
# Role of NMNAT2 Regulation in Cortical Primary Neurons

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March 2019

This thesis is submitted for the degree of Master by Research

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## Abstract

Nicotinamide mononucleotide adenylyl transferase 2 (NMNAT2) is a key neuronal maintenance factor which provides potent neuroprotection in several models of neurological disorders. NMNAT2 is significantly reduced in Alzheimer's, Huntington's, Parkinson's diseases.

MicroRNAs have emerged as key regulators of biological processes in animals. The microRNA system is part of the cell's epigenetic arsenal that shapes the formation and tight regulation of gene networks during the establishment and the remodelling of the brain by controlling both levels and translation of messenger RNA. Deregulation of miRNA function is associated with a plethora of human diseases and they are considered a key molecular tool for the diagnosis and prognosis of human diseases. Up to date, NMNAT2 regulation by microRNAs has not been investigated.

Here we investigate NMNAT2 role in axonal growth of primary cortical neurons. Consequently, we identify four putative miRNAs that target NMNAT2 and study their effect on axonal growth and NMNAT2 mRNA expression. We identify miR-129-3p as a regulator of NMNAT2 in cortical neurons as it both limits their axonal length and reduces the mRNA levels of NMNAT2.

Understanding microRNA regulation of NMNAT2 can build a strong momentum to encourage studies of miRNAs as molecular tools for the diagnosis and prognosis of NMNAT2 related pathologies as long as mechanistic studies to develop microRNA-targeted therapies.

## *Aknowledgements*

I would like to thank each and every one who supported me during my Master's.

Firstly, my supervisor, Dr. Federico Dajas-Bailador, who guided and supported me especially during the writing of my thesis.

A big thanks to my lab mates, Alex, Cristiano, Raquel, Matt, Teresa, Alice and Asta for their friendship and their help.

A special thanks to my partner Kostas for putting up with me during the writing of my thesis.

Finally, but most importantly, I want to thank my family Maria, Marouso and Kostis for always standing by me, believing in me and arming me with strength.

# Chapter 1: INTRODUCTION

## 1.1 The neuron: Growth and Development

Neurons alongside with glial cells, are the basic building blocks of the nervous system. The mature brain comprises billions of neurons which can be classified into at least a thousand different types and even more glial cells whereas the precursor of the brain, the neural plate, comprises a few hundred of cells. This significant difference indicates that regulation of the proliferation of nervous cells plays a major role since the beginning of the nervous system's development (Kandel *et al.*, 2013).

Cognition and behaviour majorly depend on the organisation of neurons in complex circuits with precise structure and function rather than on their number and variety (Kandel *et al.*, 2013).

The typical neuron has four morphologically defined regions: the cell body, dendrites, axon, and presynaptic terminals. The cell body or soma contains the nucleus, and the endoplasmic reticulum, where the majority of the cell's proteins are synthesized. The cell body usually projects into two kinds of processes, dendrites and axons, which vary vastly in their length, branching pattern, thickness and molecular composition. The neuronal polarisation is a synergistic process that requires the regulation of gene transcription, the cytoskeleton and membrane structure and dynamics (da Silva and Dotti, 2002).

Dendrites, as their name indicates, sprout like tree branches out of the soma and are the main apparatus for receiving signals from other neurons. The growing axon extends to some distance from the soma of the neuron and carries signals to other nerve cells. It is truly remarkable that neurons can

grow to the length of 30 metres in blue whales and more than 1 metre in the human body in (Goldberg, 2003; Smith, 2009).

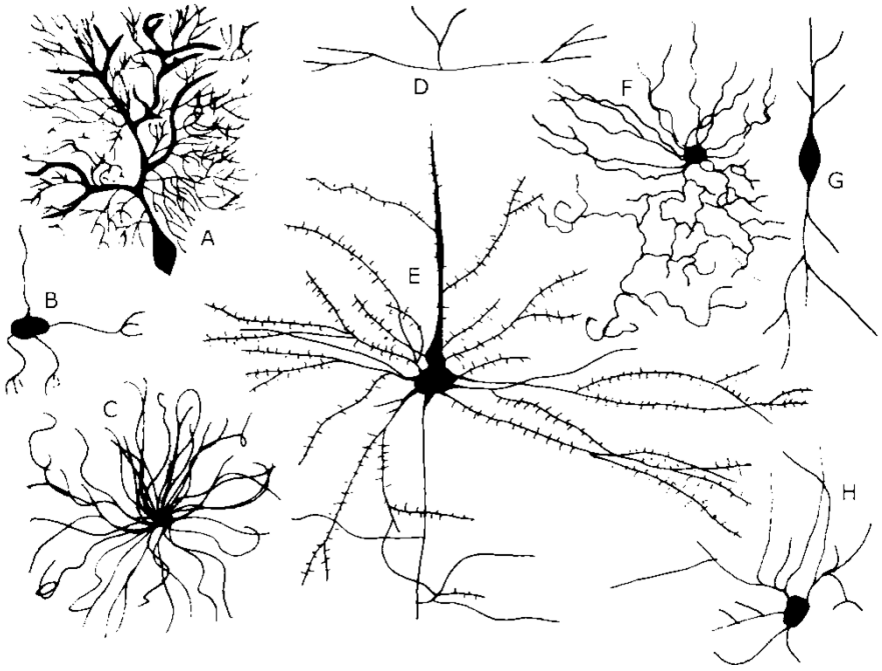



**Figure 1: Different Types of Neurons.** A. Purkinje cell B. Granule cell C. Motor neuron D. Tripolar neuron E. Pyramidal Cell F. Chandelier cell G. Spindle neuron H. Stelate Cell (Ferris Jabr;Based on constructions and drawings by Cajal).

### 1.1.1 Neuronal Polarisation, an introduction

Cell polarisation is essential for the development and function of various cell types that acquire morphological asymmetry in response to intrinsic and extrinsic cues. Naturally, neurons are among the most highly polarised cells as they extend from the cell body, structurally and functionally different

processes (axons and dendrites) to mediate the flow of information through the nervous system.

Cultured primary neurons are very useful for developmental studies because they initially do not show morphological signs of polarisation, and they acquire their specialised features gradually. After initial plating, extension of several short similar processes occurs and soon, one of these processes is established as the axon and the rest of the processes acquire dendritic features (Figure 2).

Much of our knowledge on the cellular and molecular mechanisms behind neuronal polarization derives from work performed on rodents' brains and cell cultures (Dotti, Sullivan and Banker, 1988; da Silva and Dotti, 2002). Our understanding of the molecular mechanisms responsible for mammalian neuronal polarisation has been studied predominantly through studies on cultured primary hippocampal neurons (Dotti, Sullivan and Banker, 1988; Tahirovic and Bradke, 2009). More recent studies have been focusing on the roles of extracellular and intracellular signalling molecules on neuronal polarisation *in vivo*, by using electroporation and knock-in and knockout mice (Funahashi *et al.*, 2014; Takano *et al.*, 2015).It has been shown that both extracellular signalling and intrinsic mechanisms modulate the generation and maintenance of neuronal polarity.

A central role in polarisation is provided by cytoskeletal proteins which maintain elongated processes and drive growth (da Silva and Dotti, 2002; Lewis, Courchet and Polleux, 2013). A destabilisation of the actin filaments in a young neurite and cytoskeletal rearrangements are essential steps in driving a neurite to become the axon whereas the rest of the neurites become dendrites.

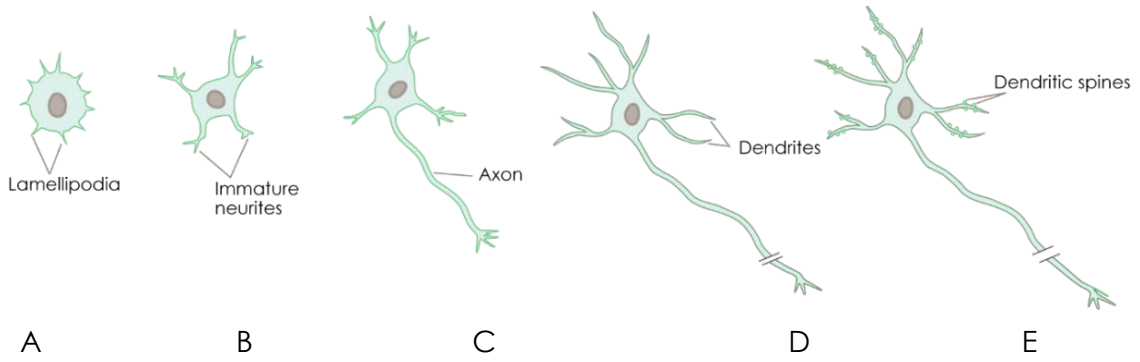



**Figure 2: Four stages in the in vitro polarisation of a neuron.** A. Lamellipodia Formation (Shortly after plating) B. Neurite Formation(0.5-1.5 days) C. One neurite becomes the axon, (1.5-3 days) D. The other neurites become Dendrites (4-7 days) E. Maturation (>7 days). Based on an image from *Principles of Neural Science, 5th edition, McGraw-Hill, 2013*

### 1.1.2 Axon: Growth and Guidance

An axon is typically a single long process that transmits signals to other neurons by releasing neurotransmitters. Dendrites are composed of multiple branched processes and dendritic spines, which contain neurotransmitter receptors to receive signals from other neurons. The formation and maintenance of such distinct cellular compartments are essential for the proper development and physiology of the nervous system.

The axonal growth and guidance require a precisely choreographed sequence of events in the nervous system. Firstly, neurons and their surrounding target tissues must be specified in expressing the correct nexus of receptors and guidance cues respectively (Lewis, Courchet and Polleux, 2013). Secondly, receptors must be assembled into the correct complexes and localised to the axonal or dendritic growth cones, while guidance cues must be correctly shipped to and localised within the extracellular environment. Lastly, signaling mechanisms must be in place to integrate and propagate signals from the surface receptors into alterations in the growth cone actin

and tubulin cytoskeleton resulting in steering decisions (Dent and Gertler, 2003).

The key neuronal element responsible for axonal growth is a specialized structure at the tip of the axon called the growth cone. Both axons and dendrites use growth cones for elongation, but the axonal ones have been studied more extensively. The growth cone is both a sensory structure that receives directional cues from the environment as well as a motor structure that drives axon elongation (Lowery and Van Vactor, 2009).
During development, growth cones follow specific pathways and navigate through a plethora of decision points to find their correct targets. At each point, growth cones face a number of guidance cues in their extracellular environments (Dickson, 2002).

Several families of phylogenetically conserved families of guidance cues and receptors have been discovered. These include semaphorins (semas) and their plexin (Plex) and neuropilin receptors (Pasterkamp and Kolodkin, 2003), netrins and their deleted in colorectal carcinoma (DCC) and UNC5 receptors for netrins (Li *et al.*, 2002), slits and their roundabout (Robo) receptors (Brose and Tessier-Lavigne, 2000) and ephrins and their Eph receptors (Kullander and Klein, 2002).

### 1.1.3 Axonal Transport

Axonal transport is an essential process in neurons because of the significant geometry, polarity and size of these cells. Anterograde transport supplies distal axons with newly synthesised proteins and lipids, synaptic components required to maintain presynaptic activity and mitochondria for local energy requirements. Retrograde transport is required to maintain homeostasis by

removing misfolded, aggregated and aging proteins as well as organelles from the distal axon for degradation and recycling of components. Retrograde axonal transport is also involved in intracellular transport of distal trophic signals to the soma and thus plays a major role in neurotrophic and injury response signalling. Until recently, it was broadly believed that the axon, which can extend up to metres from the cell body, depended exclusively on the biosynthetic and degradative activities of the soma which highlighted the necessity and importance of active transport. Genetic evidence though confirms the crucial role for active transport in the neuron as defects in many of the proteins involved, such as kinesins and dynein lead to diseases that are characterized by axonal degeneration (Blackstone, O'Kane and Reid, 2011; Holzbaur and Scherer, 2011).

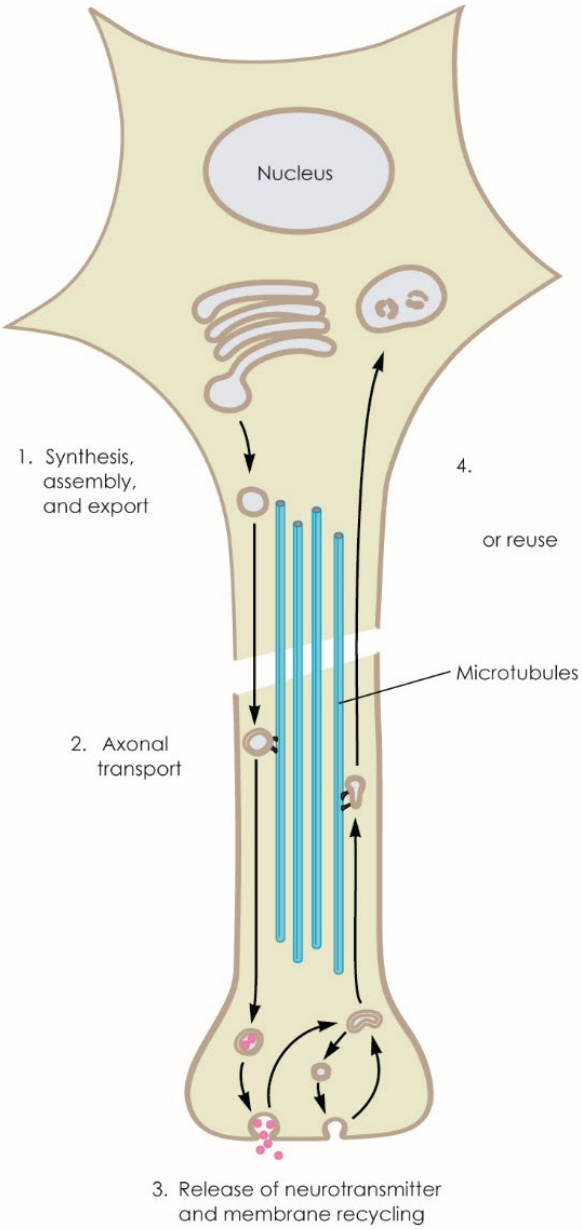



**Figure 3: Membrane trafficking in the neuron.** 1. Proteins and lipids of secretory organelles are synthesized in the endoplasmic reticulum and transported to the Golgi complex, where large dense-core vesicles (peptide-containing secretory granules) and synaptic vesicle precursors are assembled. 2. Large dense-core vesicles and transport vesicles that carry synaptic vesicle proteins travel down the axon via axonal transport. 3. At the nerve terminals the synaptic vesicles are assembled and loaded with nonpeptide neurotransmitters. Synaptic vesicles and large dense-core vesicles release their contents by exocytosis. 4. Following exocytosis, large dense-core vesicle membranes are returned to the cell body for reuse or degradation. Synaptic vesicle membranes undergo many cycles of local exocytosis and endocytosis in the presynaptic terminal. *Edited from Principles of Neural Science, 5th edition, McGraw-Hill, 2013*

### 1.1.4 Local Translation

RNA localisation and localised translation are conserved mechanisms of spatiotemporal control of protein expression. This could prove especially important for highly polarised and morphologically complex cells such as neurons (Zappulo *et al.*, 2017) in which local translation allows axons and dendrites to remodel their proteome precisely in response to local demand.

Cell-cell signalling depends on the cell's ability to adjust their local proteome with spatiotemporal precision in response to extracellular signals. Many mRNAs present specific subcellular localisation patterns in mammals (Mili, Moissoglu and Macara, 2008) as well as in *Drosophila melanogaster*(Lécuyer *et al.*, 2007), which implies that local mRNA translation may have a general function in regulation of the local proteome.

Local protein synthesis has several advantages over the transport of pre-existing proteins from one part of the cell to another (Jung, Yoon and Holt, 2012). To begin with, translationally silent forms of mRNAs can be locally stored and when needed, be used to make several copies of a protein. Secondly, during protein transport, the ectopic presence of proteins in other parts of the cell is avoided. Moreover, mRNAs can reach different subcellular compartments using 'spatial' information encrypted in their untranslated regions (UTRs) without changes in structure and function of the proteins they encode. Lastly, there are properties unique to newly produced proteins which may provide further signalling information.

Local mRNA translation has been shown to mediate synaptic plasticity in dendritic spines and there are studies that provide valuable insight on the underlying mechanisms of regulation of local mRNA synthesis by extrinsic signals (Sutton and Schuman, 2006; Wang, Martin and Zukin, 2010). The essential

components of the translation machinery are present in developing and mature axons and axonal protein synthesis has been shown to be involved in several physiological and pathological processes such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA) and Alzheimer's disease (AD) (Batista and Hengst, 2016; Spaulding and Burgess, 2017; Costa and Willis, 2018). Through axonal RNA-sequencing, thousands of mRNAs have been identified (Baleriola *et al.*, 2014; Minis *et al.*, 2014; Kar, Lee and Twiss, 2018). These vary between neuronal subtypes (Kar, Lee and Twiss, 2018), axonal subdomains and throughout the axon's lifetime (Zivraj *et al.*, 2010; Gumy *et al.*, 2011). A cell-type specific genome-wide analysis of the axonal translatome further elucidated the dynamic nature of local translation during the development and maintenance of neuronal wiring *in vivo*. Through the analysis there were identified axonally translated mRNAs which encoding functionally linked regulators who show developmental co-regulation (Shigeoka *et al.*, 2016).

The cohabitation of several different mRNAs in the same subcellular compartments raises the question of how specific groups of mRNAs are translated in response to specific cues. Axon guidance cues along with neurotrophins stimulate kinases and release their associated mRNAs for local translation (Hörnberg and Holt, 2013). The mRNA specificity of axonal translation may require a spatiotemporal combination of signalling pathways.

It has been suggested that a multiple cue integration can lead in signaling cooperation and crosstalk by multiple pathways which can fine-tune local translation for particular physiological and developmental situations. (Morales and Kania, 2017).

## 1.2 microRNAs, tiny but mighty

### 1.2.1 miRNA Biogenesis

The human genome project revealed that protein-coding sequences constitute ~1.5 % of the entire genome and the remaining ~98.5 % of the genome contains introns, regulatory DNA sequences, interspersed elements and non-coding RNA (ncRNA) molecules (Lander *et al.*, 2001). The majority of mammalian genomes are transcribed into ncRNAs, many of which are alternatively spliced or processed into smaller products. To this day, two types of non-coding RNAs have been identified—short non-coding RNA and long non-coding RNA molecules (Hüttenhofer, Schattner and Polacek, 2005). The short non-coding RNA molecules include microRNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), piwi-interacting RNA (pi-RNA), transferRNA (tRNA), ribosomal RNA (rRNA) and other uncharacterized small molecules (Hüttenhofer, Schattner and Polacek, 2005; Kaikkonen, Lam and Glass, 2011). MicroRNAs (miRNAs) are very small (18-23 nucleotides) yet powerful regulators. They are endogenous, non-coding RNAs which mediate significant gene-regulatory events by binding to the 3' untranslated region (3'UTR) of mRNAs of protein-coding genes directing their translational repression and/or degradation. The repression of these regulatory targets leads to decrease in translational efficiency and/or mRNA levels (Guo *et al.*, 2010). MicroRNAs are highly conserved across organisms including vertebrates and were first discovered in *Caenorhabditis elegans*. In 1991, Gary Ruvkun's lab observed that the deletion of two small sequences in the 3'untranslated region (3'UTR) of the lin-14 mRNA caused lin-14 protein to accumulate (Wightman *et al.*, 1991).

These deletions had no effect in the stability or function of lin-14 protein, suggesting they function in post-transcriptional regulation of lin-14 mRNA. It was

hypothesised that an unidentified regulatory factor binds to these sequences and represses the encoded protein's synthesis. A couple of years later, the source of this translational repression was identified as a new class of regulatory molecules: microRNAs. In recent years, miRNAs have gathered avid scientific interest which is illustrated by the fact that up to date miRbase contains 38589 entries potentially representing hairpin precursor miRNAs in 271 species (Kozomara and Griffiths-Jones, 2014).

As mentioned above, miRNA-binding sites are usually located in the 3′ UTR of mRNAs. The domain at the 5′ end of miRNAs that extends from nucleotide position 2 to 7 is essential for target recognition and is termed as the "miRNA seed". The downstream nucleotides of miRNA (principally nucleotide 8 and less importantly nucleotides 13–16) also contribute to binding with the target mRNAs (Bartel, 2009a).

In several species, there are multiple miRNA loci with related sequences as a result of gene duplication (Hertel *et al.*, 2006; Berezikov, 2011). In most cases miRNAs with identical sequences at nucleotides 2-8 of the mature miRNA belong to the same miRNA family. For example, the human genome contains 14 paralogous loci that belong to the let-7 family, a highly conserved, amongst bilaterian animals, miRNA family that suppresses cell proliferation and promotes cell differentiation. Some miRNAs share an evolutionary origin but differ in the miRNA seed (Berezikov, 2011; Moran *et al.*, 2017).

miRNA genes are transcribed by RNA polymerase II (Pol II) producing a long primary transcript that has a local hairpin structure where miRNA sequences are embedded. Transcription is controlled by RNA Pol II- associated transcription factors and epigenetic regulators (CAI, Hagedorn and Cullen, 2004). Some viral miRNAs are transcribed by RNA Pol III (Pfeffer *et al.*, 2005) In

addition some endogenous miRNA-like small RNAs are issued from tRNAs that are transcribed from RNA Pol III (Babiarz *et al.*, 2008). miRNA expression has been shown to be positively or negatively regulated by transcription factors, such as p53, MYC, ZEB2 and MYOD1 (Kim, Han and Siomi, 2009; Krol, Loedige and Filipowicz, 2010). There has also been described epigenetic control, such as histone modifications and DNA methylation (Davis-Dusenbery and Hata, 2010).

After transcription, the primary miRNA (pri-miRNA) is subjected to maturation. pri-miRNA is long (over 1 kb) and contains a local stem-loop structure in which mature miRNA sequences are embedded. A pri-miRNA typically comprises a stem of 33-35 bp, a terminal loop and single-stranded RNA segments at 5' and 3' sides. Maturation process is initiated by the nuclear RNase III Drosha which crops the stem-stem loop to release a small hairpin-shaped RNA of ~65 nucleotides in length (pre-miRNA)(Lee *et al.*, 2003). Along with its essential cofactor DGCR8, Drosha forms a complex called Microprocessor (Denli *et al.*, 2004). Drosha and DGCR8 are extremely important for cell proliferation and differentiation and are conserved in animals (Filippov *et al.*, 2000; Wu *et al.*, 2000; Fortin, Nicholson and Nicholson, 2002). After the Drosha processing, pre-mi-miRNA is exported into the cytoplasm by the protein exportin 5 which forms a transport complex with GTP-binding nuclear protein RAN.GTP and the pre-miRNA (Yi *et al.*, 2003; Lund *et al.*, 2004). Following translocation via the nuclear pore complex, GTP is hydrolysed, the complex disassembles and the pre-miRNA is released into the cytosol. Upon export to the cytoplasm, Dicer cleaves pre-miRNA near the terminal loop, liberating a small RNA duplex consisting a 'passenger' and a 'guide' strand (Ha and Kim, 2014). Dicer binds to pre-miRNA with a preference for a two-nucleotide-long 3' overhang that was initially generated by Drosha (Zhang *et al.*, 2004).

Following Dicer processing, the RNA duplex is released and subsequently loaded onto human and Argonaut protein (AGO 1-4) to form an effector complex called RNA-induced silencing complex (RISC) (Hammond *et al.*, 2001) RISC assembly comprises two steps: the loading of the RNA duplex and its subsequent unwinding (Kawamata and Tomari, 2010).

