Hamilton et al. 2011) and they rely on oxidative metabolism to sustain the high metabolic needs of myelination, but in the adult stage they switch to a more glycolytic state (Rao, Khan et al. 2017). Ablation of COX10 in OLs, an essential component of the mitochondrial cytochrome C oxidase, blocks oxidative metabolism after myelination has occurred, forcing OLs to rely only on glycolysis (Funfschilling, Supplie et al. 2012). These cells are perfectly able to maintain myelin and axonal integrity, but they produce larger amounts of lactate than controls, which can only be observed under isoflurane anesthesia. When the anesthetic is removed, lactate quickly decreases probably because oxidation-competent neurons can take it up for their own use. Like astrocytes, OLs also express MCT1 (Lee, Morrison et al. 2012). Mice lacking one copy of the MCT1 gene (*Slc16a1*), or mice injected with an shRNA against MCT1 in the optic nerve or corpus callosum, show axonal degeneration without changes in myelin thickness and no signs of demyelination. The authors also found reduced levels of MCT1 in human and mouse samples of ALS, and they proposed that reduced support from OLs or oligodendroglial dysfunction may contribute to the phenotype. It is also important to mention that lactate release from OLs can be induced by stimulation of NMDA receptors (Saab, Tzvetavona et al. 2016). Upon treatment with NMDA, cultured mouse OLs increase the expression of glucose transporter GLUT1 at their surface. This leads to increased glucose uptake and lactate release (which is not due to increased MCT1 expression). Mutant mice lacking expression of NMDA receptor subunit NR1 show reduced incorporation of GLUT1 into myelin sheaths and a transient delay in myelination, suggesting NMDA signaling regulates glucose availability during myelin assembly. In the absence of NR1, optic nerves are more sensitive to oxygen-glucose deprivation *ex vivo*, as their ability to recover compound action potentials is half of the control when exposed to glucose. However, both controls and mutants recover to the same level when exposed to lactate. Furthermore, mutant mice showed signs of neurodegeneration starting at around 1 year of age, with inflammation, astrogliosis and motor deficits. This suggests that neuronal activity can modulate OL myelination and metabolic support via regulation of glucose uptake and lactate release, and the impairment of this interaction contributes to neuronal pathology.

6.3 SCHWANN CELLS AS METABOLIC SUPPORTERS OF AXONS

In the PNS, SCs also seem to support axons in myelin-independent ways. Similarly to OLs, some mutations in SC-specific MPZ lead to an axonal form of peripheral neuropathy without major changes to myelin structure (Misu, Yoshihara et al. 2000, Bienfait, Faber et al. 2006), suggesting other aspects of the SC-axon interaction besides myelin are crucial for proper axonal function.

Experimental interference with SC metabolism has translated into axonal distress in different ways. Viader and collaborators (Viader, Golden et al. 2011, Viader, Sasaki et al. 2013) studied a mouse model with conditional knockout of the mitochondrial transcriptional factor *Tfam* specifically in SCs. These animals presented reduced oxygen consumption (evidence of impaired OXPHOS) but they developed normally and showed normal myelin thickness and

structure in early adulthood (P30). However, the mutant mice developed a progressive neuropathy characterized by decreased nerve conduction velocity, muscle atrophy, demyelination and axon degeneration, starting with small unmyelinated C fibers. Interestingly, SC proliferation and survival were unaffected by the mitochondrial defect and they were able to promote axon regeneration after injury but they could not remyelinate as the controls. In the second study, the researchers found that mutant SCs present activation of integrated stress response, which can drive SC dysfunction, and abnormal lipid metabolism, with accumulation of toxic lipid species that can induce axonal injury. The previously mentioned *Cnp-Cre Cox10* mutant (Funfschilling, Supplie et al. 2012) shows a stronger effect in the PNS, as compared to the CNS. Mutant SCs were able to survive but they showed defects in sorting and myelination, leading to conduction blocks and axonal loss. These studies, together with the LKB1 mutant model (Beirowski, Babetto et al. 2014) (section 4.3 of this thesis), suggest that SCs depend on mitochondrial metabolism for their functions, but they can survive on glycolytic metabolism.

Similarly to astrocytes and OL, SCs express monocarboxylate transporters MCT1 and MCT4 (MCT2 has only been detected at the mRNA level) (Domenech-Estevez, Baloui et al. 2015). The subcellular localization of each of these transporters and their relative affinities for monocarboxylates suggest different functions: MCT1 has been detected in non-compact myelin structures such as the Schmidt-Lantermann incisures and paranodal regions while MCT4 is found in the perinuclear region, outer cytoplasmic mesaxonal line and Cajal bands. The heterozygous elimination of MCT1 leads to impaired recovery from a nerve crush injury to the sciatic nerve (Morrison, Tsingalia et al. 2015). This is due to reduced ability of SCs to remove myelin debris and promote axon regeneration. Upon axotomy, SCs switch to a highly glycolytic metabolism and provide injured axons with lactate to sustain their survival (Babetto, Wong et al. 2020). Pharmacological inhibition of MCTs *in vitro* accelerates axonal disintegration, while treatment with a cell membrane-permeable form of pyruvate protects axons. MCT1 and MCT4 expression in SCs is greatly upregulated after injury, together with glycolytic enzymes, and these effects seem to be mediated by the mTORC1 pathway. In myelinating SCs, glycogen breakdown and conversion to lactate can sustain the activity of large myelinated A fibers, while unmyelinated C fibers rapidly lose their conduction ability unless other energy substrates are provided (Brown, Evans et al. 2012, Rich and Brown 2018). C fibers can directly take up and use substrates such as fructose, while myelinated fibers depend on SCs to convert fructose to lactate. These observations support the idea that SCs, too, may participate in a glia-neuron lactate shuttle. The conditional elimination of MCT1 in SCs (Jha, Lee et al. 2020) seems to lead to progressive myelin thinning in sensory nerves (despite normal development) accompanied by sensory deficits, partly due to changes in myelin lipid synthesis and a reversal of SC maturation state. It is likely that MCT1 and lactate may also be important for SC metabolism and myelin maintenance. Our own study of MCT1 (and MCT4) elimination in SCs can be found in Paper I of this thesis (Boucanova, Pollmeier et al. 2020).

SCs express a complement of cell surface transporters and receptors that may enable them to respond to axonal needs (Samara, Poirot et al. 2013). In addition to MCTs, SCs also express GLUT1 and 3 for glucose uptake (Magnani, Cherian et al. 1996) and connexins

(Altevogt, Kleopa et al. 2002), which along with cytoplasmic channels may allow the communication between the extracellular and the periaxonal spaces. Additionally, the presence of neurotransmitter receptors such as NMDAR (Campana, Mantuano et al. 2017) and metabotropic glutamate receptors, mGluR (Saitoh, Wakatsuki et al. 2016) may be the missing pieces to connect neuronal signaling to SC support: glutamate or NMDA injection into the sciatic nerve can activate ERK signaling, while in vitro stimulation of SCs with NMDA triggers ERK, AKT and PI3K pathways (Campana, Mantuano et al. 2017). Higher stimulation by glutamate via mGluRs, on the other hand, may mediate the effects of excitotoxicity and regulate response to injury (Saitoh, Wakatsuki et al. 2016). Nevertheless, whether SCs can provide metabolic support to axons in an activity-dependent manner remains to be elucidated.

6.4 MONOCARBOXYLATE TRANSPORTERS

Monocarboxylate transporters, or MCTs, are members of the solute carrier family 16 (SLC16) (Halestrap 2013). MCT1-4 are transmembrane proteins that facilitate the transport of pyruvate, lactate and ketone bodies along their concentration gradient. Other members of this family carry other substrates, such as MCT8 that transports thyroid hormone or MCT10 that carries aromatic amino-acids.

