LOCAL AXONAL TRANSLATION: REGULATION OF NEURONAL POLARITY, AXON DEVELOPMENT AND SURVIVAL

Cristiano Lucci, MSc

School of Life Sciences

University of Nottingham

August 2019

This dissertation is submitted for the degree of
Doctor of Philosophy

Abstract

Both the establishment of neuronal polarity and axonal growth are critical steps in the development of the nervous system, allowing neurons to fulfil their functional role, transmitting and receiving electrical signals. The local translation of mRNAs in the axon provides fine regulation of protein expression, and is now known to participate in axon development, homeostasis and degeneration. In this context, microRNAs play a fundamental role in the spatiotemporal regulation of axonal translation and, by doing so, can regulate almost every aspect nervous system development, physiology and disease.

This thesis focuses on elucidating the mechanisms by which local protein translation in the axon can regulates axon development and survival. I show how axonal protein synthesis contributes in supplying the needs of the axon and maintaining its homeostasis. Repression of protein translation restricted to the axonal compartment of microfluidic chambers triggers axon degeneration in mouse sensory neurons. Moreover, I identified four microRNAs as potential candidate regulators of axon degeneration pathways. I then investigated the role of a single microRNA, miR-26a, in early stage primary cortical neuron development. I show that miR-26a is highly expressed in neuronal cultures and regulates both neuronal polarity and axon growth. Specifically, inhibition of miR-26a reduces the number of polarised neurons, whilst its over-expression produces the opposite phenotype and increases the number of neurons with multiple axon-like processes *via* the targeting of GSK3β.

Using compartmentalised microfluidic neuronal cultures, I also identified a local role for miR-26a in the axon, where the repression of local synthesis of GSK3β controls axon development and growth. Removal of this repression in

the axon triggers local translation of GSK3β protein and subsequent transport to the soma, where it can impact axonal growth mechanism.

These results demonstrate how the axonal miR-26a can regulate local protein translation in the axon to facilitate retrograde communication to the soma and amplify neuronal responses, in a mechanism that influences axon development.

Acknowledgments

I would like to thank all those people who made this thesis possible and these years of PhD an unforgettable experience.

First of all, I would like to express my sincere gratitude to my supervisor Dr Federico Dajas-Bailador for his precious support and encouragement. I will always be thankful for his understanding, his helpful discussions and his ability for quite supervision.

I owe so much to Dr Raquel Mesquita-Ribeiro and Dr Andrea Loreto. Where I am in science today is largely thanks to them. My thank goes also to all my lab mates, past and present, especially to Alex, Matt, Teresa, Alice and Katerina for their precious support and friendship shown at work and outside the lab. Working with them has been a real pleasure. Special thanks to Tim Self, Chris Gell and Ian Ward for the use of imaging facilities and for technical support. Thanks also to the staff at the BSU, and especially to Susan Ecob, the "mouse whisperer". Special thanks too to Prof Michael Coleman for useful discussions and for providing *Sarm1*$^{-/-}$ ganglia.

My deepest gratitude is for my family who have always unconditionally encouraged and supported me. I hope that what I have achieved makes them happy and proud.

Last but not the least, a huge thank you to my better half, Ludovica, for her immense support and love throughout these years despite the difficulties. Together we demonstrated that long distance "cell-cell" communication does not just apply to biology.

Publications arising from the work behind this thesis

Lucci C, Mesquita-ribeiro R, Rathbone A, Dajas-bailador F. 2020. Spatiotemporal regulation of GSK3 β levels by miRNA-26a controls axon development in cortical neurons. *Development* **147**(3). doi:10.1242/dev.180232

List of abbreviations

α-SYN	α-synuclein protein
AD	Alzheimer Disease
AGO	Argonaute
AIS	Axon initial segment
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
APP	Amyloid precursor protein
ATF4	Activating transcription factor 4
Aβ	β-amyloid
BAF53a	Brg/Brm associated factor 53a
BDNF	Brain-derived neurotrophic factor
CAMKK2	Calcium/Calmodulin Dependent Protein Kinase 2
cAMP	Cyclic AMP
CDC42	Control protein 42 homologue
cDNA	Complementary DNA
CLASP2	Cytoplasmic linker associated protein 2
CMT2B	Charcot-Marie-Tooth disease type 2B
CNS	Central nervous system
COXIV	Cytochrome c oxidase IV
CP	Cortical plate
Cplx1	Complexin-1
CRE	cAMP-responsive element
CREB	cAMP response element-binding protein
CRMP2	Collapsin response mediator protein 2
C_T	Cycle threshold
CTDSP	C-terminal domain small phosphatases
DCC	Deleted in colorectal carcinoma
DG	Dentate gyrus
DGCR8	DiGeorge syndrome critical region 8
DISC1	Disrupted in schizophrenia 1
DIV	Days *in vitro*
DLK	Dual leucine zipper kinase

DMEM	Dulbecco's Modified Eagle's Medium
DNase I	Deoxyribonuclease I
DRG	Dorsal root ganglion
dsRNA	Double-stranded RNA
EB1	End binding protein 1
Efnb3	Ephrin B3
eIF2B2	Eukaryotic translation initiation factor-2B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4G2	Eukaryotic translation initiation factor 4G2
Eph	Ephrin receptors
Ephb2	Ephrin type receptor B 2
EV	Extracellular vesicles
F-actin	Actin filaments
FGF	Fibroblast growth factor
Fmr1	Fragile X Mental Retardation gene 1
FMRP1	Drosophila homologous of FMRP
GAKIN	Guanylate kinase-associated kinesin
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
GTPase	GTP hydrolase
HBSS	Hanks Balanced Salt Solution
HSN	Hermaphrodite-specific motorneuron
IGF-1	Insulin-like growth factor 1
Impa-1	Myo-inositol monophosphatase 1
IUE	*In utero* electroporation
IZ	Intermediate zone
JIP1	c-Jun N-terminal protein kinase-interacting protein 1
JNK	c-Jun N-terminal protein kinase
KAP3	Kinesin superfamily-associated protein 3
KLC	Kinesin light chains
Limk1	LIM domain kinase 1
Lis1	Lissencephaly-1
LNA	Locked nucleic acid
LP	Leading process
LTP	Long-term potentiation

LZK	Leucine zipper kinase
MAP1B	Microtubule-associated protein 1B
MAP2	Microtubule-associated protein 2
MAP3K	Mitogen-activated protein kinase kinases
MAPK	Mitogen-activated protein kinase
MAPs	Microtubule-associated proteins
MARK2	Microtubule affinity-regulating kinase 2
MHB	Midbrain-hindbrain boundary
miRNAs	microRNAs
miRNA-seq	miRNA sequencing
MMZ	Multipolar morphology zone
mRNA	Messenger RNA
mRNPs	mRNA-containing protein complexes
mTOR	Mammalian target of rapamycin
MZ	Marginal zone
NAD	Nicotinamide
NCad	Neural cadherin
NFAT	Nuclear factor of activated T-cells
NGF	Nerve growth factor
NGS	Next-Generation Sequencing
NKCC1	Sodium-potassium-chloride cotransporter 1
NLS	Nuclear localisation signal
NMNAT	Nicotinamide Nucleotide Adenylyltransferase
NPCs	Neural Precursor Cells
NRSF	Neuron-restrictive silencer factor
NSCs	Neural stem cells
Nsf	N-ethylmaleimide-sensitive factor
NT3	Neurotrophin 3
PAR3	Partitioning defective homologue 3
PCR	Polymerase chain reaction
PD	Parkinson's disease
PI3K	Phosphatidyl inositol 3-kinase
PIP3	Phosphatidyl inositol-3,4,5-trisphosphate
PLM	Posterior lateral microtubule neuron
PLO	poly-L-ornithine
PNS	Peripheral nervous system
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA

PTBP1	Polypyrimidine Tract RNA-binding protein
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative PCR
Rap1	Ras-related GTPase
RAP1B	Ras-related protein 1B
Rasa1	Endogenous p120RasGAP
RATFs	Regeneration-associated transcription factors
Rb1	Retinoblastoma protein
RBPs	RNA-binding proteins
RE1	Repressor element 1
REST	RE1 silencing transcription factor REST
RGCs	Radial glia cells
RGCs	Retinal ganglion cells
Rheb	Ras-homolog enriched in brain
Rho	Ras-homolog gene family
RISC	miRNA-induced silencing complex
RNAi	RNA interference
RNase	Ribonuclease
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RSK3	Ribosomal S6 kinase 3
RT-PCR	Real-time PCR
RT-qPCR	Real-time qPCR
Sarm1	Sterile Alpha and TIR Motif Containing
SCG	Superior cervical ganglion
SEM	Standard error of the means
SEMA3A	Semaphorin 3A
SENS	Senseless transcription factor
SEPT9	Microtubule-associated septin 9
SHH	Sonic Hedgehog
siRNA	Small interfering RNA
SLIT2	Slit homolog 2 protein
Slit-2	Slit Guidance Ligand 2
SMA	Spinal muscular atrophy
SMN1	Survival of motorneuron 1
SNP	Single nucleotide polymorphism
SOP	Single organ precursor

SOX2	SRY-box 2
SRY	Sex-determining region
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
SWI/SNF	SWItch/Sucrose Non-Fermentable complex also called BAF
Syt1	Synaptotagmin-1
TCF	T cell factor
TGF-β	Transforming growth factor β
TGIF	Transcriptional repressor TG-interacting factor
TLR	Toll-like receptor
TLX	Nuclear receptor tailless
TP	Trailing process
TRIM32	Tripartite motif-containing protein 32
TrkB	Tropomyosin receptor kinase B
TrkC	Tropomyosin receptor kinase C
TSC2	Tuberous sclerosis 2
UBE2	Ubiquitin conjugating enzymes E2
UBE4B	Ubiquitination factor E4B
UNC-6	Netrin unc-6
UPS	Ubiquitin-proteasome system
VZ	Ventricular zone
WLD^s	Wallerian degeneration slow
Wwp	WW-Containing Protein
XPO5	Exportin-5

List of figures

List of tables

Table of Contents

CHAPTER 1:

General introduction

Introduction

Brain function relies on the formation of highly structured connections between neurons in the brain. By means of their dendrites, neurons gather and process signals coming from other neuronal cells and they re-transmit the resulting output through the axon to their post-synaptic targets. However, what sounds like a relatively simple process, turns out to be an exquisitely complex mechanism, given that the human brain consists of about 80 billion neurons, an almost equal number of non-neuronal glial cells, and the neuronal networks comprising about 100 trillion connections (Azevedo *et al.*, 2009). Moreover, the huge diversity of neuronal cell types only adds a further layer of complexity that also reflects the impressive computational properties of the mammalian nervous system. How can each one of them send its axon to the proper target cell and how neurons connect with each other to establish functional circuitry? More than a century ago, Santiago Ramón y Cajal and colleagues tried to answer this question and mapped with astonishing details the complexity of neuronal networks [**Figure 1a**] and modern developmental neuroscientists follow in Ramón y Cajal's footsteps, trying to unravel the processes underlying the formation of neural circuits.

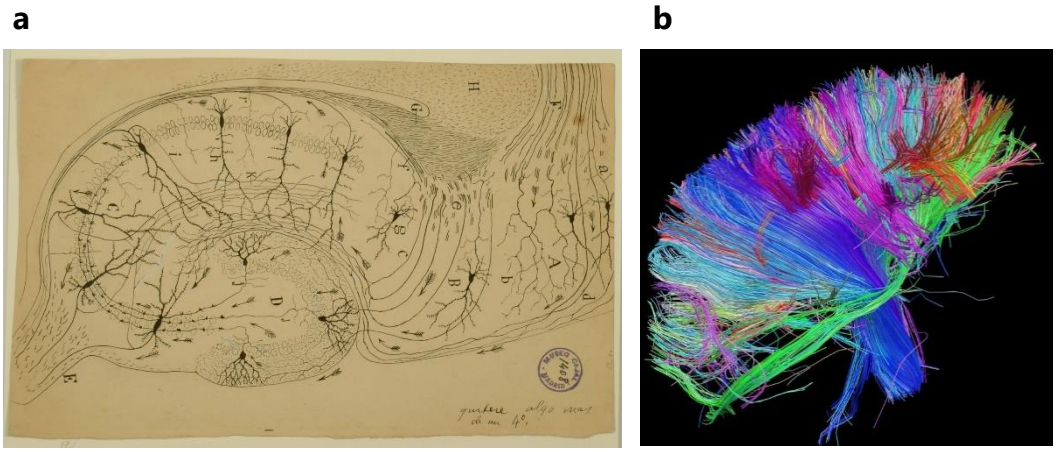

Figure 1: Neuronal connections over the century. (**a**) Cajal's sketch of the structure and connections of Ammon's horn. (**b**) White matter fibre architecture of the brain. Measured from diffusion spectral imaging. The fibres are color-coded by direction: red = left-right, green = anterior-posterior, blue = up-down. From www.humanconnectomeproject.org

Despite the remarkable improvement of imaging techniques [**Figure 1b**] and the striking advances in understanding the molecular basis of neural development, we are still marvelled by how complex and intricate those axonal projections and the brain's connectivity can be. These advances have converged to our current understanding of neuronal connectivity and we know now that the precise construction of neural circuits requires orderly transition of sequential events that ranges from the establishment of the axon-dendrite polarity, axon outgrowth, pathfinding, branching, to synaptogenesis.

Understanding how those connections are made and eventually how they can be lost in neuron degeneration is therefore essential to understand the workings of the brain, as well as to develop efficient therapeutic strategies.

1.1 Neuronal polarisation and axon specification

1.1.1 Neuronal polarity in vivo

Neurons are undoubtfully the most complex and specialised cells within our body. As said above, with their long axon and dendrites, they can form an intricate network with hundreds of millions of connections known as synapses. After all, the whole mammalian neocortex is in turn an exquisitely complex, highly organised, six-layered structure that contains hundreds of different neuronal cell types and a diverse range of glia (Ramon y Cajal, 1995). In fact, heterogenous populations of projection neurons are located in specific cortical layers and areas, have unique morphological features, express a plethora of transcription factors, and ultimately perform different functions (Molyneaux et al., 2007).

This complexity and diversity makes any classification scheme particularly challenging, and the most accurate system should probably incorporate a combination of morphology, electrophysiological properties and patterns of

gene expression (Migliore and Shepherd, 2005). However, the most basic way to classify projection neurons is according to their connectivity and projection patterns, with three major classes falling under this classification: associative, commissural and corticofugal. Associative projection neurons comprise those neurons that extend axonal projections within a single cerebral hemisphere, connecting local areas or proximal gyri. Commissural projection neurons instead, are primarily located in layers II/III, V and VI and they extend their axons from one hemisphere to neurons in the contralateral hemisphere. Then, the axons can extend projections either through the corpus callosum, the major commissural connection between the hemispheres, or through the anterior or posterior commissures. Finally, corticofugal projection neurons extend axonal projections 'away' from the cortex. These include sub-cerebral projection neurons, which are primarily located in deep-layer V and extend axons to the brainstem and spinal cord, and corticothalamic neurons, located in layer VI that project subcortically to different nuclei of the thalamus (Molyneaux et al., 2007). Depending on layer location and type of projection, morphology of excitatory neurons can vary. However, many excitatory neurons resemble so-called "pyramidal cells".

But how are these various projection neuron subtypes generated during corticogenesis? Although it's now clear that neurons in the mammalian brain are not a homogenous entity, coming in different types and varying in their morphological and functional properties, they all have to break the symmetry, and to polarise into distinct functional domains, which are normally defined as the axon and the somatodendritic compartment (Dotti *et al.*, 1988). These domains are both extremely important for the neuronal functionality but the mechanisms underlying this polarisation have only started to be elucidated in the last 30 years (Esch *et al.*, 1999). Nowadays, it is known that behind neuronal polarisation there is a coordinated reorganisation of cell-surface subdomains,

the cytoskeleton, cellular organelles and proteins, and is usually triggered by external cues (Nelson, 2003).

During mouse brain development, excitatory neurons undergo extensive migration from the neocortical ventricular zone (VZ) radially towards the pia to their final locations in the cortical plate (CP) (Anderson *et al.*, 2002; Gorski *et al.*, 2002). On the other hand, inhibitory neurons, so-called interneurons, establish local connections and contribute to intracortical information processing by modulating excitability and thus shaping cortical output (Hatten, 2002; Marín and Rubenstein, 2003). Most of these interneurons are born from germinal zones outside the neocortex, such as the medial and caudal ganglionic eminences in the ventral telencephalon. However, smaller percentages of interneurons might be produced in the lateral ganglionic eminence and septal area and migrate tangentially to reach their final destination and cortex (Wonders and Anderson, 2006). Regardless of their origin, their overall migration pattern appears really similar: in general, immature neurons migrate tangentially over long distances toward the cortex (Tanaka et al., 2003). They enter the CP from the subventricular zone (SVZ), pass through it, and reach the marginal zone (MZ), where they further execute multidirectional tangential migration and become dispersed throughout the cortex, reaching their final positions postnatally (Tanaka et al., 2009).

In the mammalian cortex, most of neurons are the excitatory type, and in this thesis, I will mostly discuss this population of cells.

Excitatory neurons are produced by cortical neural stem cells (NSCs) which generate most of cell types in the cortex: excitatory neurons, astrocytes and oligodendrocytes (Gallo and Deneen, 2014; Gorski *et al.*, 2002). During embryonic day E9.5-E11.5, NSCs undergo a series of symmetric divisions in order to self-renew and increase their number (Takahashi, 1995), but soon after, with the onset of neurogenesis, NSCs start producing another type of

apical stem cells called radial glia cells (RGCs). This population of cells in turn is capable of both self-renewal and production of postmitotic neurons. (Noctor *et al.*, 2004). Cortical neurons are generated between E11-E17 in the VZ of the mouse neocortex and they have a long basal (radial) process attached to the basal membrane at the pial surface and a short apical process on the ventricle side. Upon cell cycle exit, the postmitotic neuron goes through a multipolar transition in the SVZ where multiple neurites emerge from the cell body until one major process is generated in the radial direction and becomes the leading process (LP). At this point, the neuron initiates radial translocation along a radial glial process and leaves behind a trailing process (TP), which elongates tangentially in the intermediate zone (IZ) (it can last until around postnatal day P7 in mouse corticofugal axons with distant targets like the spinal cord). The cell body continues to migrate toward the CP while the axon rapidly elongates whilst the leading process gives rise to the apical dendrite, which initiates local branching in the MZ in post-natal development [**Figure 2**] (Barnes and Polleux, 2009). Once they have reached the CP, the immature neurons leave the RGC process and start differentiating into neurons of their specific cortical layer. Hence, the neocortex is formed in an inside-out fashion, with early born neurons forming the deep layers while the later born neurons generating the upper layers. Inhibitory neurons also extend a single axon and multiple dendrites, but their morphologies are highly heterogenous: they include basket cells, chandelier cells, Martinotti cells, double bouquet cells, neurogliaform cells, and at least 10 others (Kubota, 2014). However, so far only a few reports have described how axons or dendrites are established in these neurons (Kawaguchi, 1993).

Upon neurogenesis completion, a 'gliogenic switch' occurs and they begin differentiating into astrocytes, oligodendrocytes and ependymal cells. This transition is linked with a downregulation of the Golgi-derived apical

trafficking and VZ NSCs lose tight junctions while keeping intact the adherens junctions (Götz and Huttner, 2005). This is followed by the gradual expression in the mouse of astroglial markers, such as glial fibrillary acidic protein (GFAP) (Malatesta et al., 2003). Although the mechanisms of the neurogenic to gliogenic phase transition are not clearly understood, numerous secreted signals such as Sonic hedgehog (Shh), fibroblast growth factors (FGFs), Wnts, Notch, bone morphogenetic proteins (BMPs) and cytokines act together to spatiotemporally control cell fate, leading to the appearance of specific domains that selectively generate either astrocytes or oligodendrocytes (Zuchero and Barres, 2013).

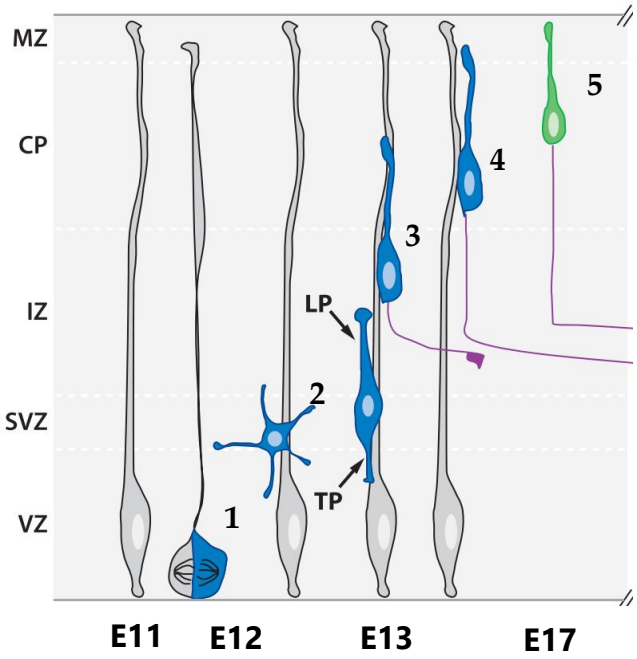

Figure 2: Polarisation of cortical neurons in vivo. Adapted from Barnes *et al.*, 2009. Neurons generate from radial glial cells (grey) between E11 and E17 in the ventricular zone (VZ) of the mouse neocortex. Upon cell cycle exit through asymmetric cell division (1), the postmitotic neuron (blue) goes through a multipolar transition in the subventricular zone (SVZ) where multiple neurites emerge rapidly from the cell body (2) before one major process forms in the radial direction and becomes the leading process (3, LP). At this point, the neuron initiates radial translocation along a radial glial process and leaves behind a trailing process (4, TP), which elongates tangentially in the intermediate zone (IZ) (purple). The cell body continues to translocate toward the cortical plate (CP) whilst the axon rapidly elongates and the leading process gives rise to the apical dendrite (5, green) which initiates local branching in the marginal zone (MZ).

Conventionally, cultures of dissociated hippocampal and cortical neurons represent a valuable model to study the cell biology and molecular mechanisms behind neuronal polarity *in vitro* (Arimura and Kaibuchi, 2007). Experiments involving this type of approach have demonstrated that isolated neurons are still capable of adopting spatially and functionally distinct axonal and dendritic domains (Dotti *et al.*, 1988) despite being outside their three-dimensional environment. However, it should be noted that most neurons in culture are post-mitotic neurons upon dissociation and therefore, the neuronal polarisation that we can observe in this *in vitro* model corresponds to a 're-polarisation' of previously polarised neurons *in vivo*. Moreover, morphological changes seen in immature neurons in different brain areas are not necessarily identical *in vivo* and *in vitro.* The axon-dendrite axis emergence differs deeply in each type of neuron. For example, the Purkinje cells in the cerebellum, similarly to pyramidal cells in the neocortex or hippocampus, are produced near the VZ, extend an axon without any minor processes towards the basal surface, and then migrate toward pre-determined positions following the leading processes that enwrap the glial fibres (Solecki et al., 2006). At the same time, young cerebellar granule cells migrate along the pia matter, and then form axons bilaterally and migrate towards the inner layer. In both Purkinje cells and cerebellar granule cells, nascent dendrites develop at a later stage (Solecki et al., 2006). In both mouse and zebrafish retinal ganglion cells and bipolar cells, post-mitotic neurons directly inherit the intrinsic apico-basal polarity of progenitors which is transformed into axon-dendrite polarity upon cell cycle exit (Zolessi et al., 2006). In fact, shortly after their terminal division, axons begin to extend from the basal process as the cell body migrates towards the basal lamina, whereas dendrite formation happens at opposite pole of the cell body, by transforming the apical process into early dendritic structures (Randlett et al., 2011b; Zolessi et al., 2006).

The vertebrate retina is indeed a valuable model to study neuronal polarisation *in vivo*. Being an outpocketing of the CNS on the surface of the embryo, the retina provides a system that can be easily accessed by experimental techniques and high-resolution in embryo microscopy.

Retinal neurons are generated by neuroepithelial progenitor cells at the apical surface of the neuroepithelium and can differentiate into one of the five neuronal cell types. Photoreceptors reside in the apical surface of the retina, with light sensitive outer segments pointing apically, and extend axons to form synapses onto bipolar cells, which have their dendrites pointing apically and have axons basally. Retinal ganglion cells establish synapses with BCs and extend axons along the basal surface of the retina, which collect at the optic disk and leave the eye to find partner neurons in the optic tectum. These three cell types form the excitatory pathway of the retina, whereas amacrine cells and horizontal cells, form the inhibitory retinal pathway and modulate the visual information that flows through the excitatory pathways (Randlett et al., 2011a).

Considering the plethora of polarised morphologies and the different modes of neuronal migration in every organism, it is reasonable that cell-type-specific components play a crucial part. Therefore, at present, it is difficult to explain every type of neuronal polarization by components and pathways identified from studies of hippocampal cultures *in vitro*.

Nevertheless, one of the greatest advantages of this approach is to be in a controlled and simpler environment than the *in vivo* situation. Very few extracellular signals are present in dissociated cell cultures and the neurons break symmetry randomly, allowing the identification of several intracellular signalling pathways important for the polarisation of the axon/dendrite axe *in vitro* (Arimura and Kaibuchi, 2007), some of which will be discussed in more detail below.

1.1.2 Neuronal polarity in vitro

In this *in vitro* system and only a few hours after plating, neurons first extend several immature neurites during what is called stages 1 and 2 [**Figure 3**]. Half a day after plating starts a phase of asymmetric growth (stage 3) during which one of the neurites begins to extend rapidly, becoming longer than any neurites until it differentiates into an axon (Jacobson *et al.*, 2006; Ruthel and Hollenbeck, 2000). Meanwhile, all the remaining processes continue to undergo a series of mechanical stretching between growth and retraction cycles for up to a week, during which their net length is maintained, and they become mature dendrites (stage 4). During this process, dendrites become thicker and shorter than the axon and begin to establish dendritic components and to construct premature dendritic spines (stage 5) (Banker, 2018; Dotti *et al.*, 1988).

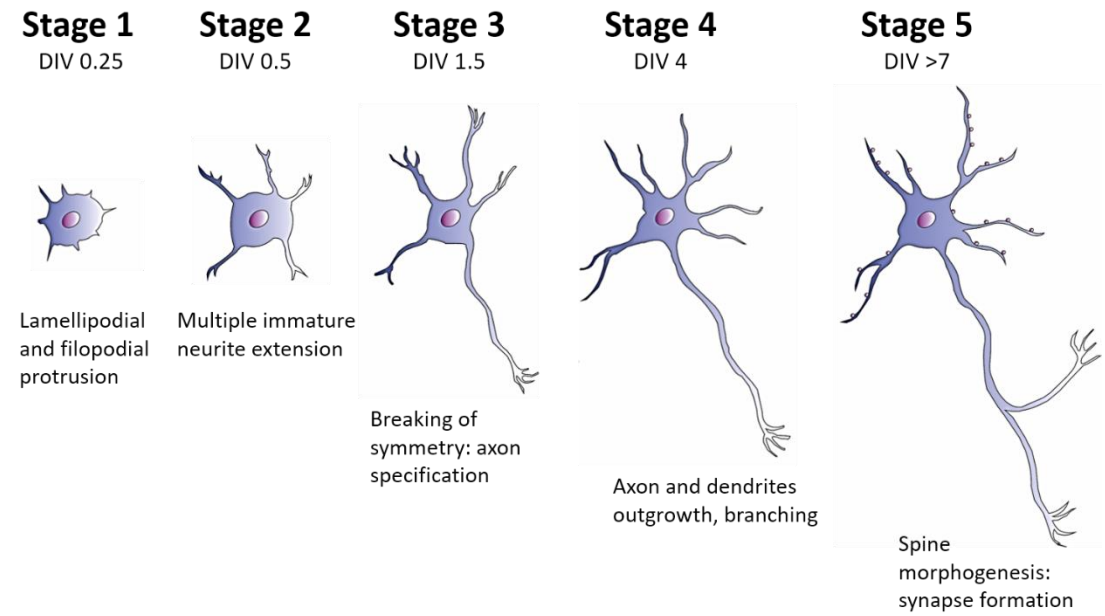

Figure 3: Polarisation of cortical neurons in vitro. In dissociated cultures, postmitotic cortical neurons display specific transitions as described for hippocampal neurons by Dotti *et al.* (1988). At stage 1, immature neurons possess intense lamellipodial and filopodial protrusive activity, which culminates to the emergence of multiple immature neurites (stage 2). Stage 3 represents a critical step when neuronal symmetry breaks and a single neurite starts growing faster to become the axon, whilst the other neurites acquire dendritic identity. Stage 4 is characterised by rapid axon and dendritic outgrowth. Finally, stage 5 neurons are terminally differentiated pyramidal neurons harbouring dendritic spines.

How a single axon is specified among equally potential neurites is still an area of active investigation (Takano *et al.*, 2019). It has been suggested that the symmetry breaking can only be achieved by generating a bi-directional feedback loop between both processes (Andersen and Bi, 2000). In this model, the neurites extension is driven by four positive forces: higher intracellular vesicular transport (Bradke and Dotti, 1997; Futerman and Banker, 1996), an increase in the dynamics of actin filaments (Bradke and Dotti, 1999), the enhancement of microtubule formation (Baas, 2004) and activation of signalling molecules (such as phosphatidyl inositol 3-kinase (PI3K) and Rho GTPase) (Apenstrom, 1999; Ueyama, 2019). These four forces create a growth-promoting-positive-regulation, which is counteracted by signalling molecules such as GTPase-activating proteins and phosphatases that, by inducing microtubule catastrophe, decreasing actin dynamics and the amount of plasma membrane (Shelly *et al.*, 2010), generate in turn a negative regulation. Before polarisation, therefore, positive and negative signals seem to be perfectly balanced: when this balance is broken by a positive cue, such as the accumulation in the growth cone of a growth-promoting protein, the activation of a continuous self-activation system (positive feedback loop) is triggered (Andersen and Bi, 2000; Goslin and Bank, 1989) and a single neurite elongates to become an axon. Concurrently, this positive feedback loop system generates a strong negative feedback signals that prevent other neurites from forming a second axon (Andersen and Bi, 2000; Takano *et al.*, 2017).

What exactly makes the axon so different from the somatodendritic domain at this stage of development? Dendrites and the axon are compartments with clearly defined borders from both a structural and a molecular point of view. The cytoskeleton underlying these domains is composed of microtubules, actin filaments, and intermediate filaments (also called neurofilaments) along with their associated binding proteins. Microtubules in turn are composed of

α- and β-tubulin subunits that polymerise to form a long filament by the means of the addition of tubulin subunits to only one side of the growing filament called the plus end, while on the opposite side depolymerisation occurs. However, the cytoskeleton that forms the backbone of the developing axon has a unique protein signature.

It was discovered more than thirty years ago that the axon of a neuron contains a very uniform distribution of microtubules with the plus ends all facing away from the cell body (Heidemann *et al.*, 1981). Whereas this observation was confirmed through the years in many neuron cell types, it was also determined that dendrites are characterised by rather complex array of microtubule orientations, which differs from the axonal microtubule orientation with their plus end pointing outward (Baas, 2004; Kapitein and Hoogenraad, 2011; Rao and Baas, 2018; Yau *et al.*, 2016). In fact, current research shows that proximal dendrites are mainly composed of minus-end out microtubules, whilst more distal dendrites shift from an equal distribution of minus-end out and plus-end out microtubules to mainly plus-end out microtubules (Ori-McKenney *et al.*, 2012; Park and Roll-Mecak, 2018; Stone *et al.*, 2007).

Another hallmark that discriminates the axonal compartment from the somatodendritic compartment is the composition in microtubule-associated proteins (MAPs), and which enrich microtubules to regulate their bundling, dynamic properties and stability (Hirokawa *et al.*, 2010). Indeed, microtubules in the axon are associated with Tau and Microtubule-associated protein 1B (MAP1B), whereas microtubules in the dendrites are labelled by proteins of the MAP2a-c family (Avila *et al.*, 1994).

The actin polymerisation into actin filaments (F-actin) also plays an important role in characterising the axonal domain. Several groups have shown that the disruption of actin polymerisation allows dendritic proteins to inaccurately enter the axonal domain (Mellman *et al.*, 1999; Song *et al.*, 2009). One possible

explanation is that a dense F-actin meshwork creates a cytoplasmic diffusion barrier shortly after polarisation, which partially separates the axonal compartment from the rest of the cell. Two recent works showed *via* high resolution imaging techniques that the axon has indeed a unique F-actin network that has not been found in dendrites (Watanabe *et al.*, 2012; Xu *et al.*, 2013), formation of which appears to directly precede the formation of the axon initial segment (AIS; Song *et al.*, 2009). Live-imaging experiments have also demonstrated that vesicles carrying dendritic proteins enter both axons and dendrites with a similar rate. However, once they have reached the AIS, almost all vesicles carrying dendritic proteins halt, whilst others reverse direction, in an actin- and Myosin-Va (involved in transport in the synaptic regions) dependent manner (Al-Bassam *et al.*, 2012). In contrast, vesicles carrying axonal or non-specifically localised proteins move undisturbed through the AIS. These observations strongly suggest that this actin-dependent barrier could be regulating the trafficking of proteins specifically to the axonal or somatodendritic membrane, allowing neurons to establish and maintain polarised compartments with distinct morphology and function. Notable, the knockdown of AnkyrinG, a key component of the AIS, causes axons to acquire molecular characteristics of dendrites (Hedstrom *et al.*, 2008). Another vesicles-sorting mechanism came out from a recent study, in which a microtubule-associated septin (SEPT9), acts as membrane traffic regulator on the plus-end-out subsets of MT (Karasmanis *et al.*, 2018). In fact, SEPT9 by localising specifically in dendrites, slows down and halts axonally destined vesicles of kinesin1/KIF5 whilst it mobilises and speeds up dendritic-destined kinesin-3/KIF1A vesicles during entry into dendrites, *via* the recognition of the lysine-rich patch in the L12 loop present in the motor domain of kinesin-3/KIF1A (Karasmanis *et al.*, 2018). The role and function of some kinesins will be discussed in more detail below.

1.1.3 Extracellular signals and polarity

As mentioned above, axon and dendrites *in vivo* develop within a defined temporal sequence and stereotyped growth directions in a three-dimensional environment. Thus, symmetry breaking *in vivo* is likely triggered by extracellular cues released into the surrounding extracellular matrix, that provide the compass for the polarity establishing intracellular events.

In *Caenorhabditis elegans,* the axon guidance molecule cue UNC-6 (also known as netrin in mammals) is required not only for axon guidance (Bellon and Mann, 2018) but also for orienting initial polarised emergence of the hermaphrodite-specific motorneuron (HSN) axon (Adler *et al.*, 2006). The secreted protein LIN-44/Wnt controls the polarisation of another *C. elegans* neuron, the mechanosensory posterior lateral microtubule neuron (PLM; Hilliard and Bargmann, 2006; Prasad and Clark, 2006). This type of neuron extends a long anterior process that forms a chemical synapse, and a shorter posterior process that does not form a synapse. In *lin-44*/*wnt* or *lin-17*/*fz* mutants, the anterior PLM process extends posteriorly to its cell body instead of anteriorly. WNT signalling cascades impair the activity of glycogen synthase kinase 3β (GSK3β) during planar polarisation which is the coordinated organisation of cells within the plane of a single-layered sheet of cells (He *et al.*, 2018; Logan and Nusse, 2004).

In mammals, the Transforming Growth Factor beta (TGF-β) released from the VZ, is the main diffusible cue that induces axon formation in developing cortical neurons (Yi *et al.*, 2010). In fact, TGF-β receptors are enriched in the axons, and their elimination induces the formation of axon-less cortical neurons. Local application of TGF-β *in vitro* is sufficient to trigger axon specification and growth (Yi *et al.*, 2010).

Other diffusible cues that are involved in axon specification and growth are Neurotrophins, such as brain-derived neurotrophic factor (BDNF) and

neurotrophin 3 (NT3). In fact, knockout of the pan-neurotrophin receptor $p75^{NTR}$ leads to failure to initiate an axon in cortical neurons and during adult hippocampal neurogenesis, and expression of dominant-negative neurotrophin receptors TrkB and TrkC (tropomyosin receptor kinase B and C) disrupts the multipolar to bipolar transition (Nakamuta *et al.*, 2011; Zuccaro *et al.*, 2014). A repulsive guidance cue, semaphorin 3A, is also known to act as a dendritic chemoattractant agent and regulates the orientation of apical dendrites in cortical neurons (Whitford *et al.*, 2002).

The extracellular protein Reelin has been shown to be involved in polarising the movement of multipolar neurons towards the CP while they are migrating through the multipolar morphology zone (MMZ, comprising the SVZ and the lower part of the IZ; Jossin and Cooper, 2011). In this study, Reelin, which is known for its role in neuron lamination in the cortical plate (Jossin, 2004), activates Ras-related GTPase (Rap1) in multipolar neurons in the IZ. Activation of Rap1 regulates and increases the level of neural cadherin (NCad) on the surface which is needed to orient the migration of multipolar neurons toward the CP (Jossin and Cooper, 2011). More recently, another small GTPase, Rab 23 has been considered as important player in the radial migration of cortical neurons by influencing the expression of NCad during migration (Hor and Goh, 2018).

Finally, ephrin guidance factors and their Eph receptors can also regulate the directionality of multipolar migration (Dimidschstein *et al.*, 2013). Ephrins are cell-surface proteins with a pivotal role in axon guidance (Drescher, 2011) that trigger a forward signal when binding to Eph family receptors present on other cells, grouped into class A and class B based on their degree of sequence similarity and binding affinities, with ephrin-A binding to EphA receptors and ephrin-B binding to EphB receptors (Seiradake *et al.*, 2016). It was first shown that the Ephrin- A/EphA forward signalling controls the lateral distribution of

neurons by promoting a wider tangential migration during the multipolar stage (Torii *et al.*, 2009). Later, it was demonstrated that ephrin-B1 reverse signalling may have an opposite effect, restricting the tangential migration of multipolar neurons at the MMZ (Dimidschstein *et al.*, 2013).

1.1.4 Intracellular signalling and polarity

Dissociated hippocampal and cortical neurons *in vitro* are still able to break the symmetry and to polarise even if they are "dragged" outside their physiological environment and in absence of extracellular cues, suggesting that axon specification is also an intrinsic cellular mechanism driven by cell-autonomous signalling cascades. Over-expression and downregulation studies of candidate genes involved in polarity have unravelled over the years an intricate and complex array of signalling molecules that orchestrate the formation of the axonal domain (Takano *et al.*, 2019). These molecules might act in distinct types of neurons at specific stages of development, but it is also possible that they form an "intricate" web that modulate neural development in a coordinated manner.

PI3K-Akt and GSK3β.

Activation of PI3K and the accumulation of its lipid product, phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the tip of the new-born axon is considered a hallmark for the induction of neuronal polarisation (Ménager *et al.*, 2004). Accordingly, inhibition of PI3K activity prevents axon specification (Ménager *et al.*, 2004; Shi *et al.*, 2003). Activation of PI3K–Akt (also known as protein kinase B) pathway phosphorylates GSK3β, a constitutively active kinase, on Serine 9, leading to the inactivation of the protein (Arimura and Kaibuchi, 2007; Eun-MI Hur and Zhou, 2010). As Akt localises to the tips

of the axons in hippocampal neurons, phosphorylated GSK3β is also restricted at the axon terminal (Jiang *et al.*, 2005; Yoshimura *et al.*, 2005) which explains why overexpression of constitutively active GSK3β impairs axon elongation. On the other hand, knockdown of GSK3β and the use of specific inhibitors or transfection of the constitutively active form of Akt cause the formation of multiple axons (Jiang *et al.*, 2005; Yoshimura *et al.*, 2006). Moreover, ectopic expression of Phosphatase and Tensin homolog (PTEN), a phosphatase that rescues PI3K activity, prevented axon specification, and this effect was counteracted by a GSK3 inhibitor (Jiang *et al.*, 2005). Conversely, knocking down *Pten* induced the formation of neurons with multiple axons, which was prevented by expression of GSK3β-Ser9Ala, a mutant of GSK3β which is unresponsive to Akt (Jiang *et al.*, 2005). GSK3β also phosphorylates Tau (Hanger *et al.*, 1992; Mandelkow *et al.*, 1992), MAP1B (Lucas *et al.*, 1998) and Adenomatous Polyposis Coli (APC; Zumbrunn *et al.*, 2000). These three substrates, when unphosphorylated, have a microtubule stabilisation role, but this function is disrupted by GSK3β-mediated activity. (Mandelkow *et al.*, 1995; Zumbrunn *et al.*, 2000). One study reported that hippocampal neurons derived from mice lacking both Tau and MAP1B show axon loss at stage 3 (Takei *et al.*, 2000). As microtubule stabilisation and protrusion into the actin network at the distal area of the growth cone generally promote axon elongation (Baas, 1999; Bradke and dotti, 1999; Dent and Gertler, 2003), GSK3β seems to have a central role as a negative regulator of neuronal polarisation but its function in nervous system development, will be discussed in more detail in Part 4 of this thesis [**Figure 4**].

PI3K and RAP1B.

Alongside the activation of Akt, PI3K also recruits RAS-related protein 1B (RAP1B), a Ras superfamily GTPase that localises to the tips of the future axons

and activates cell division control protein 42 homologue (CDC42), which in turn recruits and activates the partitioning defective homologue 3 (PAR3) – PAR6–atypical protein kinase C (aPKC) complex (Schwamborn and Püschel, 2004). Over-expression of *Rap1b* leads to the formation of multiple axon-like neurites and the accumulation of the Par complex in each neurite. Inhibition of *Rap1b* expression by RNA interference (RNAi) causes the complete loss of axons (Lova *et al.*, 2003). The loss of axons induced by *Rap1b* RNAi is partially counteracted by expressing an active form of Cdc42, whereas axonal loss in response to treatment with a PI3K inhibitor is rescued by the active form of RAP1B. So, RAP1B seems to function upstream of Cdc42 and the Par complex, and downstream of PI3K (Lova *et al.*, 2003).

PAR complex.

The Par protein complex (PAR3–PAR6–aPKC), was identified for the first time in *C. elegans* for their roles in directing asymmetric cell division during early development (Cowan and Hyman, 2004). In pyramidal neurons and before polarisation, the Par complex is localised at the tips of all processes, but later they become selectively concentrated into the developing growth cone during the transition between stages 2 and 3 (Nishimura *et al.*, 2004; Shi *et al.*, 2003). Inhibition of aPKC activity prevents axon formation (Shi *et al.*, 2003) whereas phosphorylated (active) aPKC can be seen at the tips of developing axons (Schwamborn and Püschel, 2004). Inhibition of PI3K impairs polarisation and cause an impaired localisation of both PAR3 and PAR6 (Shi *et al.*, 2003), suggesting that the Par complex is acting downstream of PI3K in the regulation of neuronal polarity. Microtubule affinity-regulating kinase 2 (MARK2) has also been suggested to function downstream of the Par complex to control neuronal polarisation (Chen *et al.*, 2006). Depletion of MARK2 by RNAi induces the formation of multiple axons in hippocampal neurons (Chen

et al., 2006), whereas its over-expression increases Tau phosphorylation and prevents axon formation. This effect can be rescued by over-expressing Par complex, as aPKC phosphorylates MARK2 on threonine 595 and inactivates its kinase activity (Suzuki *et al.*, 2004).

PI3K-Akt and the TSC-mTOR pathway

Akt activation also induces an inhibitory phosphorylation of tuberous sclerosis 2 (TSC2), reducing the GTPase activating protein (GAP) activity of TSC2 towards the Ras homolog enriched in brain (Rheb) and consequently activating the mammalian target of rapamycin (mTOR) signalling. Although phosphorylation of TSC2 by Akt inhibits TSC1 and TSC2 activity (TSC1/2), phosphorylation of TSC2 by means of GSK3 has the opposite effect (Inoki *et al.*, 2006). Thus, inhibition of GSK3 downstream of either PI3K or Wnt signalling would reduce GSK3-dependent stimulatory phosphorylation of TSC2 and thereby increase Rheb-GTP levels. This leads to the activation of mTOR-mediated translation. Activation of mTOR signalling in the axon induces local translation of the GSK3 substrates collapsin response mediator protein 2 (CRMP2) and Tau (Morita and Sobuě, 2009).

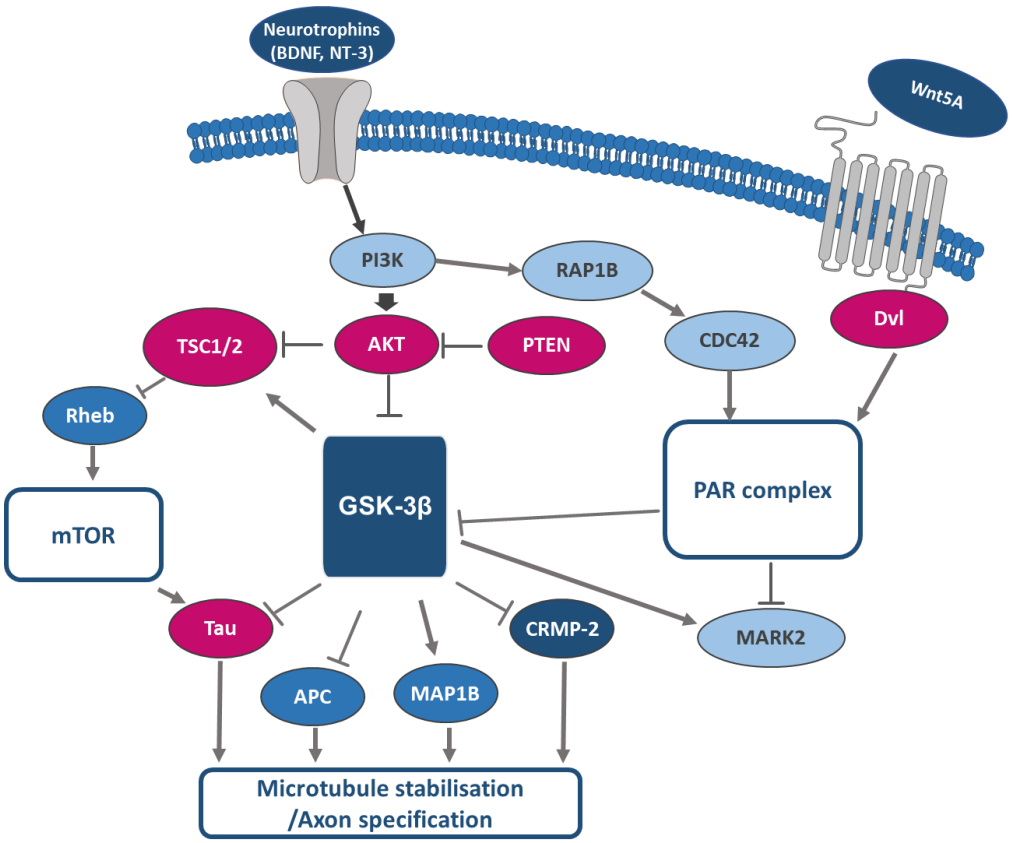

Figure 4: Key signalling pathways in neuronal polarity. PI3K activation triggers a series of signalling events: Akt activation and GSK3β inhibition stabilise microtubules. Activation of RAP1B and cdc42 promotes the PAR complex assembly and inhibition of TSC1/2 induce the activation of mTOR signalling and local translation of CRMP2 and Tau. The Wnt pathway may regulate polarity *via* Dvl by inhibiting GSK3.

1.1.5 Other polarity effectors

Centrosomes. The position of centrosomes inside the cell body was suggested to play a role in axon formation by influencing where the future axon will eventually protrude. This was based on the observation that the centrosome consistently appeared to be repositioned to the base of the newly emerging axon during transition from a unipolar to bipolar morphology in granule cells (Lefcort, 2004; Zmuda and Rivas, 1998). However, other studies suggested that the positioning of the centrosome is not necessary for neuronal polarisation (Distel *et al.*, 2010; Nguyen *et al.*, 2011) and its localisation to the site of axon formation is not correlated to the emergence of the axon in retinal ganglion cells in zebrafish (Zolessi *et al.*, 2006). Centrosome localisation is likely constrained by microtubule organisation within the cell, and therefore the centrosome position within the cell changes dynamically during different stages of polarisation (Sakakibara *et al.*, 2014). Hence, the specific centrosome localisation may not be the cause for axon formation but rather a result and by-product of axon-inducing cues (Arimura and Kaibuchi, 2007).

Actin waves. Actin waves are periodically occurring and move across the neurite shaft, being associated with protein transport and increased neurite outgrowth (Flynn *et al.*, 2009; Toriyama *et al.*, 2006; Winans *et al.*, 2016). Importantly, the frequency of actin waves is higher in the future axon than in any other projections (Flynn *et al.*, 2009). How is this preferential occurrence of actin waves in the future axon achieved? Waves are regulated by microtubule based transport (Winans *et al.*, 2016) and wave frequency is reduced upon Kinesin 12 knockdown (Liu *et al.*, 2010). Kinesin-mediated transport can be in turn regulated by microtubule stability (Hammond *et al.*, 2010). Therefore, microtubule stability could ultimately regulate actin wave frequency. This would be the perfect link between actin wave frequency and neuronal polarisation, since microtubule stability is increased in the nascent

axon (Witte *et al.*, 2008). Moreover, microtubule-based transport produces the accumulation of several polarity effectors to the growth cone during axon specification.

Kinesins and cargoes. As it will also be described in more detail in the relative section below (1.3), in order to establish and maintain polarity, neurons also employ active transport driven by cytoskeletal motor proteins, which allows cargo sorting between axons and dendrites. These motors can move in a specific direction over either microtubules (kinesins, dynein) or actin filaments (myosins) (Hirokawa *et al.*, 2010). In fact, Kinesin 1 is a conventional member of the kinesin family and a microtubule plus-end-directed motor (Vale, 2003), which is characterised by two kinesin heavy chains (KIF5; also known as KHC) and two kinesin light chains (KLC; Bloom *et al.*, 1988). KIF5 contains the motor domain and KLC contains the binding domain for the cargo and when one neurite begins to adopt the fate of an axon, KIF5 strictly concentrates in the growing axon (Nakata and Hirokawa, 2003). It has been shown that Kinesin 1 can associate with vesicles directly, or indirectly by interacting with various adaptor proteins, which determine selective transport of cargos in neurons and, among these, c-Jun N-terminal protein kinase (JNK)-interacting protein 1 (JIP1), a scaffold protein for JNK signalling pathways (Whitmarsh, 2006), mediates axonal transport of several cargos (Koushika, 2007). Interestingly, JIP1 specifically localise to a single neurite and, after axonal specification, it accumulates in the emerging axon (Dajas-Bailador *et al.*, 2008). It can also regulate axonal growth dependent upon its binding to kinesin-1 and c-Abl tyrosine kinase (Dajas-Bailador *et al.*, 2008), a well-established regulator of cytoskeletal dynamics (Lanier and Gertler, 2000). Consistent with the fact that KIF5 motor domain preferentially localises to axonal tips rather than dendrites, several key molecules that are involved in axon formation accumulate into the distal parts or tips of axons. Among them, the transport

of amyloid precursor protein (APP) has been extensively investigated as it may be involved in the progression of Alzheimer disease (AD) (Gunawardena and Goldstein, 2001; Kamal *et al.*, 2000). KIF5 can interact with APP vesicles through JIP1 (Muresan and Muresan, 2005). KIF5 also transports TrkB vesicles through the CRMP2-Slc1 complex, loading and unloading of which is regulated by Rab27 (member of the Rab subfamily of GTPases) and GSK3β (Arimura *et al.*, 2009). The CRMP2–kinesin 1 complex is conserved from worms to mammals, and regulates the transport of tubulin heterodimers to the distal part of the growing axon to influence the organisation of microtubules and actin filaments (Kimura *et al.*, 2005). As described above, during axonal specification, PIP3 accumulates at one neurite tip, recruits Akt and specifies one neurite to become an axon. It has been reported that PIP3 is transported by the guanylate kinase-associated kinesin (GAKIN) to the prospective axon (Horiguchi *et al.*, 2006), whilst another important neuronal polarity regulators, the PAR complex, is instead transported towards the axon by kinesin 2 (which comprises KIF3 and kinesin superfamily-associated protein 3 -KAP3-), through the direct binding of PAR3 to KAP3 (Nishimura *et al.*, 2004). More recently, another member of the kinesin family, KIFC1, a molecular motor well characterised in mitosis, has been reported to deeply influence on the organisation of microtubules in a number of different functional contexts (Muralidharan and Baas, 2019). Specifically, experimental manipulations of KIFC1, which had never been studied in the nervous system, elicit morphological changes in the axon as well as changes in the organisation, distribution and polarity orientation of its microtubules (Muralidharan and Baas, 2019). Taken together, these studies reinforce the important role of kinesin family in establishing neuronal polarity by recruiting molecules into a single neurite.

1.2 Axon growth and growth cone

After axon specification, growth represents another important process of axon development, and is deeply linked to axon guidance towards post-synaptic targets (Stoeckli, 2018). Axons grow at their tips by means of the growth cone, a highly dynamic structure capable of sensing and integrating a plethora of signals that, by converging on cytoskeletal dynamics, eventually lead the axons to their targets (Lowery and Vactor, 2009).

The growth cone structure is comprised of a central domain (C-domain) enriched of microtubule bundles entering from the axon shaft, a peripheral domain (P-domain) dominated by dynamic actin structures such as *lamellipodia* and *filopodia*, and lastly an intermediary transition zone (T-zone) where myosin II produces condensed actomyosin structures called actin arcs, by generating contractions of F-actin, (Dent and Gertler, 2003; Schaefer *et al.*, 2002) [**Figure 5**].

Growth cones detect extracellular signals through a rod-like, actin-rich, highly dynamic membrane-limited structures called filopodia. Their surface membranes are enriched of receptors for the molecules that serve as directional cues for the axon. When filopodia sense environmental signals, they contract and pull the growth cone forward. At this point, actin filaments polymerise at the leading edge of a filopodium, disassemble at the trailing edge, and interact with myosin along the way. Then, the net force generated by both actin polymerisation and the retrograde movement of actin, pushes the filopodium forward. Meanwhile, exocytosis adds membrane to the leading edge of the filopodium and provides new adhesion receptors to maintain the traction. The combined action of these motors creates an actin depleted space that is filled by the advance of microtubules coming from the C-domain, creating a new section of axonal shaft (Mitchison and Kirschner, 1988).

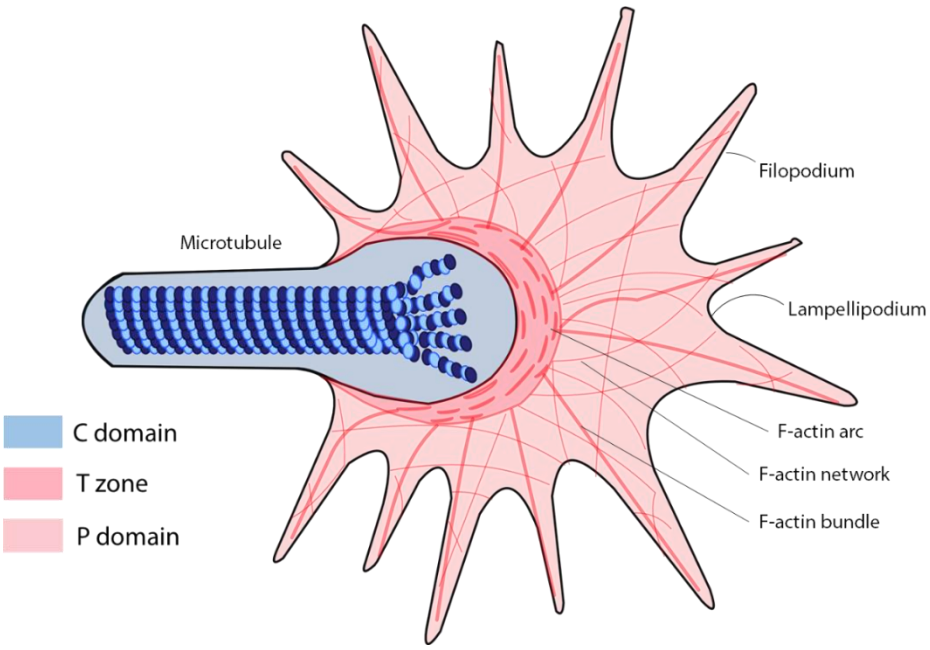

Figure 5: The structure of the growth cone. Growth cones are comprised of a central domain (C-domain) enriched of microtubule bundles entering from the axon shaft, a peripheral domain (P-domain) dominated by dynamic actin structures such as lamellipodia and filopodia, and lastly an intermediary transition zone (T-zone) where myosin II produces condensed actomyosin structures called actin arcs.

Coordinated actin and microtubule dynamics seem to be fundamental for the proper functioning of the growth cone. However, it has been shown that actin-disrupting agents have limited consequences on axon elongation and are rather involved in axon guidance *in vitro* (Marsh and Letourneau, 1984; Ruthel and Hollenbeck, 2000) and *in vivo* (Bentley and Toroian-Raymond, 1986) Local disruption of actin organisation in the growth cone of minor neurites allows them to turn into axons (Bradke and dotti, 1999; Kunda et al., 2001), indicating that the dense actin network present at the periphery of immature neurites may prevent microtubule protrusion and elongation necessary for axon specification. It is still unclear how the force is generated to drive axon growth. Several studies have provided evidence in support of the traction forces described above (Athamneh *et al.*, 2017; Bard *et al.*, 2008; Buck *et al.*, 2017; Garcia *et al.*, 2015; Toriyama *et al.*, 2013). Indeed, in hippocampal neurons,

growth does not correlate with traction (Koch *et al.*, 2012) and established neurites can still grow without filopodia and lamellipodia (Bentley and Toroian-Raymond, 1986; Bradke and dotti, 1999; Marsh and Letourneau, 1984), structures necessary for actin-mediated force generation. It remains possible that microtubules are actively contributing during neurite growth and so an axon-originating force (Lu *et al.*, 2013; Recho *et al.*, 2016; Roossien *et al.*, 2013). Axonal transport also plays a fundamental role on axon elongation (Dent and Gertler, 2003; Letourneau *et al.*, 1987; Suter and Miller, 2011). In fact, cytoskeletal elements are transported along the axon through slow axonal transport (Xia *et al.*, 2003; Yabe *et al.*, 1999), but it is still not clear whether tubulin and other cytoskeletal components are transported along the axon as monomers and/ or as polymers (Roy *et al.*, 2000; Terada *et al.*, 2000; Wang *et al.*, 2000). Either way, disruption of the slow transport of tubulin impairs the pushing force resulting from microtubule polymerisation, affecting axon elongation (Suter and Miller, 2011). Therefore, it is not surprising that axon growth is affected *in vitro* and *in vivo* by disruption of plus-end microtubule-binding proteins such as APC (Chen *et al.*, 2011; Shi *et al.*, 2004; Yokota *et al.*, 2009; Zhou *et al.*, 2004) or End binding protein 1 (EB1) (Jiménez-Mateos *et al.*, 2005; Zhou *et al.*, 2004) and microtubule-associated proteins such as MAP1B (Dajas-Bailador *et al.*, 2012; Takei *et al.*, 2000; Tortosa *et al.*, 2013; Villarroel-Campos and Gonzalez-Billault, 2014).

1.2.1 Intracellular signals and axon elongation

Proper pathfinding can occur only if both the motor component and sensory activity of growth cones are properly linked. It is then crucial that the recognition proteins on the filopodia are signal-inducing receptors capable of affecting the organisation of the cytoskeleton, and in this way modulate both

direction and movement of the growth cone, and not exclusively by means of binding moieties that facilitate adhesion.

It is reasonable to suppose that many of the molecules associated with axon specification are also involved in axon growth and elongation toward postsynaptic targets. In fact, the aforementioned GSK3β plays again a key role in this cellular process, since several of its substrates have the potential to regulate tubulin polymerisation and microtubule stability (Hur *et al.*, 2011; Kim *et al.*, 2006; Zhou and Snider, 2005), a key issue for efficient axon elongation. These include the microtubule plus-end binding proteins APC and the Cytoplasmic Linker Associated Protein 2 (CLASP2), CRMP-2, which is localised to axon tips and may play a role in cargo delivery and tubulin polymerisation, and both MAP1B and Tau (Kim *et al.*, 2006; Yoshimura *et al.*, 2005; Zhou and Snider, 2006). CLASPs are homologous proteins that were believed to possess overlapping activity in neurons (Hur *et al.*, 2011). However, a recent work reported that they may have different functions, as CLASP1 stimulates neurite outgrowth and CLASP2 acts as a break (Sayas *et al.*, 2019). Specifically, they differ in their accumulation at MT plus-ends and display different sensitivity to GSK3 activity, and hence regulation (Sayas *et al.*, 2019). Pharmacological inhibition of GSK3 specifically affects CLASP2 but not CLASP1 phosphorylation (Sayas *et al.*, 2019). Indeed, many studies indicate that local inhibition of GSK3 signalling is essential for promoting microtubule polymerisation at the level of the growth cone (Jiang *et al.*, 2005; Yoshimura *et al.*, 2005; Zhou *et al.*, 2004). However, other studies have shown how inhibition of GSK3 can block axon growth (Garrido *et al.*, 2007; Kim *et al.*, 2006; Shi *et al.*, 2004). To reconcile these contradictory results, Kim *et al.* (2006) hypothesised a model in which inhibition of GSK3 can both promote and inhibit axon growth at the same time, depending on the substrates involved. In fact, another interesting aspect of GSK3s is that before being

phosphorylated by this enzyme, some of the substrates need to be first phosphorylated by a different kinase through a process called priming, whilst others can be directly activated by GSK3s. During axon elongation, GSK3 activity in the growth cone seems to be accurately controlled, so that the kinase activity towards primed substrates is blocked whilst its activity towards unprimed substrates is preserved (Kim *et al.*, 2006). In fact, inhibition of GSK3 activity with regards to CRMP2 and APC, which are primed substrates, allows both molecules to bind and stabilise microtubules (Fukata *et al.*, 2002; Yoshimura *et al.*, 2005). By contrast, GSK3's activity towards MAP1B, an unprimed substrate, is preserved in the growth cone (Kim *et al.*, 2006). Phosphorylation of MAP1B maintains microtubules in a dynamic state, which is essential for axon growth (Dent and Gertler, 2003; Zhou and Snider, 2005). Consistently, phosphorylated MAP1B is enriched at the distal ends of growing axons (Trivedi, 2005). In this way, GSK3 can orchestrate several properties of microtubules to guarantee optimal microtubule assembly in axons.

Abundant *in vitro* evidence depicted RAS and its downstream signalling RAF/MEK/ERK cascade as important players in the modulation of axon growth (Zhou and Snider, 2006). Several studies with the pharmacological inhibitors show strong inhibition of axon growth mediated by neurotrophins and other molecules acting *via* receptor tyrosine kinases (all reviewed in Zhou and Snider, 2006). Further, gene manipulation studies *in vitro* suggested that Ras, Raf, and ERK/MAPK possess axon growth promoting activity (Markus *et al.*, 2002).

Experimental findings have also depicted an important role JNK signalling, classically considered a degenerative signal (Brecht *et al.*, 2005; Kuan *et al.*, 1999; Sabapathy *et al.*, 1999), in the regulation of axonal outgrowth. In particular, deletion of a single isoform, JNK1, disrupts axon tract maintenance *in vivo* (Bjorkblom, 2005; Chang *et al.*, 2003). Moreover, several studies suggest

this pathway is also important for axon elongation (Ciani and Salinas, 2007; Eminel *et al.*, 2008; Xiao *et al.*, 2006). JNKs are constitutively active within neurons and phosphorylate several cytoskeletal proteins that are in turn involved in axon growth, including MAP1B, MAP2, Tau, and the superior cervical ganglion 10 (SCG10) (Ciani and Salinas, 2007; Tararuk *et al.*, 2006; Yamauchi *et al.*, 2006). Axonal transport is modulated by JNK, and it has been proposed that JNK triggers the release of cargoes, such as tubulin, from kinesin complexes (Horiuchi *et al.*, 2007; Stagi *et al.*, 2006). It is thus clear that the repertoire of JNK substrates is well-suited to mediate many aspects of axon growth.

Activation of mTORC1 signalling also plays a role in axon elongation (Park and He, 2008) and combining mTORC1 activation with dysregulation of other pathways including Stat3, B-raf, and c-Myc confer additional axon growth benefit over mTORC1 activation alone (Belin *et al.*, 2015; O'Donovan *et al.*, 2014). More recently, deletion of TSC2, a negative regulator of mTORC1 signalling, enhanced axon regeneration by upregulating expression of regeneration-associated transcription factors (RATFs) known to be both necessary and sufficient for promoting axon growth (Carlin *et al.*, 2019).

