Universidade de Aveiro
2021

Departamento de Ciências Médicas

JOSÉ JOÃO MACEDO FELGUEIRAS

UNRAVELING THE AXONAL PROTEOME OF HIPPOCAMPAL NEURONS

CARACTERIZAÇÃO DO PROTEOMA AXONAL DE NEURÓNIOS DO HIPOCAMPO

Universidade de Aveiro
2021

Departamento de Ciências Médicas

JOSÉ JOÃO MACEDO FELGUEIRAS

UNRAVELING THE AXONAL PROTEOME OF HIPPOCAMPAL NEURONS

Dissertation submitted to the University of Aveiro to fulfil the requirements for obtaining a master's degree in Molecular Biomedicine, held under the scientific guidance of professor Ramiro Daniel Carvalho de Almeida, assistant professor at the Department of Medical Sciences, University of Aveiro, and scientific co-supervising of Professor Rui Miguel Pinheiro Vitorino.

To my family,
For their patience and faith

the jury

president	Prof Dr. Bruno Miguel Rodrigues das Neves, Assistant Professor at the Department of Medical Sciences, University of Aveiro, Portugal
arguer	Dr. Bruno José Fernandes Oliveira Manadas, Principal Investigator at the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal
supervisor	Prof. Dr. Ramiro Daniel Carvalho de Almeida, assistant professor at the Department of Medical Sciences, University of Aveiro, Portugal

Acknowledgments

I would like to express my deepest and sincere gratitude to my supervisor, Professor Ramiro Almeida, for the support and generosity with which he received me into his group. I also wanted to express my heartfelt thanks for giving me a new chance and for all the efforts to provide me a way to finish my master's, despite all the adversities during these unusual times. It meant a lot to me and I won't forget that gesture. I would also like to thank him for helping me develop this work and all the valuable contributions, without which this thesis would not be the same. It was a great privilege to work and study under his guidance and I am extremely grateful for everything he has offered me.

I would also like to address my thanks to my co-supervisor, Professor Rui Vitorino, who despite the unusual circumstances, and the distance did his best to teach me everything that I know about proteomics and bioinformatics. I am extremely grateful for his availability and willingness to help me during this work, sharing his knowledge.

I also express thanks to those who shared their journey with me, and who, in some way not only made this phase of my life so special and peculiar but also taught me and helped me to build the person that I am today. At the end of the day, I keep doing all these things for myself but also because I am surrounded by all these amazing and impressive people, who motivate me, believe in me, and show me that I can do better.

I am also very much thankful to my family, for their love, caring, and sacrífices for educating me and preparing me for my future life. They did the best they could so that I can have a better future and, therefore, this work also has a bit of a victory flavor to them. Thank you for supporting me, for always being there, and for not letting me give up. Throughout my life, they never stopped fighting and I will always take that with me for the rest of my life. Thank you.

palavras-chave

Proteoma Axonal, Síntese Proteica Local, Proteómica Quantitativa, Análise Bioinformática, Doenças Neurológicas

resumo

Os axónios são longas projeções das células nervosas, responsáveis por conduzem impulsos elétrico-químicos. Tanto a longa extensão que os axónios podem atingir, como também a complexidade morfológica e plasticidade neuronal, tornam especialmente desafiante para os neurónios a manutenção de uma função adequada. O transporte ao longo dos neurónios vem dar resposta às necessidades supramencionadas. No entanto, este mecanismo celular enfrenta ainda alguns obstáculos, como os longos períodos de tempo que as cargas levam a ser transportadas, tendo em conta a dimensão e arquitetura neuronal. Hoje em dia, é já aceite uma sinergia entre o transporte axonal e a tradução local, que atuam estreitamente para regular o proteoma axonal e, por conseguinte, funções axonais vitais, como a plasticidade sináptica, regeneração neuronal e projeções axonais. Assim, o proteoma axonal é composto pelo reportório das proteínas presentes nos axónios num determinado momento, estando estas também sujeitas a um processo contínuo e dinâmico de mudança. Embora grandes esforços tenham vindo a ser feitos, a maioria dos estudos disponíveis na literatura utilizam técnicas transcriptómicas, que podem diferir significativamente da expressão celular proteica. Neste estudo, descrevemos pela primeira vez o proteoma axonal em neurónios do hipocampo de rato. Adicionalmente, para obter uma compreensão sistemática e funcional das proteínas presentes no axónio, realizamos uma análise de alto rendimento recorrendo a várias ferramentas de bioinformática para catalogar e caracterizar extensivamente o proteoma axonal dos neurónios do hipocampo. Além disso, executamos uma análise de enriquecimento de dados e investigamos o *fingerprints* de diferentes doenças neurológicas, usando várias bases de dados. Desta forma permitiu-nos criar um perfil de associação de doenças com o proteoma axonal com vista a identificar possíveis futuros candidatos de pesquisa ou translação clínica.