MCTs present 12 transmembrane domains, a long intracellular loop between domains 6 and 7 and a C-terminal tail (Poole, Sansom et al. 1996). They possess a conserved lysine residue in domain 1 accompanied by conserved arginine and aspartate residues in domain 8 which, together, mediate substrate binding and translocation (Wilson, Meredith et al. 2009). MCTs can transport a monocarboxylate anion together with a proton or they can exchange one monocarboxylate with another. The latter mechanism is faster than the former and called trans-acceleration (Deuticke 1982): higher concentration of one substrate in one cellular compartment can force the movement of another substrate from another compartment. It can be used in experimental settings to deplete intracellular lactate by a forced pyruvate gradient, for example (San Martin, Ceballo et al. 2013, Machler, Wyss et al. 2016). For MCTs to be expressed at the plasma membrane they require an accessory protein - basigin or embigin, depending on the transporter: MCT1 can bind either chaperone, MCT2 is paired with embigin and MCT4 with basigin. Overexpression of MCT1 or 4 in *Xenopus* oocytes without a chaperone causes accumulation in the ER, but co-expression with basigin targets both transporters correctly to the plasma membrane (Wilson, Meredith et al. 2005). Organomercurial agents such as pCMBS (p-chloromercuribenzene sulfonate) attack a disulfide bond in basigin and interfere with its interaction with MCT1/4, inhibiting their function.

MCTs have different affinities for different substrates, which are summarized in *Table 1* (Halestrap 2013). The different affinities may help explain the cell type-specificity observed in mammals (Halestrap 2013). For example, in skeletal muscle, MCT1 is expressed in slow-twitch oxidative fibers (type I) while MCT4 is expressed in fast-twitch glycolytic fibers (type II) (Bonen, Miskovic et al. 2000). In the testis, Sertoli cells produce lactate and express MCT4

whereas spermatids express MCT1 and 2 and oxidize lactate for energy (Boussouar, Mauduit et al. 2003, Bernardino, D'Souza et al. 2019, Yu, Sun et al. 2019). These are similar patterns as the one observed in the brain and strengthen the evidence of metabolic coupling between cell types: glycolytic cells tend to express lower affinity transporters (exporting lactate due to higher cytosolic concentration) while oxidative cells express higher affinity MCTs (both import and export at lower concentrations).

Table 1 Estimated Km of MCTs for different substrates and their plasma concentration. Reproduced from Halestrap (2013).

	Estimated Km in mM (expression in *Xenopus* oocytes or endogenous expression in cancer cells*)			Plasma concentration in healthy humans (mM)
Substrate	MCT1 (medium affinity)	MCT2 (highest affinity)	MCT4 (lowest affinity)	
L-Lactate	3.5	0.74	28	1.51
Pyruvate	1.0	0.08	153	0.064
Acetoacetate	5.5*	0.80	216	0.041

Other inhibitors of MCTs have been identified and they are used in experimental settings: α-cyano-4-hydroxycinnamate (Halestrap and Denton 1974) and phloretin (Wang, Poole et al. 1993) are unspecific but reversible (unlike pCMBS), ARC-155858 is a specific and reversible inhibitor of MCT1 (Ovens, Davies et al. 2010), syrosingopine targets MCT1 and 4 (Benjamin, Robay et al. 2018) and BAY-8002 inhibits MCT1 and 2 (Quanz, Bender et al. 2018). These inhibitors have been studied in the context of cancer research. As a therapeutic approach, MCT inhibitors take advantage of cancer cells' dependence on glycolysis and use it against them. For example, treatment of cancer cell lines with BAY-8002 increases intracellular lactate and reduces proliferation (Quanz, Bender et al. 2018), while a combination of syrosingopine and metformin (an inhibitor of mitochondrial NADH dehydrogenase) depletes intracellular NAD^+, blocking glycolysis and leading to cell death (Benjamin, Robay et al. 2018). In studies of neuronal-glial metabolic coupling, use of MCT inhibitors allows the quantification of intracellular lactate (San Martin, Ceballo et al. 2013, Sotelo-Hitschfeld, Niemeyer et al. 2015) and the inhibition of lactate shuttling between cells (Babetto, Wong et al. 2020).

7 RESEARCH AIMS

The aims of this thesis are to study the development of neurons and glia in the peripheral nervous system and their long-term interactions.

Paper I

The aim of study I was to assess the role of glial monocarboxylate transporters MCT1 and MCT4 in the PNS. For this, we established conditional knockout mouse models lacking MCT1 or MCT4 specifically in SCs and evaluated their ability to establish and maintain myelin, as well as long-term maintenance of target-tissue innervation (skin and muscle) by histological, behavioral and molecular techniques.

Paper II

The aim of this study was to evaluate the role of epigenetic regulator *Prdm12* in the embryonic development of peripheral sensory neurons in mice, a gene known to cause congenital insensitivity to pain in humans when mutated. We studied constitutive knockout mouse models and assessed the development of nociceptive, mechanoreceptive and proprioceptive neurons by histochemical techniques.

Paper III

The aim of this paper was to study the consequences of a constitutively active form of transcription factor *NFATc4* in myelinating glia in mice. We generated a transgenic mouse model overexpressing an active form of human *NFATc4* in SC (and OL) and evaluated the effect of this genetic modification in SC development by electrophysiological, histological and molecular techniques.

8 METHODOLOGICAL AND ETHICAL CONSIDERATIONS

8.1 GENETICALLY MODIFIED MOUSE MODELS

In this thesis, we studied several genetically modified mouse models generated using different approaches. In Paper I, we conditionally eliminated *Slc16a1* (MCT1) or *Slc16a3* (MCT4) specifically in SC using the *Mpz-Cre* driver. This enabled us to evaluate the contribution of SC to metabolic support of axons (as "donors" of energy substrates, so to speak), while maintaining the neurons' ability to take-up monocarboxylates from the extracellular environment. In Paper II, we studied two full body knockout mouse models lacking the exon 2 of the *Prdm12* gene, leading to complete ablation of *Prdm12* expression. This approach demonstrated the importance of the gene, as the homozygous knockout animals died soon after birth, while the heterozygous littermates were undistinguishable from controls. Finally, in Paper III, we used the promoter of *Cnp*, a myelin constituent protein, to drive the overexpression of constitutively active human *NFATc4* in myelinating cells, SC and OL. This decision would allow us to assess the effects of active NFATc4 in both cell types.

From studying these three different approaches, and comparing them to relevant literature, an important point stands out for consideration. Special attention should be paid to the promoter used for Cre-mediated recombination or tissue-specific overexpression. Gene activation at earlier stages of development may produce a more robust elimination/expression of the gene but may also have unintended effects by targeting other cells derived from the same precursor population. This is the case for example with the *Wnt-Cre* line, used by Kao and colleagues (Kao, Wu et al. 2009) for the elimination of calcineurin subunit CnB: the elimination of *CnB* in *Wnt*-expressing neural crest cells leads to perinatal lethality of transgenic mice. The postnatal development of the PNS cannot be studied in this model and it is therefore unknown if the phenotype observed reflects a delay in SC maturation or a complete arrest. Similarly, the *Cnp* promoter we used in our study of constitutively active NFATc4 presents a strong CNS phenotype (frequent seizures) that limits the long-term study of the animals, not to mention the ethical aspects of maintaining a line of severely affected animals. In addition, the full body elimination of *Prdm12* in mice made evident the differences in gene expression in mice compared to humans (it can be detected in other neuronal populations in the mouse while it seems to be restricted to DRG in humans). Such effects can be overcome by selecting promoter genes that are more cell type-specific, expressed later in embryonic development or inducible.