1.2.2 Transcription factors and axon growth

Transcription is an important aspect of axon growth, targeting, and regeneration upon injury (Polleux *et al.*, 2007). One elegant mechanism has been defined involving the anaphase promoting complex, which, along with its activator protein, Cdh1, promote degradation of the transcription factor SnoN in postmitotic neurons (Konishi *et al.*, 2004; Stegmüller *et al.*, 2006). SnoN is also regulated by the TGF-β/SMAD-2 axis (Stegmuller *et al.*, 2008). Knockdown experiments *in vivo* show that a decrease of Cdh1 promote axon elongation of cerebellar granule cells and, on the other hand, suppression of

SnoN expression reduces axon growth. The scaffolding protein Cdc1 is an important target of SnoN, that controls JNK activity (Ikeuchi *et al.*, 2009). It has been reported recently that activation of TGF-β/SMAD-2 signalling pathway inhibits neurite elongation of human induced pluripotent stem cell-derived neurons (Nakashima *et al.*, 2018). Mechanistic-wise, activated Smads form a complex with transcriptional repressor TG-interacting factor (TGIF), and downregulate the expression of CRMP2 (Nakashima *et al.*, 2018).

Other transcriptions factors that have been documented to control axon elongation include CREB and NFAT transcription complexes (Graef *et al.*, 2003; Lonze *et al.*, 2002). The role of the NFATc family is particularly effective *in vivo*. Indeed, in mice lacking 3 NFATc family members, extension of peripheral axon is almost completely impaired at early developmental stages (Graef *et al.*, 2003; Lonze *et al.*, 2002).

More recently, an interesting study identified *Sox11* from a screen of transcription factors that could activate genes involved in cytoskeletal remodelling and axon growth (Norsworthy *et al.*, 2017). Remarkably, over-expression of *Sox11* killed adult α-retinal ganglion cells (RGCs), which preferentially regenerate after *Pten* deletion, whilst promoted regeneration of non α-RGCs, which are instead refractory to *Pten* deletion-induced regeneration (Norsworthy *et al.*, 2017). In the same model, phosphorylation of STAT3 significantly promoted optic nerve axon regeneration after activation of NF-κB signalling pathway upon peripheral axotomy (Ma *et al.*, 2019). In another work conducted almost in parallel, co-expression of STAT3 with KLF6, a member of the Kruppel-like factors (KLF) family and a known pro-regenerative transcription factor (Qin *et al.*, 2013; Z. Wang *et al.*, 2017), synergistically promoted axon growth *in vitro* in central nervous system (CNS) neurons (Wang *et al.*, 2018).

1.2.3 Axon pathfinding and guidance cues

Several developmental processes, such as neuronal polarity, migration and axonal outgrowth, shape precise patterns of connections in the nervous system, but none is more important than the guidance of axons from their origin to appropriate targets. To reach their destinations and establish connections with synaptic partners, axons need in fact to grow long distances and avoid numerous targets along the way. To do so, axons receive along their way guidance signals which, by means of their growth cones, can be integrated and converted into instructions that steer the structure.

As already described in previous sections of this thesis, growth-cone motility is deeply linked with the extension and retraction of filopodia along with the extension and retraction of lamellipodia between the filopodia. These latter have a central role in axon pathfinding, as they are the first structure of the growth cone to come into contact with guidance molecules and they are equipped with the molecular machinery to detect and respond to these signals (Gupton and Gertler, 2007). Filopodia react to guidance cues either by stabilising when they sense an attractive guidance cue, or by retracting when they encounter a repulsive cue. If this occurs differentially on one side of the growth cone, then the growth cone will steer towards the attractive cue or away from a repulsive guidance molecule. Such growth-cone turning is a key mechano-sensory event in axon pathfinding and occurs specifically at choice points. Thus, the growth cone relays the information conveyed by guidance molecules into changes in motility that result in steering manoeuvres and thus, a change of route during their travel towards post-synaptic targets (Kahn and Baas, 2016).

Today, a large number of axon guidance cues and receptors have been identified and in general they can be subdivided into attractive and repulsive cues that act either over long distances or locally, in a contact-dependent

manner (de Ramon Francàs et al., 2017). A combination of both genetic and biochemical approaches has led to the identification of 4 well-characterised classes of axon guidance molecules and their receptors: Ephrins, Netrins, Semaphorins and Slits.

***Ephrins/Eph*:**

Ephrins are cell surface-associated guidance cues that bind to Eph receptor tyrosine kinases in trans on opposing cells. During nervous system development, the Eph–ephrin signalling system can mediate several cellular responses including contact-mediated attraction or repulsion, adhesion or de-adhesion, and migration. A peculiar feature of Eph-ephrin complexes consist on their ability to trigger bidirectional signals that can affect both the receptor-expressing and ephrin-expressing cells (Pasquale, 2005). To mediate their function, Eph receptors and ephrins use intracellular signalling effectors, such as Src family kinases and Ras/Rho family GTPases, which are known to modulate both the organisation of the actin cytoskeleton and cell adhesion, thus influencing growth cone motility (Pasquale, 2008). A textbook example in which the role of Ephrins has been well characterised is the retinotopic mapping and proper migration of RGC axons from the retina to specific regions of the superior colliculus (Suetterlin et al., 2012). Projection patterns of RGC axons are in fact perfectly in line with the expression gradient of ephrinAs and EphAs in both the retina and tectum. The two gradient systems in the retinotectal projection are, firstly, the EphA gradient in the retina (Nasal < Temporal) and the corresponding ephrinA gradient in the tectum (Anterior < Posterior), and, secondly, the ephrinA gradient in the retina (Nasal > Temporal) and the EphA gradient in the tectum (Anterior > Posterior). Based on knockout/in experiments the model is that temporal axons with high EphA concentrations project on the anterior tectum, because they are repelled from the posterior tectum with high ephrinA concentration. In turn, nasal axons

with high ephrinA concentrations are projecting on the posterior tectum because they are repelled from projecting onto the anterior tectum expressing high EphA concentrations (Suetterlin et al., 2012).

Netrins:

Since its first discovery (Serafini et al., 1994), netrin-1 has been one of the most well-studied member of this class of proteins, playing a fundamental role in guiding axons through the midline (Raper and Mason, 2010). Interestingly, even though the majority of the axon guidance cues can mainly have either an attractive or repulsive activity, and act as either diffusible/chemotactic molecule, function of netrin-1 has never fallen into one single category. In fact, Netrin binding to its receptor, deleted in colorectal cancer (DCC) results in attractive responses, *via* homodimerization of DCC (Fazeli et al., 1997), whereas heterodimerization between DCC and receptor uncoordinated locomotion 5 (UNC5) results into repulsion (Finci et al., 2014). Mechanistically, the intracellular domain of DCC is a hotspot for interaction with several binding proteins that are involved in cytoskeletal and membrane remodelling, such as the unconventional myosin X (MyoX), the non-receptor tyrosine kinase FAK, the E3 ubiquitin ligase TRIM9, F-actin binding ezrin-radixin-moesin (ERM) proteins, and p120RasGAP (Boyer and Gupton, 2018). Rho family GTPases are also regulated downstream of Unc5. In fact, in mouse neuroblastoma cells, RhoA is activated by netrin-1 binding to UNC5A, and to a lesser extent, Rac1 and Cdc42 are activated (Picard et al., 2009).

Semaphorins

They constitute a large family of more than twenty members that activate complexes of cell-surface receptors called Plexins and Neuropilins, with Sema3A as the most studied family member. The first functional evidence of its activity was the ability of this molecule to act as a repulsive factor on

chicken DRG neurons by inducing the collapse and retraction of their growth cones (Luo et al., 1993). Most of the semaphorins discovered so far have a repulsive activity but some members have a growth promoting effect on specific neuronal subpopulations (Bagnard et al., 1998).

Knockout experiments shown several defects such as abnormal projections of sensory axons, abnormal cortical neurites orientation (Behar et al., 1996) or distorted odor map (Taniguchi et al., 2003) in Sema3A-deficient mice. In many cases, the most severe phenotype was the loss of axons in absence of Sema3A signalling (Taniguchi et al., 1997). Several defects in projections in the hippocampus, mid brain, forebrain and in the PNS of Sema3F deficient-mice have also been described (Sahay et al., 2003). The diversity of the guidance effects elicited by semaphorins is thus consistent with their role in the complex wiring of several brain regions.

Mechanistically and consistent with both other guidance cues and the considerable amount of evidences collected on Sema3A, the intracellular pathways recruited upon co-receptors activation relate to Rho GTPases (Hu and Zhu, 2018).

Slits

Slits are secreted proteins that bind to Roundabout (Robo) receptors and they are known for mediating axon repulsive activity in during nervous system development, especially in providing commissural axons with the right trajectory from one (ipsilateral) side of the CNS to the other (contralateral) (Blockus and Chédotal, 2016). In the mouse spinal cord, commissural interneurons are generated in the dorsal part of the neural tube and their axon crosses the ventral midline by E10.5-11. All three vertebrate Slits are expressed in the ventral midline, and *Slit1;2;3* triple knockouts show re-crossing and axon stalling phenotypes at the midline (Long et al., 2004). Robo2 on its own

does not contribute to midline crossing in the spinal cord, and only the *Robo1*−/− mutant partially mimics the triple Slit mutants. *Robo1;2* double mutants display similar axon stalling phenotypes as *Slit1;2;3* triple mutants (Jaworski and Tessier-Lavigne, 2012) indicating that Slits may also act independently of Robos in commissural axons and that others Slit receptors may be at play. Data also suggests that Slit-Robo signalling interplays with the Netrin-1/DCC axis. When Slit is present, both Robo1 and DCC interact in *Xenopus* spinal cord commissural axons, in turn quenching attraction to Netrin-1 (Stein and Tessier-Lavigne, 2001). However, Slit-Robo repulsive function is an active process that can occur in the absence of DCC signalling, at least in *Drosophila* (Garbe and Bashaw, 2007), weakening hypothesis that the sole function of Robo1/2 is to silence attraction.

Once again, the major effectors of Robo-triggered signalling pathway are cytoplasmic kinases and both actin and microtubule cytoskeleton regulators. The cytoplasmic kinase Abelson (Abl) plays a fundamental role and, through its effectors, influences both the actin and microtubule cytoskeleton (Blockus and Chédotal, 2016). Several studies related to different types of cancer, show that Slit and Robo are also implicated in the regulation of E-cadherin (cadherin 1)- dependent adhesion *via* the Wnt signalling axis, along with GSK3β and β-catenin (Prasad et al., 2008; Zhou et al., 2011). Slit-Robo signalling also involves GTPases, which are small GTP-binding proteins that rearrange the cytoskeleton and thus regulating cell polarity and motility.

Considering the complexity of neural circuits, and even if axon guidance cues can be shared among several classes of neurons, the number of guidance molecules is surprisingly small. This suggests that the regulation of axon guidance signalling involves all possible mechanisms of regulation: transcriptional and translational regulation, vesicles trafficking, protein-protein interactions as well as protein stability. Moreover, the link between

receptor-ligands interaction with the observed phenotypes of growth cones is still missing (Stoeckli, 2018). There is also a very small understanding of the association between surface receptors and the regulation of cytoskeletal dynamics responsible for steering growth cones (Gomez and Letourneau, 2014).

1.3 Axonal transport

Transport of proteins and organelle cargoes is essential for mammalian cells function, but for neurons this can be considered an understatement. In fact, neurons are extremely polarised cells with an altogether different proteome between the axon and dendrites, and because most neuronal proteins are synthesised in cell bodies, mechanisms are required to direct axonal vs. dendritic transport. Even within the axonal domain, cargoes must be targeted to specific locations, e.g., sodium channels are enriched at nodes of Ranvier, whereas synaptic proteins are targeted to the axon terminal. Thus, intracellular transport processes become extremely important when it comes to the neuronal structure. The two major molecular motors involved in transport machinery have been already mentioned in previous sections of this thesis and are *kinesin*, that by unidirectionally moving toward the microtubule plus end it mostly mediates transport toward the synapse (anterograde), and *dynein* that moves toward the microtubule minus end and, accordingly, mediates transport of cargoes toward the cell body (retrograde; Vale, 2003; Welte, 2004). In both the axon and dendrites, microtubules and neurofilaments constitute the main cytoskeletal structure through which kinesins and dyneins move along. In the synaptic regions, such as presynaptic terminals and postsynaptic spines, the cytoskeletal architecture is mainly composed of actin filaments, where myosins convey the cargos (Hirokawa *et al.*, 2010). As mentioned above, in the axon and dendrites transport occurs bidirectionally, from the cell body

to the periphery (anterograde transport) and from the periphery to the cell body (retrograde transport) depending on the microtubule's polarity; the latter is extremely uniform in both axon and distal part of dendrites, with all the plus-end microtubules pointing distally toward their own terminal parts. In contrast, microtubules in the proximal region of dendrites lack an overall polarity. Actin filaments have a polarity too, as the barbed end (the growing end) points to the plasma membrane in the presynaptic and postsynaptic regions (Leterrier *et al.*, 2017).

Classically, axonal transport is divided into fast and slow axonal transport depending on the bulk speeds of cargo movement. Cargoes such as vesicles, mitochondria and mRNA-containing protein complexes (mRNPs) (Ling *et al.*, 2004; Ohashi *et al.*, 2002) move by fast axonal transport, a form of transport that is faster than 400 mm per day. The organelles moved by retrograde fast axonal transport are primarily endosomes generated by endocytic activity at nerve endings, mitochondria, and elements of the endoplasmic reticulum, and many of these components are degraded by lysosomes. Retrograde fast transport also delivers signals that regulate gene expression in the neuron's nucleus. In effect, neurotrophins have been shown to signal retrogradely in peripheral neurons after triggering the local translation of specific effectors and/or transcription factors (Cox *et al.*, 2008; Walker *et al.*, 2018; Willis *et al.*, 2007).

Cytosolic proteins and cytoskeletal proteins are instead moved from the cell body by slow axonal transport which occurs only in the anterograde direction at speeds of ~1 mm/day. However, it is now established that both types of transport are mediated by the same "engines" of the fast axonal transport (kinesin and dynein) and that the slower overall speed of slow axonal transport is due to prolonged pauses between movements (Roy *et al.*, 2000; Wang *et al.*, 2000).

1.4 Local protein synthesis in the axon

As previously described, the signalling pathways underlying neuronal polarisation and axon development rely on regulatory mechanisms that modulate the formation of the axon, its elongation, and its guidance towards the synaptic targets. These processes require dynamic changes of the local proteome in order to rapidly respond to extracellular cues, thus providing the rationale for local protein synthesis in the developing axon (Mili *et al.*, 2008; Zivraj *et al.*, 2010).

Even though it is now recognised that axons do have the capacity for local protein synthesis, and that this capacity is even retained into adulthood (Costa and Willis, 2018; Gumy *et al.*, 2011; Shigeoka *et al.*, 2016) full acceptance of this mechanism as a key molecular process in axon development and function has taken decades. Indeed, early data in the 1950s suggested that local translation in the axon was an unlikely scenario for neurons, lacking both mRNA and protein synthesis apparatus. Palay and Palade demonstrated at the time the apparent lack of ribonucleoprotein (RNP) in histochemical preparations of neurons, concluding that the minimum necessary for protein production was absent (Palay and Palade, 1955). A similar conclusion was also reached by Deitch, Murray, and others, suggesting that there was no organisation of protein producing superstructures in axons (Deitch and Moses, 1957; Deitch and Murray, 1956).

The dogma that proteins were produced only in the cell bodies persisted into the mid-1960s and early 70s when it was reaffirmed that protein synthesis was unlikely to occur in mature axons as little or no ribosomal RNA was found in the axoplasm (Lasek *et al.*, 1973). This interpretation started being disputed only later, when ribosomes were identified by electron microscopy in embryonic peripheral sensory axons *in vivo* (Tennyson, 1970), in sympathetic neuronal axons (Bunge, 1973) and in embryonic cortical neurons (Bassell *et al.*,

1998). Results obtained using more sensitive biochemical methods finally showed the presence of ribosomal RNAs (Giuditta *et al.*, 1980), mRNAs (Giuditta *et al.*, 1986) and actively translating polysomes in squid giant axons (Giuditta *et al.*, 1991).

More recent immunohistological studies showed that mature PNS axons contain both ribosomal proteins and RNAs and that are localised in the peripheral axoplasm close to the plasma membrane (Benech et al., 1982; Koenig et al., 2000; Koenig and Martin, 1996; Kun et al., 2007; Sotelo-Silveira et al., 2008, 2006).

Probably the most important evidence of axonal local translation came from metabolic labelling experiments in which unmyelinated axons without their cell bodies were still capable of synthesising proteins (Eng *et al.*, 1999; Koenig, 1991; Koenig and Adams, 1982; Tobias and Koenig, 1975).

In the last years, thousands axonal mRNAs have been identified by *in situ* hybridisation, small axonal cDNA libraries, microarrays (Taylor *et al.*, 2009; Yoon *et al.*, 2009) and genome-wide transcriptome analyses (Poulopoulos et al., 2019; Shigeoka et al., 2016) exposing an ever-growing list of axonal mRNAs that included transcripts encoding for cytoskeletal proteins such as β-tubulin, β-actin, MAP1B, Tau, but also cell signalling molecules like RhoA and transmembrane receptors such as EphB2 (Yoon, Byung C. & Holt, 2009).

Technically, local protein synthesis confers several advantages over the transport of pre-existing proteins from one part of the cell to another (Holt and Bullock, 2009), such as the storage of translationally inert forms of mRNAs which may be used to synthesise a protein when needed, thereby providing an "energetic" advantage. For this reason, highly polarised cells like neurons would benefit greatly from local mRNA translation. Indeed, local mRNA translation is known to mediate long-lasting synaptic plasticity in dendritic

spines and to be regulated by extracellular signals (Sutton and Schuman, 2006).

Axonal mRNA translation allows the developing axon and its growth cone to locally and rapidly respond to cues coming from their environment (Harris *et al.*, 1987), often, even without enough time to communicate with the soma. In fact, axons severed from their cell bodies, can still properly pathfind *in vivo* (Ming *et al.*, 2002).

Among these extracellular cues there are semaphorin 3A (SEMA3A) (Campbell and Holt, 2001), nerve growth factor (NGF) (Hengst *et al.*, 2009), BDNF (Yao *et al.*, 2006) and NT3 (Zhang *et al.*, 1999). All these cues commonly activate the translational machinery on the side of the growth cone (Campbell and Holt, 2001). According to the nature of cues, only specific set mRNAs are translated, depending on whether it is an attractive or repulsive cue (Holt *et al.*, 2019). This can also determine the direction of growth cone turning. In particular, application of attractive cues, such as netrin 1 and BDNF, leads to synthesis of β-actin on the side close to the source of the cue, which in turn may lead to actin polymerisation and the turning of the growth cone toward the aforementioned cue (Leung *et al.*, 2006; Yao *et al.*, 2006). On the other hand, repulsive cues such as SEMA3A and Slit homolog 2 protein (SLIT2), activate the axonal translation of the actin-depolymerising proteins RhoA and Cofilin, causing a turn of the growth cone toward the opposite direction, preventing the axon from "meeting" with incorrect targets (Piper *et al.*, 2006; Wu *et al.*, 2005). Recent proteomic analyses have enlarged this differential translation model and revealed that a single cue typically triggers regulation of ~100 proteins, and different cues induce distinct proteomic signatures (Cagnetta *et al.*, 2018).

Axonal mRNAs and local protein translation also play a role in axon elongation during development, as for the case of PAR3, which is required for

NGF-induced and netrin 1-induced axon outgrowth (Hengst *et al.*, 2009). β-thymosin, which prevents actin polymerisation, is also locally synthesised in cultured mollusc neurites treated with brain lysate, and inhibiting its translation in isolated neurites promotes their elongation (van Kesteren, 2006). More recently, local translation of TC10, a small GTPase required for exocyst function, is essential for NGF-induced axon growth and membrane expansion (Gracias *et al.*, 2014).

Notably, mRNAs encoding for proteins involved in branching and synaptic vesicle release were enriched in mature growth cones when compared to younger developing ones (Zivraj *et al.*, 2010) of cultured *Xenopus laevis* neurons. This leads to the speculation that these mRNAs might be transported to the growth cone in sync with target arrival and might be translated in response to target-derived cues. Indeed, presynaptic protein synthesis is essential for BDNF- and NT3-induced potentiation of synaptic vesicle release in *X. laevis* lower motor neurons (Wang *et al.*, 2011; Zhang and Poo, 2002).

It is clear from the previously described studies that axonal translation is important for several aspects of neuronal development, and it is thus not surprising that axonal protein synthesis has roles in axonal signalling too. For example, local translation can relay signals coming from growth cones to the nucleus, thereby influencing gene transcription. This can be achieved by means of the local synthesis of transcription factors that are retrogradely transported to the cell body. Local synthesis CREB, CCAAT/enhancer-binding protein (CEBP-1), signal transducer and activator of transcription 3 (STAT3), importins and SMAD transcription factors have all been linked to retrograde signalling mediated by NGF, bone morphogenetic protein 4 (BMP4) and nerve lesion (Ben-Yaakov *et al.*, 2012; Cox *et al.*, 2008; Hanz *et al.*, 2003; Ji and Jaffrey, 2012; Yan *et al.*, 2009). These studies show that the consequences of local

translation are not restricted to localised responses but can also influence other subcellular compartments, such as the nucleus.

Despite their function, hundreds of different mRNAs can coexist in the same subcellular locations at the same time. Axonal transcriptome analyses from different types of neurons — including microarrays studies in *X. laevis*, mouse embryonic retinal ganglion cells (Zivraj *et al.*, 2010), rat embryonic and perinatal cortical and hippocampal neurons (Taylor *et al.*, 2009), rat embryonic and adult peripheral sensory neurons (Gumy *et al.*, 2011), and a subcellular RNA–proteome mapping on the developing callosal projection of the mouse cerebral cortex (Poulopoulos *et al.*, 2019)— identified thousands of different mRNAs in their axons.

After the initial findings, supporting data emerged from few studies *in vivo*, although *in vivo* visualisations of protein synthesis are relatively rare, due to the extreme difficulty of the experiments and anatomical restrictions (Holt et al., 2019). Nonetheless, several proteins that can encode for cytoskeletal regulators, cell-adhesion molecules, axon guidance receptors and signalling molecules, have been found to be locally synthesised also during axon growth *in vivo* (Shigeoka et al., 2016).

Evidence demonstrating the need for these locally synthesised proteins for axon pathfinding *in vivo* is again sparse due to the technical limitations associated with blocking protein synthesis exclusively in the axonal compartment. However, few studies in the mammalian spinal cord show that specific axon guidance receptors (e.g. EphA2, Robo3.2) are synthesised in growing axons at the midline, implying a role for local translation in the switches of commissural growth cone responsiveness along their journey (Brittis et al., 2002; Colak et al., 2013). Moreover, i*n vivo* inhibition of a microRNA caused defects in pathfinding and target entry in small subsets of RGCs (Bellon et al., 2017).

Breakthrough Fluorescence Recovery After Photobleaching (FRAP) experiments have demonstrated the a de novo β-actin synthesis at the bases and tips of new branches *in vivo* by using single axons in the *Xenopus* visual system (Wong et al., 2017). Moreover, inhibition of axonal β-actin mRNA translation impairs arborisation, demonstrating for the first time a requirement for local protein translation in building arbor complexity and assembling neural circuits (Wong et al., 2017).

The complexity of the dynamic mRNA subpopulations in the axon raises the question of what type of mechanisms regulate local axonal protein translation (Holt *et al.*, 2019). To date, at least four regulation mechanisms have been reported:

1. Extracellular cues involved in axon guidance and neurotrophins can stimulate and subsequently phosphorylate RBPs which release their associated mRNAs for local translation (Lepelletier *et al.*, 2017; Leung *et al.*, 2006; Sasaki *et al.*, 2010; Yao *et al.*, 2006). The same cues can also increase local translation in the axon *via* mammalian target of rapamycin complex 1 (mTORC1) activation of cap-dependent translation. Although mTORC1 generally controls global protein synthesis, it can also selectively trigger the translation of subsets of mRNAs, including eukaryotic translation initiation factor 4E (eIF4E)-sensitive and 5′ terminal oligopyrimidine (TOP) mRNAs (Campbell and Holt, 2003, 2001; Leung *et al.*, 2006; Piper *et al.*, 2006). Of note, modulation of the phosphorylation of eIF2α has been reported to regulate the nascent proteome in the axonal compartment of RGCs *via* differential eIF2B activity (Cagnetta *et al.*, 2019).
2. Post-transcriptional modifications of mRNAs, among which n6-methyladenosine (m^6A) is the most common one, has recently been suggested to regulate axonal translation (Yu et al., 2017). In fact, axonal

GAP-43 mRNA is modified by m^6A and is a substrate of the demethylase enzyme fat mass and obesity-associated protein (FTO). FTO itself can be axonally synthesised and depleting this enzyme in axons increases m^6A modification of GAP-43 mRNA, thereby repressing its local translation (Yu et al., 2017).

3. Another mechanism of local translation regulation is the direct binding between the translational machinery and a guidance cue receptor. Thus, cue stimulation can release this machinery and increases translation. In fact, DCC (deleted in colorectal carcinoma), a netrin 1 receptor, directly binds to ribosomal protein L5, a component of the 60S ribosomal subunit. Binding of netrin 1 to DCC activates translational initiation and subsequently releases the ribosome–mRNA complex from DCC, thereby allowing more ribosomes to form polysomes in the vicinity of receptor activation (Tcherkezian *et al.*, 2010).
4. MicroRNAs (miRNA) also play a fundamental role in the spatiotemporal regulation of local translation (Davis *et al.*, 2015). As will be described in more detail in the next section and in chapter 5, axons contain a huge diversity of miRNAs that also differ among several neuronal populations (Wang and Bao, 2017). miRNAs are known to repress translation by binding to 3'UTRs, but inhibition or activation of specific miRNAs by extracellular signals can also trigger a selective stimulation or repression of subsets of mRNAs in axons. Several studies (Bellon *et al.*, 2017; Dajas-Bailador *et al.*, 2012; Sasaki *et al.*, 2014) have shown how regulation of local translation by specific miRNAs can control different biological processes of both central and peripheral neurons. For example, in CNS neurons, miR-9-5p was shown to locally control axon development by targeting MAP1B (Dajas-Bailador *et al.*, 2012) and miR-338 controls axonal synthesis of two functionally linked

mRNAs for the nuclear- encoded mitochondrial proteins COXIV and ATP5G1 (Aschrafi *et al.*, 2012, 2008). This process not only can regulate the global axonal proteome, but also respond to specific needs in restricted subdomains as was recently reported for miR-182 in response to Slit2 in RGC growth cones (Bellon *et al.*, 2017). In a recent and remarkable study, it has been demonstrated that precursors miRNAs are actively transported to distal axons *via* late endosomes/lysosomes and that upon exposure to Sema3A, these pre-miRNAs are converted into mature miRNAs *in vivo* (Corradi et al., 2020). Moreover, one of this microRNAs, miR-181a can regulate growth cone steering *via* the targeting of locally translated βIII-tubulin (Corradi et al., 2020).

It is clear the axonal transcriptome is complex and highly regulated to serve different roles at different stages of neuronal development. Understanding the function and the role of miRNAs in the regulation of local translation in developing axons is the focus of the present thesis.

1.5. MicroRNAs: function and biogenesis

Cells have at their disposal a plethora of mechanisms capable of regulating protein expression. Hypothetically, any step of gene expression can be modulated, ranging from transcriptional initiation, to RNA processing and to the post-translational modification of a protein. Among those mechanisms, microRNAs have emerged in the last decade as dominant players as suppressors of unwanted transcripts.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with a length of approximately 22 nucleotides, which act as post-transcriptional regulators of gene expression. First found in *Caenorhabditis elegans*, miRNAs are

endogenously expressed in almost all eukaryotes, except for the model organisms *Saccharomyces cerevisiae,* and are transcribed mainly by RNA polymerase II. In fact, the majority of miRNA sequences are normally located within introns of non-coding or coding transcripts, but some miRNAs are encoded by exonic regions (Lee *et al.*, 2002). Moreover, miRNA genes can produce primary transcripts containing one individual miRNA but the vast majority of miRNA genes are clustered in introns of protein-coding genes and only one large primary transcript is synthesised together with the host mRNA (Bartel, 2004).

The process of miRNA biogenesis in animals (canonical pathway, **Figure 6**) can be briefly simplified into three fundamental steps. First, double-stranded primary miRNA (pri-miRNA) short hairpin structures are transcribed by RNA polymerase II (Kim, 2005). Secondly, a RNase III enzyme, Drosha, and its partner *D*i*G*eorge syndrome *c*ritical *r*egion 8 (DGCR8; also known as Pasha in *Drosophila*) help to defines one end of the pri-miRNA duplex and cleave double-stranded RNA (dsRNA) transcripts into approximately 70 nucleotides stem loops called precursor mRNAs (pre-miRNAs) (Lee *et al.*, 2003).

Some pre-miRNAs are produced from very short introns (mirtrons) as a result of slicing and debranching, thereby bypassing the Drosha– DGCR8 step (non-canonical pathway, **Figure 6**). In either case, these pre-miRNAs are then exported to the cytoplasm by Exportin-5 (XPO5) (Yi *et al.*, 2003) where the RNase III Dicer enzyme along with TAR RNA-binding protein 2 (TRBP) cleaves off the loop of the hairpin and generates a short-lived, 21 nucleotides long double stranded miR-3p/miR-5p duplex (Li and Rana, 2014). In subsequent steps, the miRNA duplex is unwound and one strand give rise to the mature miRNA and it is incorporated into a miRNA-protein complex referred to as RISC (miRNA-induced silencing complex) which is a complex of Argonaute (AGO) and other proteins (Krol *et al.*, 2010) whilst the other

strand is released and degraded. After that, the miRISC complex scans the transcriptome for complementary mRNA sequences, and the miRNA then associates with a target mRNA by imperfect base-pairing, on the most part, to its 3′UTR mediating post-transcriptional repression or decay of specific mRNA targets [**Figure 6**] (Pasquinelli, 2012).

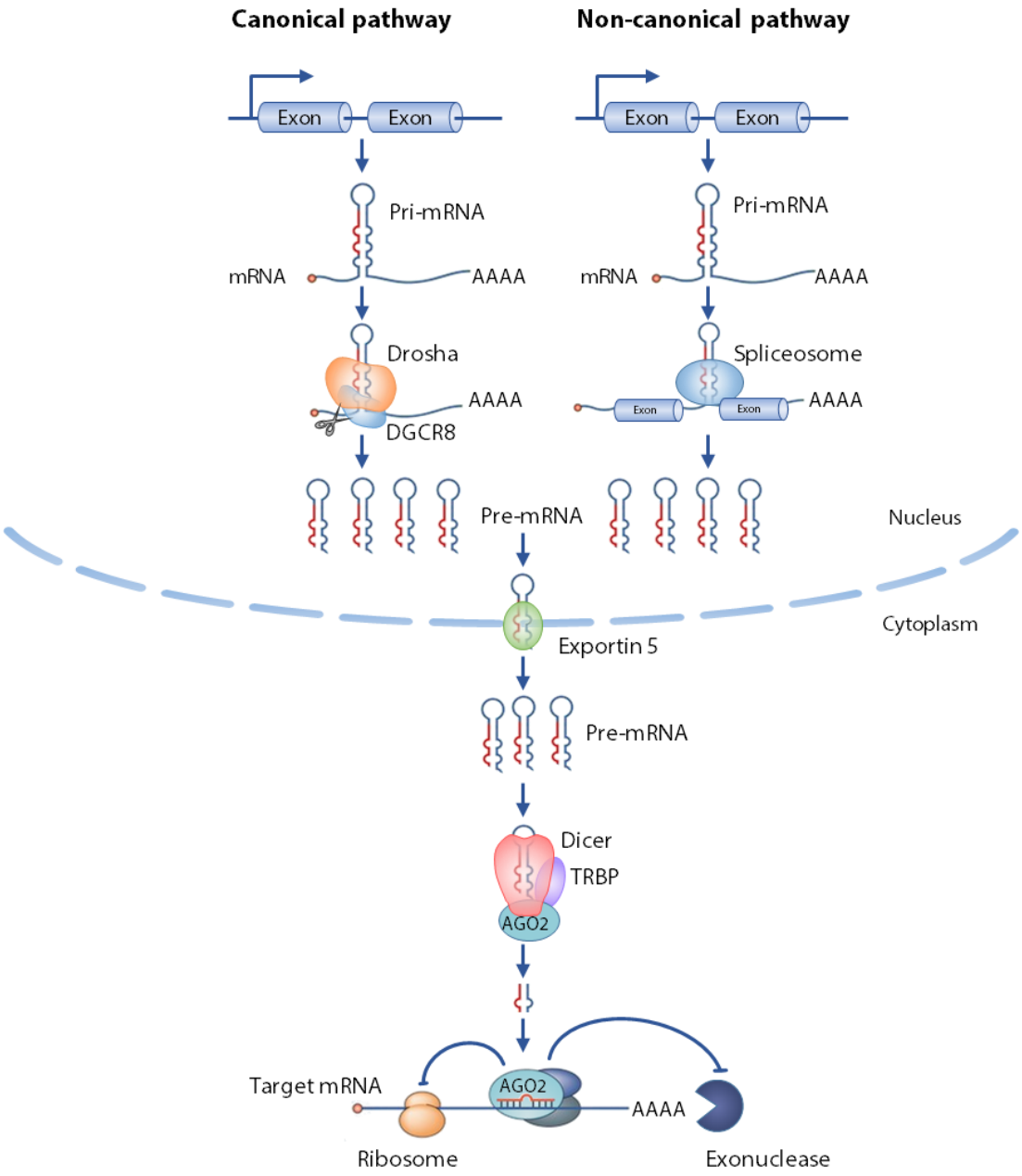

Figure 6: Canonical and non-canonical miRNA biogenesis pathways. (Adapted from Li *et al.*, 2014). In the canonical pathway, RNA polymerase II (not shown) generates pri-miRNA transcripts containing hairpins and the complex composed of DGCR8 and Drosha cleaves (red arrowheads) the pri-miRNA at the stem of the hairpin, releasing a pre-miRNA. The non-canonical pathway differs at this step, as pre-miRNAs are generated by the mRNA splicing machinery. In both pathways, Exportin 5 binds pre-miRNAs and mediates their transport to the cytoplasm, where they are further cleaved by the Dicer-TRBP complex and loaded into AGO2-containing RISCs to regulate protein translation.

The degree of complementarity between the miRNA and the target RNA influences how a miRISC acts on the target RNA. Target RNAs that have perfect or nearly perfect complementary sequence to a miRNA are cleaved in an RNAi-like manner. In this case the miRNA functions exactly like a short interfering RNA (siRNA) and guide the miRISC complex to the target for sequence-specific cleavage. Then Ago2, the endoribonuclease in mammals, cleaves the target mRNA. Perfect complementary target sites are frequently found in plants but in animals, however, perfect complementarity in miRNA target sites is extremely rare (Meister and Tuschl, 2004). In the majority of cases, miRNAs imperfectly base-pair with sequences in the 3′-UTR of target mRNAs, and regulate translation by either suppressing protein synthesis or promoting mRNA deadenylation and decay (Bartel, 2004). This means that a single miRNA has the potential to regulate the expression of hundreds of genes. Indeed, mRNA recognition is determined through nucleotides 2–8 of the 5′ end or "*seed*" region of miRNAs and the presence of GU pairs, mismatches and bulges in the seed region affects repression. However, an A residue across position 1 of the miRNA, and an A or U across position 9, improve the site efficiency, although they do not need to base pair with the miRNA nucleotides (Filipowicz *et al.*, 2008). Apart from these key sites, the rest of the miRNA sequence contacts the mRNA only partially (Bartel, 2009).

Today, two models have been proposed to show how miRNAs act on translation. One possibility is that miRNAs, since they co-sediment with polyribosomes, could act on the level of translational elongation, because ribosomes can initiate in the presence of miRNAs. The other possibility is that miRNAs function at the level of translational initiation. Indeed, it has been seen that miRNAs interact with the translation initiation machinery and inhibit mRNA circularisation by preventing interaction between the poly(A) tail and the 5'end of the mRNA (Filipowicz *et al.*, 2008).

As mentioned above, a specific miRNA could target multiple genes, that in turn they could have multiple miRNA-binding sites in their 3'UTRs and, therefore, multiple miRNA families potentially control their expression (Bartel, 2009). In addition, considering the ability of miRNAs of being temporarily and spatially differently expressed, it is not surprising that they have established a sophisticated and widespread control of gene regulatory networks. As a proof of that, miRNAs are now considered to regulate the expression of up to 50 % of all genes at the level of mRNA translation and/or stability, showing to be required for several key biological processes, such as cell differentiation and development of several type of tissues (Sayed and Abdellatif, 2011), stem cell self-renewal ability (Gangaraju and Lin, 2009) and cell proliferation in cancer (Jansson and Lund, 2012).

Moreover, research in this field has seen a huge increase in recent years, illustrated by the latest miRBase release (v22), which contains 38589 entries representing hairpin precursor microRNAs from 271 organisms, capable of producing a total of 48860 mature microRNA sequences (Kozomara *et al.*, 2019). Those numbers only indicate the extension that microRNAs could reach as fundamental regulatory players of biological processes.

1.6 MicroRNAs in the nervous system development

The capacity of the nervous system to respond to a plethora of environmental stimuli requires a well-conserved and, at the same time, flexible repertoire of molecular mechanisms. In the last decade, miRNAs have been confirmed to be key gene regulators, functioning by being spatiotemporally expressed (Chen and Qin, 2015), fine-tuning a wide range of biological processes and regulating the expression of at least one-third of all human genes (Lewis *et al.*, 2005).

Additionally, microRNAs are abundant in the brain (Fiore *et al.*, 2011) and not surprisingly, recent works have largely highlighted their diverse functions in the CNS, including neural differentiation, development and synapse formation (Barry, 2014; Fiore and Schratt, 2007; Shinde *et al.*, 2013).

The first evidence of microRNAs' involvement in nervous system development *in vivo* came from an experiment with a Dicer knockout zebrafish model. As described in the previous section, Dicer is an essential component in the microRNAs-producing-machinery and, without it, hairpin precursor miRNAs cannot be processed into their mature counterparts causing severe malformations in the brain and spinal cord (Giraldez *et al.*, 2005). Re-introduction of the miR-430 family could rescue the brain defects observed in these mutants. Similarly, the same Dicer knockout experiments in murine models led to impaired brain development and embryonic death (De Pietri Tonelli *et al.*, 2008; Huang *et al.*, 2010).

Interestingly, evidences from deletion of Dicer in specific postmitotic neurons led to opposing results. In fact, the Dicer knockout in excitatory forebrain neurons (Davis et al., 2008), midbrain dopaminergic neurons (Kim et al., 2007), and Purkinje cells produced apoptosis (Schaefer et al., 2007). However, Dicer loss of function in striatal dopaminoceptive neurons and olfactory neurons did not recapitulate the same phenotype (Cuellar et al., 2008). It is worth noting that, even though dopaminoceptive neurons survived over the life of the animal, their biological function was particularly affected, and the animals showed several phenotypes, including ataxia, reduced brain size, and smaller neurons (Cuellar et al., 2008).

Moreover, early absence of Dicer in the cells forming retina and optic chiasm produced severe axon pathfinding defects of RGCs at the midline (Pinter and Hindges, 2010). Using a conditional deletion approach in mice, Pinter and colleagues found a drastic increase of ipsilateral projections, RGC axons

extending outside the optic chiasm, the formation of a secondary optic tract and a substantial number of RGC axons projecting aberrantly into the contralateral eye. In addition, the mutant mice display a microphthalmia phenotype (Pinter and Hindges, 2010).

It is known that Dicer also processes other small non-coding RNAs involved in gene silencing, such as siRNAs, rising questions regarding the actual specificity of the phenotypes obtained by Dicer knockout animals. However, deletion of DGCR8, a more specific component of the microprocessor complex of miRNAs, resulted in diminished cognitive performance and deficit in dendritic arborisation in a mouse model of schizophrenia (Stark *et al.*, 2008). Deletion of other key players in the biogenesis of miRNA such as *Ago2* and *Dicer1* also produced embryonic lethality (Alisch et al., 2007; Morita et al., 2007).

As much as these studies on the knockdown of miRNA biogenesis machinery components in the brain gave us a valuable hint on the role of microRNAs in the nervous system development, they do not shed light on the function and impact of specific miRNAs. The silencing of single miRNA does not have as drastic an effect as knocking down all the miRNAs in the organism (Park et al., 2010). This may be due to the redundancy of miRNA:mRNA interactions the lack of one miRNA would be rescued by others. In the future, the use of conditional mice lacking single miRNAs in specific neuronal lineages could thus help to resolve this issue. However, even though *in vitro* models have provided much insight into the role of miRNAs in neural development, *in vivo* models that examine miRNA function at the organismic level are still lacking. More recent results from miRNA KO models are highly reassuring and indicate that the loss of specific miRNAs can have rather strong consequences for the development of neural circuits and animal behaviour (Amin et al., 2015; Feinberg et al., 2013).

Applying CRISPR-Cas technology to analyse miRNA function in the brain will drastically contribute and accelerate efforts to investigate the physiological function of specific miRNA-target interactions.

1.6.1 MicroRNAs in neurogenesis

Among the upregulated miRNAs upon NSC differentiation, miR-9, miR-124 and let-7 are the most enriched in the brain (Krichevsky *et al.*, 2003; Smirnova *et al.*, 2005) and so the most investigated miRNAs in the context of neurogenesis.

The let-7 family of miRNAs is among the first microRNA to be identified and one of the most conserved throughout the animal Phyla and studied especially as suppressor of cancer cells proliferation (Roush and Slack, 2008). In NSCs let-7 maturation is tightly controlled given that the RNA-binding protein LIN28 regulates its biogenesis by inhibiting let-7 maturation at both the pri-miRNA (Newman *et al.*, 2008) and pre-miRNA (Rybak *et al.*, 2008) processing steps. In fact, let-7 expression increases during neuron differentiation and its overexpression interferes with proliferation and elicits differentiation of neural stem cells in the mouse brain (Rybak *et al.*, 2008; Wulczyn *et al.*, 2007; Zhao *et al.*, 2009a). Another confirmation of the involvement of let-7 family in maintaining a balance between the proliferation of neuronal progenitor and neurogenesis came from a study in which Tripartite motif-containing protein 32 (TRIM32) (Schwamborn *et al.*, 2009) and more recently the transcription factor SRY (sex-determining region)-box 2 (SOX2) (Cimadamore *et al.*, 2013) influence let-7 levels to maintain cells in a proliferative state.

miR-9 is another well-conserved miRNA that has been shown to be a major player in the regulation of neurogenesis. Gain- and loss-of-function

experiments showed that miR-9, *via* the targeting of different components of the fibroblast growth factor (FGF) signalling pathway, promotes progression of neurogenesis and diminishes the midbrain-hindbrain boundary (MHB) progenitor pool (Leucht *et al.*, 2008). In mouse neural stem cells instead, overexpression of miR-9 leads to premature neuronal differentiation through the inhibition of the orphan nuclear receptor tailless (TLX), which is an important regulator of neural stem cell renewal (Zhao *et al.*, 2009b). Interestingly, TLX negatively regulates miR-9 expression levels, suggesting a sophisticated feedback mechanism that precisely regulates the switch between neural stem cell proliferation and differentiation (Zhao *et al.*, 2009b).

In combination with TLX, miR-9 also negatively regulates multiple transcription factors linked to neuronal differentiation processes (Shibata *et al.*, 2011). In fact, mice embryos lacking both miR-9-2 and miR-9-3 exhibited several defects in the telencephalic structures related to enhanced proliferation of neural progenitors and development abnormalities, including the suppression of basal ganglia development (Shibata *et al.*, 2011). miR-9 in combination with miR-124 can also convert human fibroblasts into physiologically functional neurons (Yoo *et al.*, 2011). This pro-neurogenic function of miR-9 (and miR-124) is achieved through regulation of the actin-related protein Brg/Brm associated factor 53a (BAF53a), components of the ATP-dependent chromatin-remodelling complex BAF (also called mammalian SWItch/Sucrose Non-Fermentable complex, SWI/SNF). This complex has the ability to switch different subunits, allowing the cell to regulate the expression of specific genes at different stages of development and specifically, BAF53a subunit promotes neural progenitor proliferation. Upon commitment to the neuronal cell fate, the level of both miR-124 and miR-9 increases, which in turn downregulates *BAF53a* gene in postmitotic neurons. This suggests that the neuron-specific transcriptional signature observed upon miR-9/miR-124

expression is a result of an extensive epigenetic alteration (Yoo *et al.*, 2011). To further complicate things, miR-9 is negatively regulated my another microRNA, miR-107, *via* the targeting of Dicer in the zebrafish MHB (Ristori *et al.*, 2015). In the same study, *in situ* hybridisation has also revealed that the localisation of miR-107 and Dicer is mutually exclusive along the hindbrain VZ, thus determining the border between the progenitor pool and differentiated neurons (Ristori *et al.*, 2015).

In the PNS of *Drosophila*, each external sensory organ develops from the division of a single sensory organ precursor (SOP) cell. In this model, in contrast to the mouse, miR-9a acts as anti-neurogenic factor and inhibits neuronal fate in non-SOP cells, including those that are adjacent to SOPs within pro-neural clusters *via* the targeting of the proneuronal zinc-finger transcription factor Senseless (SENS). The overexpression of miR-9a resulted in severe loss of SOPs, whereas depletion of miR-9a led to production of extra SOPs (Li *et al.*, 2006). This suggests that miR-9a regulates the formation of a precise number of neuronal precursor cells during neural development.

miR-124 has a key role in neurogenesis, too. In addition to the above described role of this microRNA, miR-124 has been linked to the transition of a non-neuronal to a neuronal-specific pattern of alternative splicing. In particular, miR-124 targets the Polypyrimidine Tract RNA-binding protein (PTBP1) (Makeyev *et al.*, 2007), described as a repressor of nervous system-specific splicing. The reduced expression of PTBP1 coincides with an increased expression of its nervous system-enriched homolog, PTBP2, triggering a neuronal-specific splicing program that ultimately leads to neuronal differentiation (Makeyev *et al.*, 2007). Moreover, knockdown of endogenous miR-124 preserved cells from the SVZ, a neurogenic area in the adult brain, as dividing precursors, whereas ectopic expression of the microRNA led to precocious and increased neuron formation (Cheng *et al.*, 2009).

Ablation of the miR-17-92 cluster in NSCs in a conditional transgenic mouse line significantly reduced both the number of proliferating NSCs and neuroblasts and neuronal differentiation in the dentate gyrus (DG) of the hippocampus (Pan *et al.*, 2019).

1.6.3 MicroRNAs in dendritogenesis and post-synapse

The main function of the nervous system is to store information coming from other neurons and relay the signal to post-synaptic targets. Information between different neurons is transmitted through specialised junctions known as synapses, which consist of a presynaptic part (axon terminal) and a postsynaptic part (dendrite). They are very dynamic structures that can modulate their strength in response to external stimuli, a process better known as synaptic plasticity. Given the compartmentalised nature of neurons and that synapse formation depends on the local synthesis of proteins (Holt *et al.*, 2019), it is not surprising that microRNAs could have a role in this process, too. Indeed, as will be reported for the axonal domain in chapter 5, miRNAs are also enriched in dendrites, as shown by a comparative analysis by laser capture multiplex RT-PCR (Kye *et al.*, 2007; Sambandan *et al.*, 2017), or by the characterisation of miRNAs enriched in the synaptoneurosome (a biochemical fraction highly enriched in synaptic proteins and membranes) across several regions of the mammalian brain (Pichardo-Casas *et al.*, 2012). Moreover, since the distribution of the synaptoneurosomal miRNA population was different in each brain region explored, it has been suggested that a particular miRNA might have a specific role in synaptic plasticity depending on its location in the brain (Pichardo-Casas *et al.*, 2012).

Among the most enriched miRNAs in dendrites, the brain-specific miR-134 was seen to negatively modulate spines size by inhibiting the local synthesis of LIM domain kinase 1 (Limk1), a kinase that promotes actin polymerisation

in spines. Moreover, this effect is activity dependant given that miR-134-mediated repression of Limk1 was rescued upon BDNF application (Schratt *et al.*, 2006).

In addition to miR-134, miR-132 was also found to regulate neuronal morphogenesis in developing neurons by repressing translation of the Ras-homolog gene family (Rho) GTPase-activating protein, p250GAP (Marler *et al.*, 2014; Remenyi *et al.*, 2013; Wayman *et al.*, 2008). In addition, besides its role in neurogenesis and axon development, as will be described in chapter 4, miR-9 has also been reported to be necessary for dendrite development in both mouse brain (Giusti *et al.*, 2014) and in sensory neurons of *Drosophila* (Y. Wang *et al.*, 2016).

Several miRNAs have also been implicated in synaptic transmission and synaptogenesis. Among these, miR-137 is the most studied and of particular interest, since a single nucleotide polymorphism (SNP) located in the *MIR137* gene was one of the most significant SNP associated with schizophrenia (Consortium, 2011). Using neuronal-like SH-SY5Y cells, it has been reported that inhibition of miR-137 led to up-regulation of a subset of genes involved in synaptogenesis and neuronal transmission (Strazisar *et al.*, 2015). In line with this, miR-137 gain of function downregulates three presynaptic target genes, complexin-1 (*Cplx1*), N-ethylmaleimide-sensitive factor (*Nsf*) and synaptotagmin-1 (*Syt1*), causing impairment in synaptic vesicle trafficking and alterations in synaptic plasticity (Siegert *et al.*, 2015).

In an elegant work by Lippi *et al.* (2016), miR-101 was shown to coordinate pre- and post-synaptic functions during neural circuit development. Specifically, transient inhibition of miR-101 activity in post-natal mice induces a switch from excitatory to inhibitory synaptic transmission *via* the targeting of the sodium-potassium-chloride cotransporter 1 (NKCC1) (Lippi *et al.*, 2016).

Specific roles of microRNAs in the axon will be described in the introductory section of chapter 5.

1.7 Aims of this thesis

Local translation of mRNAs in the axon provides precise regulation of protein expression and has a pivotal role in axon development, homeostasis and degeneration (Cioni *et al.*, 2018). In this scenario, the present thesis aims to expand our current knowledge of the mechanisms controlling axon specification, elongation and degeneration.

Along this line, I focus on understanding how microRNAs, a class of small non-coding RNAs with the ability to regulate gene networks, can modulate the molecular signalling programs underlying these cellular processes.

Specifically, Chapter 3 discusses how axonal local protein translation contributes in supplying the needs of the axon and maintaining its homeostasis. Moreover, this chapter identifies four microRNAs as potential candidate regulators of axon degeneration pathways.

The role of a single microRNA, miR-26a, in modulating two distinct but also sequentially related developmental processes, axon specification and growth *via* the targeting of GSK3β, will be addressed in Chapter 4.

I then test whether the same microRNA is also controlling axon outgrowth locally in the axon of cortical neurons and I elucidate a novel molecular mechanism in which inhibition of miR-26a leads to a remarkable process of long-distance signalling, where both local axon translation of GSK3β and its transport to the soma are required for the regulation of axon development (Chapter 5).

CHAPTER 2:

Materials and Methods

The materials and methods used in this thesis are described below. The numbers in brackets in the headings denote the chapter of this thesis in which the method was used.

2.1 Animals

(Chapters 3, 4 and 5)

Mice (C57/BL6) were housed, bred and treated in compliance with the ethics and animal welfare in place in the University of Nottingham, in accordance to the *Animal* (*Scientific Procedures*) *Act 1986.*

2.2 Mouse Dorsal root ganglia (DRG) cultures

(Chapter 3)

2.2.1 Cultures of Dorsal root ganglia (DRG) explants

C57/BL6 (referred to as wild-type) mouse DRGs explants were dissected from E16.5 mouse embryos. Cleaned explants were placed in the centre of 3.5 cm tissue culture dishes pre-coated with poly-L-lysine (20 μg/ml for 1–2 h; Sigma) and laminin (20 μg/ml for 1–2 h; Sigma). Explants were cultured in Dulbecco's Modified Eagle's Medium (DMEM D6546, Sigma) with 2 mM glutamine, 1% penicillin/streptomycin, 50 ng/ml 2.5 S NGF (all Invitrogen), 50 ng/ml GDNF (Sigma-Aldrich) and 2% B27 (Gibco). 4 μM aphidicolin (Sigma-Aldrich) (hereafter referred to as supplemented DMEM) was used to reduce proliferation and viability of small numbers of non-neuronal cells. Culture media was replenished every 3 days. Axons were allowed to extend for 7 days before performing the experiments.

2.2.2 Cultures of dissociated DRGs in compartmentalised microfluidic chambers

Mouse DRG explants isolated from wild-type and *Sarm1*$^{-/-}$ mice (the latter kindly provided by Prof. Michael Coleman, University of Cambridge) were incubated in 0.025 % trypsin (Sigma-Aldrich) in PBS (without $CaCl_2$ and $MgCl_2$) for 30 min followed by 0.2 % collagenase type II (Gibco) in PBS for 30 min. Ganglia were then gently triturated using a pipette. DRG dissociated cells were cultured for 5-7 d in microfluidic devices with 150 μm long microgrooves between opposing channels (Xona Microfluidics, SND150) [**Figure 7a**] or in three channel devices with two 500-μm microgroove channels and a 500-μm central chamber (Xona Microfluidics, TCND500) [**Figure 7b**]. Both type of chambers allows the fluidic isolation and functional compartmentalisation of the axon and somal compartments. The devices were prepared as described previously (Garcez *et al.*, 2016). Briefly, ethanol sterile devices were mounted onto 35mm culture dishes (Nunc, Thermo Fisher Scientific) coated with poly-L-lysine (100 μg/ml for 1–2 h) and all the channels were equilibrated for 1 h with laminin (20 μg/ml for 1–2 h; Sigma) and supplemented DMEM. Following collection of excess media from the devices' reservoirs, dissociated cells were added onto to the designated somal channel at a seeding density of $5x10^6$ cells/ml and incubated for 4 h (37°C, 5% CO_2) to allow for cell attachment. The devices' reservoirs were then topped up with supplemented DMEM and incubated at 37°C, 5% CO_2. Axons were allowed to extend and cross the microgrooves to the axonal channels. Functional experiments were performed after 5-6 days *in vitro*.

To suppress protein synthesis, emetine (Sigma-Aldrich) at 10 μM (Gilley and Coleman, 2010; Milde et al., 2013) was added to the axon side of the microfluidic device at the beginning of the experiment.

A difference of volume of 100 µl was maintained at all times between the soma and axonal channels in order to maintain fluidic isolation. The opposite experimental setup was performed for the soma channel to control for non-local effects. Live imaging of the axons in the axonal channel was performed at different time points (as indicated in the figures and/or figure legends) after addition of the drug on Axiovert 200M microscope (Zeiss) under a 10x phase contrast lens.

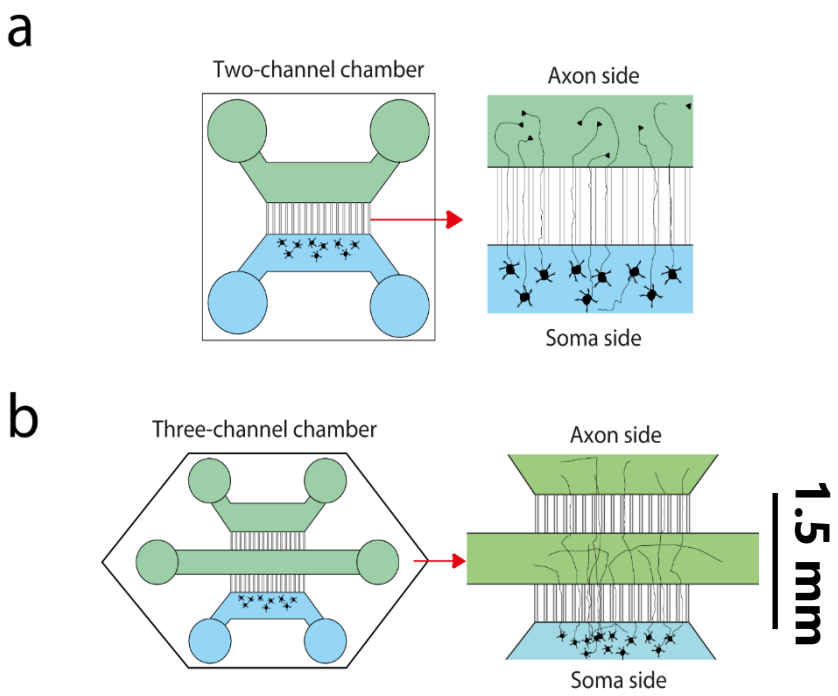

Figure 7: Schematic representation of microfluidic devices. (**a**) Two-channel device. In these devices, neurons are plated into one of the lateral compartments (soma side) and extend their axons through 150 µm long microgrooves into the opposite compartment (axon side). (**b**) Three-channel device. DRG neurons are plated into the bottom compartment (somal side) and extend their axons into the top compartment (axon side) through two barriers of 500 µm long microgrooves and a 500 µm thick central chamber. In both cases, microgroove channels ensure total fluidic isolation of the axonal and somal compartments.

2.3 Axon degeneration assay

(Chapter 3)

2.3.1 DRG explants axotomy experiments

Wild-type DRG axons were cut around the cell bodies using a disposable scalpel under a dissection microscope [**Figure 8**]. Emetine (Sigma-Aldrich) at 10 μM was administered immediately after axotomy. The time of pre-incubation and the concentrations used for every experiment are indicated in the figures and/or figure legends.

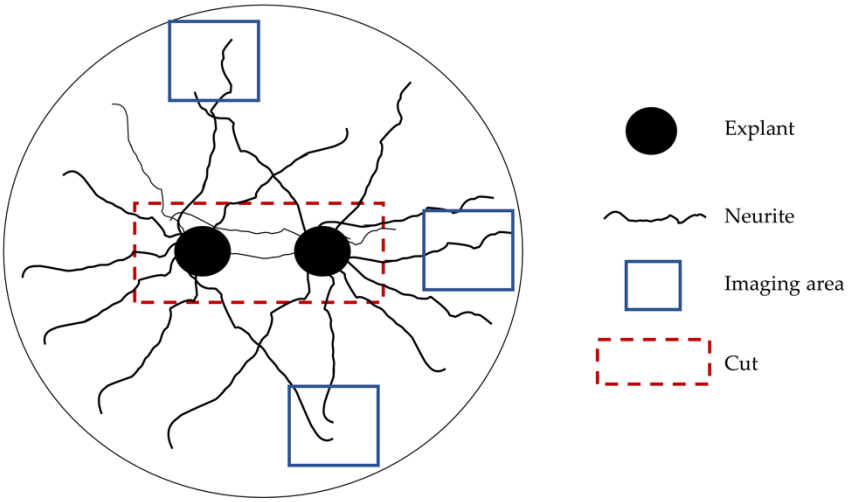

Figure 8: Schematic representation of an axotomy experiment.

2.3.2 Acquisition of phase contrast/bright field images and quantification of axon degeneration

Phase contrast/bright field images were acquired under a 10x objective of a widefield fluorescence microscope (Axiovert 200M, Zeiss), coupled to a CCD camera (Photometrics CoolSnap MYO) and Micro-Manager software 1.4.21 (Stuurman et al., 2010). To measure the axon degeneration index, an ImageJ

plugin was used (Schneider *et al.*, 2012) which calculates the ratio of fragmented axonal area over total axonal area after binarisation of the pictures and subtraction of the background (**Figure 9** and Sasaki *et al.*, 2009). Axons that detached from the dish were considered completely degenerated and scored as 1.

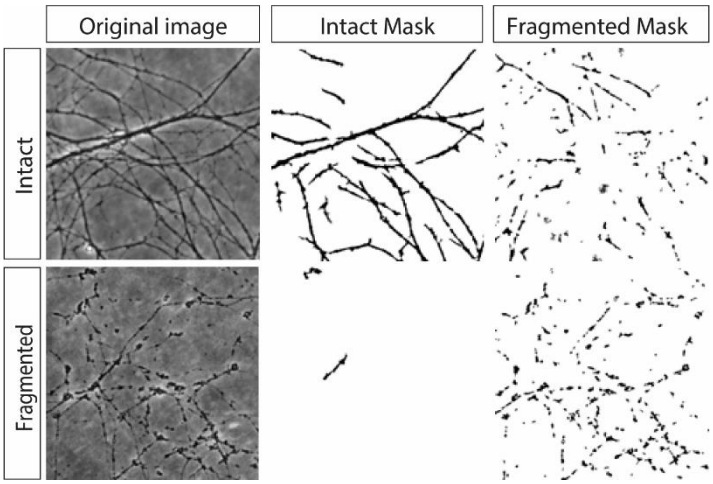

Figure 9: Quantitative axon degeneration assay. Representative pictures of original images, intact and fragmented masks images from intact and degenerated DRG axons.

2.4 Primary mouse cortical cultures

(Chapter 4 and 5)

C57/BL6 mouse embryos at E16.5 stage of development were culled and their brains removed. The brain cortices were dissected and the meninges separated under a dissection microscope. The tissue was further incubated in Hanks Balanced Salt Solution (HBSS, Ca^{2+} and Mg^{2+}-free; Gibco) with 1mg/ml trypsin and 5 mg/ml DNase I (Sigma-Aldrich) at 37°C for 30'. Following the addition of 0.05% (v/v) soybean trypsin inhibitor (Sigma-Aldrich), the tissue was mechanically dissociated in Neurobasal media (Invitrogen) supplemented with 1X GlutaMax and 2% B-27 (Gibco).

2.4.1 Primary cortical cultures

Following mechanic trituration of the digested tissue, dissociated neurons were resuspended in supplemented Neurobasal media to a final cell density of $10x10^6$ cells/mL. For functional assays and RNA extraction, neurons were plated at a final seeding density of $1.75x10^5$ cells/cm^2 in 6-well plates (Corning) with or without 22x22mm glass coverslips (Menzel Glaser) and incubated at 37°C, 5% CO_2. Glass coverslips or 6-well plates were previously coated with 50 µg/ml poly-L-ornithine (PLO; Sigma-Aldrich) and washed twice with sterile water. For experiments that required over 7 days in culture, media was replenished with ¼ of its volume every 2-3 days.

2.4.2 Primary cortical neurons in compartmentalised microfluidic chambers

Primary cortical neurons were cultured for 5 d in two-channel-microfluidic devices (SND150; Xona Microfluidics) [**Figure 7a**]. The devices were prepared as described above and mounted onto PLO-coated 35mm culture dishes (Nunc, Thermo Fisher Scientific) and both channels equilibrated for 1 h with supplemented Neurobasal media. Following collection of excess media from the devices' reservoirs, cortical neurons were added onto to the designated somal channel at a seeding density of $4x10^6$ cells/ml and incubated for 30' (37°C, 5% CO_2) to allow for cell attachment. The devices' reservoirs were then topped up with supplemented Neurobasal media and incubated at 37°C, 5% CO_2. Axons were allowed to extend and cross the microgrooves to the axonal channel. Functional experiments were performed after 5-6 days *in vitro.*

2.5 MicroRNAs functional analysis tools

(Chapter 4 and 5)

In order to investigate microRNAs functionals mechanism, and specifically miR-26a with regards to axon development, I used commercially available miRNA mimics and inhibitors (miRCURY LNA miRNA Mimics and Inhibitors, Qiagen), a tool widely used in the field and in our laboratory (Dajas-Bailador *et al.*, 2012; Garcez *et al.*, 2016). These miRNA mimics and inhibitors are oligonucleotides that contain locked nucleic acid monomers, in which the ribose moiety is modified with an extra bridge connecting the 2' oxygen and 4' carbon that "locks" the ribose in a rigid conformation, improving their binding specificity and making them resistant to nuclease degradation (Kaur *et al.*, 2006).

miRNA mimics

Mimics are designed to "mimic" the natural activity of endogenous miRNAs They are characterised by three RNA-LNA strands: one strand is an unmodified RNA strand (guide strand) bearing a sequence identical to the endogenous mature miRNA of interested, whilst the passenger strand is divided into two locked nucleic acids (LNA)-enhanced RNA strands. The segmented nature of the passenger strand ensures that only the miRNA strand is loaded into the RNA-induced silencing complex (RISC) with no resulting miRNA activity from the two complementary passenger strands (Bramsen *et al.*, 2007).

miRNA inhibitors

miRNA silencing was instead achieved using miRNA inhibitors, which are antisense oligonucleotides bearing a complementary sequence of a specific miRNA. When introduced into the cells, miRNA inhibitors strongly bind to

and sequester the endogenous miRNA of interest, leading to the blocking of normal miRNA-mediated activity (Naguibneva *et al.*, 2006).

miRNA power-inhibitors

In this work (chapter 5) I also used cell-permeable miRNA inhibitors that incorporate phosphorothioate modifications to their LNA backbone (miRCURY LNA miRNA Power inhibitors, Qiagen) which markedly improves their stability in culture and a transfection-free incorporation into the cell.