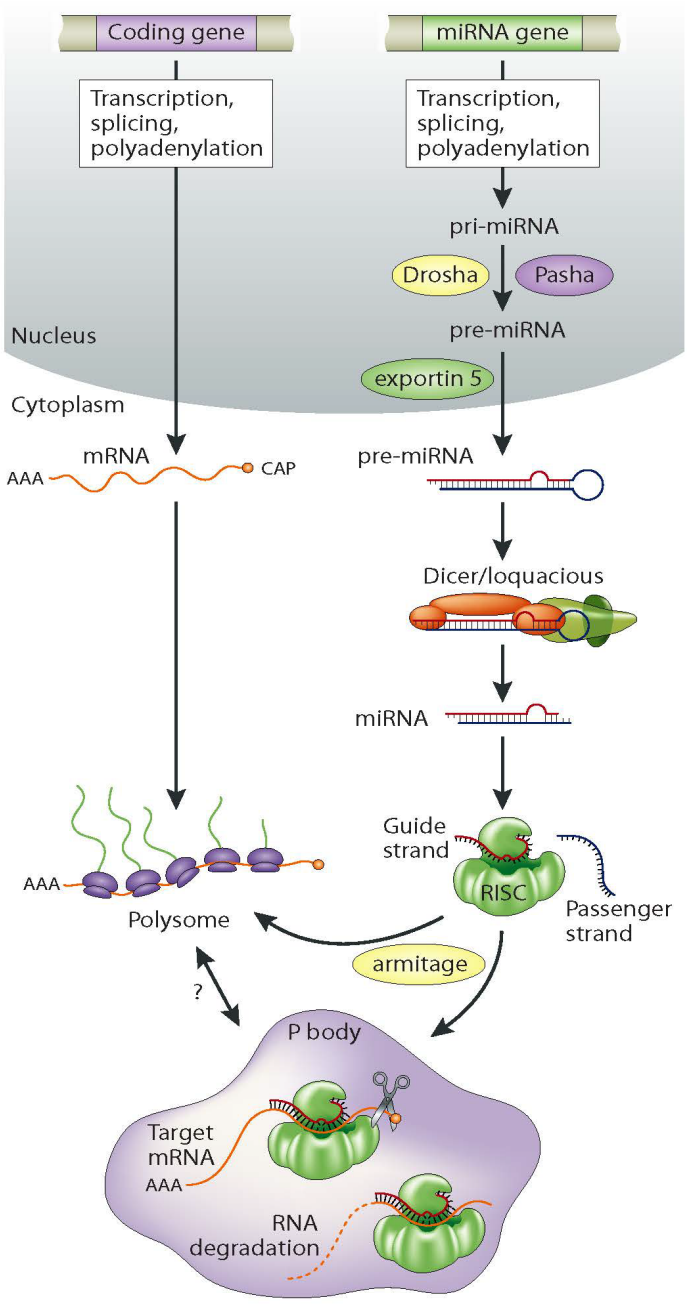


**Figure 4: microRNA biogenesis pathway**. microRNAs (miRNAs) are genomically encoded. miRNA genes generally have RNA polymerase Pol II promoters, and their transcripts can undergo splicing and polyadenylation. The transcript is referred to as the primary miRNA (pri-miRNA). This transcript is cleaved in the nucleus by an enzyme complex that contains the RNaseIII enzyme Drosha, and the double-stranded RNA binding-domain protein Pasha. The product of this cleavage is the precursor miRNA (pre-miRNA), which is exported to the cytoplasm by exportin 5. Once in the cytoplasm, the pre-miRNA is cleaved by an enzyme complex that is functionally similar to the nuclear cleavage complex, and comprises the proteins Dicer and loquacious. The resulting miRNA duplex is then unwound, (possibly by the helicase armitage). The guide strand is translocated to the RNA-induced silencing complex (RISC) where it binds to a target mRNA, whereas the passenger strand is destroyed. The cellular structure most closely associated with the RISC, and visible at the light microscopic level, is the P body. Cytoplasmic destinations of miRNAs include both P bodies and polysomes; however, exchange between these compartments is speculative. Armitage might also control whether or not a miRNA/mRNA duplex is translated (Kosik, 2006).

Consequently, miRNA duplex loading, the pre-RISC (in which AGO proteins associate with RNA duplexes) removes the passenger strand to generate a mature RISC. RISC loading of small RNA duplexes is an active process that requires ATP, whereas the release of the passenger strand is ATP-independent (Tomari *et al.*, 2004; Kawamata, Seitz and Tomari, 2009; Yoda *et al.*, 2010). The guide strand is determined during the AGO loading step, based on the relative thermodynamic stability of the two ends of the small RNA duplex (Khvorova, Reynolds and Jayasena, 2003; Schwarz *et al.*, 2003). The strand with an unstable terminus at the 5' side is typically selected as the guide strand. Another factor that acts as a determinant for strand choice is the first nucleotide sequence since AGO proteins select for guide strands with a U at nucleotide position 1. Key characteristics of human miRNA guide strands are a U-bias at the 5'-end and an excess of purines, whereas the passenger

strands have a C-bias at the 5'-end and an excess of pyrimidines (Meijer, Smith and Bushell, 2014). Until recently it was considered that the passenger strand undergoes quick degradation which results in a strong bias in favour of the guide strand in the mature miRNA pool. Although, the strand that is not favoured can also get selected and the less abundant passenger strand can also have a silencing function although less potently than the more abundant guide strand. Arm switching, the phenomenon where the mature miRNA switches between one of the two arms of precursor miRNA has been observed to occur in different species, tissue types, or development stages (Chen *et al.*, 2018). In conclusion, the miRISC protein complex is composed by Argonaut 2 and other regulatory proteins (Fabian and Sonenberg, 2012) and allows binding on specific sequences in the 3'UTR of targeted mRNAs.

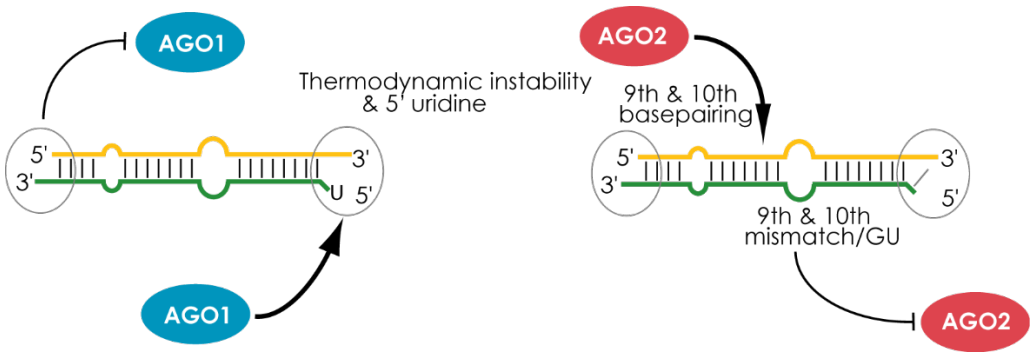



**Figure 5: Model for Differential miRNA Strand Selection by AGO1 and AGO2.** AGO1 strand selection is influenced by 5'uridine in addition to the previously proposed thermodynamic rule, while it is insensitive to the positions of central mismatches. In contrast, AGO2 strand selection is highly sensitive to 9th and 10th base-pairing status. Edited from (Okamura, Liu and Lai, 2009)

### 1.2.2 Mechanisms of miRNA-mRNA interaction and function

The binding of a miRNA to its target mRNA results in repression of protein synthesis by degradation of the transcript or inhibition of protein translation. The specificity of interaction is determined by the complementarity between a core sequence of ~6-8 nucleotides in the microRNA and their specific seed sequence in the target 3'UTR allowing the recognition of multiple mRNAs 3' UTRs by a single microRNA and the downregulation of each mRNA by multiple microRNAs (Bartel, 2009b).

All nuclear transcribed eukaryotic mRNAs contain a 5′ m7GpppN structure (where N is any nucleotide) termed the 5′ cap, which interacts with the eukaryotic translation initiation factor eIF4E (reviewed in ref. 4). eIF4E, along with other eIFs, in turn recruits ribosomes to initiate mRNA translation (Fabian and Sonenberg, 2012). The miRISC has been shown to inhibit cap-dependent translation at both initiation (by interfering with ribosome recruitment) (Humphreys *et al.*, 2005; Wang, Yanez and Novina, 2008) and post-initiation steps(Maroney *et al.*, 2006; Petersen *et al.*, 2006)

Although the miRNA sequence indicates the mRNAs it can potentially interact with, it is the proteins of the miRISC that implement the targets' silencing. The miRISC also engenders deadenylation and subsequent decapping and decay of target mRNAs (Fig. 1a, bottom)14–18.

### 1.2.3 MicroRNAs in nervous system

#### 1.2.3.1 MicroRNAs and neuronal development

Considering the cellular and transcriptional complexity of the nervous system, it is not surprising that miRNAs are highly abundant in this tissue (Kosik, 2006). Even within a single neuron, its complex functional architecture and compartmentalisation can be regulated by different sets of miRNAs (McNeill and Van Vactor, 2012).

Soon after their discovery, and given their ability to regulate gene expression, it was proposed that miRNAs could regulate specific phases of development (regarding their embryonic or adult-specific expression patterns) and that they could have tissue-specific functions (based on their organ and cell-specific expression patterns). This was noticeably evident in the nervous system, where individual miRNAs or families of miRNAs have been found to regulate gene expression in particular neuronal cell types, at specific stages of development, and even in specific compartments of the cell. For example, in highly polarised neurons, miRNA-dependent regulation of gene expression can occur both at the level of the entire cell or in specific subcellular compartments, such as axons or dendrites. These many studies show that miRNAs are significantly involved in the spatiotemporal regulation of neuronal gene expression which is essential for neural differentiation, circuit development and modification.

miRNAs are involved in determining the fate of the two major cell types, neurons and glia, that are present in the central nervous system (CNS) as well as in the peripheral nervous system (PNS). Neurons and glia emerge from the same type of neuronal precursor cell (NPC). Although neuronal

differentiation occurs mainly during embryonic development when many miRNAs are enriched, glial differentiation continues in the early postnatal nervous system. These processes of neurogenesis and gliogenesis involve many intermediate cell types as reviewed in (Paridaen and Huttner, 2014). miRNAs have also been associated with the regulation of adult neurogenesis as well, a process that is limited to few regions in the adult brain (e.g. the subgranular zone of the hippocampus) and likely plays a role in learning and memory (Luikart, Perederiy and Westbrook, 2012; Schouten *et al.*, 2012).

Monitoring of miRNA expression during neurogenesis in vivo has identified time-specific, spatially restricted (ventral midline/midbrain dopaminergic progenitor pool) or cell type-specific miRNAs, suggesting that different sets of miRNAs might be involved in neuronal versus glial differentiation (Rajman and Schratt, 2017). This is backed by the discovery of 116 miRNAs (out of 351) being differentially expressed in primary cultures enriched for neurons, astrocytes, oligodendrocytes and microglia (Jovicic *et al.*, 2013).

The key role of miRNAs in specific processes can be proven by disruption of the machinery responsible for their synthesis. Studies in the nervous system assessed the consequences depleting Dicer gene (which leads to absence of mature miRNAs) on neurogenesis. In Zebrafish, the complete absence of Dicer leads to major defects in the general morphology of both the CNS and PNS, as well as impairment of neuronal differentiation (Giraldez *et al.*, 2005).

#### 1.2.3.2 miRNAs and Neuron Connectivity

Neurons upon migration along the cortical plate, start growing axons and dendrites to establish functional connections. Axons comprise the presynaptic compartment and are essential for long distance information transmission. MiRNA screens have shown that miRNAs are present in axons, with

specific miRNAs being enriched in axons in comparison with the neuronal cell body (Natera-Naranjo *et al.*, 2010; Iyer, Bellon and Baudet, 2014; Sasaki *et al.*, 2014).

A comparison between miRNA in the cell bodies and neurites of rodent hippocampal neurons showed graded distribution across the candidates and selective enrichment of miRNAs in dendrites versus somata (Kye *et al.*, 2007). These discoveries suggest that miRNAs reach axons by active transportation and that they participate in the local regulation of axonal protein synthesis through controlling processes such as axonal branching and guidance (Jung, Yoon and Holt, 2012). It has been shown that BDNF-promoted axonal branching in the developing murine retina ganglia cells, relies on elevated levels of miR-132 which induces axonal branching by inhibiting the translation of Rho family GTPase-activating protein, p250GAP (Arhgap32) (Marler *et al.*, 2014). Axonal branching has been shown to be induced by others miRNAs such as miR-124 in hippocampal neurons and miR-29a in cortical neurons (Franke *et al.*, 2012; Li *et al.*, 2014).

#### 1.2.3.3 miRNAs and Axon Guidance

miRNAs are also involved in axon guidance, the directional growth of axons. There have been studies on several model organisms and it has been shown that correct axon guidance is regulated by guidance cues which are secreted by targets and depends on local protein synthesis (Campbell and Holt, 2001; Brittis, Lu and Flanagan, 2002). That implicates the function of miRNAs. For example, in primary rat cortical neurons, two axonally localized miRNAs (miR-338, miR-181c) debilitate axonal outgrowth by regulating the expression of transcripts involved in the axon guidance (Kos *et al.*, 2016, 2017). There is evidence that miRNAs can regulate not only intrinsic axon growth

programmes but axonal growth stimulated by specific guidance cues as well. Other findings suggest that miRNAs may be involved in mediating the spatiotemporal effects of guidance factors during axonal growth. For example, microtubule-associated protein 1B (MAP1B) expression, which has an important function in outgrowth and branching (Bouquet *et al.*, 2004;) can be locally regulated by miR-9 in mouse cortical neurons (Dajas-Bailador *et al.*, 2012) and by miR-181 in mouse peripheral sensory neurons (Wang *et al.*, 2015). Interestingly, it was shown that the levels of miR-9 and miR-181 in these conditions and by expansion the translation of *Map1b* mRNA, are regulated by BDNF (Dajas-Bailador *et al.*, 2012) and NGF respectively (Wang *et al.*, 2015) These findings explain the spatiotemporal dependence of neurotrophin action in axon guidance.

Several screenings have demonstrated that miRNAs can be specifically localized to the synaptodendritic (Kye *et al.*, 2007; Siegel *et al.*, 2009; Sambandan *et al.*, 2017)compartment and also have an important function in dendritogenesis. For example, miR-132 has been shown to regulate the dendritic growth and branching of mouse and chick young hippocampal neurons *in vitro* and *in vivo* by repressing p250GAP (Magill *et al.*, 2010; Remenyi *et al.*, 2010; Marler *et al.*, 2014) Mir-134 is required for the activity-induced dendritic growth of rat hippocampal neurons *in vitro* by targeting the RNA-binding protein Pum2 In dendrites (Fiore *et al.*, 2009). miR-134 when overexpressed, reduced cortical pyramidal neuron dendritogenesis in cultured mouse brains (Christensen *et al.*, 2010). Mir-9 has been shown to be essential for proper dendrite development in the mouse brain, by targeting the transcriptional repressor REST (Giusti *et al.*, 2014) and in Drosophila melanogaster sensory neurons (Wang *et al.*, 2016), results that suggest that miR-9's function in dendrite development is conserved.

Most of the studies on the roles of miRNAs in neuronal development have focused on Bilateria, organisms that develop from three germ layers (ectoderm, mesoderm and endoderm). Nevertheless, miRNA pathways are not limited to these triploblastic animals and are likely to be present in the diploblasts, as in the phyla of cnidaria and porifera.

Experiments that demonstrate a neurodevelopmental function for miRNAs have followed two directions: in the first approach the full complement of miRNAs is disrupted by interfering with their biogenesis, while in the second approach the role of a single miRNA is investigated. The issue regarding the experiments that disrupt miRNA biogenesis, is the difficulty to understand to what extent the disruption of cellular functions affects the phenotype and to what extent the organism can bypass a single gene knockout or knockdown.

One defect of disrupting a single miRNA gene is that many miRNAs are members of a family of related miRNAs whose functions could be redundant. Another challenge in phenotypic discovery using miRNA gene disruption techniques is the unknown manner in which multiple miRNAs interact to carry out functions in the cell.

#### 1.2.3.4 microRNAs as regulators of local translation

MicroRNAs have been proposed to be negative regulators of local translation in the nervous system precisely controlling physiological and morphological changes in neurons (Schratt *et al.*, 2006; Murashov *et al.*, 2007; Aschrafi *et al.*, 2008; Maes *et al.*, 2009; Siegel *et al.*, 2009; Weinberg and Wood, 2009; Liu and Xu, 2011; Zhou *et al.*, 2011; Davies *et al.*, 2018). Inhibition or activation of specific miRNAs by extracellular cues can selectively stimulate or repress

subsets of mRNAs in axons. The highly conserved miR-9 has been found to control axonal extension and branching by regulating the levels of microtubule-associated protein 1b (Map1b), an important protein for microtubule stability. miR-9 represses Map1b translation and is a functional target for the BDNF-dependent control of axon extension and branching (Dajas-Bailador *et al.*, 2012). MiR-388 for example modulates axonal synthesis of the mRNAs that encode the mitochondrial proteins COXIV and ATP5G1 (Aschrafi *et al.*, 2008, 2012). This process can control the total axonal proteome and respond to urgent needs in restricted regions. An example of this is the regulation from miR-182 of Slit2-mediated axon guidance by the modulation of local translation of the respective mRNA (Bellon *et al.*, 2017). MiRNA-132 has been found to be enriched in developing axons where it locally regulates Rasa1 mRNA promoting axon extension (Hancock *et al.*, 2014). A recent study has revealed that axonal transcripts have significantly longer 3'UTRs (Andreassi *et al.*, 2017) so for miRNAs, which are able to repress translation by binding to 3'UTRs, longer 3'UTRs allow for more rigid and even more axon-specific regulation by miRNAs.

## 1.3 Neuronal degeneration and death

### 1.3.1 Basic Mechanisms of neuronal cell death

Karl Vogt was the first to note the requirement for physiological cell death in 1842 while studying metamorphosis of amphibians (Vogt, 1842). Neuronal cell death was initially noticed as a loss of neurons during development. The first reports from 1890 and 1892 by Beard described a loss during development of a specific population of sensory neurons in the spinal cord of *Raja*

*batis* (Chondrichthyes) and *Lepisosteus osseus* (Osteichthyes) (Beard, 1890, 1892).

Cell death is an extensively regulated and crucial homeostatic mechanism necessary to maintain tissues, organ size and normal function. Neuronal cells are a special type of cells that are excluded from the everyday flux of cellular birth and death. Post-mitotic neurons, following their developmental period are required to live long in order to maintain proper circuits. During the developmental period though, cell death affects both mitotic neuronal precursor and postmitotic differentiated neuronal populations (Sadoul, 1998; Kuan *et al.*, 2000; Buss *et al.*, 2006). Developmental programmed cell death holds an important role in the establishment of functional circuits within the nervous system through several mechanisms such as the elimination of neurons migrating to ectopic positions or innervating wrong targets, and competition of neurons for limiting supplies of pro-survival factors produced by targets to reach optimal target innervation (Buss *et al.*, 2006).

There are several mechanisms that execute neuronal cell death. Firstly, there is apoptosis which can be triggered by two principal pathways: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. The extrinsic apoptosis pathway is triggered by the ligation of tumour necrosis factor (TNF)-family death receptors on the cell surface. Receptor ligation can lead to the recruitment of Fas-associated death domain protein (FADD), which subsequently binds pro-caspase-8 molecules, which leads to autoproteolysis and activation of caspase-8.

Upon its activation, caspase-8 may activate downstream effector caspases directly through proteolytic cleavage or indirectly by cleavage of the BH3-only protein Bid to produce tBid which translocates to mitochondria to induce Bax activation and mitochondrial outer membrane permeabilization (MOMP) (Haase *et al.*, 2008).

The intrinsic apoptosis pathway centres on the regulation of MOMP by the *Bcl-2* family proteins (Sadoul, 1998; Cheng *et al.*, 2001). Proteins of the *Bcl-2* family share homology within at least one of up to four Bcl-2 homology (BH) domains that are necessary for the homo- and heterotypic interactions that determine the decision to undergo MOMP. The pro-apoptotic Bax and Bak contain BH1–3 and are essential for the execution of apoptosis via the intrinsic pathway (Cheng *et al.*, 2001; Wei *et al.*, 2001; Zong *et al.*, 2001), though it has come to light a new but yet unknown Bax/Bak-independent intrinsic apoptotic pathway (Zamorano *et al.*, 2012). In neurons Bak is expressed as an alternately transcribed product (N-Bak) that is translationally repressed and does not participate in apoptosis (Jakobson *et al.*, 2013b). Therefore, the induction of intrinsic apoptosis in neurons is completely dependent on Bax expression and activation. Deletion and inhibition of Bax has been shown to prevent aberrant neuronal cell death in several in vitro and in vivo models of neurodegeneration (Deckwerth *et al.*, 1996; Miller *et al.*, 1997; Xiang *et al.*, 1998; Johnson *et al.*, 1998; Gibson *et al.*, 2001; Wong *et al.*, 2005; Gould *et al.*, 2006; Smith and Deshmukh, 2007; Tehranian *et al.*, 2008; Jakobson *et al.*, 2013a; D'Orsi *et al.*, 2015, 2016).

Autophagic cell death, paraptosis and phagoapoptosis are different mechanisms of neuronal cell death (Fricker *et al.*, 2018),the analysis of which does not serve a particular goal concerning this thesis.

### 1.3.2 Neuronal Death induced by stimuli

Apart from mechanisms leading to it, neuronal cell death can also be induced by various important stimuli. To begin with, neuronal cell death can be induced by the lack of neurotrophic factors (NTFs). According to the neurotrophic hypothesis neurons are generated in excess and their survival

depends on competition for limiting amounts of NTFs synthesised by their target fields or lack of these factors if the axons go off course (Purves, 1986). Catalytic role to the proposal and validation of this hypothesis played the discovery of NGF by Rita Levi-Montalcini in the 50s (Levi-Montalcini, 1964; Aloe, 2004)and later of the brain derived-neurotrophic factor (BDNF), NT3 and NT4/5 (Davies, 1988)and their specific Trk tyrosine kinase receptors (Korsching, 1993). Later important discoveries were that of glial-derived neurotrophic factor (GDNF) family along with their specific receptor subunits and signaling receptor Ret tyrosine kinase (Airaksinen, Titievsky and Saarma, 1999; Baloh *et al.*, 2000), the LIF/CNTF(ciliary neurotrophic factor) cytokine family that is substantial after injury and their composite receptors (Richardson, 1994; Baloh *et al.*, 2000) and insulin-like growth factor I(IGF-1) (Nikolić, Gardner and Tucker, 2013).

A neuron's death can lead to death of neurons connected to it. This process is called transneuronal degeneration or secondary neuronal loss and the degeneration can occur either anterograde or retrograde. Transneuronal degeneration results from loss of synaptic inputs to neurons while retrograde transneuronal degeneration results from the loss of synaptic outputs (Fricker *et al.*, 2018).

Cell death can also be induced by aberrant mitosis, also called "mitotic catastrophe" (Castedo *et al.*, 2004; Vakifahmetoglu, Olsson and Zhivotovsky, 2008; Vitale *et al.*, 2011)a process that has been suggested to contribute to neuronal death in neurodegenerative diseases (Yurov, Vorsanova and Iourov, 2011; Seward *et al.*, 2013; Tokarz, Kaarniranta and Blasiak, 2016).

Excitotoxicity, cell death due to high levels of excitatory neurotransmission (Olney, 1971) and "oxidative glutamate toxicity" or "oxytosis", glutamate-induced cell death mediated by inhibition of the cystine/glutamate antiporter

and consequent oxidative stress (Schubert and Piasecki, 2001) have been reported.

The accumulation of aggregated/misfolded proteins is a major cause of neuronal dysfunction and death in neurodegenerative disorders including Alzheimer's disease (tau and amyloid β), Parkinson's disease (a-synuclein), Huntington's disease (huntingtin), prion diseases (prion protein), and ALS (superoxide dismutase 1, TDP-43, FUS) (Fricker *et al.*, 2018).

Other causes of neuronal cell death are ROS and RNS, reactive molecules derived from oxygen and reactive molecules derived from NO respectively. ROS and RNS have been implicated in neuronal death in various ways and in almost all types of neuropathology and neurodegenerative diseases (Cobb and Cole, 2015).

Microglia, the macrophages of the brain, are able to orchestrate the immunological response by interacting with infiltrating immune cells. They are essential in neuronal survival as they produce neurotrophic factors and phagocytose dead cells, cellular debris, protein aggregates and pathogens. In the healthy brain, microglia are in a state of "rest", immobile with long, motile processes but if they detect inflammatory stimuli, they become activated, motile and express iNOS and cytokines (Wolf, Boddeke and Kettenmann, 2017). Activated microglia can kill neurons with release of TNF-a, glutamate, cathepsin B and/or reactive oxygen and nitrogen species (RONS) which can induce apoptotic, excitotoxic and necrotic death of the surrounding neurons (Block, Zecca and Hong, 2007; Brown and Vilalta, 2015; Kim, Kim and Yenari, 2015).

Lastly, axon death and cell death can be induced by axotomy which leads to different death mechanisms in different neuronal compartments. A widely studied and well understood mechanism is Wallerian degeneration which is described in the following section.