Another important aspect is the relationship between different genes. In the case of the MCT1 and MCT4 cKO mice, the mild phenotype observed in both lines may indicate the ability of the remaining transporters to compensate for the absent gene. In this case, a combination of several or all genes could yield more robust results. Not to be ignored also is the contribution of extracellular metabolites to the support of neuronal metabolism. In this case, the elimination of the gene(s) in the neuronal compartment, alone or in combination with the glial cKO, is another relevant model to study.

Working with genetically modified mouse models should always give the researcher some pause. We should ask ourselves, why am I using this model and are there alternatives? An experimental model should be adjusted to the scientific question asked, as some questions can be addressed in a culture dish, while others require the study of the full organism. The utilitarian perspective would argue that the ends justify the means. The possibility of developing a cure for a human ailment, minimizing the suffering of millions of humans, is a worthy goal that justifies the sacrifice of non-human animals. Do we not use the same rationale when we consume animals for food? However, anyone who has ever had a pet knows that animals are sentient creatures, they feel pleasure, pain, fear, we could even say some have personalities. It is therefore the humane attitude to minimize animal suffering throughout all aspects of model development and research. This can be done by establishing humane endpoints, providing environmental enrichment and symptomatic treatment. We should also refine our methods in order to reduce the number of animals used in a study. For these reasons, it is essential to consult with an ethical committee, which will judge our experimental approaches and recommend better courses of action.

If we choose to completely avoid animal experimentation, what other options do we have? *In vitro* studies can be suitable, but the problem of cell origin remains. We can use cell lines, which are immortalized and can be passaged multiple times, but these cells present tumor-like properties and will not reproduce the metabolism of a healthy tissue. Alternatively, primary cell cultures are a more faithful representative, but they still require the sacrifice of dozens of animals. Additionally, certain cell types can only be obtained from embryos meaning they will not necessarily behave as adult cells. Whether *in vitro* or *in vivo*, one should also remember that the biology of a mouse is not the same as a human, and results may not be directly translatable. To overcome this difficulty, great advances have been made to the development of induced pluripotent stem cell technology. This allows the use of donated human tissue from a small skin biopsy to produce stem cells that can differentiate into a variety of tissues and cell types. Nevertheless, these methods are still in their infancy and every differentiation protocol must be optimized and well characterized before drawing conclusions from experiments. However, *in vitro* approaches have limitations when it comes to the study of long-term cellular interactions and complex systemic diseases, as well as late-onset disorders or ageing. Often, an *in vitro* approach will require a strong inductive stimulus that is not representative of a physiological state. In these situations, it may be required to resort to animal models instead.

In this thesis we opted to study mouse models because we deem the topic of axon-SC interactions to be very complex, as certain aspects can only be assessed at the behavioral level, for example. The intimate relationship between neurons and SCs cannot be fully represented *in vitro*, namely the developmental and ageing aspects. *In vitro* studies can also carry high variability and minor phenotypes may be easily missed. Additionally, animal studies allow experimental readouts such as innervation of target tissues, like muscle and skin, that cannot yet be established *in vitro.*

9 PRELIMINARY RESULTS

9.1 MULTI-MCT CKO MOUSE MODELS

Upon the observation that the MCT1 and MCT4 cKO mice presented a mild phenotype, we crossed both lines to obtain a double cKO (dCKO). These animals were characterized at the level of motor and sensory behavior, and preliminary nerve conduction velocity recordings were performed.

Methods

All tests were performed as described in Paper I. Briefly, one-year old animals were anesthetized with ketamine and xylazine, and motor nerve conduction velocity (MNCV) was recorded by inserting needle electrodes into the interdigital muscles of the hind-paw. The muscles were stimulated first at the base of the gastrocnemius and then at the sciatic notch, and the difference in latency was divided by the distance between the two stimulation points to obtain the conduction velocity. Three measurements per leg were averaged for each individual datapoint and results are represented as mean of 3-6 individuals ± standard deviation. Similarly, sensory nerve conduction velocity (SNCV) was determined on the tail by recording subdermally at the base of the tail and stimulating at a distance of 3 cm. Each measurement consisted of 10 sweeps and results are presented as mean of 3 individuals ± standard deviation.

The sensory behavioral tests consisted of modified Hargreaves box (for heat stimulation), acetone drop test (for cold stimulation) and von Frey filaments test (for mechanical stimulation). The Catwalk system (Noldus) was used to evaluate motor behavior and multiple parameters were recorded. Animals were allowed to habituate to testing conditions prior to recording of results and the experimenter was blinded to animals' genotypes during testing. For details please refer to Paper I of this thesis.

Results

Preliminary data on the double conditional knockout of MCT1 and MCT4 shows preserved motor and sensory nerve conduction, which suggests presence of intact myelin in these animals (Figure 9-1a). Behavioral tests indicate mostly preserved sensory function, with only a small increase of 2 s in latency to respond heat stimulation in dCKO males (Figure 9-1b). Motor function assessed by the Catwalk did not identify differences between dCKO and control animals (Figure 9-1c).

These results suggest that simultaneous elimination of MCT1 and MCT4 may not be sufficient to impair SC-derived support to axons in a way that affects gross behavior and conduction velocity. The remaining transporter expressed by SC, high-affinity MCT2, and/or axonal MCTs may be the critical molecules for this function. It is also possible that it is still too early to detect changes in axonal maintenance, and that later timepoints may present a stronger phenotype.