2.6 DNA constructs and oligos

(Chapter 4)

For the pcDNA-GSK3β and pcDNA-PTEN constructs, both GSK3β and PTEN cDNAs were PCR-amplified from a replication construct (pMD18-TSimple (Sino Biological) and pCMV-Sport6 respectively (Source Biosciences) with primers containing the appropriate restriction sites: GSK3β fwd – 5′ CTC CAT TGG CTA GCT ATG TCG GGG CGA CCG AGA ACC TCC TT 3′; GSK3β rev – 5′ GCG GTC TCT AGA TCA GGT GGA GTT GGA AGC TGA TGC AGA AGC 3′; PTEN fwd - 5′ CTC CAT TGG GAT CCA TGA CAG CCA TCA AAG AG 3′; PTEN rev – 5′ GCG GTC TCT AGA TCA GAC TTT TGT AAT TTG TGA ATG 3′. The amplicons were cloned into pcDNA3.1/Zeo(+) vector (a kind gift from Dr Simon Dawson, University of Nottingham), using Nhe/XbaI (GSK3β) and BamHI/XbaI (PTEN) restriction sites. The miRCURY LNA miRNA Inhibitor Control (sequence: TAACACGTCTATACGCCCA; catalogue number YI00199006) and the negative control miRCURY LNA miRNA mimic control (sequence: UCACCGGGUGUAAAUCAGCUUG; catalogue number YM00479902) were from Qiagen, as well as the miR-26a-5p miRCURY LNA miRNA Inhibitor (sequence: GCCTATCCTGGATTACTTGA; catalogue

number YI04102930) and the miR-26a-5p miRCURY LNA miRNA Mimic (sequence: UUCAAGUAAUCCAGGAUAGGCU; catalogue number YM00471417). The miRCURY LNA miRNA Power Inhibitor Control (catalogue number YI00199006-DDA) and the miR-26a-5p miRCURY LNA miRNA Power Inhibitor (catalogue number YI04102930-DDA) were also obtained from Qiagen.

2.7 Neuronal incorporation of microRNAs

(Chapter 4)

Neuronal transfections with miRCURY LNA miRNA Mimics and Inhibitors were performed 4 h, 24 h or 5 d after plating using 5 μL/well of Lipofectamine 2000 reagent and 250 μl/well of Opti-MEM reduced serum media (Thermo Fisher Scientific), in accordance to manufacturer instructions. miRCURY LNA microRNA inhibitor [50 nM], inhibitor control [50 nM], mimic [20 nM] and mimic control [20 nM] of miR-26a (all Qiagen) were used for transfections. In all cases, 1 μg pmaxFP-Green-C (Lonza) was co-transfected for visualisation of transfected neurons. In protein overexpression studies, neurons were transfected with 1 μg pmaxGFP (hereafter referred to as GFP) and either 1 μg of pcDNA3.1/Zeo (+) or 1 μg of pcDNA-GSK-3β.

Other concentrations for both miR-26a inhibitor and mimic were also investigated alongside the concentrations stated above, such as microRNA inhibitor [25 nM, 75 nM] and mimic [10 nM, 50 nM]. Among these, both miR-26a inhibitor [75 nM] and miR-26a mimic [50 nM] had a cytotoxic effect, whereas the lowest concentrations tested for both miR-26a inhibitor and mimic resulted in a lower effect compared to the concentrations chosen for all the experiments. [**Figure 47**].

To rescue the effects of miR-26a inhibition, cortical neurons were co-transfected with 1 μg GFP and LNA inhibitor control or LNA miR-26a inhibitor 50 nM, whilst the GSK-3 inhibitor SB415286 (Tocris) was used at a concentration of 1 μM (Dajas-Bailador et al., 2014) and added to the culture 24 h after plating. The same inhibitor was also tested at a concentration of 10 μM (Jiang et al., 2005) but since its effect with regards to axon length was the same as that of lower concentration [**Figure 48**], for consistency with previously published work in our lab (Dajas-Bailador et al., 2014) the lowest concentration was chosen to carry out all the experiments.

In all the experiments, cortical neurons were fixed in 4% paraformaldehyde 72 h after transfection and washed in PBS before direct visualisation and/or immunostaining. Microscope imaging was done using a widefield fluorescence microscope (Axiovert 200M, Zeiss), coupled to a CCD camera (Photometrics CoolSnap MYO) and Micro-Manager software (Stuurman et al., 2010).

2.8 RNA extraction

(Chapters 4 and 5)

In standard cultures, cells were seeded at a density of 1.75x105/cm2 in 6-well plates and cultured as described above. Total RNA was isolated from cortical cultures at 4h, 24h after plating and then at day *in vitro* (DIV) 5 and 9 by the phenol-chloroform extraction method using TRIzol Reagent (Invitrogen) for extraction of total RNA, in accordance to manufacturer's instructions. Briefly, culture media was removed, the cells washed with ice cold PBS (ThermoFisher), and then scraped and collected in 250 μl of TRIzol® Reagent (Fisher Scientific) per well into LoBind tubes (Eppendorf), homogenised and mixed with 1/5 volume of chloroform (ThermoFisher). Following centrifugation at 12000x g/ 4°C for 15 min, the aqueous phase was mixed with

equal volume of isopropanol (ThermoFisher) and incubated at -20°C overnight. Total RNA was precipitated at 12000x g/ 4°C for 30 min and the pellet further washed twice in 75 % (v/v) ethanol (12000x g/ 4°C, for 30 min; ThermoFisher). RNA pellet was left to dry until gel-like appearance and resuspended in Hyclone nuclease-free water (GE Healthcare).

Axonal RNA from microfluidic cultures was obtained following the procedure described in (Garcez *et al.*, 2016) with few modifications. Microfluidic cortical cultures were grown for 8 d, when the average dendrite length is ~40% lower than the 150 μm microgrooves and Map2 (Microtubule-associated protein 2) staining shows no contamination by dendrite projections [**Figure 10**]. Device channels were washed twice with PBS and 20 μL of TRIzol was added to each reservoir of the axonal channel and incubated for 2 min at room temperature, while 100 μL PBS was kept in the soma reservoirs to prevent contamination from cell body RNA. Following collection of axonal fractions, the somal fraction was obtained in the same manner. Fractions from 40-50 devices were collected for each independent experiment and total RNA was extracted as described above. All steps were conducted in an RNAse-free environment, using nuclease-free tubes and reagents, filtered pipette tips and, where adequate, on ice to minimise the rapid RNAse-dependant degradation of the samples. All materials, equipment and surfaces were decontaminated with RNAZap solution (Sigma) before commencing the procedure.

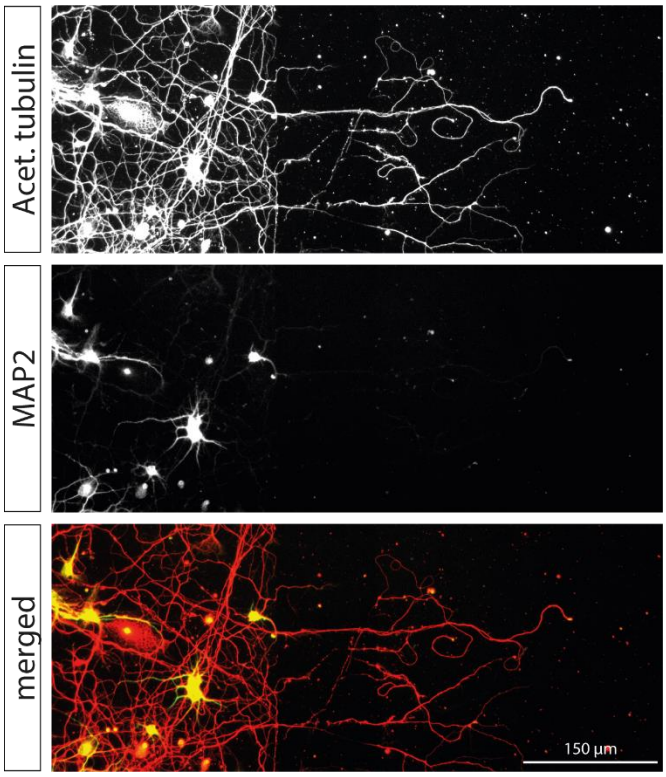

Figure 10: MAP2 staining in microfluidic chamber. Representative images of cortical neurons cultured in microfluidic chambers for 8 days and immunolabelled with acetylated tubulin (Cambray-Deakin and Burgo, 1987) and MAP2, used as axonal and dendritic marker respectively. The panel shows that only axons extend through microgrooves and are able to reach the axonal compartment. Dendrites (MAP2 positive) are restricted to the somal compartment, as they are on average ~60 µm shorter than the 150-µm-long microgrooves. In our study the average dendrite length was 91.53 ± 2.7 µm.

2.9 Quantification by real-time qPCR

(Chapters 4 and 5)

2.9.1 miRNA qPCR

cDNA was synthesised from mature miRNAs using the miRCURY LNA™ Universal cDNA synthesis kit (Qiagen, UK) as per manufacturer's instructions, using 10 ng of total RNA. For each timepoint 5 biological samples were run in duplicate using the following miRCURY LNA™ (Qiagen, UK) primers [**Table 1**].

Table 1: microRNA primers

mmu-miR-26a-5p	5'UUCAAGUAAUCCAGGAUAGGCU
mmu-miR-100-5p	5'AACCCGUAGAUCCGAACUUGUG
mmu-miR-128-3p	5'UCACAGUGAACCGGUCUCUUU
mmu-miR-134-5p	5'UGUGACUGGUUGACCAGAGGGG
mmu-miR-434-3p	5'UUUGAACCAUCACUCGACUCCU
mmu-let-7a-5p	5'UGAGGUAGUAGGUUGUAUAGUU

qPCR was undertaken using the ExiLENT SYBR® Green master mix kit (Qiagen, UK), and the Applied Biosystems Step One Plus thermocycler was used in standard mode with cycling parameters recommended by Qiagen. Data was acquired using Applied Biosystems SDS2.3 programme. Passive reference dye ROX™ (Fisher Scientific - UK Ltd) was included in all reactions. Expression of miR-26a-5p was analysed by relative quantification using the comparative Ct method (2-ΔΔCt). The choice of reference miRNA genes was advised by pilot the RT-qPCR studies previously conducted in the lab and the geometric mean of miR-100-5p, miR-128-3p, miR-134-5p, miR-434-3p and

let7a-5p was used as reference due to their stable expression across developmental stages (Bustin *et al.,* 2009) and miR-26a-5p levels expressed as relative expression to 4 h. miRNAs used for endogenous reference were selected in accordance to previous in-house qPCR studies on the development of cortical neurons. Data are expressed as fold change to 4 h +/- SEM.

2.9.2 mRNA qPCR

cDNA was synthesised from 100ng total RNA using SuperScript IV™ and Oligo(dT)20 primer (Invitrogen) as per manufacturer's instructions. Q-PCR was undertaken using the PowerUp™ SYBR™ Green (Applied Biosystems) using 1.5 μL cDNA per replicate and 400 nM primers. PCR amplification was carried out in the same thermocycling system using the fast mode cycling parameters recommended by Applied Biosystems. For each time point 5 biological samples were run in duplicate using primers for GSK3β (Sino Biological Inc.) and the reference genes GAPDH (F- 5′ CTGCACCACCAACTGCTTAG 3′ and R- 5′ ACAGTCTTCTGGGTGGCAGT 3′), UBE2 (F- 5′ TGCCTGAGATTGCTCGGATCT 3′ and R- 5′ TCGCATACTTCTGAGTCCATTCC 3′) and ROX™ (Fisher Scientific). Expression of GSK3β was analysed by relative quantification using the comparative Ct method (2-ΔΔCt). The geometric mean of GAPDH and UBE2 was used as reference due to their stable expression across developmental stages and GSK3β levels expressed as relative expression to 4 h. Data are expressed as fold change to 4 h +/- SEM.

2.10 Immunofluorescence

(Chapters 4 and 5)

Cortical neurons cultured on coverslips or microfluidic devices were fixed using 4% paraformaldehyde (w/v) (ThermoFisher) for 30', washed with 10 mM Glycine in PBS, permeabilised in PBS/Glycine-Triton (1x PBS, 10 mM glycine, 0.2% Triton X-100; Sigma),blocked with 3% bovine serum albumin in PBS (BSA; Sigma) and further incubated with the following primary antibodies overnight: anti-JIP1 (1:100, Santa Cruz) anti-GSK-3β (1:100, BD Biosciences), anti-acetylated tubulin (1:300, Sigma-Aldrich), anti-MAP2 (1:100; Abcam) and anti-βIII tubulin (1:100; Abcam). Following PBS-Triton 0.1% washes, cells were incubated with secondary antibodies (Alexa Fluor 488 and 568; 1:300 Molecular Probes) and mounted with Vectashield Hardset mounting media with Dapi (Vectorlabs).

2.11 Data analysis

(Chapters 4 and 5)

Measurement of axons in primary cortical neurons. For quantification of axon length, an axon was defined as a neurite that was at least 3 times the length of any other neurite and measured from the cell body to the distal extent of the central region of the growth cone using Fiji software [**Figure 11**] (Dajas-Bailador *et al.*, 2008; Schindelin *et al.*, 2012). Data are expressed as percentages of respective controls (~300 axons measured for each condition from 4-6 independent experiments). Data are expressed as mean +/- SEM.

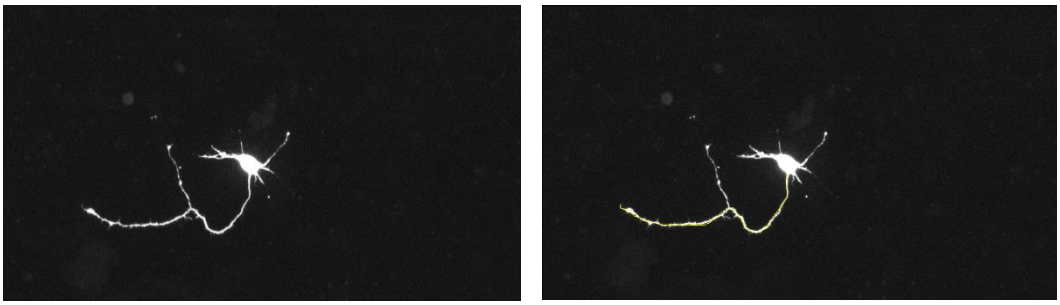

Figure 11: Measurement of axonal projection in ImageJ.

Measurement of average neurites length. Neurite length was assessed by measuring the length from the cell body to the distal tip of all the projections in each GFP-positive cell. Data are expressed as percentages of respective controls (~700 projections measured for each condition from 4 independent experiments). Data are expressed as mean +/- SEM.

Polarity assessment. The aforementioned criteria for the definition of axon was also used to define a neuron as a polarised cell. Neuronal polarisation in culture was then assessed by determining the percentage of polarised cells with respect to the total number GFP-positive cells. The data set of 5 independent experiments was normalised to respective control and expressed as percentage of control (mean ± SEM). Multi polar neurons were identified as neurons bearing more than one axon, defined as a neurite with JIP1-positive tips (Dajas-Bailador *et al.*, 2014, 2008; Deng *et al.*, 2014; Fu and Holzbaur, 2013). The data set of 5 individual experiments was normalised to respective control and expressed as percentage of control (mean ± SEM).

Quantification of fluorescence signal. Neurons stained for GSK3β were imaged at 63x and images were further processed with Fiji software. Cell bodies and growth cones were manually selected and the area, mean grey value and integrated density were measured. In order to correct for background in each image, 3 empty areas were selected around every cell body/growth cone. Total

cell fluorescence (C.F.) per cell was calculated as the measured integrated density corrected for background, according to the formula:

C.F.= Integ.Density – [Area of cell body X Average (mean grey value of background)].

For quantification of endogenous GSK3β in culture, approximately 150 cell bodies and 75 growth cones were measured in each condition from 3 independent experiments. Data was normalised to the average C.F. of the control expressed in percentage as mean ± SEM.

Measurement of axon length in microfluidic cortical cultures. Cortical neurons seeded in microfluidic devices were cultured for 5-6 days to allow axons to extend through the microgrooves into the axonal channel. Cell-permeable Power inhibitor miR-26a or Power inhibitor control at 100 nM was added to the axon side of the microfluidic device at day 5. A difference of volume of ~100 µl was maintained at all times between the soma and axonal channels in order to maintain fluidic isolation. Live imaging of the axons in the axonal channel was performed at different time points (0h, 24h and 48h) after addition of inhibitors on Axiovert 200M microscope (Zeiss) under a 10x phase contrast lens. To rescue the local effects of miR-26a inhibition, the axonal channel was treated with GSK3 inhibitor SB415286 at 1 µM together with the cell-permeable inhibitor of miR-26a-5p or Power inhibitor control at 100 nM. and live imaging of the axons in the axonal channel was performed at 0 h and 24 h after addition of drugs/inhibitors. In all the experiments, the length of the axons was measured in Fiji software by tracing at least ~125 axons in each condition from 4 independent experiments; each axon was traced from the edge of microgrooves to the growth cone of the longest axonal branch [**Figure 12**]. Data for the different timepoints in each chamber was normalised to t 0 and expressed as a percent of respective controls (mean ±SEM).

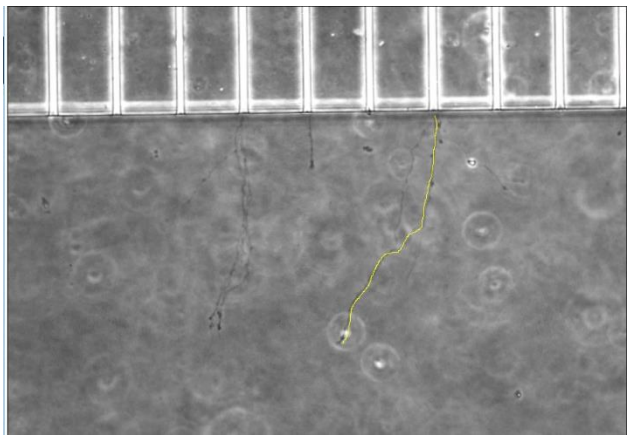

Figure 12: Measurement of axonal projections in microfluidic chambers in ImageJ.

Quantification of fluorescence signal in microfluidic cortical culture and disruption of axonal transport. To impair axonal transport, nocodazole (0.1 μM, Sigma-Aldrich) was added 18 h after the addition of the cell-permeable miR-26a inhibitor. Following 6 h of nocodazole incubation (24 h in total after addition of miRNA inhibitors/controls), devices were removed, and neurons fixed and immunolabelled for GSK3β protein and βIII tubulin. During the protocol optimisation for the axonal transport impairment, I have titrated nocodazole effects to make sure axonal integrity was not affected in the experimental window to be tested , in agreement with previous studies using nocodazole as an inhibitor of retrograde transport (Twelvetrees et al., 2016). Moreover, use of mitotracker as a new experimental control to demonstrate how addition of 100 nM nocodazole dramatically inhibits axonal transport in my cultures (Lucci et al., 2020). For quantification of endogenous GSK-3β levels, ~200 cell bodies and ~200 growth cones were measured in each condition from 4 independent experiments. Data was normalised to the average C.F. of the control expressed in percentage as mean ± SEM.

2.12 Statistical analysis

(Chapters 3, 4 and 5)

In all statistical tests, "n" refers to the number of independent experimental repeats, which varied from 4-7 depending on experimental model (see specific section for details). Data analysis was done using Prism v7.0 (GraphPad Software) and all data groups shown are expressed as mean +/- SEM. The probability distribution of the data set was analysed before further statistical analysis (Shapiro–Wilk test). Statistical evaluation between two groups was performed using unpaired Student's t-test. Analysis of more than 2 groups were carried out using ANOVA with Bonferroni post hoc analysis. Kruskal-Wallis' test followed by a Dunn's multiple comparisons test was used for non-parametric distributions. For all tests, $p<0.05$ was used as threshold for significant difference. For all tests P values are two-tailed. When less than 3 experiments were performed, data are indicated as preliminary in the main text and no statistical analysis test was carried out.

CHAPTER 3:

Local protein translation in axonal survival

3.1 Introduction

As described in Chapter 1, axonally synthesised proteins are important for the spatially and temporally sensitive events that occur during development, including axon growth and guidance (Jung *et al.*, 2012). Developing neurons face a challenge unique to their cellular identity, which is to successfully and accurately meet with their post-synaptic contacts. To do so, both central nervous system and peripheral nervous system neurons locally synthesise proteins in their axons as they knit their way to their targets (Batista and Hengst, 2016). Once the neuron has formed its synapse, constant work is still needed to mature and maintain the synapse both during the rest of development and throughout the life of the organism (Meems *et al.*, 2006). This would suggest that given the complex polarised morphology, a tightly controlled level of protein synthesis must be sustained over the large cytoplasmic volume that compose the long axonal connections.

Despite the evidence available for the local axonal synthesis of multiple proteins, many others appear to be synthesised only in the cell body and rely on axonal transport to reach their site of action in the axon or synapse (Cioni *et al.*, 2018). This constant supply process is extremely demanding, and not surprisingly, any impairment affects axonal function or survival (all reviewed in De Vos, Grierson, Ackerley, & Miller, 2008).

In this chapter, I will address the possibility that local synthesis in the axon of survival factors may contribute to regulate their levels in the axon and influence their long-term viability. In the next sections, I will discuss in more detail what is currently known about local protein translation in axonal survival, regeneration and neurodegenerative diseases and I will introduce NMNAT2 and SARM1 as axonal proteins involved in survival and pro-degenerative processes respectively and their role in Wallerian degeneration.

3.1.1 Local protein synthesis in injury response

As previously mentioned, the axonal mRNA population is really dynamic across the developmental stages and life span. After development, the quantity of rRNA and mRNA in axons decreases (Bassell *et al.*, 1994; Hengst and Jaffrey, 2007), and so does the ability to regrow after axotomy (Gumy *et al.*, 2010). Typically, CNS neurons show decreased regenerative potential as compared with PNS axons, as the latter are partially facilitated by some of the same locally translated proteins that help a developing neuron to find its synaptic target and establish connections (Deglincerti and Jaffrey, 2012; Verma *et al.*, 2005). However, CNS axons are still capable of local synthesis (Shigeoka *et al.*, 2016) and the effects of local translation following an injury are twofold, providing both the materials for axon regrowth and the proteins that act as retrograde signals, reporting on the status of the injury and subsequent recovery (Ben-Yaakov *et al.*, 2012; Costa and Willis, 2018). Local protein translation amplifies and accentuates nervous system-wide signals, facilitating communication between the axon-soma axis. Following axonal injury there is a complex and orchestrated response within the axon designed to generate a signal that allows the neuron to survive and ultimately regenerate (Cioni *et al.*, 2018; Terenzio *et al.*, 2018; Verma *et al.*, 2005; Zheng *et al.*, 2001). The signalling cascade induced by nerve injury has been extensively studied in peripheral axons and some of the locally synthesised proteins and signalling cascades have been uncovered (Gumy *et al.*, 2010; Rishal and Fainzilber, 2010). An initial calcium wave triggers a first round of translation of several sensor mRNAs, forming a signalling complex that is retrogradely trafficked and influences transcription (Rishal and Fainzilber, 2014). Importin β, whose typical function is to facilitate nuclear import of nuclear localisation signal (NLS)-bearing proteins, is locally translated in the injury site and a core component of the injury signalling complex (Hanz *et al.*, 2003). Its upregulation

leads to the formation of a NLS binding complex that associates with the motor protein dynein and travels retrogradely to the cell body (Hanz *et al.*, 2003). Deleting axonal localisation sequence in the importin β1 transcript in mice causes a subcellular loss of both importin β1 mRNA and protein in axons, affects the transcriptional response, and delays functional recovery to nerve injury (Perry *et al.*, 2012), confirming its significance *in vivo*.

The local synthesis of transcription factors, such as signal transducer and activator of transcription 3 (STAT 3), has also been shown as a relevant mechanism in the retrograde communication from the periphery to the cell body, in this case leading to the mounting of an injury response (Ben-Yaakov *et al.*, 2012). Overall, boosting protein synthesis appears to restore the regenerative potential of CNS axons (Park and He, 2008), and importantly, mRNAs and protein synthetic machinery managed to localise to regenerating mature CNS axons when they are provided with a growth supporting substrate (Kalinski *et al.*, 2015).

3.1.2 Local protein synthesis in neuronal survival and homeostasis

Local translation might also contribute to axonal homeostasis and survival by ensuring a constant supply of functional mitochondria. In fact, considering the extensive axonal arborisation that some type of neurons have, such as nigrostriatal dopamine neurons that are capable of forming up to 245,000 synapses (Matsuda *et al.*, 2009), and given that mitochondria are enriched at synapses, the cell body might not be able to produce enough copies of nuclear-encoded mitochondrial proteins at a rate to sustain a constant supply of axonal mitochondria (Court and Coleman, 2012; Schwarz, 2013). Moreover, any mutations that impair either mitochondrial function or transport lead to degeneration (Pease and Segal, 2014; Schwarz, 2013). To reinforce this hypothesis, rat superior cervical ganglia (SCG) axons contain several

mitochondrial mRNAs and suppression of axonal protein synthesis impairs mitochondrial membrane potential (Hillefors *et al.*, 2007). mir-338 is also present in the axon, which is known to target COXIV (Aschrafi *et al.*, 2008 and see Chapter 1). The mimicking of miR-338 downregulates COXIV and reduces both the mitochondrial oxygen consumption and ATP levels (Aschrafi *et al.*, 2008). Local protein translation may also guarantee a local pool of axon survival factors, such as neurotrophins. In fact, *in vitro* application of NGF induces the local synthesis of pro-survival transcription factors such as cAMP-responsive element (CRE)-binding protein (CREB) and its activator myo-inositol monophosphatase 1 (Impa-1) for retrograde transport, while its selective silencing decreased nuclear CREB activation and induced axonal degeneration (Andreassi *et al.*, 2010). NGF application also triggers the axonal synthesis of the dynein regulators Lissencephaly-1 (Lis1) and $p150^{Glued}$ and thus mediates the transport of vesicles that are presumed to contribute to axon survival (Villarin *et al.*, 2016). To reinforce this theory, local synthesis of Lys1 was also shown to be necessary for the retrograde transport of a pro-apoptotic signal upon NGF- deprivation (Villarin *et al.*, 2016). NGF application regulates transcription of the anti-apoptotic gene *bcl-w* with its consequent transport to the axon and local synthesis (Cosker *et al.*, 2013). Loss of axonal *bcl-w* mRNA has recently been linked to neuropathy (Pease-Raissi *et al.*, 2017). Since the use of protein synthesis inhibitors "nullify" the protective effects of neurotrophins, other axonal survival factors might be at play (T. Kim *et al.*, 2009; Pease and Segal, 2014). A good candidate is nicotinamide nucleotide adenylyltransferase 2 (NMNAT2), an essential axon survival factor with a half-life of only few hours (Gilley and Coleman, 2010). Importantly, even if NMNAT2 was transported at the fastest rate by axonal transport (1 μm per second), the protein would take 11.6 days to reach distal axon terminals in large mammals

(1 m away) (Maday *et al.*, 2014). As a result, neurons may require different balances of transported *vs*. locally translated NMNAT2.

The protective effects of NMNAT2 in axon degeneration is contrasted by the destruction program mediated by SARM1, which triggers a soma-independent axon destruction program by counteracting *Nmnat* function (Gerdts *et al.*, 2016) , and it is highly translated *in vivo* during the axon pruning phase of development, but not in adults (Shigeoka *et al.*, 2016). A possible explanation for this switch of the axonal translatome from a degenerative to a survival mode at the end of development is that while the developing axon might need components of axon degeneration pathways for selective branch pruning, adult axons would keep them at low levels to maintain long term connectivity (Shigeoka *et al.*, 2016). The function of SARM1 will be discussed in more detail below.

3.2 SARM1

A genetic screening in *Drosophila melanogaster* revealed another important effector of the Wallerian degeneration pathway, a protein better known as Sterile α and TIR motif–containing protein 1 (SARM1 in mammals, dSARM in *Drosophila*). SARM1 is a Toll-like receptor (TLR) adaptor family member and plays a role in the innate immunity response (Carty et al., 2006; Mink et al., 2001). Its suppression drastically delays degeneration in both PNS and CNS neurons and in both mice and *Drosophila melanogaster* (Osterloh *et al.*, 2012). Moreover, the degree of protection obtained with SARM1/dSARM deletion after injury is similar to that of Wallerian degeneration mutant mouse (WLDs/NMNATs) (Gerdts *et al.*, 2013; Gilley *et al.*, 2017; Osterloh *et al.*, 2012), mechanisms of which will be described in the next section. *Sarm1*$^{-/-}$ axons can also be protected from the toxicity caused by vincristine and NGF withdrawal (Gerdts *et al.*, 2013). Mice lacking SARM1 are healthy and do not show any

sign of abnormality (Gilley et al., 2015), making SARM1 an really promising target for therapy and its mechanism of action is thus the source of intensive studies.

Since both SARM1 and NMNAT2 loss trigger axon degeneration, it would be interesting to determine whether they are both members of a common pathway impinging on Wallerian degeneration, or if they act independently. A relatively recent study demonstrated that SARM1 could be acting downstream of NMNAT2 loss or in a parallel branch of a convergent pathway (Gilley *et al.*, 2015; Loreto *et al.*, 2015). Similar to the effects seen after expression of WLD^{S}, SARM1 deficiency fully corrects axonal defects and perinatal lethality caused by NMNAT2 loss (Gilley *et al.*, 2015). In fact, NMNAT2-deficient embryos show truncation of peripheral nerve axons, leading to perinatal lethality (Gilley *et al.*, 2013). On the other hand, double NMNAT2 and SARM1 knock out mice are healthy into adulthood (Gilley *et al.*, 2015). These results reinforce the presence of a strong crosstalk between SARM1 and NMNAT2 to regulate axon degeneration.

SARM1 deletion ameliorates axonal damage in a model of traumatic brain injury (Henninger *et al.*, 2016; Hill *et al.*, 2016) and in a genome-wide association study, variants of SARM1 have been associated to Amyotrophic lateral sclerosis (ALS) (Fogh *et al.*, 2014).

3.3 NMNAT2 is a critical survival factor for axons

Multiple mechanisms can control the health and homeostasis of axons throughout life and be part of distress/degenerative processes during aging. Both injury and disease induce axon degeneration by compromising maintenance mechanisms and promoting active self-destruction pathways. In this context, Wallerian degeneration, first described by the neurophysiologist

Augustus Waller in 1850, is defined as the degeneration of the axon distal to a site of physical injury (Waller, 1850). In this model, axon degeneration is initiated by an injury at a defined site/time and simultaneously affects all axons. Expression of the Wallerian degeneration slow (WLD^S) mutant protein, a chimeric fusion of the nuclear NAD+ biosynthetic enzyme Nicotinamide Nucleotide Adenylyltransferase 1 (NMNAT1) and a fragment of the ubiquitination factor E4B (UBE4B) (Conforti *et al.*, 2009), delays the Wallerian degeneration process (Waller, 1850) induced by numerous pathological insults (reviewed in Conforti, Gilley, & Coleman, 2014). WLD^S is predominantly nuclear, reflecting the nuclear localisation of NMNAT1. This led to the suggestion that it has a nuclear axon-protective action (Araki *et al.*, 2004), however, small amounts of WLD^S are also present in axons, and multiple lines of evidence indicate that this is the location where WLD^S acts to delay injury-induced degeneration (Beirowski *et al.*, 2009; Cohen *et al.*, 2012; Conforti *et al.*, 2009).

An interesting model to explain WLD^S axonal protection has been proposed (Gilley and Coleman, 2010). When WLD^S is present in the injured axon, it provides the enzymatic NAD synthesis capacity *via* its NMNAT1 activity, with a much longer half-life compared to NMNAT2, which is the cytosolic NMNAT isoform normally present in the axon. This action of WLDs substitutes for the rapid loss of endogenous NMNAT2 in the axon after injury, thus maintaining NMNAT activity for a prolonged period (Gilley *et al.*, 2013).

In support of this model, downregulation of NMNAT2 caused spontaneous degeneration of non-injured-axons, indicating that NMNAT2 acts as an endogenous survival factor (Gilley and Coleman, 2010). As far as it is known, NMNAT2 is synthesised in the cell bodies and constantly delivered to the axon and axon terminal by fast axonal transport. However, the short half-life of NMNAT2 means that when axons are injured or axonal transport is impeded,

its axonal levels quickly decrease, triggering degeneration. Neither nuclear NMNAT1 nor mitochondrial NMNAT3 can compensate for the loss of NMNAT2 (Gilley *et al.*, 2013; Gilley and Coleman, 2010). To further support the role of this protein in axon survival, strong overexpression of the enzyme delays Wallerian degeneration *in vitro*, and this protective effect is dependent on its enzymatic activity (Yan *et al.*, 2010). In addition, NMNAT2 is required for normal axon growth in embryos (Gilley *et al.*, 2013).

As a consequence of its very short half-life (around 4h) (Milde *et al.*, 2013), any disruption in NMNAT2 axonal transport could result in spontaneous axon degeneration, raising the important question of how neurons are able to supply enough quantities of this enzyme without triggering degeneration.

A possible explanation relies on the local synthesis of NMNAT2 in the axon. However, several large-scale studies in the past identified hundreds of axonal mRNAs but have so far failed to detect *NMNAT2* mRNA (Gumy *et al.*, 2011; Zivraj *et al.*, 2010). Only recently, *NMNAT2* mRNA has been found *in vivo* in both sensory neuron axons (Dr. Jose Sotelo-Silveira, personal communication) and in RGC axons (Shigeoka *et al.*, 2016), indicating a possible involvement of local translation in the more subtle regulation of local NMNAT2 levels.

Despite the prediction of molecular mechanisms and these recent findings, the axonal synthesis of NMNAT2 has not received much attention likely due to the fact that cell body but not axonal protein synthesis is apparently required for axon survival (Gilley and Coleman, 2010). This indicates that the majority of the axonal NMNAT2 pool is supplied by the cell body, at least *in vitro*. Nonetheless, it is worth noting that the specific experiment in Gilley and Coleman (2010) did not monitor axonal survival for longer than 24 hours. Indeed, local transport seems unlikely to be the only mechanisms involved, especially in a long human peripheral nerve of one-meter length, with fast

axonal transport taking several days to arrive at the distal end (Spaulding and Burgess, 2017).

3.4 MicroRNAs in axon degeneration and homeostasis

Despite the growing number of studies demonstrating the importance of miRNAs in neuronal development (Rajman and Schratt, 2017; Swanger and Bassell, 2011) evidence for their role in axon degeneration and homeostasis has been largely missing (Aschrafi *et al.*, 2008). High-throughput technologies, such as microarray and Next-Generation Sequencing (NGS), have depicted several deregulated miRNAs in different types of injuries and neurodegenerative disorders (Rajgor, 2018; Bhalala *et al.*, 2013; Foggin *et al.*, 2019; Maciotta *et al.*, 2013; Reddy *et al.*, 2017), however only few investigations attempted to link those miRNAs-expression profiles to function of specific microRNAs in neurodegenerative disorders.

An example comes from the well-known brain-enriched miR-9, which targets a number of proteins involved in Alzheimer's disease (AD) pathogenesis pathways, including Sirtuin-1 (a protein involved in reducing amyloid beta - Aβ- peptides and anti-aging) and Calcium/Calmodulin Dependent Protein Kinase Kinase 2 (CAMKK2) (Chang *et al.*, 2014; Schonrock *et al.*, 2012). Specifically, the latter is capable of phosphorylating Tau and its activity is higher in hippocampal neurons treated with Aβ peptides, leading to dendritic spine loss (Mairet-Coello *et al.*, 2013), while over-expression of miR-9 can rescue this phenotype (Chang *et al.*, 2014). In another study, miR-26b has been found to be upregulated in human post-mortem brains at early stages of AD and remains elevated during the disease progression (Absalon *et al.*, 2013). Mechanistically, over-expression of miR-26b in rat primary cortical neurons

led to DNA replication and aberrant cell cycle entry *via* the targeting of Retinoblastoma protein (Rb1), with increased Tau phosphorylation and ultimately cell death (Absalon *et al.*, 2013).

After AD, Parkinson's disease (PD) is the second most common neurodegenerative disorder and approximately 30% of PD cases are caused by mutation in the α-synuclein protein (α-SYN) (Capriotti and Terzakis, 2016). Interestingly, both miR-34b and miR-34c target α-SYN and their levels are downregulated in the brain of patients suffering from PD (Kabaria *et al.*, 2015). Moreover, their suppression in human SH-SY5Y cells increased α-SYN levels and triggered aggregate formation (Kabaria *et al.*, 2015).

Very recently, both miR-181a/b have been involved in global regulation of mitochondrial by controlling a group of genes involved in their biogenesis, function and redox balance (Indrieri *et al.*, 2019). Downregulation of these two miRNAs preserve mitochondrial homeostasis and ameliorates the phenotype of three different animal models of mitochondrial disease. (Indrieri *et al.*, 2019).

The studies described above are just representative examples and it is clear that much work remains to be done in order to elucidate and define the various roles of multiple miRNAs in pathological pathways, mainly due to multiple miRNA-target interaction. However, the potential use of circulating miRNAs detected in biological fluids as early biomarkers of disease onset or their use as therapeutic targets may provide an important breakthrough in neurodegenerative disease therapies.

3.5 Aims of the chapter

Highly polarised cells like neurons must face the huge logistical challenge of sustaining homeostasis over the long distance that may separate the cell body from its distal axonal part (Spaulding and Burgess, 2017). Decades of research

have revealed axonal translation as an indispensable tool of axonal development and homeostasis (Sahoo *et al.*, 2018). However, its impact in *in vitro* models of primary neurons, as well its spatiotemporal regulation remains poorly understood.

In this context, the axonal trafficking dynamics of the NAD-synthetic enzyme NMNAT2 represents an interesting experimental model, given that it's constant supply from the cell body into axons is required for axon maintenance (Gilley and Coleman, 2010), but its rapid turnover (Milde *et al.*, 2013) makes it unlikely that protein levels reach distal axons in sufficient quantities to ensure viability. Considering the observation that *NMNAT2* mRNA is present in the axon of peripheral neurons, and based on the evidence discussed above, the aims of this chapter are as follows:

- To address the possibility that local protein synthesis can contribute towards axonal survival *in vitro*;

- To test whether deletion of *Sarm1* can impact axon survival upon local translation suppression;

- To identify for microRNAs that could target NMNAT2 and regulate its expression in the axon

3.6 Results

3.6.1 Somatic protein synthesis suppression induces axon degeneration

Towards the aim of addressing whether local protein synthesis is required for axonal survival *in vitro*, I first addressed one question: Is general protein synthesis suppression in intact primary neuronal cultures sufficient to trigger axon degeneration? In fact, my main hypothesis predicts that if protein synthesis is required for survival, blocking of protein translation is expected to trigger Wallerian-like degeneration even without injury, similar to that induced by blocking axonal transport (**Figure 13** and Wang *et al.*, 2001),

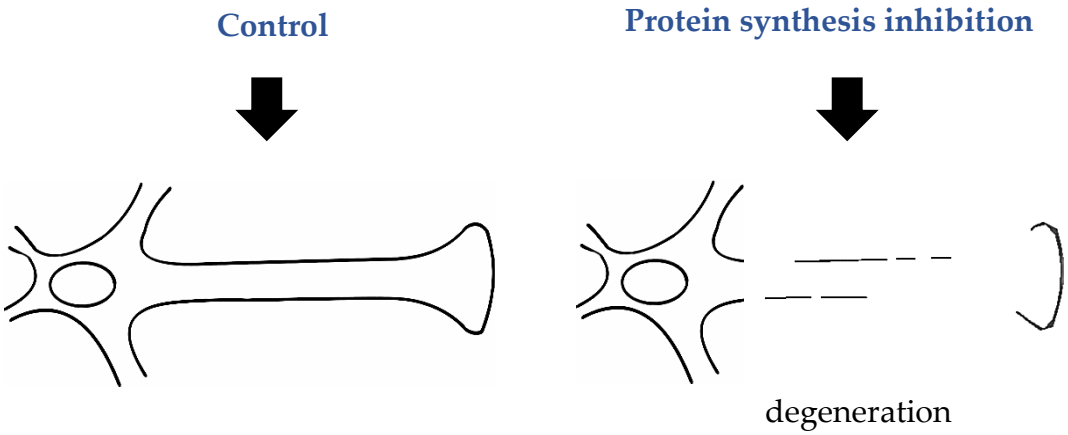

Figure 13: Diagram of the expected mechanism of action of global protein synthesis suppression in intact axons.

To test this, I used DRG primary neuronal cultures, a widely used *in vitro* model in the field and in our laboratory. DRG neurons can either be cultured as explants or they can be dissociated into separate neuron cultures. In both conditions, DRG neurons extend long axons, hereafter referred as neurites (see Materials and Methods for additional details on this *in vitro* model). The first experimental approach was to inhibit all protein translation in DRGs explant cultures using emetine at 10 μM (Gilley and Coleman, 2010; Milde et al., 2013).

As expected and consistent with previous results (Gilley and Coleman, 2010), addition of 10 μM emetine to uninjured explants induced neurite degeneration by 24 h [**Figure 14a-b**], whilst neurites in control cultures continued to grow and appeared morphologically normal.

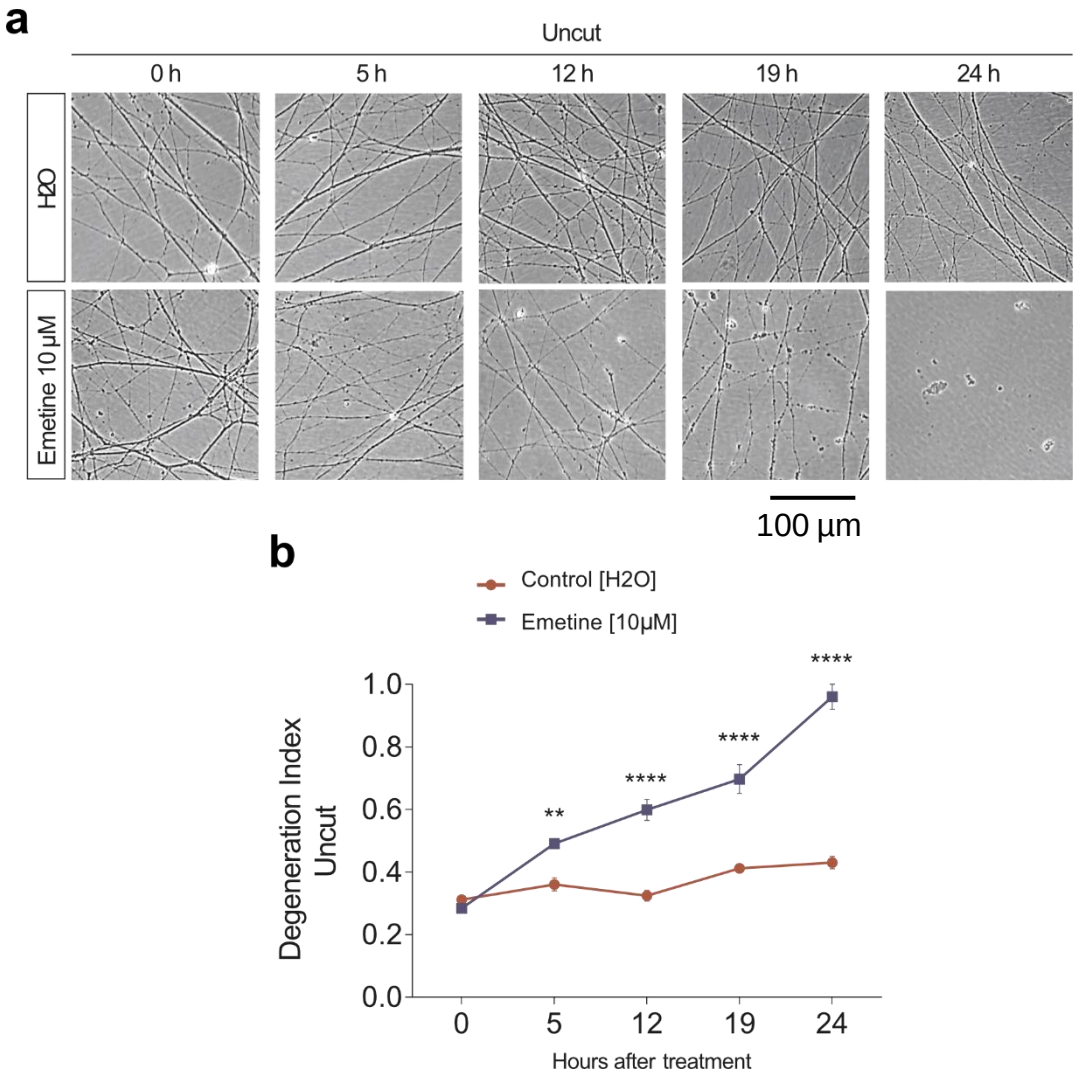

Figure 14: Protein synthesis suppression induces rapid Wallerian-like degeneration of DRG neurites. Representative phase contrast images of distal uninjured neurites from wild-type (C57) mouse DRG explant cultures treated with H_2O as control and 10 μM emetine as indicated. Images were captured at the times indicated on top. (**b**) Degeneration index was calculated from three fields per condition in 3 independent experiments. Bar graphs expressed as mean ± SEM; two-way ANOVA followed by Bonferroni post-hoc test: **: P≤0.01, ****: P≤0.0001, compared with untreated at the same time point.

Beyond the expected confirmation that global inhibition of protein synthesis leads to axon/neurite degeneration, the previous experimental approach does not discriminate between the effects caused by suppression of somal vs axonal protein synthesis. To directly test whether local protein translation in the axon contributes to axonal survival, I employed an axotomy model in which all the neurites were separated from their cell bodies by cutting around the explant with a scalpel (see methods and Di Stefano *et al.*, 2015). Considering the effects seen in the previous experiment, and if it is true that axonal protein synthesis has a role in axon survival, then addition of emetine in injured neurites should trigger faster degeneration rate compared to injured controls [**Figure 15**].

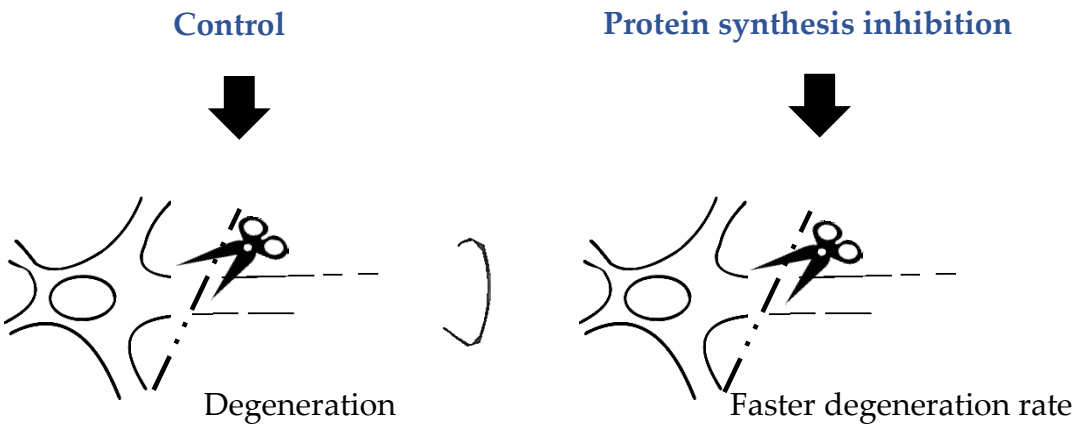

Figure 15: Diagram of the expected mechanism of action of global protein synthesis suppression in the axotomy model.

Unexpectedly, the inhibition of protein synthesis in the axotomised neurites did not accelerate the process of degeneration, but it was instead significantly slower than the control [**Figure 16a-b**], at least in the earlier timepoints. Although multiple factors might be at play in this process, a feasible explanation for the slower rate of degeneration [**Figure 16b**] is that blocking protein synthesis could inhibit the axonal translation of putative pro-degenerative factors. This is an interesting proposition, as it suggests that local

synthesis in the axon might also have a role in active degeneration alongside survival *in vitro*. However, it also reflects a technical limitation of the cut/injury model, in which the presence of the physical injury, with the consequent induction of many simultaneous changes, might confuse the final observations.

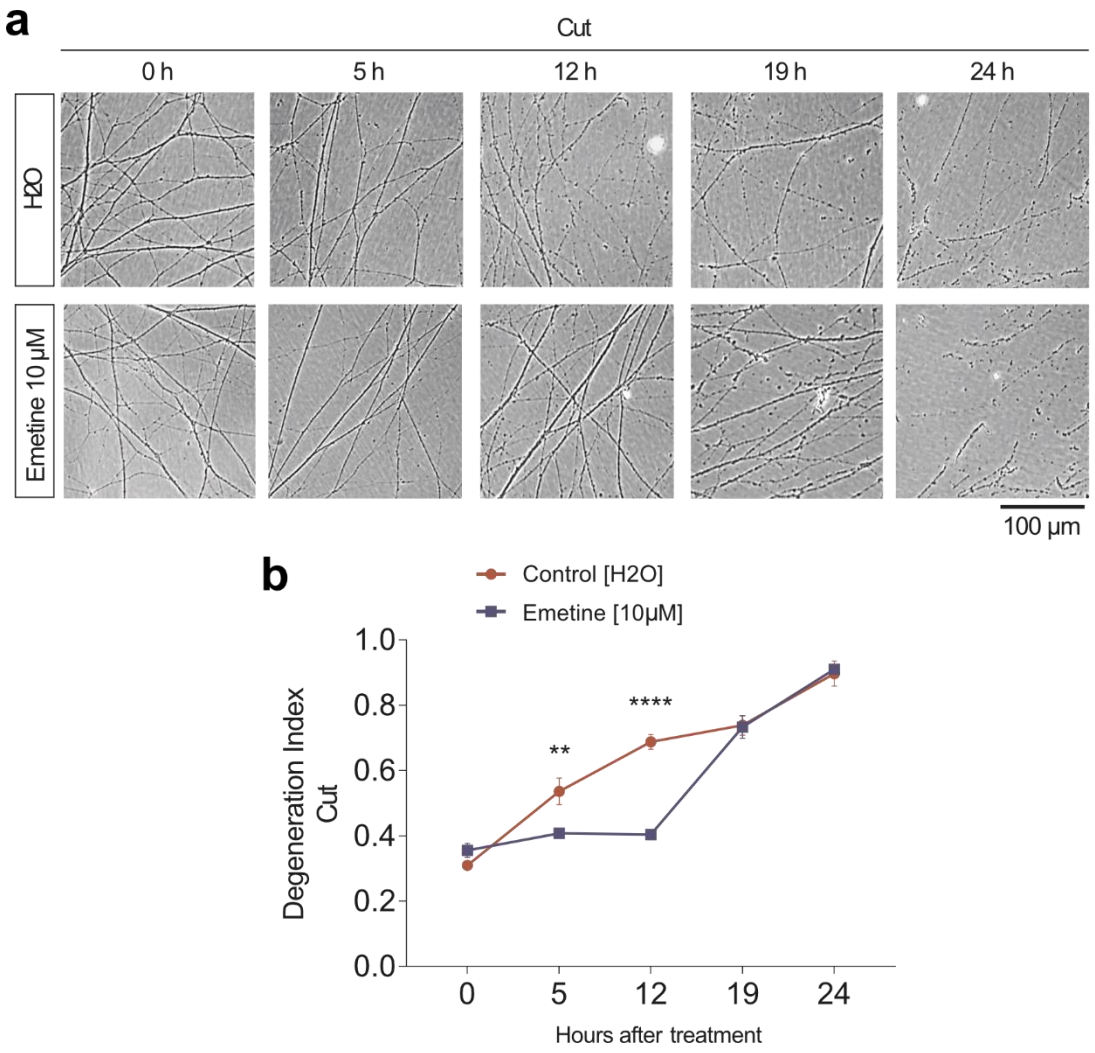

Figure 16: Protein synthesis suppression delays axon degeneration in axotomy model. Representative phase contrast images of distal injured neurites from wild-type (C57) mouse DRG explant cultures. Axons were cut and then treated as indicated immediately after cut. Images were acquired at the times indicated on top. (**b**) Degeneration index was calculated from three fields per condition in 3 independent experiments. Bar graphs expressed as mean ± SEM; two-way ANOVA followed by Bonferroni post-hoc test: **: P≤0.01, ****: P≤0.0001, compared with untreated at the same time point.

3.6.2 Neurite degenerates after suppression of protein synthesis in the cell body

The fact that the previously employed experimental approach had some limitations, such as the need of axotomy to isolate the axons from the cell bodies, with the consequent trigger of axon degeneration pathways and induction of many simultaneous changes, prompted me to use compartmentalised cell cultures in microfluidic chambers. Their greatest advantage compared to other models or even other compartmentalised culture devices is the ability to fluidically isolate the axonal (axon side) from the somato-dendritic domain (somal side) of cultured neurons, exploiting differential hydrostatic pressure. This allows for selective manipulations either in the somas or in the axons, without affecting the other compartment (**Figure 7** and Garcez *et al.*, 2016; Taylor *et al.*, 2005).

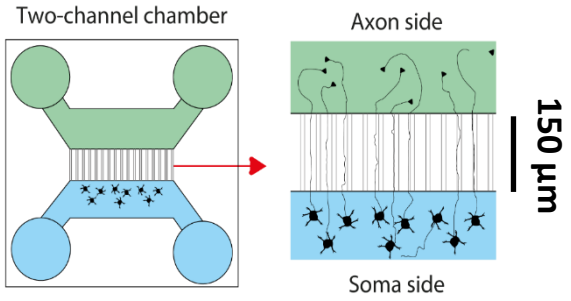

Figure 17: Schematic representation of microfluidic devices. Two-channel device. In these devices, neurons are plated into one of the lateral compartments (soma side) and extend their axons through 150 µm long microgrooves into the opposite compartment (axon side). Microgroove channels ensure total fluidic isolation of the axonal and somal compartments.

So, in order to test directly whether axonal protein synthesis is required for axon survival, I cultured dissociated DRG neurons in two-channel

microfluidic culture platforms (SND150, **Figure 17**) for 5 d and applied the same protein synthesis inhibitor as before in either the somal or axonal compartment of these microfluidic devices.

Consistent with the previous experiment, in which cell bodies were exposed to the translation blocker, our initial experiments with standard two-channel microfluidic chambers showed that neurites degenerated in less than 24 h only when the inhibitor was applied to the compartment containing neuronal cell bodies and proximal neurites [**Figure 18a**]. However, translation inhibitor applied only to the axonal compartment caused no significant degeneration within this timeframe **[Figure 18b**]. Indeed, neurites continued to grow as well as in the control conditions. This is in agreement with previous findings using Campenot chambers, in which SCG neurites degenerated only when protein

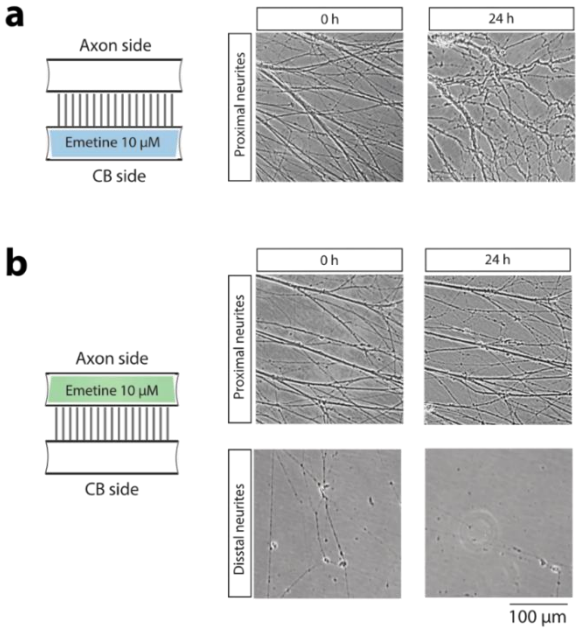

Figure 18: Neurites degenerate when suppression of protein synthesis is restricted to the cell body side of microfluidic chamber (**a**) Representative bright-field images from the axonal side of a microfluidic chambers in which 10 µM emetine was added to either the cell bodies side or (**b**) the axonal side. Phase-contrast images of the same field were acquired just after emetine addition (0 h) and 24 h later. For all the experiments, schematics of the microfluidic chambers (upper right corner) depict where drugs were added. Application to the axon and the cell body (CB) side is illustrated in green and blue, respectively.

synthesis inhibitors were applied to the compartment containing neuronal cell bodies and proximal neurites (Gilley and Coleman, 2010). Nonetheless, it is worth noting that even if no quantification of axon degeneration was carried out for this preliminary experiment, at 24 h after the drug addition, clear signs of degeneration started appearing in the most distal part of neurites of the axonal side of the devices [**Figure 18**], suggesting that if local translation is needed, tip of neurites farther from the cell bodies might be more sensitive to axonal transport impairment.

3.6.3 Axonal protein synthesis suppression induces degeneration in triple chamber devices

The fact that the distal part of neurites started degenerating at 24 h after treatment suggested that the farther the axonal tips are from the cell bodies, the more susceptible to local protein synthesis inhibition they must be. To test this hypothesis, dissociated DRG neurons were cultured in three-channel chamber devices, a more sophisticated device that consists of one 500 μm wide central chamber and two lateral compartments/channels separated from each other by 500 μm microgroove barriers. This means that neurites are allowed

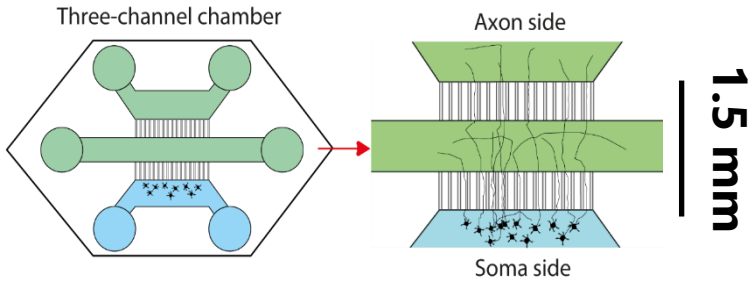

Figure 19: Schematic representation of microfluidic three-channel devices. DRG neurons are plated into the bottom compartment (somal side) and extend their axons into the top compartment (axon side) through two barriers of 500 μm long microgrooves and a 500 μm thick central chamber. In both cases, microgroove channels ensure total fluidic isolation of the axonal and somal compartments.

to extend up to 1.5 mm across the microgroove barriers and compartmentalised channels [**Figure 19**].

As described in the methods and in **Figure 19** DRG neurons were seeded in one of the lateral compartments and their axons were let to grow long enough to pass through the first microgroove barrier, reaching the central compartment, usually within 2-3 days. After 5-6 days in cultures, axons also reach the further lateral compartment. In order to have a representative number of neurites, quantification of degeneration was performed by taking 4 representative images of the central compartment for each technical replicate.

As previously observed in the two-channel device, when the addition of the protein synthesis inhibitor was restricted to the cell bodies compartment, significant axonal degeneration was observed within 24 h and almost complete loss at 72 h [**Figure 20a-b**].

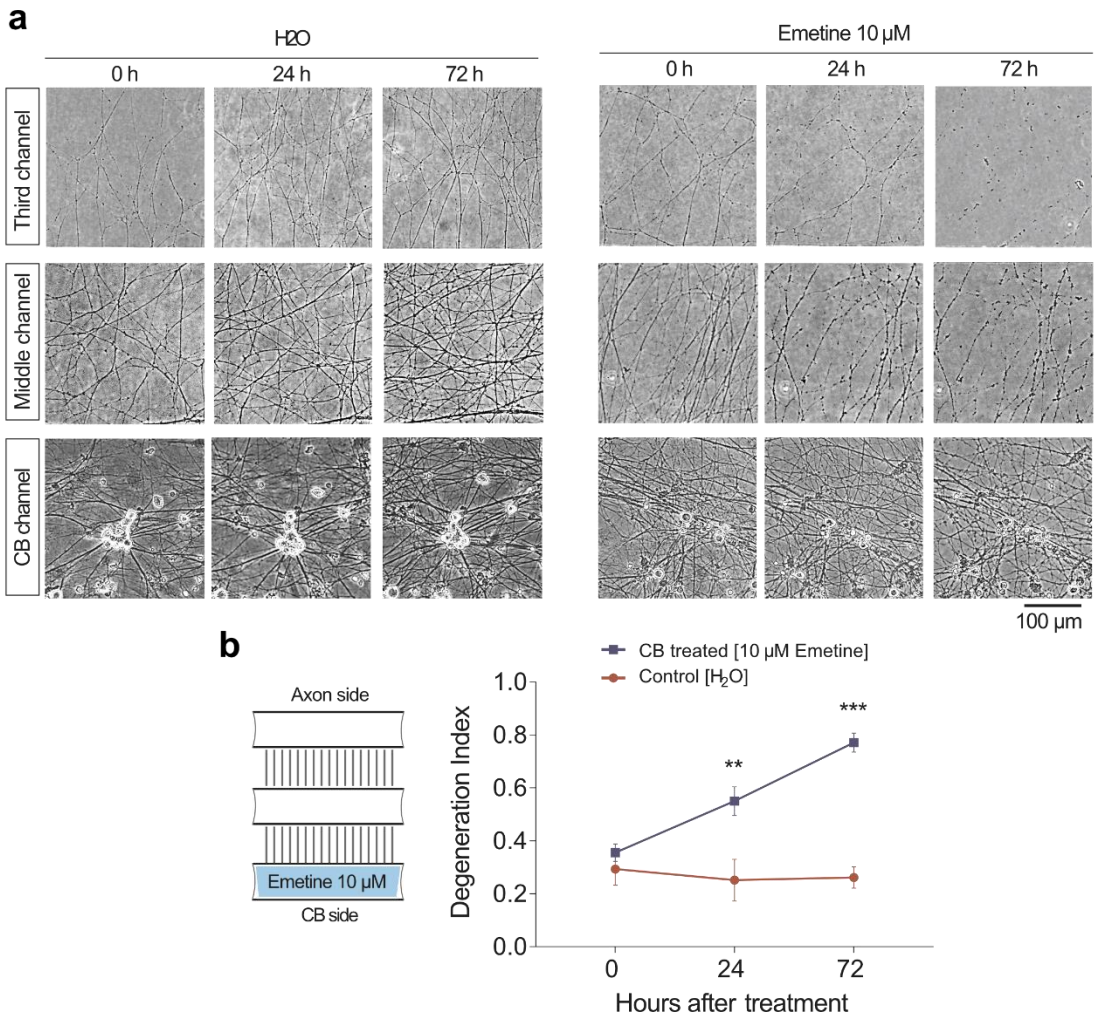

Figure 20: Somal protein synthesis suppression induces degeneration in triple chamber devices. (**a**) Representative phase-contrast images of the same field from the cell bodies (CB) channel, middle and third channel of three-channel microfluidic devices in which 10 µM emetine was added to the bottom somal compartment. Images were captured just after emetine addition (0 h) and at the times indicated on top. (**b**) Degeneration index was calculated from three fields of the middle channel per condition in 3 independent experiments. For all the experiments, schematics of the microfluidic chambers (above the graphs) depict where drugs were added. Bar graphs expressed as mean ± SEM; two-way ANOVA followed by Bonferroni post-hoc test: **: P≤0.01, ***: P≤0.001, compared with untreated at the same time point.

When emetine was applied to the axonal compartments instead, distal neurites in the middle axonal compartment started degenerating after 24 h, until the degeneration was complete at 144 h after treatment [**Figure 21a-c**]. Overall, these results indicate that local translation is needed for the survival of long axonal projections.