### 1.3.1 Wallerian Degeneration, a deathly pathway

As previously described, neurons acquire at least two self-destruction programmes. They have an intracellular death programme for apoptosis when they are injured, infected or no longer needed. Moreover, they seem to have a molecularly distinct self-destruct programme in their axon. These independent and different death pathways that somata and axons develop can be activated, more than one of which, in the same cell (Raff, Whitmore and Finn, 2002). In 1850, Augustus Waller first described the degeneration sequence of separated nerve fibres from their cell bodies. This pathological process in which axon degeneration is triggered by axonal injury at a specific site and time and simultaneously affects all axons is called Wallerian degeneration (Waller, 1851). This process begins with rapid axonal disintegration of axoplasm and axolemma, breakdown of myelin sheath and activation of microglia followed by clearance of the tissue debris by Schwann cells and invading macrophages (Buss *et al.*, 2004). In the peripheral nervous system, Wallerian degeneration is characterised by three distinct phases. Firstly, minutes after the transection, proximal and distal to the injury site short axonal stumps are affected by acute degeneration. This process is defined as acute axonal degeneration (AAD) (Kerschensteiner *et al.*, 2005). This is followed by a latent phase where no morphological changes take place and the axons remain electrically excitable. This lag between injury and degeneration varies amongst different species from 20 to 48 hours in young rats (Miledi and Slater, 1970) and mice (Beirowski *et al.*, 2005) to several days in primates including humans (Gilliatt and Hjorth, 1972; Chaudhry and Cornblath, 1992). The latent phase precedes a rapid disintegration phase characterised by the development of axonal swellings and followed by

fragmentation and the presence of ovoids of degenerating myelin (Conforti, Gilley and Coleman, 2014).

Interestingly, the Wallerian mechanisms is not limited to injury. Uninjured, wild-type axons degenerate in a Wallerian like manner after NGF withdrawal, exposure to vincristine, blockage of axonal transport, inflammation, ischemia and several other stresses. A common denominator of many of these stresses in the impaired delivery of nicotinamide mononucleotide adenylyl transferase 2 (NMNAT2) to distal axons (Conforti, Gilley and Coleman, 2014).

### 1.3.2 The WLD$^S$ mouse

After decades of study, Wallerian Degeneration was thought to be a passive degenerative process, the inevitable consequence of axons separating and losing support from their cell bodies. In mammals within hours to days of axonal injuries, explosive degeneration of the axonal cytoskeleton in the distal nerve stump takes place. Examples of prolonged survival of truncated axons had been found only in invertebrates and poikilotherms (Bittner, 1991). This belief came to change after the serendipitous discovery of a spontaneous dominant mutation in mice whose transected axons remain intact ten times longer than normal (Lunn *et al.*, 1989). The mouse was later named Wallerian Degeneration Slow (WLD$^S$). PNS or CNS axons of the Wld$^s$ mouse survive for up to 4 weeks and support action potential and the bidirectional redistribution of proteins for at least 2 weeks (Lunn *et al.*, 1989; Glass and Griffin, 1994). Wld$^s$ derived SCG and DRG primary neuronal cultures show protection for more than 72 hours when wild-type axons survive for 6-16 hours Synaptic terminals are preserved in the Wld$^s$ mice but after axotomy,(Beirowski *et al.*, 2005; Conforti *et al.*, 2009) synapse withdrawal is faster than the degeneration of the axon shaft especially in older animals (Adalbert *et al.*, 2005; Gillingwater *et al.*, 2006). The Wld$^s$ mouse does not show clear abnormality

apart from a nerve degeneration defect and reduced plasticity following injury that is secondary to the delayed degeneration (Bisby and Chen, 1990; Collyer *et al.*, 2014). Evidence show that the Wld$^{s}$ abnormality is intrinsic to neuronal axons rather than monocyte or glia cells (Crawford *et al.*, 1995). The precise contribution of glial cells in Wallerian Degeneration is still under investigation.

### 1.3.3 The WLD$^{S}$ gene and protein

Studies showed that the slow Wallerian Degeneration phenotype is caused by the over-expression of the WLD$^{S}$ fusion protein. The Wld$^{s}$ chimeric gene is mapped to mouse chromosome 4 where a tandem triplication of an 85-kb genomic region has been identified. The resulting WLD$^{S}$ protein contains the full length nicotinamide mononucleotide adenylyl transferase 1 (NMNAT1) at the C-terminal end, fused with 70 N-terminal amino acids of the N-terminus portion of ubiquitin ligase E4B (Conforti *et al.*, 2000). The two domains of the WLD$^{S}$ protein are linked by an 18 amino acid sequence (the epitope of the specific Eld18 antibody) which was originally part of NMNAT1 5' untranslated region (UTR) (Samsam *et al.*, 2003; Coleman and Freeman, 2010). It has been shown that the protective phenotype is dose-dependent in axons as well as in synaptic terminals (Adalbert *et al.*, 2005). It is interesting that the protective effects of NMNAT/WLD$^{S}$ are maintained even in axons empty of mitochondria (Kitay *et al.*, 2013). Transgenic expression of the WLD$^{S}$ gives protection to mice, rats, zebrafish, Drosophila and primary human neuronal cultures (Lunn *et al.*, 1989; MacDonald *et al.*, 2006; Martin *et al.*, 2010; Kitay *et al.*, 2013). The presence of NMNAT1 gives WLD$^{S}$ intrinsic NMNAT enzymatic activity (Mack *et al.*, 2001). On the other hand, the fusion protein contains only 6% of the UBE4B sequence and the catalytic U-box sequence is excluded.

Consequently, $WLD^S$ is unlikely to have ubiquitin ligase activity (Coleman and Freeman, 2010; Conforti, Gilley and Coleman, 2014).

## 1.4 NAD metabolism and axon degeneration

### 1.4.1 NMNATs and $NAD^+$ metabolism

The importance of $NAD^+$ metabolism for axonal survival emerged from the discovery of $WLD^S$ which contains NMNAT1, an enzyme that catalyses $NAD^+$ synthesis from ATP and NMN (Magni *et al.*, 2004a). Apart from its action as a co-enzyme in redox reactions $NAD^+$ and its metabolites have key signalling roles. ADP ribose (ADPR), cyclic ADPR and nicotinic acid adenine dinucleotide phosphate (NAADP) act as key second messengers in $Ca^{2+}$ signalling (Koch-Nolte *et al.*, 2009). ADP also contributes to post-translational protein modifications such as mono or poly-ADP ribosylation, which is catalysed by mono (ADP ribose) transferases (ARTs) and poly (ADP ribose) polymerases (PARPs), and deacetylation which is catalysed by sirtuins.

NMNAT enzymes can be found in all organisms. They catalyse the final step of NAD biosynthesis in *de novo* and salvage pathways. There are three salvage pathways each of which uses a different substrate: Nicotinic Acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR) which are all members of the vitamin $B_3$ family and are found in plenty of foods such as eggs, fish, meat, dairy, vegetables, wheat and corn (Bieganowski and Brenner, 2004; Bogan and Brenner, 2008; de Figueiredo *et al.*, 2011).

In the NA pathway, also known as Preiss and Handler pathway (Preiss and Handler, 1958), NA is converted to NaMN which is then catalysed by NMNATs

to produce nicotinic acid adenine dinucleotide (NaAD). In the next step NAD synthetase converts NaAD to $NAD^+$. In the second salvage pathway, NAM is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyl transferase (NAMPT). Following that, NMN is converted to $NAD^+$ by NMNATs. In the de novo pathway or kynurenine pathway, the essential aromatic amino acid tryptophan is converted to NaMN and then to $NAD^+$ through the Preiss-Handler pathway (Magni *et al.*, 2004a; Di Stefano and Conforti, 2013). Tryptophan though, is inadequate to fulfil $NAD^+$ needs on its own and emphasizes the importance of the salvage pathways (de Figueiredo *et al.*, 2011).

Interestingly, Di Stefano et al. found that blocking NAMPT proved to be axon-protective despite lowering $NAD^+$ levels. They showed that after axon injury, NMN accumulates and promotes degeneration. They also found that NMN promotes Wallerian-like degeneration without physical injury. (Di Stefano *et al.*, 2015)

Concerning mammals, $NAD^+$ is hydrolysed by several enzymes such as mono-ADP-ribosyl transferases (MARTs), poly-ADP-ribose polymerases (PARPs), sirtuins (SIRTs), and ADP-ribosyl cyclases producing NAM (Rongvaux *et al.*, 2003; Hassa *et al.*, 2006; Bogan and Brenner, 2008; Di Stefano and Conforti, 2013; Mori *et al.*, 2014; Imai, 2016).

$NAD^+$ takes part in a plethora of intracellular processes. It is a cofactor in redox reactions and plays an important role in glycolytic and mitochondrial ATP synthesis. It also has a function in the regulation of post-translational protein modifications and $Ca^{2+}$ signalling (Belenky, Bogan and Brenner, 2007; Chiarugi *et al.*, 2012; Di Stefano and Conforti, 2013). Moreover, it has been shown that the decline in systemic $NAD^+$ biosynthesis can be a trigger for pathophysiological changes that contribute to aging. NAMPT-mediated

$NAD^+$ biosynthesis deteriorates with age in multiple organs and tissues, such as pancreas, adipose tissue, skeletal muscle, liver, and brain through reduction of the activities of SIRT1 and other sirtuins in those organs and tissues (Imai, 2016).

### 1.4.2 NMNAT

#### 1.4.2.1 The NMNAT2 protein

The necessity of the enzymatically active NMNAT1 for $WLD^S$ neuroprotective function shed light to the link between NMNATs/NAD biosynthetic pathway and axon degeneration. Mammals have three NMNAT isoforms all of which have the same enzymatic activity but are distributed in different cellular compartments. NMNAT1, which is present in $WLD^S$, is located in the nucleus (Magni *et al.*, 2004b). NMNAT2 can be located in the cytoplasm and the axons associated with Golgi-derived vesicles while NMNAT3 is found in mitochondria (Berger *et al.*, 2005). In Drosophila there has been only one NMNAT gene which produces dNMNAT that is widely distributed in the cell (Zhai *et al.*, 2006).

Amongst the three isoforms, only the depletion of NMNAT2 by a siRNA induces degeneration in uninjured SCG axons in vitro. $WLD^S$ protects the neuron in this experimental condition, while endogenous NMNAT1 and NMNAT3 fail to do so. Inhibiting the protein synthesis leads to quick depletion of NMNAT2, which suggests a fast turnover rate and consequent axonal degeneration (Gilley and Coleman, 2010). Under physiological conditions NMNAT2 is mainly synthesized in the soma and reaches the axons through fast axonal transport in association with Golgi-derived vesicles (Milde, Gilley and Coleman, 2013). NMNAT2 mRNA has been detected in axons (personal

communication with Dr. Sotelo-Silveira) and in human tibial nerve (Ray *et al.*, 2018) Injury disrupts the delivery of soma-synthesised NMNAT2 which explains the observed depletion. $WLD^S$ is significantly more stable than NMNAT2 and is hence not quickly depleted in transected axons (Gilley and Coleman, 2010). Yet, inhibition of protein synthesis in the somata using compartmentalised cultures leads to distal axon degeneration.

NMNAT2 is a remarkably labile protein with a particularly short half-life (<1 hour) and its turnover is mediated by the ubiquitin-proteasome system (Milde, Gilley and Coleman, 2013). NMNAT2 overexpression has been found to be protective in SCG neurons in vitro (Gilley and Coleman, 2010).

#### 1.4.2.2 Nmnat2 is a survival factor for the axon

Axotomy has been found to lead to depletion of several axonal proteins but NMNAT2 appears to be critical and sufficient to induce degeneration. NMNAT2's role in axonal health seems to extend beyond regulation of Wallerian Degeneration. Indeed, studies reveal an essential role of NMNAT2 in axon growth and maintenance (Hicks *et al.*, 2012; Gilley *et al.*, 2013). Knocking out NMNAT2 leads to serious defects in perinatal lethality and peripheral innervation in mice. Primary SCG and DRG neuronal cultures of NMNAT2 null mice are not able to develop full length axons which reflects deficiency of axon elongation and not dying-back degeneration, axons gradually degenerating from the synaptic towards the cell body (Gilley *et al.*, 2013). NMNAT2, as mentioned before, is transported through attachment on Golgi-derived vesicles. Notably, disruption of its palmitoylation-dependent attachment to vesicles results in expanded NMNAT2 cytoplasmic distribution and consequently increased protection, both *in vitro* and *in vivo*. These variants, which

show increased stability, can reach the axons and protect them (Milde *et al.*, 2013; Milde, Gilley and Coleman, 2013; Milde and Coleman, 2014).

There is though controversy regarding the mechanism of NMNAT-mediated neuroprotection. The prevailing hypothesis is that NMNAT overexpression provides continuous enzyme activity in injured neurons and thus prevents the consequent decrease in $NAD^+$ and the accumulation of the precursor NMN (Gerdts *et al.*, 2016). Recent studies though, revealed that $NAD^+$ depletion following axon injury is due to massive depletion of $NAD^+$ by SARM1 (sterile alpha and TIR motif-containing 1). The overexpression of cytoplasmic Nmnat1, rather than altering $NAD^+$ synthesis, blocks SARM1-dependent $NAD^+$ , (Sasaki *et al.*, 2016). So, NMNAT-mediated axon protection relies on its ability to block SARM1 signaling, but this does not rely on enzymatic conversion of NMN to $NAD^+$.

Interestingly, Di Stefano et al. showed that NMN accumulates after nerve injury promoting axon degeneration. Despite lowering $NAD^+$, inhibitors of NAMPT are neuroprotective in injured axons and synapses but exogenous NMN abolishes this protection. This suggests that NMN accumulation in axons after NMNAT2 degradation might promote degeneration. In conclusion, they proposed that the mechanism by which NMNAT2 and $Wld^S$ protein confer axon survival is by the limitation of NMN accumulation (Di Stefano *et al.*, 2015).

On a more recent work, it was shown that NMN deamidase, a bacterial enzyme, can also delay axon degeneration in zebrafish larvae and transgenic mice. Similarly to overexpressed NMNATs, NMN deamidase decreases NMN accumulation in injured mouse sciatic nerves and preserves some axons up to three weeks, even when it is expressed at a low level. Moreover, NMN deamidase rescues axon outgrowth and perinatal lethality in a dose-dependent way in NMNAT2-depleted mice. These findings further support a pro-degenerative effect of accumulating NMN in axons in vivo (Di Stefano et al.,

2017). Loreto et al showed that *in vitro* and *in vivo* axotomy leads to an increase of intra-axonal $Ca^{2+}$ which can be suppressed by genetic or pharmacological reduction of NMN levels. NMN requires SARM1 to induce $Ca^{2+}$ influx and axon degeneration. Although inhibition of NMN synthesis and SARM1 deletion block rise of $Ca^{2+}$ and preserve axonal integrity, they do not prevent early mitochondrial dynamic changes. Their work indicates that NMN and SARM1 act in a common pathway climaxing in intra-axonal $Ca^{2+}$ increase and fragmentation and that mitochondrial dysfunctions are not causative in NMN-induced degeneration (Loreto *et al.*, 2015).
An alternative is that NMNAT provides neuroprotection via an enzyme-independent function, which leads to the hypothesis that NMNAT mediated protection is chaperone-dependent (Zhai *et al.*, 2008; Brazill *et al.*, 2017).

#### 1.4.2.3 NMNAT2 and the brain

NMNAT2 is highly expressed in the mammalian brain, and NMNAT2 mRNA levels have been found reduced in Parkinson's Disease (PD), Huntington's Disease (HD), Alzheimer Disease (AD) and tauopathy patients (Ali *et al.*, 2016). The NMNAT2 mRNA levels show a positive correlation with cognitive function and a negative one with pathological features of AD (Ali *et al.*, 2016). In AD brains NMNAT2 mRNA and protein levels are reduced and NMNAT2 co-localises with aggregated tau proteins (Ali *et al.*, 2016). Overexpressing NMNAT2 leads to the reduction of the pathological accumulation of hyperphosphorylated tau without altering total tau levels (Ljungberg *et al.*, 2012; Ali *et al.*, 2016). NMNAT2 has been shown to prevent protein denaturation and to promote protein refolding with similar activity to Hsp70. Interestingly, the enzymatically-dead NMNAT2 mutants which lack NAD synthetic activity, also reduce hyperphosphorylated tau levels. Furthermore, deletion

of NMNAT2 increases the susceptibility of cortical neurons to proteotoxic stress (Ali *et al.*, 2016).

Single nucleotide polymorphisms within the NMNAT2 gene genomically associate it with human cognition (Davies *et al.*, 2018). A positive correlation between NMNAT2 mRNA levels and cognition has also been identified (Ali *et al.*, 2016). Reduced mRNA levels have also been observed in Parkinson's, Alzheimer's and Huntington's disease patients (Ali *et al.*, 2013, 2016) and *Nmnat2* mRNA levels are down-regulated prior to neurodegeneration in a mouse model of human tauopathy (Ljungberg *et al.*, 2012). These findings suggest that a low or decreasing expression of NMNAT2, a key regulator of axon health, may be conducive to clinical and subclinical phenotypes.

Crucially, NMNAT2 mRNA levels appear to vary widely between individuals in the human population (Ali *et al.*, 2016), so a better understanding of how reduced (but not absent) NMNAT2 expression influences axon survival, either constitutively or in response to neurodegenerative stresses, is needed.

Interestingly, the neuroprotective action of NMNATs expands beyond their NAD synthase enzymatic activity they act as molecular chaperones (Ali *et al.*, 2013). Early studies demonstrating the chaperone function of NMNATs noticed that loss of *Drosophila* NMNAT (dNMNAT) caused severe and rapid neurodegeneration, which could be rescued by enzymatically inactive dNMNAT (Zhai *et al.*, 2006). Moreover, dNMNAT overexpression in *Drosophila* turned out to be protective against spinocerebellar ataxia 1 (SCA1)-induced neurodegeneration, a disorder characterised by the aggregation of misfolded proteins (Zhai *et al.*, 2008). These findings unveiled the molecular chaperone function of dNMNAT independent of the NAD synthase function (Zhai *et al.*, 2006, 2008).

In vertebrates, a recent study described a molecular chaperone role for NMNAT2 in regulating protein homeostasis in neurodegeneration-related scenarios. The study showed that overexpression of NMNAT2 in the brain reduced pathological signs of neurodegeneration in rTg4510 mice, a transgenic model of frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), which expresses the human protein Tau with the missense mutation P301L (Ljungberg *et al.*, 2012). These results support the theory that NMNAT2 might be acting as a molecular chaperone protecting against neurodegeneration by, still unknown mechanisms (Ljungberg *et al.*, 2012).

## 1.5 Aim of the Thesis

As previously stated, only NMNAT2 out of the three NMNAT isoforms in mammals, seems to have the biggest influence over axon survival under physiological conditions as solely depletion of this isoform causes a primary axonal phenotype either *in vitro* or *in vivo*.

Although, NMNAT2 protein has been studied extensively in the peripheral nervous system, we still need to elucidate its role and function in central nervous system.

In this work we decided to study NMNAT2 protein's effect on cortical neurons as well as its putative regulation by microRNAs. In order to do so, we set three objectives:

Firstly, the investigation of the role of NMNAT2 overexpression in mouse primary cortical neurons in axonal growth and degeneration.

Secondly, the identification of miRNAs with the potential to target NMNAT2 using bioinformatic methods and relevant existing literature.

Lastly, the investigation of the effect of overexpression of these microRNAs on axonal development and degeneration.

# Chapter 2: Materials and Methods

## 2.1 Cell cultures

### 2.1.1. Animals and primary cortical cultures

All animal studies conformed to the institution's ethical requirements in accordance with the 1986 Animals (Scientific Procedures) Act.
C57/BL6 mouse embryos in E16.5 developmental stage were culled and their brains were removed. The brain cortices were dissected and the meninges separated under a stereoscopic microscope. The acquired tissue was incubated in Hanks Balanced Salt Solution (HBSS, $Ca^{2+}$ and $Mg^{2+}$ free; Gibco) with 1mg/ml trypsin and 5mg/ml DNAseI (both Sigma) at 37°C/5% $CO_2$ for 30 min. After treatment with 0.05% (v/v) trypsin inhibitor (Life Technologies), the tissue was washed in Neurobasal media (Gibco) and 5mg/ml DNAseI was added before mechanical dissociation of the tissue. The cells were finally washed in Neurobasal media and spun down at 250x g for 5 min. Dissociated cells were then resuspended and plated in Neurobasal media supplemented with 1x GlutaMax and 2% (v/v) B-27 (Gibco), according to experimental requirements. The procedure was conducted in a class II cabinet apart from the dissection, which was performed under the stereoscopic microscope.

#### 2.1.1.1 Dissociated cortical cultures

The dissociated cortical cells were resuspended in supplemented Neurobasal media to a final density of 10 x $10^6$ cells/ml. The cortical cell suspension was diluted down and plated in 6-well plates (Corning) containing 22x22mm glass coverslips (Menzel Glaser) at a final plating density of 1.75 x $10^5$

cells/cm$^2$ and incubated at 37°C, 5% $CO^2$. Prior to plating, glass coverslips were coated with 50µg/ml poly-L-ornithine (PLO; Sigma-Aldrich) overnight and washed twice with sterile distilled water. Neurons were allowed to grow for 24 hours before being transfected.

#### 2.1.1.2 Functional assays of axonal growth

For these experiments, cortical primary cultures in 6-well configuration (1.75x105 cells/cm2) were transfected 24h after plating using: 5µL/well of Lipofectamine 2000 (LF 2000; Life Technologies) and 250µl/well of Opti-MEM reduced serum media (Life Technologies), in accordance with the manufacturer's instructions. In short, LF 2000 was incubated with 125µl/well of Opti-MEM for 15 min and, in parallel, pmaxGFP plasmid (Lonza) and miRNA mimics or plasmid constructs were incubated with 125µl/well of Opti-MEM. The reaction mixes were then mixed together and incubated for 30 minutes at RT to allow nucleic acid-loaded liposome formation before they were finally loaded to the cell wells. In all functional assays of axonal growth cells were transfected for 72 hours prior to analysis.

##### 2.1.1.2.1 Transfection with miRNAs

To test the effect of overexpression of mmu-miR-494-3p, mmu-miR-132-3p, mmu-miR-129-1-3p and mmu-miR-181a-5p on axonal length, primary cortical cultures were transfected with miRCURY LNA microRNA mimics of the aforementioned microRNAs and microRNA mimic Control (Qiagen). These are short oligonucleotides with a Locked Nucleic Acid (LNA). Briefly, LNAs are modified RNA nucleotides in which the 2'-O and 4'-C atoms of the ribose are joined through a methylene bridge. This additional bridge limits their flexibility, essentially locking the structure into a rigid conformation. They are also

known as 'inaccessible RNA' (Koshkin et al., 1998). Cells were co-transfected with 1 µg of pmaxGFP (Lonza and each microRNA mimic at a final concentration of 20 nM. MicroRNAs were all tested at the concentration of 20 nM.

#### 2.1.1.2.2 Transfection with NMNATs

To test the effect of overexpression of NMNAT2, enzymatically-dead NMNAT2 and NMNAT1 on axonal length, primary cortical cultures were co-transfected with 1 µg pmaxGFP and 1 µg of empty pCMV-TAG-2B vector or pCMV-TAG-2B-NMNAT2, pCMV-TAG-2B-NMANT2 (enzymatically-dead) and pCMV-TAG-2B-NMNAT1 respectively. The plasmids were a gift from Professor Coleman, University of Cambridge.

### 2.1.2. Mammalian cell lines

Neuro2a cells were maintained and grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Penicillin-Streptomycin (both Sigma) in a humidified incubator at 37°C, 5% $CO_2$. Before each experiment, cells were detached with 1x trypsin-EDTA (Sigma) and washed with supplemented DMEM.

#### 2.1.2.1 Transfection of Neuro2A cells with microRNAs

Neuro2A cells have significantly higher transfection efficiency compared to primary cortical neurons and this makes them an ideal model to test the effects of miRNA mimics in NMNAT2 mRNA levels, which would otherwise be

extremely challenging. Neuro2A cell cultures were seeded at a density of $1x10^5$ cells per well in 12-well plates (Greiner) and transfected after 24h with miR-129, miR-181 mimics and mimic negative control (to final concentration of 50 nM), 1.5 µL of Lipofectamine 2000 and 200 µL of Opti-MEM, following the procedure described above for transfection of cortical neurons. All experiments were conducted in duplicates.

Total RNA was extracted 48h and 72h for quantification of NMNAT2 and GAPDH mRNA levels by RT-qPCR as described on the next chapter.