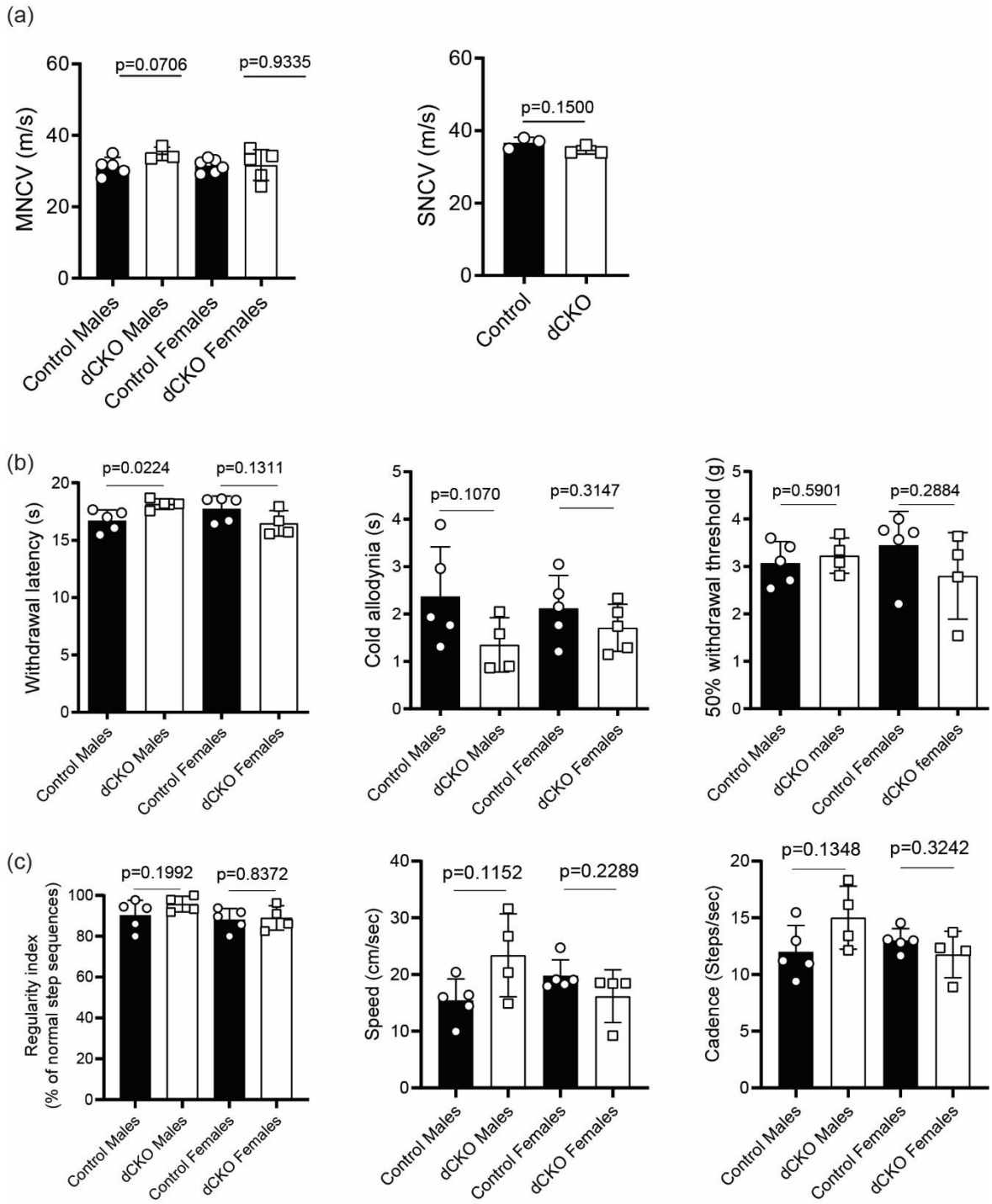

Figure 9-1 Electrophysiological and behavioral characterization of one-year old double MCT1/MCT4 conditional knockout mice (dCKO). a) Motor (left) and sensory (right) nerve conduction velocity measurements indicate preservation of conduction in motor and sensory nerves, suggesting preserved myelin structure. b) Sensory behavioral tests suggest that sensory function is preserved in these animals, with only a small increase in latency to respond to heat stimulation by dCKO males (left panel – Hargreaves test, middle panel – acetone drop test, right panel – von Frey filaments test). c) Motor behavior was assessed using the Catwalk system (Noldus) and results suggest that motor function is not impaired in one-year old dCKO mice.

In addition, we established a line carrying a floxed *Slc16a7* (MCT2) allele, which was crossed with an *Advillin-Cre* driver for gene elimination in peripheral sensory neurons. The same floxed allele was then introduced into the double cKO to generate a triple cKO with *Mpz-Cre* driver for elimination in SCs. In parallel, the double *Mct1/Mct4* floxed alleles were combined into the *Mct2 Advillin-Cre* to produce a triple cKO in sensory neurons. The triple cKO lines have recently been made available to other researchers in the field for further study.

10 RESULTS AND DISCUSSION

10.1 PAPER I

In study I of this thesis we hypothesized that monocarboxylate transporters MCT1 and MCT4 expressed by SCs are important for metabolic support of axons, in a mechanism similar to the ANLS. To assess the importance of these transporters, we conditionally deleted the expression of MCT1 or MCT4 in SC specifically, using *Mpz-Cre* driver.

After verifying the depletion of both mRNA and protein expression in sciatic nerves, we assessed the function of the PNS in these mice at the behavioral and electrophysiological level. No major differences were detected at 1 year of age, suggesting that the elimination of either MCT1 or MCT4 alone in SC do not impair sensory or motor behavior nor nerve conduction (selected results in Figure 10-1). We then evaluated the structure of the sciatic nerve but were unable to detect differences in myelin thickness (g-ratio) or Remak bundle structure, which indicates that both MCT1 and MCT4 are dispensable for SC myelination/myelin maintenance and axon sorting.

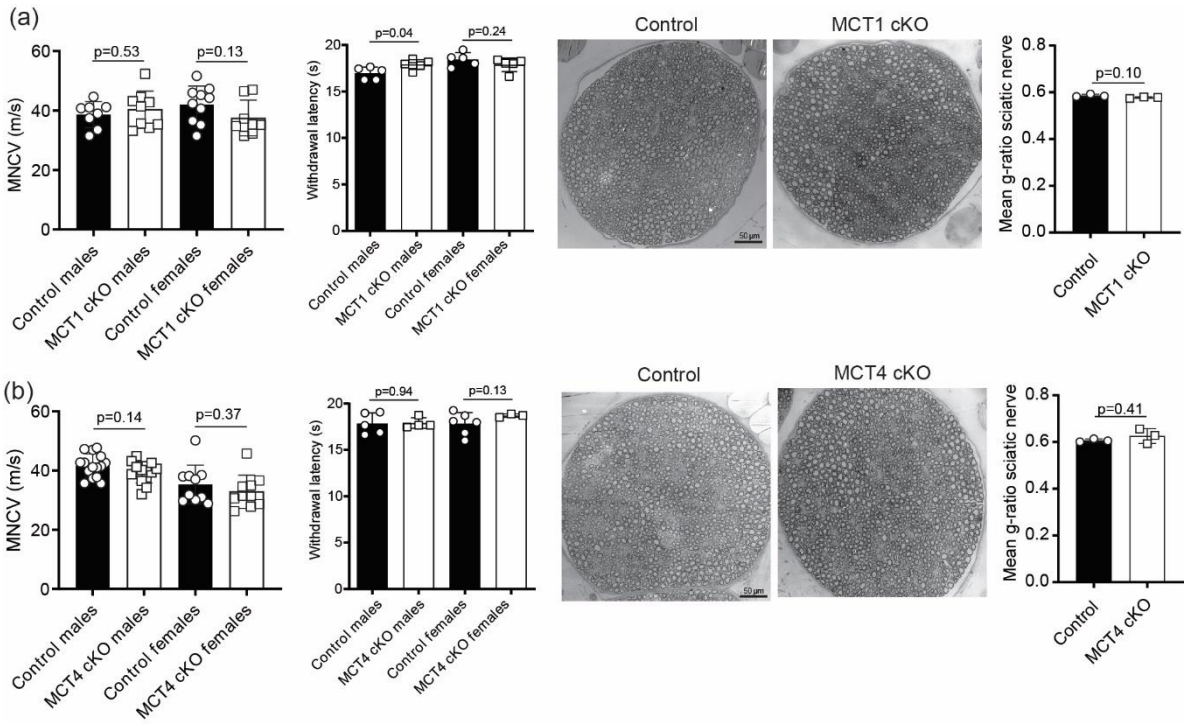

Figure 10-1 In the absence of MCT1 (a) or MCT4 (b), the sensorimotor function of the PNS is preserved, as well as its structure. Adapted from Bouçanova et al. (2020).

Since MCT1 is more robustly expressed by SC, as compared to MCT4, we decided to focus our analysis on the MCT1 cKO line. We hypothesized that there might be changes in the innervation of target tissues that had not yet manifested at the behavior level. We, therefore, quantified the intraepidermal nerve density (free nerve endings in the skin) and the innervation status of neuromuscular junctions in the gastrocnemius muscle. We found no difference in skin innervation, but there was a shift in the innervation status of NMJs, with a reduction in fully

innervated NMJs accompanied by an increase in both fully denervated and partially denervated NMJs. This observation prompted us to question whether spinal motoneurons might be affected at the molecular level and we performed laser capture microdissection-RNA sequencing (LCM-Seq). We found changes in genes related to cytoskeleton, mitochondria and regulation of transcription, among others (selected results in Figure 10-2).