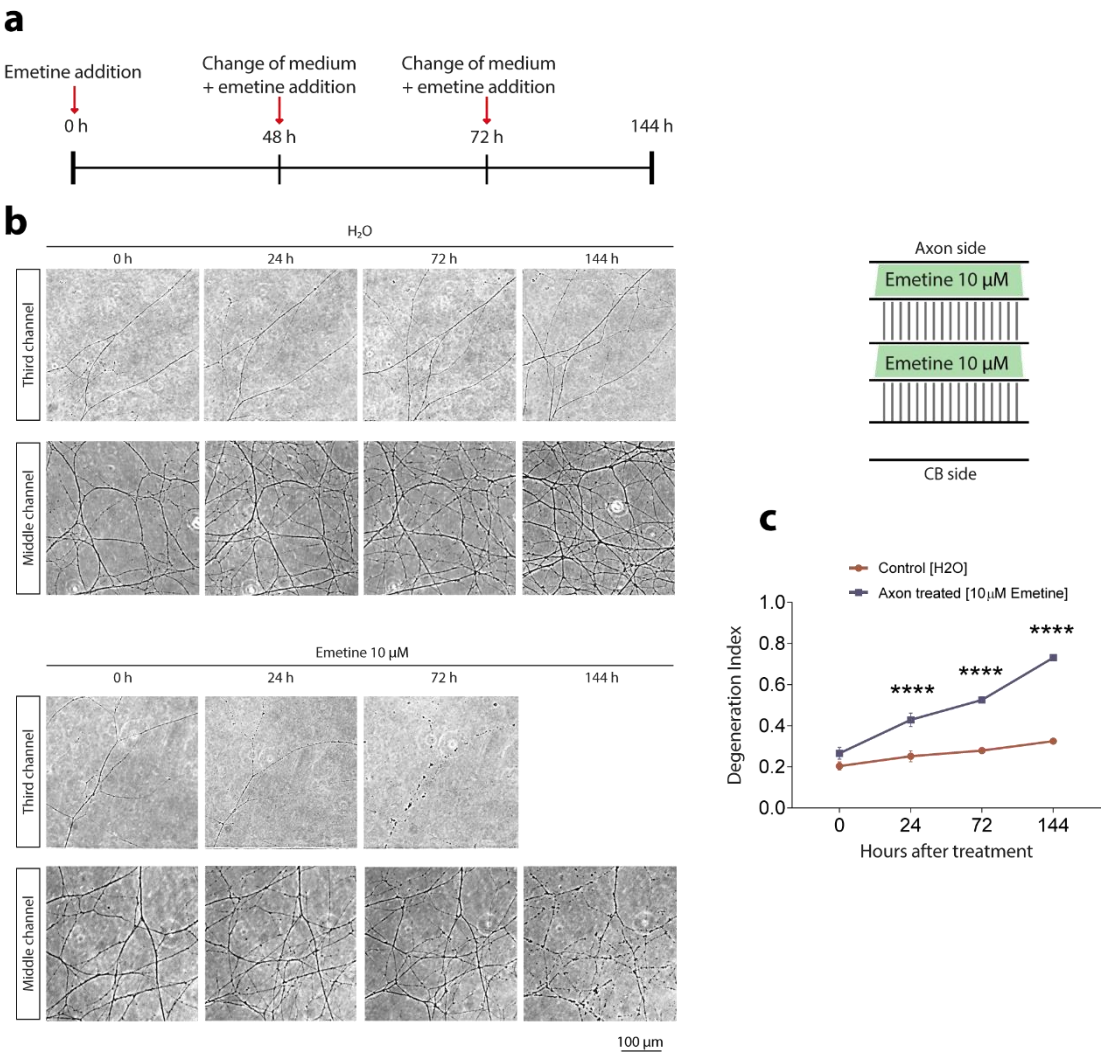

Figure 21: Axonal protein synthesis suppression induces degeneration in triple chamber devices. (**a**) Schematic representation of the experimental design. (**b**) Representative phase-contrast images of the same field from both the middle channel and third channel of three-channel microfluidic devices in which 10 µM emetine was added in both axonal compartments. (**c**) Degeneration index was calculated from three fields of the middle channel per condition in 3 independent experiments. For all the experiments, schematics of the microfluidic chambers (above the graphs) depict where drugs were added. Bar graphs expressed as mean ± SEM; two-way ANOVA followed by Bonferroni post-hoc test: ****: P≤0.0001, compared with untreated at the same time point.

3.6.4 Axonal protein synthesis suppression induces degeneration in Sarm1$^{-/-}$ DRG neurons

As mentioned in the introduction to this chapter, axon survival/degeneration can be regulated through at least two main pathways: one involves the capacity to maintain axonal protein/energy homeostasis *via* NMNAT2, and the other requires the tight regulation of SARM1-dependent axon degeneration pathway. SARM1 appears to act downstream of NMNAT2 loss to promote axon degeneration, and its depletion is, to date, one of the most valuable tools for investigating Wallerian-like-degeneration mechanisms (Gilley *et al.*, 2015; Loreto *et al.*, 2015). Considering that a decrease in NMNAT2 levels (*via* inhibition of its local translation) could be one of the cellular mechanisms impacted by the addition of emetine to the axon compartment, I therefore hypothesised whether the previously described protection provided by SARM1 deletion in axon degeneration triggered by NMNAT loss (Gilley *et al.*, 2017, 2015) could also work in the experimental model used here. To this purpose, I cultured dissociated DRG neurons from *Sarm1*$^{-/-}$ mice in triple-channel-microfluidic devices as above, and then evaluated axon degeneration in the axonal channels. Pilot studies found a 24 h delay in the degeneration rate of Sarm1$^{-/-}$ axons compared to the experiments with wild-type DRG neurons, when emetine was added in the axonal side of microfluidic chambers [**Figure 22a-c**].

Taken together, these preliminary data suggest that the degeneration of axons following proteins translation impairment can be delayed by regulators of Wallerian degeneration, but more experiments are needed to confirm this, alongside a direct comparison with wild-type DRG neurons and treatments in the somal side of compartmentalised devices for non-local effects.

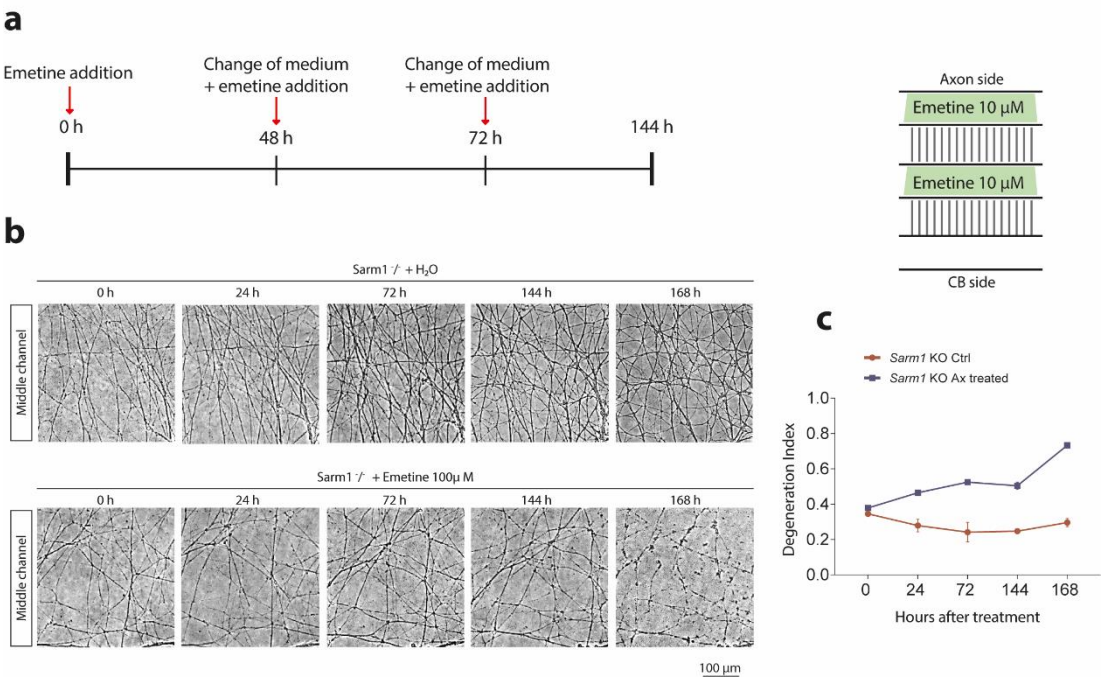

Figure 22: Loss of Sarm1 delays emetine-induced axon degeneration. (**a**) Schematic representation of the experimental design. (**b**) Representative phase-contrast images of the same field from the middle channel of three-channel microfluidic devices in which 10 µM emetine was added to the axonal compartment. Images were captured just after emetine addition (0 h) and at the times indicated on top. (**c**) Degeneration index was calculated from three fields of the middle channel per condition in 1 independent experiment. Bar graphs expressed as mean ± SEM. Preliminary data and no statistical analysis test was carried out.

3.6.5 In silico screening for NMNAT2 regulatory microRNAs

After finding that the pharmacological inhibition of protein synthesis spatially restricted to the axonal compartment can induce axon degeneration and thus local protein synthesis contributes towards axonal survival, the next step was to identify the molecular mechanism underlying this process. In particular, the fact that the same protein synthesis suppression could delay the degeneration rate in absence of SARM1 [**Figure 22b-c**] and after injury [**Figure 16c-d]**, made me speculate about the presence of a regulatory mechanism that is in turn controlling the NMNAT2:SARM1-axon degeneration-mediated pathway and its major players. Interestingly, a previous report and, more recently, a preliminary study conducted in our laboratory, showed that NMNAT2 is a developmentally regulated and low abundance neuronal protein,

overexpression of which can be toxic to CNS neurons (data from Katerina Konstatoulaki, MRes student in the lab). This observation suggests that despite its well-known protective properties, endogenous protein levels must be tightly regulated (Mayer *et al.*, 2010), in order to avoid potential detrimental effects. Overall, these results also provide a rationale for the evolutionary development of an axon survival protein (NMNAT2) with a very short half-life (~4 hours), given that longer half-life would be initially a beneficial property of proteins that require long-distance transport along the axon.

As mentioned in Chapter 1, since miRNAs are fundamental regulatory players in most biological processes, targeting at least 60% of the genes in the human genome (Zhang and Wang, 2017), it is plausible to speculate that NMNAT2 could be one of them. For this reason, I carried out a standard bioinformatics workflow (Riffo-Campos *et al.*, 2016) to identify potential microRNAs that could target *NMNAT2* mRNA. First, two extensively used miRNA prediction tools, TargetScan (Agarwal *et al.*, 2015) and DIANA-microT-CDS (Paraskevopoulou *et al.*, 2013), were employed to create a computational prediction list of potential microRNAs targeting the *NMNAT2* transcript. As it will be also described in the next chapter of this thesis, both prediction tools generate a list of miRNAs scored by strength of predicted NMNAT2-miRNA site interaction. Specifically, I found 692 miRNAs in TargetScan (TargetScanMouse 7.1) and 92 miRNAs in DIANA-microT-CDS (miTG score threshold >0.7). To increase the likelihood of identifying biologically valid miRNA candidates, I overlapped the two miRNA datasets and further narrowed it down by selecting only conserved sites for miRNA families in turn conserved among vertebrates and mammals. In this way I obtained a subset of 11 candidates common to both TargetScan and Diana prediction analysis [**Figure 23a-b]**. Next step was to manually curate in NCBI the list of miRNAs for expression in nervous system and pinpoint candidates whose regulatory

activity has a function in axonal outgrowth, development and/or degeneration. This workflow resulted in a shortlist of 4 candidate miRNAs: mmu-miR-494-3p, mmu-miR-132-3p, mmu-miR-129-3p and mmu-miR-181a-5p [**Table 2**].

Future work now is needed to experimentally validate the interaction mRNA:miRNA *in vitro* through luciferase reporter assays, whereby the binding is probed by measuring the capacity of a candidate miRNA sequence to repress activity of luciferase linked to the mRNA binding site. In parallel, phenotype rescue experiments carried out by re-expressing the targeted gene product alongside with the selected miRNA can provide a further confirmation of the specificity of the miRNA activity (Cullen, 2006).

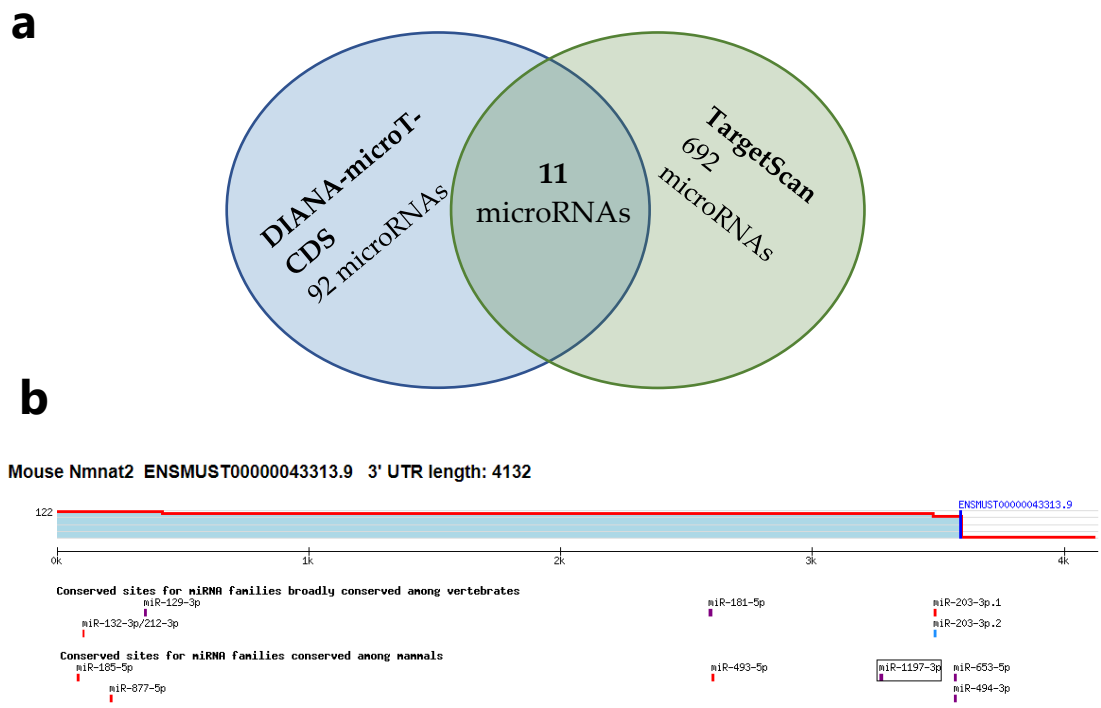

Figure 23: microRNAs predicted to target NMNAT2. Venn diagram representing the number of common microRNAs predicted to target NMNAT2 between TargetScan and DIANA micro-T-CDS computational lists. (**b**) miRNA target sites in the NMNAT2 3'UTR sequence predicted using TargetScan.

Table 2: List of microRNAs predicted to target NMNAT2.

miRNAs	Position in the 3'UTR	seed match
miR-185-5p	66-72	7mer-m8
miR-132-3p	101-107	7mer-m8
miR-212-3p	101-107	7mer-m8
miR-877-5p	198-204	7mer-m8
miR-129-3p	346-353	8mer
miR-181a-5p	2591-2598	8mer
miR-493-5p	2594-2600	7mer-m8
miR-1197-3p	3263-3270	8mer
miR-203-3p.1	3484-3490	7mer-m8
miR-203-3p.2	3484-3490	7mer-1A
miR-494-3p	3557-3564	8mer
miR-653-5p	3558-3565	8mer

3.7 Discussion

Local mRNA translation is widely used to maintain subcellular autonomy in both axons and dendrites (Bellon *et al.*, 2017; Dajas-Bailador *et al.*, 2012; Jung *et al.*, 2012), suggesting that this mechanism might also contribute to the maintenance of the steady state of the local proteome in the distal axons (Holt *et al.*, 2019). The work presented in this chapter focuses on determining whether local protein synthesis in the axon is needed to maintain axonal homeostasis and survival in primary DRG neurons. To this purpose, I combined the use of protein synthesis inhibitor along with an axotomy model first, and then with microfluidic compartmentalised devices. Our data shows that inhibition of protein translation restricted to the axonal compartment of microfluidic chambers triggers axon degeneration in mouse sensory neurons and that this process might be delayed after deletion of SARM1. Next, I attempted to dissect the molecular mechanisms underlying this process by investigating the possibility of a NMNAT2 post-transcriptional regulatory mechanism mediated by microRNAs.

Since axon requires local protein synthesis to maintain and control its function (Cioni *et al.*, 2018), it is plausible to think that axons severed from their cell bodies will also synthesise proteins to counteract the potential effects of Wallerian-like degeneration and inhibition of translation in axons would be thus expected to accelerate the degeneration rate. However, our findings with axotomised neurites revealed an unforeseen outcome in which protein synthesis suppression did not produced such a degeneration-promoting effect, at least at early stages of the experiment but it rather had a protective role. As already hypothesised in the results section, a possible explanation for the observed axonal protection window might lie in the broad-spectrum consequences of inhibiting protein synthesis as a whole. Only one concentration of emetine was tested for consistency with previously published

data (Gilley and Coleman, 2010), but there is the possibility that other concentrations may provide a more tailored inhibition of protein translation. Moreover, the use of leucine incorporation assays would be needed to confirm and determine the level of protein synthesis inhibition. In fact, it is reasonable to speculate that alongside the suppression of putative axon survival factors, repression on pro-degenerative factors is also at stake and thereby the prevention of degeneration-activating signals.

In this context, a good candidate as pro-degenerative factor might be SARM1. In effect SARM1 is an essential component of the axon degeneration mechanism, and a defining molecule in this program, whose activation triggers an irreversible commitment to axon destruction (Gerdts *et al.*, 2013; Osterloh *et al.*, 2012). Moreover, SARM1 activation seems to occur within the first 4 hours upon injury *in vitro* (Gerdts *et al.*, 2016, 2013) and this is in line with the later degeneration observed in our axotomy experiments. To support this hypothesis, not only its depletion but also the small interfering RNA (siRNA)-mediated inhibition of SARM1 can attenuate Wallerian-Like Degeneration of RGC axons (Massoll *et al.*, 2013). The next crucial step would be identifying the signal that triggers SARM1 activation. As mentioned in the introductory section of this chapter, relatively recent findings have placed SARM1 downstream of NMNAT2 loss-dependent degeneration (Gilley *et al.*, 2015). NMNAT2 may represent a "survival factor" whose depletion can trigger the axon destruction cascade, as knockdown of NMNAT2 in cultured neurons is sufficient to cause axon degeneration in the absence of injury (Gilley and Coleman, 2010). The tight link between positive and negative axon stability mechanisms, exemplified by NMNAT2 and SARM1 respectively, may then ensure that in healthy axons, degeneration signalling is closely maintained in an "off" state, thus preventing spontaneous axon degeneration. This relationship also reflects the difficulty in standardising interpretation of

the axotomy approach, reinforcing the need to dissect the effects caused by disruption of axon survival from degeneration.

An experimental challenge in demonstrating the role of local translation in neurons is in fact the need to differentiate local translation in the axon from somatic translation with protein transport to distal axonal parts (Holt *et al.*, 2019). Recent advances in cell culture technologies has allowed to morphologically and functionally separate axons from their cell bodies (Dajas-Bailador *et al.*, 2012; Garcez *et al.*, 2016; Hengst *et al.*, 2009; Taylor *et al.*, 2005), and thus provide experimental models to study local mRNA translation in axons. Previous studies have in fact attempted to test whether a critical axon survival factor(s) has to be synthesised and delivered from cell bodies by using compartmentalised SCG cultures with Campenot chambers, suggesting that somal but not axonal protein synthesis is the only apparent requirement for axon survival *in vitro* (Gilley and Coleman, 2010). However, as already mentioned in the introduction, Gilley and colleagues monitored the effects of axon translation inhibition for only 24 h and over a distance of 150 μm. In our experiments using three-channel microfluidic chambers we observed a later effect, in which more distal neurites start undergoing degeneration. In fact, it is tempting to hypothesise that the longest axons, may be the most reliant on mRNA transport and local translation for homeostasis. Confirmation of this hypothesis was provided by blocking axonal protein synthesis using microfluidic three-channel devices. In this experimental paradigm, neurites could extend themselves up to 2 mm and, as predicted, more distal neurites started degenerating after 24 h post translation inhibition in axons. This is an important observation, at it suggests a physiological significance of axonal translation in nervous system maintenance. Of note, evidence of neurodegeneration as a result of dysregulation of mRNA transport is found with mutations in the RNA binding protein Survival of Motor Neuron 1

(SMN1), which cause spinal muscular atrophy (E. T. Wang *et al.*, 2016). More recently, a really elegant work has described how axonal RNAs are co-transported with late endosomes, and suggests that late endosomes function as platforms for local synthesis of proteins responsible for axonal integrity and survival (Cioni *et al.*, 2019). Interestingly, disruption of endosomal RNA association triggered dysfunction within mitochondria (Cioni *et al.*, 2019).

Among multiple interconnected cellular mechanisms, two cellular processes might be key in axon survival: the maintenance of a healthy supply of functional mitochondria (Cosker *et al.*, 2016; Pease and Segal, 2014) and the inhibition of a destruction program mediated by SARM1 (Gerdts *et al.*, 2015; Gilley *et al.*, 2017, 2015). Whilst both mechanisms are at play after axon injury (Gerdts *et al.*, 2016; Loreto *et al.*, 2015), our preliminary study in which protein synthesis was suppressed in the axons of *Sarm1*$^{-/-}$ mice initially suggested only a partial contribution to axon survival from this player. In fact, other effectors might be operating upstream to counteract the absence of SARM1-mediated protection, in a process that also likely requires local axon translation. As before, considering its role as gatekeeper of axonal survival, one of the players might be NMNAT2.

In this scenario, even if it has not been addressed directly, the axonal trafficking dynamics of the NAD-synthesising enzyme NMNAT2 represents a challenging and interesting open question. As said in the introductory section of this chapter, NMNAT2 is critical for axon survival in PNS primary cultures and its depletion may contribute to axon degeneration in a variety of neurodegenerative disorders (Conforti *et al.*, 2014; Gilley and Coleman, 2010). On the other hand, this enzyme is also actively transported along the axon, but, due to a short half-life of only 4 h (Milde *et al.*, 2013), its levels in cut axons drop prior to any visible sign of fragmentation, suggesting it may be a trigger for axon degeneration.

How neurons manage to supply enough copies of extremely labile NMNAT2 into axons, in order to avoid spontaneous axon degeneration? The scale of the problem becomes even more significant in human peripheral nerves that can be one-meter long, with fast axonal transport taking several days to arrive at the distal end (Holt *et al.*, 2019). Data presented in our study showed that blocking translation exclusively in the axonal compartment only triggers degeneration at 24 h, and not within the precise timeframe of the NMNAT2 half-life *in vitro.* A possibility that has not been addressed so far is that NMNAT2 turnover might be faster in axons after cut. A longer half-life in uninjured axons would allow sufficient copies of NMNAT2 to reach the distal ends of axons and so ensuring axon maintenance. In this scenario, the local synthesis of this enzyme in the axon might constitute an additional mechanism of action that should be considered. We have recently obtained data from RNA extracted from sciatic nerve of adult rats, where *NMNAT2* RNA was detected *in vivo* in axons (collaboration with Sotelo-Silveira lab at the IIBCE Institute, Uruguay). Thus, the potential for locally translated NMNAT2 in the axon constitutes a tantalising possibility that has not been previously investigated and requires further consideration. Moreover, potential off-target effects related to the use of a protein synthesis inhibitor in this study need to be contemplated, whereas a more selective silencing of NMNAT2 in the axon, by means of a cell-permeable siRNA technology might be a more direct approach.

Whilst NMNAT2 neuroprotective activity has been largely investigated in the peripheral nervous system, its role in the central nervous system is still largely unexplored. Mice lacking NMNAT2 die at birth with severe axon defects in the CNS (Gilley *et al.*, 2013), but, paradoxically, Mayer and co-workers found that overexpression of the exogenous enzyme was toxic to primary cortical neurons and led to massive cell death (Mayer *et al.*, 2010). This is also in agreement with recent results in our lab, where over-expression of NMNAT2

resulted to be toxic in mouse primary cortical neurons. Whilst these data may seem contradictory with its role in peripheral axons, they essentially support a scenario in which NMNAT2 is tightly regulated and any impairment on its levels (either up or down) would lead to harmful outcomes for the cell.

NMNAT2 is also axonally transported on Golgi-derived vesicles, to which it is anchored by palmitoylation, but surprisingly, its protective efficacy is greatly increased if it is detached from these vesicles (Milde, 2013) through a mechanism of reduction of ubiquitin-proteasome mediated degradation. More recently, the pharmacological inhibition of the mitogen-activated protein kinase kinase kinases (MAP3Ks) dual leucine zipper kinase (DLK) and leucine zipper kinase (LZK) increased NMNAT2 abundance and protected axons from injury-induced degeneration (Summers *et al.*, 2018). Mechanistically, MAPK signalling selectively promoted degradation of palmitoylated NMNAT2, suggesting that this lipid modification is a component of a broader strategy for regulating protein degradation of neuronal proteins. (Summers *et al.*, 2018). So, as another option, post-translational modifications could target NMNAT2 to multiple vesicle sub-populations with different outcomes on its half-life, reinforcing the need of fine-tuning the regulation of this protein.

microRNAs and NMNAT2: the need for precise regulation of protein expression levels

Considering the pivotal role of miRNAs in regulating almost every aspect of central nervous system function (Fiore *et al.*, 2011), it is somehow surprising that only few investigations attempted to link miRNAs-mediated mechanisms to axon homeostasis and survival (Aschrafi *et al.*, 2008; Indrieri *et al.*, 2019). Even more, a putative miRNA-mediated regulation of NMNAT2 has not been addressed yet. Our *in-silico* screening of miRNAs potentially targeting NMNAT2 has yielded a list of 4 potential candidate miRNAs: mmu-miR-129-

3p, mmu-miR-132-3p, mmu-miR-494-3p, and mmu-miR-181a-5p. As mentioned in the results section, both the expression and function of these miRNAs in nervous system were used as search parameters to identify plausible candidates. For example miR-129 has been recently placed into a regulatory network capable of regulating axon regeneration of DRG neurons *via* the targeting of the Insulin-like growth factor 1 (IGF-1), providing further insight into the regulatory role of miRNAs in peripheral nerve regeneration (Zhu *et al.*, 2018). Moreover, in developing cortical neurons, over-expression studies of miR-129 showed that this miRNA also owns the ability to impair both migration and transition from multipolar to bipolar through Fragile X Mental Retardation gene 1 (Fmr1), which is mutated in the autism spectrum disorder fragile X syndrome (Wu *et al.*, 2019).

Besides the already mentioned protective role of miR-181 inhibition on mitochondrial disease models (Indrieri *et al.*, 2019), the inhibition of this miRNA has been also found to reduce apoptosis and mitochondrial dysfunction in astrocytes *via* the targeting of Bcl-2 family members (Ouyang *et al.*, 2012), and its levels are upregulated in the hippocampus of triple AD transgenic mice (3xTg-AD) (Rodriguez-Ortiz *et al.*, 2014).

Gain of function studies with miR-494 instead, significantly decreased the level of DJ-1, an oxidative sensor that participates in both familial and sporadic PD, and rendered a neuronal cell line more susceptible to oxidative stress (Xiong *et al.*, 2014). Very recently, an elegant work reported that levels of miR-494 were down-regulated in extracellular vesicles (EV) derived from iAstrocytes of ALS patients (Varcianna *et al.*, 2019). Of note, restoring miR-494-3p levels increased motor neurons survival *in vitro* (Varcianna *et al.*, 2019).

Finally, miR-132, an old acquaintance of the nervous system development, is also noticeably dysregulated AD (Pichler *et al.*, 2017) and plays a key role in Tau metabolism, as it regulates exon splicing of Tau (Hébert *et al.*, 2012).

Further studies are now needed to confirm this interaction, and this is part of current work in our lab. Specifically, it would be informative to determine the phenotypes obtained after manipulating the levels of those miRNAs and if their over-expression can mimic the effects seen after the loss of this key regulator of axon health.

CHAPTER 4:
Role of miR-26a in neuronal polarity and axon development

4.1 Introduction

The establishment of cell polarity in pyramidal neurons through the development of multiple dendrites and a long axonal projection is one of the most complex structural and functional challenges faced by any cell type. As described in the general introduction of this thesis, several cellular and molecular mechanisms underlying this process have been extensively investigated, leading to the identification of many important intracellular signalling pathways and molecules with sometimes convergent actions orchestrating both axon establishment and elongation (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; Hapak *et al.*, 2018).

In this scenario, local translation of mRNAs in the axon offers the capacity for precise temporal and spatial regulation of protein expression (Cioni *et al.*, 2018; Costa and Willis, 2018). As extensively described in previous sections of this thesis, axonal protein synthesis is now considered a fundamental part of the neuron's biology, playing key roles in several processes including development, growth, pathfinding, formation of pre-synaptic terminals and maintenance (Batista *et al.*, 2017; Campbell and Holt, 2001; Deglincerti *et al.*, 2015; Gracias *et al.*, 2014; Hengst *et al.*, 2009; Hengst and Jaffrey, 2007; Jung *et al.*, 2012; Piper *et al.*, 2006; Sasaki *et al.*, 2010; Yao *et al.*, 2006). Considering these vast arrays of cellular processes at play, it is important to understand the complexity and diversity of the axonal translatome, and to elucidate the regulatory mechanisms controlling axonal mRNAs.

As described in the introductory section of this thesis, miRNAs have undoubtfully emerged in recent years as important players in multiple cellular processes, such as neurogenesis, axon development, pathfinding and neuron connectivity (Bellon *et al.*, 2017; Dajas-Bailador *et al.*, 2012; Hancock *et al.*, 2014; Kaplan *et al.*, 2013; Reh and Hindges, 2018).

The identification of axonal miRNAs has been previously carried out using microarray expression profiling and/or RT-qPCR (Natera-Naranjo *et al.*, 2010; Sasaki *et al.*, 2014). Although new technologies have greatly increased the quality and sensitivity of axonal miRNAs profiling studies, the lack of reproducibility derived from different sample preparations and detection methods is still a weakness of this type of studies, making the consolidation of data a particularly demanding task.

For this reason, my lab has been focussing in the last few years on shedding light on the role of miRNAs in axon biology. In particular, preliminary work aimed to outline the axonal miRNA content in the axon of mouse cortical neurons using NGS, as a more sensitive and unbiased method. This study made the starting point of the results presented in this chapter, in which the role of an axon-enriched miRNA was further investigated. Thus, this section aims to briefly describe the background work leading into the identification of these miRNAs of interest, and to provide a more complete background on the scientific questions that will discussed in the following chapters.

In order to obtain a pure axonal RNA fraction, cortical neurons were cultured for 8-10 days in compartmentalised microfluidic devices (Garcez *et al.*, 2016; Taylor *et al.*, 2005) which allow for the separation of the axons from the cells bodies [**Figure 24**]. The RNA extracted from both the cell body and soma side of ~ 40 chambers was used for axonal miRNA sequencing (miRNA-seq) allowing us to identify a complex axonal miRNA subpopulation of over 100 miRNAs. Considering this long list of potential candidates to study, we decided to refine it by making a hierarchical list based on miRNA axon enrichment (axon/cell body), which allowed the identification of ~20 axonally enriched miRNAs [**Table 3**]. These miRNAs were further validated by qPCR. Among the validated results, we could identify both miR-9 and miR-16, two microRNAs previously described to have a role in axon outgrowth (Dajas-

Bailador *et al.*, 2012; Kar *et al.*, 2013), thus confirming the validity of our approach. Additionally, the majority of axon-enriched miRNAs had Ct values < 32, consistent with previous evidence showing that mature miRNAs present in low copy numbers have Ct values that are normally higher than 35-36 (Natera-Naranjo *et al.*, 2010; Schmittgen and Livak, 2008).

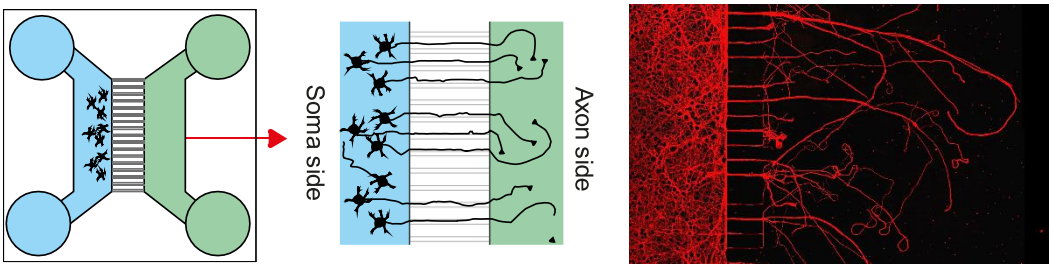

Figure 24: miRNAs enriched in developing cortical axons. Schematic representation of a microfluidic device used to separate axons from cell bodies and primary cortical neurons cultured in these devices and immunostained with acetylated tubulin antibody (Red), depicting the compartmentalisation of the culture. The table below depicts a list of the top 20 miRNAs most enriched in the axons as compared to the soma fraction.

Table 3: Axonal-enriched microRNAs

Top 20 axonal-enriched microRNAs			
miR-9-5p	miR-16-5p	miR-191-5p	miR-125b-3p
miR-151-3p	miR-134-5p	miR-181c-5p	miR-181b-5p
miR-434-3p	**miR-26a-5p**	miR-182-5p	miR-708-5p
miR-3470b_1	miR-146a-5p	miR-27-3p	miR-143-3p
miR-99a-5p	miR-25-3p	miR-146b-5p	miR-30a-5p

As depicted in **Table 3** one of the top axon-enriched miRNAs identified by this preliminary study and with the lowest Ct values in the qPCR experiments is the mmu-miR-26a-5p (hereafter referred to as miR-26a) and, in this chapter of thesis, I will focus on its role in axon. First, I will briefly describe the family of this miRNA and what is known to date about its functions in neurons, followed by an overview of one of its validated targets, GSK3β, with regards to its role during neuronal system development.

4.1.1 miR-26a

The miR-26 family is widely conserved across vertebrates (Lagos-Quintana *et al.*, 2001) and harbours two homologs, miR-26a and miR-26b, which are transcribed from three different genomic loci, miR-26a-1, miR-26a-2 and miR-26b. The mature miRNA of miR-26a-1 and miR-26a-2 possesses the same sequence, with the exception of 2 different nucleotides in mature miR-26b. In mammals, miR-26 family members reside in introns of the C-terminal domain small phosphatases (*CTDSP*) genes (miR-26a-1 (*CTDSPL*), miR-26a-2 (*CTDSP2*), miR-26b (*CTDSP1*) a class of phosphatase that, by acting synergistically with repressor element 1 (RE1) silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) protein complex, inhibits RNA Polymerase II and suppress neuronal gene expression in NSCs (Chen et al., 1998; Yeo et al., 2005).

These miRNAs can target their host transcripts and so creating an intrinsic inhibitory feedback loop of regulation (Dill *et al.*, 2012), and in fact, this feedback loop is fundamental in zebrafish, where *via* the targeting of its own host gene *ctdsp2* miR-26b promotes neurogenesis. Interestingly, mature miR-26b is not constitutively co-expressed with its CTDSP2 host but rather kept in an inactive form in NPCs and in non-neuronal cells, which represses the negative feedback loop. This is achieved through the inhibition of miR-26 processing in Neural Precursor Cells (NPCs) and in non-neuronal cells (Dill *et al.*, 2012). Consistently, miR-26a is highly expressed in the mouse cerebral cortex at embryonic day 12 and throughout cortical development, where it has been shown to regulate neural progenitor differentiation and cell-cycle progression, too (Lambert *et al.*, 2018; Zhang *et al.*, 2018).

Beyond its role in differentiation, miR-26a can also modulate other processes in the nervous system. For example, the knocking down of miR-26a in peripheral sensory neurons leads to impaired axon regeneration, indicating

that miR-26a had a role in the decrease of GSK3β needed for axon re-growth (Jiang *et al.*, 2015). In another study that used rat neonatal cortical cultures for neurite growth assays, inhibition of miR-26a also showed an effect in neuritic/dendritic growth, *via* the targeting of PTEN (Li and Sun, 2013). Moreover, in an elegant study, miR-26a along with miR-384-5p have been implicated in the long-term maintenance of long-term potentiation (LTP) and spine enlargement of rat hippocampal neurons *via* the targeting of ribosomal S6 kinase 3 (RSK3).

4.1.2 GSK3β: one master key to neuronal development

As mentioned in the general introduction of this thesis, among the neuronal polarity effectors, GSK3β has been positioned at a signalling crossroad able to coordinate the complex emergence of axon/dendrite axis in neurons (Kim and Snider, 2011).

GSK3 proteins are serine/threonine kinases that have been originally described as important enzymes capable of regulating glucose metabolism (Woodgett and Cohen, 1984). There are two mammalian isoforms, GSK3*α* and GSK3β, encoded by separate genes, which show high sequence homology with each other across species, with 95% identity in the catalytic domains from flies to humans (Woodgett, 2018). GSK3s have been also described to coordinate several signalling pathways, among which the Wnt signalling pathway is the most studied (Woodgett, 2003). Considering the Wnt proteins function in the nervous system, especially during development, it is not surprising that evidence point to GSK3s as key regulators of several neurodevelopmental processes, including neurogenesis, the already described neuronal polarisation and axon growth.

GSK3 signalling in neurogenesis. In a key study using a conditional knockout strategy in a *Gsk3a* null background to specifically target *Gsk3b* in neuronal progenitors, it has been found that GSK3 deletion has a pivotal role in the regulation of progenitor proliferation and differentiation by impairing homeostasis in neural progenitors and shifting the balance toward self-renewal and away from neurogenesis (W.-Y. Kim *et al.*, 2009). Moreover, the observed phenotype was associated with the dysregulation of β-catenin, Sonic Hedgehog (SHH), Notch and FGF signalling that are in turn all GSK substrates (Doble and Woodgett, 2003; Espinosa *et al.*, 2003; Shimizu *et al.*, 2008) and regulators of neural progenitor proliferation (Iwata and Hevner, 2009; Machold *et al.*, 2003; Yoon and Gaiano, 2005). Deletion of both *Gsk3a* and *Gsk3b* in new-born neurons at later stages instead, impairs a correct migration and dendritic arborisation of excitatory neurons (Morgan-Smith et al., 2014). More recently, suppression of either GSK3*α* or GSK3β enhanced the proliferation of neural progenitor cells in the VZ, according to previous findings (W.-Y. Kim et al., 2009) but at later stages, deletion of each isoform resulted in distinct outcomes (Ma et al., 2017). Specifically, transition of radial progenitors to intermediate progenitor cells was triggered in GSK3*α*-depleted cells, but prevented from doing so in GSK3β-depleted cells (Ma et al., 2017).

Further proof of the involvement of GSK3 signalling in neurogenesis was obtained by manipulating in neuronal progenitors both Disrupted in schizophrenia 1 (DISC1) and PAR3, which are two upstream regulators of GSK3. (Bultje *et al.*, 2009; Mao *et al.*, 2009). Indeed, over-expression of these two regulators promoted proliferation of progenitors and inhibited neuronal differentiation, consistently with the *Gsk3* knockout mice. Specifically, in the first study, ectopic expression of DISC1 triggered the activation of the Wnt pathway and through an interaction with GSK3 prevented it from phosphorylating β-catenin and targeting it for ubiquitination (Mao *et al.*, 2009).

In the second work, although a direct involvement of GSK3 was not assessed, overexpression of PAR3 activated Notch signalling, as it was also observed in Gsk3 knockout mice (W.-Y. Kim *et al.*, 2009).

The discovery that during mitotic cell division GSK3-phosphorylated β-catenin, which is normally targeted for degradation, is inherited by only one daughter cell (Fuentealba *et al.*, 2008), led to the hypothesis that during asymmetrical division of RGCs, the two daughter cells may inherit a different level of GSK3 activity because of asymmetrical distribution of upstream GSK3 regulators, such as PAR3. The daughter cell with low levels of GSK3 activity will accumulates β-catenin up (and perhaps other pro-proliferative proteins such as c-myc) and maintains its progenitor status. On the other hand, the daughter cell with higher GSK3 activity will target these proteins for ubiquitination and then differentiate into either a neuron or an IPC (Eun-Mi Hur and Zhou, 2010). To support this model, there are studies suggesting the ability of GSK3 to regulate the stability of a wide range of proteins through the ubiquitin-proteasome system (UPS) (N. Kim *et al.*, 2009; Xu *et al.*, 2009). It has been also seen the axin-GSK3β interaction is fundamental for the amplification of the intermediate progenitors' pool, whereas the Axin-β-catenin interaction promotes neuronal differentiation (Fang et al., 2013).

GSK3 signalling in axon outgrowth. Beyond its ability to control cytoskeletal properties in the axon tip, which has been already described in the general introduction of this thesis, GSK3 has also been involved in the transcriptional regulation of axon elongation. In the canonical Wnt pathway, inhibition of GSK3β leads to the accumulation of β-catenin which in turn, after entering the nucleus, activates T cell factor (TCF)-mediated gene transcription and promotes axon growth (Lu *et al.*, 2004). Others transcription factors involved in axonal outgrowth are nuclear factor of activated T-cells (NFAT) proteins. Extracellular cues such as neurotrophins and netrins can trigger

Ca^{2+}/calcineurin-dependent nuclear translocation of NFAT family of proteins where they induce transcription of genes involved in axon growth (Graef *et al.*, 2003). However, since NFAT are rapidly re-shuttled back into the cytoplasm through a GSK3-mediated phosphorylation, GSK3 is likely to regulate NFAT-mediated gene transcription in axon elongation (Beals *et al.*, 1997). Neurotrophins can also induce the phosphorylation of CREB which in turn promotes the assembly of the transcriptional complex (Vo and Goodman, 2014). Given the fact that CREB can be phosphorylated by GSK3 impairing its DNA-binding activity (Grimes and Jope, 2001), one could speculate that GSK3 signalling is capable of regulating CREB activity.

An interesting and still open question regards how GSK3 can control and coordinate such a plethora of developmental processes. This broad regulatory capacity of GSK3 can of course be explained by its long list of functional substrates. However, unlike many other kinases, GSK3 proteins are normally active in resting cells, with several regulatory mechanisms controlling their activity, including protein-protein interactions, spatial regulation and phosphorylation (Etienne-Manneville and Hall, 2003; Hengst *et al.*, 2009; Thornton *et al.*, 2008; Wu *et al.*, 2009). The constitutive activity, together with the complex array of post-translational mechanisms that can control substrate specific actions (Beurel *et al.*, 2015), suggests the need for tight regulatory mechanisms that can control GSK3 levels. Despite this, the protein levels of GSK3β do not appear to be regulated by proteasome activity during the establishment of neuronal polarity (Yan *et al.*, 2006), highlighting the potential importance of GSK3β translation regulation.

4.2 Aims of this chapter

Despite the growing number of studies demonstrating the importance of miRNAs in axon and synapse development (Rajman and Schratt, 2017; Swanger and Bassell, 2011), evidence for their role in axon specification and neuronal polarisation has been largely missing. In this scenario, miR-26a is a well conserved miRNA with an already established function in the nervous system, and our preliminary screening depicted miR-26a as one of the highly enriched miRNAs in developing axons, reinforcing the case for its potential function in neuronal development. Experiments described in this chapter will explore the role of miR-26a in the establishment of neuronal polarity and axon elongation in cultured mouse cortical neurons. Hence, the specific aims of this part of the thesis are as follows:

- Examine miR-26a expression over cortical neurons development;
- Investigate the regulatory effects of miR-26a in neuronal polarity and axon outgrowth in developing cortical axons in culture;
- Identify the regulatory target genes that mediate miR-26a activity in both neuronal polarity and axonal outgrowth;
- Address roles for the functional target genes of miR-26a in axon specification and growth, using gain and loss-of-function approaches.

4.3 Results

4.3.1 miR-26a regulates neuron polarisation and axonal growth in cortical primary neurons

As described in the general introduction, the preliminary miRNA profiling performed in our lab identified miR-26a as one of the most enriched miRNAs in developing axons when compared to the cell body fraction of neurons cultured in microfluidic chambers (see Materials and Methods for additional details). I first addressed two questions: **i)** is miR-26a expressed over the development of mouse primary cortical neuron cultures? **ii**) What is the role of miR-26a in the development of CNS neurons *in vitro*? To test these two questions, first, the relative abundance of miR-26a was quantified in whole cortical cultures at 4 h post plating, and then at 2 days *in vitro* (DIV2), 5 (DIV5) and 9 (DIV9) *in vitro* by RT- qPCR. In agreement with previous observations (Li and Sun, 2013) I found miR-26a being expressed in cortical neurons at 4 h post plating, with a trend towards decrease in more developed cultures up to DIV9 [**Figure 25a-b**]. Despite the slight decrease in expression over the timepoints measured, particularly at later stages, miR-26a is consistently expressed in cortical neurons over the cortical neuron's development in culture.

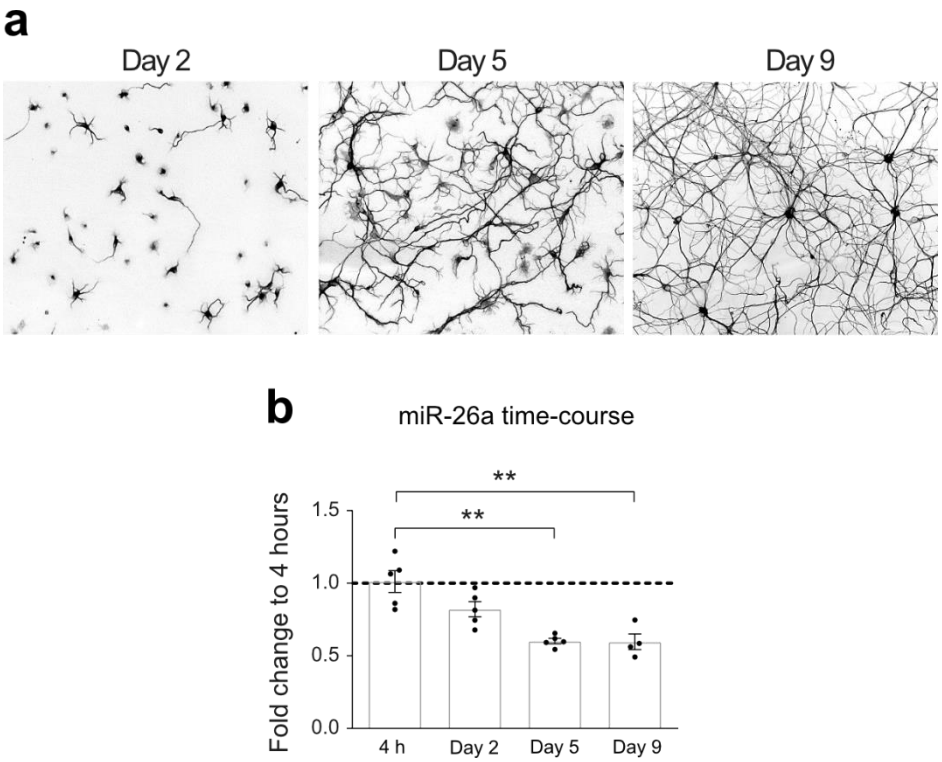

Figure 25: miR-26a is expressed in primary cortical neurons. (**a**) Acetylated tubulin staining depicts the increasing axonal complexity of a cortical culture over development. (**b**) Quantification of miR-26a levels over development of cortical primary cultures, from 4 h to 9 days *in vitro*. Expression of miR-26a-5p was analysed by relative quantification using the comparative Ct method (2-ΔΔCt) and the geometric mean of miR-100-5p, miR-128-3p, miR-134-5p, miR-434-3p and let7a-5p used as reference; mean ± SEM of 5 independent experiments. One-way ANOVA with Bonferroni's multiple comparison post-hoc tests: **: $p \leq 0.01$.

Next, as an initial approach to determine miR-26a neuronal role, I transfected mouse primary cortical neurons with a specific miRNA inhibitor (locked nucleic acid technology, LNA, from Exiqon) of miR-26a and evaluated axonal growth after three days in culture. For this, neurons were transfected 4 h after plating using Lipofectamine 2000 and their development was evaluated 72 h later. To inhibit endogenous miR-26a and at the same time label transfected neurons, the LNA miR-26a inhibitor (50 nM) was co-transfected with GFP. LNA inhibitor control at 50 nM along with GFP was used as an experimental control. Consistent with previous results, (Jiang *et al.*, 2015; Li and Sun, 2013), inhibition of miR-26a generated a significant decrease in the axonal length of

cortical primary neurons. At this timepoint, as depicted in **Figure 26a-b,** inhibition of miR-26a decreased axonal length up to 20 % compared to non-targeting control probes. In addition to this effect on length, a closer morphological examination of the transfected primary cortical cultures also revealed that inhibition of miR-26a produced a significant reduction in the proportion of polarised neurons [**Figure 26c-e**], (*i.e*. those with a distinct axon projection, defined as a neurite that was at least 3 times the length of any other neurite (Dajas-Bailador *et al.*, 2008).

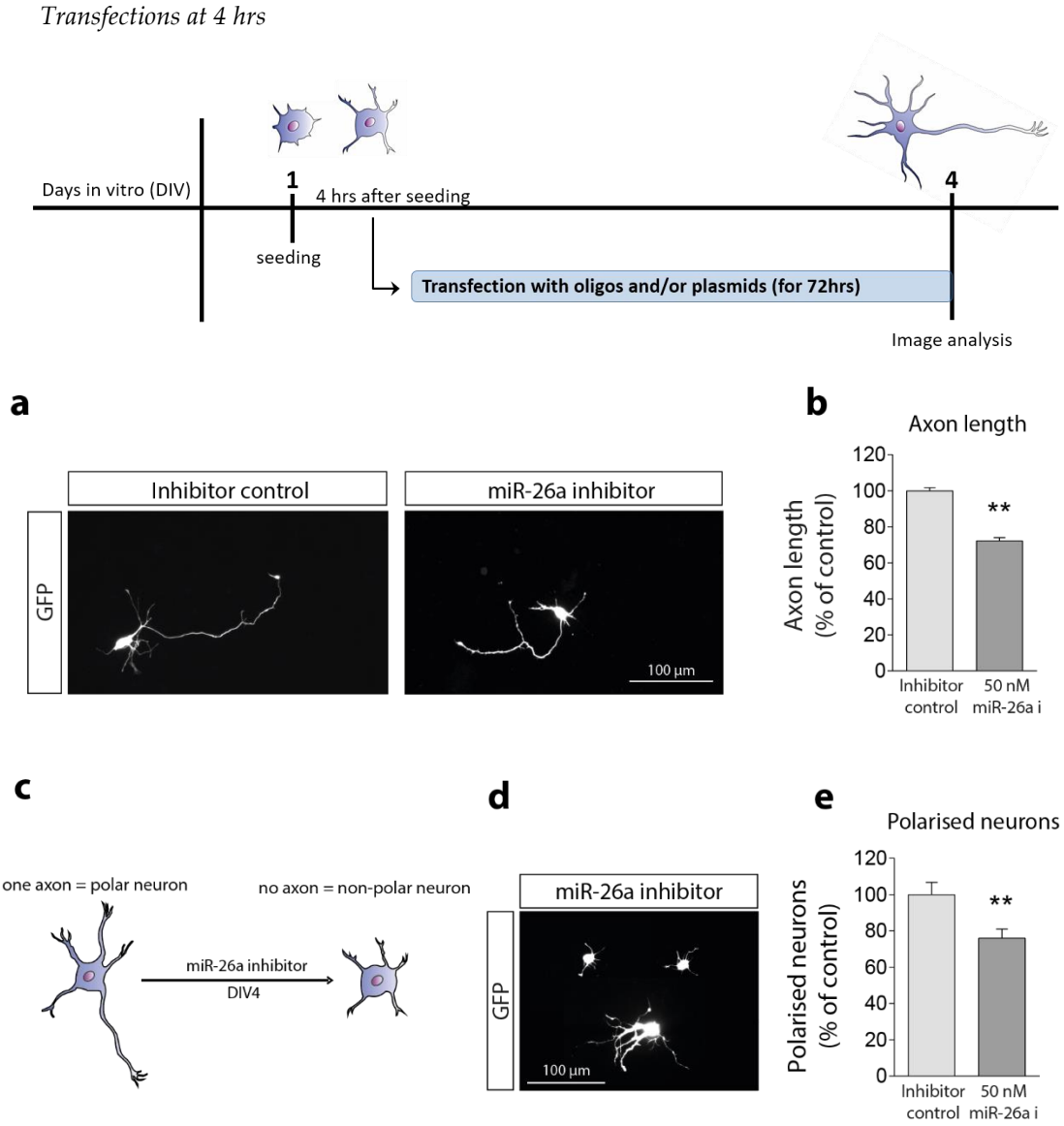

Figure 26: miR-26a regulates neuronal polarisation and axonal outgrowth. **(top)** Schematic representation of the experimental design. (**a**) Representative images of polarised cortical neurons after transfection with GFP plus a miR-26a inhibitor. (**b**) Quantification of axon length after inhibition of miR-26a (50 nM miR-26a i), showing up to 25 % decrease compared to a non-targeting control, n=5. (**c-d**) Schematic representation and images of polarity changes induced by miR-26a inhibitor on cortical neurons. (**e**) Quantification of the number of polarised neurons after inhibition of miR-26a, expressed as a percent of neurons transfected with non-targeting control, n=5. Data is expressed as mean ± SEM; Student's t-test: **: $p \leq 0.01$.

4.3.2 Over-expression of miR-26a induces axonal outgrowth and formation of multiple axon-like processes

To further investigate the potential role of miR-26a in neuron polarisation and axon growth, we carried-out overexpression studies using a miR-26a mimic (see Methods section for details on the mechanism of action of miRNA mimics). Cortical neurons were transfected 4 h after dissection in an analogous manner of the previous experiment. MiR-26 mimic were used at 20 nM, compared to LNA mimic and co-transfected with GFP to label transfected neurons. Subsequently, cortical neurons were cultured for 4 days *in vitro* and then analysed for their ability to polarise and axonal length.

The rise in miR-26a levels in cortical neurons produced a significant increase in axonal growth, in agreement with previous reports in peripheral sensory neurons (Jiang *et al.*, 2015). In fact, as depicted in [**Figure 27a-b**]**,** over-expression of miR-26a promoted axonal length up to almost 40 % compared to non-targeting control oligonucleotides.

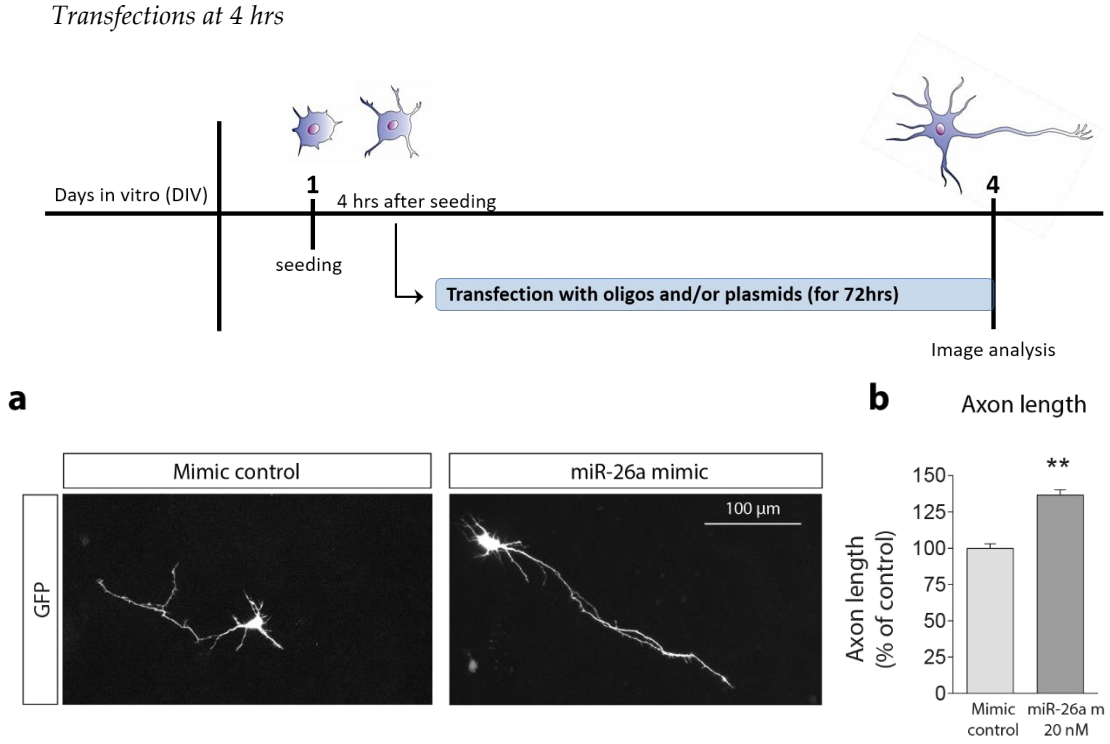

Figure 27: miR-26a overexpression induces axonal outgrowth. **(top)** Schematic representation of the experimental design. (**a**) Representative images of polarised cortical neurons after transfection with GFP plus a miR-26a mimic. (**b**) Quantification of axon length in polarised neurons after over-expression of miR-26a (miR-26a m 20 nM), showing an increase in axon length up to almost 40 % compared to a non-targeting control, n=8. Data is expressed as mean ± SEM; Student's t-test: **: $p \leq 0.01$.

Crucially, transfection with miR-26a mimic also induced a dramatic increase in the number of neurons with multiple axon-like processes [**Figure 28a-b**], identified by the presence of the axonal marker JIP-1 (Dajas-Bailador *et al.*, 2014, 2008; Deng *et al.*, 2014; Fu and Holzbaur, 2013). This multi-polar neuronal phenotype was accompanied by an overall increase in the length of projections, which were approximately 40% longer than the average neurite in similar cultures [**Figure 28c**]. Overall, these results demonstrate that in addition to affecting axonal growth per se, miR-26a can control neuronal polarity.

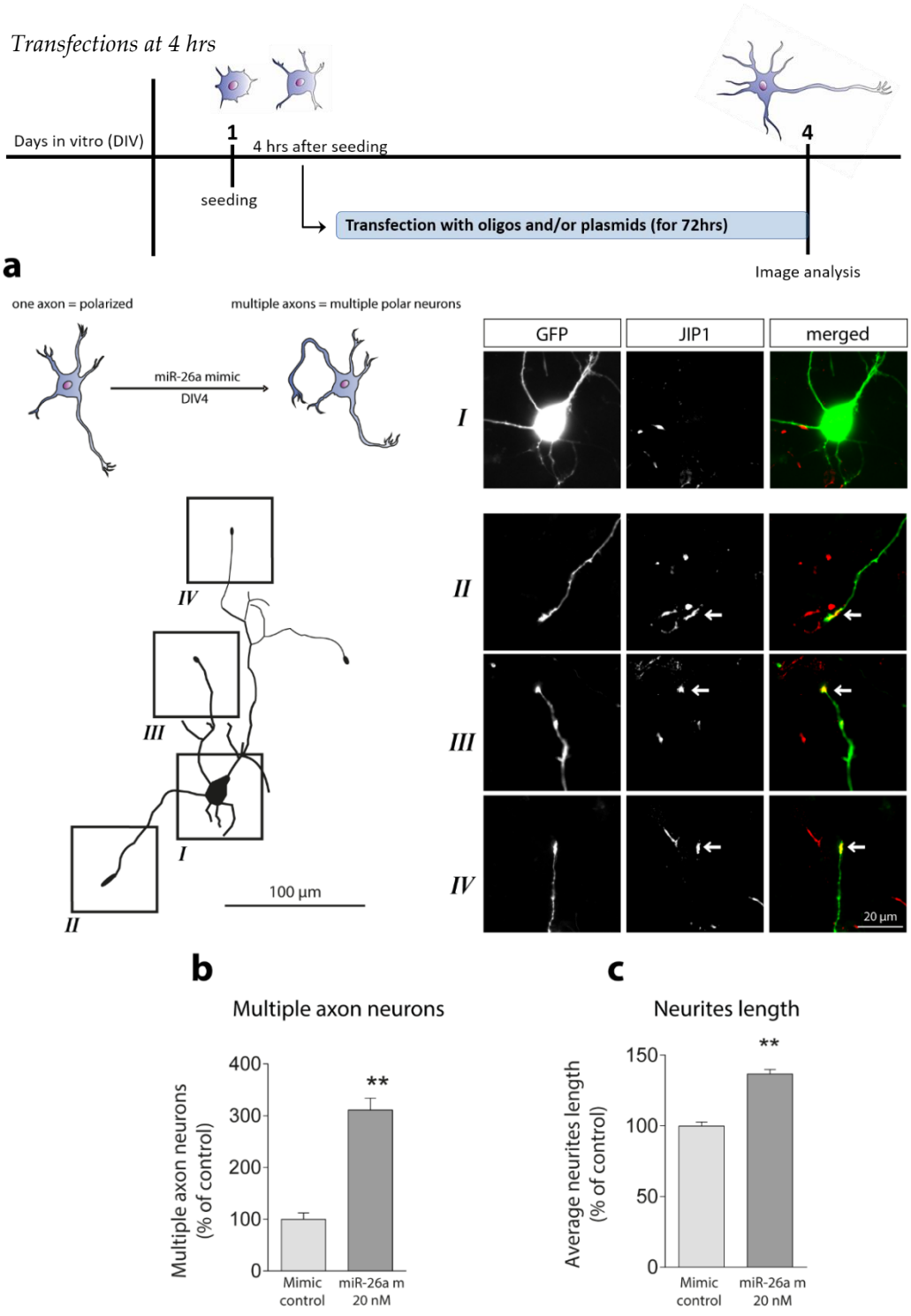

Figure 28: miR-26a overexpression induces formation of multiple axon-like processes. **(top)** Schematic representation of the experimental design. (**a**) Schematic representation and trace to demonstrate the polarity changes induced by miR-26a mimic, showing the appearance of neurons with multiple axons. Squares (I-IV) on the trace correspond to images from soma and neurite terminals of cortical neuron. Arrows indicate JIP1 labelling, which was used as an established marker of axonal growth cones. (**b**) Quantification of the number of neurons with multiple axon-like processes after over-expression of miR-26a and expressed as a percent of neurons transfected with a non-targeting control, n=5. (**c**) Quantification of the overall length of all projecting neurites, n=4. Data is expressed as mean ± SEM; Student's t-test: **: $p \leq 0.01$.

4.3.3 miR-26a regulation of neuronal polarity over cortical neurons development

The capacity of miR-26a to control axon specification and growth, two cellular processes that are intrinsically linked in early stage neuron polarisation, made us speculate whether miR-26a could retain the ability to control both neuronal polarity and growth at different stages of neuron development in culture. To assess this, I transfected cortical neurons at two further time-points (24 h and DIV5) with either miR-26a inhibitor or mimic and analysed them for their ability to develop/maintain polarity 72 h later. I found that when transfected at 24 h, inhibition of miR-26a still decreased both the number of polarised neurons and the growth of the developing axons [**Figure 29a-c**].

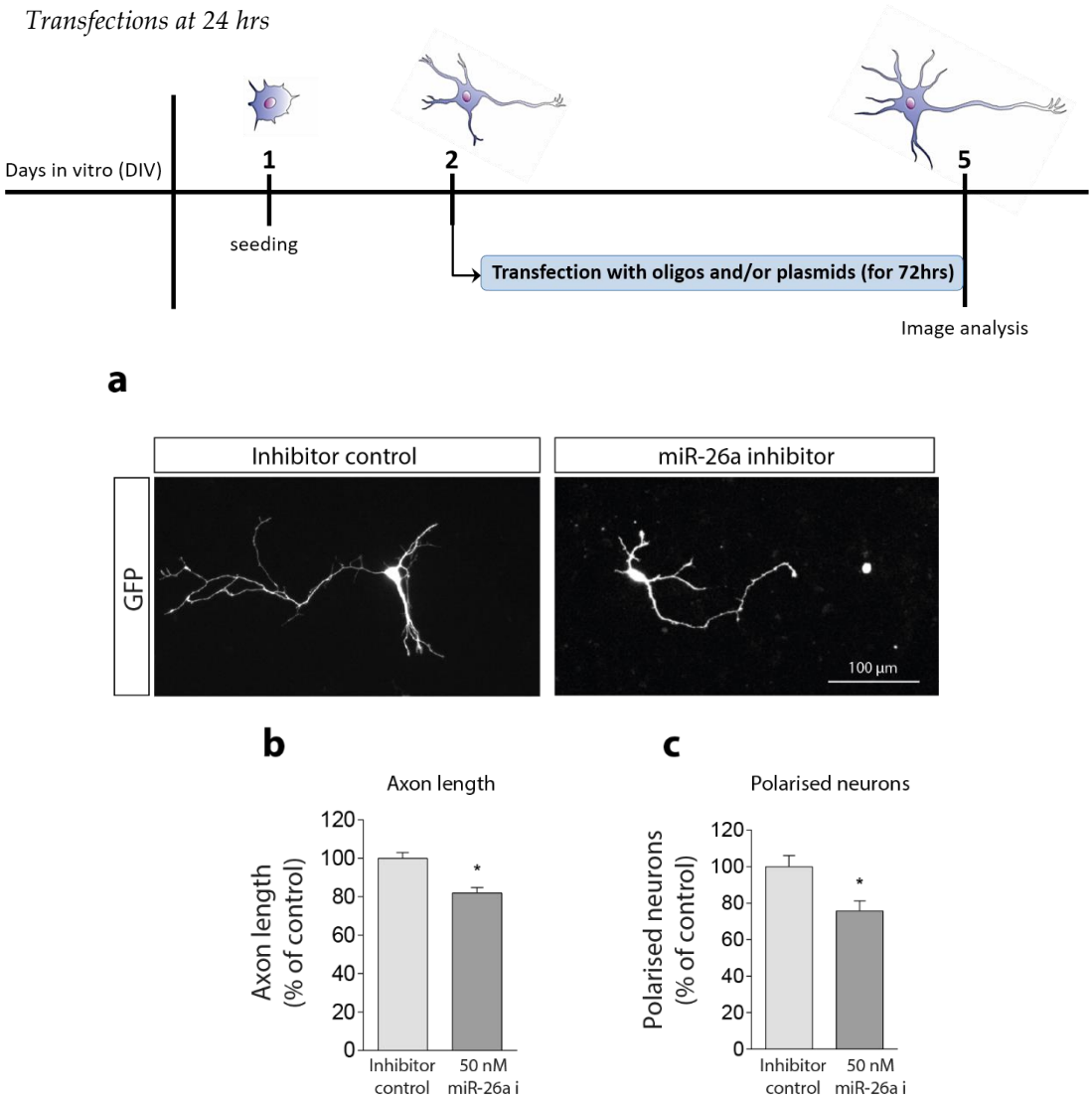

Figure 29: miR-26a affects neuronal polarity and growth when inhibited after 24 h. **(top)** Schematic representation of the experimental design. (**a**) Representative images of cortical neurons after transfection with GFP plus a miR-26a inhibitor 24 h after plating. (**b-c**) Quantification of axon length and percent of polarised cells after inhibition of miR-26a (50 nM miR-26a i), showing up to 20 % decrease for both metrics compared to non-targeting controls, n=4. Data is expressed as mean ± SEM; Student's t-test: *: $p < 0.05$.