## 2.2 Microscopy and Image analysis

Cortical neurons cultured on coverslips were fixed in 4% (w/v) paraformaldehyde (3.6% sucrose (w/v), 1x PBS, 5mM $MgCl_2$, pH 7.4; ThermoFisher) for 25min at Room Temperature, washed with 1x PBS three times and mounted with Vectashiled Hardset mounting media with Dapi (vector Labs). In each independent experiment, axons of approximately 50 GFP-positive neurons were measured per condition. The axon was defined as the neurite being at least 3 times the length of any other neurite projecting from the cell(Dajas-Bailador *et al.*, 2012) and was measured using Fiji software (Schindelin *et al.*, 2012). Intact axons of healthy neurons were traced from the soma to the tip of the longest projection as shown in Figure 6. Axon length measured on DIV5 as later on, it becomes extremely difficult to measure axonal length of individual neurons as axons from several neurons cross each other giving a rather unclear image under the microscope. Data were collected from 3 to 6 individual experiments, normalised to the average of the control and expressed as percentage of control, mean±s.e.m.

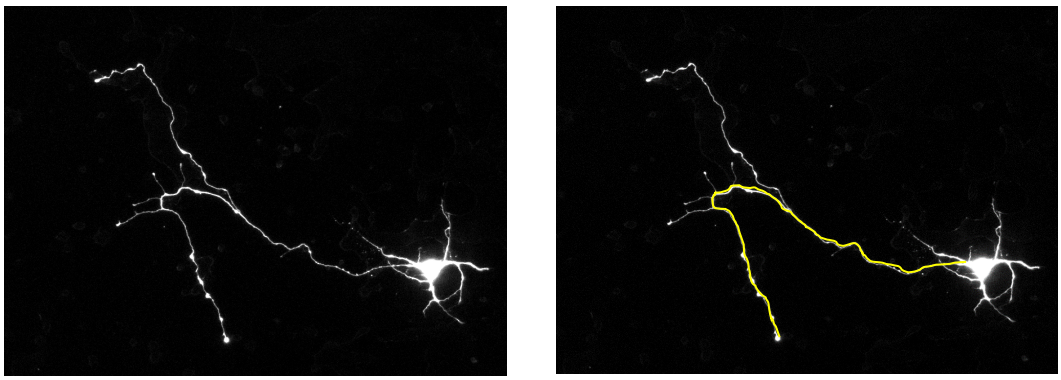


**Figure 6: Measurement of axonal projection on ImageJ**

## 2.3 Molecular Analysis

### 2.3.1 RNA extraction

Total RNA was isolated from Neuro2A cultures at 48 and 72h after transfection by the phenol-chloroform extraction method using TRIzol Reagent (Invitrogen) for extraction of total RNA, according to manufacturer's instructions. In brief, culture media were removed and the cells were washed with PBS (ThermoFisher), the cells were then treated with 250μl of TRIzol (per well), were homogenized, scrapped off and put into LoBind tubes (Eppendorf) were a mix ed with 1/5 volume of chloroform (ThermoFisher) followed. Afterwards, centrifugation was performed at 12000xg/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 at 12000xg/4°C, for 30 min (ThermoFisher). RNA pellet was left to dry until it appeared like a clear jell and then was resuspended in Hyclone nuclease-free water (GE Healthcare). All steps were conducted in an RNAse-free

environment, using nuclease-free tubes and reagents, filtered pipette tips and, where adequate, on ice to further control the RNAse-dependant degradation of the samples.

### 2.3.2 Quantification of NMNAT2 mRNA levels by real time qPCR

Reverse transcription for cDNA synthesis was performed using 100 ng of total RNA extracted from N2A cells using SuperScript IV reverse transcriptase and Oligo(dT)$_{20}$ primers (both Invitrogen), in accordance with the manufacturer's instructions. PCR amplification was performed using PowerUp SYBR Green (Applied Biosystems) following manufacturer's instructions, using 1.5µL of template cDNA or nuclease free water (non-template control) per amplification reaction.

| RT reaction | 20 µL |
|---|---|
| Oligo d(T)20 primer | 1 µL |
| 10 mM dNTP mix | 1 µL |
| RNA (20 ng/µL) | 5 µL |
| Hyclone water | 6 µL |
| Primer Annealing | 65°C, 5 min |
| 5x Buffer | 4 µL |
| DTT (100 mM) | 1 µL |
| RNaseOUT Rnase Inhibitor | 1 µL |
| Superscript IV | 1 µL |
| RT reaction | 50°C, 10min |
| Heat Inactivation | 80°C, 10min |

| PCR amplification | |
|---|---|
| Initial denaturation | 98°C, 30s |
| Amplification | 35 cycles |
| | 98°C, 10s |
| | 60°C, 30s |
| | 72°C, 30s |
| Extension | 72°C, 2min |

**Table 1: PCR reaction and amplification program of NMNAT2 cDNA**

As for miRNA RT-qPCR, amplification reactions were set up in duplicate in a 96-well configuration and run on a StepOne Plus Real-Time PCR instrument, with melting curve analysis being conducted in standard instrument conditions, showing single melt curves for all primer pairs. Primer sequences targeting *Nmnat2* transcript were selected from (Cheng *et al.*, 2013) with the following sequences Forward: 5'-GTGATTGGCG GGATTGTCTCT-3' and Reverse: 5'-TCTTCATCAGGTC TCGAT GGT-3' (IDT). As a reference gene we used GADPH with the following primers Forward: 5'-CTGCACCAC-CAACTGCTTAG-3' and Reverse: 5'-ACAG CTTCTGGGTGGCAGT-3'.

### 2.3.3 Real-Time qPCR analysis

The threshold was set at the same level for all targets and Ct (Cycle Threshold) values were acquired using StepOne Software v2.3 (Applied Biosystems). Data was analysed using the comparative $2^{-\Delta\Delta Ct}$ method for calculation of the relative expression of each target gene based on the formula:

Fold difference = $2^{-\Delta\Delta C_t}$

where

$\Delta C_{t\ sample} - \Delta C_{t\ calibrator} = \Delta\Delta C_t$

$C_{t\ NMNAT2}{}^{S} - C_{t\ GADPH}{}^{S} = \Delta C_{t\ sample}$

$C_{t\ NMNAT2}{}^{C} - C_{t\ GADPH}{}^{C} = \Delta C_{t\ calibrator}$

For studying *Nmnat2* expression in N2A cells transfected with microRNA mimics, NMNAT2 levels were normalised to the levels of GAPDH which is a commonly used as reference gene as its $\Delta Ct<1.5$ amongst all samples within each experiment.

## 2.4 Statistical analysis

Data are expressed as Mean ± SEM. Statistical analysis was performed using Shapiro-Wilk test to evaluate normality and one-way ANOVA with Dunnett's post-hoc analysis or One sample t-test, where applicable, with P-values less than 0.05 being considered as significant for any set of data. Stars represent statistical significance with p= * <0.05, ** <0.01, *** <0.001, ****<0.0001. Data were analysed using GraphPad Prism version 7.0 and Microsoft Excel (Microsoft Office Professional Plus 2019).

# Chapter 3: Results

## 3.1 Nmnat2 and Mouse Primary Cortical Neurons

### 3.1.1 Introduction

Several studies have shown that the NMNAT2 transcript is enriched in the brain in comparison to other tissues (Raffaelli *et al.*, 2002; Yalowitz *et al.*, 2004) though it has also been detected in muscle and heart (Yalowitz *et al.*, 2004). NMNAT2 mRNA levels seem to vary broadly among individuals in human population (Ali *et al.*, 2016), hence the need for a deeper understanding of how *Nmnat2 levels can be controlled and how they can* influence axon survival and function both constitutively and in response to neurodegenerative stress. Overexpression of Nmnat1, -2, or -3 in neurons has been demonstrated to slow the rate of axon degeneration in vivo and in vitro(Sasaki, Araki and Milbrandt, 2006; Conforti *et al.*, 2009; Wen *et al.*, 2011; Kitaoka *et al.*, 2013; Sasaki *et al.*, 2016) especially after axon injury in PNS neurons.

NMNAT2 function has been broadly studied in the peripheral nervous system but a deeper understanding of its role in the central nervous system is still lacking. In PNS neurons, NMNAT2 overexpression has been shown to be protective against axon degeneration and by extension neurodegeneration. However, Mayer et all found that overexpression of exogenous Nmnat2 was toxic to primary cortical neurons and led to pervasive cell death. More specifically, they infected primary cortical neurons with lentivirus expressing either Nmnat2-FLAG or Nmnat2 C164S/C165S-FLAG. 4 days after infection when the cultures were fixed and probed with an anti-FLAG antibody, they observed that Nmnat2, but not Nmnat2 C164S/C165S, was toxic to neurons. Their experiments focused on the Expression, Localization, and Biochemical

Characterization of *Nmnat2* and they did not test other NMNATs (Mayer et al., 2010).

Overall these studies demonstrate the need for further studies of the role of NMNAT2 in CNS neurons, as results reported might indicate the need for a tight regulation of NMNAT2 levels in neuronal models in general.

### 3.1.2 Overexpression of NMNATs in cortical neurons

As a first approach to test the effect of controlling NMNAT2 levels in cortical neurons, we decided to overexpress the protein in primary cortical neurons by transfecting with a NMNAT2 plasmid. For this experiment, we co-transfected cortical neurons with pmaxGFP as a reporter and pCMV-NMNAT2 at final concentration of 1 µg per well.

The overexpression of NMNAT2 resulted in neurons with axons significantly shorter than the control by 28.58% (P value=0.008). This is an interesting result considering that up to date, a plethora of studies have mainly indicated the protective role of Nmnat2 in neurons of the PNS but had not focused on axonal growth. As NMNAT2 function in the PNS is associated with its cytoplasmic/axonal function, we decided to test whether the effect on axonal growth is indeed limited to its cytoplasmic *properties*. To test this, we took advantage of the capacity to overexpress NMNAT1, which shares enzymatic activity with NMNAT2 but has a nuclear localization. Unlike its cytoplasmic isoform, co-transfecting cortical neurons with GPF and pCMV-NMNAT1 had no effect on the length of developing axons. After finding that only NMNAT2 has an effect on neurons we tested whether the enzymatic activity of thecytoplasmic *Nmnat2* was responsible for the observed phenotype. To study that, we transfected the cortical neurons with an enzymatically-dead NMNAT2 which resulted in neurons with significantly shorter 17.7% axons (P value= 0.0227), which falls in between the NMNAT2 effect and the control

and may suggest the existence of a dual function mechanism. A one sample t-test between NMNAT2 and enzymatically-dead NMNAT2 transfected neurons' axonal growth does not indicate significant difference (P=0.0767).

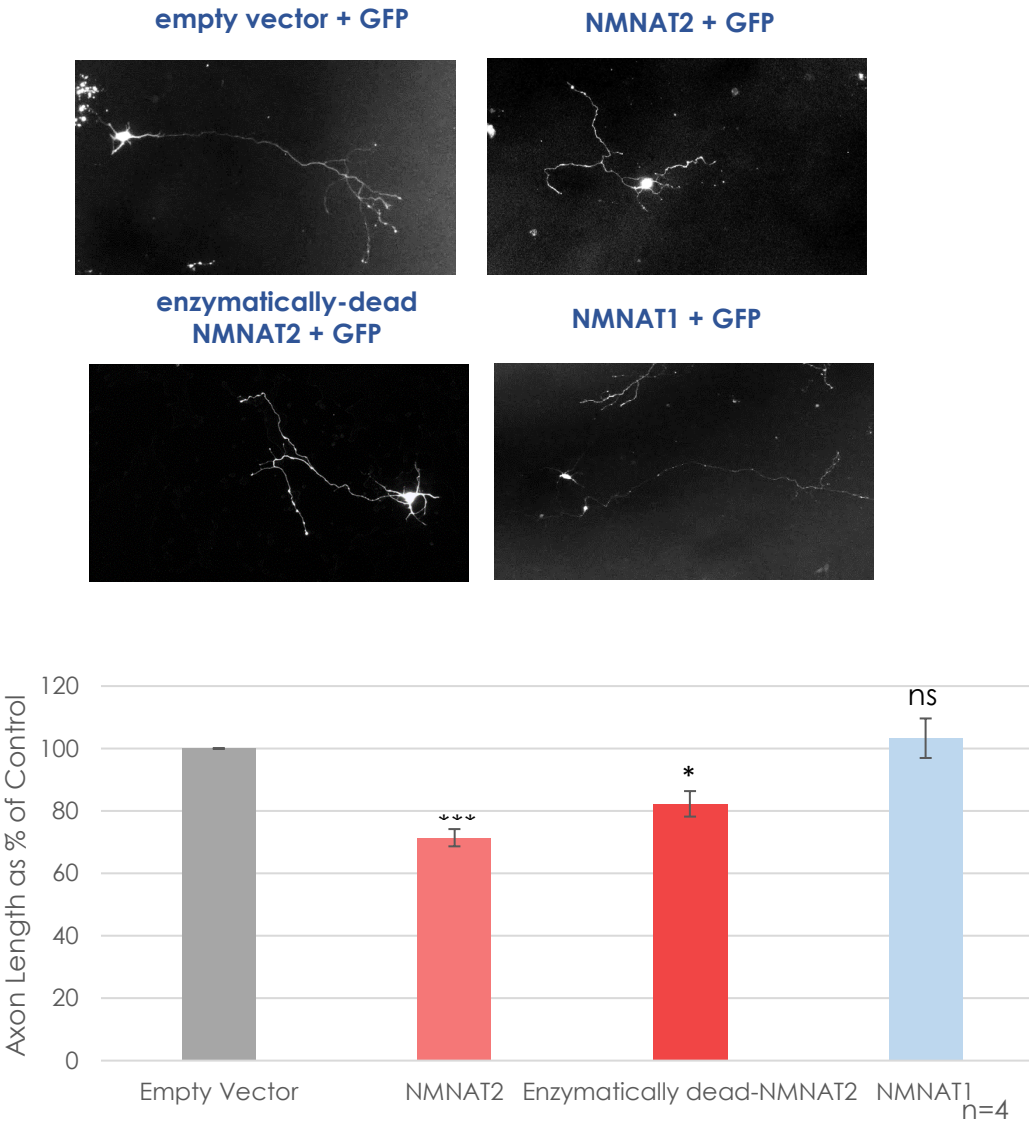
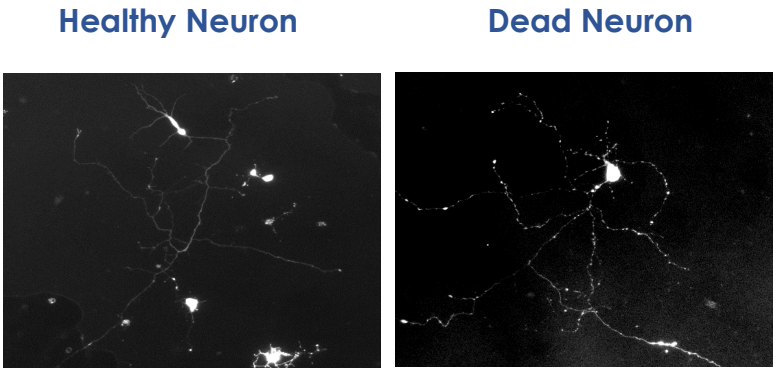



**Figure 7 Overexpression of NMNATs in cortical neurons.** Developing Cortical neurons were co-transfected with 1µg of pmaxGFP and 1µg of pCMV-NMNAT2 or pCMV-enzymatically-dead-NMNAT2 or pCMV-NMNAT1 (DIV2). The length of axons of GFP-positive neurons measured on DIV5 was 71.4%~2,764, 82.3%~4,087 and 103%~6,334 of the control (pCMV empty vector) respectively. Data are expressed as percentage of the average length of control axons and presented as mean±SEM from four independent experiments. One-way ANOVA was performed, P= 0,0003. Representative images of GFP-positive neurons for all conditions are shown above.

Since the loss of axonal growth could be the outcome of a decrease in cell viability, we decided to test neuronal death. In order to measure neuronal mortality, we focused on axon degeneration. As degenerated, we considered axons that had axonal swellings, appeared bead-like in part or all of their length. We counted alive and dead cells in all conditions and found no



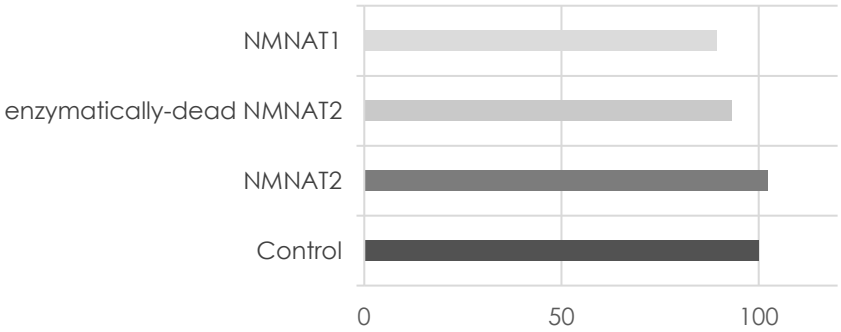




**Figure 8: None of the NMNATs proved to be toxic when overexpressed in cortical neurons.** On the pictures above we can see from left to right, healthy neuron with an intact axon representative of those measured and a dead neuron with a degenerating axon. The graph represents the percentage of neurons with degenerating axons as a percentage of the control.

significance between the control and the experimental conditions. None of the overexpressed plasmid constructs had an effect on cellular mortality as it is shown on figure 8. In all experiments presented in this section, as control

we used the empty pCMV-TAG-2B vector which had no effect on axonal length and cell viability.

### 3.1.3 Conclusion

Due to its essential neuronal maintenance role of NMNAT2 and its neuroprotective properties, understanding the regulation of NMNAT2 levels is crucial to unlocking its therapeutic potential (Brazill *et al.*, 2017). It is critical than neurons maintain sufficient levels of NMNAT2 as its loss is an initiating factor in Wallerian degeneration and has been shown to be consistent in progression of neurodegenerative diseases (Milde, Gilley and Coleman, 2013).

Our findings that overexpression of NMNAT can lead to a decrease in axonal growth, can point towards a novel functional mechanism regulated by NMNAT2 levels during the development of neuron connectivity in the CNS. Interestingly, this functional role appears to depend on both enzymatic and non-enzymatic properties of NMNAT2, an intriguing possibility that has been found to be important in other models (Brazill *et al.*, no date; Zhai *et al.*, 2008; Ali *et al.*, 2013)

Overall, findings reported here can be interpreted in the context of those described for NMNAT2 in the PNS, and underline the importance for neurons to finetune the levels of NMNAT2 to the cell's needs as the nervous system needs to adapt to many conditions and circumstances. MicroRNAs are powerful regulators and thus an interesting field to focus on.

## 3.2 microRNAs and Neuronal Development

Given *Nmnat2* short half-life and the presence of NMNAT2 mRNA in axons that may require local translation and thus repression on its trip to the axon

terminal, it is somewhat surprising that there have not been studies investigating the regulation of NMNAT2 by microRNAs.

### 3.2.1 Bioinformatic Analysis

#### 3.2.1.1 microRNAs targeting NMNAT2

After discovering the effect of *Nmnat2* on developing neurons we decided to focus on the protein's regulation by miRNAs. For that matter we performed a bioinformatic and bibliographic analysis to identify those miRNAs that could target NMNAT mRNA.

The target prediction tool Targetscan mouse (Agarwal *et al.*, 2015) was used in order to identify the putative miRNAs targeting the NMNAT2 transcript, producing a large list of miRNA families. In brief, TargetScan is a tool which predicts biological targets of miRNAs by searching for conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA (Lewis, Burge and Bartel, 2005) In mammals, predictions are ranked by the predicted efficacy of targeting which is calculated using cumulative weighted context++ scores (CS) of the sites (Agarwal *et al.*, 2015). Moreover, predictions can also be ranked based on their probability of conserved targeting ($P_{CT}$) (Friedman *et al.*, 2009).

We focused on the miRNA families that were conserved firstly among vertebrates and secondly among mammals as shown in figure 9. Targetscan predicted a total of 692 microRNA families targeting NMNAT2 twelve of which were broadly conserved and specifically five among vertebrates and six among mammals (Table 3).

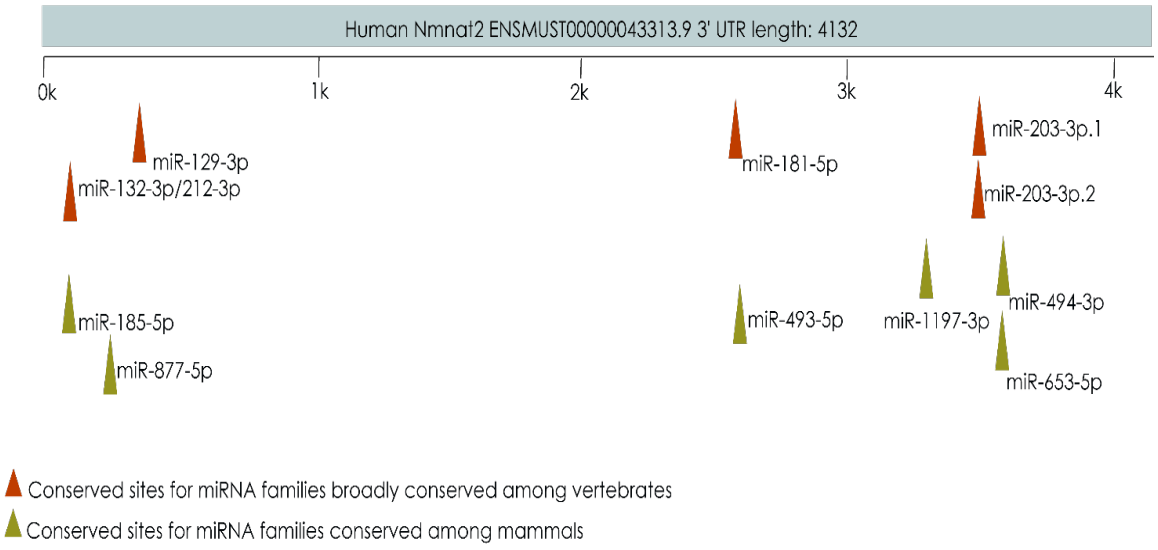



**Figure 9: 3'UTR of NMNAT2 and conserved sites for miRNA families.** 3'UTR length is 4132 kb. In the figure there are depicted the broadly conserved sites for miRNA families among vertebrates in brown and among mammals in green along the 3'UTR of the gene.

To increase probabilities of identifying biologically significant miRNAs, we curated manually the list of miRNA families shown on the table below, focusing on expression in the nervous system and function on neuronal growth, development and/or degeneration. That way, our initial list was narrowed down to four microRNAs: mmu-miR-494-3p, mmu -miR-132-3p, mmu-miR-129-3p and mmu -miR-181a-5p shown in bold on table 2 below.

| miRNA conserved sites | Position in the 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-7035-5p | 324-331 | 8mer |
| **miR-129-1-3p** | 346-353 | 8mer |
| miR-129-2-3p | 346-353 | 8mer |
| miR-181c-5p | 2591-2598 | 8mer |
| **miR-181a-5p** | 2591-2598 | 8mer |
| miR-181b-5p | 2591-2598 | 8mer |
| miR-181d-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 |

**Table 2: miRNA conserved sites binding on NMNAT2.** We can distinguish 12 miRNA families binding on 11 different sites.

#### 3.2.1.2 Functional profile of the microRNAs

##### 3.2.1.2.1 miR-129-3p family and the nervous system

Mir-129 has been shown to have the capacity to control function in axonal regeneration by regulating insulin-growth factor-1 in peripheral injury with its overexpression impairs outgrowth ability in DRG neurons. (Zhu *et al.*, 2018). Both miR-129-3p and miR-129-5p have been found to be under-expressed in

pilocytic astrocytoma in comparison with healthy tissue (Ho *et al.*, 2013) microRNA-129-1 has been proposed to act as a negative regulator of Insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3) and Mitogen Activated Protein Kinase 3 MAPK1 and also as a cell cycle arrest inducer in glioblastoma multiforme cells (GBM) cells . miR-129-3P has also been shown to downregulate CDK6 by potentially targeting its 3'-UTR (Ouyang *et al.*, 2016).