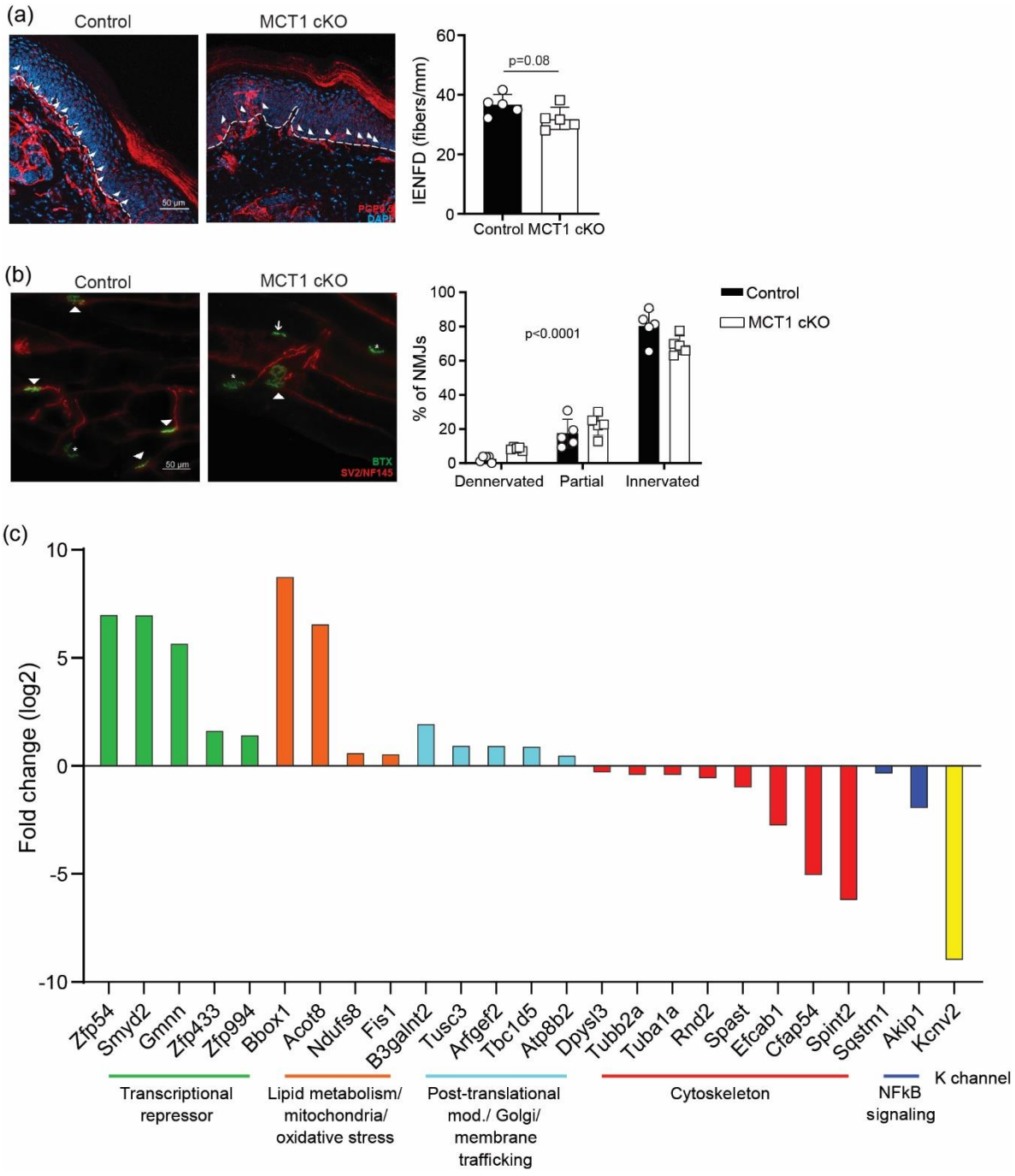

Figure 10-2 Ablation of MCT1 in SC does not affect long-term maintenance of skin innervation (a) but it leads to a small shift in the innervation status of NMJs (b). LCM-seq of lumbar motoneurons reveals a few changes in gene transcription, namely an increase in transcriptional repressors and genes related to mitochondria, as well as a reduction in cytoskeletal genes, among others. Adapted from Bouçanova et al. (2020).

No specific pathway emerged as significantly enriched, which may reflect the mild phenotype observed. It is possible that we have only detected the onset of a slow-developing neuropathy and that the analysis of older animals could yield more robust results. Additionally, Lee *et al.* (2012) showed that reduced MCT1 in OLs causes degeneration in only a small subset of neurons in the optic nerve.

Comparison of our findings with those of Jha et al. (2019) are limited by the differences in methodology. Jha and collaborators found sensory deficits in mutant mice, together with progressive thinning of myelin and altered lipid metabolism. Conversely, we looked at the sciatic nerve, which presents mixed sensory and motor axons, and we did not find alterations in myelin. However, when comparing myelination in spinal roots we did not find any differences either. Nevertheless, our method of SNCV measurement is likely less precise than Jha's and may have missed possible subtle differences. Finally, our method of assessing mechanical sensitivity is also different (we evaluated the median stimulus intensity, which could trigger a response in 50% of trials, whereas Jha et al. looked at the frequency of paw withdrawal at specific stimuli intensities). In common, both studies indicate that removing MCT1 from SCs has limited effect on the underlying axon, suggesting that either MCT1 is not the only or not the main route of glial-derived metabolic support. We believe that the presence of MCT2 in SC and/or neurons (not analyzed in this study) could play an important role in mediating import/export of energetic metabolites by the two cell types, and that models lacking all MCTs are required to truly assess their relevance.

10.2 PAPER II

Mutations in the *PRDM12* gene were identified in 2015 as a cause of congenital insensitivity to pain in human patients and, in frogs, this gene seems to participate in sensory neuron development (Chen, Auer-Grumbach et al. 2015). In Paper II we studied the role of mouse *Prdm12* in the embryonic development of neurons in the DRG.

First, we characterized the pattern of *Prdm12* expression in developing sensory neurons in wild-type mice and found that it can be detected initially in migratory SOX10^{+} precursor cells, but later becomes restricted to TRKA^{+} neurons. We could detect a few TRKC^{+} cells also expressing *Prdm12* mRNA but we could not detect it in the remaining neuronal subtypes (TRKB^{+} and RET^{+}). Next, we studied *Prdm12* knockout mice and discovered that the ablation is likely lethal, as we could not obtain any newborn *Prdm12*$^{-/-}$ pups. Nevertheless, the *Prdm12*$^{+/-}$ heterozygous littermates developed normally and we were able to collect knockout embryos up to E18.5, suggesting post-natal lethality. In the knockout embryos, we found that the DRG is significantly smaller in size and that this is due to a total absence of TRKA^{+} neurons, while the remaining neuronal populations were relatively unchanged (selected results in Figure 10-3a-e). We could also detect reduced peripheral innervation, and an absence of epidermal free nerve endings (nociceptive skin receptors) (selected results in Figure 10-3f and g).