As depicted in **Figure 29b-c,** quantification of axon length and the percentage of polarised cells after inhibition of miR-26a at 50 nM showed up to 20 % decrease for both analyses compared to non-targeting controls. However, when I analysed neurons for their ability to polarise 72 h after transfections with miR-26a inhibitor at DIV 5, I found no significant changes in the polarity of cortical neurons. Unlike the effect seen at 24 h, miR-26a inhibitor after 5 days of culture did not decrease the percentage of polarised cells [**Figure 30a-b**]. More importantly, even the over-expression of the microRNA at this later time point did not significantly increase the number of neurons with multiple axons [**Figure 30c-d**].

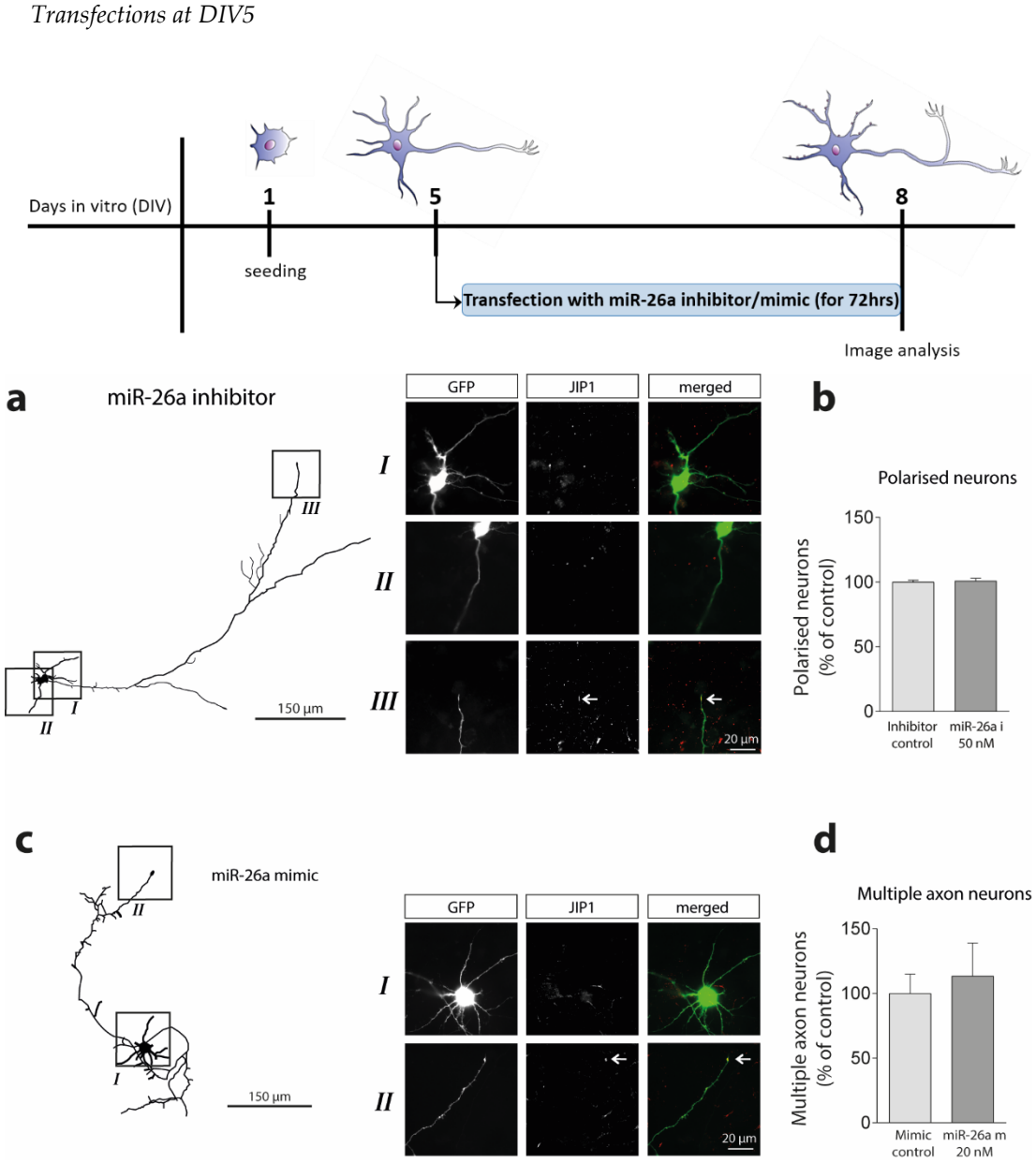

Figure 30: miR-26a does not affect polarity at later stage of development. **(top)** Schematic representation of the experimental design. (**a**) Trace of a representative neuron after miR-26a inhibition at DIV5. Squares (I-III) on the trace correspond to images from soma and neurite terminals of cortical neuron. Arrows indicate JIP1 labelling, which was used as an established marker of axonal growth cones. (**b**) Quantification of the number of polarised neurons after inhibition of miR-26a, expressed as a percent of neurons transfected with non-targeting control, n=3. (**c**) Representative traces of a neuron after over-expression of miR-26a at DIV5. Squares (I-II) on the trace correspond to images from soma and neurite terminals of cortical neuron. Arrows indicate JIP1 labelling, which was used as an established marker of axonal growth cones. (**d**) Quantification of the number of neurons with multiple axon-like processes after over-expression of miR-26a and expressed as a percent of neurons transfected with a non-targeting control, n=3. Data is expressed as mean ± SEM.

4.3.4 The search for a target of miR-26a: the GSK3β hypothesis

A critical next step after finding a function for a specific miRNA is to identify the regulatory networks through which the miRNA is acting. However, as described in the general introduction, the miRNA:mRNA interaction is not unique, and a single mRNA could bear multiple miRNA binding sites in its 3'UTRs and *vice versa* (Bartel, 2009), making the identification of miRNA-dependent regulatory pathways particularly challenging. A way to solve this problem is usually through the use of computational prediction tools and subsequent experimental validation of these miRNA:mRNA interactions (Peterson et al., 2014; Riffo-Campos et al., 2016). For this reason, I exploited the use of miRNA target prediction tools in order to generate a list of miR-26a putative target genes that were further refined according to their function and expression in the nervous system.

Firstly, I used two of the most common miRNA target prediction tools, TargetScan (Agarwal *et al.*, 2015) and DIANA-microT-CDS (Paraskevopoulou *et al.*, 2013), in order to produce a list of miR-26a putative target genes. Both tools produced a ranked list of genes scored by strength of predicted miR-26a-mRNA site interaction: 1046 targets in TargetScan (TargetScanHuman 7.1) and 1568 targets DIANA-microT-CDS (miRNA targeted genes [miTG] score threshold >0.7). To increase the likelihood of identifying biologically valid target candidates, I produced an overlap of the two gene datasets, obtaining a subset of 747 targets genes common to both TargetScan and Diana prediction analysis (Riffo-Campos *et al.*, 2016; Sethupathy *et al.*, 2006). As the list of putative targets was still extensive, which is not uncommon for widely conserved microRNA families (Bartel, 2009), I decided to further refine the list by matching it with miRTarBase, a curated database that provides information about experimentally validated miRNA-target interactions (Chou *et al.*, 2018).

The result narrowed down the list of putative targets to 139 validated targets of miR-26a [**Figure 31**].

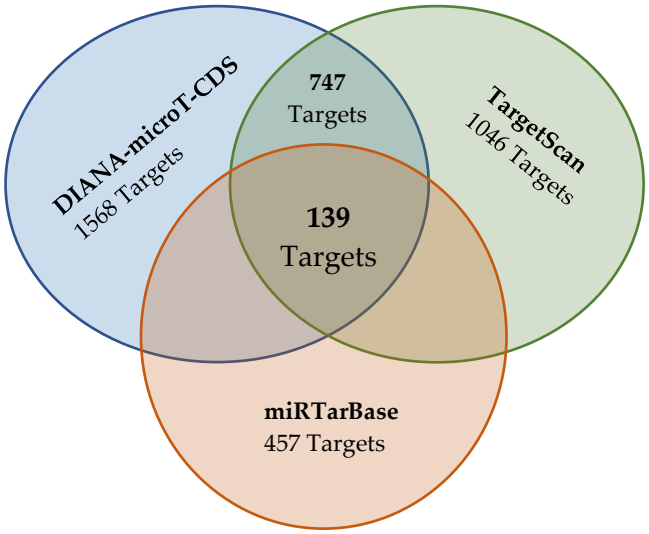

Figure 31: miR-26a predicted target genes. Venn diagram representing the number of common targets between TargetScan, DIANA micro-T-CDS and miRTarBase lists of predicted/validated targets.

After this step, I wanted to identify the molecular pathways potentially targeted by miR-26a by performing a pathway analysis of the 139 putative target transcripts. For this, I used PANTHER-Pathways (PANTHER 13.1 release; Mi *et al.*, 2017), a bioinformatics tool for analysis of curated pathways. As shown in **Figure 32**, the 10 top pathways obtained from the analysis include some of the most well studied pathways in neuronal function, such as EGF receptor signalling pathway that has been involved in axon outgrowth (Evangelopoulos *et al.*, 2009; Goldshmit *et al.*, 2004), FGF signalling pathway that plays a role in both axonal specification and elongation (Barnes and Polleux, 2009; Williams *et al.*, 1994) and the PI3K pathway, already described to have a pivotal role in the establishment of axon-dendrite axis (Ménager *et al.*, 2004; Shi *et al.*, 2003; Yoshimura *et al.*, 2006).

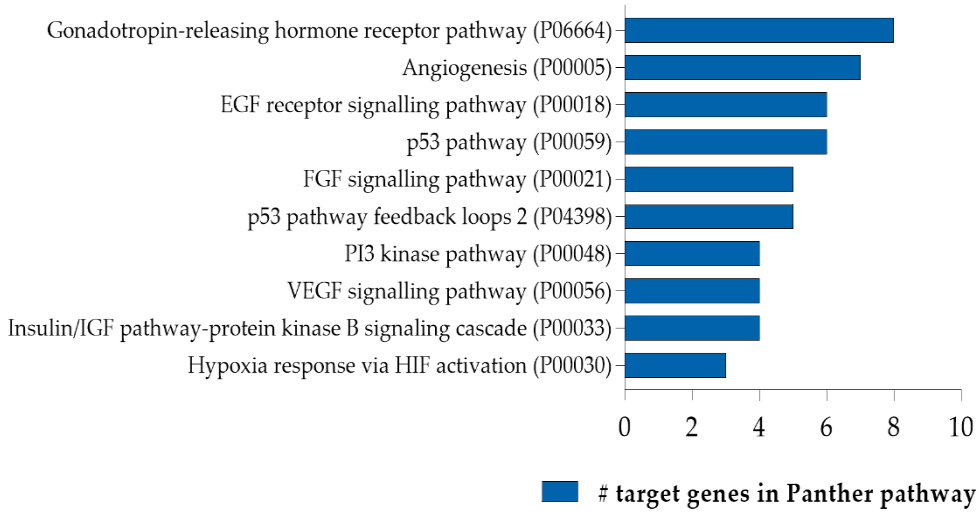

Figure 32: Pathway analysis of miR-26a regulatory networks. Top 10 pathways targeted by miR-26a obtained from PANTHER Pathway analysis of miR-26a predicted target genes. Pathways were ranked by number of target genes (bars) belonging to each Panther pathway depicted in the graph.

Lastly, by means of manual curation of the literature, I investigated the role of each target in neuronal function and development. This allowed me to both gather hints on the molecular pathways through which miR-26a could be acting in neurons and, more importantly, to identify the targets whose already described function in the nervous system development fit with the observed phenotype of miR-26a in cortical axons. As seen in the functional data discussed in **Figure 26** and **Figure 27**, miR-26a acts as a growth promoting factor in developing cortical neurons, therefore the transcript predicted to be targeted by miR-26a is expected to promote growth and regulate axon specification. This led us to investigate both PTEN and GSK3β as targets of miR-26a. They both respectively bear three and two highly conserved miR-26a-binding site sequences in their 3'UTRs **[Figure 33a-b]** and both have been previously described as a functional target of miR-26a (Cui *et al.*, 2015; Jiang *et al.*, 2015; Li and Sun, 2013). Key to my findings, both PTEN and GSK3β play a

fundamental role in multiple neurodevelopmental processes, including neuronal polarisation and axon growth.

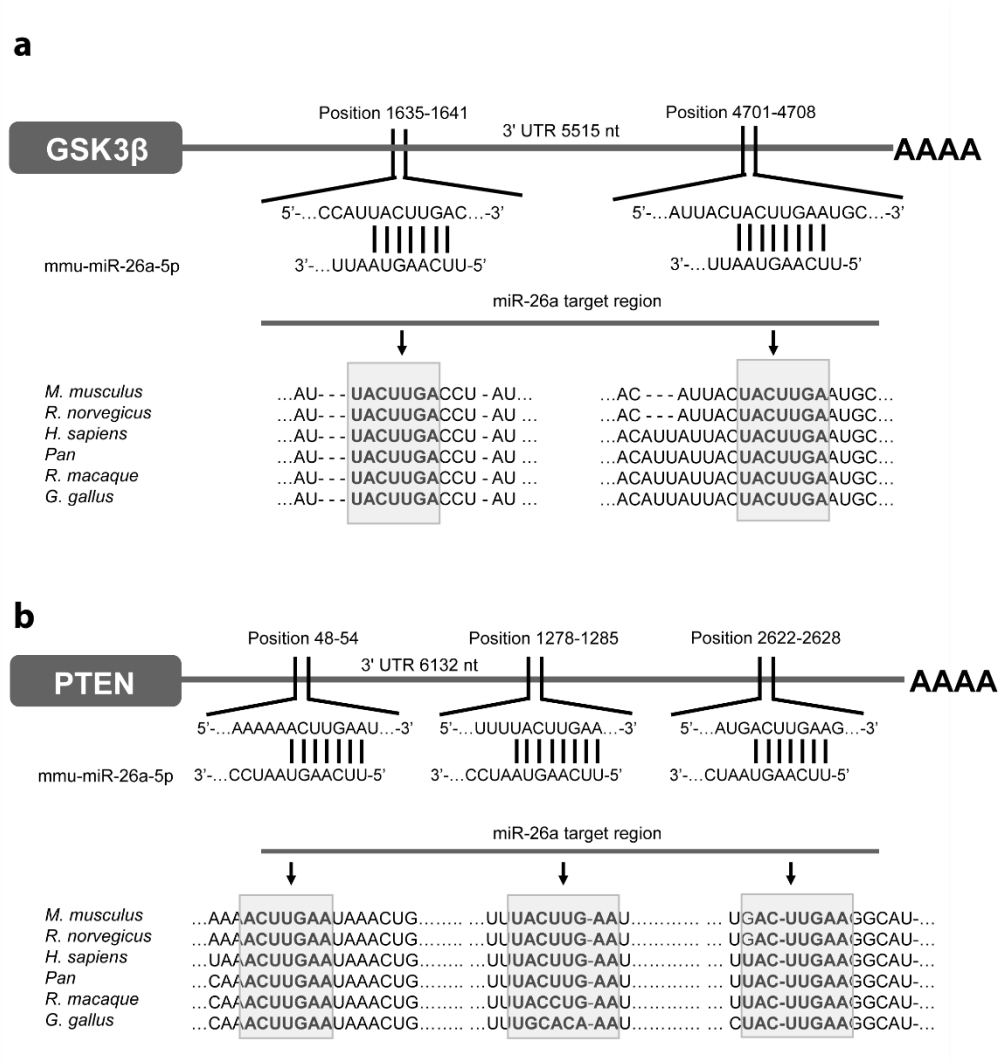

Figure 33: Gsk3β and Pten are targets of miR-26a. (**a**) Diagram of miR-26a sites within the 3′UTR of *Gsk3b* predicted by TargetScan, showing the complementary binding to miR-26a seed sequence and the conservation of miR-26a binding site across vertebrates. (**b**) Diagram of miR-26a sites within the 3′UTR of *Pten* predicted by TargetScan, showing the complementary binding to miR-26a seed sequence and the conservation of miR-26a binding site across vertebrates.

Over-expression of constitutively active GSK3β and PTEN disrupts axon formation and elongation (Jiang *et al.*, 2005), whereas knockdown of GSK3β and the use of specific inhibitors cause the formation of multiple axons (Gartner *et al.*, 2006; Jiang *et al.*, 2005). As depicted by Jiang *et al.* (2005), GSK3β

manipulations prevail over PTEN on neuronal polarity, indicating that PTEN acts upstream of GSK3β in polarity formation. For this reason, I decided to focus on the latter and test my hypothesis of whether GSK3β is a functional target of miR-26a in cortical primary neurons.

4.3.5 miR-26a regulates the expression levels of GSK3β protein in primary cortical neurons

To begin to elucidate the potential functions of GSK3β in cortical neuronal development, I first examined its expression in cortical neurons over development at the transcriptional level. Firstly, the same total RNA samples extracted from primary cortical cultures at four different timepoints to match with miR-26a levels, were again used to investigate GSK3β expression patterns in young neurons at 4 h, DIV2, DIV5 and DIV9 in culture. Relative quantification by RT-qPCR revealed that GSK3β is expressed in developing cortical neurons *in vitro* across all the timepoints analysed (average Ct of 20.91 from all samples tested), with a trend towards increase at DIV5 [**Figure 34**].

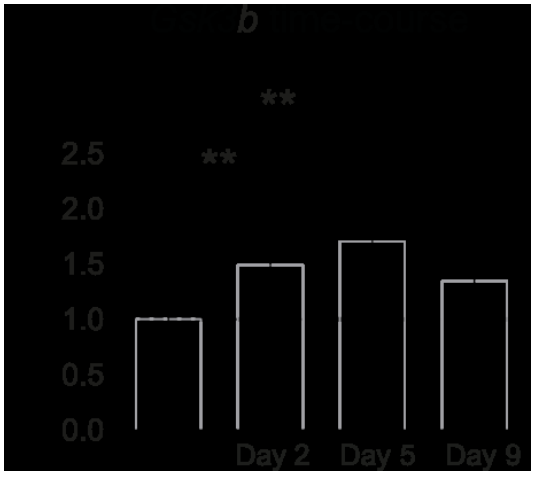

Figure 34: Gsk3b expression levels in cortical neurons. Quantification of *Gsk3b* expression levels over development of cortical primary cultures, from 4 h to 9 days *in vitro*. Expression of *Gsk3b* was analysed by relative quantification using the comparative Ct method (2-ΔΔCt). The geometric mean of Gapdh and Ube2 was used as reference; mean ± SEM of 5 independent experiments. One-way ANOVA with Bonferroni's multiple comparison post-hoc tests: **: $p \leq 0.01$.

Taking advantage of the morphological polarisation of cortical neurons *in vitro*, the next step was to investigate whether miR-26a can directly regulate

the expression levels of GSK3β protein in neuronal somas and/or growth cones by quantitative immunostaining. If GSK3β is an actual target of miR-26a in our model, the mimicking of miR-26a activity in cortical neurons should lead to a decrease in GSK3β levels. As shown in **Figure 35a-b**, over-expression of miR-26a drastically decreased GSK3β levels in both the soma and axonal growth cones, resulting in a significant ~40% decrease in the protein levels in both somas and growth cones when compared to non-targeting control oligos.

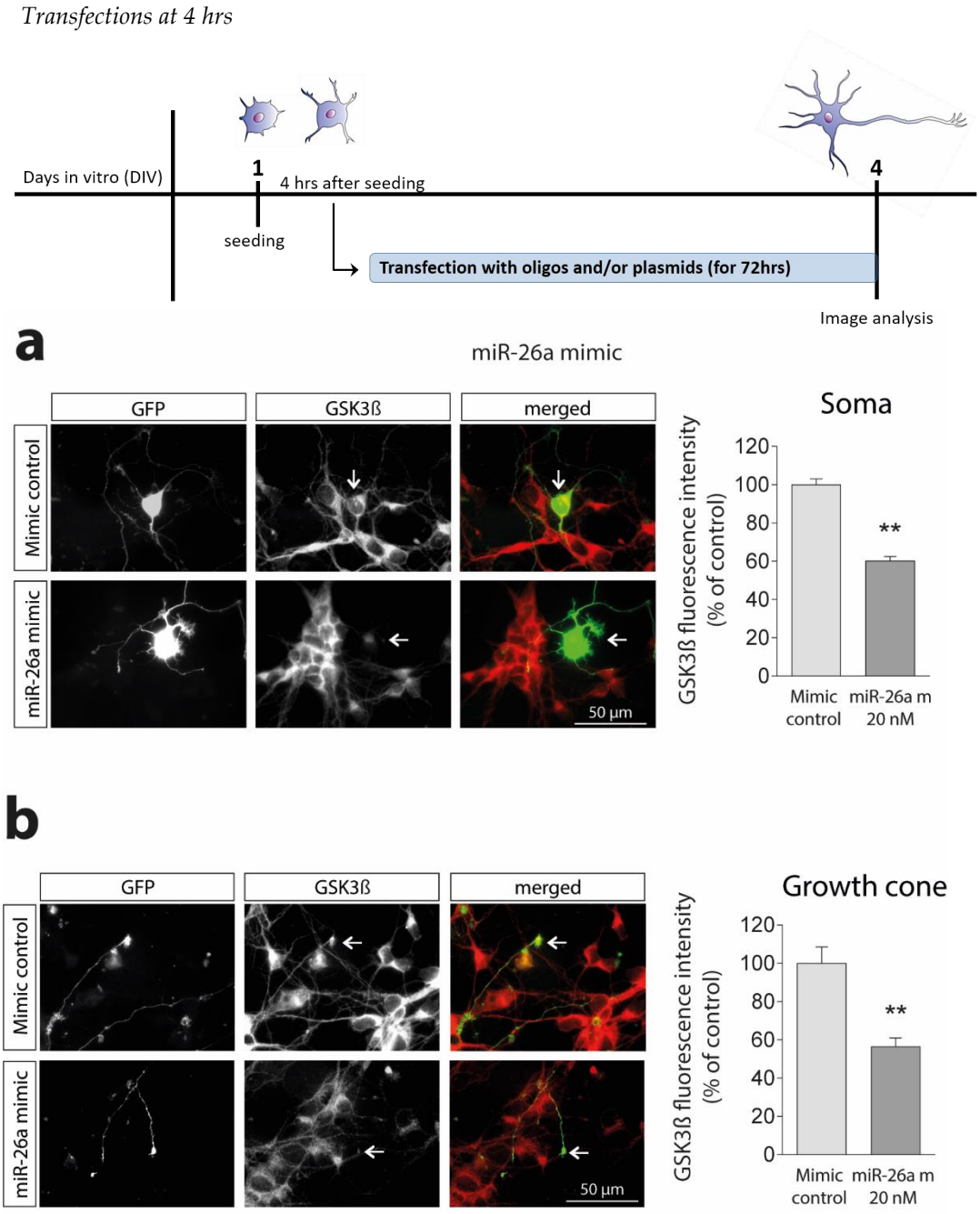

Figure 35: Over-expression of miR-26a regulates the expression levels of GSK3β protein in neuronal somas and growth cones. **(top)** Schematic representation of the experimental design**. (a**) Representative images of the soma of cortical neurons and (**b**) growth cones, after transfection with GFP plus a miR-26a mimic and immunostaining with GSK3β (red). Bar charts represent the quantification of GSK3β protein levels expressed as a percent of mimic controls, n=4. Data is expressed as mean ± SEM; Student's t-test: **: $p \leq 0.01$.

Conversely, inhibition of endogenous miR-26a drastically raised the levels of the GSK3β protein up to 50% in both morphological domains [**Figure 36a-b**]. This is an important observation as it may suggest a local effect of this microRNA in the axon compartment, as previously reported with other microRNAs (Bellon *et al.*, 2017; Dajas-Bailador *et al.*, 2012; Hancock *et al.*, 2014; Y. Zhang *et al.*, 2015). Further experiments using *in situ* hybridisation (FISH) could help better understand miR-26a mechanism of action at the transcript level. FISH detection of miR-26a effect on *Gsk3b* mRNA levels in cortical neurons could help reinforcing the results obtained by immunofluorescence.

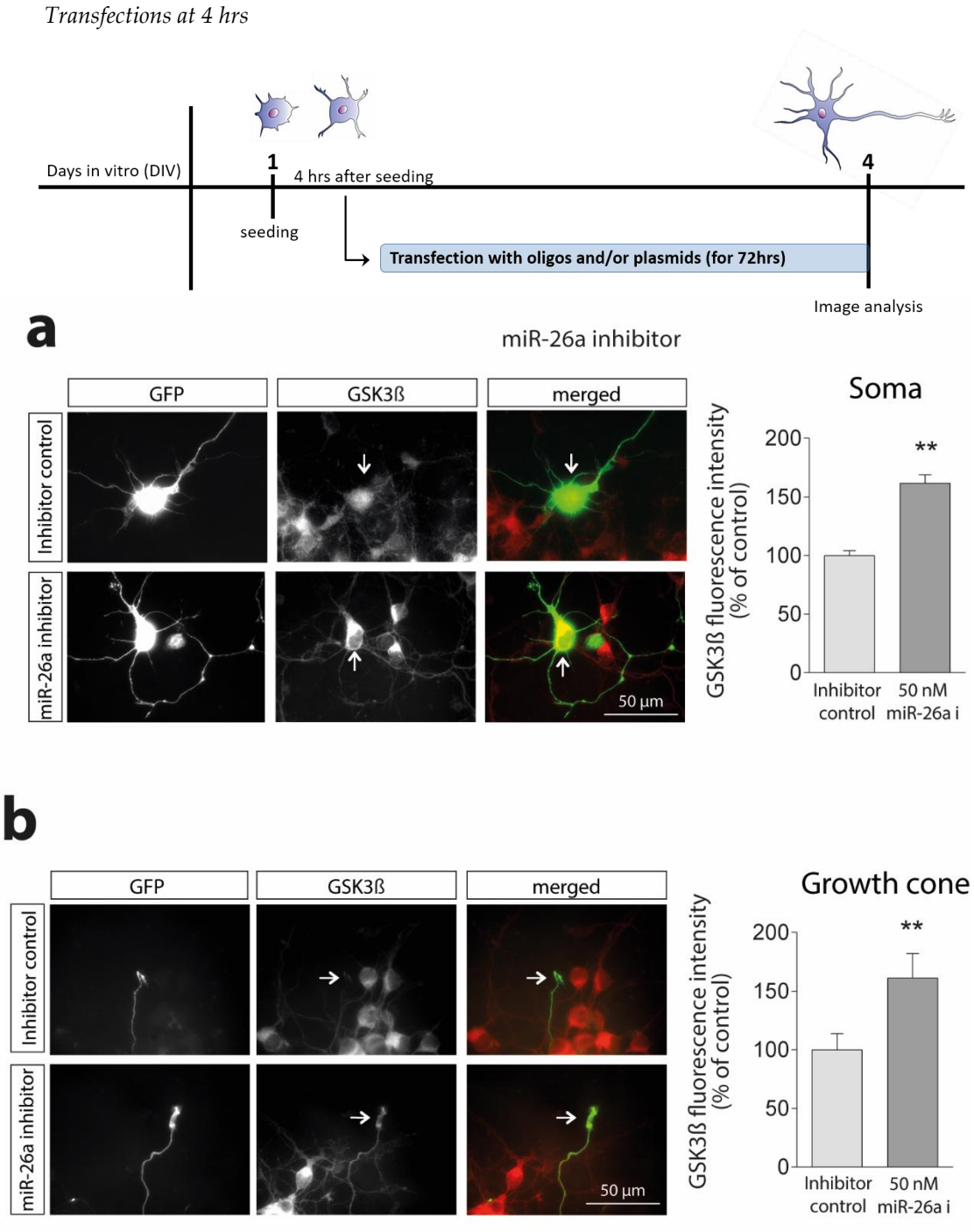

Figure 36: Inhibition of miR-26a regulates the expression levels of GSK3β protein in neuronal somas and growth cones. **(top)** Schematic representation of the experimental design**.** (**a**) Representative images of the soma of cortical neurons and (**b**) growth cones after transfection with GFP plus a miR-26a inhibitor and immunostaining with GSK3β (red). Bar charts represent the quantification of GSK3β protein levels expressed as a percent of non-targeting control, n=3. Data is expressed as mean ± SEM; Student's t-test: **: $p \leq 0.01$.

4.3.6 GSK3β mediates the functional effects of miR-26a in neuron polarisation and growth

Considering the capacity for miR-26a to control GSK3β protein levels in primary cortical neurons, the next obvious question that I wanted to address is whether miR-26a can in fact modulate endogenous GSK3β to regulate its actions in the developing axon of cortical neurons. Toward this aim, the first approach was to conduct gain- and loss-of-function experiments in primary cortical neurons to examine the impact of this protein alone in both axon specification and elongation. These experiments were performed at the same developmental stage selected for miR-26a functional assays. Mouse GSK3β coding sequence was subcloned into the pcDNA 3.1(+) vector (pcDNA-GSK3β) and co-transfected with a GFP plasmid into primary cortical neurons at 4 h after plating, whereas co-transfection with pcDNA 3.1(+) vector was used as control (pcDNA). At DIV4, GFP positive neurons were imaged and assessed for their ability to polarise and their axons measured following the same methodology as for previous miR-26a functional experiments. As depicted in **Figure 37b-d**, overexpression of GSK3β induced a decrease of ~25% in both axon length and % of polarised cells when compared to control. **Figure 37a-c** shows representative images of cortical neurons in both conditions and illustrates the validation of GSK3β overexpression conducted by immunostaining of the protein, which confirmed GSK3β overexpression by a striking increase in the protein fluorescence intensity.

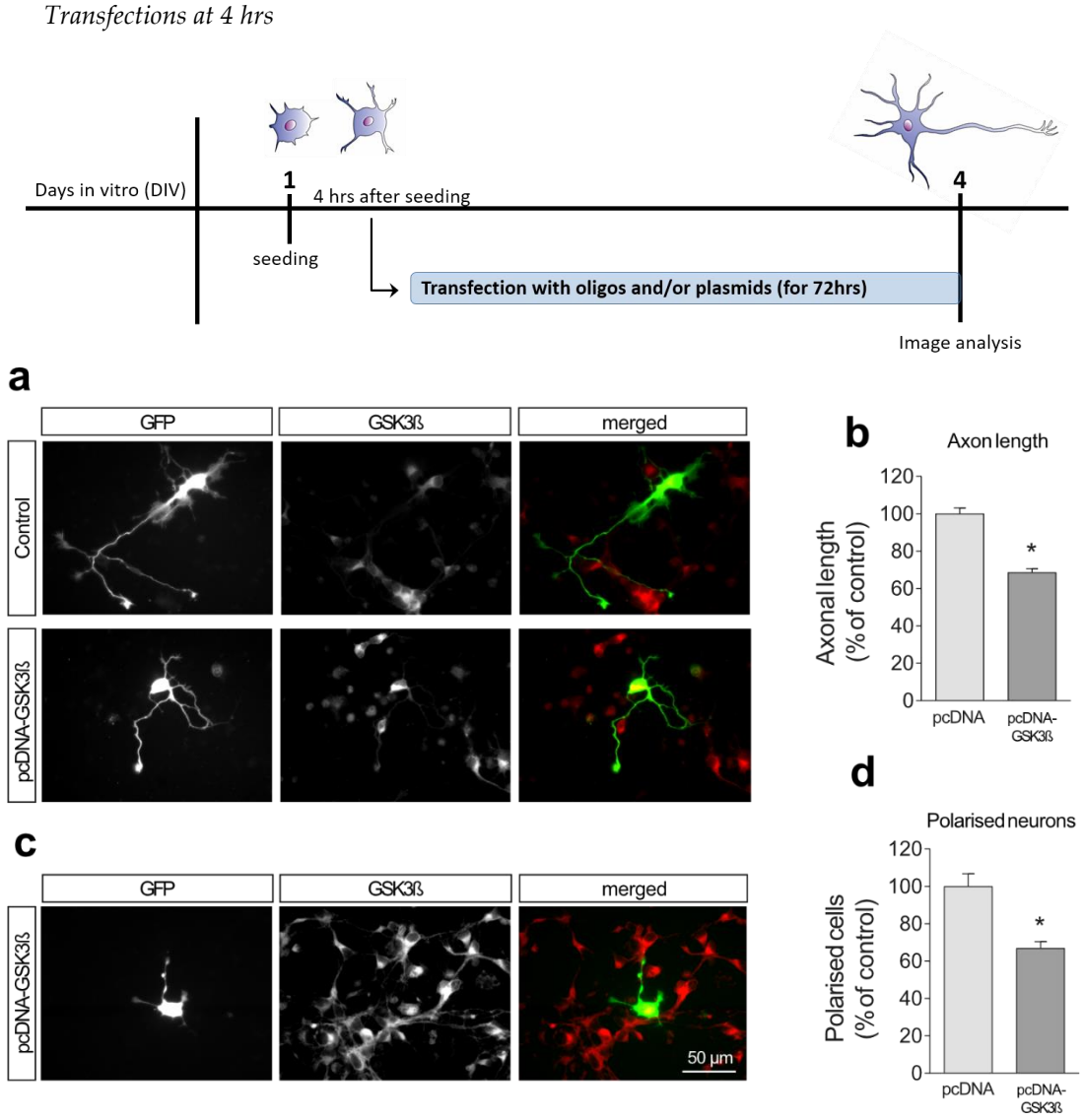

Figure 37: Over-expression of GSK3β in cortical neurons represses neuronal polarity and axonal outgrowth. **(top)** Schematic representation of the experimental design. (**a**) Representative images of cortical neurons after transfection with GFP plus either empty vector or pcDNA-GSK3β and immunostaining with GSK3β. (**b**) Quantification of axon length after over-expression of GSK3β showing up to 25 % decrease compared to the empty vector. (**c-d**) Representative images of cortical neurons showing the polarity changes induced by GSK3β over-expression and quantification of the number of polarised neurons after transfection with GFP plus either empty vector or pcDNA-GSK3β, n=4. Bar graphs expressed as mean ± SEM; Student's t-test: *: $p < 0.05$.

To inhibit GSK3β and evaluate the effect of its decreased activity in axon development, I employed SB415286, an extensively used and selective GSK3 pharmacological inhibitor that competes with ATP (Gobrecht et al., 2014; Guo et al., 2017; Jiang et al., 2005). As before, transfections with a GFP plasmid

were performed at 4 h after plating, whilst GSK3 inhibitor was used at a concentration of 1 μM and added to the culture 24 h after plating. Neurons were then analysed at DIV4 for their axon growth and morphological differentiation. As depicted in **Figure 38a-b**, inhibition of GSK3 produced a similar effect in increasing the number of polarised neurons and promoting axon outgrowth as observed in **Figure 27a-b** by mimicking miR-26a activity.

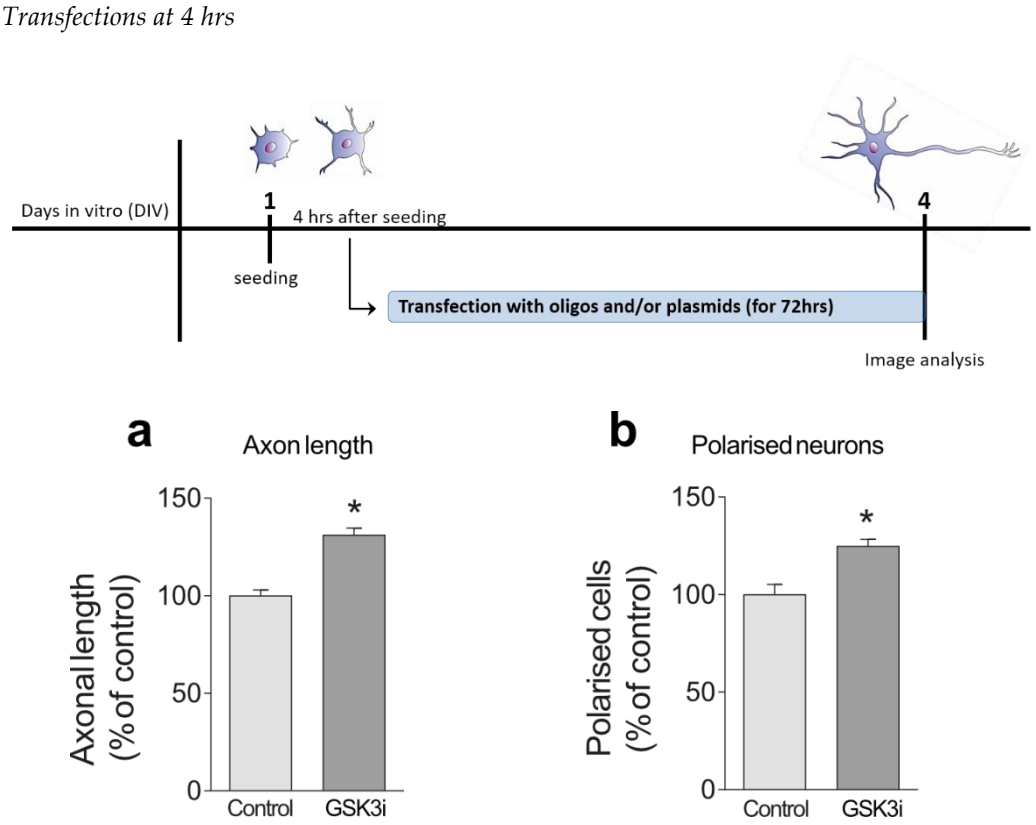

Figure 38: Inhibition of GSK3 promotes axon specification and extension in cortical neurons. **(top)** Schematic representation of the experimental design. (**a**) Quantification of axon length upon addition of GSK3 inhibitor (SB415286) 24 h after transfection with GFP, n=4. (**b**) Quantification of the number of polarised neurons upon application of GSK3 inhibitor (SB415286) 24 h after transfection with GFP, n=4. Bar graphs expressed as mean ± SEM; Student's t-test: *: $p < 0.05$

Taken together, these data confirmed the role of GSK3β in the specification and growth of axons in cortical neurons and are consistent with a scenario where miR-26a modulation of neuronal polarity and axonal outgrowth in primary cortical neurons occurs through repression of GSK3β.

A canonical experimental setup for confirmation of the specificity and validity of RNAi-activity-based data are phenotype rescue experiments through the expression of a siRNA-resistant sequence of the target gene (Cullen, 2006). Since this same principle can also be extended to miRNA activity and its target, I therefore directly tested whether GSK3β mediates the functional effects of miR-26a in neuron polarisation and growth by attempting functional rescue experiments after the inhibition and overexpression of miR-26a. For this, I exploited again the use of both GSK3 inhibitor and the pcDNA-GSK3β plasmid deprived of its 3'UTR in overexpression studies.

Pharmacological inhibition of GSK3 reversed the effect of the miR-26a inhibitor with regards to neuronal polarity. For this, transfections were again performed at 4 h after plating, with SB415286 (1 μM) being added 24 h after transfections. The inhibition of GSK3 abolished the drop in 23% of polarised cells after inhibition of miR-26a, returning to those seen in control conditions [**Figure 39a-b]**. The effect of pharmacological inhibition of GSK3 was not restricted to polarity, and also reverted the decrease in axon length after inhibition of miR-26a [**Figure 39c**]. Conversely, we found that over-expression of GSK3β (pcDNA-GSK3β) counterbalanced the increase in axon length after transfection with miR-26a mimic, whilst the empty vector (pcDNA) failed to affect the growth-promoting actions of miR-26a mimic [**Figure 39d-e**]. Thus, this observation suggests that miR-26a effects in the outgrowth of developing axons is mediated by its direct target, GSK3β.

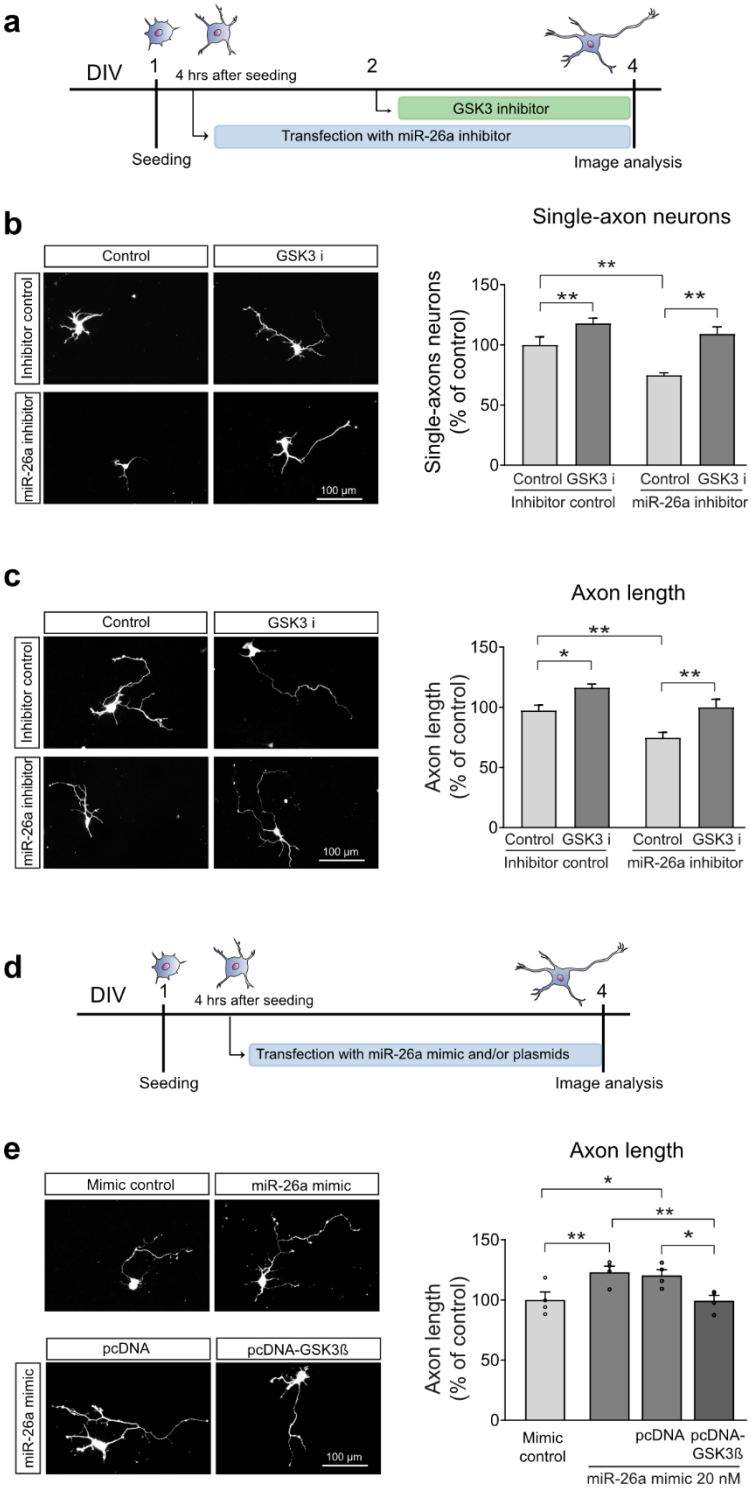

Figure 39: GSK3β mediates the functional effects of miR-26a in neuron polarisation and growth. (**a**) Diagrammatic representation of the experimental design used in **b** and **c**. (**b**) Representative images and quantification of the number of single-axon neurons after inhibition of miR-26a and the addition of GSK3 inhibitor (SB415286, 1 µM) 24 h after transfections, n=7. (**c**) Representative images and quantification of axon length after inhibition of miR-26a and the addition of GSK3 inhibitor (SB415286, 1 µM) 24 h after transfections, n=7. (**d**) Diagrammatic representation of the experimental design used in **e**. (**e**) Representative images and quantification of axon length after overexpression of both miR-26a and GSK3β, n=4. Data are mean±s.e.m. one-way ANOVA with Bonferroni's multiple comparison post-hoc tests, *P<0.05, **P≤0.01.

As mentioned at the beginning of this section, another validated target of miR-26a that could be mediating the effect observed in our model after miR-26a manipulations, is PTEN. Although GSK3β seems to be acting downstream of PTEN in axon-dendrite polarity formation and growth (Jiang *et al.*, 2005), I still wanted to investigate the impact of this protein on our system and whether the targeting of the protein is still necessary for miR-26a to affect neuronal polarity and growth. Hence, I employed the same experimental approach described above for GSK3β and subcloned mouse PTEN into the pcDNA 3.1(+) vector (pcDNA-PTEN) and performed gain-of-function experiments. Similar to GSK3β, over-expression of PTEN affected both axon specification and elongation [**Figure 40a-b**] and phenotype rescue experiments also confirmed that over-expression of PTEN compensated the rise in axon outgrowth after overexpression of miR-26a [**Figure 40c**]. Therefore, all together these results suggest that miR-26a is an important regulator of axon development through the PI3K–Akt–GSK3β signalling pathway [**Figure 4**].

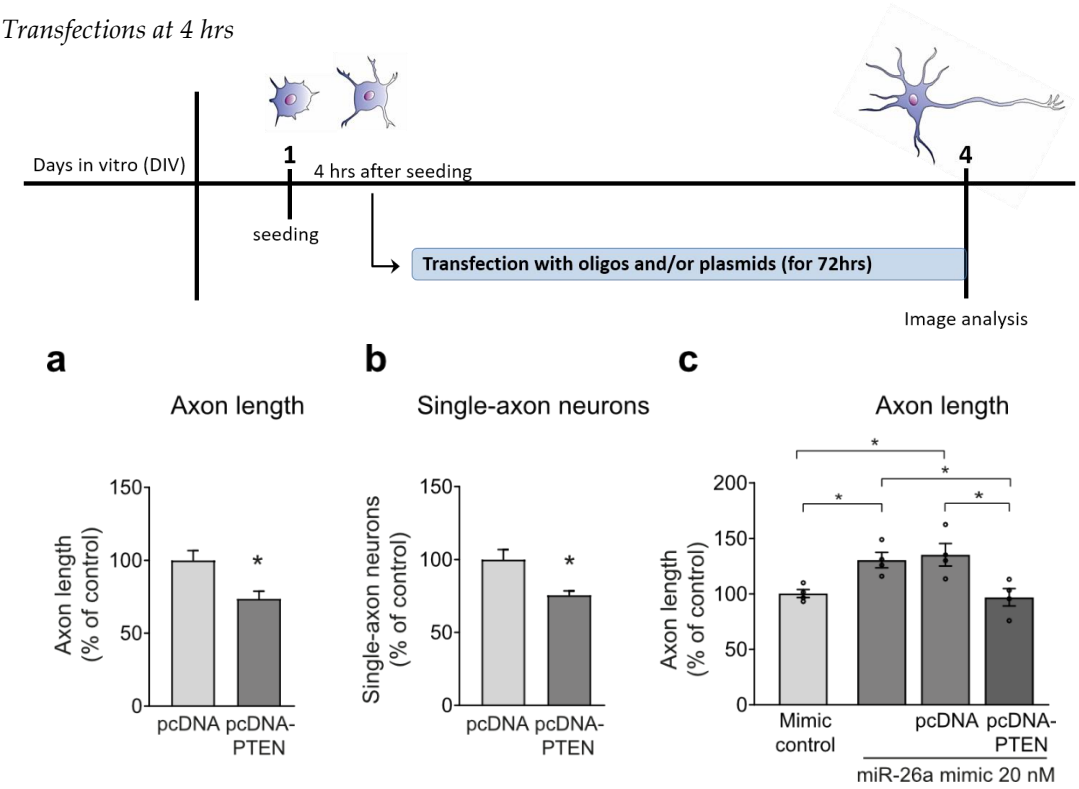

Figure 40: miR-26a regulates axon outgrowth via the targeting of Pten. **(top)** Schematic representation of the experimental design. (**a**) Quantification of axon length after over-expression of PTEN showing up to 20 % decrease compared to the empty vector n=4. (**b**) Quantification of the number of polarised neurons after transfection with GFP plus pcDNA-PTEN, n=3. (**c**) Quantification of axon length after overexpression of both miR-26a and PTEN, n=4. Data is expressed as mean ± SEM; Student's t test (a-b), one-way ANOVA with Dunnett's multiple comparisons post hoc tests (c): *: $p < 0.05$

4.4 Discussion

The tight control of multiple signalling pathways allows the development of axon/dendrite polarity in neurons and provides the structural platform for the establishment of neuronal communication in the nervous system (Barnes and Polleux, 2009; Namba *et al.*, 2015). The work presented in this chapter, applied bioinformatics, miRNA functional analysis and immunofluorescence approaches in mouse primary cortical cultures to examine the role of miR-26a, a microRNA previously found to be enriched in developing cortical axons, in neuronal polarity and axon elongation. Specifically, the data showed that this microRNA can modulate alone two distinct but also sequentially related cellular processes, axon specification and growth, *via* the targeting of GSK3β. Importantly, functional rescue experiments placed GSK3β at the centre of miR-26a-mediated actions in the specification and outgrowth of developing cortical axons, with GSK3β activity inhibition or overexpression leading to the phenotypic rescue of both the neuronal polarity and axonal growth defects caused by inhibiting or mimicking miR-26a activity.

The capacity for miR-26a to regulate these neuronal processes supports previous experimental evidence that axonal growth is not just a consequence of axonal specification (Jiang *et al.*, 2005). As such, the ablation of axons in order to eliminate length differences can reset axon-dendrite polarity (Bradke and Dotti, 2000; Dotti and Banker, 1987), while promoting neurite growth can lead to axon specification (Lamoureux *et al.*, 2002; Nakamuta *et al.*, 2011). It is still unclear how neurons can generate only one axon and multiple dendrites and the molecular mechanisms underlying the maintenance of neuronal polarity remain particularly elusive. The aforementioned studies have shown that neuronal polarity *in vitro* can be easily manipulated and reverted (Bradke and Dotti, 2000; Jiang *et al.*, 2005; Lamoureux *et al.*, 2002), but how can the formation of multiple axons, after an axon has been already specified, be

explained? A possible mechanism for this is that the transport of polarity effectors may not be fixed to the axon. Increasing the length of another neurite may function as force to redistribute polarity effectors. While neurite growth is usually impaired in minor neurites after axon specification (Arimura and Kaibuchi, 2007; Schelski and Bradke, 2017), this inhibition can be relieved by for example stabilising microtubules (Witte *et al.*, 2008) . This would trigger the redistribution of both polarity effectors and vesicle recycling (Bradke and Dotti, 2000; Jiang *et al.*, 2005) and cause the formation of multiple axons.

Although the molecular mechanisms that could differentiate axon specification *vs.* growth are the focus of active study, research has also shown that several molecules are actively involved in both processes (Arimura and Kaibuchi, 2007; Lewis *et al.*, 2013). Among them, GSK3β has the ability to crosstalk with most of the pathways reported to control these biological mechanisms, at the transcription, translation and cytoskeleton level, suggests that it may function as a central node in the coordination and integration of neural development and the establishment and maintenance of polarity (Beurel *et al.*, 2015; Guo *et al.*, 2016; Eun-MI Hur and Zhou, 2010; Inoki *et al.*, 2006; Kim and Snider, 2011).

The role of GSK3β in axonal growth has been demonstrated both at the developmental level (Eun-MI Hur and Zhou, 2010; Hur *et al.*, 2011; Kim *et al.*, 2006) and in regenerative processes following axonal injury (Iekmann and Fischer, 2015). In this regard, although the control of axonal growth has been long recognised (Kim and Snider, 2011), its precise role in regeneration has been more controversial (Leibinger *et al.*, 2017), mainly due to the fact that a multitude of regulatory pathways and targets can be involved in GSK3β activity. In effect, the capacity of GSK to control such an array of cellular functions may arise from the multiple sophisticated mechanisms that regulate its action and protein expression, ensuring that it can only phosphorylate

substrates at the precise time and in discreet subcellular compartments (Beurel *et al.*, 2015).

The unique ability of miR-26a to control both polarisation and axonal growth is mainly achieved *via* the targeting of GSK3β, which is a known regulator of both processes. Additionally, the fact that miR-26a can also control these processes *via* PTEN, an upstream member of the GSK3β signalling pathway, reinforces its role as an important regulator of axon development.

Maintaining local inactivation of GSK3β at the nascent axon is crucial for polarisation (Jiang *et al.*, 2005; Shi *et al.*, 2003; Yoshimura *et al.*, 2005) and inhibition of miR-26a breaks this equilibrium and impairs axon specification. On the other hand, global inhibition of GSK3 by small-molecule inhibitors or knocking down of *Gsk3b* induces the formation of multiple axons (Jiang *et al.*, 2005) and the over-expression of miR-26a was able to reproduce the same multiple "axon-like" neurites phenotype. It would be interesting to test, in future experiments, whether those "supernumerary" axons obtained after the over-expression of miR-26a are also electrically active. A recent study demonstrated that the multiple axons induced by GSK-3 inhibition have relatively intact AIS structures and they are capable of initiating action potentials before the recruitment of soma-dendritic components (Guo *et al.*, 2017), thus it is not unreasonable to speculate that this could also happen in our model and help to rebuild neuronal network activity after axon injury.

Once an axon has been specified, inactive GSK3β is restricted at its tips in cultured hippocampal neurons (Jiang *et al.*, 2005). Given the fact that our preliminary screening depicted miR-26a as axonal-enriched microRNA, and that its inhibition could increase the levels of the GSK3β protein in the growth cones of our cultures, it would be compelling to investigate whether this microRNA can regulate GSK3β in the axon and thus modulate its intrinsic development. From our data emerged that inhibition of miR-26a does not exert

a function on axon specification at later time points of cortical development (DIV5-DIV8). This is not totally unexpected, since this temporal window of cortical development corresponds to a period in which most of the axons have been already specified (Banker, 2018; Dotti *et al.*, 1988). However, due to technical limitation of the culture system, we could not establish whether this also applies with regards to axon elongation or if miR-26a preserves a function in this process. The use of microfluidic chamber in the same temporal window might help to address this question, and, at the same time, it would confirm with spatiotemporal accuracy the theory of an axonal role of miR-26a. This hypothesis will be tested directly in chapter 5.

Despite the growing number of studies demonstrating the importance of miRNAs in axon and synapse development (Rajman and Schratt, 2017; Swanger and Bassell, 2011), evidence for their role in axon specification and neuronal polarisation has been largely missing. Only recently, miR-338 was shown to have a role in neuronal placement and polarisation in the cortical plate, controlling neuronal polarity, migration and or cortical placement cues (Kos *et al.*, 2017a). Furthermore, a recent paper by Ambrozkiewicz *et al.* (2018) demonstrated the capacity of miR-140 to act synergistically with its host gene E3 ubiquitin ligase WW-Containing Protein 2 (*Wwp2)* and *Wwp1* in the establishment of axon-dendrite polarity of developing cortical neurons *in vivo.* An aspect that has not been addressed so far is whether the effects of miR-26a with regards to neuronal polarity *in vitro* can also be translated and reproduced *in vivo*. As Kos and co-workers showed, the manipulation of miRNAs levels using a sequence-specific miR-sponge through *In utero* electroporation (IUE) technique, it might be a feasible experimental approach to delineate the *in vivo* function of a specific RNA in corticogenesis (Kos *et al.*, 2017a). Moreover, this approach overcome the limitations of a global miRNA-depletion approach, in which the conditional deletion of essential genes for

miRNA biogenesis such as *Dicer* causes gross anatomical abnormalities and overwhelming amount of apoptosis (Davis *et al.*, 2008; Makeyev *et al.*, 2007).

However, in the case of miR-26a, even its specific inhibition through *in utero* electroporation approaches could become ambitious. In fact, a yet unpublished study identified the miR-26 family, including miR-26a, as a regulatory RNA network required for neurogenesis, thus, inhibition of miR-26a *in vivo* might disrupt neuronal migration and differentiation, making the unification of results a seemingly impossible task (M. Sauer, 2017).

CHAPTER 5:

Axonal miR-26a spatiotemporally regulates GSK3β

5.1 Introduction

As described in the general introduction, neuronal miRNAs were initially implicated in early stages of nervous system development, but only relatively recently their functions in postmitotic neurons have started to be investigated. Because of the compartmentalised nature of neurons, the dendritic localisation of proteins belonging to the miRNA biogenesis pathway in mature neurons (Lugli *et al.*, 2005) and the isolation of several miRNAs associated with the translational machinery of postmitotic neurons (Kim et al., 2004) provided the rationale for miRNAs to operate locally within specific neuronal compartments to regulate the expression of a subset of mRNAs. This allows neurons to control protein synthesis with temporal and spatial resolution in fundamental neuronal processes, such as neurite outgrowth, axon guidance, synapse formation, and, ultimately cognitive brain function (McNeill and Van Vactor, 2012). In effect, studies have depicted a pivotal role of miRNAs in fine tuning the axonal translation of local mRNAs over several steps of axon development (Iyer *et al.*, 2014; McNeill and Van Vactor, 2012). In chapter 4, we showed that the axon-enriched miR-26a is highly expressed in neuronal cultures and regulates both neuronal polarity and axon growth *via* the targeting of GSK3β. Moreover, we hinted at the possibility that this microRNA might have a local effect in the axon of cortical neurons, given the fact that inhibition of the endogenous miR-26a can raise the GSK3β protein levels in growth cones [**Figure 36b]**. In the final experimental chapter of this thesis, I will explore this possibility. First, I will give a brief introduction of the function of miRNAs compartmentalised in the axon of different neurons with regards to axon development.

5.1.1 MicroRNAs in the axon

A large investigation of the miRNA expression profile in axons revealed around 137 miRNAs in distal axons, four of which were highly enriched in the axon in comparison to the cell body (Natera-Naranjo *et al.*, 2010). In this elegant work, superior cervical ganglia neurons were cultured in a compartmentalised Campenot culture chamber (Eng *et al.*, 1999) in which axons are isolated from their cell bodies. miRNAs were identified from the pure axonal RNA fraction by microarray analysis and further validated by qPCR (Natera-Naranjo *et al.*, 2010). In a more recent study, using a "neuronal ball" for an efficient spatial separation of large amounts of purified axons, seven axon-enriched miRNAs were found to be localised to distal axons and growth cones (Sasaki *et al.*, 2014).

Although the repertoire of axonal miRNAs has been greatly expanded by profiling studies, very little is known about the function of specific miRNAs in the developing axon. Using mice cortical neurons, Dajas-Bailador and co-workers first revealed that a miRNA, miR-9, modulates the translational repression of Map1b and that this process can be regulated by BDNF-dependent signalling processes in the axon. Inhibition of miR-9 affected axonal growth only when applied locally in axons, suggesting that BDNF affects this developmental process *via* local, miRNA-mediated translational control of a cytoskeletal regulator (Dajas-Bailador *et al.*, 2012). A further confirmation of these local mechanisms came later with the axon-enriched miR-132, which promotes embryonic DRG axon outgrowth by targeting endogenous p120RasGAP (Rasa1), a protein involved in cytoskeletal regulation (Hancock *et al.*, 2014). In this work, miR-132 induced the increase in axonal Rasa1 protein levels and the process was dependent on local protein synthesis, demonstrated by the abolishment of this process when a translation inhibitor was applied to severed axons (Hancock *et al.*, 2014). In another work conducted in parallel

BDNF, has been also found to promote axonal branching in the developing mouse retina through up-regulation of miR-132, which in turn downregulates its known target Rho family GTPase-activating protein, p250GAP (Marler *et al.*, 2014).

miR-16, a brain and axon-enriched miRNA (Natera-Naranjo *et al.*, 2010) has been described as regulator of the local protein synthesis machinery in distal axons, *via* the targeting of eukaryotic initiation factor-2B (eIF2B2) and the eukaryotic translation initiation factors 4 gamma 2 (eIF4G2) (Kar *et al.*, 2013). Transfection of the precursor miRNA in the axon modulated both mRNA and protein levels of eIF2B2 and eIF4G2 as well as axon growth. After metabolic labelling studies, downregulation of axonal eIF2B2 and eIF4G2 suppressed both local axonal protein synthesis and axon outgrowth (Kar *et al.*, 2013).

Axonal over-expression of the miR-17-92 cluster in microfluidic compartmentalised culture of embryonic cortical neurons promoted axonal outgrowth, whereas axonal inhibition of endogenous miR-19a, a key component of this cluster, supressed outgrowth by regulating local expression of PTEN (Y. Zhang *et al.*, 2013). More recent research has identified miR-181d as an axon-enriched miRNA that regulates axon elongation by locally targeting two mRNAs, MAP1B and calmodulin, in the microfluidic culture of embryonic DRG neurons (Wang *et al.*, 2015)

Finally, as already mentioned, microRNAs can also regulate correct axon guidance (Holt *et al.*, 2019). In a fish model of axonal growth, the knockdown of miR-204 leads to misguided growth of RGC axons into retinal layers *via* the targeting of ephrin type receptor B 2 (Ephb2) and ephrin B3 (Efnb3) (Conte *et al.*, 2014). On the other hand, over-expression of miR-204 rescued these defects (Conte *et al.*, 2014). More recently, miR-182 was described to regulate growth cone responsiveness to Slit Guidance Ligand 2 (Slit-2) in the same model. Both i*n vitro* and *in vivo* experiments demonstrated that miR-182 is locally

suppressing the axonal translation of cofilin-1, a cytoskeleton regulator. Accordingly, loss of miR-182 caused RGC axon targeting defects *in vivo* and impaired Slit2-induced growth cone repulsion (Bellon *et al.*, 2017).

5.2 Aims of this chapter

As mentioned in different parts of this thesis, the subcellular localisation and translation of mRNAs in the axon are essential for axon elongation, branching, and survival. Recent studies have denoted the vast diversity of axonal miRNAs and have identified miRNA-based functions in the translational control of local protein synthesis. However, the localisation, function, and regulatory mechanism of numerous miRNAs in the axon remain unknown.

Our preliminary screening depicted miR-26a as one of the highly enriched miRNAs in developing axons, and data in chapter 4 suggested that it might exert a function in the axonal compartment. Hence, the specific aims of this part of the thesis are as follows:

- Address the axonal localisation of miR-26a and its function in axon growth;
- Investigate whether miR-26a is locally controlling axon elongation through GSK3β;
- Elucidate the molecular mechanisms through with miR-26a is regulating axonal length locally in the axon compartment.

5. Results:

5.1 Localised inhibition of miR-26a in the axon can regulate axonal growth

The capacity for miRNAs to regulate axon development, and to do so by localising to the axon compartment is a relatively new area of investigation (Bellon *et al.*, 2017; Dajas-Bailador *et al.*, 2012; Wang and Bao, 2017). As described in the introductory section of chapter 4, a deep sequencing screening identified miR-26a in a subset of axon-enriched microRNAs. Moreover, since inhibition of endogenous miR-26a drastically raised the levels of the GSK3β protein up to 50% in the axonal domain [**Figure 36b**], it is possible to speculate that miR-26a might have a local effect in the axon, controlling axon outgrowth through the regulation of GSK3β. To address if this potential mechanism was relevant in the effects observed for miR-26a, I first assessed the presence of *Gsk3b* mRNA in the axons of cortical primary neurons. For this, I cultured neurons in compartmentalised microfluidic chambers, which allow the morphological and functional separation of axons from somas (Taylor *et al.*, 2005). As shown in **Figure 41**, I could detect both miR-26a and *Gsk3b* mRNA in qPCR experiments using axonal RNA (Poulopoulos *et al.*, 2019).

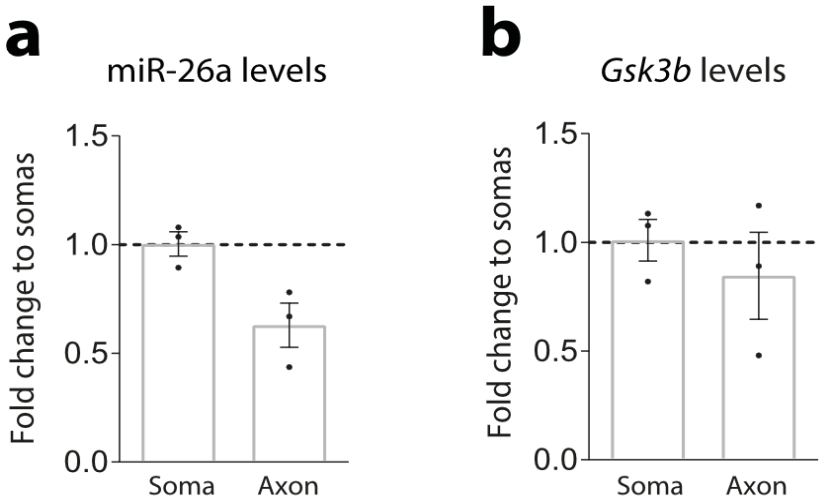

Figure 41: Expression of Gsk3b in the axons of cortical neurons. *(a)* Quantification of miR-26a levels in the axonal fraction of cortical primary cultures relative to soma. Expression of miR-26a-5p was analysed by relative quantification using the comparative Ct method (2-ΔΔCt) and the geometric mean of miR-100-5p, miR-128-3p, miR-434-3p and let7a-5p used as endogenous reference. miR-26a detection levels in the axons were within the range of detection for mature miRNAs (Average C_T value = 29.95) and comparable to previous miRNA qPCR quantification experiments in cortical and DRG axons (Natera-Naranjo *et al.*, 2010; Zhang *et al.*, 2013); mean±SEM of 3 independent experiments. (**b**) Quantification of Gsk3b expression levels in the axonal fraction of cortical primary cultures relative to soma. Expression of Gsk3b was analysed by relative quantification using the comparative Ct method (2-ΔΔCt). The geometric mean of Gapdh and Ube2 was used as endogenous reference; mean ± SEM of 3 independent experiments.