#### 3.2.1.2.2 miR-181 family and the nervous system

MiR-181 is highly expressed in the brain (Chen *et al.*, 2004) and has been implicated in apoptosis and inflammation, the regulation of lymphocyte development and function (Ouyang, Lu, Yue and Giffard, 2012; Ouyang, Lu, Yue, Xu, *et al.*, 2012; Hutchison *et al.*, 2013; Xu *et al.*, 2013; Zhang *et al.*, 2013) and particularly, miR-181c has been shown to suppress the activation of $CD4^+$ T cells (Xue *et al.*, 2011) . MiR-181-a/b/c is downregulated in the rat brain after transient focal ischemia (Jeyaseelan, Lim and Armugam, 2008).

miR-181-1 has been predicted a potential regulator in Parkinson's disease mechanisms due to its interaction with known PD genes (Chandrasekaran and Bonchev, 2013). It has been demonstrated that members of the miR-181 family are involved in the regulation of microglial activation (Ye *et al.*, 2018). miR-181a, which is enriched in the synaptodendritic compartment of the nucleus accumbens, has been proved to post-transcriptionally regulate the glutamate receptor 2 subunit (GluA2) of AMPA-Rs. AMPA-Rs dynamics are crucially involved in synaptic plasticity and neuroadaptations to drugs of abuse and therefore miR-181a may pay play a role in addiction related neuroplastic changes and their therapeutic approach (Saba *et al.*, 2012). miR181a and miR181b were found to be involved in alteration of synaptic

plasticity associated with neuropathology in a murine model of AD with finding indicating a connection between miR-181 and proteins involve in synaptic plasticity and memory processing (Rodriguez-Ortiz et al., 2014). In a recent study showed that mir-181a interacts with ataxin's 3' UTR and whose expression is dysregulated in human Machado-Joseph disease (MJD) neurons and in other MJD cell and animal models. Moreover, overexpression of miR-181ain mice resulted in reduction of mutATXN3 levels, aggregate counts, and neuronal dysfunction (Carmona *et al.*, 2017).

#### 3.2.1.3.3 microRNA-132 and the nervous system

miR-132 is one of the best characterised miRNAs in the nervous system, and is known for its functions in dendrite morphology and synapse function (Vo, Cambronne and Goodman, 2010; Siegel, Saba and Schratt, 2011; Wanet *et al.*, 2012). Two of the miR-132 targets that have been studied extensively in dendrites are the mRNAs for Rho GTPase activator p250GAP, and transcription factor MeCP2. miR-132 is present in the somatodendritic compartment and at synapses (Kye *et al.*, 2007; Lugli *et al.*, 2008). MiR-132 is noticeably dysregulated in major neurodevelopmental, psychiatric, and neurodegenerative diseases such as Alzheimer's Disease, Schizophrenia and Autism Spectrum Disorder (Mellios and Sur, 2012; Pichler *et al.*, 2017).

In AD brains, miR132-3p levels are decreased in neurons enriched with hyper-phosphorylated Tau (Lau *et al.*, 2013). Various genes involved in Tau network, including the transcription factor (TF) FOXO1a, were identified as miR132-3p targets by in silico methods (Lau *et al.*, 2013). Knocking out the

miR132/212 cluster results in rise of Tau phosphorylation and aggregation. In an AD mouse model, miR132 treatment rescued memory deficits (Wanet *et al.*, 2012).

In new-born hippocampal neurons miR-132 plays an important role by influencing dendritic branching and synaptic integration (Magill *et al.*, 2010; Luikart *et al.*, 2011). Growth factors such as brain-derived neurotrophic factor (BDNF) are known to interfere with miRNA expression and function. For example, BDNF can induce the expression of miR-132 and co-transcribed miR-212 (Remenyi *et al.*, 2010). Hippocampal miR-132 mediates stress-induced cognitive deficits by suppressing acetylcholinesterase (Haramati *et al.*, 2011). miR-132 has been implicated in neuropathic pain after chronic constriction injury (CCI) (Arai *et al.*, 2013). On a similar note, spinal cord miR-132 has been proposed as a mediator of neuropathic pain following spared nerve injury (SNI) (Zhang *et al.*, 2015).

Overexpression of the miR132 precursor in primary cortical neurons increases neurite sprouting in comparison to cells transfected with pmaxGFP alone (Vo *et al.*, 2005). It has also been shown that neurons secrete miR-132-containing exosomes to regulate brain vascular integrity, a finding that contributes to the role of miR-132 of miR-132 in neural development and function (Xu *et al.*, 2017). In vivo inhibition of miR-132 function in visual cortex resulted in increased levels of spine inhibitor p250GAP and reduced dendritic spine density and immature morphology (Mellios *et al.*, 2011). On the other hand, in vivo upregulation of miR-132 expression promoted spine maturation (Tognini *et al.*, 2011). Both of these manipulations affected ocular dominance plasticity and thus suggest that balanced levels of miR-132 are required for brain plasticity, and that too high or too low levels of miR-132 expression can result in precocious or delayed cortical maturation. Multiple studies have shown that miR-132 expression is induced in other brain regions

in an experience-dependent manner (Nudelman *et al.*, 2009). miR-132 represses Rasa1 mRNA (a Ras GTPase activator), in a mechanism that operates locally within the axon. miR-132 levels in DRGs peaked in the period of maximum axon growth in vivo which suggests it has a role as a developmental timer. (Hancock *et al.*, 2014).

#### 3.2.1.2.4 microRNA-494 and the nervous system

Mir-494, a broadly conserved family among mammals microRNA, is not particularly studied in the nervous system yet. Xiong et al. showed that miR-494, which is abundantly present in substantia nigra pars compacta (SNpc), targets DJ-1. Gain of miR-494 function led to decreased DJ-1 protein levels and caused increased oxidative stress as well as loss of dopaminergic (DA) neurons in both in vitro and in vivo studies, thus confirming miR-mediated regulation of DJ-1 in PD pathogenesis (Xiong *et al.*, 2014). MiR-494-3p has been found to be a modulator of the progression of *in vitro* and *in vivo* PD models by targeting sirtuin 3 (SRT3). It has been shown to be upregulated in macaques suffering from bovine spongiform encephalopathies-infected macaques compared to non-infected animals (Saba *et al.*, 2008).

A recent study showed that mir-494 interacts with ataxin's 3' UTR, whose expression is dysregulated in human Machado-Joseph disease (MJD) neurons and in other MJD cell and animal models and that the overexpression of miR-494in mice resulted in reduction of mutATXN3 levels, aggregate counts, and neuronal dysfunction (Carmona et al., 2017). MiR-494-3p is a negative regulator of semaphorin 3A (SEMA3A) and other targets associated with axonal maintenance. Recently, in C9ORF72-ALS iAstrocytes EV and miRNA cargo

were found to be dysregulated which affects neurite network maintenance. In particular a downregulation of miR-494-3p was identified and it was shown that by restoring miR-494-3p levels through expression of an engineered miRNA mimic Sema3A levels in motor neurons (MN) can be downregulated resulting in an increase of MN survival in vitro (Varcianna et al., 2019).

### 3.2.2 Conclusion

Combining the bioinformatic tools and manual curation allowed us to select four microRNAs targeting NMNAT2 with a potential role in the nervous system and effect on *Nmnat2* levels. Given that *Nmnat2* is a critical protein for neuronal survival and maintenance, we proceeded to investigate the role of these four microRNAs in axon growth,

## 3.3 Analysis of selected microRNAs and their role in axon development

### 3.3.1 Functional Analysis of microRNAs in cortical neurons

After the identification of the four miRNAs by bioinformatic tools, manual curation on NCBI and extensive bibliographic review, we proceeded to test and analyse their functional effect on primary cortical neurons.

### 3.3.1.1 miR-129-3p

Following the prediction of miR-129-3p as conserved amongst vertebrates microRNA that targets NMNAT2 we decided to test its putative role in neuronal development and axonal outgrowth in vitro. Consequently, in order to assess the effect of increasing miR-129-1-3p levels in developing axons, cortical neurons were co-transfected with pmaxGFP and miR-129-1-3p mimic or a non-targeting oligonucleotide control (mimic negative control) at DIV2 and axon length was measured on DIV5. As it is shown in figure 10, overexpression of miR-129-1-3p leads to axonal shortening of 21% compared to control. Both the control mimic and the experimental mimic were

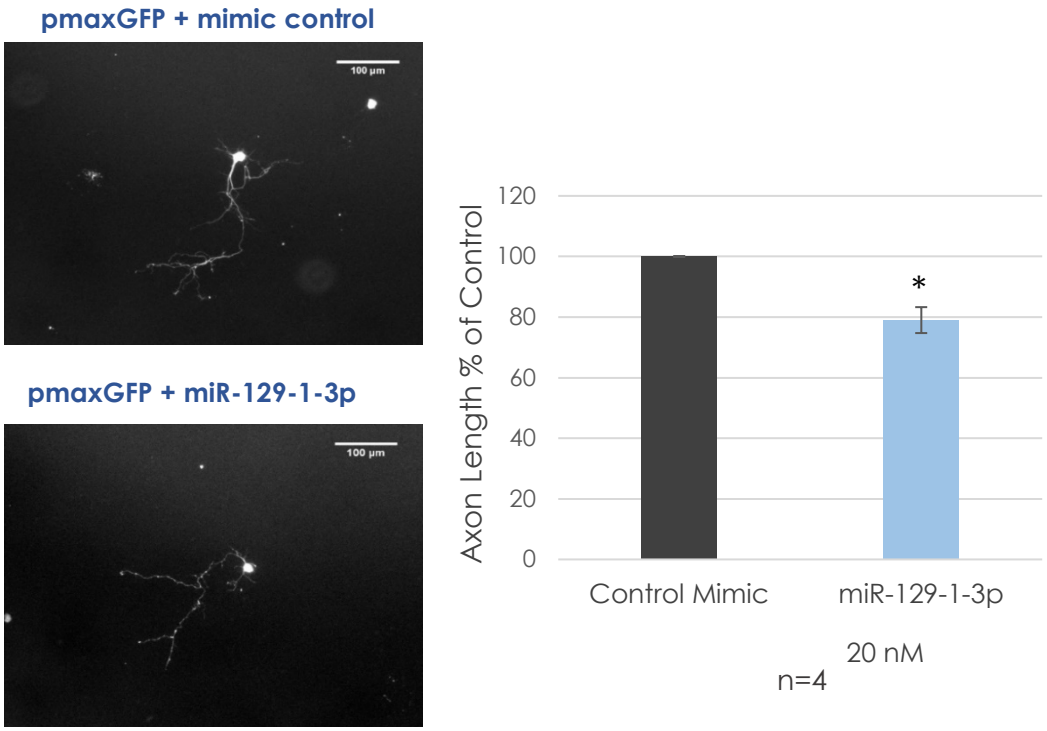



**Figure 10. miR-129-1-3p induces decrease of axonal length in cortical neurons.** Developing Cortical neurons were transfected with miR-129-1-3p mimic (DIV2), the length of axons of GFP-positive neurons measured on DIV5 was 79%~4,28 the control (both at 20mM). Neurons were co-transfected with GFP for visualisation of transfection. Control neurons were transfected with non-targeting oligonucleotides. Data are expressed as percentage of the average length of control axons and presented as mean±SEM from five independent experiments. One sample t-test. P= 0,0162. Representative images of GFP-positive neurons for both conditions are shown above.

transfected at 20 nM final concentration. We followed the one sample t-test statistical analysis and our findings have a p-value of 0,0162<0.05 which dictates that our results are statistically significant.

#### 3.3.1.2 microRNA-181

Similarly, after predicting miR-181-a-5p as conserved among vertebrates microRNA that targets NMNAT2 we decided to test its putative role in neuronal development and axonal outgrowth in vitro. In order to assess the effect of increasing miR-181-a-5p levels in developing axons, cortical neurons were co-transfected with pmaxGFP and the miR-181-a-5p mimic or a non-targeting oligonucleotide control (mimic negative control) both at final concentration 20 nM at DIV2 and axon length was measured on DIV5. As it is shown in figure 9. overexpression of miR-181-a-5p has no significant effect on the outgrowth of cortical neurons. The average axon length of the experimental condition was by 15.78% shorter but the decrease in axonal length was not statistically significant. We performed a one sample t-test and got a P value of 0.0902>0.05.

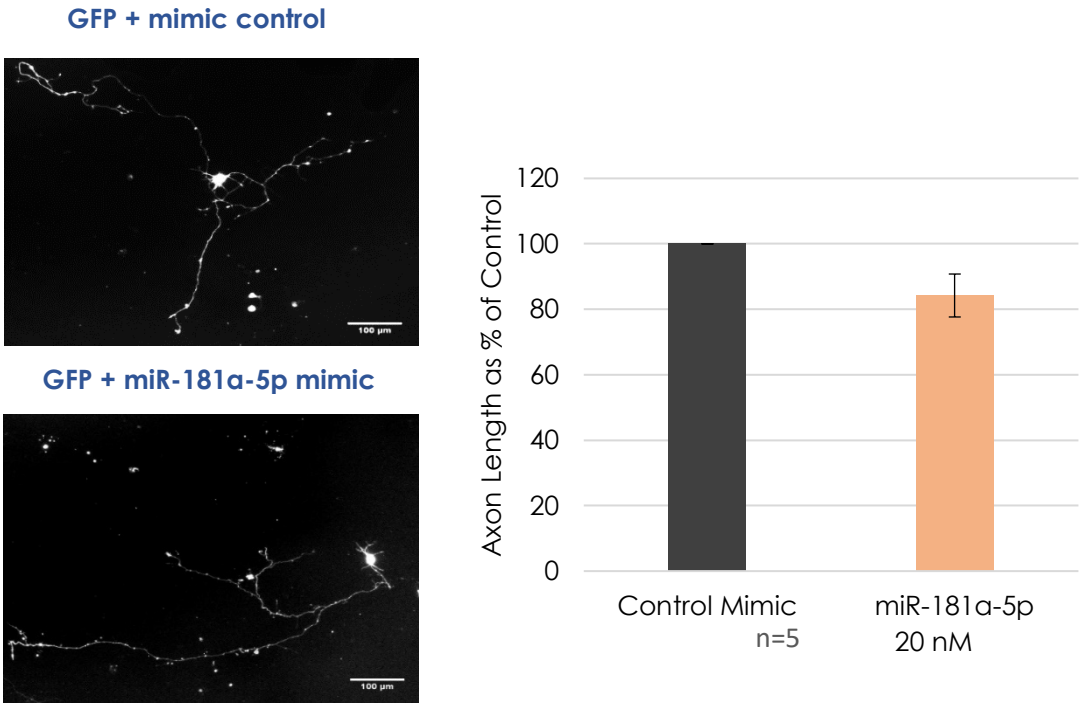



**FIGURE 9: miR-181a-5p does not have an effect on axonal length of cortical neurons**. Developing Cortical neurons were transfected with miR-181a-5p mimic (DIV2), the length of axons of GFP-positive neurons measured on DIV5 was 84.22%~6,562 of the control (both at 20mM). Neurons were co-transfected with GFP for visualisation of transfection. Control neurons were transfected with non-targeting oligonucleotides. Data are expressed as percentage of the average length of control axons and presented as mean±SEM from five independent experiments. One sample t-test. P=0,0902. Representative images of GFP-positive neurons for both conditions are shown above.

### 3.3.1.3 microRNA-132-3p

Mir-132-3p was likewise predicted as a conserved amongst vertebrates' miRNA targeting NMNAT2. To test its effect when overexpressed in cortical neurons primary cortical cultures were co-transfected with pmaxGFP and the miR-132-3p mimic

or a non-targeting oligonucleotide control (mimic negative control) both at final concentration 20 nM at DIV2 and axon length was measured on DIV5. As it is depicted in figure 10 overexpression of miR-132-3p has no significant effect on axonal outgrowth. The average axon length of the experimental condition was by 5.26% shorter. We performed a one sample t-test and got a P value of 0.2542>0.05.

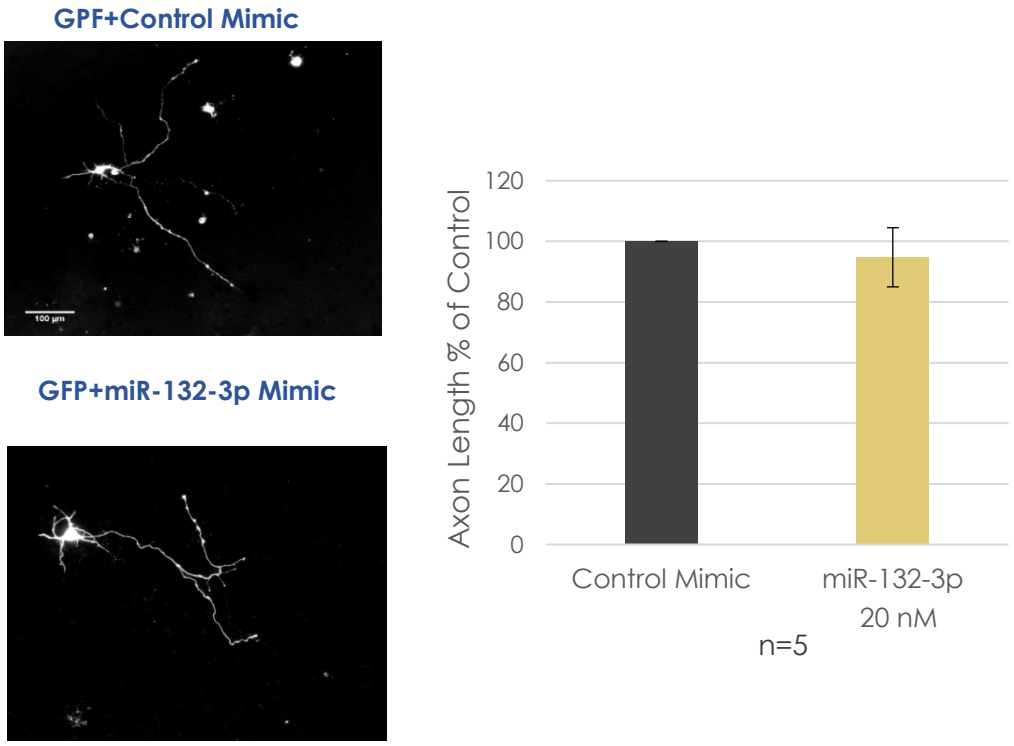



**Figure 10: miR-132-3p has no significant effect in axonal length of cortical neurons.** Developing Cortical neurons were transfected with miR-132-3p mimic (DIV2), the length of axons of GFP-positive neurons measured on DIV5 was 88.21%~9,762 of the control (both at 20mM). Neurons were co-transfected with GFP for visualisation of transfection. Control neurons were transfected with non-targeting oligonucleotides. Data are expressed as percentage of the average length of control axons and presented as mean±SEM from five independent experiments. One sample t-test. P=0.2542. Representative images of GFP-positive neurons for both conditions are shown above.

### 3.3.1.4 miR-494-3p

Mir-494-3p was predicted as conserved amongst mammalian miRNA targeting NMNAT2. To test its effect when overexpressed in cortical neurons primary cortical cultures were co-transfected with pmaxGFP and the miR-494-3p mimic or a non-targeting oligonucleotide control (mimic negative control) both at final concentration 20 nM at DIV2 and axon length was measured as on DIV5. As it is depicted in figure 11 overexpression of miR-494-3p has no significant effect on axonal outgrowth. The average axon length of the experimental condition was by 5.24% shorter but the results were not significant. We performed a one sample t-test and got a P value of 0.6141>0.05.

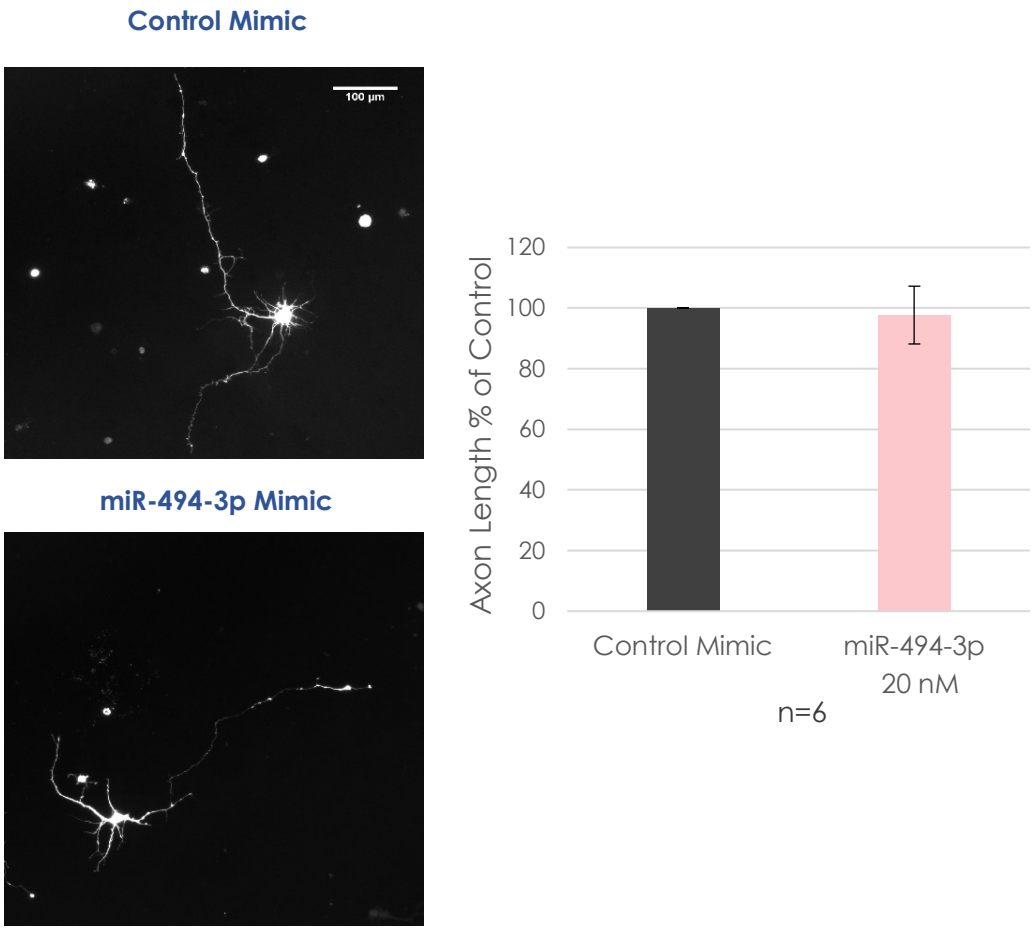


**FIGURE 11 miR-494-3p has no significant effect in axonal length of cortical neurons.** Developing Cortical neurons were transfected with miR-494-3p mimic (DIV2), the length of axons of GFP-positive neurons measured on DIV5 was 97.70%~9,678 of the control (both at 20mM). Neurons were co-transfected with GFP for visualisation of transfection. Control neurons were transfected with non-targeting oligonucleotides. Data are expressed as percentage of the average length of control axons and presented as mean±SEM from five independent experiments. One sample t-test. P=0,8188. Representative images of GFP-positive neurons for both conditions are shown above.

#### 3.3.1.5 Conclusion

Our results show that most of the miRNAs we tested had no effect on axonal growth. However, overexpression of miR-129-1-3p resulted in significantly shorter axons and thus suggests that regulation by this miRNA may have an effect on axon development. Future work could include functional rescues with NMNAT2 overexpression to test whether the miRNA has indeed an effect that is mediated by NMNAT2. However, the effect may not be mediated by the targeting of NMNAT2 only or exclusively. In order to investigate this further we decided to test the expression of endogenous NMNAT2 mRNA levels in cells transfected with two of the microRNAs, miR-129-1-3p (which phenotypically had an effect) and miR-181a-5p.

### 3.3.2 Expression of NMNAT2 in N2A cells after overexpression of miRNAs

Following the functional analysis of the four miRNAs, where miR-129-1-3p showed to have a shortening effect in developing axons when overexpressed, we decided to assess the NMNAT2 transcript levels in cortical neurons transfected with miR-129-1-3p and miR-181a-5p which appeared to have no effect on developing neurons. In order to do that, we used Neuro2A

cells as they have significantly higher transfection efficiency compared to primary cortical neurons, making them an ideal model to test the effects of miRNA mimics in NMNAT2 mRNA levels, which would otherwise be extremely challenging. In order to identify these microRNAs effect on NMNAT2 mRNA levels we quantified the NMNAT2 transcript's levels in N2A cells where either miR-129-1-3p or miR-181a-5p had been overexpressed.

The cells were transfected with the mimic control or the miRNA mimics at 50nM final concentration,24h after plating and the NMNAT2 mRNA levels were measured by Real Time qPCR 48h and 72h after transfection. Relative expression at each time point was calculated following the comparative ΔΔCt method as shown in the diagrams below. Regarding miR-129-1-3p transfected cells, fold change was measured 0.406~0,194 after 24 hours, 1,131~0,136 after 48 hours and 0,746 after 72 hours. In miR-181a-5p transfected cells, fold change was measured 1,130~0,351 after 48 hours and 0,900 after 72 hours. The results suggest that the expression levels begin to alter 3 days after transfection as we begin to see minor differences compared to the control. More experiments need to be conducted regarding that at the 72h time point, as only one experiment was performed per condition (hence the relatively high variability) and because the 48h time point proved to be rather early for any safe conclusions. The error bar on the miR-181a-5p on the 72h time point reflects experimental repeats and not biological repeats.