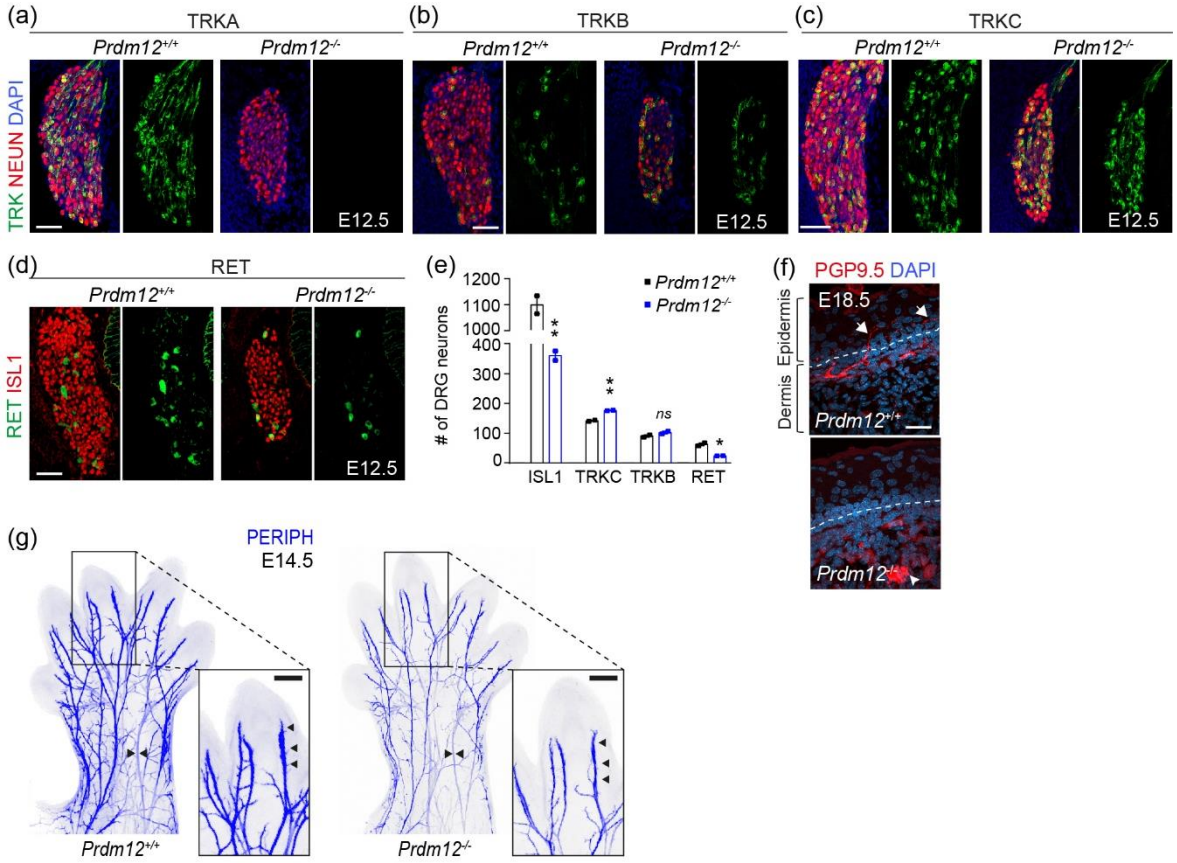

Figure 10-3 Deletion of Prdm12 in mice leads to complete absence of TRKA^{+} nociceptive neurons. (a) Immunostaining of E12.5 DRGs from control and Prdm12 KO mice with anti-NeuN (red, pan-neuronal marker) and anti-TRKA (green, nociceptor marker), showing a complete absence of TRKA staining. Immunostaining with other markers of sensory neurons

(TRKB (b), TRKC (c) and RET (d)) show these subpopulations are not affected by Prdm12 deletion (scale bars 50µm). (e) Quantification of number of neurons (ISL1) and number of A-fiber low-threshold mechanoreceptors (TRKC, TRKB and RET) indicate a robust decrease in the total number of neurons, with minor changes in TRKC$^+$ and RET$^+$ cells. f) Innervation of the epidermis by pain-sensing free nerve endings is absent in Prdm12-/- E18.5 embryos (arrows), but mechanoceptive Meissner corpuscles are still present in the dermis(arrowhead). Dotted line indicates the interface between dermis and epidermis (scale bar 20µm). g) Whole-mount immunohistochemistry of E14.5 forelimbs indicates reduced innervation and branching, inset is a higher magnification of a digit (PERIPH - peripherin, scale bar 100µm). Adapted from Bartesaghi et al. (2019).

The reduced number of neurons was traced back to a reduced proliferation of SOX10$^+$ progenitors but not increased apoptosis (selected results in Figure 10-4a and b). Since the mechanoreceptor and proprioceptor subpopulations were neither increased nor decreased, we questioned if forcing the expression of *Prdm12* would be sufficient to induce a nociceptor fate. We electroporated chicken embryos with either a control construct driving the expression of eGFP or a construct for FLAG-tagged *Prdm12* overexpression (selected results in Figure 10-4c). We discovered that the FLAG tag could only be detected in TRKA$^+$ neurons or neurons lacking additional subtype-specific markers (while the GFP control was detected in neurons and other cell types) (selected results Figure 10-4d). The total proportion of TRKA$^+$ neurons was the same in both conditions (selected results Figure 10-4e) but no TRKC$^+$ cells were positive for the FLAG-tagged construct (selected results Figure 10-4f). This indicates that *Prdm12* expression can repress the differentiation of other sensory neuron populations but alone it is not sufficient to induce a nociceptor development program.

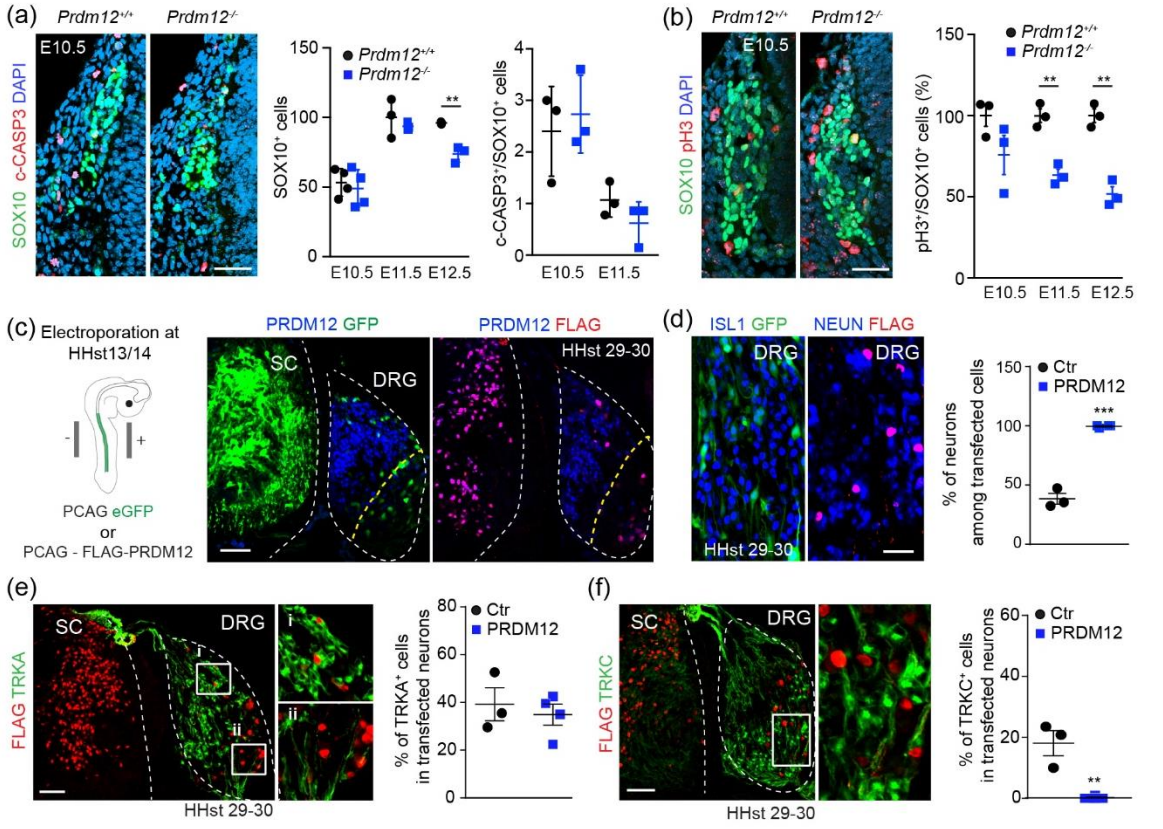

Figure 10-4 Prdm12 expression limits the development of sensory neurons to the nociceptor lineage. (a) Deletion of Prdm12 leads to reduced numbers of SOX10$^+$ progenitor cells. This is not due to increased apoptosis but to reduced proliferative cells starting at E11.5 (b) (scale bar 50µm). (c) and (d) Overexpression of a FLAG-tagged Prdm12 construct is restricted to neurons, while a control eGFP construct targets neurons and other cell types (scale bar 50µm in (c) and 25µm in (d)).