Considering the presence in the axon of both miR-26a and GSK3β, the following step was to carry out functional assays in microfluidic compartmentalised cortical cultures, with the aim to determine the role of intra-axonal miR-26a in axon growth. As described in Chapter 3, one of advantages of these microfluidic chambers compared to other compartmentalised models is their ability to fluidically isolate the axonal (axon side) from the somato-dendritic compartment (soma side) by employing differential hydrostatic pressure. This allows for the selective manipulation in

either the axons or somas, without affecting the other compartment (Taylor *et al.*, 2005).

Accordingly, neurons were seeded in the soma side and grown for 5-6 days in order to allow a significant number of axons to cross into the axonal side of the device. At this point, the cell permeable inhibitor of miR-26a was added to either the soma or axon compartment of the chambers and the length of the axons was recorded at 24 h and 48 h after application (0 h) of either the cell-permeable miR-26a inhibitor or inhibitor control (100 nM). As depicted in **Figure 42a**, axonal outgrowth is drastically reduced when the miR-26a inhibitor is applied exclusively in the axonal side of microfluidic chambers at both 24 h and 48 h after application. Importantly, this effect on axonal growth is not observed when the miR-26a inhibitor was added to the soma side [**Figure 42b**]. Since the reduction in axon length was already significantly visible at 24 h after the selective axonal inhibition of miR-26a, I therefore used this time point as a cut off to evaluate axonal outgrowth in future experiments, thus facilitating the analysis and minimising off-target effects of pharmacological inhibitors that will be described in the following sections. Overall, these results indicate that miR-26a controls axonal growth in cortical neurons up to 8 days after plating by acting in the axon compartment.

Time course of compartmentalised inhibition of miR-26a

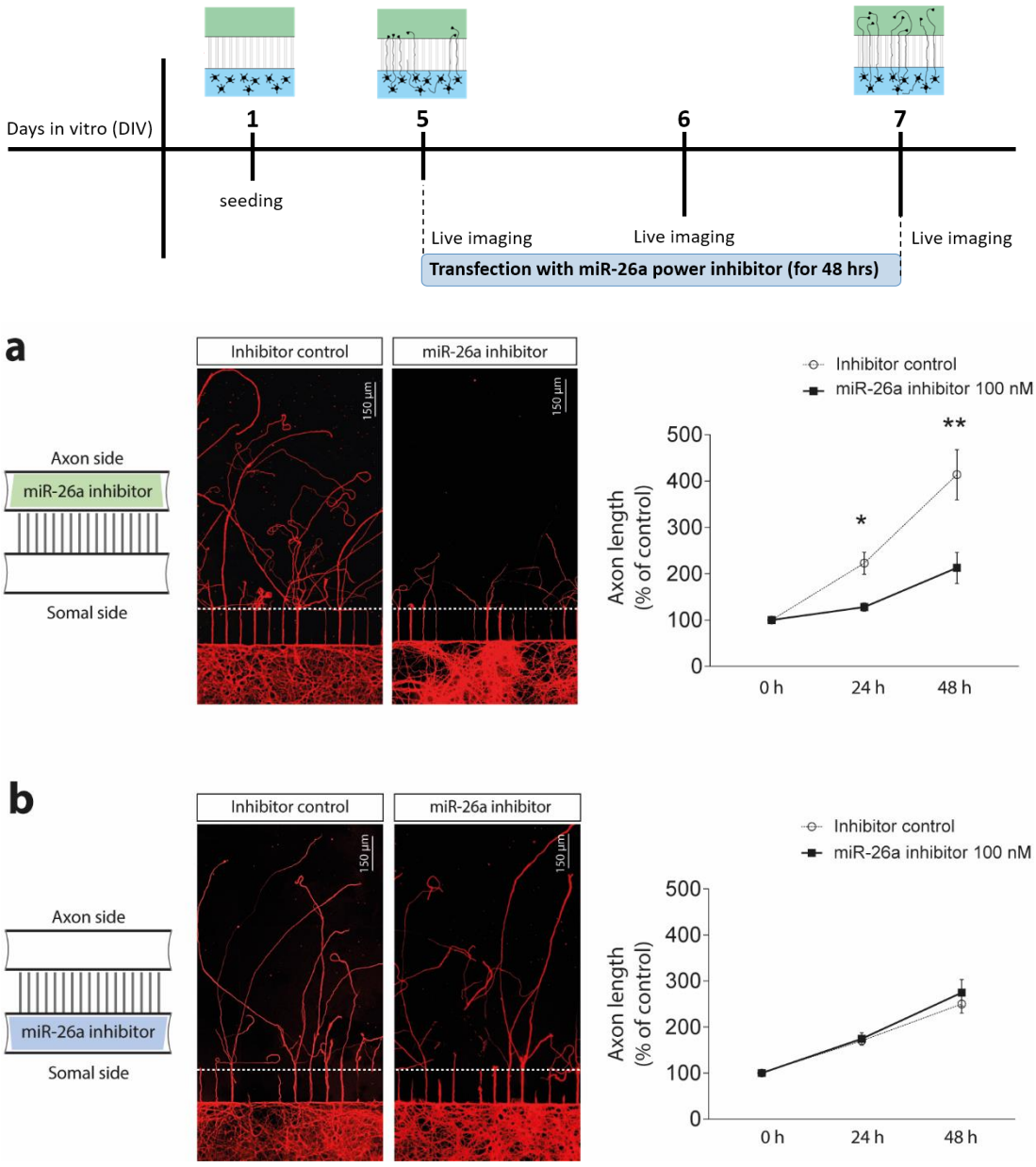

Figure 42: Localised inhibition of miR-26a in the axon can regulate axonal growth. Cell-permeable miR-26a inhibitor or inhibitor negative control was applied to compartmentalised cultures at DIV5 and axons growing in the axonal side were measured at 0 h, 24 h and 48 h after application. (**top**) Schematic representation of the experimental design. (**a**) Inhibition of endogenous axonal miR-26a by application of a cell-permeable miR-26a inhibitor to the axonal compartment induced a decrease in axon length in comparison to a cell-permeable inhibitor negative control, 48 h after application. (**b**) When the cell-permeable miR-26a inhibitor or inhibitor negative control were applied to the somal compartment, no significant effect was observed in the length of axons growing in the axonal side. For all the experiments, schematics of the microfluidic chambers (left corner) depict where drugs were added. Application to the axon and the soma side is illustrated in green and blue, respectively. Data normalised to axon length at T=0 h (t0) and presented as percentage of t0, n=4. Data is shown as mean ± SEM; one-way ANOVA with Bonferroni pots-hoc test, *: $p < 0.05$, **: $p \leq 0.01$

5.2 Localised inhibition of miR-26a in the axon regulate axonal growth via GSK3β signalling

To further test the local effects of miR-26a effects and considering the presence of *Gsk3b* mRNA in the axon, I hypothesised that axonal application of the GSK3 inhibitor would rescue the locally-mediated decrease in axon growth induced by inhibition of miR-26a. For this, I cultured cortical neurons in microfluidic chambers for 5-6 days as previously described and applied the cell-permeable miR-26a inhibitor in the axon side for 24 h, together with the GSK3 inhibitor (SB415286). As predicted, addition of SB415286 into the axonal compartment abolished the decrease in axonal growth mediated by local application of the miR-26a inhibitor [**Figure 43a-b**]. However, unlike the experiments with miR-26a inhibitor alone, which failed to affect axonal growth when applied to the soma, the addition of the GSK3 inhibitor to the soma side also rescued the decrease in axon length observed after axonal inhibition of miR-26a function [**Figure 43c**]. The fact that GSK3 inhibition on its own [**Figure 43b-c**] did not increase axonal length when applied on the soma or axon sides would suggest that in conditions of active axonal growth, the GSK3β activity controlling this process is relatively low. The observation that GSK3β activity in the soma is necessary to prevent axon growth after miR-26a inhibition is in agreement with previous findings (Jiang *et al.*, 2015). However, unlike Jiang *et al*, our results also demonstrate that a local effect of miR-26a present in the axon is required for this to occur. Overall, these set of results suggest the interesting possibility that GSK3β might be locally translated in the axon and that this process is a pre-requisite for a functional outcome in the cell body. According to this hypothesis, although newly synthesised GSK3β may still act on axon-local mechanisms, likely impacting on cytoskeletal dynamics, it also undergoes retrograde transport towards the soma where it activates further regulatory mechanisms controlling axonal growth. This retrograde transport

of locally synthesised proteins as a mechanism of axonal signalling has been only demonstrated for a small number of transcription factors (Cox *et al.*, 2008; Ji and Jaffrey, 2012; Willis *et al.*, 2007), and very recently in a neurodegenerative CNS neuronal model (Walker *et al.*, 2018). However, this functional mechanism has not been shown for intra-axonal miRNA-regulated translation.

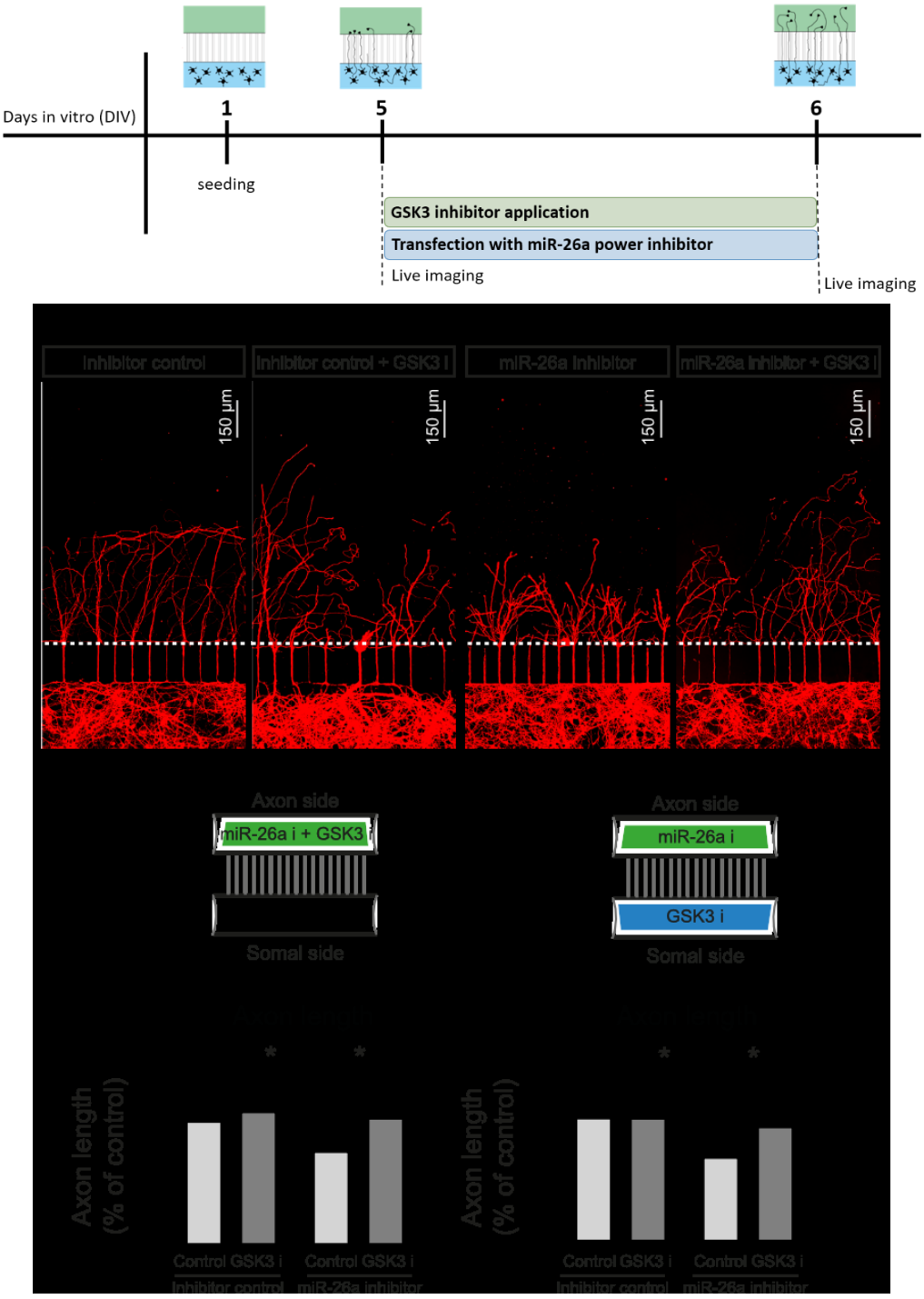

Figure 43: Localised inhibition of miR-26a in the axon regulate axonal growth via GSK3β signalling. **(top)** Schematic representation of the experimental design. (**a**) Representative images and (**b**) quantification of axonal length after using both miR-26a and GSK3 inhibitor (SB415286) applied to the axonal side of microfluidic chambers, n=5. (**c**) Quantification of axonal length after application of miR-26a in the axon side and GSK3 inhibitor in the somal side of microfluidic chambers, n=4. For all experiments, schematics of the microfluidic chambers (above the graph) depict where drugs were added. Application to the axon and the soma side is illustrated in green and blue, respectively. Data is expressed as mean ± SEM; Kruskal-Wallis with Dunn's multiple comparison test (**b**), one-way ANOVA with Bonferroni's multiple comparison post-hoc tests (**c**): *: $p < 0.05$.

5.3 Retrograde transport of locally translated GSK3β is required for the regulation of axonal growth after inhibition of miR-26a in the axon

In order to test this hypothesis, I first decided to investigate the levels of GSK3β protein in the axon and soma of cortical neurons after compartmentalised application of the miR-26a inhibitor. Neurons were seeded in the soma side and grown for 5-6 days and the cell permeable inhibitor of miR-26a was added to either the soma or axon compartment of the chambers. At 24 h after application of the inhibitor, the levels of GSK3β protein in neuronal somas and/or growth cones were quantified by quantitative immunostaining. As shown in **Figure 44a-b** inhibition of miR-26a only in the axon compartment of microfluidic chambers produced a significant increase in GSK3β protein levels, both in the axon and soma of cortical neurons. Conversely, application of the inhibitor only to the soma side, failed to produce an increase in GSK3β protein, both in the soma and axons when compared to non-targeting oligonucleotides **[Figure 44c-d]**. The latter result is particularly interesting as it supports the hypothesis of an axon-exclusive regulation of GSK3β translation *via* miR-26a. Moreover, it also suggests that passive diffusion of the inhibitor along the axon is not a likely explanation for the observed effects. Overall, these findings support the idea that miR-26a can regulate GSK3β levels in the axon, but to achieve its full functional effect, it requires an increase in GSK3β levels in the soma that is axon dependent.

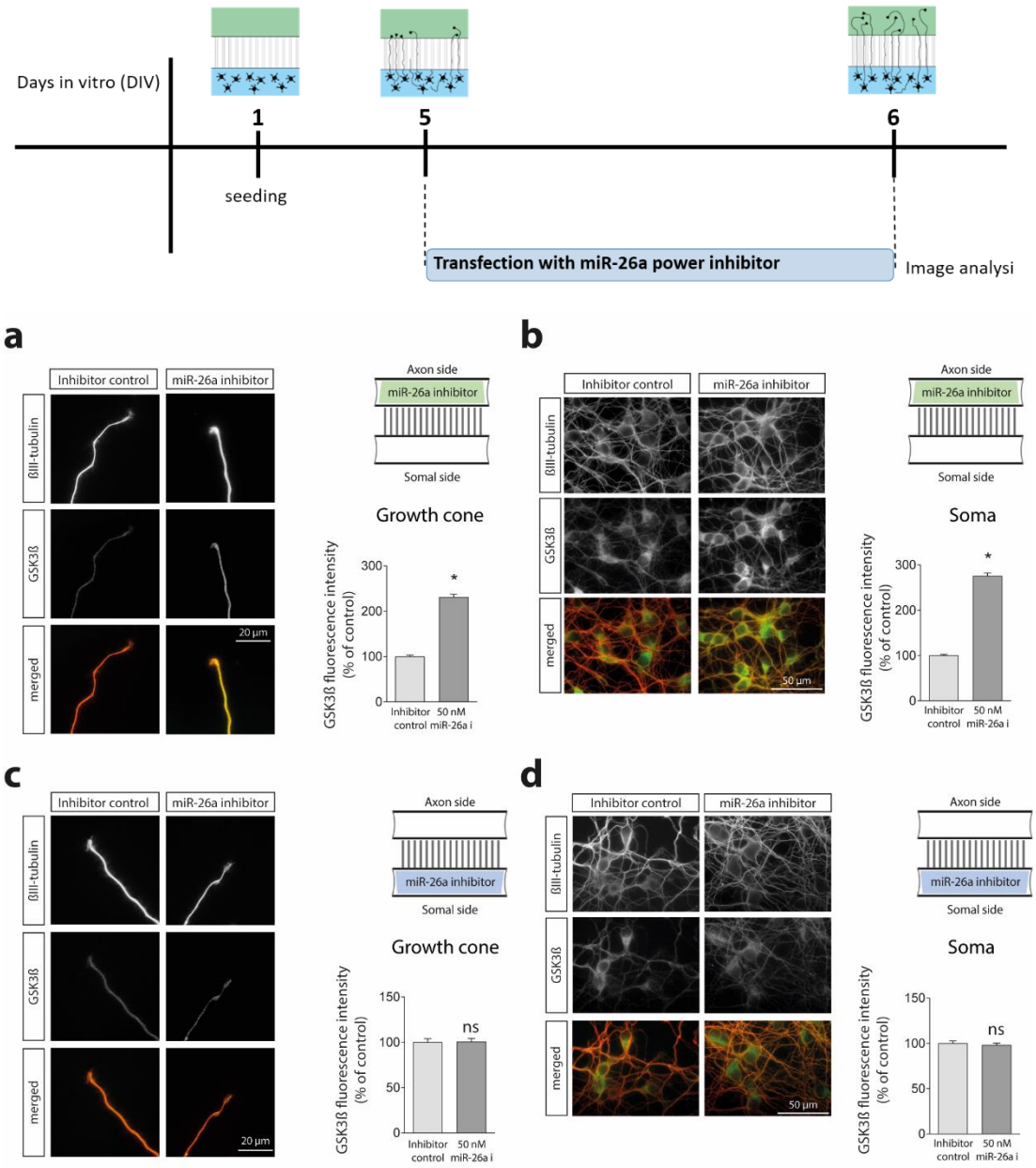

Figure 44: miR-26a regulates the expression levels of GSK3β protein in neuronal somas and growth cones only when is inhibited in the axon. (**top**) Schematic representation of the experimental design. Representative images and quantification of GSK3β protein levels in the (**a**) growth cones and (**b**) somas of cortical neurons after local application of cell-permeable miR-26a inhibitor in the axon side of microfluidic chambers, n=5. Representative images and quantification of GSK3β protein levels in the (**c**) growth cones and (**d**) somas of cortical neurons after local application of cell-permeable miR-26a inhibitor in the somal side of microfluidic chambers, n=4. Data is expressed as mean ± SEM; Student's t-test: *: $p < 0.05$.

If this is true, blocking axonal transport exclusively in the axonal compartment might further increase the levels of GSK3β protein in the growth cones and consequently impair the previously described somatic accumulation of the protein after axonal inhibition of miR-26a. To test the validity of this novel mechanism, a compartmentalised culture model where axonal transport was disrupted was implemented. For this I used a microtubule-destabilising drug, nocodazole, which has been previously demonstrated to impair axonal transport without dramatically impacting the neuron's viability (Gobrecht *et al.*, 2014; Saijilafu *et al.*, 2013). In this experiment, cortical axons were treated with nocodazole 18 h after axonal application of miR-26a inhibitor and imaged 6 h later. As shown in **Figure 45a**, addition of nocodazole after inhibition of miR-26a in the axon led to a further increase in axonal GSK3β levels, but crucially, prevented the previously observed increase in the soma [**Figure 45b**]. Overall, when these experiments are put together with our functional studies, they provide demonstration of two key mechanisms. First, local synthesis of GSK3β in the axon is regulated by axonal miR-26a, which is normally repressing its translation. Secondly, regulation of axonal growth by GSK3β after release of miR-26a repression requires the transport of newly synthesised GSK3β to the somas of cortical neurons.

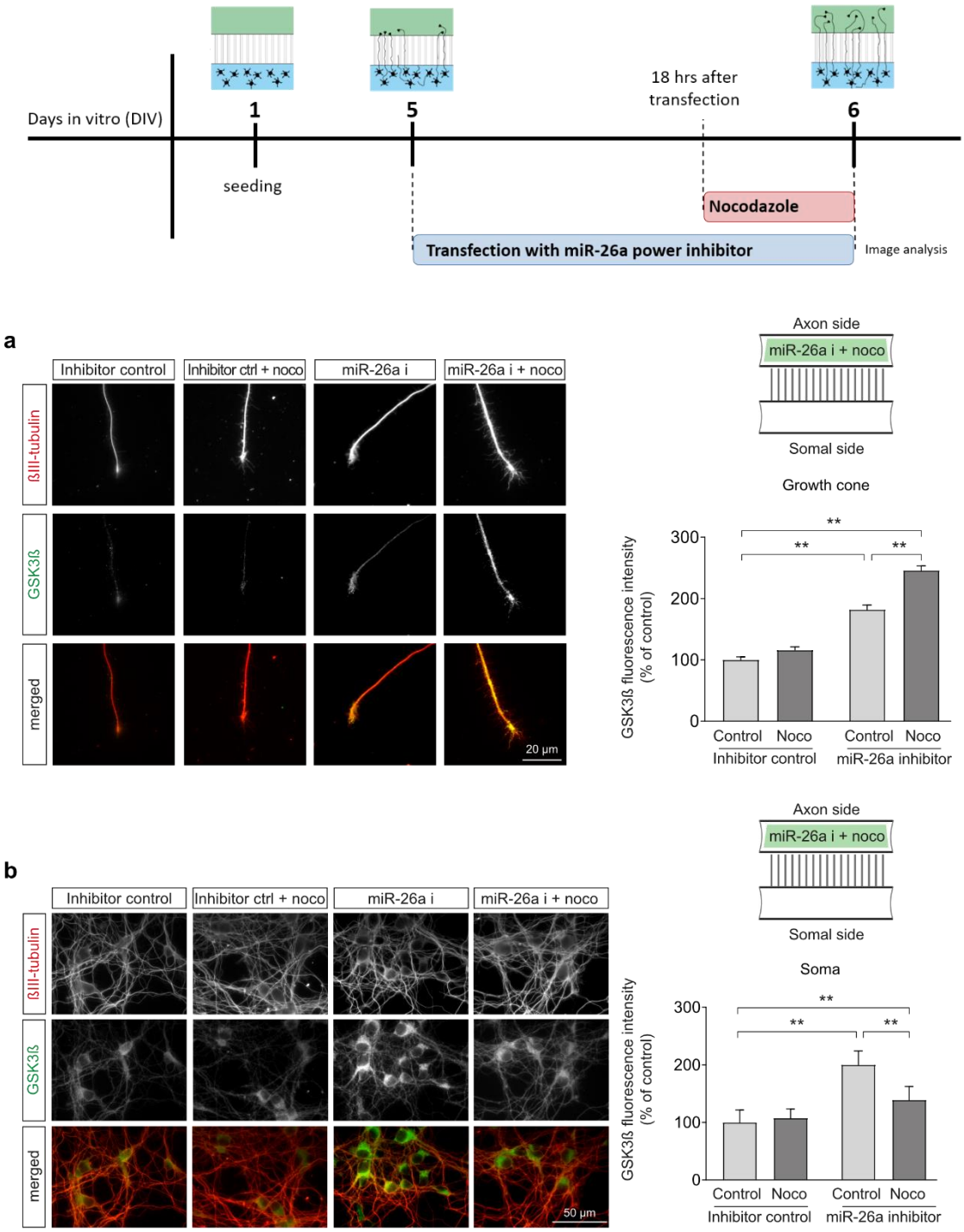

Figure 45: Retrograde transport of locally translated GSK3β is required for the regulation of axonal growth after inhibition of miR-26a in the axon. (**top**) Schematic representation of the experimental design. (**a-b**) Representative images and quantification of GSK3β protein levels in both growth cones and somas of cortical neurons treated with nocodazole 18 h after axonal application of miR-26a inhibitor and imaged 6 h later. For all the panels, schematics of the microfluidic chambers (upper right corner) depict where drugs were added. Application to the axon and the soma side is illustrated in green and blue, respectively, n=4. Data is expressed as mean ± SEM one-way ANOVA with Bonferroni's multiple comparison post-hoc tests, **P≤0.01.

5.4 Discussion

As fundamental regulators of protein translation in the nervous system (Davis *et al.*, 2015), the investigation of miRNAs and their specific role in the axon compartment of neurons has rapidly expanded in recent years (Kaplan *et al.*, 2013; Wang *et al.*, 2015; Y. Zhang *et al.*, 2015). Studies from us and others (Bellon *et al.*, 2017; Dajas-Bailador *et al.*, 2012; Sasaki *et al.*, 2014) have shown how regulation of local translation by specific miRNAs can control energy metabolism, growth and branching of axons in culture models of both central and peripheral neurons. For example, Kar *et al.* (2013), elegantly showed how axonal transfection of miR-16 or miR-16 inhibitor in rat sympathetic neurons was able to regulate mRNA levels of two of its targets (eIF2B2 and eIF4G2) in the axon, whereas no effect on the neuron's soma levels was observed. In CNS neurons, miR-9-5p was shown to locally control axon development by targeting the microtubule associated protein Map1b (Dajas-Bailador *et al.*, 2012). In chapter 4, we identified miR-26a as key player in the regulation of neuronal polarity and axon outgrowth *via* the targeting of GSK3β. The data in this chapter have revealed for the first time a local role of miR-26a in the regulation of axon development, in a process that requires the repression of local synthesis of GSK3β. Removal of miR-26a-mediated repression in the axon, triggers the local translation of GSK3β protein along with its subsequent transport to the neuronal soma, where its activity further regulates axonal growth.

Crucially, our study has also unravelled a previously unknown mechanism for neuronal information processing and GSK3β signalling in developing CNS neurons. In effect, local inhibition of miR-26a in the axon produced a significant increase in GSK3β protein levels and a decrease in axonal growth. Although axonal inhibition of miR-26a increased GSK3β protein levels in both the axon and soma of cortical neurons, this was not observed when inhibition

of this microRNA was restricted to the soma side of compartmentalised microfluidic chambers, indicating an axon-exclusive regulation of GSK3β translation *via* miR-26a.

We suggested a model in which GSK3β activity is needed in both soma and axon compartments, since the decrease in axonal length observed after inhibition of miR-26a in the axon was prevented by local application of the GSK3 inhibitor (SB415286) in either the soma or axon side of microfluidic chambers. The GSK3β expression studies reveal a molecular mechanism where local translation of GSK3β in the axon is normally repressed by the presence of miR-26a. In neuronal cultures, this promotes axonal development and growth. However, when miR-26a function is inhibited in the axon, local translation of GSK3β is triggered, followed by transport to the soma of cortical neurons. Although the activity of GSK3β is required in the axon and soma, the somatic increase in GSK activity capable of regulating axon function is dependent on its translation in the axon compartment [**Figure 46**].

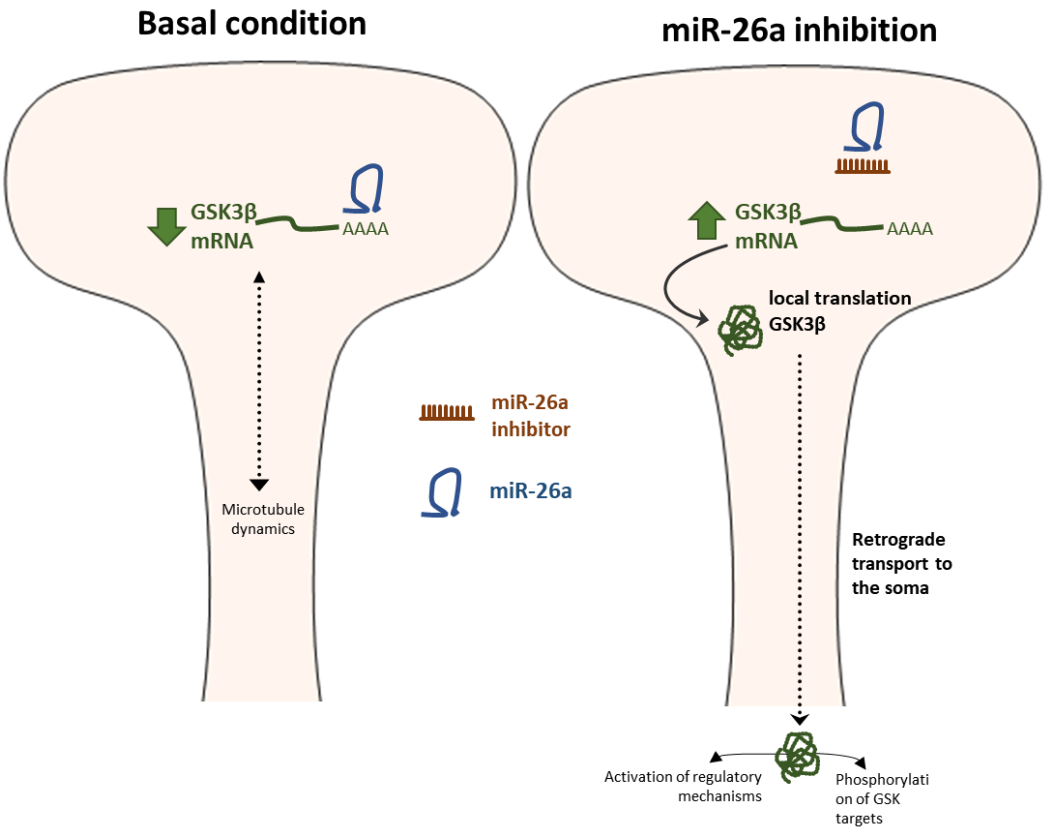

Figure 46: Proposed miR-26a-mediated-mechanism of action. In condition of active growth, the GSK3β activity controlling this cellular process are relatively low and miR-26a is normally repressing its translation. When miR-26a function is inhibited in the axon, local translation of GSK3β is triggered, followed by transport to the soma of cortical neurons where it activates further regulatory mechanisms or targets controlling axonal growth.

Confirmation of this mechanism was provided by the application of nocodazole in the axon side of microfluidic chambers. In this experimental paradigm, disruption of microtubule structure impairs axonal transport (Gobrecht *et al.*, 2014), and as predicted, it also prevented the increase in GSK3β protein levels that was observed in the soma after axonal inhibition of miR-26a. Reassuringly, nocodazole application did not stop the local translation of GSK3β in the axon once miR-26a translational repression was removed locally. However, the use of dynein inhibitors (i.e. ciliobrevin; Walker et al., 2018) would be needed to confirm the specific molecular mechanism involved in its retrograde transport to the soma. Moreover, levels

of GSK3β protein after the concurrent inhibition of miR-26a in the axon and block of retrograde transport indicates that 6 h are sufficient to produce a 20% increase in GSK3β protein levels in the axon. This likely reflects the high rate of GSK3β axon translation and transport following release of miR-26a repression.

In recent years, local protein synthesis has been confirmed as a cellular process that can provide the structural and regulatory components that are specifically needed in the axon, either during development, synaptic maturation or regeneration (Batista *et al.*, 2017; Batista and Hengst, 2016; Costa and Willis, 2018), with an ever-growing list of components being locally translated (Campbell and Holt, 2001; Si *et al.*, 2003; Verma *et al.*, 2005; Yoon *et al.*, 2012). It remains unclear why a protein would be locally synthesised just to be transported back to the soma. A possible answer might be that local synthesis and retrograde transport of signalling molecules allow to tightly control a signalling event. In fact, a similar mechanism has been previously shown only with transcription factors (Cox *et al.*, 2008; Ji and Jaffrey, 2012) such as CREB, which can be retrogradely transported to the nucleus to promote neuronal survival. In this way, local axon translation can facilitate and amplify communication between the axon and the neuronal soma, allowing the transport of newly synthesised "protein messengers" from the distal ends of a neuron (Cox *et al.*, 2008). More recently, exposure of axons to oligomeric $A\beta_{1-42}$ generates a retrograde signalling complex, inhibition of which can prevent the normal cell body response to $A\beta_{1-42}$ (Walker *et al.*, 2018). Considering that so far this mechanism has been only shown in peripheral neurons and after injury (Ben-Yaakov *et al.*, 2012; Terenzio *et al.*, 2018; Walker *et al.*, 2018), our study, for the first time, shows that the retrograde transport of a locally translated signalling molecule can trigger a functional outcome in developing CNS neurons. Further experiments are now needed to determine why GSK3β

retrograde transport is necessary to regulate polarity and axonal growth. To address this question, it would be informative to depict what are the targets that GSK3β phosphorylates after being transported back to the soma and/or if GSK3 has a role in NFAT- or CREB-mediated gene transcription during axon growth.

Our results demonstrate how a single miRNA can use the spatiotemporal control of axonally originated protein synthesis to impact events globally in the soma. This is a significant observation that challenges the prevalent view of miRNAs as only fine tuners of protein translation. In fact, localised regulation by specific miRNAs can dramatically change protein levels in defined neuronal compartments. However, it would be interesting to investigate about the mechanisms through which miR-26a can be in turn regulated. Expression of microRNAs can be regulated on multiple levels, such as at both the transcriptional and post-transcriptional level (all reviewed in Ha and Kim, 2014). But an alternative possibility, as it has been previously described for some microRNAs reported to have a function in axon development, is that extrinsic signals are involved in their regulation. For example, axonal treatment with a low concentration of BDNF reduces the level of axonal miR-9 and increases axon extension by inducing local synthesis of MAP1B, whereas prolonged treatment with a high concentration of BDNF increases the level of the miR and increases axon branching by repressing axonal synthesis of MAP1B.(Dajas-Bailador *et al.*, 2012). Another axonal miRNA that responds to local signalling is miR-181d, which is involved in the NGF-mediated axon elongation of embryonic DRG neurons. However, unlike the BDNF-mediated regulation of miR-9, NGF treatment does not change the level of miR-181d but regulates the dissociation of targets from miR-181d-repressing granules (Wang *et al.*, 2015). Considering the fact that miR-26a needs to constantly control the levels of GSK3β to modulate axon growth,

further experimental studies of the mechanisms behind the miR-26a expression are crucial.

In conclusion, our findings have placed miR-26a at a junction of regulatory mechanisms able to impinge on neuronal polarity and axon development *via* the control of GSK3β levels.

CHAPTER 6:

General discussion and outlook

6.1 Local protein translation in disease

As stated in previous sections of this thesis, the mRNA transcripts that are transported along the axon are highly heterogeneous, being sensitive to both developmental cues and pathophysiological conditions (Costa and Willis, 2018). Besides prompting further investigations about the function, role and regulatory mechanisms of the axonal translatome (Cioni *et al.*, 2018; Jung *et al.*, 2012), the complexity of the axonal mRNA population raised also interest in evaluating the functional consequences when the process of local protein translation is lost. The work presented in chapter 3 investigates this question using cultures of primary DRG neurons.

Local translation is thought to be particularly important in the distal parts of long axons, such as sensory and motor neurons, because it supplies new proteins to meet local demand far from the soma and rapidly respond to extracellular stimuli. In this regard, the data presented in chapter 3 represents a proof of concept for this mechanism, confirming the important physiological role of local translation in preserving both axon survival and homeostasis. Specifically, we found how distal portions of axons/neurites were also more susceptible to degeneration when the translation machinery was locally disrupted. Recent data might support this notion, given the fact that after disruption of axonal mRNA-trafficking through the expression of Charcot-Marie-Tooth disease type 2B (CMT2B)-linked Rab7a mutants, local protein synthesis of mitochondrial protein was impaired with the consequent loss of axon integrity (Cioni *et al.*, 2019).

Accumulating evidence has associated aberrant axonal localisation of mRNAs and disruption of translation to several neurodevelopmental disorders, such as fragile X mental retardation and autism, which seem to have underlying local translation deficits (Bear *et al.*, 2008; Kar *et al.*, 2014). Fragile X mental retardation protein (FMRP) is a well-documented plasticity regulator in

dendritic spines (Bassell and Warren, 2008). It is found in growth cones and axons (Antar *et al.*, 2006; Christie *et al.*, 2009), where it regulates the presynaptic proteome (Akins *et al.*, 2012). Several nuclear-encoded mitochondrial mRNAs are localised within axons, and, not surprisingly, clinical phenotypes are often associated with the disruption of their transport or translation (Aschrafi *et al.*, 2010). In fact, exogenous expression of the Cytochrome C oxidase IV (COXIV) in cultured SCG neurons results in the reduction of local ATP levels and increases levels of reactive oxygen species (ROS) in the axon, and this increase correlates with an anxiety- and depression-like phenotype that is reminiscent of neuropsychiatric disease in humans (Kar *et al.*, 2014). Dysregulation of microRNAs that regulate these mitochondrial mRNAs also have been linked to neuropsychiatric disorders. Deletions within 22q11 are linked with schizophrenia and one of the deleted genes embedded in this region encodes for DGCR8, a fundamental component of the microRNA's biogenesis machinery. This is also associated with depletion of miR-338, a known regulator of COXIV (Aschrafi *et al.*, 2008).

Once the transcripts are made and then stabilised by binding to RBPs in granules, they are prepared to make the long journey to the axon terminal. Thus, it is not surprising that mutations of RNA-binding proteins (RBPs) have been connected with neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Bassell *et al.*, 2011). Mutations of RNA-binding proteins in fact, lead to both ALS and some types of frontotemporal lobar degeneration and impair axonal trafficking of mRNA granules (Alami *et al.*, 2014). SMA is instead caused by deletion or mutation(s) of the survival motor neuron (SMN) protein. SMN is present in all cell types, and its total deletion is lethal, but somehow motor neurons are more sensitive to its reduction (Fallini *et al.*, 2016) This might be because a decrease in SMN

causes the reduction in the axonal localisation of several mRNAs (Rage *et al.*, 2013), and inhibits mTOR activity in axons (Kye *et al.*, 2014).

Perhaps more interesting was the discovery for a role of axonal protein synthesis in the response to β-amyloid (Aβ) stimulation, indicating a functional role for local translation in the pathogenesis Alzheimer's disease (AD) (Baleriola *et al.*, 2014). In this study, compartmentalised hippocampal cultures were used for the application of oligomeric Aβ1-42 specifically to axons, and this elicited the recruitment and axonal translation of many mRNAs, including the transcript for the transcription factor activating transcription factor 4 (ATF4). Locally synthesised ATF4 is retrogradely transported to the cell soma, where it changes nuclear transcription, ultimately leading to cell death.

The discussion above highlights, once again, the importance of axonal protein synthesis and the associated regulatory mechanisms. It is also clear that the centre-stage is now moving towards the clinical consequences that the functional loss of this process might induce. However, further investigation into the underlying molecular mechanisms are still needed, thus allowing the selective control and coordination of axonal translation and the design of new strategies for therapies aimed at neurodevelopmental and neurodegenerative diseases. In this context, the PI3K-Akt-GSK3β axonal growth regulation pathway has a long history of study in the context of axon regeneration (Dill *et al.*, 2008; Saijilafu *et al.*, 2013), and in the data presented in chapter 5 of this thesis, we demonstrate how local translation of GSK3β in a specific cellular compartment, the developing axon, can have functional effects that influence distant cellular domains. Whilst the wider implications of this cellular mechanism still need to be further explored, it provides an additional mode of spatiotemporal regulation of GSK3β as master regulator of axon development and regeneration, with potentially broad physiological implications.

6.2 MicroRNAs: tiny but mighty regulators of nervous system.

The data presented in this thesis demonstrate that miRNAs exert an important influence in the molecular networks that regulate the formation of the nervous system. The establishment of neuronal polarity is a very dynamic process, as well as a prerequisite for the correct development of both the axon and the somatodendritic compartment, which in turn provide the underpinning for the transmission and reception of electric signals and overall neuronal communication in the brain (Schelski and Bradke, 2017). For correct development to take place, this biological process requires the coordinated action of extracellular signals, receptors and intracellular signalling pathways, in a process made possible by the tight control of regulatory mechanisms. In this context, miRNAs have been considered as attractive candidates to regulate both signalling pathways and the neuronal translatome, due to their spatiotemporal- and tissue-specific-expression patterns (Bartel, 2004; Lagos-quintana *et al.*, 2001). Now, these post-translational regulators are recognised to be fundamental players of virtually all aspects of CNS development, physiology and disease (Cao *et al.*, 2016). According to its target, a single miRNA can either promote or inhibit a specific developmental process. As the target for a given miRNA can change as a function of time and space (Bartel, 2009), miRNA activity is often context specific, as nicely illustrated by findings that one miRNA can play different roles in different stages of neuronal development or in different regions of the brain (Rajman and Schratt, 2017).

The work presented in chapter 4 and 5 of this thesis explores miRNA function in post-mitotic neurons at the period of neuronal wiring, when axons are being specified and extend towards specific post-synaptic partners to form functional connections (Polleux and Snider, 2010). Indeed, how axons manage to accurately follow specific paths to reach their partners is one of the highly

investigated questions in the axon development field, and the regulation layer provided by miRNA activity might play an important part. As neuronal polarisation and the development of neuron connectivity involves a temporally regulated series of cellular processes that include axon specification and outgrowth, pathfinding and the formation of pre-synaptic structures, it has now become clear that such tightly regulated events need the coordinated expression of miRNAs and their target genes.

Although different experimental approaches and biological systems have been used to detect miRNA levels, very few studies have profiled miRNA expression directly within the developing axon (including growth cones). The study of their differential localisation and enrichment in these compartments (Hancock *et al.*, 2014; Natera-Naranjo *et al.*, 2010; Sasaki *et al.*, 2014), could generate important information regarding the functional roles of local translation and axonal miRNAs.

Beyond their identification in the axon compartment, very few studies have demonstrated a specific role for local miRNAs in the control of intrinsic axon growth pathways as well as guidance cues-stimulated axon outgrowth (Dajas-Bailador *et al.*, 2012; Hancock *et al.*, 2014; Reh and Hindges, 2018; Wang and Bao, 2017), or the spatiotemporal effects of such guidance cues during axonal elongation (Bellon *et al.*, 2017). In effect, apart from these specific studies, which have been described in different sections of this thesis, the function and regulatory mechanisms of miRNAs in the axon remain largely unknown. Another open question in the field, and not necessarily limited to neural development or to nervous system in general, is to determine when miRNAs act as "master regulators" or "switches" and when as fine-tuners of gene expression. miRNAs that are highly expressed in early neurogenesis (e.g. miR-124, miR-9) can be classified as switch genes that control cell fate (Coolen *et al.*, 2013; Makeyev *et al.*, 2007), but more modestly expressed miRNAs involved at

later stages of neuronal development seem to act instead as fine-tuner of gene expression in response to the activity state of the network. Although some miRNAs have crucial targets, regulation of which is enough to give rise to a specific phenotype (Dajas-Bailador *et al.*, 2012; Kos *et al.*, 2017b; W. M. Wang *et al.*, 2017), many other miRNAs contribute instead to the regulation of up to a few hundred different targets, often in combination with other co-expressed miRNAs (Yi Zhang *et al.*, 2013), making the process to assign particular biological roles particularly hard.

Such a complicated view of the miRNA regulatory system comes from some studies demonstrating that the repression exerted by one single miRNA is not sufficient to influence an entire biological pathway (Baek *et al.*, 2008; Selbach *et al.*, 2008), as already showed in neocortical development studies (Barca-Mayo and De Pietri Tonelli, 2014). However, the possibility to investigate miRNAs function in localised cellular environments (Dajas-Bailador *et al.*, 2012; Kos *et al.*, 2016; Yi Zhang *et al.*, 2013), where the consequence of their actions could be more marked, has provided further functional insight that is difficult to obtain with other cellular and culture models. In this context, the localised action of miR-26a might constitute a representative example, given the fact that its axonal suppression radically altered the levels of GSK3β in the axon [**Figure 44**] and whole neuronal cultures [**Figure 35**].

Another hypothesis that might explain how miRNAs can manage simultaneously and precisely to control several biological processes is the convergent activity of multiple microRNAs. One or more individual miRNAs might act on different seed regions in one or more target 3'UTR, thus resulting in a regulatory effect (Barca-Mayo and De Pietri Tonelli, 2014), or converge on a functional outcome by acting at different intracellular signalling pathways that share the same biological function. Finally, microRNAs could act on a single target or on a pathway by targeting different molecules belonging to the

same pathway. One could speculate that the latter might be again the case of miR-26a, as it can control neuronal polarity and axon outgrowth *via* the targeting of both PTEN and GSK3β [**Figure 39** and **Figure 40**]. Thus, advanced technologies such as high-throughput RNA sequencing for axonal RNAs present at low levels may provide an integrated map for the miRNA-mediated regulation of mRNAs during axon development.

Another layer of complexity in the microRNA regulatory network might come from cell-cell communication, with the potential to modulate regulatory networks away from the cells of origin (Prada *et al.*, 2018). Brain function depends on coordinated interactions between neurons and glial cells (Allen and Barres, 2009), with the latter providing a wide range of functions, from metabolic support to myelination, immune defence, and engagement in synapse formation and plasticity (Johanne and Linda, 2012). Recent evidence indicates that these cells also release endosome-derived microvesicles termed exosomes and carry specific proteins and RNA cargoes including microRNAs (Frühbeis *et al.*, 2012). Exosomes can thus interact with neighbouring cells, mediate signalling between brain cells and facilitate the delivery of bioactive molecules, (Frühbeis *et al.*, 2012). Not surprisingly, miRNAs within exosomes were shown to be actively released into the extracellular space and subsequently uptaken to exert regulatory actions in the recipient cells (L. Zhang *et al.*, 2015), opening up an entirely novel field in exosome study. Even though exosome biology is still in its infancy (J. Zhang *et al.*, 2015), the possibility to use exosomes and deliver their cargoes as a clinical tool to diagnose and monitor diseases, perhaps even for gene therapy, is really tantalising. Interestingly, a recent report suggested that miR-26a is enriched in astrocytes-derived exosomes and thus, it has the potential to be released and internalised by recipient cells (Lafourcade *et al.*, 2016). These observations, along with the novel functions described for miR-26a in axon development

(chapter 4-5) raise only more questions. What could be the regulatory function of miR-26a in the recipient cell? Could exosomes-derived miR-26a be exerting regulatory actions in the specification and elongation of axons of neighbouring neurons? If this is true, could then miR-26a be uptaken by axons and produce the local effects described here?

6.3 Implications of miRNAs in Neurological Diseases

Considering the fundamental role of microRNAs in every stage of neuronal development, it is not surprising that a growing number of studies are linking their dysregulation to the pathology of neurological diseases such as neurodevelopmental disorders, neuropsychiatric disorders, and neurodegenerative disorders (Cao *et al.*, 2016; Wang *et al.*, 2012). One of the most extensively studied neurodevelopmental disorders for which miRNA dysfunction plays a key role is schizophrenia and, as I have already described in the general introduction of this thesis, miR-137 is the most well-documented microRNA implicated with the disease (Siegert *et al.*, 2015).

Interestingly, as for aberrant axonal localisation of mRNAs, fragile X mental retardation has been also associated with microRNAs. In Drosophila, it was showed that phenotypes caused by overexpression of miR-124a could be partially rescued by inactivation of dFMR1 (*Drosophila* homologous of FMRP) (Xu *et al.*, 2008), whilst in mouse, it has been seen that FMRP was required for miR-125b and miR-132 effects on spine morphology changes (Edbauer *et al.*, 2010).

miRNA profiling studies have shown that circulating miRNAs could be used as potential biomarkers for neurodegenerative disorders, as the expression of miRNAs is dysregulated in patient brain compared to normal brain (Cardo *et*

al., 2013; Grasso *et al.*, 2014; Leidinger *et al.*, 2013). In this context, the relatively high levels of miR-26a expression in mature neuronal cultures and CNS raises potentially relevant questions about its role in adult brain. There is now a clear understanding of how the loss of axon and neuron connectivity constitutes a fundamental step in the early and progressive degradation of network information capacity (Coleman, 2005; Conforti *et al.*, 2007). Interestingly, both miR-26a, as part of a signature group of miRNAs known to be deregulated in Alzheimer's disease (Cogswell *et al.*, 2008; Leidinger *et al.*, 2013), and GSK3β, which has shown increased activity leading to Tau hyperphosphorylation in various Alzheimer's disease models, have been implicated in neurodegenerative processes (Dargahi *et al.*, 2015; Hooper *et al.*, 2008). Future work will need to establish whether the spatiotemporal control of GSK3β molecular mechanisms that are regulated by axonal miR-26a in developing neurons, could also have an impact in neuronal function in the mature and ageing brain.

***

Appendix

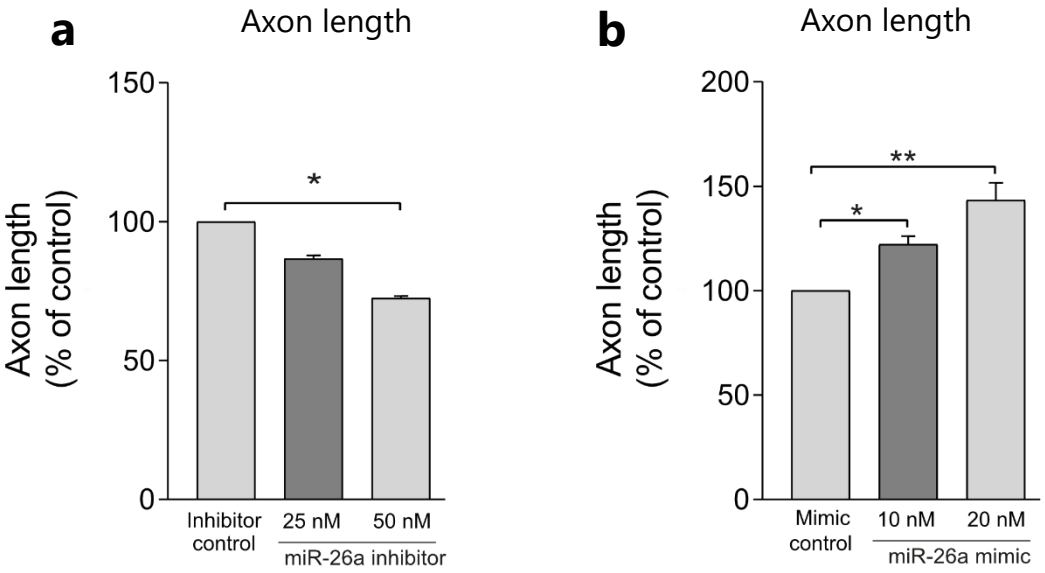

Figure 47: Test of different concentrations for both miR-26a inhibitor and mimic. (**a**) Quantification of axon length in polarised neurons after inhibition of miR-26a (miR-26a inhibitor 25 and 50 nM), showing a dose-dependent decrease in axon compared to a non-targeting control, n=2. Data is expressed as mean ± SEM; (**b**) Quantification of axon length in polarised neurons after over-expression of miR-26a (miR-26a m 10 and 20 nM), showing a dose-dependent increase in axon length up to almost 40 % compared to a non-targeting control, *n*=3. Data are mean±s.e.m. one-way ANOVA with Bonferroni's multiple comparison post-hoc tests, *P<0.05, **P≤0.01.

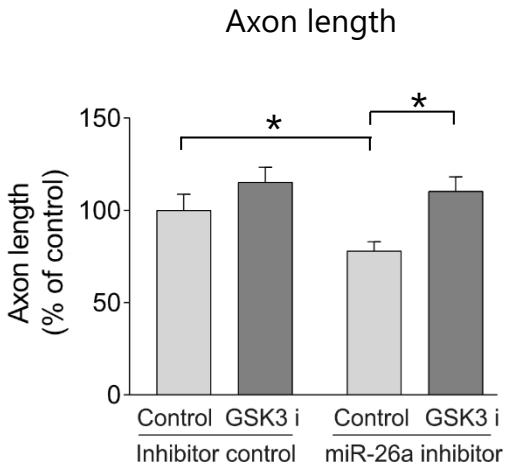

Figure 48: Quantification of axon length after inhibition of miR-26a and the addition of GSK3 inhibitor (SB415286, 10 μM) 24 h after transfections, n=3. Data are mean±s.e.m. one-way ANOVA with Bonferroni's multiple comparison post-hoc tests, *P<0.05

References

Absalon S, Kochanek DM, Raghavan V, Krichevsky AM. 2013. MiR-26b, Upregulated in Alzheimer's Disease, Activates Cell Cycle Entry, Tau-Phosphorylation, and Apoptosis in Postmitotic Neurons. *J Neurosci* **33**:14645–14659. doi:10.1523/JNEUROSCI.1327-13.2013

Adler CE, Fetter RD, Bargmann CI. 2006. UNC-6/Netrin induces neuronal asymmetry and defines the site of axon formation. *Nat Neurosci* **9**:511–518. doi:10.1038/nn1666

Agarwal V, Bell GW, Nam J-W, Bartel DP. 2015. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4**:1–38. doi:10.7554/elife.05005

Akins MR, Leblanc HF, Stackpole EE, Chyung E, Fallon JR. 2012. Systematic mapping of fragile X granules in the mouse brain reveals a potential role for presynaptic FMRP in sensorimotor functions. *J Comp Neurol* **520**:3687–3706. doi:10.1002/cne.23123

Al-Bassam S, Xu M, Wandless TJ, Arnold DB. 2012. Differential trafficking of transport vesicles contributes to the localization of dendritic proteins. *Cell Rep* **2**:89–100. doi:10.1016/j.celrep.2012.05.018

Alami NH, Smith RB, Carrasco MA, Williams LA, Winborn CS, Han SSW, Kiskinis E, Winborn B, Freibaum BD, Kanagaraj A, Clare AJ, Badders NM, Bilican B, Chaum E, Chandran S, Shaw CE, Eggan KC, Maniatis T, Taylor JP. 2014. Axonal Transport of TDP-43 mRNA Granules Is Impaired by ALS-Causing Mutations. *Neuron* **81**:536–543. doi:10.1016/j.neuron.2013.12.018

Alisch RS, Jin P, Epstein M, Caspary T, Warren ST. 2007. Argonaute2 is essential for mammalian gastrulation and proper mesoderm formation.

PLoS Genet **3**:2565–2571. doi:10.1371/journal.pgen.0030227

Allen NJ, Barres BA. 2009. Glia - more than just brain glue. *Nat Neurosci* **457**:675–677. doi:10.1038/457675a

Ambrozkiewicz MC, Schwark M, Kishimoto-Suga M, Borisova E, Hori K, Salazar-Lázaro A, Rusanova A, Altas B, Piepkorn L, Bessa P, Schaub T, Zhang X, Rabe T, Ripamonti S, Rosário M, Akiyama H, Jahn O, Kobayashi T, Hoshino M, Tarabykin V, Kawabe H. 2018. Polarity Acquisition in Cortical Neurons Is Driven by Synergistic Action of Sox9-Regulated Wwp1 and Wwp2 E3 Ubiquitin Ligases and Intronic miR-140. *Neuron* **100**:1097-1115.e15. doi:10.1016/j.neuron.2018.10.008

Amin ND, Bai G, Klug JR, Bonanomi D, Pankratz MT, Gifford WD, Hinckley CA, Sternfeld MJ, Driscoll SP, Dominguez B, Lee KF, Jin X, Pfaff SL. 2015. Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure. *Science (80-)* **350**:1525–1529. doi:10.1126/science.aad2509

Andersen SSL, Bi GQ. 2000. Axon formation: A molecular model for the generation of neuronal polarity. *BioEssays* **22**:172–179. doi:10.1002/(SICI)1521-1878(200002)22:2<172::AID-BIES8>3.0.CO;2-Q

Anderson SA, Kaznowski C, McConnel SK. 2002. Distinct Origins of Neocortical Projection Neurons and Interneurons In Vivo. *Cereb Cortex* **12**:702–709. doi:10.1093/cercor/12.7.702

Andreassi C, Zimmermann C, Mitter R, Fusco S, Devita S, Saiardi A, Riccio A. 2010. An NGF-responsive element targets myo-inositol monophosphatase-1 mRNA to sympathetic neuron axons. *Nat Neurosci* **13**:291–301. doi:10.1038/nn.2486

Antar LN, Li C, Zhang H, Carroll RC, Bassell GJ. 2006. Local functions for FMRP in axon growth cone motility and activity-dependent regulation of

filopodia and spine synapses. *Mol Cell Neurosci* **32**:37–48. doi:10.1016/j.mcn.2006.02.001

Apenstrom P. 1999. Effectors for the Rho GTPases Pontus Aspenström WASP. *Curr Opin Cell Biol* **11**:95–102. doi:10.1016/s0955-0674(99)80011-8

Araki T, Sasaki Y, Milbrandt J. 2004. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science (80-)* **305**:1010–1013. doi:10.1126/science.1098014

Arimura N, Kaibuchi K. 2007. Neuronal polarity: from extracellular signals to intracellular mechanisms. *Nat Rev Neurosci* **8**:194–205. doi:10.1038/nrn2056

Arimura N, Kimura T, Nakamuta S, Taya S, Funahashi Y, Hattori A, Shimada A, Ménager C, Kawabata S, Fujii K, Iwamatsu A, Segal RA, Fukuda M, Kaibuchi K. 2009. Anterograde Transport of TrkB in Axons Is Mediated by Direct Interaction with Slp1 and Rab27. *Dev Cell* **16**:675–686. doi:10.1016/j.devcel.2009.03.005

Aschrafi A, Kar AN, Natera-Naranjo O, MacGibeny MA, Gioio AE, Kaplan BB, Natera-Naranjo O, Gioio AE, Kaplan BB. 2012. MicroRNA-338 regulates the axonal expression of multiple nuclear-encoded mitochondrial mRNAs encoding subunits of the oxidative phosphorylation machinery. *Cell Mol Life Sci* **69**:4017–4027. doi:10.1007/s00018-012-1064-8

Aschrafi A, Natera-Naranjo O, Gioio AE, Kaplan BB. 2010. Regulation of axonal trafficking of cytochrome c oxidase IV mRNA. *Mol Cell Neurosci* **43**:422–430. doi:10.1016/j.mcn.2010.01.009

Aschrafi A, Schwechter AD, Natera-Naranjo O, Gioio AE, Mameza MG, Kaplan BB. 2008. MicroRNA-338 Regulates Local Cytochrome c Oxidase IV mRNA Levels and Oxidative Phosphorylation in the Axons of Sympathetic Neurons. *J Neurosci* **28**:12581–12590.

doi:10.1523/jneurosci.3338-08.2008

Athamneh AIM, He Y, Lamoureux P, Fix L, Suter DM, Miller KE. 2017. Neurite elongation is highly correlated with bulk forward translocation of microtubules. *Sci Rep* **7**:1–13. doi:10.1038/s41598-017-07402-6

Avila J, Dominguez J, Diaz-Nido J. 1994. Regulation of microtubule dynamics by microtubule-associated protein expression and phosphorylation during neuronal development. *Int J Dev Biol* **38**:13–25.

Azevedo F a C, Carvalho LRB, Grinberg LT, Farfel JM, Ferretti REL, Leite REP, Filho WJ, Lent R, Herculano-Houzel S. 2009. Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up pAzevedo, F. a C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., … Herculano-Houzel, S. (2009). *J Comp Neurol* **513**:532–541. doi:10.1002/cne.21974

Baas PW. 2004. The transport properties of axonal microtubules establish their polarity orientation. *J Cell Biol* **120**:1427–1437. doi:10.1083/jcb.120.6.1427

Baas PW. 1999. Microtubules and neuronal polarity: Lessons from mitosis. *Neuron* **22**:23–31. doi:10.1016/S0896-6273(00)80675-3

Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. 2008. The impact of microRNAs on protein output. *Nature* **455**:64–71. doi:10.1038/nature07242

Bagnard D, Lohrum M, Uziel D, Püschel AW, Bolz J. 1998. Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. *Development* **125**:5043–5053.

Baleriola J, Walker CA, Jean YY, Crary JF, Troy CM, Nagy PL, Hengst U. 2014. Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. *Cell* **158**:1159–1172. doi:10.1016/j.cell.2014.07.001

Banker G. 2018. The Development of Neuronal Polarity: A Retrospective View.