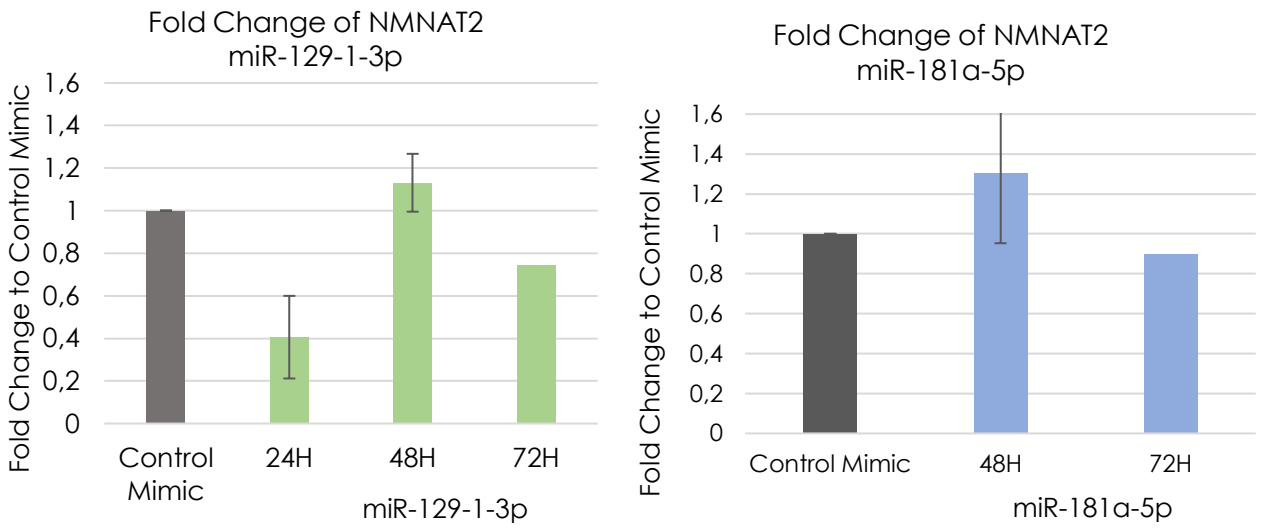



**Figure 12: Endogenous NMNAT2 after miRNAs overexpression (A)** *Nmnat2* transcript was quantified by RT-qPCR in N2A cultures 48 (n=3) and 72h(n=1) after transfection with miR-129-1-3p mimic (50nM). The graph represents *Nmnat2* expression levels relative to that of Control Mimic transfected neurons. **(B)** *Nmnat2* transcript was quantified by RT-qPCR in N2A cultures 48 (n=2) and 72h(n=1) after transfection with miR-181a-5p mimic (50nM). The graph represents *Nmnat2* expression levels relative to that of Control Mimic transfected neurons. Data is expressed as fold change to control from each time point, mean±SEM of n independent experiments (see details above), each read in duplicate.

## Conclusion

As we showed in the previous chapter, mir-129-1-3p overexpression in primary cortical neurons resulted in significantly shorter axons. qPCR results indicate that miR-129-1-3p, which showed the strongest effect among the two miRNAs we amplified, provide early confirmation that this miRNA may indeed target NMNAT2. More experiments an on later time points should be performed in order to acquire a clearer picture and to reach further conclusions.

# Chapter 4: Discussion

NMNAT2 catalyses the synthesis of NAD from NMN and ATP and is particularly important because its transcript is expressed predominately in the brain (Sood *et al.*, 2001; Raffaelli *et al.*, 2002; Yalowitz *et al.*, 2004). Axons require a constant supply of the labile axon survival factor Nmnat2 to avoid spontaneous axon degeneration while depletion of this isoform causes a primary axonal phenotype either *in vitro* or *in vivo* (Conforti *et al.*, 2011; Gilley *et al.*, 2013).

Supporting a protective role of Nmnat2 in axon survival, strong overexpression of Nmnat2 in PNS neurons, has been shown to delay Wallerian degeneration in vitro, and this protective effect is dependent on its enzymatic activity (Gilley and Coleman, 2010; Yan *et al.*, 2010). Nmnat2 role in central nervous system has been significantly less studied and for that reason we decided to investigate NMNAT2 overexpression in primary cortical cultures.

NMNAT2 mRNA has been detected in axons and given the protein's short half-life, it is reasonable to conclude that regulation must be tightly fine-tuned in order to control its levels. Part of our work attempted to shed light in Nmnat2 regulation by microRNAs in cortical neurons, which surprisingly had not been investigated yet. For that matter, we conducted a bioinformatic and bibliographic research and identified four strong miRNA candidates that putatively target NMNAT2 transcripts and repress their expression.

Overexpression of NMNAT2 in primary cortical neurons, led to significantly shorter axons. Up to date data have shown that the overexpression of NMNAT2 in PNS neurons has protective role but its effect on axonal length had not been investigated. We proceeded to overexpress the nuclear isoform of the protein, NMNAT1 in primary cortical cultures which had no effect

on axonal length. overexpression of the enzymatically-dead *NMNAT2* had a shortening effect on axons but not as robust as that of wild type NMNAT2. These findings indicate that the effect of Nmnat2 on axon growth may not rely exclusively on NMNAT2 enzyme activity. As mentioned in the introduction, Nmnat2 apart from its enzymatic activity it also acts as a chaperone. Indeed, an alternative theory suggests that NMNAT provide protection via an enzyme-independent function, leading to the hypothesis that NMNAT mediated neuroprotection is chaperone-dependent. For example, it has been shown that Nmnat2's enzyme activity is dispensable for relieving the toxic phosphorylated tau burden in a model of fronto-temporal dementia and parkinsonism 17 (FTDP-17), but Nmnat2 forms a complex with HSP90 chaperone, possibly to promote refolding of toxic tau (Ali et al., 2016).

In any case, the study of Nmnat2 regulation is of great importance as it is a protein associated with various neurodegenerative conditions. Naturally, we focused on Nmnat2 potential regulation by miRNAs which regulate gene expression by mRNA degradation or translational repression.

After the prediction of four putative regulators, miR-129-1-3p, miR-181a-5p, miR-132-3p and miR-494-3p through bioinformatic analysis and manual curation, we proceeded on functional studies on neuronal cultures. All four miRNAs were overexpressed in primary cortical neurons. Neurons transfected with miR-129-1-3p showed significant axonal shortening which is an indication that the microRNA may have a regulatory effect on Nmnat2.

Given that NMNAT2 when overexpressed cause decrease in axon growth the fact that we acquire similar results from the overexpression of miR-129-1-3p, a predicted to target NMNAT2 microRNA, appears contradictory. There are though some possible explanations behind this contradiction. There is a possibility that miR-129-1-3p does not target NMNAT2. Mir-129-3p potentially

targets other 437 genes according to Targetscan (Agarwal *et al.*, 2015) and thus, our phenotype may appear because of the miRNA's interaction of another or other genes.

Our contradictory result can also point at the fact that NMNAT2 levels, as we previously underlined, need to be tightly controlled and any change of its essential levels may lead to axonal impairments. This may explain why such a fundamental for axonal function protein has developed to be delivered to the axon and have such a short half-life. When a protein is so tightly regulated, any change in its levels, increase or decrease, would lead to harmful outcomes for the cell.

In order to investigate whether our phenotype was related with miRNA repression of Nmnat2, we measured NMNAT2 mRNA levels in Neuroblastoma2A cells, where the microRNAs had been overexpressed, by qPCR. qPCR results indicate that miR-129-1-3p targets NMNAT2 and represses its expression. The effect can be observed after 72h of transfection so more experiments on this and later time points could help us acquire a better view.

Future experiments of functional rescues with NMNAT2 overexpression could test whether the miRNA has indeed an effect on the specific protein and the phenotype is because of its repression exclusively or partially.

Regarding NMNAT2's chaperone activity, a big question that needs to be addressed is which are these proteins that NMNAT2 "chaperones" under physiological conditions. Genome editing approaches in mice could be tried to test NMNAT2's chaperone activity and to investigate if it is essential in the brain.

The regulation of NMNAT2 seems to be extremely fine-tuned due to its labile nature as a protein and it is possible that its regulators follow similarly precise

patterns. It would be informative to perform experiments of inhibition of the endogenous miRNAs and especially miR-129-1-3p which seems to have an effect on axon growth. Since Nmnat2 might be locally post-transcriptionally modified and/or translated the use of compartmentalised cultures in our studies of regulation, would provide more information regarding the mRNA and protein spatiotemporal distribution across development. Moreover, we could compare through qPCR, NMNAT2 mRNA from somata and axons of cortical to investigate whether the levels differentiate between the two sub-cellular compartments after transfection with miRNAs.

The levels of the protein per se in cortical neurons, could be measured by luciferase assay and that could be done also separately in somata and axons to check whether there is a difference in the expression. This is something we were interested in doing but unfortunately antibodies available for Nmnat2 are not entirely reliable (Coleman lab, personal communication).

MicroRNAs, depending on the specific context can have either protective or disease-promoting effects and their manipulation may offer novel therapeutic opportunities for neurodevelopmental disorders of complex genetic origins. Furthermore, the manipulation of NMNAT2 by miRNAs, which has been associated with cognition and major condition such as Parkinson's, Alzheimer's and Huntington's disease, can have important therapeutic value.

# References


Adalbert, R. *et al.* (2005) 'A rat model of slow Wallerian degeneration ( *Wld* $^{S}$ ) with improved preservation of neuromuscular synapses', *European Journal of Neuroscience*. Wiley/Blackwell (10.1111), 21(1), pp. 271–277. doi: 10.1111/j.1460-9568.2004.03833.x.

Agarwal, V. *et al.* (2015) 'Predicting effective microRNA target sites in mammalian mRNAs.', *eLife*. eLife Sciences Publications, Ltd, 4. doi: 10.7554/eLife.05005.

Airaksinen, M. S., Titievsky, A. and Saarma, M. (1999) 'GDNF Family Neurotrophic Factor Signaling: Four Masters, One Servant?', *Molecular and Cellular Neuroscience*. Academic Press, 13(5), pp. 313–325. doi: 10.1006/MCNE.1999.0754.

Ali, Y. O. *et al.* (2013) 'NMNATs, evolutionarily conserved neuronal maintenance factors', *Trends in Neurosciences*, 36(11), pp. 632–640. doi: 10.1016/j.tins.2013.07.002.

Ali, Y. O. *et al.* (2016) 'NMNAT2:HSP90 Complex Mediates Proteostasis in Proteinopathies', *PLOS Biology*. Edited by B. A. Barres, 14(6), p. e1002472. doi: 10.1371/journal.pbio.1002472.

Aloe, L. (2004) 'Rita Levi-Montalcini: the discovery of nerve growth factor and modern neurobiology', *Trends in Cell Biology*, 14(7), pp. 395–399. doi: 10.1016/j.tcb.2004.05.011.

Andreassi, C. *et al.* (2017) '3'UTR cleavage of transcripts localized in axons of sympathetic neurons'. bioRxiv. doi: 10.1101/170100.

Arai, M. *et al.* (2013) 'The miRNA and mRNA Changes in Rat Hippocampi after Chronic Constriction Injury', *Pain Medicine*, 14(5), pp. 720–729. doi: 10.1111/pme.12066.

Aschrafi, A. *et al.* (2008) 'MicroRNA-338 Regulates Local Cytochrome c Oxidase IV mRNA Levels and Oxidative Phosphorylation in the Axons of Sympathetic Neurons', *Journal of Neuroscience*, 28(47), pp. 12581–12590. doi: 10.1523/JNEUROSCI.3338-08.2008.

Aschrafi, A. *et al.* (2012) 'MicroRNA-338 regulates the axonal expression of multiple nuclear-encoded mitochondrial mRNAs encoding subunits of the oxidative phosphorylation machinery', *Cellular and Molecular Life Sciences*. SP Birkhäuser Verlag Basel, 69(23), pp. 4017–4027. doi: 10.1007/s00018-012-1064-8.

Babiarz, J. E. *et al.* (2008) 'Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs', *Genes & Development*, 22(20), pp. 2773–2785. doi: 10.1101/gad.1705308.

Baleriola, J. *et al.* (2014) 'Axonally Synthesized ATF4 Transmits a Neurodegenerative Signal across Brain Regions', *Cell*. Cell Press, 158(5), pp. 1159–1172. doi: 10.1016/J.CELL.2014.07.001.

Baloh, R. H. *et al.* (2000) 'The GDNF family ligands and receptors — implications for neural development', *Current Opinion in Neurobiology*. Elsevier Current Trends, 10(1), pp. 103–110. doi: 10.1016/S0959-4388(99)00048-3.

Bartel, D. P. (2009a) 'MicroRNAs: target recognition and regulatory functions.', *Cell*. NIH Public Access, 136(2), pp. 215–33. doi: 10.1016/j.cell.2009.01.002.

Bartel, D. P. (2009b) 'MicroRNAs: target recognition and regulatory functions.', *Cell*. NIH Public Access, 136(2), pp. 215–33. doi: 10.1016/j.cell.2009.01.002.

Batista, A. F. R. and Hengst, U. (2016) 'Intra-axonal protein synthesis in development and beyond', *International Journal of Developmental Neuroscience*. Pergamon, 55, pp. 140–149. doi: 10.1016/J.IJDEVNEU.2016.03.004.

Beard, J. (1890) 'IV. On the early development of Lepidosteus osseus . — Preliminary notice', *Proceedings of the Royal Society of London*, 46(280–285), pp. 108–118. doi: 10.1098/rspl.1889.0015.

Beard, J. (1892) 'The Transient Ganglion Cells and their Nerves in Raja batis.', *Anat Anz. 7:191–206*.

Beirowski, B. *et al.* (2005) 'The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves.', *BMC neuroscience*, 6(1), p. 6. doi: 10.1186/1471-2202-6-6.

Belenky, P., Bogan, K. L. and Brenner, C. (2007) 'NAD+ metabolism in health and disease.', *Trends in biochemical sciences*, 32(1), pp. 12–9. doi: 10.1016/j.tibs.2006.11.006.

Bellon, A. *et al.* (2017) 'miR-182 Regulates Slit2-Mediated Axon Guidance by Modulating the Local Translation of a Specific mRNA', *Cell Reports*. Cell Press, 18(5), pp. 1171–1186. doi: 10.1016/J.CELREP.2016.12.093.

Berezikov, E. (2011) 'Evolution of microRNA diversity and regulation in animals', *Nature Reviews Genetics*. Nature Publishing Group, 12(12), pp. 846–860. doi: 10.1038/nrg3079.

Berger, F. *et al.* (2005) 'Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms.', *The Journal of biological chemistry*, 280(43), pp. 36334–41. doi: 10.1074/jbc.M508660200.

Bieganowski, P. and Brenner, C. (2004) 'Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD+ in fungi and humans.', *Cell*, 117(4), pp. 495–502. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15137942 (Accessed: 27 November 2018).

Bisby, M. A. and Chen, S. (1990) 'Delayed wallerian degeneration in sciatic nerves of C57BL/Ola mice is associated with impaired regeneration of sensory axons.', *Brain research*, 530(1), pp. 117–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2271939 (Accessed: 1 November 2018).

Bittner, G. D. (1991) 'Long-term survival of anucleate axons and its implications for nerve regeneration.', *Trends in neurosciences*, 14(5), pp. 188–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1713720 (Accessed: 1 November 2018).

Blackstone, C., O'Kane, C. J. and Reid, E. (2011) 'Hereditary spastic paraplegias: membrane traffic and the motor pathway', *Nature Reviews Neuroscience*, 12(1), pp. 31–42. doi: 10.1038/nrn2946.

Block, M. L., Zecca, L. and Hong, J.-S. (2007) 'Microglia-mediated neurotoxicity: uncovering the molecular mechanisms', *Nature Reviews Neuroscience*. Nature Publishing Group, 8(1), pp. 57–69. doi: 10.1038/nrn2038.

Bogan, K. L. and Brenner, C. (2008) 'Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD+ precursor vitamins in human nutrition.', *Annual review of nutrition*, 28(1), pp. 115–30. doi: 10.1146/annurev.nutr.28.061807.155443.

Bouquet, C. *et al.* (2004) 'Microtubule-associated protein 1B controls directionality of growth

cone migration and axonal branching in regeneration of adult dorsal root ganglia neurons.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 24(32), pp. 7204–13. doi: 10.1523/JNEUROSCI.2254-04.2004.

Brazill, J. M. *et al.* (2017) 'NMNAT: It's an NAD+ synthase… It's a chaperone… It's a neuroprotector.', *Current opinion in genetics & development*. NIH Public Access, 44, pp. 156–162. doi: 10.1016/j.gde.2017.03.014.

Brazill, J. M. *et al.* (no date) 'NMNAT: It's an NAD + Synthase… It's a Chaperone… It's a Neuroprotector'. doi: 10.1016/j.gde.2017.03.014.

Brittis, P. A., Lu, Q. and Flanagan, J. G. (2002) 'Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target.', *Cell*, 110(2), pp. 223–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12150930 (Accessed: 7 February 2019).

Brose, K. and Tessier-Lavigne, M. (2000) 'Slit proteins: key regulators of axon guidance, axonal branching, and cell migration.', *Current opinion in neurobiology*, 10(1), pp. 95–102. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10679444 (Accessed: 30 January 2019).

Brown, G. C. and Vilalta, A. (2015) 'How microglia kill neurons', *Brain Research*. Elsevier, 1628, pp. 288–297. doi: 10.1016/J.BRAINRES.2015.08.031.

Buss, A. *et al.* (2004) 'Sequential loss of myelin proteins during Wallerian degeneration in the human spinal cord', *Brain*, 128(2), pp. 356–364. doi: 10.1093/brain/awh355.

Buss, R. R. *et al.* (2006) 'Neuromuscular Development in the Absence of Programmed Cell Death: Phenotypic Alteration of Motoneurons and Muscle', *Journal of Neuroscience*, 26(52), pp. 13413–13427. doi: 10.1523/JNEUROSCI.3528-06.2006.

CAI, X., Hagedorn, C. H. and Cullen, B. R. (2004) 'Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs', *RNA*, 10(12), pp. 1957–1966. doi: 10.1261/rna.7135204.

Campbell, D. S. and Holt, C. E. (2001) 'Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation.', *Neuron*, 32(6), pp. 1013–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11754834 (Accessed: 7 February 2019).

Carmona, V. *et al.* (2017) 'Unravelling Endogenous MicroRNA System Dysfunction as a New Pathophysiological Mechanism in Machado-Joseph Disease.', *Molecular therapy : the journal of the American Society of Gene Therapy*. Elsevier, 25(4), pp. 1038–1055. doi: 10.1016/j.ymthe.2017.01.021.

Castedo, M. *et al.* (2004) 'Cell death by mitotic catastrophe: a molecular definition', *Oncogene*, 23(16), pp. 2825–2837. doi: 10.1038/sj.onc.1207528.

Chandrasekaran, S. and Bonchev, D. (2013) 'A NETWORK VIEW ON PARKINSON'S DISEASE', *Computational and Structural Biotechnology Journal*, 7(8), p. e201304004. doi: 10.5936/csbj.201304004.

Chaudhry, V. and Cornblath, D. R. (1992) 'Wallerian degeneration in human nerves: Serial electrophysiological studies', *Muscle & Nerve*, 15(6), pp. 687–693. doi: 10.1002/mus.880150610.

Chen, C.-Z. *et al.* (2004) 'MicroRNAs Modulate Hematopoietic Lineage Differentiation', *Science*, 303(5654), pp. 83–86. doi: 10.1126/science.1091903.

Chen, L. *et al.* (2018) 'miRNA arm switching identifies novel tumour biomarkers.', *EBioMedicine*. Elsevier, 38, pp. 37–46. doi: 10.1016/j.ebiom.2018.11.003.

Cheng, E. H. *et al.* (2001) 'BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis.', *Molecular cell*, 8(3), pp. 705–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11583631 (Accessed: 22 February 2019).

Cheng, X.-S. *et al.* (2013) 'Nmnat2 Attenuates Tau Phosphorylation Through Activation of PP2A', *Journal of Alzheimer's Disease*, 36(1), pp. 185–195. doi: 10.3233/JAD-122173.

Chiarugi, A. *et al.* (2012) 'The NAD metabolome--a key determinant of cancer cell biology.', *Nature reviews. Cancer*, 12(11), pp. 741–52. doi: 10.1038/nrc3340.

Christensen, M. *et al.* (2010) 'Recombinant adeno-associated virus-mediated microRNA delivery into the postnatal mouse brain reveals a role for miR-134 in dendritogenesis in vivo', *Frontiers in Neural Circuits*, 3, p. 16. doi: 10.3389/neuro.04.016.2009.

Cobb, C. A. and Cole, M. P. (2015) 'Oxidative and nitrative stress in neurodegeneration', *Neurobiology of Disease*. Academic Press, 84, pp. 4–21. doi: 10.1016/J.NBD.2015.04.020.

Coleman, M. P. and Freeman, M. R. (2010) 'Wallerian Degeneration, Wld $^{S}$ , and Nmnat', *Annual Review of Neuroscience*, 33(1), pp. 245–267. doi: 10.1146/annurev-neuro-060909-153248.

Collyer, E. *et al.* (2014) 'Sprouting of axonal collaterals after spinal cord injury is prevented by delayed axonal degeneration.', *Experimental neurology*, 261, pp. 451–61. doi: 10.1016/j.expneurol.2014.07.014.

Conforti, L. *et al.* (2000) 'A Ufd2/D4Cole1e chimeric protein and overexpression of Rbp7 in the slow Wallerian degeneration (WldS) mouse.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 97(21), pp. 11377–82. doi: 10.1073/pnas.97.21.11377.

Conforti, L. *et al.* (2009) 'Wld S protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice.', *The Journal of cell biology*, 184(4), pp. 491–500. doi: 10.1083/jcb.200807175.

Conforti, L. *et al.* (2011) 'Reducing expression of NAD+ synthesizing enzyme NMNAT1 does not affect the rate of Wallerian degeneration', *FEBS Journal*, 278(15), pp. 2666–2679. doi: 10.1111/j.1742-4658.2011.08193.x.

Conforti, L., Gilley, J. and Coleman, M. P. (2014) 'Wallerian degeneration: An emerging axon death pathway linking injury and disease', *Nature Reviews Neuroscience*. Nature Publishing Group, 15(6), pp. 394–409. doi: 10.1038/nrn3680.

Costa, C. J. and Willis, D. E. (2018) 'To the end of the line: Axonal mRNA transport and local translation in health and neurodegenerative disease', *Developmental Neurobiology*, 78(3), pp. 209–220. doi: 10.1002/dneu.22555.

Crawford, T. O. *et al.* (1995) 'Prolonged axonal survival in transected nerves of C57BL/Ola mice is independent of age.', *Journal of neurocytology*, 24(5), pp. 333–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7650538 (Accessed: 1 November 2018).

D'Orsi, B. *et al.* (2015) 'Bax Regulates Neuronal Ca2+ Homeostasis', *Journal of Neuroscience*, 35(4), pp. 1706–1722. doi: 10.1523/JNEUROSCI.2453-14.2015.

D'Orsi, B. *et al.* (2016) 'Bok Is Not Pro-Apoptotic But Suppresses Poly ADP-Ribose Polymerase-Dependent Cell Death Pathways and Protects against Excitotoxic and Seizure-Induced Neuronal Injury', *Journal of Neuroscience*, 36(16), pp. 4564–4578. doi: 10.1523/JNEUROSCI.3780-15.2016.

Dajas-Bailador, F. *et al.* (2012) 'microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons', *Nature Neuroscience*, 15(5), pp. 697–699. doi: 10.1038/nn.3082.

Davies, A. M. (1988) 'The emerging generality of the neurotrophic hypothesis', *Trends in Neurosciences*. Elsevier Current Trends, 11(6), pp. 243–244. doi: 10.1016/0166-2236(88)90099-9.

Davies, G. *et al.* (2018) 'Study of 300,486 individuals identifies 148 independent genetic loci influencing general cognitive function.', *Nature communications*. Nature Publishing Group, 9(1), p. 2098. doi: 10.1038/s41467-018-04362-x.

Davis-Dusenbery, B. N. and Hata, A. (2010) 'Mechanisms of control of microRNA biogenesis.', *Journal of biochemistry*, 148(4), pp. 381–92. doi: 10.1093/jb/mvq096.

Deckwerth, T. L. *et al.* (1996) 'BAX is required for neuronal death after trophic factor deprivation and during development.', *Neuron*, 17(3), pp. 401–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8816704 (Accessed: 17 May 2019).

Denli, A. M. *et al.* (2004) 'Processing of primary microRNAs by the Microprocessor complex', *Nature*, 432(7014), pp. 231–235. doi: 10.1038/nature03049.