(e) and (f) TRKA$^+$ cells represented similar amounts of the transfected cells in both conditions (e), but TRKC$^+$ cells were not present in the FLAG-Prdm12 transfected condition (f), suggesting Prdm12 expression restricts the development of alternative neuronal subtypes (scale bar 50µm). Adapted from Bartesaghi et al. (2019).

As a final experiment, we investigated the relationship between *Prdm12* expression and that of NGN2 and NGN1, factors responsible by the first and second waves of neurogenesis, respectively. While the numbers of NGN2$^+$ and NGN1$^+$ cells were unchanged in the PRDM12$^{-/-}$ DRG at E10.5 (selected results Figure 10-5a), there was a significant reduction of NGN1 expression just 2 days later (selected results Figure 10-5b). This suggests that *Prdm12* expression may be necessary for the maintenance of NGN1. In NGN1$^{-/-}$ and NGN2$^{-/-}$ samples *Prdm12* levels were unaffected, indicating its expression is independent of NGNs (selected results Figure 10-5 c and d).

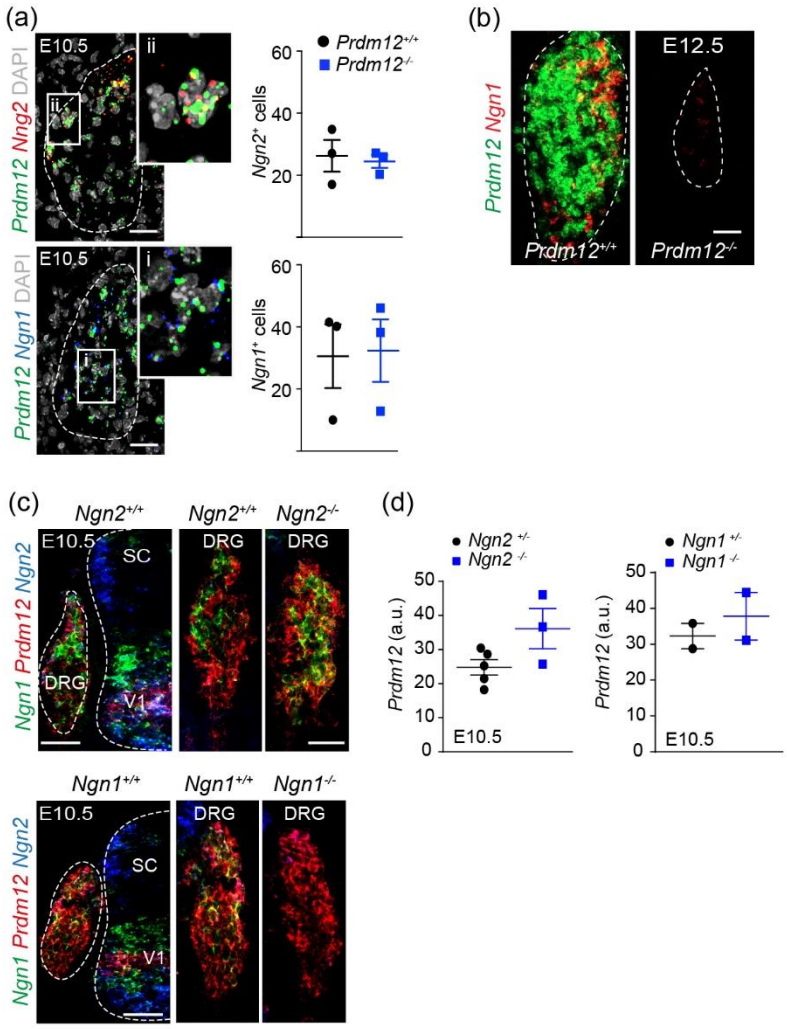

Figure 10-5 Prdm12 is necessary for the sustained expression of Ngn1 during development. (a) at E10.5, the number of cells expressing Ngn1 or Ngn2 was similar between control and Prdm12 KO embryos. However, at E12.5 (b) the amount of Ngn1 was reduced in the KO DRG. (c and d) Ngn1 and Ngn2 are not required for the expression of Prdm12. Adapted from Bartesaghi et al. (2019).

Our results indicate that *Prdm12* contributes to nociceptor progenitor proliferation, sustained NGN1 expression and fate-restriction, but it is not able to directly promote nociceptor

differentiation on its own. Desiderio and colleagues (Desiderio, Vermeiren et al. 2019) also observed that absence of *Prdm12* expression leads to selective loss of nociceptors and they state that *Prdm12* is necessary for initiation of *Ntrk1* (TRKA) expression. They also conclude that *Prdm12* is required for the expression of additional nociceptor-markers such as *Scn10a* (encoding voltage-gated sodium channel Nav1.8), as its expression is reduced even before overt loss of $TRKA^+$ cells. In *Xenopus* animal cap explants, the combined overexpression of *Prdm12* with *Ngn1* led to increased levels of *Ntrk1* and reduced *Ntrk3* (TRKC) and co-expression of *Prdm12* with *Ngn2* blocked the expression of *Ntrk2* (TRKB) and *Ntrk3.*

Differently than us, Desiderio *et al.* (2019) identified an increase in apoptosis at E12.5 by counting the total number of cleaved-caspase 3^+ cells, whereas we focused on the subset of $SOX10^+$ precursor cells. In this precursor population we did not find increased apoptosis, but it is possible that the apoptotic cells seen by Desiderio *et al.* belong to a $SOX10^-$ population such as post-mitotic neurons. This is supported by the *Advillin-Cre*-driven conditional deletion of *Prdm12*, which shows progressive loss of $TRKA^+$ cells from E13.5 onwards. Additionally, we observed a decreased percentage of phospho-histone 3^+ /$SOX10^+$ cells starting at E11.5, while Desiderio *et al.* did not find any change in the total number of phospho-histone 3^+ cells, suggesting another population of cells is contributing with increased proliferation and balancing out the total. Boundary cap cells are likely not the source of proliferative cells as they also express SOX10 (Frob, Bremer et al. 2012). Another difference between these two studies is the fact that in human iPSC-derived sensory neurons, the overexpression of *PRDM12* (using a doxycycline-inducible promoter) led to increased levels of *NTRK1* mRNA and reduced levels of *NTRK2/3* mRNA, suggesting the ability to promote the nociceptor fate in this system (Desiderio, Vermeiren et al. 2019). However, the authors did not quantify the number of $TRKA^+$, $TRKB^+$ and $TRKC^+$ cells, so it is still unclear if the changes in gene expression translate directly into an increase of the nociceptive population.