J Neurosci **38**:1867–1873. doi:10.1523/JNEUROSCI.1372-16.2018

Barca-Mayo O, De Pietri Tonelli D. 2014. Convergent microRNA actions coordinate neocortical development. *Cell Mol Life Sci* **71**:2975–2995. doi:10.1007/s00018-014-1576-5

Bard L, Boscher C, Lambert M, Mege R-M, Choquet D, Thoumine O. 2008. A Molecular Clutch between the Actin Flow and N-Cadherin Adhesions Drives Growth Cone Migration. *J Neurosci* **28**:5879–5890. doi:10.1523/JNEUROSCI.5331-07.2008

Barnes AP, Polleux F. 2009. Establishment of Axon-Dendrite Polarity in Developing Neurons. *Annu Rev Neurosci* **32**:347–381. doi:10.1146/annurev.neuro.31.060407.125536

Barry G. 2014. Integrating the roles of long and small non-coding RNA in brain function and disease. *Mol Psychiatry* **19**:410–416. doi:10.1038/mp.2013.196

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**:215–33. doi:10.1016/j.cell.2009.01.002

Bartel DP. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* **116**:281–297. doi:10.1038/nn.3695

Bassell GJ, Gitler AD, Warren ST, Hart AC, Wolozin B, Klann E, Richter JD, Liu-Yesucevitz L. 2011. Local RNA Translation at the Synapse and in Disease. *J Neurosci* **31**:16086–16093. doi:10.1523/jneurosci.4105-11.2011

Bassell GJ, Singer RH, Kosik KS. 1994. Association of poly(A) mRNA with microtubules in cultured neurons. *Neuron* **12**:571–582. doi:10.1016/0896-6273(94)90213-5

Bassell GJ, Warren ST. 2008. Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function. *Neuron* **60**:201–214. doi:10.1016/j.neuron.2008.10.004

Bassell GJ, Zhang H, Byrd AL, Femino AM, Singer RH, Taneja KL, Lifshitz LM, Herman IM, Kosik KS. 1998. Sorting of beta-actin mRNA and protein to neurites and growth cones in culture. *J Neurosci* **18**:251–65. doi:10.1523/JNEUROSCI.18-01-00251.1998

Batista AFR, Hengst U. 2016. Intra-axonal protein synthesis in development and beyond. *Int J Dev Neurosci* **55**:140–149. doi:10.1016/j.ijdevneu.2016.03.004

Batista AFR, Martínez JC, Hengst U. 2017. Intra-axonal Synthesis of SNAP25 Is Required for the Formation of Presynaptic Terminals. *Cell Rep* **20**:3085–3098. doi:10.1016/j.celrep.2017.08.097

Beals CR, Sheridan CM, Turck CW, Gardner P, Crabtree GR. 1997. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science (80-)* **275**:1930–1933. doi:10.1126/science.275.5308.1930

Bear MF, Dölen G, Osterweil E, Nagarajan N. 2008. Fragile X: Translation in action. *Neuropsychopharmacology* **33**:84–87. doi:10.1038/sj.npp.1301610

Behar O, Golden JA, Mashimo H, Schoen FJ, Fishman MC. 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature* **383**:525–528. doi:10.1038/383525a0

Beirowski B, Babetto E, Magni G, Janeckova L, Conforti L, Mazzola F, Gilley J, Ribchester RR, Coleman MP. 2009. Non-Nuclear WldS Determines Its Neuroprotective Efficacy for Axons and Synapses In Vivo. *J Neurosci* **29**:653–668. doi:10.1523/jneurosci.3814-08.2009

Belin S, Nawabi H, Wang C, Tang S, Latremoliere A, Warren P, Schorle H, Uncu C, Woolf CJ, He Z, Steen JA. 2015. Injury-Induced Decline of Intrinsic Regenerative Ability Revealed by Quantitative Proteomics. *Neuron* **86**:1000–1014. doi:10.1016/j.neuron.2015.03.060

Bellon A, Iyer A, Bridi S, Lee FCY, Ovando-Vázquez C, Corradi E, Longhi S,

Roccuzzo M, Strohbuecker S, Naik S, Sarkies P, Miska E, Abreu-Goodger C, Holt CE, Baudet ML. 2017. miR-182 Regulates Slit2-Mediated Axon Guidance by Modulating the Local Translation of a Specific mRNA. *Cell Rep* **18**:1171–1186. doi:10.1016/j.celrep.2016.12.093

Bellon A, Mann F. 2018. Keeping up with advances in axon guidance. *Curr Opin Neurobiol* **53**:183–191. doi:10.1016/j.conb.2018.09.004

Ben-Yaakov K, Dagan SY, Segal-Ruder Y, Shalem O, Vuppalanchi D, Willis DE, Yudin D, Rishal I, Rother F, Bader M, Blesch A, Pilpel Y, Twiss JL, Fainzilber M. 2012. Axonal transcription factors signal retrogradely in lesioned peripheral nerve. *EMBO J* **31**:1350–1363. doi:10.1038/emboj.2011.494

Benech C, Sotelo JR, Menéndez J, Correa-Luna R. 1982. Autoradiographic study of RNA and protein synthesis in sectioned peripheral nerves. *Exp Neurol* **76**:72–82. doi:10.1016/0014-4886(82)90102-9

Bentley D, Toroian-Raymond A. 1986. Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature* **323**:712–715. doi:10.1038/323712a0

Beurel EEE, Grieco SF, Jope RS. 2015. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacol Ther* **148**:114–131. doi:10.1016/j.pharmthera.2014.11.016

Bhalala OG, Srikanth M, Kessler JA. 2013. The emerging roles of microRNAs in CNS injuries. *Nat Rev Neurol* **9**:328–339. doi:10.1038/nrneurol.2013.67

Bjorkblom B. 2005. Constitutively Active Cytoplasmic c-Jun N-Terminal Kinase 1 Is a Dominant Regulator of Dendritic Architecture: Role of Microtubule-Associated Protein 2 as an Effector. *J Neurosci* **25**:6350–6361. doi:10.1523/jneurosci.1517-05.2005

Blockus H, Chédotal A. 2016. Slit-robo signaling. *Dev* **143**:3037–3044.

doi:10.1242/dev.132829

Bloom GS, Wagner MC, Pfister KK, Brady ST. 1988. Native Structure and Physical Properties of Bovine Brain Kinesin and Identification of the ATP-Binding Subunit Polypeptide. *Biochemistry* **27**:3409–3416. doi:10.1021/bi00409a043

Boyer NP, Gupton SL. 2018. Revisiting netrin-1: One who guides (Axons). *Front Cell Neurosci* **12**:1–18. doi:10.3389/fncel.2018.00221

Bradke F, dotti. 1999. The Role of Local Actin Instability in Axon Formation. *Science (80-)* **283**:1931–1934. doi:10.1126/science.283.5409.1931

Bradke F, Dotti CG. 2000. Differentiated neurons retain the capacity to generate axons from dendrites. *Curr Biol* **10**:1467–1470. doi:10.1016/S0960-9822(00)00807-1

Bradke F, Dotti CG. 1997. Vectorial cytoplasmic flow precedes axon formation. *Neuron* **19**:1175–1186. doi:10.1016/s0896-6273(00)80410-9

Bramsen JB, Laursen MB, Damgaard CK, Lena SW, Ravindra Babu B, Wengel J, Kjems J. 2007. Improved silencing properties using small internally segmented interfering RNAs. *Nucleic Acids Res* **35**:5886–5897. doi:10.1093/nar/gkm548

Brecht S, Kirchhof R, Chromik A, Willesen M, Nicolaus T, Raivich G, Wessig J, Waetzig V, Goetz M, Claussen M, Pearse D, Kuan CY, Vaudano E, Behrens A, Wagner E, Flavell RA, Davis RJ, Herdegen T. 2005. Specific pathophysiological functions of JNK isoforms in the brain. *Eur J Neurosci* **21**:363–377. doi:10.1111/j.1460-9568.2005.03857.x

Brittis PA, Lu Q, Flanagan JG. 2002. Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. *Cell* **110**:223–235. doi:10.1016/S0092-8674(02)00813-9

Buck KB, Schaefer AW, Schoonderwoert VT, Creamer MS, Dufresne ER, Forscher P. 2017. Local Arp2/3-dependent actin assembly modulates applied traction force during apCAM adhesion site maturation. *Mol Biol Cell* **28**:98–110. doi:10.1091/mbc.e16-04-0228

Bultje RS, Castaneda-Castellanos DR, Jan LY, Jan YN, Kriegstein AR, Shi SH. 2009. Mammalian Par3 Regulates Progenitor Cell Asymmetric Division via Notch Signaling in the Developing Neocortex. *Neuron* **63**:189–202. doi:10.1016/j.neuron.2009.07.004

Bunge MB. 1973. Fine Structure of Nerve Fibers and Growth Cones of Isolated Sympathetic Neurons in Culture. *J Cell Biol* **56**:713–735. doi:10.1083/jcb.56.3.713

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**:611–622. doi:10.1373/clinchem.2008.112797

Cagnetta R, Frese CK, Shigeoka T, Krijgsveld J, Holt CE. 2018. Rapid Cue-Specific Remodeling of the Nascent Axonal Proteome. *Neuron* **99**:29-46.e4. doi:10.1016/j.neuron.2018.06.004

Cagnetta R, Wong HHW, Frese CK, Mallucci GR, Krijgsveld J, Holt CE. 2019. Noncanonical Modulation of the eIF2 Pathway Controls an Increase in Local Translation during Neural Wiring. *Mol Cell* **73**:474-489.e5. doi:10.1016/j.molcel.2018.11.013

Cambray-Deakin MA, Burgo. 1987. Posttranslational modifications of alpha-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. *J Cell Biol* **104**:1569–1574. doi:10.1083/jcb.104.6.1569

Campbell DS, Holt CE. 2003. Apoptotic Pathway and MAPKs Differentially

Regulate Chemotropic Responses of Retinal Growth Cones type for a superfamily of conserved signaling cascades in all eukaryotes sharing the motif of three serially linked kinases: a MAPK kinase kinase (MAPKKK or. *Neuron* **37**:939–952. doi:10.1016/S0896-6273(03)00158-2

Campbell DS, Holt CE. 2001. Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* **32**:1013–1026. doi:10.1016/S0896-6273(01)00551-7

Cao D-D, Li L, Chan W-Y. 2016. MicroRNAs: Key Regulators in the Central Nervous System and Their Implication in Neurological Diseases. *Int J Mol Sci* **17**:1–28. doi:10.3390/ijms17060842

Capriotti T, Terzakis K. 2016. Parkinson Disease. *Home Healthc now* **34**:300–307. doi:10.1097/NHH.0000000000000398

Cardo LF, Coto E, De Mena L, Ribacoba R, Moris G, Menéndez M, Alvarez V. 2013. Profile of microRNAs in the plasma of Parkinson's disease patients and healthy controls. *J Neurol* **260**:1420–1422. doi:10.1007/s00415-013-6900-8

Carlin D, Halevi AE, Ewan EE, Moore AM, Cavalli V. 2019. Nociceptor Deletion of Tsc2 Enhances Axon Regeneration by Inducing a Conditioning Injury Response in Dorsal Root Ganglia. *eNeuro* **6**:1–20. doi:10.1523/ENEURO.0168-19.2019 1

Carty M, Stack J, Moynagh PN, Schröder M, Goodbody R, Bowie AG, Schröder M, Stack J, Moynagh PN, Bowie AG. 2006. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nat Immunol* **7**:1074–1081. doi:10.1038/ni1382

Chang F, Zhang LH, Xu WUP, Jing P, Zhan PY. 2014. microRNA-9 attenuates amyloidβ-induced synaptotoxicity by targeting calcium/calmodulin-dependent protein kinase kinase 2. *Mol Med Rep* **9**:1917–1922.

doi:10.3892/mmr.2014.2013

Chang L, Jones Y, Ellisman MH, Goldstein LSB, Karin M. 2003. JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. *Dev Cell* **4**:521–533. doi:10.1016/S1534-5807(03)00094-7

Chen W, Qin C. 2015. General hallmarks of microRNAs in brain evolution and development. *RNA Biol* **12**:701–708. doi:10.1080/15476286.2015.1048954

Chen Y, Tian X, Kim WY, Snider WD. 2011. Adenomatous polyposis coli regulates axon arborization and cytoskeleton organization via its n-terminus. *PLoS One* **6**:1–11. doi:10.1371/journal.pone.0024335

Chen YM, Wang QJ, Hu HS, Yu PC, Zhu J, Drewes G, Piwnica-Worms H, Luo ZG. 2006. Microtubule affinity-regulating kinase 2 functions downstream of the PAR-3/PAR-6/atypical PKC complex in regulating hippocampal neuronal polarity. *Proc Natl Acad Sci* **103**:8534–8539. doi:10.1073/pnas.0509955103

Chen ZF, Paquette AJ, Anderson DJ. 1998. NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat Genet* **20**:136–142. doi:10.1038/2431

Cheng LC, Pastrana E, Tavazoie M, Doetsch F. 2009. MiR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat Neurosci* **12**:399–408. doi:10.1038/nn.2294

Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, Huang WC, Sun TH, Tu SJ, Lee WH, Chiew MY, Tai CS, Wei TY, Tsai TR, Huang HT, Wang CY, Wu HY, Ho SY, Chen PR, Chuang CH, Hsieh PJ, Wu YS, Chen WL, Li MJ, Wu YC, Huang XY, Ng FL, Buddhakosai W, Huang PC, Lan KC, Huang CY, Weng SL, Cheng YN, Liang C, Hsu WL, Huang H Da. 2018. MiRTarBase update 2018: A resource for experimentally validated

microRNA-target interactions. *Nucleic Acids Res* **46**:D296–D302. doi:10.1093/nar/gkx1067

Christie SB, Akins MR, Schwob JE, Fallon JR. 2009. The FXG: A Presynaptic Fragile X Granule Expressed in a Subset of Developing Brain Circuits. *J Neurosci* **29**:1514–1524. doi:10.1523/jneurosci.3937-08.2009

Ciani L, Salinas PC. 2007. c-Jun N-terminal kinase (JNK) cooperates with Gsk3β to regulate Dishevelled-mediated microtubule stability. *BMC Cell Biol* **8**:1–14. doi:10.1186/1471-2121-8-27

Cimadamore F, Amador-arjona A, Chen C, Huang C, Terskikh A V. 2013. SOX2–LIN28/let-7 pathway regulates proliferation and neurogenesis in neural precursors. *pnas* **2013**:3017–3026. doi:10.1073/pnas.1220176110

Cioni JM, Koppers M, Holt CE. 2018. Molecular control of local translation in axon development and maintenance. *Curr Opin Neurobiol* **51**:86–94. doi:10.1016/j.conb.2018.02.025

Cioni JM, Lin JQ, Holtermann A V., Koppers M, Jakobs MAH, Azizi A, Turner-Bridger B, Shigeoka T, Franze K, Harris WA, Holt CE. 2019. Late Endosomes Act as mRNA Translation Platforms and Sustain Mitochondria in Axons. *Cell* **176**:56-72.e15. doi:10.1016/j.cell.2018.11.030

Cohen MS, Ghosh AK, Kim HJ, Jeon NL, Jaffrey SR. 2012. Chemical genetic-mediated spatial regulation of protein expression in neurons reveals an axonal function for wld S. *Chem Biol* **19**:179–187. doi:10.1016/j.chembiol.2012.01.012

Colak D, Ji SJ, Porse BT, Jaffrey SR. 2013. Regulation of axon guidance by compartmentalized nonsense-mediated mRNA decay. *Cell* **153**:1252. doi:10.1016/j.cell.2013.04.056

Conforti L, Gilley J, Coleman MP. 2014. Wallerian degeneration: an emerging axon death pathway linking injury and disease. *Nat Rev Neurosci* **15**:394–

409. doi:10.1038/nrn3680

Conforti L, Wilbrey A, Morreale G, Janeckova L, Beirowski B, Adalbert R, Mazzola F, Stefano M Di, Hartley R, Babetto E, Smith T, Gilley J, Billington RA, Genazzani AA, Ribchester RR, Magni G, Coleman M. 2009. Wld S protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice. *J Cell Biol* **184**:491–500. doi:10.1083/jcb.200807175

Consortium TSPG-WAS (GWAS). 2011. Genome-wide association study identifies five new schizophrenia loci. *Nat Genet* **43**:969–978. doi:10.1038/ng.940

Conte I, Merella S, Garcia-Manteiga JM, Migliore C, Lazarevic D, Carrella S, Marco-Ferreres R, Avellino R, Davidson NP, Emmett W, Sanges R, Bockett N, Van Heel D, Meroni G, Bovolenta P, Stupka E, Banfi S. 2014. The combination of transcriptomics and informatics identifies pathways targeted by miR-204 during neurogenesis and axon guidance. *Nucleic Acids Res* **42**:7793–7806. doi:10.1093/nar/gku498

Coolen M, Katz S, Bally-Cuif L. 2013. miR-9: a versatile regulator of neurogenesis. *Front Cell Neurosci* **7**:1–11. doi:10.3389/fncel.2013.00220

Corradi E, Dalla Costa I, Gavoci A, Iyer A, Roccuzzo M, Otto TA, Oliani E, Bridi S, Strohbuecker S, Santos-Rodriguez G, Valdembri D, Serini G, Abreu-Goodger C, Baudet M-L. 2020. Axonal precursor miRNAs hitchhike on endosomesand locally regulate the development of neural circuits. *EMBO J* **39**:17–24. doi:10.15252/embj.2019102513

Cosker KE, Fenstermacher SJ, Pazyra-Murphy MF, Elliott HL, Segal RA. 2016. The RNA-binding protein SFPQ orchestrates an RNA regulon to promote axon viability. *Nat Neurosci* **19**:690–696. doi:10.1038/nn.4280

Cosker KE, Pazyra-Murphy MF, Fenstermacher SJ, Segal RA. 2013. Target-derived neurotrophins coordinate transcription and transport of Bclw to

prevent axonal degeneration. *Ann Intern Med* **158**:5195–5207. doi:10.1523/JNEUROSCI.3862-12.2013

Costa CJ, Willis DE. 2018. To the end of the line: Axonal mRNA transport and local translation in health and neurodegenerative disease. *Dev Neurobiol* **78**:209–220. doi:10.1002/dneu.22555

Court FA, Coleman MP. 2012. Mitochondria as a central sensor for axonal degenerative stimuli. *Trends Neurosci* **35**:364–372. doi:10.1016/j.tins.2012.04.001

Cowan CR, Hyman AA. 2004. ASYMMETRIC CELL DIVISION IN C. ELEGANS : Cortical Polarity and Spindle Positioning . *Annu Rev Cell Dev Biol* **20**:427–453. doi:10.1146/annurev.cellbio.19.111301.113823

Cox LJ, Hengst U, Gurskaya NG, Lukyanov KA, Jaffrey SR. 2008. Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. *Nat Cell Biol* **10**:149–159. doi:10.1038/ncb1677

Cuellar TL, Davis TH, Nelson PT, Loeb GB, Harfe BD, Ullian E, McManus MT. 2008. Dicer loss in striatal neurons produces behavioral and neuroanatomical phenotypes in the absence of neurodegeneration. *Proc Natl Acad Sci U S A* **105**:5614–5619. doi:10.1073/pnas.0801689105

Cui C, Xu G, Qiu J, Fan X. 2015. Up-regulation of miR-26a promotes neurite outgrowth and ameliorates apoptosis by inhibiting PTEN in bupivacaine injured mouse dorsal root ganglia. *Cell Biol Int* **39**:933–942. doi:10.1002/cbin.10461

Cullen BR. 2006. Enhancing and confirming the specificity of RNAi experiments. *Nat Methods* **3**:677–681. doi:10.1038/nmeth913

Dajas-Bailador F, Bantounas I, Jones E V, Whitmarsh AJ. 2014. Regulation of axon growth by the JIP1-AKT axis. *J Cell Sci* **127**:230–9. doi:10.1242/jcs.137208

Dajas-Bailador F, Bonev B, Garcez P, Stanley P, Guillemot F, Papalopulu N. 2012. microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons. *Nat Neurosci* **15**:697–699. doi:10.1038/nn.3082

Dajas-Bailador F, Jones E V., Whitmarsh AJ. 2008. The JIP1 Scaffold Protein Regulates Axonal Development in Cortical Neurons. *Curr Biol* **18**:221–226. doi:10.1016/j.cub.2008.01.025

Davis GM, Haas MA, Pocock R. 2015. MicroRNAs: Not "Fine-Tuners" but Key Regulators of Neuronal Development and Function. *Front Neurol* **6**:245. doi:10.3389/fneur.2015.00245

Davis TH, Cuellar TL, Koch SM, Barker AJ, Harfe BD, McManus MT, Ullian EM. 2008. Conditional Loss of Dicer Disrupts Cellular and Tissue Morphogenesis in the Cortex and Hippocampus. *J Neurosci* **28**:4322–4330. doi:10.1523/JNEUROSCI.4815-07.2008

De Pietri Tonelli D, Pulvers JN, Haffner C, Murchison EP, Hannon GJ, Huttner WB. 2008. miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. *Development* **135**:3911–3921. doi:10.1242/dev.025080

de Ramon Francàs G, Zuñiga NR, Stoeckli ET. 2017. The spinal cord shows the way – How axons navigate intermediate targets. *Dev Biol* **432**:43–52. doi:10.1016/j.ydbio.2016.12.002

De Vos KJ, Grierson AJ, Ackerley S, Miller CCJ. 2008. Role of Axonal Transport in Neurodegenerative Diseases. *Annu Rev Neurosci* **31**:151–173. doi:10.1146/annurev.neuro.31.061307.090711

Deglincerti A, Jaffrey SR. 2012. Insights into the roles of local translation from the axonal transcriptome. *Open Biol* **2**:120079–120079.

doi:10.1098/rsob.120079

Deglincerti A, Liu Y, Colak D, Hengst U, Xu G, Jaffrey SR. 2015. Coupled local translation and degradation regulate growth cone collapse. *Nat Commun* **6**:1–12. doi:10.1038/ncomms7888

Deitch A, Moses M. 1957. The Nissl substance of living and fixed spinal ganglion cells. II. An ultraviolet absorption study. *J Biophys Biochem Cytol* **25**:449–53. doi:10.1083/jcb.3.3.449

Deitch A, Murray A. 1956. The Nissl substance of liv- ing and fixed spinal ganglion cells. I. A phase contrast study. *J Biophys Biochem Cytol* **25**:433–44. doi:10.1083/jcb.2.4.433

Deng C-Y, Lei W-L, Xu X-H, Ju X-C, Liu Y, Luo Z-G. 2014. JIP1 Mediates Anterograde Transport of Rab10 Cargos during Neuronal Polarization. *J Neurosci* **34**:1710–1723. doi:10.1523/JNEUROSCI.4496-13.2014

Dent EW, Gertler FB. 2003. Cytoskeletal dynamics and transport in growth cone motility and guidance. *Neuron* **40**:209–227. doi:10.1016/S0896-6273(03)00633-0

Di Stefano M, Nascimento-Ferreira I, Orsomando G, Mori V, Gilley J, Brown R, Janeckova L, Vargas ME, Worrell LA, Loreto A, Tickle J, Patrick J, Webster JRM, Marangoni M, Carpi FM, Pucciarelli S, Rossi F, Meng W, Sagasti A, Ribchester RR, Magni G, Coleman MP, Conforti L. 2015. A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration. *Cell Death Differ* **22**:731–742. doi:10.1038/cdd.2014.164

Dill H, Linder B, Fehr A, Fischer U. 2012. Intronic miR-26b controls neuronal differentiation by repressing its host transcript, ctdsp2. *Genes Dev* **26**:25–30. doi:10.1101/gad.177774.111

Dill J, Wang H, Zhou F, Li S. 2008. Inactivation of glycogen synthase kinase 3

promotes axonal growth and recovery in the CNS. *J Neurosci* **28**:8914–8928. doi:10.1523/JNEUROSCI.1178-08.2008

Dimidschstein J, Passante L, Dufour A, vandenAmeele J, Tiberi L, Hrechdakian T, Adams R, Klein R, Lie DC, Jossin Y, Vanderhaeghen P. 2013. Ephrin-B1 controls the columnar distribution of cortical pyramidal neurons by restricting their tangential migration. *Neuron* **79**:1123–1135. doi:10.1016/j.neuron.2013.07.015

Distel M, Hocking JC, Volkmann K, Köster RW. 2010. The centrosome neither persistently leads migration nor determines the site of axonogenesis in migrating neurons in vivo. *J Cell Biol* **191**:875–890. doi:10.1083/jcb.201004154

Doble B, Woodgett JR. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* **116**:1175–1186. doi:10.1242/jcs.00384

Dotti CG, Banker GA. 1987. Experimentally induced alteration in the polarity of developig neurons. *Nature* 0–2.

Dotti CG, Sullivan C a, Banker G a. 1988. The establishment of polarity by hippocampal neurons in culture. *J Neurosci* **8**:1454–1468. doi:10.1016/0012-1606(89)90269-8

Drescher U. 2011. Axon guidance: Push and pull with ephrins and GDNF. *Curr Biol* **21**:R30–R32. doi:10.1016/j.cub.2010.11.064

Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, Tada T, Dolan BM, Sharp PA, Sheng M. 2010. Regulation of Synaptic Structure and Function by FMRP-Associated MicroRNAs miR-125b and miR-132. *Neuron* **65**:373–384. doi:10.1016/j.neuron.2010.01.005

Eminel S, Roemer L, Waetzig V, Herdegen T. 2008. c-Jun N-terminal kinases trigger both degeneration and neurite outgrowth in primary hippocampal and cortical neurons. *J Neurochem* **104**:957–969. doi:10.1111/j.1471-

4159.2007.05101.x

Eng H, Lund K, Campenot RB. 1999. Synthesis of beta-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. *J Neurosci* **19**:1–9. doi:10.1523/JNEUROSCI.19-01-00001.1999

Esch T, Lemmon V, Banker G. 1999. Local presentation of substrate molecules directs axon specification by cultured hippocampal neurons. *J Neurosci* **19**:6417–26. doi:10.1523/JNEUROSCI.19-15-06417.1999

Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A. 2003. Phosphorylation by glycogen synthase kinase-3β down-regulates Notch activity, a link for Notch and Wnt pathways. *J Biol Chem* **278**:32227–32235. doi:10.1074/jbc.M304001200

Etienne-Manneville S, Hall A. 2003. Cdc42 regulates GSK-3β and adenomatous polyposis coli to control cell polarity. *Nature* **421**:753–756. doi:10.1038/nature01423

Evangelopoulos ME, Weis J, Krüttgen A. 2009. Mevastatin-induced neurite outgrowth of neuroblastoma cells via activation of EGFR. *J Neurosci Res* **87**:2138–2144. doi:10.1002/jnr.22025

Fallini C, Donlin-Asp PG, Rouanet JP, Bassell GJ, Rossoll W. 2016. Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons. *J Neurosci* **36**:3811–3820. doi:10.1523/jneurosci.2396-15.2016

Fang WQ, Chen WW, Fu AKY, Ip NY. 2013. Axin directs the amplification and differentiation of intermediate progenitors in the developing cerebral cortex. *Neuron* **79**:665–679. doi:10.1016/j.neuron.2013.06.017

Fazeli A, Dickinson SL, Hermiston ML, Tighe R V., Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H, Simons J, Bronson RT, Gordon JI, Tessier-Lavigne M, Weinberg RA. 1997. Phenotype of mice

lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* **386**:796–804. doi:10.1038/386796a0

Feinberg P, Mann S, Handler A, Kjems J, Surmeier DJ, Schaefer A. 2013. MicroRNA-128 Governs Neuronal Excitability and Motor Behavior in Mice. *Science (80-)* **342**:1254–1258. doi:10.1126/science.1244193

Filipowicz W, Bhattacharyya SN, Sonenberg N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? *Nat Rev Genet* **9**:102–114. doi:10.1038/nrg2290

Finci LI, Krüger N, Sun X, Zhang J, Chegkazi M, Wu Y, Schenk G, Mertens HDT, Svergun DI, Zhang Y, Wang J huai, Meijers R. 2014. The Crystal Structure of Netrin-1 in Complex with DCC Reveals the Bifunctionality of Netrin-1 As a Guidance Cue. *Neuron* **83**:839–849. doi:10.1016/j.neuron.2014.07.010

Fiore R, Khudayberdiev S, Saba R, Schratt G. 2011. MicroRNA function in the nervous system. *Prog Mol Biol Transl Sci* **102**:47–100. doi:10.1016/B978-0-12-415795-8.00004-0

Fiore R, Schratt G. 2007. MicroRNAs in synapse development: tiny molecules to remember. *Expert Opin Biol Ther* **7**:1823–1831. doi:10.1517/14712598.7.12.1823

Flynn KC, Pak CW, Shaw AE, Bradke F, Bamburg JR. 2009. Growth cone-like waves transport actin and promote axonogenesis and neurite branching. *Dev Neurobiol* **69**:761–779. doi:10.1002/dneu.20734

Foggin S, Mesquita-Ribeiro R, Dajas-Bailador F, Layfield R. 2019. Biological Significance of microRNA Biomarkers in ALS—Innocent Bystanders or Disease Culprits? *Front Neurol* **10**:1–8. doi:10.3389/fneur.2019.00578

Fogh I, Ratti A, Gellera C, Lin K, Tiloca C, Moskvina V, Corrado L, Sorarù G, Cereda C, Corti S, Gentilini D, Calini D, Castellotti B, Mazzini L, Querin

G, Gagliardi S, Del bo R, Conforti FL, Siciliano G, Inghilleri M, Saccà F, Bongioanni P, Penco S, Corbo M, Sorbi S, Filosto M, Ferlini A, Di blasio AM, Signorini S, Shatunov A, Jones A, Shaw PJ, Morrison KE, Farmer AE, Van damme P, Robberecht W, Chiò A, Traynor BJ, Sendtner M, Melki J, Meininger V, Hardiman O, Andersen PM, Leigh NP, Glass JD, Overste D, Diekstra FP, Veldink JH, Van es MA, Shaw CE, Weale ME, Lewis CM, Williams J, Brown RH, Landers JE, Ticozzi N, Ceroni M, Pegoraro E, Comi GP, D'alfonso S, Van den berg LH, Taroni F, Al-chalabi A, Powell J, Silani V. 2014. A genome-wide association meta-analysis identifies a novel locus at 17q11.2 associated with sporadic amyotrophic lateral sclerosis. *Hum Mol Genet* **23**:2220–2231. doi:10.1093/hmg/ddt587

Frühbeis C, Fröhlich D, Krämer-Albers EM. 2012. Emerging roles of exosomes in neuron-glia communication. *Front Physiol* **3**:1–7. doi:10.3389/fphys.2012.00119

Fu MM, Holzbaur ELF. 2013. JIP1 regulates the directionality of APP axonal transport by coordinating kinesin and dynein motors. *J Cell Biol* **202**:495–508. doi:10.1083/jcb.201302078

Fuentealba LC, Eivers E, Geissert D, Taelman V, De Robertis EM. 2008. Asymmetric mitosis: Unequal segregation of proteins destined for degradation. *Proc Natl Acad Sci* **105**:7732–7737. doi:10.1073/pnas.0803027105

Fukata Y, Itoh TJ, Kimura T, Ménager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K. 2002. CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* **4**:583–591. doi:10.1038/ncb825

Futerman AH, Banker GA. 1996. The economics of neurite outgrowth - The addition of new membrane to growing axons. *Trends Neurosci* **19**:144–149.

doi:10.1016/S0166-2236(96)80025-7

Gallo V, Deneen B. 2014. Glial development: The crossroads of regeneration and repair in the CNS. *Neuron* **83**:283–308. doi:10.1016/j.neuron.2014.06.010

Gangaraju VK, Lin H. 2009. MicroRNAs: key regulators of stem cells. *Nature* **10 (2)**:116–125. doi:10.1038/nrm2621

Garbe DS, Bashaw GJ. 2007. Independent functions of slit-robo repulsion and Netrin-Frazzled attraction regulate axon crossing at the midline in Drosophila. *J Neurosci* **27**:3584–3592. doi:10.1523/JNEUROSCI.0301-07.2007

Garcez PP, Guillemot FF, Dajas-Bailador F. 2016. Study of miRNA Function in the Developing Axons of Mouse Cortical Neurons: Use of Compartmentalized Microfluidic Chambers and In Utero Electroporation. *Neuromethods* 391–403. doi:10.1007/7657

Garcia M, Leduc C, Lagardère M, Argento A, Sibarita J-B, Thoumine O. 2015. Two-tiered coupling between flowing actin and immobilized N -cadherin/catenin complexes in neuronal growth cones . *Proc Natl Acad Sci* **112**:6997–7002. doi:10.1073/pnas.1423455112

Garrido JJ, Simón D, Varea O, Wandosell F. 2007. GSK3 alpha and GSK3 beta are necessary for axon formation. *FEBS Lett* **581**:1579–1586. doi:10.1016/j.febslet.2007.03.018

Gartner A, Huang X, Hall A. 2006. Neuronal polarity is regulated by glycogen synthase kinase-3 (GSK-3) independently of Akt/PKB serine phosphorylation. *J Cell Sci* **119**:3927–3934. doi:10.1242/jcs.03159

Gerdts J, Brace EJ, Sasaki Y, Diantonio A, Milbrandt J. 2015. SARM1 activation triggers axon degeneration locally via NAD+ destruction.

Gerdts J, Summers DW, Milbrandt J, DiAntonio A. 2016. Axon Self-Destruction: New Links among SARM1, MAPKs, and NAD+ Metabolism. *Neuron* **89**:449–460. doi:10.1016/j.neuron.2015.12.023

Gerdts J, Summers DW, Sasaki Y, DiAntonio A, Milbrandt J. 2013. Sarm1-Mediated Axon Degeneration Requires Both SAM and TIR Interactions. *J Neurosci* **33**:13569–13580. doi:10.1523/JNEUROSCI.1197-13.2013

Gilley J, Adalbert R, Yu G, Coleman MP. 2013. Rescue of Peripheral and CNS Axon Defects in Mice Lacking NMNAT2. *J Neurosci* **33**:13410–13424. doi:10.1523/jneurosci.1534-13.2013

Gilley J, Coleman MP. 2010. Endogenous Nmnat2 Is an Essential Survival Factor for Maintenance of Healthy Axons. *PLoS Biol* **8**:1–18. doi:10.1371/journal.pbio.1000300

Gilley J, Orsomando G, Nascimento-Ferreira I, Coleman MP. 2015. Absence of SARM1 rescues development and survival of NMNAT2-Deficient axons. *Cell Rep* **10**:1975–1982. doi:10.1016/j.celrep.2015.02.060

Gilley J, Ribchester RR, Coleman MP. 2017. Sarm1 Deletion, but Not WldS, Confers Lifelong Rescue in a Mouse Model of Severe Axonopathy. *Cell Rep* **21**:10–16. doi:10.1016/j.celrep.2017.09.027

Giraldez AJ, Cinalli RM, Glasner M, Schier AF. 2005. MicroRNAs regulate brain morphogenesis in zebrafish. *Science (80-)* **308**:833–38. doi:10.1126/science.1109020

Giuditta A, Cupellot A, Lazzarini G. 1980. Ribosomal RNA in the Axoplasm of the Squid Giant Axon. *J Neurochem* **34**:1757–1760. doi:10.1111/j.1471-4159.1980.tb11271.x

Giuditta A, Hunt T, Santella L. 1986. Rapid important paper. *Neurochem Int* **8**:435–442. doi:10.1016/0197-0186(86)90019-7

Giuditta A, Menichini E, Capano CP, Langella M, Martin R, Castigli E, Kaplan BB. 1991. Active polysomes in the axoplasm of the squid giant axon. *J Neurosci Res* **28**:18–28. doi:10.1002/jnr.490280103

Giusti SA, Vogl AM, Brockmann MM, Vercelli CA, Rein ML, Trümbach D, Wurst W, Cazalla D, Stein V, Deussing JM, Refojo D. 2014. MicroRNA-9 controls dendritic development by targeting REST. *Elife* **3**:1–22. doi:10.7554/elife.02755

Gobrecht P, Leibinger M, Andreadaki A, Fischer D. 2014. Sustained GSK3 activity markedly facilitates nerve regeneration. *Nat Commun* **5**:4561. doi:10.1038/ncomms5561

Goldshmit Y, Walters CE, Scott HJ, Greenhalgh CJ, Turnley AM. 2004. SOCS2 Induces Neurite Outgrowth by Regulation of Epidermal Growth Factor Receptor Activation. *J Biol Chem* **279**:16349–16355. doi:10.1074/jbc.M312873200

Gomez TM, Letourneau PC. 2014. Actin dynamics in growth cone motility and navigation. *J Neurochem* **129**:221–234. doi:10.1111/jnc.12506

Gorski JA, Talley T, Qiu M, Puelles L, Rubenstein JLR, Jones KR. 2002. Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J Neurosci* **22**:6309–14. doi:20026564

Goslin K, bank. 1989. Experimental observations on the development of polarity by hippocampal neurons in culture. *J Cell Biol* **108**:1507–1516. doi:10.1083/jcb.108.4.1507

Götz M, Huttner WB. 2005. The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* **6**:777–788. doi:10.1038/nrm1739

Gracias NG, Shirkey-Son NJ, Hengst U. 2014. Local translation of TC10 is required for membrane expansion during axon outgrowth. *Nat Commun* **5**:1–13. doi:10.1038/ncomms4506

Graef IA, Wang F, Charron F, Chen L, Neilson J, Tessier-Lavigne M, Crabtree GR. 2003. Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**:657–670. doi:10.1016/S0092-8674(03)00390-8

Grasso M, Piscopo P, Confaloni A, Denti MA. 2014. Circulating miRNAs as biomarkers for neurodegenerative disorders. *Molecules* **19**:6891–6910. doi:10.3390/molecules19056891

Grimes CA, Jope RS. 2001. Creb DNA binding activity is inhibited by glycogen synthase kinase-3β and facilitated by lithium. *J Neurochem* **78**:1219–1232. doi:10.1046/j.1471-4159.2001.00495.x

Gumy LF, Tan CL, Fawcett JW. 2010. The role of local protein synthesis and degradation in axon regeneration. *Exp Neurol* **223**:28–37. doi:10.1016/j.expneurol.2009.06.004

Gumy LF, Yeo GSH, Tung YCL, Zivraj KH, Willis D, Coppola G, Lam BYH, Twiss JL, Holt CE, Fawcett JW. 2011. Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. *Rna* **17**:85–98. doi:10.1261/rna.2386111

Gunawardena S, Goldstein LSB. 2001. Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in Drosophila. *Neuron* **32**:389–401. doi:10.1016/S0896-6273(01)00496-2

Guo X, Snider WD, Chen B. 2016. Gsk3β regulates AKT-induced central nervous system axon regeneration via an eIF2Bε -dependent, mTORC1-independent pathway. *Elife* **5**:1–18. doi:10.7554/eLife.11903

Guo Y, Liu Z, Chen Y kun, Chai Z, Zhou C, Zhang Y. 2017. Neurons with Multiple Axons Have Functional Axon Initial Segments. *Neurosci Bull* **33**:641–652. doi:10.1007/s12264-017-0169-3

Gupton SL, Gertler FB. 2007. Filopodia: the fingers that do the walking. *Sci*

STKE **re5**:1–8. doi:10.1126/stke.4002007re5

Ha M, Kim VN. 2014. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* **15**:509–524. doi:10.1038/nrm3838

Hammond J, Huang C, Verhey K. 2010. Posttranslational Modifications of Tubulin and the Polarized Transport of Kinesin-1 in Neurons. *Mol Biol Cell* **21**:572–583. doi:10.1091/mbc.E09

Hancock ML, Preitner N, Quan J, Flanagan JG. 2014. MicroRNA-132 Is Enriched in Developing Axons, Locally Regulates Rasa1 mRNA, and Promotes Axon Extension. *J Neurosci* **34**:66–78. doi:10.1523/JNEUROSCI.3371-13.2014

Hanger DP, Hughes K, Woodgett JR, Brion JP, Anderton BH. 1992. Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: Generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci Lett* **147**:58–62. doi:10.1016/0304-3940(92)90774-2

Hanz S, Perlson E, Willis D, Zheng JQ, Massarwa R, Huerta JJ, Koltzenburg M, Kohler M, Van-Minnen J, Twiss JL, Fainzilber M. 2003. Axoplasmic importins enable retrograde injury signaling in lesioned nerve. *Neuron* **40**:1095–1104. doi:10.1016/S0896-6273(03)00770-0

Hapak SM, Rothlin C V., Ghosh S. 2018. PAR3–PAR6–atypical PKC polarity complex proteins in neuronal polarization. *Cell Mol Life Sci* **75**:2735–2761. doi:10.1007/s00018-018-2828-6

Harris W, Holt C, Bonhoeffer F. 1987. Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibres in vivo. *Development* **101**:123–33.

Hatten M. 2002. New directions in neuronal migration. *Science (80-)* **297**:1660–1664. doi:10.1126/science.1074572

He CW, Liao CP, Pan CL. 2018. Wnt signalling in the development of axon, dendrites and synapses. *Open Biol* **8**:1–12. doi:10.1098/rsob.180116

Hébert SS, Sergeant N, Buée L. 2012. MicroRNAs and the Regulation of Tau Metabolism. *Int J Alzheimers Dis* **2012**:1–6. doi:10.1155/2012/406561

Hedstrom KL, Ogawa Y, Rasband MN. 2008. AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. *J Cell Biol* **183**:635–640. doi:10.1083/jcb.200806112

Heidemann SR, Landers JM, Hamborg MA. 1981. Polarity orientation of axonal microtubules. *J Cell Biol* **91**:661–665. doi:10.1083/jcb.91.3.661

Hengst U, Deglincerti A, Kim HJ, Jeon NL, Jaffrey SR. 2009. Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. *Nat Cell Biol* **11**:1024–1030. doi:10.1038/ncb1916

Hengst U, Jaffrey SR. 2007. Function and translational regulation of mRNA in developing axons. *Semin Cell Dev Biol* **18**:209–215. doi:10.1016/j.semcdb.2007.01.003.Function

Henninger N, Bouley J, Sikoglu EM, An J, Moore CM, King JA, Bowser R, Freeman MR, Brown RH. 2016. Attenuated traumatic axonal injury and improved functional outcome after traumatic brain injury in mice lacking Sarm1. *Brain* **139**:1094–1105. doi:10.1093/brain/aww001

Hill CS, Coleman MP, Menon DK. 2016. Traumatic Axonal Injury: Mechanisms and Translational Opportunities. *Trends Neurosci* **39**:311–324. doi:10.1016/j.tins.2016.03.002

Hillefors M, Gioio AE, Mameza MG, Kaplan BB. 2007. Axon viability and mitochondrial function are dependent on local protein synthesis in sympathetic neurons. *Cell Mol Neurobiol* **27**:701–716. doi:10.1007/s10571-007-9148-y

Hilliard MA, Bargmann CI. 2006. Wnt signals and Frizzled activity orient anterior-posterior axon outgrowth in C. elegans. *Dev Cell* **10**:379–390. doi:10.1016/j.devcel.2006.01.013

Hirokawa N, Niwa S, Tanaka Y. 2010. Molecular Motors in Neurons: Transport Mechanisms and Roles in Brain Function, Development, and Disease. *Neuron* **68**:610–638. doi:10.1016/j.neuron.2010.09.039

Holt CE, Bullock S. 2009. Subcellular mRNA Localization in Animal Cells and Why It Matter. *Science (80-)* **1212**:1212–1217. doi:10.1126/science.1176488

Holt CE, Martin KC, Schuman EM. 2019. Local translation in neurons: visualization and function. *Nat Struct Mol Biol* **26**:557–566. doi:10.1038/s41594-019-0263-5

Hor CHH, Goh ELK. 2018. Rab23 Regulates Radial Migration of Projection Neurons via N-cadherin. *Cereb Cortex* **28**:1516–1531. doi:10.1093/cercor/bhy018

Horiguchi K, Hanada T, Fukui Y, Chishti AH. 2006. Transport of PIP3 by GAKIN, a kinesin-3 family protein, regulates neuronal cell polarity. *J Cell Biol* **174**:425–436. doi:10.1083/jcb.200604031

Horiuchi D, Collins CA, Bhat P, Barkus R V., DiAntonio A, Saxton WM. 2007. Control of a Kinesin-Cargo Linkage Mechanism by JNK Pathway Kinases. *Curr Biol* **17**:1313–1317. doi:10.1016/j.cub.2007.06.062

Hu S, Zhu L. 2018. Semaphorins and their receptors: From axonal guidance to atherosclerosis. *Front Physiol* **9**:1–11. doi:10.3389/fphys.2018.01236

Huang T, Liu Y, Huang M, Zhao X, Cheng L. 2010. Wnt1-cre-mediated conditional loss of Dicer results in malformation of the midbrain and cerebellum and failure of neural crest and dopaminergic differentiation in mice. *J Mol Cell Biol* **2**:152–163. doi:10.1093/jmcb/mjq008

Hur Eun-MI, Zhou F-Q. 2010. GSK3 signaling in neural development. *Nat Rev Neurosci* **11**:539–551. doi:10.1038/nrn2870.GSK3

Hur Eun-Mi, Zhou F-Q. 2010. GSK3 signalling in neural development. *Nat Rev Neurosci* **11**:539–51. doi:10.1038/nrn2870

Hur EM, Saijilafu, Lee BD, Kim SJ, Xu WL, Zhou FQ. 2011. GSK3 controls axon growth via CLASP-mediated regulation of growth cone microtubules. *Genes Dev* **25**:1968–1981. doi:10.1101/gad.17015911

Iekmann H, Fischer D. 2015. Role of GSK3 in peripheral nerve regeneration. *Neural Regen Res* **10**:1602–1603. doi:10.4103/1673-5374.167753

Ikeuchi Y, Stegmuller J, Netherton S, Huynh MA, Masu M, Frank D, Bonni S, Bonni A. 2009. A SnoN-Ccd1 Pathway Promotes Axonal Morphogenesis in the Mammalian Brain. *J Neurosci* **29**:4312–4321. doi:10.1523/jneurosci.0126-09.2009

Indrieri A, Carrella S, Romano A, Spaziano A, Marrocco E, Fernandez-Vizarra E, Barbato S, Pizzo M, Ezhova Y, Golia FM, Ciampi L, Tammaro R, Henao-Mejia J, Williams A, Flavell RA, De Leonibus E, Zeviani M, Surace EM, Banfi S, Franco B. 2019. miR-181a/b downregulation exerts a protective action on mitochondrial disease models. *EMBO Mol Med* **11**:e8734. doi:10.15252/emmm.201708734

Inoki K, Ouyang H, He X, Harada Y, Stankunas K, Yang Q, MacDougald OA, Bennett C, You M, Lindvall C, Wang Y, Zhu T, Wang C, Zhang X, Williams BO, Guan K-L. 2006. TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth. *Cell* **126**:955–968. doi:10.1016/j.cell.2006.06.055

Iwata T, Hevner RF. 2009. Fibroblast growth factor signaling in development of the cerebral cortex. *Dev Growth Differ* **51**:299–323. doi:10.1111/j.1440-169X.2009.01104.x

Iyer AN, Bellon A, Baudet M-L. 2014. microRNAs in axon guidance. *Front Cell Neurosci* **8**:1–14. doi:10.3389/fncel.2014.00078

Jacobson C, Schnapp B, Banker GA. 2006. A change in the selective translocation of the kinesin-1 motor domain marks the initial specification of the axon. *Neuron* **49**:797–804. doi:10.1016/j.neuron.2006.02.005

Jansson MD, Lund AH. 2012. MicroRNA and cancer. *Mol Oncol* **6**:590–610. doi:10.1016/j.molonc.2012.09.006

Jaworski A, Tessier-Lavigne M. 2012. Autocrine/juxtaparacrine regulation of axon fasciculation by Slit-Robo signaling. *Nat Neurosci* **15**:367–369. doi:10.1038/nn.3037

Ji SJ, Jaffrey SR. 2012. Intra-axonal Translation of SMAD1/5/8 Mediates Retrograde Regulation of Trigeminal Ganglia Subtype Specification. *Neuron* **74**:95–107. doi:10.1016/j.neuron.2012.02.022

Jiang H, Guo W, Liang X, Rao Y. 2005. Both the establishment and the maintenance of neuronal polarity require active mechanisms: Critical roles of GSK-3?? and its upstream regulators. *Cell* **120**:123–135. doi:10.1016/j.cell.2004.12.033

Jiang J-J, Liu C-M, Zhang B-Y, Wang X-W, Zhang M, Saijilafu, Zhang S-R, Hall P, Hu Y-W, Zhou F-Q. 2015. MicroRNA-26a supports mammalian axon regeneration in vivo by suppressing GSK3β expression. *Cell Death Dis* **6**:1–9. doi:10.1038/cddis.2015.239

Jiménez-Mateos EM, Paglini G, González-Billault C, Cáceres A, Avila J. 2005. End binding protein-1 (EB1) complements microtubule-associated protein-1B during axonogenesis. *J Neurosci Res* **80**:350–359. doi:10.1002/jnr.20453

Johanne R, Linda B. 2012. The wrap that feeds neurons. *Nature* **487**:435–436. doi:10.1038/487435a

Jossin Y. 2004. Neuronal Migration and the Role of Reelin During Early Development of the Cerebral Cortex. *Mol Neurobiol* **30**:225–251. doi:10.1385/MN:30:3:225

Jossin Y, Cooper JA. 2011. Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex. *Nat Neurosci* **14**:697–703. doi:10.1038/nn.2816

Jung H, Yoon BC, Holt CE. 2012. Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. *Nat Rev Neurosci*. doi:10.1038/nrn3274

Kabaria S, Choi DC, Chaudhuri AD, Mouradian MM, Junn E. 2015. Inhibition of miR-34b and miR-34c enhances α-synuclein expression in Parkinson's disease. *FEBS Lett* **589**:319–325. doi:10.1016/j.febslet.2014.12.014

Kahn OI, Baas PW. 2016. Microtubules and Growth Cones: Motors Drive the Turn. *Trends Neurosci* **39**:433–440. doi:10.1016/j.tins.2016.04.009

Kalinski AL, Twiss JL, Sachdeva R, Lee SJ, Shah Z, Houle JD, Gomes C. 2015. mRNAs and Protein Synthetic Machinery Localize into Regenerating Spinal Cord Axons When They Are Provided a Substrate That Supports Growth. *J Neurosci* **35**:10357–10370. doi:10.1523/jneurosci.1249-15.2015

Kamal A, Stokin GB, Yang Z, Xia CH, Goldstein LSB. 2000. Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron* **28**:449–459. doi:10.1016/S0896-6273(00)00124-0

Kapitein LC, Hoogenraad CC. 2011. Which way to go? Cytoskeletal organization and polarized transport in neurons. *Mol Cell Neurosci* **46**:9–20. doi:10.1016/j.mcn.2010.08.015

Kaplan BB, Kar AN, Gioio AE, Aschrafi A. 2013. MicroRNAs in the axon and presynaptic nerve terminal. *Front Cell Neurosci* **7**:1–5.

doi:10.3389/fncel.2013.00126

Kar AN, MacGibeny MA, Gervasi NM, Gioio AE, Kaplan BB. 2013. Intra-axonal Synthesis of Eukaryotic Translation Initiation Factors Regulates Local Protein Synthesis and Axon Growth in Rat Sympathetic Neurons. *J Neurosci* **33**:7165–7174. doi:10.1523/jneurosci.2040-12.2013

Kar AN, Sun CY, Reichard K, Gervasi NM, Pickel J, Nakazawa K, Gioio AE, Kaplan BB. 2014. Dysregulation of the axonal trafficking of nuclear-encoded mitochondrial mRNA alters neuronal mitochondrial activity and mouse behavior. *Dev Neurobiol* **74**:333–350. doi:10.1002/dneu.22141

Karasmanis EP, Phan CT, Angelis D, Kesisova IA, Hoogenraad CC, McKenney RJ, Spiliotis ET. 2018. Polarity of Neuronal Membrane Traffic Requires Sorting of Kinesin Motor Cargo during Entry into Dendrites by a Microtubule-Associated Septin. *Dev Cell* **46**:204-218.e7. doi:10.1016/j.devcel.2018.06.013

Kaur H, Arora A, Wengel J, Maiti S. 2006. Thermodynamic, counterion, and hydration effects for the incorporation of locked nucleic acid nucleotides into DNA duplexes. *Biochemistry* **45**:7347–7355. doi:10.1021/bi060307w

Kawaguchi Y. 1993. Groupings of nonpyramidal and pyramidal cells with specific physiological and morphological characteristics in rat frontal cortex. *J Neurophysiol* **69**:416–431. doi:10.1152/jn.1993.69.2.416

Kim J, Inoue K, Ishii J, Vanti WB, Voronov S V., Murchison E, Hannon G, Abeliovich A. 2007. A microRNA feedback circuit in midbrain dopamine neurons. *Science (80-)* **317**:1220–1224. doi:10.1126/science.1140481

Kim J, Krichevsky A, Grad Y, Hayes GD, Kosik KS, Church GM, Ruvkun G. 2004. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. *Pnas* **101**:360–365. doi:10.1073/pnas.2333854100

Kim N, Xu C, Gumbiner B. 2009. Identification of targets of the Wnt pathway destruction complex in addition to -catenin. *Proc Natl Acad Sci* **106**:5165–5170. doi:10.1073/pnas.0810185106

Kim T, Courchesne SL, Cosker KE, Heerssen HM, Greenberg ME, Karch C, Hans A, Watson FL, Segal RA, Pazyra-Murphy MF. 2009. A Retrograde Neuronal Survival Response: Target-Derived Neurotrophins Regulate MEF2D and bcl-w. *J Neurosci* **29**:6700–6709. doi:10.1523/jneurosci.0233-09.2009

Kim VN. 2005. MicroRNA biogenesis: Coordinated cropping and dicing. *Nat Rev Mol Cell Biol* **6**:376–385. doi:10.1038/nrm1644

Kim W-Y, Snider WD. 2011. Functions of GSK-3 Signaling in Development of the Nervous System. *Front Mol Neurosci* **4**:1–13. doi:10.3389/fnmol.2011.00044

Kim W-Y, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR, Snider WD. 2009. GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci* **12**:1390–1397. doi:10.1038/nn.2408

Kim WY, Zhou FQ, Zhou J, Yokota Y, Wang YM, Yoshimura T, Kaibuchi K, Woodgett JR, Anton ES, Snider WD. 2006. Essential Roles for GSK-3s and GSK-3-Primed Substrates in Neurotrophin-Induced and Hippocampal Axon Growth. *Neuron* **52**:981–996. doi:10.1016/j.neuron.2006.10.031

Kimura T, Arimura N, Fukata Y, Watanabe H, Iwamatsu A, Kaibuchi K. 2005. Tubulin and CRMP-2 complex is transported via Kinesin-1. *J Neurochem* **93**:1371–1382. doi:10.1111/j.1471-4159.2005.03063.x

Koch D, Rosoff WJ, Jiang J, Geller HM, Urbach JS. 2012. Strength in the periphery: Growth cone biomechanics and substrate rigidity response in peripheral and central nervous system neurons. *Biophys J* **102**:452–460. doi:10.1016/j.bpj.2011.12.025

Koenig E. 1991. Evaluation of local synthesis of axonal proteins in the goldfish Mauthner cell axon and axons of dorsal and ventral roots of the rat in vitro. *Mol Cell Neurosci* **2**:384–394. doi:10.1016/1044-7431(91)90025-J

Koenig E, Adams P. 1982. Local Protein Synthesizing Activity in Axonal Fields Regenerating In Vitro. *J Neurochem* **39**:386–400. doi:10.1111/j.1471-4159.1982.tb03960.x

Koenig E, Martin R. 1996. Cortical plaque-like structures identify ribosome-containing domains in the Mauthner cell axon. *J Neurosci* **16**:1400–1411. doi:10.1523/jneurosci.16-04-01400.1996

Koenig E, Martin R, Titmus M, Sotelo-Silveira JR. 2000. Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons. *J Neurosci* **20**:8390–400. doi:10.1523/JNEUROSCI.20-22-08390.2000

Konishi Y, Stegmüller J, Bonni A. 2004. Cdh1-APC Controls Axonal Growth and Patterning in the Mammalian Brain. *Science (80-)* **303**:1026–1030. doi:10.1177/0023830917727774

Kos A, de Mooij-Malsen AJ, van Bokhoven H, Kaplan BB, Martens GJ, Kolk SM, Aschrafi A. 2017a. MicroRNA-338 modulates cortical neuronal placement and polarity. *RNA Biol* **14**:905–913. doi:10.1080/15476286.2017.1325067

Kos A, Klein-Gunnewiek T, Meinhardt J, Loohuis NFMO, van Bokhoven H, Kaplan BB, Martens GJ, Kolk SM, Aschrafi A. 2017b. MicroRNA-338 Attenuates Cortical Neuronal Outgrowth by Modulating the Expression of Axon Guidance Genes. *Mol Neurobiol* **54**:3439–3452. doi:10.1007/s12035-016-9925-z

Kos A, Loohuis NO, Meinhardt J, Bokhoven H Van, Martens G, Aschrafi A. 2016. MicroRNA-181 promotes synaptogenesis and attenuates axonal

outgrowth in cortical neurons **73**:3555–3567. doi:10.1007/s00018-016-2179-0.MicroRNA-181

Koushika SP. 2007. ‘“ JIP ”’ ing along the axon : the complex roles of JIPs in axonal transport. *BioEssays* **30**:10–14. doi:10.1002/bies.20695

Kozomara A, Birgaoanu M, Griffiths-Jones S. 2019. miRBase: from microRNA sequences to function. *Nucleic Acids Res* **47**:D155–D162. doi:10.1093/nar/gky1141

Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. 2003. A microRNA array reveals extensive regulation of microRNAs during brain development 1274–1281. doi:10.1261/rna.5980303.regulation

Krol J, Loedige I, Filipowicz W. 2010. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* **11**:597–610. doi:10.1038/nrg2843

Kuan C-Y, Yang DD, Flavell R. 1999. The Jnk1 and Jnk2 Protein Kinases Are Required for Regional Specific Apoptosis during Early Brain Development. *Neuron* **22**:667–76. doi:10.1016/s0896-6273(00)80727-8

Kubota Y. 2014. Untangling GABAergic wiring in the cortical microcircuit. *Curr Opin Neurobiol* **26**:7–14. doi:10.1016/j.conb.2013.10.003

Kun A, Leonardo O, Sotelo JR. 2007. Ribosomal Distributions in Axons of Mammalian Myelinated Fibers. *J Neurosci Res* **804**:798–804. doi:10.1002/jnr

Kunda P, Paglini G, Cáceres A. 2001. Evidence for the role of Tiam1 in axon formation. *Mol Biol Cell* **12**:2087–98. doi:10.1091/mbc.12.7.2087

Kye M-J, Liu T, Levy SF, Xu NL, Groves BB, Bonneau R, Lao K, Kosik KS. 2007. Somatodendritic microRNAs identified by laser capture and multiplex RT-PCR. *Rna* **13**:1224–1234. doi:10.1261/rna.480407

Kye MJ eon., Niederst ED, Wertz MH, Gonçalves I do CG, Akten B, Dover KZ,

Peters M, Riessland M, Neveu P, Wirth B, Kosik KS, Sardi SP, Monani UR, Passini MA, Sahin M. 2014. SMN regulates axonal local translation via miR-183/mTOR pathway. *Hum Mol Genet* **23**:6318–6331. doi:10.1093/hmg/ddu350

Lafourcade C, Ramírez JP, Luarte A, Fernández A, Wyneken U. 2016. MiRNAs in astrocyte-derived exosomes as possible mediators of neuronal plasticity. *J Exp Neurosci* **2016**:1–9. doi:10.4137/JEN.S39916

Lagos-quintana M, Rauhut R, Lendeckel W, Tuschl T. 2001. Identification of Novel Genes Coding for Small Expressed RNAs. *Sci Rep* **294**:853–858. doi:10.1126/science.1064921

Lambert MP, Terrone S, Giraud G, Benoit-Pilven C, Cluet D, Combaret V, Mortreux F, Auboeuf D, Bourgeois CF. 2018. The RNA helicase DDX17 controls the transcriptional activity of REST and the expression of proneural microRNAs in neuronal differentiation. *Nucleic Acids Res* **46**:7686–7700. doi:10.1093/nar/gky545

Lamoureux P, Ruthel G, Buxbaum RE, Heidemann SR. 2002. Mechanical tension can specify axonal fate in hippocampal neurons. *J Cell Biol* **159**:499–508. doi:10.1083/jcb.200207174

Lanier LM, Gertler FB. 2000. From Abl to actin : Abl tyrosine kinase and associated proteins in growth cone motility. *Curr Opin Neurobiol* **10**:80–87. doi:10.1016/s0959-4388(99)00058-6

Lasek R, Dabrowski C, Nordlander R. 1973. Analysis of Axoplasmic RNA from Invertebrate Giant Axons. *Nat Phys Sci* **241**:162–165. doi:10.1038/246421a0

Lee Y, Jeon K, Lee J, Kim S, Kim VN. 2002. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* **21**:4663–4670. doi:10.1093/emboj/cdf476

Lee Y, Provost P, Kim S, Ahn C, Han J, Choi H, Rådmark O, Lee J, Kim J, Yim

J, Kim VN. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**:415–419. doi:10.1038/nature01957

Lefcort F. 2004. Organization of cytoskeletal elements and organelles preceding growth cone emergence from an identified neuron in situ. *J Cell Biol* **108**:1737–1749. doi:10.1083/jcb.108.5.1737

Leibinger M, Andreadaki A, Golla R, Levin E, Hilla AM, Diekmann H, Fischer D. 2017. Boosting CNS axon regeneration by harnessing antagonistic effects of GSK3 activity. *Proc Natl Acad Sci* **114**:E5454–E5463. doi:10.1073/pnas.1621225114

Leidinger P, Backes C, Deutscher S, Schmitt K, Mueller SC, Frese K, Haas J, Ruprecht K, Paul F, Stähler C, Lang CJG, Meder B, Bartfai T, Meese E, Keller A. 2013. A blood based 12-miRNA signature of Alzheimer disease patients. *Genome Biol* **14**:R78. doi:10.1186/gb-2013-14-7-r78

Lepelletier L, Langlois SD, Kent CB, Welshhans K, Morin S, Bassell GJ, Yam PT, Charron F. 2017. Sonic Hedgehog Guides Axons via Zipcode Binding Protein 1-Mediated Local Translation. *J Neurosci* **37**:1685–1695. doi:10.1523/JNEUROSCI.3016-16.2016

Leterrier C, Dubey P, Roy S. 2017. The nano-architecture of the axonal cytoskeleton. *Nat Rev Neurosci* **18**:713–726. doi:10.1038/nrn.2017.129

Letourneau PC, Shattuck TA, Ressler AH. 1987. "Pull" and "push" in neurite elongation: observations on the effects of different concentrations of cytochalasin b and taxol. *Cell Motil Cytoskeleton* **8**:193–209. doi:10.1002/cm.970080302

Leucht C, Stigloher C, Wizenmann A, Klafke R, Folchert A, Bally-cuif L. 2008. MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. *Nat Neurosci* **11**:641–648. doi:10.1038/nn.2115

Leung KM, Van Horck FPG, Lin AC, Allison R, Standart N, Holt CE. 2006.

Asymmetrical β-actin mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat Neurosci* **9**:1247–1256. doi:10.1038/nn1775

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**:15–20. doi:10.1016/j.cell.2004.12.035

Lewis TL, Courchet J, Polleux F. 2013. Cellular and molecular mechanisms underlying axon formation, growth, and branching. *J Cell Biol* **202**:837–848. doi:10.1083/jcb.201305098

Li B, Sun H. 2013. miR-26a promotes neurite outgrowth by repressing PTEN expression. *Mol Med Rep* **8**:676–680. doi:10.3892/mmr.2013.1534

Li Y, Wang F, Lee J, Gao F. 2006. MicroRNA-9a ensures the precise specification of sensory organ precursors in Drosophila. *Genes Dev* **20**:2793–2805. doi:10.1101/gad.1466306.ample

Li Z, Rana TM. 2014. Therapeutic targeting of microRNAs: current status and future challenges. *Nat Rev Drug Discov* **13**:622–638. doi:10.1038/nrd4359

Ling S-C, Fahrner PS, Greenough WT, Gelfand VI. 2004. Transport of Drosophila fragile X mental retardation protein-containing ribonucleoprotein granules by kinesin-1 and cytoplasmic dynein. *Proc Natl Acad Sci* **101**:17428–17433. doi:10.1073/pnas.0408114101

Lippi G, Fernandes CC, Ewell LA, John D, Romoli B, Curia G, Taylor SR, Frady EP, Jensen AB, Liu JC, Chaabane MM, Belal C, Nathanson JL, Zoli M, Leutgeb JK, Biagini G, Yeo GW, Berg DK. 2016. MicroRNA-101 Regulates Multiple Developmental Programs to Constrain Excitation in Adult Neural Networks. *Neuron* **92**:1337–1351. doi:10.1016/j.neuron.2016.11.017

Liu M, Nadar VC, Kozielski F, Kozlowska M, Yu W, Baas PW. 2010. Kinesin-12, a Mitotic Microtubule-Associated Motor Protein, Impacts Axonal

Growth, Navigation, and Branching. *J Neurosci* **30**:14896–14906. doi:10.1523/jneurosci.3739-10.2010

Logan CY, Nusse R. 2004. The Wnt Signaling Pathway in Development and Disease. *Annu Rev Cell Dev Biol* **20**:781–810. doi:10.1146/annurev.cellbio.20.010403.113126

Long H, Sabatier C, Ma L, Plump A, Yuan W, Ornitz DM, Tamada A, Murakami F, Goodman CS, Tessier-Lavigne M. 2004. Conserved roles for Slit and Robo proteins in midline commissural axon guidance. *Neuron* **42**:213–223. doi:10.1016/S0896-6273(04)00179-5

Lonze BE, Riccio A, Cohen S, Ginty DD. 2002. Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. *Neuron* **34**:371–385. doi:10.1016/S0896-6273(02)00686-4

Loreto A, Stefano M Di, Gering M, Conforti L, Loreto A, Stefano M Di, Gering M, Conforti L. 2015. Wallerian Degeneration Is Executed by an NMN-SARM1-Dependent Late Ca 2 + Influx but Only Modestly Influenced by Mitochondria Article Wallerian Degeneration Is Executed by an NMN-SARM1-Dependent Late Ca 2 + Influx but Only Modestly Influenced by Mitochond. *CellReports* **13**:1–14. doi:10.1016/j.celrep.2015.11.032

Lova P, Paganini S, Hirsch E, Barberis L, Wymann M, Sinigaglia F, Balduini C, Torti M. 2003. A selective role for phosphatidylinositol 3,4,5-trisphosphate in the G i -dependent activation of platelet Rap1B. *J Biol Chem* **278**:131–138. doi:10.1074/jbc.m204821200

Lowery LA, Vactor D Van. 2009. The trip of the tip: Understanding the growth cone machinery. *Nat Rev Mol Cell Biol* **10**:332–343. doi:10.1038/nrm2679

Lu W, Fox P, Lakonishok M, Davidson MW, Gelfand VI. 2013. Initial neurite outgrowth in drosophila neurons is driven by kinesin-powered microtubule sliding. *Curr Biol* **23**:1018–1023. doi:10.1016/j.cub.2013.04.050

Lu W, Yamamoto V, Ortega B, Baltimore D. 2004. Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. *Cell* **119**:97–108. doi:10.1016/j.cell.2004.09.019

Lucas FR, Goold RG, Gordon-Weeks PR, Salinas PC. 1998. Inhibition of GSK-3beta leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J Cell Sci* **111**:1351–61.