Dent, E. W. and Gertler, F. B. (2003) 'Cytoskeletal dynamics and transport in growth cone motility and axon guidance.', *Neuron*, 40(2), pp. 209–27. doi: 10.1016/s0896-6273(03)00633-0.

Dickson, B. J. (2002) 'Molecular Mechanisms of Axon Guidance', *Science*, 298(5600), pp. 1959–1964. doi: 10.1126/science.1072165.

Dotti, C. G., Sullivan, C. A. and Banker, G. A. (1988) 'The establishment of polarity by hippocampal neurons in culture.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 8(4), pp. 1454–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3282038 (Accessed: 22 February 2019).

Fabian, M. R. and Sonenberg, N. (2012) 'The mechanics of miRNA-mediated gene silencing: A look under the hood of miRISC', *Nature Structural and Molecular Biology*. Nature Publishing Group, 19(6), pp. 586–593. doi: 10.1038/nsmb.2296.

de Figueiredo, L. F. *et al.* (2011) 'Pathway analysis of NAD $^{+}$ metabolism', *Biochemical Journal*, 439(2), pp. 341–348. doi: 10.1042/BJ20110320.

Filippov, V. *et al.* (2000) 'A novel type of RNase III family proteins in eukaryotes.', *Gene*, 245(1), pp. 213–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10713462 (Accessed: 16 January 2019).

Fiore, R. *et al.* (2009) 'Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels.', *The EMBO journal*. European Molecular Biology Organization, 28(6), pp. 697–710. doi: 10.1038/emboj.2009.10.

Fortin, K. R., Nicholson, R. H. and Nicholson, A. W. (2002) 'Mouse ribonuclease III. cDNA

structure, expression analysis, and chromosomal location.', *BMC genomics*, 3(1), p. 26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12191433 (Accessed: 16 January 2019).

Franke, K. *et al.* (2012) 'miR-124-regulated RhoG reduces neuronal process complexity via ELMO/Dock180/Rac1 and Cdc42 signalling', *The EMBO Journal*, 31(13), pp. 2908–2921. doi: 10.1038/emboj.2012.130.

Fricker, M. *et al.* (2018) 'Neuronal Cell Death', *Physiological Reviews*. American Physiological Society Bethesda, MD , 98(2), pp. 813–880. doi: 10.1152/physrev.00011.2017.

Friedman, R. C. *et al.* (2009) 'Most mammalian mRNAs are conserved targets of microRNAs.', *Genome research*. Cold Spring Harbor Laboratory Press, 19(1), pp. 92–105. doi: 10.1101/gr.082701.108.

Funahashi, Y. *et al.* (2014) 'Neuronal polarization in vivo: Growing in a complex environment', *Current Opinion in Neurobiology*. Elsevier Current Trends, 27, pp. 215–223. doi: 10.1016/J.CONB.2014.04.009.

Gerdts, J. *et al.* (2016) 'Axon Self-Destruction: New Links among SARM1, MAPKs, and NAD+ Metabolism', *Neuron*, 89(3), pp. 449–460. doi: 10.1016/j.neuron.2015.12.023.

Gibson, M. E. *et al.* (2001) 'BAX contributes to apoptotic-like death following neonatal hypoxia-ischemia: evidence for distinct apoptosis pathways.', *Molecular medicine (Cambridge, Mass.)*, 7(9), pp. 644–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11778654 (Accessed: 17 May 2019).

Gilley, J. *et al.* (2013) 'Rescue of peripheral and CNS axon defects in mice lacking NMNAT2.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(33), pp. 13410–24. doi: 10.1523/JNEUROSCI.1534-13.2013.

Gilley, J. and Coleman, M. P. (2010) 'Endogenous Nmnat2 Is an Essential Survival Factor for Maintenance of Healthy Axons', *PLoS Biology*. Public Library of Science, 8(1), p. e1000300. doi: 10.1371/journal.pbio.1000300.

Gilliatt, R. W. and Hjorth, R. J. (1972) 'Nerve conduction during Wallerian degeneration in the baloon.', *Journal of neurology, neurosurgery, and psychiatry*, 35(3), pp. 335–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4624688 (Accessed: 29 October 2018).

Gillingwater, T. H. *et al.* (2006) 'Delayed synaptic degeneration in the CNS of Wlds mice after cortical lesion.', *Brain : a journal of neurology*, 129(Pt 6), pp. 1546–56. doi: 10.1093/brain/awl101.

Giraldez, A. J. *et al.* (2005) 'MicroRNAs Regulate Brain Morphogenesis in Zebrafish', *Science*, 308(5723), pp. 833–838. doi: 10.1126/science.1109020.

Giusti, S. A. *et al.* (2014) 'MicroRNA-9 controls dendritic development by targeting REST', *eLife*, 3. doi: 10.7554/eLife.02755.

Glass, J. D. and Griffin, J. W. (1994) 'Retrograde transport of radiolabeled cytoskeletal proteins in transected nerves.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*. Society for Neuroscience, 14(6), pp. 3915–21. doi: 10.1523/JNEUROSCI.14-06-03915.1994.

Goldberg, J. L. (2003) 'How does an axon grow?', *Genes & development*. Cold Spring Harbor Laboratory Press, 17(8), pp. 941–58. doi: 10.1101/gad.1062303.

Gould, T. W. *et al.* (2006) 'Complete Dissociation of Motor Neuron Death from Motor Dysfunction by Bax Deletion in a Mouse Model of ALS', *Journal of Neuroscience*, 26(34), pp. 8774–8786. doi: 10.1523/JNEUROSCI.2315-06.2006.

Gumy, L. F. *et al.* (2011) 'Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization.', *RNA (New York, N.Y.)*. Cold Spring Harbor Laboratory Press, 17(1), pp. 85–98. doi: 10.1261/rna.2386111.

Guo, H. *et al.* (2010) 'Mammalian microRNAs predominantly act to decrease target mRNA levels', *Nature*, 466(7308), pp. 835–840. doi: 10.1038/nature09267.

Ha, M. and Kim, V. N. (2014) 'Regulation of microRNA biogenesis', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 15(8), pp. 509–524. doi: 10.1038/nrm3838.

Haase, G. *et al.* (2008) 'Signaling by death receptors in the nervous system', *Current Opinion in Neurobiology*. Elsevier Current Trends, 18(3), pp. 284–291. doi: 10.1016/J.CONB.2008.07.013.

Hammond, S. M. *et al.* (2001) 'Argonaute2, a link between genetic and biochemical analyses of RNAi.', *Science (New York, N.Y.)*, 293(5532), pp. 1146–50. doi: 10.1126/science.1064023.

Hancock, M. L. *et al.* (2014) 'MicroRNA-132 Is Enriched in Developing Axons, Locally Regulates Rasa1 mRNA, and Promotes Axon Extension', *Journal of Neuroscience*, 34(1), pp. 66–78. doi: 10.1523/JNEUROSCI.3371-13.2014.

Haramati, S. *et al.* (2011) 'microRNA as Repressors of Stress-Induced Anxiety: The Case of Amygdalar miR-34', *Journal of Neuroscience*, 31(40), pp. 14191–14203. doi: 10.1523/JNEUROSCI.1673-11.2011.

Hassa, P. O. *et al.* (2006) 'Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going?', *Microbiology and molecular biology reviews : MMBR*, 70(3), pp. 789–829. doi: 10.1128/MMBR.00040-05.

Hertel, J. *et al.* (2006) 'The expansion of the metazoan microRNA repertoire.', *BMC Genomics*, 7(1), p. 25. doi: 10.1186/1471-2164-7-25.

Hicks, A. N. *et al.* (2012) 'Nicotinamide Mononucleotide Adenylyltransferase 2 (Nmnat2) Regulates Axon Integrity in the Mouse Embryo', *PLoS ONE*. Edited by S. Strack, 7(10), p. e47869. doi: 10.1371/journal.pone.0047869.

Ho, C.-Y. *et al.* (2013) 'MicroRNA profiling in pediatric pilocytic astrocytoma reveals biologically relevant targets, including PBX3, NFIB, and METAP2.', *Neuro-oncology*. Oxford University Press, 15(1), pp. 69–82. doi: 10.1093/neuonc/nos269.

Holzbaur, E. L. F. and Scherer, S. S. (2011) 'Microtubules, Axonal Transport, and Neuropathy', *New England Journal of Medicine*, 365(24), pp. 2330–2332. doi: 10.1056/NEJMcibr1112481.

Hörnberg, H. and Holt, C. (2013) 'RNA-binding proteins and translational regulation in axons and growth cones', *Frontiers in Neuroscience*. Frontiers, 7, p. 81. doi: 10.3389/fnins.2013.00081.

Humphreys, D. T. *et al.* (2005) 'MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function.', *Proceedings of the National Academy of Sciences of the United States of America*, 102(47), pp. 16961–6. doi: 10.1073/pnas.0506482102.

Hutchison, E. R. *et al.* (2013) 'Evidence for miR-181 involvement in neuroinflammatory responses of astrocytes', *Glia*, 61(7), pp. 1018–1028. doi: 10.1002/glia.22483.

Hüttenhofer, A., Schattner, P. and Polacek, N. (2005) 'Non-coding RNAs: hope or hype?', *Trends in Genetics*, 21(5), pp. 289–297. doi: 10.1016/j.tig.2005.03.007.

Imai, S. (2016) 'The NAD World 2.0: the importance of the inter-tissue communication mediated by NAMPT/NAD+/SIRT1 in mammalian aging and longevity control', *npj Systems Biology and Applications*, 2(1), p. 16018. doi: 10.1038/npjsba.2016.18.

Iyer, A. N., Bellon, A. and Baudet, M.-L. (2014) 'microRNAs in axon guidance.', *Frontiers in cellular neuroscience*. Frontiers Media SA, 8, p. 78. doi: 10.3389/fncel.2014.00078.

Jakobson, M. *et al.* (2013a) 'Multiple mechanisms repress N-Bak mRNA translation in the healthy and apoptotic neurons.', *Cell death & disease*. Nature Publishing Group, 4(8), p. e777. doi: 10.1038/cddis.2013.297.

Jakobson, M. *et al.* (2013b) 'Multiple mechanisms repress N-Bak mRNA translation in the healthy and apoptotic neurons', *Cell Death & Disease*, 4(8), pp. e777–e777. doi: 10.1038/cddis.2013.297.

Jeyaseelan, K., Lim, K. Y. and Armugam, A. (2008) 'MicroRNA Expression in the Blood and Brain of Rats Subjected to Transient Focal Ischemia by Middle Cerebral Artery Occlusion', *Stroke*, 39(3), pp. 959–966. doi: 10.1161/STROKEAHA.107.500736.

Johnson, M. D. *et al.* (1998) 'Evidence for involvement of Bax and p53, but not caspases, in radiation-induced cell death of cultured postnatal hippocampal neurons', *Journal of Neuroscience Research*, 54(6), pp. 721–733. doi: 10.1002/(SICI)1097-4547(19981215)54:6<721::AID-JNR1>3.0.CO;2-1.

Jovicic, A. *et al.* (2013) 'Comprehensive Expression Analyses of Neural Cell-Type-Specific miRNAs Identify New Determinants of the Specification and Maintenance of Neuronal Phenotypes', *Journal of Neuroscience*, 33(12), pp. 5127–5137. doi: 10.1523/JNEUROSCI.0600-12.2013.

Jung, H., Yoon, B. C. and Holt, C. E. (2012) 'Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair', *Nature Reviews Neuroscience*, 13(5), pp. 308–324. doi: 10.1038/nrn3210.

Kaikkonen, M. U., Lam, M. T. Y. and Glass, C. K. (2011) 'Non-coding RNAs as regulators of gene expression and epigenetics', *Cardiovascular Research*, 90(3), pp. 430–440. doi: 10.1093/cvr/cvr097.

Kandel, E. R. *et al.* (2013) *Principles of Neural Science*. 5th edn. The McGraw-Hill Companies,Inc.

Kar, A. N., Lee, S. J. and Twiss, J. L. (2018) 'Expanding Axonal Transcriptome Brings New Functions for Axonally Synthesized Proteins in Health and Disease', *The Neuroscientist*, 24(2), pp. 111–129. doi: 10.1177/1073858417712668.

Kawamata, T., Seitz, H. and Tomari, Y. (2009) 'Structural determinants of miRNAs for RISC loading and slicer-independent unwinding.', *Nature structural & molecular biology*, 16(9), pp. 953–60. doi: 10.1038/nsmb.1630.

Kawamata, T. and Tomari, Y. (2010) 'Making RISC', *Trends in Biochemical Sciences*, 35(7), pp. 368–376. doi: 10.1016/j.tibs.2010.03.009.

Kerschensteiner, M. *et al.* (2005) 'In vivo imaging of axonal degeneration and regeneration in the injured spinal cord', *Nature Medicine*, 11(5), pp. 572–577. doi: 10.1038/nm1229.

Khvorova, A., Reynolds, A. and Jayasena, S. D. (2003) 'Functional siRNAs and miRNAs exhibit strand bias.', *Cell*, 115(2), pp. 209–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14567918 (Accessed: 17 January 2019).

Kim, J.-Y., Kim, N. and Yenari, M. A. (2015) 'Mechanisms and Potential Therapeutic Applications of Microglial Activation after Brain Injury', *CNS Neuroscience & Therapeutics*. John Wiley & Sons, Ltd (10.1111), 21(4), pp. 309–319. doi: 10.1111/cns.12360.

Kim, V. N., Han, J. and Siomi, M. C. (2009) 'Biogenesis of small RNAs in animals', *Nature Reviews Molecular Cell Biology*, 10(2), pp. 126–139. doi: 10.1038/nrm2632.

Kitaoka, Y. *et al.* (2013) 'Axonal protection by Nmnat3 overexpression with involvement of autophagy in optic nerve degeneration.', *Cell death & disease*. Nature Publishing Group, 4(10), p. e860. doi: 10.1038/cddis.2013.391.

Kitay, B. M. *et al.* (2013) 'Mislocalization of neuronal mitochondria reveals regulation of Wallerian degeneration and NMNAT/WLD(S)-mediated axon protection independent of axonal mitochondria.', *Human molecular genetics*. Oxford University Press, 22(8), pp. 1601–14. doi: 10.1093/hmg/ddt009.

Koch-Nolte, F. *et al.* (2009) 'Emerging Roles of NAD+ and Its Metabolites in Cell Signaling', *Science Signaling*, 2(57), pp. mr1–mr1. doi: 10.1126/scisignal.257mr1.

Korsching, S. (1993) 'The neurotrophic factor concept: a reexamination.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 13(7), pp. 2739–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8331370 (Accessed: 24 February 2019).

Kos, A. *et al.* (2016) 'MicroRNA-181 promotes synaptogenesis and attenuates axonal outgrowth in cortical neurons', *Cellular and Molecular Life Sciences*, 73(18), pp. 3555–3567. doi: 10.1007/s00018-016-2179-0.

Kos, A. *et al.* (2017) 'MicroRNA-338 Attenuates Cortical Neuronal Outgrowth by Modulating the Expression of Axon Guidance Genes', *Molecular Neurobiology*, 54(5), pp. 3439–3452. doi: 10.1007/s12035-016-9925-z.

Koshkin, A. A. *et al.* (1998) 'LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition', *Tetrahedron*. Pergamon, 54(14), pp. 3607–3630. doi: 10.1016/S0040-4020(98)00094-5.

Kosik, K. S. (2006) 'The neuronal microRNA system', *Nature Reviews Neuroscience*, 7(12), pp. 911–920. doi: 10.1038/nrn2037.

Kozomara, A. and Griffiths-Jones, S. (2014) 'miRBase: annotating high confidence microRNAs using deep sequencing data', *Nucleic Acids Research*, 42(D1), pp. D68–D73. doi: 10.1093/nar/gkt1181.

Krol, J., Loedige, I. and Filipowicz, W. (2010) 'The widespread regulation of microRNA biogenesis, function and decay', *Nature Reviews Genetics*. Nature Publishing Group, 11(9), pp. 597–610. doi: 10.1038/nrg2843.

Kuan, C. Y. *et al.* (2000) 'Mechanisms of programmed cell death in the developing brain.', *Trends in neurosciences*. Elsevier, 23(7), pp. 291–7. doi: 10.1016/S0166-2236(00)01581-2.

Kullander, K. and Klein, R. (2002) 'Mechanisms and functions of eph and ephrin signalling', *Nature Reviews Molecular Cell Biology*, 3(7), pp. 475–486. doi: 10.1038/nrm856.

Kye, M.-J. *et al.* (2007) 'Somatodendritic microRNAs identified by laser capture and multiplex RT-PCR', *RNA*, 13(8), pp. 1224–1234. doi: 10.1261/rna.480407.

Lander, E. S. *et al.* (2001) 'Initial sequencing and analysis of the human genome', *Nature*, 409(6822), pp. 860–921. doi: 10.1038/35057062.

Lau, P. *et al.* (2013) 'Alteration of the microRNA network during the progression of Alzheimer's disease', *EMBO Molecular Medicine*, 5(10), pp. 1613–1634. doi: 10.1002/emmm.201201974.

Lécuyer, E. *et al.* (2007) 'Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function.', *Cell*. Elsevier, 131(1), pp. 174–87. doi: 10.1016/j.cell.2007.08.003.

Lee, Y. *et al.* (2003) 'The nuclear RNase III Drosha initiates microRNA processing', *Nature*, 425(6956), pp. 415–419. doi: 10.1038/nature01957.

Levi-Montalcini, R. (1964) 'THE NERVE GROWTH FACTOR', *Annals of the New York Academy of Sciences*. John Wiley & Sons, Ltd (10.1111), 118(3), pp. 149–170. doi: 10.1111/j.1749-6632.1964.tb33978.x.

Lewis, B. P., Burge, C. B. and Bartel, D. P. (2005) 'Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets', *Cell*, 120(1), pp. 15–20. doi: 10.1016/j.cell.2004.12.035.

Lewis, T. L., Courchet, J. and Polleux, F. (2013) 'Cellular and molecular mechanisms underlying axon formation, growth, and branching', *Journal of Cell Biology*, 202(6), pp. 837–848. doi: 10.1083/jcb.201305098.

Li, H. *et al.* (2014) 'MicroRNA-29a modulates axon branching by targeting doublecortin in primary neurons', *Protein & Cell*, 5(2), pp. 160–169. doi: 10.1007/s13238-014-0022-7.

Li, X. *et al.* (2002) 'The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism.', *The Journal of biological chemistry*, 277(40), pp. 37788–97. doi: 10.1074/jbc.M205428200.

Liu, N.-K. and Xu, X.-M. (2011) 'MicroRNA in central nervous system trauma and degenerative disorders.', *Physiological genomics*, 43(10), pp. 571–80. doi: 10.1152/physiolgenomics.00168.2010.

Ljungberg, M. C. *et al.* (2012) 'CREB-activity and nmnat2 transcription are down-regulated prior to neurodegeneration, while NMNAT2 over-expression is neuroprotective, in a mouse model of human tauopathy', *Human Molecular Genetics*, 21(2), pp. 251–267. doi: 10.1093/hmg/ddr492.

Loreto, A. *et al.* (2015) 'Wallerian Degeneration Is Executed by an NMN-SARM1-Dependent Late Ca(2+) Influx but Only Modestly Influenced by Mitochondria.', *Cell reports*. Elsevier, 13(11), pp. 2539–2552. doi: 10.1016/j.celrep.2015.11.032.

Lowery, L. A. and Van Vactor, D. (2009) 'The trip of the tip: understanding the growth cone machinery.', *Nature reviews. Molecular cell biology*. NIH Public Access, 10(5), pp. 332–43. doi: 10.1038/nrm2679.

Lugli, G. *et al.* (2008) 'Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain.', *Journal of neurochemistry*. NIH Public Access, 106(2), pp. 650–61. doi: 10.1111/j.1471-4159.2008.05413.x.

Luikart, B. W. *et al.* (2011) 'miR-132 Mediates the Integration of Newborn Neurons into the Adult Dentate Gyrus', *PLoS ONE*. Edited by F. Tell, 6(5), p. e19077. doi: 10.1371/journal.pone.0019077.

Luikart, B. W., Perederiy, J. V. and Westbrook, G. L. (2012) 'Dentate gyrus neurogenesis, integration and microRNAs', *Behavioural Brain Research*, 227(2), pp. 348–355. doi: 10.1016/j.bbr.2011.03.048.

Lund, E. *et al.* (2004) 'Nuclear Export of MicroRNA Precursors', *Science*, 303(5654), pp. 95–98. doi: 10.1126/science.1090599.

Lunn, E. R. *et al.* (1989) 'Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve.', *The European journal of neuroscience*, 1(1), pp. 27–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12106171 (Accessed: 31 October 2018).

MacDonald, J. M. *et al.* (2006) 'The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons.', *Neuron*, 50(6), pp. 869–81. doi: 10.1016/j.neuron.2006.04.028.

Mack, T. G. *et al.* (2001) 'Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene.', *Nature neuroscience*, 4(12), pp. 1199–206. doi: 10.1038/nn770.

Maes, O. *et al.* (2009) 'MicroRNA: Implications for Alzheimer Disease and other Human CNS Disorders', *Current Genomics*, 10(3), pp. 154–168. doi: 10.2174/138920209788185252.

Magill, S. T. *et al.* (2010) 'microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus', *Proceedings of the National Academy of Sciences*, 107(47), pp. 20382–20387. doi: 10.1073/pnas.1015691107.

Magni, G. *et al.* (2004a) 'Enzymology of NAD+ homeostasis in man.', *Cellular and molecular life sciences : CMLS*, 61(1), pp. 19–34. doi: 10.1007/s00018-003-3161-1.

Magni, G. *et al.* (2004b) 'Structure and function of nicotinamide mononucleotide adenylyltransferase.', *Current medicinal chemistry*, 11(7), pp. 873–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15078171 (Accessed: 5 December 2018).

Marler, K. J. *et al.* (2014) 'BDNF Promotes Axon Branching of Retinal Ganglion Cells via miRNA-132 and p250GAP', *The Journal of Neuroscience*, 34(3), pp. 969–979. doi: 10.1523/JNEUROSCI.1910-13.2014.

Maroney, P. A. *et al.* (2006) 'Evidence that microRNAs are associated with translating messenger RNAs in human cells.', *Nature structural & molecular biology*, 13(12), pp. 1102–7. doi: 10.1038/nsmb1174.

Martin, S. M. *et al.* (2010) 'Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning.', *Development (Cambridge, England)*, 137(23), pp. 3985–94. doi: 10.1242/dev.053611.

Mayer, P. R. *et al.* (2010) 'Expression , Localization , and Biochemical Characterization of Nicotinamide Mononucleotide Adenylyltransferase 2 *', 285(51), pp. 40387–40396. doi: 10.1074/jbc.M110.178913.

McNeill, E. and Van Vactor, D. (2012) 'MicroRNAs Shape the Neuronal Landscape', *Neuron*, 75(3), pp. 363–379. doi: 10.1016/j.neuron.2012.07.005.

Meijer, H. A., Smith, E. M. and Bushell, M. (2014) 'Regulation of miRNA strand selection: follow the leader?', *Biochemical Society Transactions*, 42(4), pp. 1135–1140. doi: 10.1042/BST20140142.

Mellios, N. *et al.* (2011) 'miR-132, an experience-dependent microRNA, is essential for visual cortex plasticity.', *Nature neuroscience*. NIH Public Access, 14(10), pp. 1240–2. doi: 10.1038/nn.2909.

Mellios, N. and Sur, M. (2012) 'The Emerging Role of microRNAs in Schizophrenia and Autism Spectrum Disorders.', *Frontiers in psychiatry*. Frontiers Media SA, 3, p. 39. doi: 10.3389/fpsyt.2012.00039.

Milde, S. *et al.* (2013) 'Deletions within its subcellular targeting domain enhance the axon protective capacity of Nmnat2 in vivo', *Scientific Reports*, 3(1), p. 2567. doi: 10.1038/srep02567.

Milde, S. and Coleman, M. P. (2014) 'Identification of Palmitoyltransferase and Thioesterase Enzymes That Control the Subcellular Localization of Axon Survival Factor Nicotinamide Mononucleotide Adenylyltransferase 2 (NMNAT2)', *Journal of Biological Chemistry*, 289(47), pp. 32858–32870. doi: 10.1074/jbc.M114.582338.

Milde, S., Gilley, J. and Coleman, M. P. (2013) 'Subcellular Localization Determines the Stability and Axon Protective Capacity of Axon Survival Factor Nmnat2', *PLoS Biology*. Edited by B. A. Barres, 11(4), p. e1001539. doi: 10.1371/journal.pbio.1001539.