The early and sustained expression of PRDM12 may act as a determinant of cell fate, but it is unknown what upstream signals lead to PRDM12 expression. It is also unknown which genes are directly regulated by PRDM12, nor its cofactors. The identification of all these mechanisms may allow the development of tools for the production of stem cell-derived nociceptors and/or therapies for pain management.

10.3 PAPER III

In Paper III we overexpressed a constitutively active form of human NFATc4 in myelinating glia in mice, using the promoter *Cnp.*

Previous work suggested activation of NFATc4 was necessary for PNS development (Kao, Wu et al. 2009). Unexpectedly, overexpression of active NFATc4 seemed to impair PNS development, leading to a similar phenotype as the one observed by Kao and colleagues. NFATc4 transgenic (Tg) mice showed tremor, seizures and hind-limb clasping, indicative of a neuropathy phenotype. These mice presented reduced brain and sciatic nerve size, as well as reduced motor nerve conduction velocity. At birth, NFATc4 Tg mice showed increased unsorted axonal bundles in the sciatic nerve and an absence of fibers undergoing myelination, suggesting a delay in radial sorting and initiation of myelination.

To better understand the molecular changes caused by overexpression of constitutively active NFATc4, we performed RNA sequencing on newborn sciatic nerve mRNA. We found a dramatic alteration of the transcriptional landscape, which suggested that the presence of active NFATc4 was driving an aberrant transcription program. Among the differences detected were a decrease in myelin related genes and regulatory genes such as *Mbp, Mpz* and *Egr2/Krox20,* which fit with the phenotype observed. We analyzed the expression of these genes by qPCR at different time points and found that the expression profile of these genes was altered also in adulthood. Two other genes stood out from the list of differentially expressed transcripts: a decrease in *Ngfr/P75ntr* and an increase in *Pten*. By qPCR we showed that the changes in expression of both genes are sustained into adulthood. By Western blotting we were able to detect a reduction in $P75^{NTR}$ protein level at P0 (in the wild type, $P75^{NTR}$ is highly expressed at birth but then decreases (Cosgaya, Chan et al. 2002)), whereas PTEN protein level was only robustly increased at P10. We also verified the reduction of MBP protein expression by Western blotting. These data suggest that the timing and intensity of NFATc4 activation in SC are crucial for proper development of the sciatic nerve. The constitutive activation of NFATc4 seems to deregulate the transcriptional program, delaying or inhibiting the expression of markers of SC development, and leading to comparatively higher levels of transcripts related to embryonic development of different tissues.

A recent study (Reed, Frick et al. 2020) overcame the lethality observed by Kao et al. (2009) by eliminating *CnB* under the *Mpz-Cre* promoter. They found that calcineurin, and by extension NFAT, has a limited role in radial sorting and myelination, and its elimination does not affect expression of myelin-related genes such as *Krox20*, *Oct6* and *Mpz*. It is unclear at this point if the differences are simply due to the timing of CnB elimination or if they could be related to effects on other targets of calcineurin in SCs that have not yet been described. It is therefore necessary to clarify the role of NFATs specifically, and a SC-specific conditional knockout of one or several NFATs may be required to do so.

11 CONCLUSIONS AND FUTURE PERSPECTIVES

The experimental research presented in this thesis adds to the growing body of knowledge on neuron and glial cells in the PNS, a field that is still largely unexplored in some aspects but that has garnered increased interest in the past decade. The more we learn about the PNS, the stronger the foundation for the development of therapies in the future. It is paramount to verify if the mechanisms and pathways identified in the CNS can be translated to the PNS, and to understand how different physiological or pathological states can affect the molecular interactions between axons and SCs.

In **Paper I** we discovered that MCT1 or MCT4 ablation in SCs does not have detrimental effects on PNS development, myelination and myelin maintenance. At one year of age, animals lacking either MCT show normal sensory and motor function, but there is a small defect in muscle innervation in mice lacking MCT1 in SCs. This is accompanied by changes in mRNA expression by spinal cord motoneurons and may indicate the onset of a neuropathy. In light of recent publications (Babetto, Wong et al. 2020, Jha, Lee et al. 2020), it will be of interest to clarify the impact of MCT ablation in SCs on long-term axonal maintenance (ageing) and in pathological settings such as injury, diabetic neuropathy and hereditary disorders like CMTs. Alternatively, it is possible that the axonal ability to take up lactate from the extracellular milieu may play a more important part. The study of models with neuronal ablation of MCTs could provide clarification on this topic. We have developed two models of glial or neuronal elimination of MCT1/2/4 under the control of *Mpz-Cre* or *Advillin-Cre*, respectively that have been made available to other research groups.

In **Paper II** we identified PRDM12 as an essential factor for the development of nociceptive neurons, as its expression is required for maintenance of Ngn1 levels during the second wave of neurogenesis, it can restrict cell fate to $TRKA^+$ neurons and it can regulate the proliferation of precursor cells. However, our work did not answer the question of how exactly PRDM12 performs these functions. Efforts should be made to identify PRDM12 target genes and binding partners, as PRDM12 does not possess enzymatic activity. Chromatin immunoprecipitation could be followed by mass spectrometry to identify the co-factors of PRDM12. Additionally, sequencing of the DNA bound by PRDM12-containing complexes would allow the identification of the target genes in the developing DRG as well as in the adult. It would be highly relevant to verify if indeed PRDM12 acts as a transcriptional activator in the embryonic stage *vs* as a repressor in the adult stage (as noted by Landy et al. (2020, preprint)), and how these opposite functions are regulated. Other research avenues could include for example, the study of PRDM12 expression (or its partners) in different models of neuropathies with impaired sensory function. These studies could allow future development of therapies to treat chronic pain, as well as restore function in painless conditions such as CIP.

In **Paper III** we found that overexpression of constitutively active NFATc4 in SCs leads to impaired development of peripheral nerves and a severe neuropathy phenotype. This was due to abnormal gene expression at birth, with downregulation of SC development markers

and myelin-related genes, and upregulation of many genes related to embryonic and different tissue development, suggesting these cells may be in a delayed or abnormal developmental stage. We interpret these observations as evidence that NFATc4 activation must be regulated during development to allow proper maturation of the PNS. Results from recent literature have raised more questions than answers. The elimination of CnB in neural crest cells is clearly more detrimental than in SCs, as shown by the studies of Kao *et al.* (2009) and Reed *et al.* (2020). However, NFATc4 is not the only target of calcineurin, and the results observed could be attributed to other downstream effects of impaired calcineurin function. Our study is the only one that directly targets NFATc4, by-passing the regulatory influence of calcineurin. It would be interesting to see if there are additional target genes of NFATc4 in SCs, besides *Krox20* and *MPZ*, as well as additional co-factors. The overexpression of active NFAT led to many transcriptional changes in the newborn sciatic nerve, but we could not detect a direct proportionality (increased expression of known target genes), suggesting an issue with the stoichiometry of putative co-factors or the occurrence of unintended/ off-target effects. The use of inducible conditional knockout and/or inducible overexpression models specific to SCs could help shed light on the regulation of NFATc4 function at different developmental stages. NFATs are heavily studied in the immune system and efforts have been made to identify or develop new drugs that can influence their function. It would be interesting to assess the effects of those drugs in the PNS, as they could lead to deleterious side effects or they may be repurposed for the treatment of peripheral neuropathies.

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Pedras no caminho? Guardo-as todas. Um dia vou construir um castelo.[1]

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[1] *Stones in my path? I save them all. One day I will build a castle.* (Unknown author, commonly attributed to the Portuguese poet Fernando Pessoa)

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