Lucci C, Mesquita-ribeiro R, Rathbone A, Dajas-bailador F. 2020. Spatiotemporal regulation of GSK3 β levels by miRNA-26a controls axon development in cortical neurons. *Development* **147**:1–16. doi:10.1242/dev.180232

Lugli G, Larson J, Martone ME, Jones Y, Smalheiser NR. 2005. Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner. *J Neurochem* **94**:896–905. doi:10.1111/j.1471-4159.2005.03224.x

Luo Y, Raible D, Raper JA. 1993. Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**:217–227. doi:10.1016/0092-8674(93)80064-L

Ma J, Xu R, Qi S, Wang F, Ma Y, Zhang Hong-Cheng, Xu J, Qin X, Zhang Hao-Nan, Liu C, Li B, Chen J, Yang H, Saijilafu. 2019. Regulation of adult mammalian intrinsic axonal regeneration by NF-κB/STAT3 signaling cascade. *J Cell Physiol* 1–12. doi:10.1002/jcp.28815

Ma Y, Wang X, Chen J, Li B, Hur E-M, Saijilafu. 2017. Differential Roles of Glycogen Synthase Kinase 3 Subtypes Alpha and Beta in Cortical Development. *Front Mol Neurosci* **10**:1–16. doi:10.3389/fnmol.2017.00391

Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G. 2003. Sonic hedgehog is required for progenitor cell

maintenance in telencephalic stem cell niches. *Neuron* **39**:937–950. doi:10.1016/S0896-6273(03)00561-0

Maciotta S, Meregalli M, Torrente Y. 2013. The involvement of microRNAs in neurodegenerative diseases. *Front Cell Neurosci* **7**:1–17. doi:10.3389/fncel.2013.00265

Maday S, Twelvetrees AE, Moughamian AJ, Holzbaur ELF. 2014. Axonal Transport: Cargo-Specific Mechanisms of Motility and Regulation. *Neuron* **84**:292–309. doi:10.1016/j.neuron.2014.10.019

Mairet-Coello G, Courchet J, Pieraut S, Courchet V, Maximov A, Polleux F. 2013. The CAMKK2-AMPK Kinase Pathway Mediates the Synaptotoxic Effects of Aβ Oligomers through Tau Phosphorylation. *Neuron* **78**:94–108. doi:10.1016/j.neuron.2013.02.003

Makeyev E V., Zhang J, Carrasco MA, Maniatis T. 2007. The MicroRNA miR-124 Promotes Neuronal Differentiation by Triggering Brain-Specific Alternative Pre-mRNA Splicing. *Mol Cell* **27**:435–448. doi:10.1016/j.molcel.2007.07.015

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Götz M. 2003. Neuronal or glial progeny: Regional differences in radial glia fate. *Neuron* **37**:751–764. doi:10.1016/S0896-6273(03)00116-8

Mandelkow EM, Biernat J, Drewes G, Gustke N, Trinczek B, Mandelkow E. 1995. Tau domains, phosphorylation, and interactions with microtubules. *Neurobiol Aging* **16**:355–362. doi:10.1016/0197-4580(95)00025-A

Mandelkow EM, Drewes G, Biernat J, Gustke N, Van Lint J, Vandenheede JR, Mandelkow EM. 1992. Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett* **314**:315–321. doi:10.1016/0014-5793(92)81496-9

Mao Y, Ge X, Frank CL, Madison JM, Koehler AN, Doud MK, Tassa C, Berry

EM, Soda T, Singh KK, Biechele T, Petryshen TL, Moon RT, Haggarty SJ, Tsai LH. 2009. Disrupted in Schizophrenia 1 Regulates Neuronal Progenitor Proliferation via Modulation of GSK3β/β-Catenin Signaling. *Cell* **136**:1017–1031. doi:10.1016/j.cell.2008.12.044

Marín O, Rubenstein JLR. 2003. CELL MIGRATION IN THE FOREBRAIN. *Annu Rev Neurosci* **26**:441–483. doi:10.1146/annurev.neuro.26.041002.131058

Markus A, Zhong J, Snider WD. 2002. Raf and Akt mediate distinct aspects of sensory axon growth. *Neuron* **35**:65–76. doi:10.1016/S0896-6273(02)00752-3

Marler KJ, Suetterlin P, Dopplapudi A, Rubikaite A, Adnan J, Maiorano NA, Lowe AS, Thompson ID, Pathania M, Bordey A, Fulga T, Van Vactor DL, Hindges R, Drescher U. 2014. BDNF Promotes Axon Branching of Retinal Ganglion Cells via miRNA-132 and p250GAP. *J Neurosci* **34**:969–979. doi:10.1523/jneurosci.1910-13.2014

Marsh L, Letourneau PC. 1984. Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. *J Cell Biol* **99**:2041–2047. doi:10.1083/jcb.99.6.2041

Massoll C, Mando W, Chintala SK. 2013. Excitotoxicity upregulates SARM1 protein expression and promotes Wallerian-like degeneration of retinal ganglion cells and their axons. *Investig Ophthalmol Vis Sci* **54**:2771–2780. doi:10.1167/iovs.12-10973

Matsuda W, Kaneko T, Hioki H, Arai R, Furuta T, Fujiyama F, Nakamura KC. 2009. Single Nigrostriatal Dopaminergic Neurons Form Widely Spread and Highly Dense Axonal Arborizations in the Neostriatum. *J Neurosci* **29**:444–453. doi:10.1523/jneurosci.4029-08.2009

Mayer PR, Huang N, Dewey CM, Dries DR, Zhang H, Yu G. 2010. Expression,

localization, and biochemical characterization of nicotinamide mononucleotide adenylyltransferase 2. *J Biol Chem* **285**:40387–96. doi:10.1074/jbc.M110.178913

McNeill E, Van Vactor D. 2012. MicroRNAs Shape the Neuronal Landscape. *Neuron* **75**:363–379. doi:10.1016/j.neuron.2012.07.005

Meems R, Munno D, van Minnen J, Syed NI. 2006. Synapse Formation Between Isolated Axons Requires Presynaptic Soma and Redistribution of Postsynaptic AChRs. *J Neurophysiol* **89**:2611–2619. doi:10.1152/jn.00898.2002

Meister G, Tuschl T. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**:343–349. doi:10.1038/nature02873

Mellman I, Winckler B, Forscher P, Mellman I. 1999. A diffusion barrier maintains distribution of membrane proteins in polarized neurons. *Nature* **397**:698–701. doi:10.1038/17806

Ménager C, Arimura N, Fukata Y, Kaibuchi K. 2004. PIP3 is involved in neuronal polarization and axon formation. *J Neurochem* **89**:109–118. doi:10.1046/j.1471-4159.2004.02302.x

Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, Thomas PD. 2017. PANTHER version 11: Expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res* **45**:D183–D189. doi:10.1093/nar/gkw1138

Migliore M, Shepherd G. 2005. An integrated approach to classifying neuronal phenotypes. *Nat Rev Neurosci* **6**:810–818. doi:10.1093/acprof:oso/9780195182224.003.0010

Milde S, Gilley J, Coleman MP. 2013. Subcellular Localization Determines the Stability and Axon Protective Capacity of Axon Survival Factor Nmnat2. *PLoS Biol* **11**. doi:10.1371/journal.pbio.1001539

Mili S, Moissoglu K, Macara IG. 2008. Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. *Nature* **453**:115–119. doi:10.1038/nature06888

Ming G, Wong ST, Henley J, Yuan X, Song H, Spitzer NC, Poo M. 2002. Adaptation in the chemotactic guidance of nerve growth cones. *Nature* **417**:411–418. doi:10.1038/nature745.

Mink M, Fogelgren B, Olszewski K, Maroy P, Csiszar K. 2001. A novel human gene (SARM) at chromosome 17q11 encodes a protein with a SAM motif and structural similarity to Armadillo/β-catenin that is conserved in mouse, Drosophila, and Caenorhabditis elegans. *Genomics* **74**:234–244. doi:10.1006/geno.2001.6548

Mitchison T, Kirschner M. 1988. Cytoskeletal dynamics and nerve growth. *Neuron* **1**:761–772. doi:10.1016/0896-6273(88)90124-9

Molyneaux BJ, Arlotta P, Menezes JRL, Macklis JD. 2007. Neuronal subtype specification in the cerebral cortex. *Nat Rev Neurosci* **8**:427–437. doi:10.1038/nrn2151

Morgan-Smith M, Wu Y, Zhu X, Pringle J, Snider WD. 2014. GSK-3 signaling in developing cortical neurons is essential for radial migration and dendritic orientation. *Elife* **3**:1–24. doi:10.7554/eLife.02663

Morita S, Horii T, Kimura M, Goto Y, Ochiya T, Hatada I. 2007. One Argonaute family member, Eif2c2 (Ago2), is essential for development and appears not to be involved in DNA methylation. *Genomics* **89**:687–696. doi:10.1016/j.ygeno.2007.01.004

Morita T, Sobuě K. 2009. Specification of neuronal polarity regulated by local translation of CRMP2 and tau via the mTOR-p70S6K pathway. *J Biol Chem* **284**:27734–27745. doi:10.1074/jbc.M109.008177

Muralidharan H, Baas PW. 2019. Mitotic Motor KIFC1 Is an Organizer of

Microtubules in the Axon. *J Neurosci* **39**:3792–3811. doi:10.1523/jneurosci.3099-18.2019

Muresan Z, Muresan V. 2005. Coordinated transport of phosphorylated amyloid-β precursor protein and c-Jun NH2-terminal kinase-interacting protein-1. *J Cell Biol* **171**:615–625. doi:10.1083/jcb.200502043

Naguibneva I, Ameyar-Zazoua M, Nonne N, Polesskaya A, Ait-Si-Ali S, Groisman R, Souidi M, Pritchard LL, Harel-Bellan A. 2006. An LNA-based loss-of-function assay for micro-RNAs. *Biomed Pharmacother* **60**:633–638. doi:10.1016/j.biopha.2006.07.078

Nakamuta S, Funahashi Y, Namba T, Arimura N, Picciotto MR, Tokumitsu H, Soderling TR, Sakakibara A, Miyata T, Kamiguchi H, Kaibuchi K. 2011. Local Application of Neurotrophins Specifies Axons and Ca 2 + / Calmodulin – Dependent Protein Kinases. *Sci Signal* **4**:1–14. doi:10.1126/scisignal.2002011

Nakashima H, Tsujimura K, Irie K, Ishizu M, Pan M, Kameda T, Nakashima K. 2018. Canonical TGF-β Signaling Negatively Regulates Neuronal Morphogenesis through TGIF/Smad Complex-Mediated CRMP2 Suppression. *J Neurosci* **38**:4791–4810. doi:10.1523/JNEUROSCI.2423-17.2018

Nakata T, Hirokawa N. 2003. Microtubules provide directional cues for polarized axonal transport through interaction with kinesin motor head. *J Cell Biol* **162**:1045–1055. doi:10.1083/jcb.200302175

Namba T, Funahashi Y, Nakamuta S, Xu C, Takano T, Kaibuchi K. 2015. Extracellular and Intracellular Signaling for Neuronal Polarity. *Physiol Rev* **95**:995–1024. doi:10.1152/physrev.00025.2014

Natera-Naranjo O, Aschrafi A, Gioio AE, Kaplan BB. 2010. Identification and quantitative analyses of microRNAs located in the distal axons of

sympathetic neurons. *RNA* **16**:1516–29. doi:10.1261/rna.1833310

Nelson WJ. 2003. Adaptation of core mechanisms to generate cell polarity. *Nature* **422**:766–73. doi:doi:10.1038/nature01602 1.

Newman MA, Thomson JM, Hammond SM. 2008. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* **14**:1539–1549. doi:10.1261/rna.1155108.has

Nguyen MM, Stone MC, Rolls MM. 2011. Microtubules are organized independently of the centrosome in Drosophila neurons. *Neural Dev* **6**:1–16. doi:10.1186/1749-8104-6-38

Nishimura T, Kato K, Yamaguchi T, Fukata Y, Ohno S, Kaibuchi K. 2004. Role of the PAR-3–KIF3 complex in the establishment of neuronal polarity. *Nat Cell Biol* **6**:328–334. doi:10.1038/ncb1118

Noctor SC, Martinez-Cerdeño V, Ivic L, Kriegstein AR. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci* **7**:136–144. doi:10.1038/nn1172

Norsworthy MW, Bei F, Kawaguchi R, Wang Q, Tran NM, Li Y, Brommer B, Zhang Y, Wang C, Sanes JR, Coppola G, He Z. 2017. Sox11 Expression Promotes Regeneration of Some Retinal Ganglion Cell Types but Kills Others. *Neuron* **94**:1112-1120.e4. doi:10.1016/j.neuron.2017.05.035

O'Donovan KJ, Ma K, Guo H, Wang C, Sun F, Han SB, Kim H, Wong JK, Charron J, Zou H, Son Y-J, He Z, Zhong J. 2014. B-RAF kinase drives developmental axon growth and promotes axon regeneration in the injured mature CNS. *J Exp Med* **211**:801–814. doi:10.1084/jem.20131780

Ohashi S, Ohara S, Omori A, Kobayashi S, Ichinose S, Koike K, Sato T-A, Anzai K. 2002. Identification of mRNA/Protein (mRNP) Complexes Containing Purα, mStaufen, Fragile X Protein, and Myosin Va and their Association with Rough Endoplasmic Reticulum Equipped with a Kinesin Motor. *J*

Biol Chem **277**:37804–37810. doi:10.1074/jbc.m203608200

Ori-McKenney KM, Jan LY, Jan YN. 2012. Golgi Outposts Shape Dendrite Morphology by Functioning as Sites of Acentrosomal Microtubule Nucleation in Neurons. *Neuron* **76**:921–930. doi:10.1016/j.neuron.2012.10.008

Osterloh JM, Yang J, Rooney TM, Fox AN, Adalbert R, Powell EH, Sheehan AE, Avery MA, Hackett R, Logan MA, MacDonald JM, Ziegenfuss JS, Milde S, Hou Y-J, Nathan C, Ding A, Brown RH, Conforti L, Coleman M, Tessier-Lavigne M, Zuchner S, Freeman MR. 2012. dSarm/Sarm1 Is Required for Activation of an Injury-Induced Axon Death Pathway. *Science (80-)* **337**:481–484. doi:10.1126/science.1223899

Ouyang YB, Lu Y, Yue S, Giffard RG. 2012. MiR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes. *Mitochondrion* **12**:213–219. doi:10.1016/j.mito.2011.09.001

Palay BYSL, Palade GE. 1955. THE FINE STRUCTURE OF NEURONS. *J Cell Biol* **1**:69–88. doi:10.1083/jcb.1.1.69

Pan WL, Chopp M, Fan B, Zhang R, Wang X, Hu J, Zhang XM, Zhang ZG, Liu XS. 2019. Ablation of the microRNA-17-92 cluster in neural stem cells diminishes adult hippocampal neurogenesis and cognitive function. *FASEB J* **33**:5257–5267. doi:10.1096/fj.201801019R

Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, Filippidis C, Dalamagas T, Hatzigeorgiou AG. 2013. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic Acids Res* **41**:169–173. doi:10.1093/nar/gkt393

Park CY, Choi YS, McManus MT. 2010. Analysis of microRNA knockouts in mice. *Hum Mol Genet* **19**:169–175. doi:10.1093/hmg/ddq367

Park JH, Roll-Mecak A. 2018. The tubulin code in neuronal polarity. *Curr Opin*

Neurobiol **51**:95–102. doi:10.1016/j.conb.2018.03.001

Park K, He Z. 2008. Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. *Science (80-)* **322**:1673–1675.

Pasquale EB. 2008. Eph-Ephrin Bidirectional Signaling in Physiology and Disease. *Cell* **133**:38–52. doi:10.1016/j.cell.2008.03.011

Pasquale EB. 2005. Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol* **6**:462–475. doi:10.1038/nrm1662

Pasquinelli AE. 2012. MicroRNAs and their targets: Recognition, regulation and an emerging reciprocal relationship. *Nat Rev Genet* **13**:271–282. doi:10.1038/nrg3162

Pease-Raissi SE, Pazyra-Murphy MF, Li Y, Wachter F, Fukuda Y, Fenstermacher SJ, Barclay LA, Bird GH, Walensky LD, Segal RA. 2017. Paclitaxel Reduces Axonal Bclw to Initiate IP3R1-Dependent Axon Degeneration. *Neuron* **96**:373-386.e6. doi:10.1016/j.neuron.2017.09.034

Pease SE, Segal RA. 2014. Preserve and protect: Maintaining axons within functional circuits. *Trends Neurosci* **37**:572–582. doi:10.1016/j.tins.2014.07.007

Perry RBT, Doron-Mandel E, Iavnilovitch E, Rishal I, Dagan SY, Tsoory M, Coppola G, McDonald MK, Gomes C, Geschwind DH, Twiss JL, Yaron A, Fainzilber M. 2012. Subcellular Knockout of Importin β1 Perturbs Axonal Retrograde Signaling. *Neuron* **75**:294–305. doi:10.1016/j.neuron.2012.05.033

Peterson SM, Thompson JA, Ufkin ML, Sathyanarayana P, Liaw L, Congdon CB. 2014. Common features of microRNA target prediction tools. *Front Genet* **5**:1–10. doi:10.3389/fgene.2014.00023

Picard M, Petrie RJ, Antoine-Bertrand J, Saint-Cyr-Proulx E, Villemure JF,

Lamarche-Vane N. 2009. Spatial and temporal activation of the small GTPases RhoA and Rac1 by the netrin-1 receptor UNC5a during neurite outgrowth. *Cell Signal* **21**:1961–1973. doi:10.1016/j.cellsig.2009.09.004

Pichardo-Casas I, Goff LA, Swerdel MR, Athie A, Davila J, Ramos-Brossier M, Lapid-Volosin M, Friedman WJ, Hart RP, Vaca L. 2012. Expression profiling of synaptic microRNAs from the adult rat brain identifies regional differences and seizure-induced dynamic modulation. *Brain Res* **1436**:20–33. doi:10.1016/j.brainres.2011.12.001

Pichler S, Gu W, Hartl D, Gasparoni G, Leidinger P, Keller A, Meese E, Mayhaus M, Hampel H, Riemenschneider M. 2017. The miRNome of Alzheimer's disease: consistent downregulation of the miR-132/212 cluster. *Neurobiol Aging* **50**:167.e1-167.e10. doi:10.1016/j.neurobiolaging.2016.09.019

Pinter R, Hindges R. 2010. Perturbations of microRNA function in mouse dicer mutants produce retinal defects and lead to aberrant axon pathfinding at the optic chiasm. *PLoS One* **5**:1–12. doi:10.1371/journal.pone.0010021

Piper M, Anderson R, Dwivedy A, Weinl C, Van Horck F, Leung KM, Cogill E, Holt C. 2006. Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones. *Neuron* **49**:215–228. doi:10.1016/j.neuron.2005.12.008

Polleux F, Ince-Dunn G, Ghosh A. 2007. Transcriptional regulation of vertebrate axon guidance and synapse formation. *Nat Rev Neurosci* **8**:331–340. doi:10.1038/nrn2118

Polleux F, Snider W. 2010. Initiating and growing an axon. *Cold Spring Harb Perspect Biol* **2**:1–20. doi:10.1101/cshperspect.a001925

Poulopoulos A, Murphy AJ, Ozkan A, Davis P, Hatch J, Kirchner R, Macklis JD. 2019. Subcellular transcriptomes and proteomes of developing axon

projections in the cerebral cortex. *Nature* **565**:356–63. doi:10.1038/s41586-018-0847-y

Prada I, Gabrielli M, Turola E, Iorio A, D'Arrigo G, Parolisi R, De Luca M, Pacifici M, Bastoni M, Lombardi M, Legname G, Cojoc D, Buffo A, Furlan R, Peruzzi F, Verderio C. 2018. Glia-to-neuron transfer of miRNAs via extracellular vesicles: a new mechanism underlying inflammation-induced synaptic alterations. *Acta Neuropathol* **135**:529–550. doi:10.1007/s00401-017-1803-x

Prasad A, Paruchuri V, Preet A, Latif F, Ganju RK. 2008. Slit-2 induces a tumor-suppressive effect by regulating β-catenin in breast cancer cells. *J Biol Chem* **283**:26624–26633. doi:10.1074/jbc.M800679200

Prasad BC, Clark SG. 2006. Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in C. elegans. *Development* **133**:1757–1766. doi:10.1242/dev.02357

Qin S, Zou Y, Zhang CL. 2013. Cross-talk between klf4 and stat3 regulates axon regeneration. *Nat Commun* **4**:1–9. doi:10.1038/ncomms3633

Rage F, Boulisfane N, Rihan K, Soret A. 2013. Genome-wide identification of mRNAs associated with the protein SMN whose depletion decreases their axonal localization. *Rna* **19**:1755–1766. doi:10.1261/rna.040204.113

Rajgor D. 2018. Macro roles for microRNAs in neurodegenerative diseases. *Non-coding RNA Res* **3**:154–159. doi:10.1016/j.ncrna.2018.07.001

Rajman M, Schratt G. 2017. MicroRNAs in neural development: from master regulators to fine-tuners. *Development* **144**:2310–2322. doi:10.1242/dev.144337

Ramon y Cajal S. 1995. Histology of the Nervous System. *N Engl J Med* 333–1088. doi:10.1056/NEJM199510193331619

Randlett O, Norden C, Harris WA. 2011a. The vertebrate retina: A model for neuronal polarization in vivo. *Dev Neurobiol* **71**:567–583. doi:10.1002/dneu.20841

Randlett O, Poggi L, Zolessi FR, Harris WA. 2011b. The Oriented Emergence of Axons from Retinal Ganglion Cells Is Directed by Laminin Contact In Vivo. *Neuron* **70**:266–280. doi:10.1016/j.neuron.2011.03.013

Rao AN, Baas PW. 2018. Polarity Sorting of Microtubules in the Axon. *Trends Neurosci* **41**:77–88. doi:10.1016/j.tins.2017.11.002

Raper J, Mason C. 2010. Cellular strategies of axonal pathfinding. *Cold Spring Harb Perspect Biol* **2**:1–22. doi:10.1101/cshperspect.a001933

Recho P, Jerusalem A, Goriely A. 2016. Growth, collapse, and stalling in a mechanical model for neurite motility. *Phys Rev E* **93**:1–13. doi:10.1103/PhysRevE.93.032410

Reddy PH, Williams J, Smith F, Bhatti JS, Kumar S, Vijayan M, Kandimalla R, Kuruva CS, Wang R, Manczak M, Yin X, Reddy AP. 2017. MicroRNAs, Aging, Cellular Senescence, and Alzheimer's Disease. *Prog Mol Biol Transl Sci* **146**:127–171. doi:10.1016/bs.pmbts.2016.12.009

Reh TA, Hindges R. 2018. MicroRNAs in Retinal Development. *Annu Rev Vis Sci* **4**:25–44. doi:10.1146/annurev-vision-091517-034357

Remenyi J, van den Bosch MWM, Palygin O, Mistry RB, McKenzie C, Macdonald A, Hutvagner G, Arthur JSC, Frenguelli BG, Pankratov Y. 2013. miR-132/212 Knockout Mice Reveal Roles for These miRNAs in Regulating Cortical Synaptic Transmission and Plasticity. *PLoS One* **8**:1–14. doi:10.1371/journal.pone.0062509

Riffo-Campos ÁL, Riquelme I, Brebi-Mieville P. 2016. Tools for sequence-based miRNA target prediction: What to choose? *Int J Mol Sci* **17**:1–18. doi:10.3390/ijms17121987

Rishal I, Fainzilber M. 2014. Axon-soma communication in neuronal injury. *Nat Rev Neurosci* **15**:32–42. doi:10.1038/nrn3609

Rishal I, Fainzilber M. 2010. Retrograde signaling in axonal regeneration. *Exp Neurol* **223**:5–10. doi:10.1016/j.expneurol.2009.08.010

Ristori E, Lopez-Ramirez MA, Narayanan A, Hill-Teran G, Moro A, Calvo CF, Thomas JL, Nicoli S. 2015. A Dicer-miR-107 Interaction Regulates Biogenesis of Specific miRNAs Crucial for Neurogenesis. *Dev Cell* **32**:546–560. doi:10.1016/j.devcel.2014.12.013

Rodriguez-Ortiz CJ, Baglietto-Vargas D, Martinez-Coria H, Laferla FM, Kitazawa M. 2014. Upregulation of miR-181 decreases c-Fos and SIRT-1 in the hippocampus of 3xTg-AD mice. *J Alzheimer's Dis* **42**:1229–1238. doi:10.3233/JAD-140204

Roossien DH, Lamoureux P, Van Vactor D, Miller KE. 2013. Drosophila growth cones advance by forward translocation of the neuronal cytoskeletal meshwork in vivo. *PLoS One* **8**:1–12. doi:10.1371/journal.pone.0080136

Roush S, Slack FJ. 2008. The let-7 family of microRNAs. *Trends Biochem Sci* **18**:505–516. doi:10.1016/j.tcb.2008.07.007

Roy S, Coffee P, Smith G, Liem RK, Brady ST, Black MM. 2000. Neurofilaments are transported rapidly but intermittently in axons: implications for slow axonal transport. *J Neurosci* **20**:6849–61. doi:10.1523/JNEUROSCI.20-18-06849.2000

Ruthel G, Hollenbeck PJ. 2000. Growth Cones Are Not Required for Initial Establishment of Polarity or Differential Axon Branch Growth in Cultured Hippocampal Neurons. *J Neurosci* **20**:2266–2274. doi:10.1523/jneurosci.20-06-02266.2000

Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R, Wulczyn FG.

2008. A feedback loop comprising lin-28 and let-7 controls pre- let-7 maturation during neural stem-cell commitment. *Nat Cell Biol* **10**:987–93. doi:10.1038/ncb1759

Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, Wagner EF. 1999. Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev* **89**:115–124. doi:10.1016/S0925-4773(99)00213-0

Sahay A, Molliver ME, Ginty DD, Kolodkin AL. 2003. Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. *J Neurosci* **23**:6671–6680. doi:10.1523/jneurosci.23-17-06671.2003

Sahoo PK, Smith DS, Perrone-Bizzozero N, Twiss JL. 2018. Axonal mRNA transport and translation at a glance. *J Cell Sci* **131**:jcs196808. doi:10.1242/jcs.196808

Saijilafu, Hur EM, Liu CM, Jiao Z, Xu WL, Zhou FQ. 2013. PI3K-GSK3 signalling regulates mammalian axon regeneration by inducing the expression of Smad1. *Nat Commun* **4**:1–14. doi:10.1038/ncomms3690

Sakakibara A, Sato T, Ando R, Noguchi N, Masaoka M, Miyata T. 2014. Dynamics of centrosome translocation and microtubule organization in neocortical neurons during distinct modes of polarization. *Cereb Cortex* **24**:1301–1310. doi:10.1093/cercor/bhs411

Sambandan S, Akbalik G, Kochen L, Heckel A, Rinne J, Alvarez-Castelao B, Kahlstatt J, Tushev G, Glock C, Schuman EM. 2017. Activity-dependent spatially localized miRNA maturation in neuronal dendrites. *Science (80-)* **355**:634–637. doi:10.1126/science.aaf8995

Sasaki Y, Gross C, Xing L, Goshima Y, Bassell GJ. 2014. Identification of axon-enriched microRNAs localized to growth cones of cortical neurons. *Dev*

Neurobiol **74**:397–406. doi:10.1002/dneu.22113

Sasaki Y, Vohra BPS, Lund FE, Milbrandt J. 2009. Nicotinamide Mononucleotide Adenylyl Transferase-Mediated Axonal Protection Requires Enzymatic Activity But Not Increased Levels of Neuronal Nicotinamide Adenine Dinucleotide. *J Neurosci* **29**:5525–5535. doi:10.1523/jneurosci.5469-08.2009

Sasaki Y, Welshhans K, Wen Z, Yao J, Xu M, Goshima Y, Zheng JQ, Bassell GJ. 2010. Phosphorylation of Zipcode Binding Protein 1 Is Required for Brain-Derived Neurotrophic Factor Signaling of Local -Actin Synthesis and Growth Cone Turning. *J Neurosci* **30**:9349–9358. doi:10.1523/jneurosci.0499-10.2010

Sayas CL, Basu S, van der Reijden M, Bustos-Morán E, Liz M, Sousa M, van IJcken WFJ, Avila J, Galjart N. 2019. Distinct Functions for Mammalian CLASP1 and -2 During Neurite and Axon Elongation. *Front Cell Neurosci* **13**:1–17. doi:10.3389/fncel.2019.00005

Sayed D, Abdellatif M. 2011. MicroRNAs in Development and Disease. *Physiol Rev* **91**:827–887. doi:10.1152/physrev.00006.2010

Schaefer A, O'Carroll D, Chan LT, Hillman D, Sugimori M, Llinas R, Greengard P. 2007. Cerebellar neurodegeneration in the absence of microRNAs. *J Exp Med* **204**:1553–1558. doi:10.1084/jem.20070823

Schaefer AW, Kabir N, Forscher P. 2002. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J Cell Biol* **158**:139–152. doi:10.1083/jcb.200203038

Schelski M, Bradke F. 2017. Neuronal polarization: From spatiotemporal signaling to cytoskeletal dynamics. *Mol Cell Neurosci* **84**:11–28. doi:10.1016/j.mcn.2017.03.008

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-YJ-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A, Liceiri K, Tomancak P, A. C. 2012. Fiji: An open source platform for biological image analysis. *Nat Methods* **9**:676–682. doi:10.1038/nmeth.2019.Fiji

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3**:1101–8. doi:10.1038/nprot.2008.73

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**:671–5. doi:10.1038/nmeth.2089

Schonrock N, Humphreys DT, Preiss T, Götz J. 2012. Target gene repression mediated by miRNAs miR-181c and miR-9 both of which are down-regulated by amyloid-β. *J Mol Neurosci* **46**:324–335. doi:10.1007/s12031-011-9587-2

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. 2006. A brain-specific microRNA regulates dendritic spine development. *Nature* **439**:283–289. doi:10.1038/nature04367

Schwamborn JC, Berezikov E, Knoblich JA. 2009. The TRIM-NHL Protein TRIM32 Activates MicroRNAs and Prevents Self-Renewal in Mouse Neural Progenitors. *Cell* **136**:913–925. doi:10.1016/j.cell.2008.12.024

Schwamborn JC, Püschel AW. 2004. The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat Neurosci* **7**:923–929. doi:10.1038/nn1295

Schwarz TL. 2013. Mitochondrial trafficking in neurons. *Cold Spring Harb Perspect Med* **3**:1–16. doi:10.1101/cshperspect.a011304

Seiradake E, Jones EY, Klein R. 2016. Structural Perspectives on Axon Guidance. *Annu Rev Cell Dev Biol* **32**:577–608. doi:10.1146/annurev-cellbio-111315-125008

Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. 2008. Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**:58–63. doi:10.1038/nature07228

Serafini T, Kennedy TE, Gaiko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* **78**:409–424. doi:10.1016/0092-8674(94)90420-0

Sethupathy P, Megraw M, Hatzigeorgiou AG. 2006. A guide through present computational approaches for the identification of mammalian microRNA targets. *Nat Methods* **3**:881–886. doi:10.1038/nmeth954

Shelly M, Lim BK, Cancedda L, Poo M. 2010. Local and Long-range Reciprocal Regulation of cAMP and cGMP in Axon/Dendrite Formation. *Science (80-)* **20**:113–122. doi:10.7897/2277-4343.06493

Shi SH, Cheng T, Jan y. 2004. APC and GSK-3b Are Involved in mPar3 Targeting to the Nascent Axon and Establishment of Neuronal Polarity. *Curr Biol* **14**:189–190. doi:10.1016/j

Shi SH, Jan LY, Jan YN. 2003. Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* **112**:63–75. doi:10.1016/S0092-8674(02)01249-7

Shibata M, Nakao H, Kiyonari H, Abe T, Aizawa S. 2011. MicroRNA-9 Regulates Neurogenesis in Mouse Telencephalon by Targeting Multiple Transcription Factors. *J Neurosci* **31**:3407–3422. doi:10.1523/JNEUROSCI.5085-10.2011

Shigeoka T, Jung H, Jung J, Turner-Bridger B, Ohk J, Lin JQ, Amieux PS, Holt CE. 2016. Dynamic Axonal Translation in Developing and Mature Visual Circuits. *Cell* **166**:181–192. doi:10.1016/j.cell.2016.05.029

Shimizu T, Kagawa T, Inoue T, Nonaka A, Takada S, Aburatani H, Taga T.

2008. Stabilized -Catenin Functions through TCF/LEF Proteins and the Notch/RBP-J Complex To Promote Proliferation and Suppress Differentiation of Neural Precursor Cells. *Mol Cell Biol* **28**:7427–7441. doi:10.1128/MCB.01962-07

Shinde S, Arora N, Bhadra U. 2013. A Complex Network of MicroRNAs Expressed in Brain and Genes Associated with Amyotrophic Lateral Sclerosis. *Int J Genomics* **2013**:1–12. doi:10.1155/2013/383024

Si K, Giustetto M, Etkin A, Hsu R, Janisiewicz AM, Miniaci MC, Kim JH, Zhu H, Kandel ER. 2003. A Neuronal Isoform of CPEB Regulates Local Protein Synthesis and Stabilizes Synapse-Specific Long-Term Facilitation in Aplysia. *Cell* **115**:893–904. doi:10.1016/S0092-8674(03)01021-3

Siegert S, Seo J, Kwon EJ, Rudenko A, Cho S, Wang W, Flood Z, Martorell AJ, Ericsson M, Mungenast AE, Tsai LH. 2015. The schizophrenia risk gene product miR-137 alters presynaptic plasticity. *Nat Neurosci* **18**:1008–1016. doi:10.1038/nn.4023

Smirnova L, Gräfe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. 2005. Regulation of miRNA expression during neural cell specification. *Eur J Neurosci* **21**:1469–1477. doi:10.1111/j.1460-9568.2005.03978.x

Solecki DJ, Govek EE, Tomoda T, Hatten ME. 2006. Neuronal polarity in CNS development. *Genes Dev* **20**:2639–2647. doi:10.1101/gad.1462506

Song A hong, Wang D, Chen G, Li Y, Luo J, Duan S, Poo M ming. 2009. A Selective Filter for Cytoplasmic Transport at the Axon Initial Segment. *Cell* **136**:1148–1160. doi:10.1016/j.cell.2009.01.016

Sotelo-Silveira J, Crispino M, Puppo A, Sotelo JR, Koenig E. 2008. Myelinated axons contain β-actin mRNA and ZBP-1 in periaxoplasmic ribosomal plaques and depend on cyclic AMP and F-actin integrity for in vitro translation. *J Neurochem* **104**:545–557. doi:10.1111/j.1471-4159.2007.04999.x

Sotelo-Silveira JR, Calliari A, Kun A, Koenig E, Sotelo JR. 2006. RNA trafficking in axons. *Traffic* **7**:508–515. doi:10.1111/j.1600-0854.2006.00405.x

Spaulding EL, Burgess RW. 2017. Accumulating Evidence for Axonal Translation in Neuronal Homeostasis. *Front Neurosci* **11**:312. doi:10.3389/fnins.2017.00312

Stagi M, Gorlovoy P, Larionov S, Takahashi K, Neumann H. 2006. Unloading kinesin transported cargoes from the tubulin track via the inflammatory c-Jun N-terminal kinase pathway. *FASEB J* **20**:2573–2575. doi:10.1096/fj.06-6679fje

Stark KL, Xu B, Bagchi A, Lai WS, Liu H, Hsu R, Wan X, Pavlidis P, Mills AA, Karayiorgou M, Gogos JA. 2008. Altered brain microRNA biogenesis contributes to phenotypic deficits in a 22q11-deletion mouse model. *Nat Genet* **40**:751–760. doi:10.1038/ng.138

Stegmuller J, Huynh MA, Yuan Z, Konishi Y, Bonni A. 2008. TGF -Smad2 Signaling Regulates the Cdh1-APC/SnoN Pathway of Axonal Morphogenesis. *J Neurosci* **28**:1961–1969. doi:10.1523/JNEUROSCI.3061-07.2008

Stegmüller J, Konishi Y, Huynh MA, Yuan Z, DiBacco S, Bonni A. 2006. Cell-Intrinsic Regulation of Axonal Morphogenesis by the Cdh1-APC Target SnoN. *Neuron* **50**:389–400. doi:10.1016/j.neuron.2006.03.034

Stein E, Tessier-Lavigne M. 2001. Hierarchical organization of guidance receptors: Silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science (80-)* **291**:1928–1938. doi:10.1126/science.1058445

Stoeckli ET. 2018. Understanding axon guidance: are we nearly there yet? *Development* **145**:1–10. doi:10.1242/dev.151415

Stone MC, Roegiers F, Rolls MM. 2007. Microtubules Have Opposite Orientation in Axons and Dendrites of Drosophila Neurons. *Mol Biol Cell* **19**:308–317. doi:10.1091/mbc.E07

Strazisar M, Cammaerts S, Van Der Ven K, Forero DA, Lenaerts AS, Nordin A, Almeida-Souza L, Genovese G, Timmerman V, Liekens A, De Rijk P, Adolfsson R, Callaerts P, Del-Favero J. 2015. MIR137 variants identified in psychiatric patients affect synaptogenesis and neuronal transmission gene sets. *Mol Psychiatry* **20**:472–481. doi:10.1038/mp.2014.53

Stuurman N, Edelstein A, Amodaj N, Hoover K, Vale R. 2010. Computer control of microscopes using manager. *Curr Protoc Mol Biol* 1–22. doi:10.1002/0471142727.mb1420s92

Suetterlin P, Marler KM, Drescher U. 2012. Axonal ephrinA/EphA interactions, and the emergence of order in topographic projections. *Semin Cell Dev Biol* **23**:1–6. doi:10.1016/j.semcdb.2011.10.015

Summers DW, Milbrandt J, DiAntonio A. 2018. Palmitoylation enables MAPK-dependent proteostasis of axon survival factors. *Proc Natl Acad Sci* **115**:E8746–E8754. doi:10.1073/pnas.1806933115

Suter DM, Miller KE. 2011. The emerging role of forces in axonal elongation. *Prog Neurobiol* **94**:91–101. doi:10.1016/j.pneurobio.2011.04.002

Sutton MA, Schuman EM. 2006. Dendritic Protein Synthesis, Synaptic Plasticity, and Memory. *Cell* **127**:49–58. doi:10.1016/j.cell.2006.09.014

Suzuki A, Hirata M, Ohno S. 2004. aPKC Acts Upstream of PAR-1b in Both the Establishment and Maintenance of Mammalian Epithelial Polarity. *Curr Biol* **14**:1425–35. doi:10.1016/j

Swanger SA, Bassell GJ. 2011. Making and breaking synapses through local mRNA regulation. *Curr Opin Genet Dev* **21**:414–421. doi:10.1016/j.gde.2011.04.002

Takahashi T. 1995. The Cell Cycle of the Pseudostratified Embryonic Murine Cerebral Wall. *J Neurosci* **15**:6046–6057.

Takano T, Funahashi Y, Kaibuchi K. 2019. Neuronal Polarity: Positive and Negative Feedback Signals. *Front Cell Dev Biol* **7**:1–10. doi:10.3389/fcell.2019.00069

Takano T, Wu M, Nakamuta S, Naoki H, Ishizawa N, Namba T, Watanabe T, Xu C, Hamaguchi T, Yura Y, Amano M, Hahn KM, Kaibuchi K. 2017. Discovery of long-range inhibitory signaling to ensure single axon formation. *Nat Commun* **8**:1–17. doi:10.1038/s41467-017-00044-2

Takei Y, Teng J, Harada A, Hirokawa N. 2000. Defects in Axonal Elongation and Neuronal Migration in Mice with Disrupted tau and map1b Genes. *J Cell Biol* **150**:989 LP – 1000.

Tanaka D, Nakaya Y, Yanagawa Y, Obata K, Murakami F. 2003. Multimodal tangential migration of neocortical GABAergic neurons independent of GPI-anchored proteins. *Development* **130**:5803–5813. doi:10.1242/dev.00825

Tanaka DH, Yanagida M, Zhu Y, Mikami S, Nagasawa T, Miyazaki JI, Yanagawa Y, Obata K, Murakami F. 2009. Random walk behavior of migrating cortical interneurons in the marginal zone: Time-lapse analysis in flat-mount cortex. *J Neurosci* **29**:1300–1311. doi:10.1523/JNEUROSCI.5446-08.2009

Taniguchi M, Nagao H, Takahashi YK, Yamaguchi M, Mitsui S, Yagi T, Mori K, Shimizu T. 2003. Distorted odor maps in the olfactory bulb of semaphorin 3A-deficient mice. *J Neurosci* **23**:1390–1397. doi:10.1523/jneurosci.23-04-01390.2003

Taniguchi M, Yuasa S, Fujisawa H, Naruse I, Saga S, Mishina M, Yagi T. 1997. Disruption of semaphorin III/D gene causes severe abnormality in

peripheral nerve projection. *Neuron* **19**:519–530. doi:10.1016/S0896-6273(00)80368-2

Tararuk T, Östman N, Li W, Björkblom B, Padzik A, Zdrojewska J, Hongisto V, Herdegen T, Konopka W, Courtney MJ, Coffey ET. 2006. JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length. *J Cell Biol* **173**:265–277. doi:10.1083/jcb.200511055

Taylor AM, Berchtold NC, Perreau VM, Tu CH, Li Jeon N, Cotman CW. 2009. Axonal mRNA in Uninjured and Regenerating Cortical Mammalian Axons. *J Neurosci* **29**:4697–4707. doi:10.1523/jneurosci.6130-08.2009

Taylor AM, Blurton-Jones M, Jeon NL, Rhee SW, Cribbs DH, Cotman CW, Jeon NL. 2005. A microfluidic culture platform for CNS axonal injury, regeneration and and Transport. *Nat Methods* **2**:599–605. doi:10.1038/nmeth777

Tcherkezian J, Brittis PA, Thomas F, Roux PP, Flanagan JG. 2010. Transmembrane Receptor DCC Associates with Protein Synthesis Machinery and Regulates Translation. *Cell* **141**:632–644. doi:10.1016/j.cell.2010.04.008

Tennyson VM. 1970. The fine structure of the axon and growth cone of the dorsal root neuroblast of the rabbit embryo. *J Cell Biol* **44**:62–79. doi:10.1083/jcb.44.1.62

Terada S, Kinjo M, Hirokawa N. 2000. Oligomeric tubulin in large transporting complex is transported via kinesin in squid giant axons. *Cell* **103**:141–155. doi:10.1016/S0092-8674(00)00094-5

Terenzio M, Koley S, Samra N, Rishal I, Zhao Q, Sahoo PK, Urisman A, Marvaldi L, Oses-Prieto JA, Forester C, Gomes C, Kalinski AL, Di Pizio A, Doron-Mandel E, Perry RB, Koppel I, Twiss JL, Burlingame AL, Fainzilber M. 2018. Locally translated mTOR controls axonal local translation in

nerve injury. *Science (80-)* **359**:1416–1421. doi:10.1126/science.aan1053

Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Sabio G, Davis RJ, Matthews DE, Doble B, Rincon M. 2008. Phosphorylation by p38 MAPK as an alternative pathway for GSK3β inactivation. *Science (80-)* **320**:667–670. doi:10.1126/science.1156037

Tobias GS, Koenig E. 1975. Influence of nerve cell body and neurolemma cell on local axonal protein synthesis following neurotomy. *Exp Neurol* **49**:235–245. doi:10.1016/0014-4886(75)90207-1

Torii M, Hashimoto-Torii K, Levitt P, Rakic P. 2009. Integration of neuronal clones in the radial cortical columns by EphA and ephrin-A signalling. *Nature* **461**:524–528. doi:10.1038/nature08362

Toriyama M, Kozawa S, Sakumura Y, Inagaki N. 2013. Conversion of a signal into forces for axon outgrowth through pak1-mediated shootin1 phosphorylation. *Curr Biol* **23**:529–534. doi:10.1016/j.cub.2013.02.017

Toriyama M, Shimada T, Kim KB, Mitsuba M, Nomura E, Katsuta K, Sakumura Y, Roepstorff P, Inagaki N. 2006. Shootin 1: A protein involved in the organization of an asymmetric signal for neuronal polarization. *J Cell Biol* **175**:147–157. doi:10.1083/jcb.200604160

Tortosa E, Galjart N, Avila J, Sayas CL. 2013. MAP1B regulates microtubule dynamics by sequestering EB1/3 in the cytosol of developing neuronal cells. *EMBO J* **32**:1293–1306. doi:10.1038/emboj.2013.76

Trivedi N. 2005. Glycogen synthase kinase-3 phosphorylation of MAP1B at Ser1260 and Thr1265 is spatially restricted to growing axons. *J Cell Sci* **118**:993–1005. doi:10.1242/jcs.01697

Twelvetrees AEE, Pernigo S, Sanger A, Guedes-Dias P, Schiavo G, Steiner RAA, Dodding MPP, Holzbaur ELLF. 2016. The Dynamic Localization of Cytoplasmic Dynein in Neurons Is Driven by Kinesin-1. *Neuron* **90**:1000–

1015. doi:10.1016/j.neuron.2016.04.046

Ueyama T. 2019. Rho-Family Small GTPases: From Highly Polarized Sensory Neurons to Cancer Cells. *Cells* **8**:92. doi:10.3390/cells8020092

Vale RD. 2003. The molecular motor toolbox for intracellular transport. *Cell* **112**:467–80. doi:10.1016/s0092-8674(03)00111-9

van Kesteren RE. 2006. Local Synthesis of Actin-Binding Protein -Thymosin Regulates Neurite Outgrowth. *J Neurosci* **26**:152–157. doi:10.1523/jneurosci.4164-05.2006

Varcianna A, Myszczynska MA, Castelli LM, O'Neill B, Kim Y, Talbot J, Nyberg S, Nyamali I, Heath PR, Stopford MJ, Hautbergue GM, Ferraiuolo L. 2019. Micro-RNAs secreted through astrocyte-derived extracellular vesicles cause neuronal network degeneration in C9orf72 ALS. *EBioMedicine* **40**:626–635. doi:10.1016/j.ebiom.2018.11.067

Verma P, Chierzi S, Codd AM, Campbell DS, Ronald L, Holt CE, Fawcett JW. 2005. Axonal Protein Synthesis and Degradation Are Necessary for Efficient Growth Cone Regeneration. *J Neurosci* **25**:331–342. doi:10.1523/jneurosci.3073-04.2005

Villarin JM, McCurdy EP, Martínez JC, Hengst U. 2016. Local synthesis of dynein cofactors matches retrograde transport to acutely changing demands. *Nat Commun* **7**:1–14. doi:10.1038/ncomms13865

Villarroel-Campos D, Gonzalez-Billault C. 2014. The MAP1B case: An old MAP that is new again. *Dev Neurobiol* **74**:953–971. doi:10.1002/dneu.22178

Vo N, Goodman RH. 2014. CREB-binding Protein and p300 in Transcriptional Regulation. *J Biol Chem* **276**:13505–13508. doi:10.1074/jbc.r000025200

Walker CA, Randolph LK, Matute C, Alberdi E, Baleriola J, Hengst U. 2018. Aβ $_{1–42}$ triggers the generation of a retrograde signaling complex from

sentinel mRNAs in axons. *EMBO Rep* **19**:e45435. doi:10.15252/embr.201745435

Waller A. 1850. Experiments on the Section of the Glossopharyngeal and Hypoglossal Nerves of the Frog, and observations of the alterations produced thereby in the Structure of their Primitive Fibres. *Philos Trans R Soc London* **140**:432–429.

Wang B, Bao L. 2017. Axonal microRNAs: localization, function and regulatory mechanism during axon development. *J Mol Cell Biol* **9**:82–90. doi:10.1093/jmcb/mjw050

Wang B, Pan L, Wei M, Wang Q, Liu WW, Wang N, Jiang XY, Zhang X, Bao L. 2015. FMRP-Mediated Axonal Delivery of miR-181d Regulates Axon Elongation by Locally Targeting Map1b and Calm1. *Cell Rep* **13**:2794–2807. doi:10.1016/j.celrep.2015.11.057

Wang ET, Taliaferro XJM, Lee XJ, Sudhakaran XIP, Rossoll W, Gross XC, Moss KR, Bassell GJ. 2016. Dysregulation of mRNA Localization and Translation in Genetic Disease. *J Neurophysiol* **36**:11418–11426. doi:10.1523/JNEUROSCI.2352-16.2016

Wang L, Ho C, Sun D, Liem RKH, Brown A. 2000. Rapid movement of axonal neurofilaments interrupted by prolonged pauses. *Nat Cell Biol* **2**:137–141. doi:10.1038/35004008

Wang MS, Wu Y, Culver DG, Glass JD. 2001. The gene for slow Wallerian degeneration (Wlds) is also protective against vincristine neuropathy. *Neurobiol Dis* **8**:155–161. doi:10.1006/nbdi.2000.0334

Wang W, Kwon EJ, Tsai L-H. 2012. MicroRNAs in learning, memory, and neurological diseases. *Learn Mem* **19**:359–368. doi:10.1101/lm.026492.112

Wang WM, Lu G, Su XW, Lyu H, Poon WS. 2017. MicroRNA-182 Regulates Neurite Outgrowth Involving the PTEN/AKT Pathway. *Front Cell*

Neurosci **11**:1–14. doi:10.3389/fncel.2017.00096

Wang Y, Je H, Wu W, Lu B, Ji Y, Yang F. 2011. Presynaptic protein synthesis required for NT-3-induced long-term synaptic modulation. *Mol Brain* **4**:1–8. doi:10.1186/1756-6606-4-1

Wang Y, Wang H, Li X, Li Y. 2016. Epithelial microRNA-9a regulates dendrite growth through Fmi-Gq signaling in Drosophila sensory neurons. *Dev Neurobiol* **76**:225–237. doi:10.1002/dneu.22309

Wang Z, Mehra V, Simpson MT, Maunze B, Chakraborty A, Holan L, Eastwood E, Blackmore MG, Venkatesh I. 2018. KLF6 and STAT3 co-occupy regulatory DNA and functionally synergize to promote axon growth in CNS neurons. *Sci Rep* **8**:1–16. doi:10.1038/s41598-018-31101-5

Wang Z, Winsor K, Nienhaus C, Hess E, Blackmore MG. 2017. Combined chondroitinase and KLF7 expression reduce net retraction of sensory and CST axons from sites of spinal injury. *Neurobiol Dis* **99**:24–35. doi:10.1016/j.nbd.2016.12.010

Watanabe K, Al-Bassam S, Miyazaki Y, Wandless TJ, Webster P, Arnold DB. 2012. Networks of Polarized Actin Filaments in the Axon Initial Segment Provide a Mechanism for Sorting Axonal and Dendritic Proteins. *Cell Rep* **2**:1546–1553. doi:10.1016/j.celrep.2012.11.015

Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, Cheng H-YM, Marks D, Obrietan K, Soderling TR, Goodman RH, Impey S. 2008. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. *Proc Natl Acad Sci* **105**:9093–9098. doi:10.1073/pnas.0803072105

Welte MA. 2004. Bidirectional transport along microtubules. *Curr Biol* **14**:525–537. doi:10.1016/j.cub.2004.06.045

Whitford KL, Dijkhuizen P, Polleux F, Ghosh A. 2002. Molecular Control of

Cortical Dendrite Development. *Annu Rev Neurosci* **25**:127–149. doi:10.1146/annurev.neuro.25.112701.142932

Whitmarsh AJ. 2006. The JIP family of MAPK scaffold proteins. *Biochem Soc Trans* **34**:828–832. doi:10.1042/BST0340828

Williams EJ, Furness J, Walsh FS, Doherty P. 1994. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron* **13**:583–594. doi:10.1016/0896-6273(94)90027-2

Willis DE, Van Niekerk EA, Sasaki Y, Mesngon M, Merianda TT, Williams GG, Kendall M, Smith DS, Bassell GJ, Twiss JL. 2007. Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J Cell Biol* **178**:965–980. doi:10.1083/jcb.200703209

Winans AM, Collins SR, Meyer T. 2016. Waves of actin and microtubule polymerization drive microtubule-based transport and neurite growth before single axon formation. *Elife* **5**:1–22. doi:10.7554/eLife.12387

Witte H, Neukirchen D, Bradke F. 2008. Microtubule stabilization specifies initial neuronal polarization. *J Cell Biol* **180**:619–632. doi:10.1083/jcb.200707042

Wonders CP, Anderson SA. 2006. The origin and specification of cortical interneurons. *Nat Rev Neurosci* **7**:687–696. doi:10.1038/nrn1954

Wong HHW, Lin JQ, Ströhl F, Roque CG, Cioni JM, Cagnetta R, Turner-Bridger B, Laine RF, Harris WA, Kaminski CF, Holt CE. 2017. RNA Docking and Local Translation Regulate Site-Specific Axon Remodeling In Vivo. *Neuron* **95**:852-868.e8. doi:10.1016/j.neuron.2017.07.016

Woodgett JR. 2018. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J* **9**:2431–2438. doi:10.1002/j.1460-2075.1990.tb07419.x

Woodgett JR. 2003. Judging a Protein by More Than Its Name: GSK-3. *Sci Signal* **2001**:re12–re12. doi:10.1126/stke.2001.100.re12

Woodgett JR, Cohen P. 1984. Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthease kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochim Biophys Acta (BBA)/Protein Struct Mol* **788**:339–347. doi:10.1016/0167-4838(84)90047-5

Wu C, Zhang X, Chen P, Ruan X, Liu W, Li Y, Sun C, Hou L, Yin B, Qiang B, Shu P, Peng X. 2019. MicroRNA-129 modulates neuronal migration by targeting Fmr1 in the developing mouse cortex. *Cell Death Dis* **10**:1–13. doi:10.1038/s41419-019-1517-1

Wu G, Huang H, Abreu JG, He X. 2009. Inhibition of GSK3 phosphorylation of β-catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. *PLoS One* **4**. doi:10.1371/journal.pone.0004926

Wu KY, Hengst U, Urquhart ER, Macosko EZ, Cox LJ, Jeromin A, Jaffrey SR. 2005. Local translation of RhoA regulates growth cone collapse. *Nature* **436**:1020–1024. doi:10.1038/nature03885

Wulczyn FG, Smirnova L, Rybak A, Brandt C, Kwidzinski E, Ninnemann O, Strehle M, Seiler A, Schumacher S, Nitsch R. 2007. Post-transcriptional regulation of the let-7 microRNA during neural cell specification. *FASEB J* 415–426. doi:10.1096/fj.06-6130com

Xia CH, Roberts EA, Her LS, Liu X, Williams DS, Cleveland DW, Goldstein LSB. 2003. Abnormal neurofilament transport caused by targeted disruption of neuronal kinesin heavy chain KIF5A. *J Cell Biol* **161**:55–66. doi:10.1083/jcb.200301026

Xiao J, Pradhan A, Liu Y. 2006. Functional role of JNK in neuritogenesis of PC12-N1 cells. *Neurosci Lett* **392**:231–234. doi:10.1016/j.neulet.2005.09.024

Xiong R, Wang Z, Zhao Z, Li H, Chen W, Zhang B, Wang L, Wu L, Li W, Ding

J, Chen S. 2014. MicroRNA-494 reduces DJ-1 expression and exacerbates neurodegeneration. *Neurobiol Aging* **35**:705–714. doi:10.1016/j.neurobiolaging.2013.09.027

Xu C, Kim NG, Gumbiner BM. 2009. Regulation of protein stability by GSK3 mediated phosphorylation. *Cell Cycle* **8**:4032–4039. doi:10.4161/cc.8.24.10111

Xu K, Zhong G, Zhuang X. 2013. Actin, Spectrin, and Associated Proteins Form a Periodic Cytoskeletal Structure in Axons. *Science (80-)* **339**:452–457. doi:10.1126/science

Xu X-L, Li Y, Wang F, Gao F-B. 2008. The Steady-State Level of the Nervous-System-Specific MicroRNA-124a Is Regulated by dFMR1 in Drosophila. *J Neurosci* **28**:11883–11889. doi:10.1523/jneurosci.4114-08.2008

Yabe JT, Pimenta A, Shea TB, Vale RD, Goldman RD. 1999. Kinesin-mediated transport of neurofilament protein oligomers in growing axons. *J Cell Sci* **112 (Pt 2**:3799–814. doi:10.1242/jcs.104729

Yamauchi J, Miyamoto Y, Sanbe A, Tanoue A. 2006. JNK phosphorylation of paxillin, acting through the Rac1 and Cdc42 signaling cascade, mediates neurite extension in N1E-115 cells. *Exp Cell Res* **312**:2954–2961. doi:10.1016/j.yexcr.2006.05.016

Yan D, Guo L, Wang Y. 2006. Requirement of dendritic Akt degradation by the ubiquitin-proteasome system for neuronal polarity. *J Cell Biol* **174**:415–424. doi:10.1083/jcb.200511028

Yan D, Wu Z, Chisholm AD, Jin Y. 2009. The DLK-1 Kinase Promotes mRNA Stability and Local Translation in C. elegans Synapses and Axon Regeneration. *Cell* **138**:1005–1018. doi:10.1016/j.cell.2009.06.023

Yan T, Feng Y, Zheng J, Ge X, Zhang Y, Wu D, Zhao J, Zhai Q. 2010. Nmnat2 delays axon degeneration in superior cervical ganglia dependent on its

NAD synthesis activity. *Neurochem Int* **56**:101–106. doi:10.1016/j.neuint.2009.09.007

Yao J, Sasaki Y, Wen Z, Bassell GJ, Zheng JQ. 2006. An essential role for β-actin mRNA localization and translation in Ca2+-dependent growth cone guidance. *Nat Neurosci* **9**:1265–1273. doi:10.1038/nn1773

Yau KW, Schatzle P, Tortosa E, Pages S, Holtmaat A, Kapitein LC, Hoogenraad CC. 2016. Dendrites In Vitro and In Vivo Contain Microtubules of Opposite Polarity and Axon Formation Correlates with Uniform Plus-End-Out Microtubule Orientation. *J Neurosci* **36**:1071–1085. doi:10.1523/jneurosci.2430-15.2016

Yeo M, Lee S, Lee B, Ruiz E, Pfaff S, Gill G. 2005. Small CTD phosphatases function in silencing neuronal gene expression. *Science (80-)* **307**:596–600. doi:10.1126/science.1100801 ARTICLE

Yi JJ, Barnes AP, Hand R, Polleux F, Ehlers MD. 2010. TGF-β Signaling Specifies Axons during Brain Development. *Cell* **142**:144–157. doi:10.1016/j.cell.2010.06.010

Yi R, Qin Y, Macara IG, Cullen BR. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs — Genes & Development. *Genes Dev* **17**:3011–3016. doi:10.1101/gad.1158803.miRNA

Yokota Y, Kim WY, Chen Y, Wang X, Stanco A, Komuro Y, Snider W, Anton ES. 2009. The Adenomatous Polyposis Coli Protein Is an Essential Regulator of Radial Glial Polarity and Construction of the Cerebral Cortex. *Neuron* **61**:42–56. doi:10.1016/j.neuron.2008.10.053

Yoo AS, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, Lee-messer C, Dolmetsch RE, Tsien RW, Crabtree GR. 2011. MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature* **476**:228–32. doi:10.1038/nature10323

Yoon BC, Jung H, Dwivedy A, O'Hare CM, Zivraj KH, Holt CE. 2012. Local translation of extranuclear lamin B promotes axon maintenance. *Cell* **148**:752–764. doi:10.1016/j.cell.2011.11.064

Yoon BC, Zivraj KH, Holt CE. 2009. Local Translation and mRNA Trafficking in Axon Pathfinding. *Reaults Probl Cell Differ* **48**:269–88. doi:10.1007/400_2009_5

Yoon K, Gaiano N. 2005. Notch signaling in the mammalian central nervous system: Insights from mouse mutants. *Nat Neurosci* **8**:709–715. doi:10.1038/nn1475

Yoshimura T, Arimura N, Kawano Y, Kawabata S, Wang S, Kaibuchi K. 2006. Ras regulates neuronal polarity via the PI3-kinase/Akt/GSK-3β/CRMP-2 pathway. *Biochem Biophys Res Commun* **340**:62–68. doi:10.1016/j.bbrc.2005.11.147

Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K. 2005. GSK-3β regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* **120**:137–149. doi:10.1016/j.cell.2004.11.012

Yu J, Chen M, Huang H, Zhu Junda, Song H, Zhu Jian, Park J, Ji SJ. 2017. Dynamic m6A modification regulates local translation of mRNA in axons. *Nucleic Acids Res* **46**:1412–1423. doi:10.1093/nar/gkx1182

Zhang F, Wang D. 2017. The pattern of microRNA binding site distribution. *Genes (Basel)* **8**:1–11. doi:10.3390/genes8110296

Zhang H, Zhang L, Sun T. 2018. Cohesive Regulation of Neural Progenitor Development by microRNA miR-26, Its Host Gene Ctdsp and Target Gene Emx2 in the Mouse Embryonic Cerebral Cortex. *Front Mol Neurosci* **11**:1–12. doi:10.3389/fnmol.2018.00044

Zhang HL, Singer RH, Bassell GJ. 1999. Neurotrophin regulation of b-actin mRNA and protein localization within Growth Cones. *J Cell Biol* **147**:59–

70. doi:10.1083/jcb.147.1.59

Zhang J, Li S, Li L, Li M, Guo C, Yao J, Mi S. 2015. Exosome and exosomal microRNA: Trafficking, sorting, and function. *Genomics, Proteomics Bioinforma* **13**:17–24. doi:10.1016/j.gpb.2015.02.001

Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, Li P, Li M, Wang X, Zhang C, Wang H, Ellis K, Cheerathodi M, McCarty JH, Palmieri D, Saunus J, Lakhani S, Huang S, Sahin AA, Aldape KD, Steeg PS, Yu D. 2015. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature* **527**:100–104. doi:10.1038/nature15376

Zhang X, Poo M. 2002. Localized Synaptic Potentiation by BDNF Requires Local Protein Synthesis in the Developing Axon. *Neuron* **36**:675–688. doi:10.1016/S0896-6273(02)01023-1

Zhang Y, Chopp M, Liu XS, Kassis H, Wang X, Li C, An G, Zhang ZG. 2015. MicroRNAs in the axon locally mediate the effects of chondroitin sulfate proteoglycans and cGMP on axonal growth. *Dev Neurobiol* **75**:1402–1419. doi:10.1002/dneu.22292

Zhang Y., Ueno Y, Liu XS, Buller B, Wang X, Chopp M, Zhang ZG. 2013. The MicroRNA-17-92 Cluster Enhances Axonal Outgrowth in Embryonic Cortical Neurons. *J Neurosci* **33**:6885–6894. doi:10.1523/JNEUROSCI.5180-12.2013

Zhang Yi, Ueno Y, Liu XS, Buller B, Wang X, Chopp M, Zhang ZG. 2013. The MicroRNA-17–92 Cluster Enhances Axonal Outgrowth in Embryonic Cortical Neurons **33**:6885–6894. doi:10.1523/JNEUROSCI.5180-12.2013

Zhao C, Sun G, Li S, Lang M, Yang S, Li W, Shi Y. 2009a. MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. *pnas* **107**:1876–81.

doi:10.1073/pnas.0908750107

Zhao C, Sun G, Li S, Shi Y. 2009b. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nat Struct Mol Biol* **16**:365–371. doi:10.1038/nsmb.1576

Zheng JQ, Kelly TK, Chang B, Ryazantsev S, Rajasekaran AK, Martin KC, Twiss JL. 2001. A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. *J Neurosci* **21**:9291–303. doi:10.1523/JNEUROSCI.21-23-09291.2001

Zhou F-Q, Snider WD. 2006. Intracellular control of developmental and regenerative axon growth. *Philos Trans R Soc B Biol Sci* **361**:1575–1592. doi:10.1098/rstb.2006.1882

Zhou F-Q, Snider WD. 2005. GSK-3 and Microtubule Assembly in Axons. *Science (80-)* **308**:211–214. doi:10.1126/science.1110301

Zhou FQ, Zhou J, Dedhar S, Wu YH, Snider WD. 2004. NGF-induced axon growth is mediated by localized inactivation of GSK-3β and functions of the microtubule plus end binding protein APC. *Neuron* **42**:897–912. doi:10.1016/j.neuron.2004.05.011

Zhou WJ, Geng ZH, Chi S, Zhang W, Niu XF, Lan SJ, Ma L, Yang X, Wang LJ, Ding YQ, Geng JG. 2011. Slit-Robo signaling induces malignant transformation through Hakai-mediated E-cadherin degradation during colorectal epithelial cell carcinogenesis. *Cell Res* **21**:609–626. doi:10.1038/cr.2011.17

Zhu H, Xue C, Yao M, Wang H, Zhang P, Qian T, Zhou S, Li S, Yu B, Wang Y, Gu X. 2018. MIR-129 controls axonal regeneration via regulating insulin-like growth factor-1 in peripheral nerve injury. *Cell Death Dis* **9**:1–17. doi:10.1038/s41419-018-0760-1

Zivraj KH, Tung YCL, Piper M, Gumy L, Fawcett JW, Yeo GSH, Holt CE. 2010.

Subcellular Profiling Reveals Distinct and Developmentally Regulated Repertoire of Growth Cone mRNAs. *J Neurosci* **30**:15464–15478. doi:10.1523/JNEUROSCI.1800-10.2010

Zmuda JF, Rivas RJ. 1998. The Golgi apparatus and the centrosome are localized to the sites of newly emerging axons in cerebellar granule neurons in vitro. *Cell Motil Cytoskeleton* **41**:18–38. doi:10.1002/(SICI)1097-0169(1998)41:1<18::AID-CM2>3.0.CO;2-B

Zolessi FR, Poggi L, Wilkinson CJ, Chien C-BB, Harris WA. 2006. Polarization and orientation of retinal ganglion cells in vivo. *Neural Dev* **1**:1–21. doi:10.1186/1749-8104-1-2

Zuccaro E, Bergami M, Vignoli B, Bony G, Pierchala BA, Santi S, Cancedda L, Canossa M. 2014. Polarized Expression of p75NTR specifies axons during development and adult neurogenesis. *Cell Rep* **7**:138–152. doi:10.1016/j.celrep.2014.02.039

Zuchero JB, Barres BA. 2013. Intrinsic and extrinsic control of oligodendrocyte development. *Curr Opin Neurobiol* **23**:914–920. doi:10.1016/j.conb.2013.06.005

Zumbrunn J, Kinoshita K, Nathke IS. 2000. Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3® phosphorylation. *Curr Biol* **11**:44–49. doi:10.1126/science.196.4292.830