Miledi, R. and Slater, C. R. (1970) 'On the degeneration of rat neuromuscular junctions after nerve section.', *The Journal of physiology*, 207(2), pp. 507–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5499034 (Accessed: 29 October 2018).

Mili, S., Moissoglu, K. and Macara, I. G. (2008) 'Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions', *Nature*, 453(7191), pp. 115–119. doi: 10.1038/nature06888.

Miller, T. M. *et al.* (1997) 'Bax Deletion Further Orders the Cell Death Pathway in Cerebellar Granule Cells and Suggests a Caspase-independent Pathway to Cell Death', *The Journal of Cell Biology*, 139(1), pp. 205–217. doi: 10.1083/jcb.139.1.205.

Minis, A. *et al.* (2014) 'Subcellular transcriptomics-Dissection of the mRNA composition in the axonal compartment of sensory neurons', *Developmental Neurobiology*. John Wiley & Sons, Ltd, 74(3), pp. 365–381. doi: 10.1002/dneu.22140.

Morales, D. and Kania, A. (2017) 'Cooperation and crosstalk in axon guidance cue integration: Additivity, synergy, and fine-tuning in combinatorial signaling', *Developmental Neurobiology*. John Wiley & Sons, Ltd, 77(7), pp. 891–904. doi: 10.1002/dneu.22463.

Moran, Y. *et al.* (2017) 'The evolutionary origin of plant and animal microRNAs.', *Nature ecology & evolution*. Europe PMC Funders, 1(3), p. 27. doi: 10.1038/s41559-016-0027.

Mori, V. *et al.* (2014) 'Metabolic profiling of alternative NAD biosynthetic routes in mouse tissues.', *PloS one*. Edited by V. de Crécy-Lagard, 9(11), p. e113939. doi: 10.1371/journal.pone.0113939.

Murashov, A. K. *et al.* (2007) 'RNAi pathway is functional in peripheral nerve axons', *The FASEB Journal*, 21(3), pp. 656–670. doi: 10.1096/fj.06-6155com.

Natera-Naranjo, O. *et al.* (2010) 'Identification and quantitative analyses of microRNAs located in the distal axons of sympathetic neurons.', *RNA (New York, N.Y.)*. Cold Spring Harbor Laboratory Press, 16(8), pp. 1516–29. doi: 10.1261/rna.1833310.

Nikolić, M., Gardner, H. A. R. and Tucker, K. L. (2013) 'Postnatal neuronal apoptosis in the cerebral cortex: Physiological and pathophysiological mechanisms', *Neuroscience*. Pergamon, 254, pp. 369–378. doi: 10.1016/J.NEUROSCIENCE.2013.09.035.

Nudelman, A. S. *et al.* (2009) 'Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo', *Hippocampus*, 20(4), p. NA-NA. doi: 10.1002/hipo.20646.

Okamura, K., Liu, N. and Lai, E. C. (2009) 'Distinct mechanisms for microRNA strand selection by Drosophila Argonautes.', *Molecular cell*. NIH Public Access, 36(3), pp. 431–44. doi: 10.1016/j.molcel.2009.09.027.

Olney, J. W. (1971) 'GLUTAMATE-INDUCED NEURONAL NECROSIS IN THE INFANT MOUSE HYPOTHALAMUS', *Journal of Neuropathology and Experimental Neurology*. Oxford University Press, 30(1), pp. 75–90. doi: 10.1097/00005072-197101000-00008.

Ouyang, Q. *et al.* (2016) 'Neurotensin signaling stimulates glioblastoma cell proliferation by upregulating c-Myc and inhibiting miR-29b-1 and miR-129-3p', *Neuro-Oncology*. Oxford University Press, 18(2), p. 216. doi: 10.1093/NEUONC/NOV114.

Ouyang, Y.-B., Lu, Y., Yue, S., Xu, L.-J., *et al.* (2012) 'miR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo', *Neurobiology of Disease*, 45(1), pp. 555–563. doi: 10.1016/j.nbd.2011.09.012.

Ouyang, Y.-B., Lu, Y., Yue, S. and Giffard, R. G. (2012) 'miR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes', *Mitochondrion*, 12(2), pp. 213–219. doi: 10.1016/j.mito.2011.09.001.

Paridaen, J. T. and Huttner, W. B. (2014) 'Neurogenesis during development of the vertebrate central nervous system', *EMBO reports*, 15(4), pp. 351–364. doi: 10.1002/embr.201438447.

Pasterkamp, R. J. and Kolodkin, A. L. (2003) 'Semaphorin junction: making tracks toward neural connectivity.', *Current opinion in neurobiology*, 13(1), pp. 79–89. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12593985 (Accessed: 30 January 2019).

Petersen, C. P. *et al.* (2006) 'Short RNAs Repress Translation after Initiation in Mammalian Cells', *Molecular Cell*, 21(4), pp. 533–542. doi: 10.1016/j.molcel.2006.01.031.

Pfeffer, S. *et al.* (2005) 'Identification of microRNAs of the herpesvirus family', *Nature Methods*, 2(4), pp. 269–276. doi: 10.1038/nmeth746.

Pichler, S. *et al.* (2017) 'The miRNome of Alzheimer's disease: consistent downregulation of the miR-132/212 cluster', *Neurobiology of Aging*, 50, pp. 167.e1-167.e10. doi: 10.1016/j.neurobiolaging.2016.09.019.

Preiss, J. and Handler, P. (1958) 'Biosynthesis of diphosphopyridine nucleotide. II. Enzymatic aspects.', *The Journal of biological chemistry*, 233(2), pp. 493–500. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13563527 (Accessed: 27 November 2018).

Purves, D. (1986) 'The trophic theory of neural concentrations', *Trends in Neurosciences*. Elsevier Current Trends, 9, pp. 486–489. doi: 10.1016/0166-2236(86)90155-4.

Raff, M. C., Whitmore, A. V and Finn, J. T. (2002) 'Axonal self-destruction and neurodegeneration.', *Science (New York, N.Y.)*, 296(5569), pp. 868–71. doi: 10.1126/science.1068613.

Raffaelli, N. *et al.* (2002) 'Identification of a novel human nicotinamide mononucleotide adenylyltransferase.', *Biochemical and biophysical research communications*, 297(4), pp. 835–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12359228 (Accessed: 17 February 2019).

Rajman, M. and Schratt, G. (2017) 'MicroRNAs in neural development: from master regulators to fine-tuners', *Development*. Oxford University Press for The Company of Biologists Limited, 144(13), pp. 2310–2322. doi: 10.1242/DEV.144337.

Ray, P. *et al.* (2018) 'Title: Comparative transcriptome profiling of the human and mouse dorsal root ganglia: An RNA-seq-based resource for pain and sensory neuroscience research Short Title: Human and mouse DRG comparative transcriptomics', *PAIN, 159(7):1325 - 1345*. doi: 10.1101/165431.

Remenyi, J. *et al.* (2010) 'Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins', *Biochemical Journal*, 428(2), pp. 281–291. doi: 10.1042/BJ20100024.

Richardson, P. M. (1994) 'Ciliary neurotrophic factor: A review', *Pharmacology & Therapeutics*. Pergamon, 63(2), pp. 187–198. doi: 10.1016/0163-7258(94)90045-0.

Rodriguez-Ortiz, C. J. *et al.* (2014) 'Upregulation of miR-181 Decreases c-Fos and SIRT-1 in the Hippocampus of 3xTg-AD Mice', *Journal of Alzheimer's Disease*, 42(4), pp. 1229–1238. doi: 10.3233/JAD-140204.

Rongvaux, A. *et al.* (2003) 'Reconstructing eukaryotic NAD metabolism.', *BioEssays : news and reviews in molecular, cellular and developmental biology*, 25(7), pp. 683–90. doi: 10.1002/bies.10297.

Saba, R. *et al.* (2008) 'A miRNA signature of prion induced neurodegeneration.', *PloS one*. Public Library of Science, 3(11), p. e3652. doi: 10.1371/journal.pone.0003652.

Saba, R. *et al.* (2012) 'Dopamine-Regulated MicroRNA MiR-181a Controls GluA2 Surface Expression in Hippocampal Neurons', *Molecular and Cellular Biology*, 32(3), pp. 619–632. doi: 10.1128/MCB.05896-11.

Sadoul, R. (1998) 'BCL-2 family members in the development and degenerative pathologies of the nervous system', *Cell Death & Differentiation*. Nature Publishing Group, 5(10), pp. 805–815. doi: 10.1038/sj.cdd.4400438.

Sambandan, S. *et al.* (2017) 'Activity-dependent spatially localized miRNA maturation in neuronal dendrites.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 355(6325), pp. 634–637. doi: 10.1126/science.aaf8995.

Samsam, M. *et al.* (2003) 'The Wlds mutation delays robust loss of motor and sensory axons in a genetic model for myelin-related axonopathy.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(7), pp. 2833–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12684470 (Accessed: 5 November 2018).

Sasaki, Y. *et al.* (2014) 'Identification of axon-enriched MicroRNAs localized to growth cones of cortical neurons', *Developmental Neurobiology*, 74(3), pp. 397–406. doi: 10.1002/dneu.22113.

Sasaki, Y. *et al.* (2016) 'NMNAT1 inhibits axon degeneration via blockade of SARM1-mediated NAD+ depletion', *eLife*, 5. doi: 10.7554/eLife.19749.

Sasaki, Y., Araki, T. and Milbrandt, J. (2006) 'Stimulation of Nicotinamide Adenine Dinucleotide Biosynthetic Pathways Delays Axonal Degeneration after Axotomy', *Journal of Neuroscience*, 26(33), pp. 8484–8491. doi: 10.1523/JNEUROSCI.2320-06.2006.

Schindelin, J. *et al.* (2012) 'Fiji: an open-source platform for biological-image analysis', *Nature Methods*, 9(7), pp. 676–682. doi: 10.1038/nmeth.2019.

Schouten, M. *et al.* (2012) 'New Neurons in Aging Brains: Molecular Control by Small Non-Coding RNAs.', *Frontiers in neuroscience*. Frontiers Media SA, 6, p. 25. doi: 10.3389/fnins.2012.00025.

Schratt, G. M. *et al.* (2006) 'A brain-specific microRNA regulates dendritic spine development', *Nature*, 439(7074), pp. 283–289. doi: 10.1038/nature04367.

Schubert, D. and Piasecki, D. (2001) 'Oxidative glutamate toxicity can be a component of the excitotoxicity cascade.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(19), pp. 7455–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11567035 (Accessed: 24 February 2019).

Schwarz, D. S. *et al.* (2003) 'Asymmetry in the assembly of the RNAi enzyme complex.', *Cell*, 115(2), pp. 199–208. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14567917 (Accessed: 17 January 2019).

Seward, M. E. *et al.* (2013) 'Amyloid-β signals through tau to drive ectopic neuronal cell cycle re-entry in Alzheimer's disease.', *Journal of cell science*. The Company of Biologists Ltd, 126(Pt 5), pp. 1278–86. doi: 10.1242/jcs.1125880.

Shigeoka, T. *et al.* (2016) 'Dynamic Axonal Translation in Developing and Mature Visual Circuits', *Cell*. Cell Press, 166(1), pp. 181–192. doi: 10.1016/J.CELL.2016.05.029.

Siegel, G. *et al.* (2009) 'A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis', *Nature Cell Biology*, 11(6), pp. 705–716. doi: 10.1038/ncb1876.

Siegel, G., Saba, R. and Schratt, G. (2011) 'microRNAs in neurons: manifold regulatory roles at the synapse', *Current Opinion in Genetics & Development*, 21(4), pp. 491–497. doi: 10.1016/j.gde.2011.04.008.

da Silva, J. S. and Dotti, C. G. (2002) 'Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis.', *Nature reviews. Neuroscience*, 3(9), pp. 694–704. doi: 10.1038/nrn918.

Smith, D. H. (2009) 'Stretch growth of integrated axon tracts: extremes and exploitations.', *Progress in neurobiology*. NIH Public Access, 89(3), pp. 231–9. doi: 10.1016/j.pneurobio.2009.07.006.

Smith, M. I. and Deshmukh, M. (2007) 'Endoplasmic reticulum stress-induced apoptosis requires bax for commitment and Apaf-1 for execution in primary neurons', *Cell Death & Differentiation*, 14(5), pp. 1011–1019. doi: 10.1038/sj.cdd.4402089.

Sood, R. *et al.* (2001) 'Cloning and Characterization of 13 Novel Transcripts and the Human RGS8 Gene from the 1q25 Region Encompassing the Hereditary Prostate Cancer (HPC1) Locus', *Genomics*, 73(2), pp. 211–222. doi: 10.1006/geno.2001.6500.

Spaulding, E. L. and Burgess, R. W. (2017) 'Accumulating Evidence for Axonal Translation in Neuronal Homeostasis', *Frontiers in Neuroscience*. Frontiers, 11, p. 312. doi: 10.3389/fnins.2017.00312.

Di Stefano, M. *et al.* (2015) 'A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration', *Cell Death & Differentiation*. Nature Publishing Group, 22(5), pp. 731–742. doi: 10.1038/cdd.2014.164.

Di Stefano, M. *et al.* (2017) 'NMN Deamidase Delays Wallerian Degeneration and Rescues Axonal Defects Caused by NMNAT2 Deficiency In Vivo', *Current Biology*, 27(6), pp. 784–794. doi: 10.1016/j.cub.2017.01.070.

Di Stefano, M. and Conforti, L. (2013) 'Diversification of NAD biological role: the importance of location.', *The FEBS journal*, 280(19), pp. 4711–28. doi: 10.1111/febs.12433.

Sutton, M. A. and Schuman, E. M. (2006) 'Dendritic Protein Synthesis, Synaptic Plasticity, and Memory', *Cell*, 127(1), pp. 49–58. doi: 10.1016/j.cell.2006.09.014.

Tahirovic, S. and Bradke, F. (2009) 'Neuronal Polarity', *Cold Spring Harbor Perspectives in Biology*, 1(3), pp. a001644–a001644. doi: 10.1101/cshperspect.a001644.

Takano, T. *et al.* (2015) 'Neuronal polarization', *Development*, 142(12), pp. 2088–2093. doi: 10.1242/dev.114454.

Tehranian, R. *et al.* (2008) 'Disruption of Bax protein prevents neuronal cell death but produces cognitive impairment in mice following traumatic brain injury.', *Journal of neurotrauma*. Mary Ann Liebert, Inc., 25(7), pp. 755–67. doi: 10.1089/neu.2007.0441.

Tognini, P. *et al.* (2011) 'Experience-dependent expression of miR-132 regulates ocular dominance plasticity.', *Nature neuroscience*. Europe PMC Funders, 14(10), pp. 1237–9. doi: 10.1038/nn.2920.

Tokarz, P., Kaarniranta, K. and Blasiak, J. (2016) 'Role of the Cell Cycle Re-Initiation in DNA Damage Response of Post-Mitotic Cells and Its Implication in the Pathogenesis of Neurodegenerative Diseases', *Rejuvenation Research*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA , 19(2), pp. 131–139. doi: 10.1089/rej.2015.1717.

Tomari, Y. *et al.* (2004) 'RISC assembly defects in the Drosophila RNAi mutant armitage.', *Cell*, 116(6), pp. 831–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15035985 (Accessed: 17 January 2019).

Vakifahmetoglu, H., Olsson, M. and Zhivotovsky, B. (2008) 'Death through a tragedy: mitotic catastrophe', *Cell Death & Differentiation*. Nature Publishing Group, 15(7), pp. 1153–1162. doi: 10.1038/cdd.2008.47.

Varcianna, A. *et al.* (2019) 'Micro-RNAs secreted through astrocyte-derived extracellular vesicles cause neuronal network degeneration in C9orf72 ALS', *EBioMedicine*. Elsevier. doi: 10.1016/J.EBIOM.2018.11.067.

Vitale, I. *et al.* (2011) 'Mitotic catastrophe: a mechanism for avoiding genomic instability', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 12(6), pp. 385–392. doi: 10.1038/nrm3115.

Vo, N. *et al.* (2005) 'From The Cover: A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis', *Proceedings of the National Academy of*

*Sciences*, 102(45), pp. 16426–16431. doi: 10.1073/pnas.0508448102.

Vo, N. K., Cambronne, X. A. and Goodman, R. H. (2010) 'MicroRNA pathways in neural development and plasticity', *Current Opinion in Neurobiology*, 20(4), pp. 457–465. doi: 10.1016/j.conb.2010.04.002.

Vogt, K. C. (1842) *Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte ... - Carl I Vogt - Βιβλία Google*. Available at: https://books.google.co.uk/books/about/Untersuchungen_über_die_Entwicklungsges.html?id=FjtOAAAAcAAJ&redir_esc=y (Accessed: 21 February 2019).

Waller, A. (1851) 'Experiments on the Section of the Glosso-Pharyngeal and Hypoglossal Nerves of the Frog, and Observations of the Alterations Produced Thereby in the Structure of Their Primitive Fibres.', *Edinburgh medical and surgical journal*. Biomedical Journal Digitization Project, 76(189), pp. 369–376. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30332247 (Accessed: 29 October 2018).

Wanet, A. *et al.* (2012) 'miR-212/132 expression and functions: within and beyond the neuronal compartment', *Nucleic Acids Research*, 40(11), pp. 4742–4753. doi: 10.1093/nar/gks151.

Wang, B. *et al.* (2015) 'FMRP-Mediated Axonal Delivery of miR-181d Regulates Axon Elongation by Locally Targeting Map1b and Calm1', *Cell Reports*, 13(12), pp. 2794–2807. doi: 10.1016/j.celrep.2015.11.057.

Wang, B., Yanez, A. and Novina, C. D. (2008) 'MicroRNA-repressed mRNAs contain 40S but not 60S components.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(14), pp. 5343–8. doi: 10.1073/pnas.0801102105.

Wang, D. O., Martin, K. C. and Zukin, R. S. (2010) 'Spatially restricting gene expression by local translation at synapses', *Trends in Neurosciences*, 33(4), pp. 173–182. doi: 10.1016/j.tins.2010.01.005.

Wang, Y. *et al.* (2016) 'Epithelial microRNA-9a regulates dendrite growth through Fmi-Gq signaling in *Drosophila* sensory neurons', *Developmental Neurobiology*, 76(2), pp. 225–237. doi: 10.1002/dneu.22309.

Wei, M. C. *et al.* (2001) 'Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 292(5517), pp. 727–30. doi: 10.1126/science.1059108.

Weinberg, M. S. and Wood, M. J. A. (2009) 'Short non-coding RNA biology and neurodegenerative disorders: novel disease targets and therapeutics', *Human Molecular Genetics*, 18(R1), pp. R27–R39. doi: 10.1093/hmg/ddp070.

Wen, Y. *et al.* (2011) 'Nmnat exerts neuroprotective effects in dendrites and axons.', *Molecular and cellular neurosciences*. NIH Public Access, 48(1), pp. 1–8. doi: 10.1016/j.mcn.2011.05.002.

Wightman, B. *et al.* (1991) 'Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development.', *Genes & development*, 5(10), pp. 1813–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1916264 (Accessed: 21 January 2019).

Wolf, S. A., Boddeke, H. W. G. M. and Kettenmann, H. (2017) 'Microglia in Physiology and Disease', *Annual Review of Physiology*, 79(1), pp. 619–643. doi: 10.1146/annurev-physiol-

022516-034406.

Wong, H. K. *et al.* (2005) 'Mutually exclusive subsets of BH3-only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenite.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 25(19), pp. 8732–47. doi: 10.1128/MCB.25.19.8732-8747.2005.

Wu, H. *et al.* (2000) 'Human RNase III is a 160-kDa protein involved in preribosomal RNA processing.', *The Journal of biological chemistry*, 275(47), pp. 36957–65. doi: 10.1074/jbc.M005494200.

Xiang, H. *et al.* (1998) 'Bax involvement in p53-mediated neuronal cell death.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(4), pp. 1363–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9454845 (Accessed: 17 May 2019).

Xiong, R. *et al.* (2014) 'MicroRNA-494 reduces DJ-1 expression and exacerbates neurodegeneration', *Neurobiology of Aging*, 35(3), pp. 705–714. doi: 10.1016/j.neurobiolaging.2013.09.027.

Xu, B. *et al.* (2017) 'Neurons secrete miR-132-containing exosomes to regulate brain vascular integrity.', *Cell research*. Nature Publishing Group, 27(7), pp. 882–897. doi: 10.1038/cr.2017.62.

Xu, Z. *et al.* (2013) 'MicroRNA-181 Regulates CARM1 and Histone Aginine Methylation to Promote Differentiation of Human Embryonic Stem Cells', *PLoS ONE*. Edited by A. Wutz, 8(1), p. e53146. doi: 10.1371/journal.pone.0053146.

Xue, Q. *et al.* (2011) 'Human activated CD4+ T lymphocytes increase IL-2 expression by downregulating microRNA-181c', *Molecular Immunology*, 48(4), pp. 592–599. doi: 10.1016/j.molimm.2010.10.021.

Yalowitz, J. A. *et al.* (2004) 'Characterization of human brain nicotinamide 5'-mononucleotide adenylyltransferase-2 and expression in human pancreas.', *The Biochemical journal*, 377(Pt 2), pp. 317–26. doi: 10.1042/BJ20030518.

Yan, T. *et al.* (2010) 'Nmnat2 delays axon degeneration in superior cervical ganglia dependent on its NAD synthesis activity', *Neurochemistry International*, 56(1), pp. 101–106. doi: 10.1016/j.neuint.2009.09.007.

Ye, Y. *et al.* (2018) 'A lincRNA-p21/miR-181 family feedback loop regulates microglial activation during systemic LPS- and MPTP- induced neuroinflammation.', *Cell death & disease*. Nature Publishing Group, 9(8), p. 803. doi: 10.1038/s41419-018-0821-5.

Yi, R. *et al.* (2003) 'Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs.', *Genes & development*, 17(24), pp. 3011–6. doi: 10.1101/gad.1158803.

Yoda, M. *et al.* (2010) 'ATP-dependent human RISC assembly pathways', *Nature Structural & Molecular Biology*, 17(1), pp. 17–23. doi: 10.1038/nsmb.1733.

Yurov, Y. B., Vorsanova, S. G. and Iourov, I. Y. (2011) 'The DNA replication stress hypothesis of Alzheimer's disease.', *TheScientificWorldJournal*. Hindawi, 11, pp. 2602–12. doi: 10.1100/2011/625690.

Zamorano, S. *et al.* (2012) 'A BAX/BAK and Cyclophilin D-Independent Intrinsic Apoptosis Pathway', *PLoS ONE*. Edited by D. Chandra. Public Library of Science, 7(6), p. e37782. doi: 10.1371/journal.pone.0037782.

Zappulo, A. *et al.* (2017) 'RNA localization is a key determinant of neurite-enriched proteome', *Nature Communications*. Nature Publishing Group, 8(1), p. 583. doi: 10.1038/s41467-017-00690-6.

Zhai, R. G. *et al.* (2006) 'Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity.', *PLoS biology*. Public Library of Science, 4(12), p. e416. doi: 10.1371/journal.pbio.0040416.

Zhai, R. G. *et al.* (2008) 'NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration', *Nature*. Nature Publishing Group, 452(7189), pp. 887–891. doi: 10.1038/nature06721.

Zhang, H. *et al.* (2004) 'Single processing center models for human Dicer and bacterial RNase III.', *Cell*, 118(1), pp. 57–68. doi: 10.1016/j.cell.2004.06.017.

Zhang, Q. *et al.* (2013) 'MicroRNA-181a suppresses mouse granulosa cell proliferation by targeting activin receptor IIA.', *PloS one*. Public Library of Science, 8(3), p. e59667. doi: 10.1371/journal.pone.0059667.

Zhang, R. *et al.* (2015) 'MeCP2 Plays an Analgesic Role in Pain Transmission through Regulating CREB / miR-132 Pathway', *Molecular Pain*, 11, pp. s12990-015–0015. doi: 10.1186/s12990-015-0015-4.

Zhou, S. *et al.* (2011) 'Early changes of microRNAs expression in the dorsal root ganglia following rat sciatic nerve transection', *Neuroscience Letters*, 494(2), pp. 89–93. doi: 10.1016/j.neulet.2011.02.064.

Zhu, H. *et al.* (2018) 'miR-129 controls axonal regeneration via regulating insulin-like growth factor-1 in peripheral nerve injury.', *Cell death & disease*. Nature Publishing Group, 9(7), p. 720. doi: 10.1038/s41419-018-0760-1.

Zivraj, K. H. *et al.* (2010) 'Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(46), pp. 15464–78. doi: 10.1523/JNEUROSCI.1800-10.2010.

Zong, W. X. *et al.* (2001) 'BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak.', *Genes & development*. Cold Spring Harbor Laboratory Press, 15(12), pp. 1481–6. doi: 10.1101/gad.897601.