keywords

Axonal proteome, Local protein synthesis, Quantitative proteomics, Bioinformatic analysis, Neurological disorders

abstract

Axons are long projections of a nerve cell responsible to conduct electrochemical impulses. The length of axons, their morphological complexity long axonas and neuronal plasticity poses a challenge to maintain cellular function. Transport along the neurons can address the mentioned hurdles. However, this cellular mechanism has some drawbacks associated, such as the long time to transport cargos from one point to another due to the neuronal size and architecture. Nowadays, a synergy between axonal transport and local translation is well accepted, both working tightly to regulate the axonal proteome. Therefore, these mechanisms have a crucial role in vital axonal functions, such as synaptic plasticity, axonal pathfinding, and regeneration. The axonal proteome is composed of the entire repertoire of the present proteins, in the axons at a given moment, and is in a continuous and dynamic changing process. Although great efforts have been made, the majority of studies available in literature use transcriptomic techniques, which can significantly differ from protein cell expression. In the present study, we reported for the first time the axonal proteome in developing hippocampal rat neurons. Additionally, to gain a systemic understanding of the functional and dynamic role of these proteins, we perform a high-throughput analysis, using multiple bioinformatic tools to catalogue and extensively characterize the axonal proteome. In addition, we run an enrichment analysis and search for different neurological disorders fingerprints among the proteome, using several databases to create a disease-association profile, and ultimately identify possible future research candidates to integrate in clinical translation.

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

ACTB - Actin Beta

AD - Alzheimer's disease

ALDOA – Aldolase A

ALS - Amyotrophic Lateral Sclerosis

APOE - Apolipoprotein E

APP – beta Amyloid Precursor Protein

ARF5 - ADP Ribosylation Factor 5

ASD - Autism Spectrum Disorders

ATF4 - Activating Transcription Factor 4

ATP5F1B - ATP Synthase F1 Subunit Beta

BDNF - Brain Derived Neurotrophic Factor

BP - Biological Process

CC - Cellular Component

CCT2 - Chaperonin Containing TCP1 Subunit 2

CLU – Clusterin

CREB1 - Cyclic AMP-Responsive Element-Binding Protein 1

CRYM - Crystallin Mu

CSF - Cerebrospinal Fluid

DBN1 - Drebrin 1

DGR - Dorsal Root Ganglion Cells

DIGE - Differential In-Gel Electrophoresis

DYNC1H1 - Dynein Cytoplasmic 1 Heavy Chain 1

eEF1 - Eukaryotic translation elongation factor 1

eIF4 - Eukaryotic Initiation Factor 4

eIF4A2 - Eukaryotic Translation Initiation Factor 4A2

FMRP - Fragile X mental retardation protein

GAP43 - Growth Associated Protein 43

GDI1 - GDP Dissociation Inhibitor 1

GO - Gene Ontology

hESC - human Embryonic stem cells

HMGB1 - High mobility group protein B1

HNRNPA1 - Heterogeneous nuclear ribonucleoprotein A1

IGF1 - Insulin Like Growth Factor 1

IDE - Insulin-Degrading Enzyme

iPSC - Induced Pluripotent Stem Cells

iTRAQ - Isobaric tags for relative and absolute quantitation

KEGG - Kyoto Encyclopedia of Genes and Genomes

KPNB1 - Importin Subunit beta-1

L1CAM - L1 cell adhesion molecule

LC - Liquid chromatography

LC-MS/MS - Liquid Chromatography-Tandem Mass Spectrometry

LCM – Laser Capture Microdissection

LMNB1 - Lamin B1

LRP1 - Low Density Lipoprotein Receptor-Related Protein 1

LTD - long-term depression

LTP - long-term potentiation

MAP2 - Microtubule-associated protein 2

MAP2K1 - Dual Specificity Mitogen-Activated Protein Kinase Kinase 1

MAPT - Microtubule Associated Protein Tau

MATR3 - Matrin 3

MF - Molecular Function

miRNA - Micro RNA

mRNA - Messenger RNA

mRNP - mRNA Ribonucleoprotein

MS - Mass Spectrometry

MS/MS -Tandem Mass Spectrometry

MYH10 - Myosin Heavy Chain 10

NME1 - Nucleoside Diphosphate kinase A

OPRM1 - Opioid Receptor Mu 1

PD - Parkinson's disease

PDMS - Polydimethylsiloxane

PRPH - Peripherin

PTN - Pleiotrophin

RBP - RNA Binding Proteins

RGC - Retinal Ganglion Cell

RNA - Ribonucleic Acid

RNP - Ribonucleoprotein Particles

RPL18 - Ribosomal Protein L18

RPSA - Ribosomal Protein SA

SCN1A - Sodium Voltage-Gated Channel Alpha Subunit 1

SILAC - Stable Isotope-Labelling Method

SOD1 - Superoxide Dismutase 1

STAT3 - Signal transducer and activator of transcription 3

tRNA - transfer RNA

TUBA1A - Tubulin Alpha 1a

uORF - Upstream Open Reading Frame

UTR - Untranslated Regions

VCP - Valosin Containing Protein

ZBP1 - Zipcode Binding Protein-1

CHAPTER I: INTRODUCTION

NEURONAL CELLS AND CELLULAR TRANSPORTATION CHALLENGES

Neurons, or nerve cells, are very specialized cells from the nervous system responsible for conducting an electrochemical impulse, allowing communication and interaction with the environment through specialized connections called synapses. These very specialized and polarized cells have a distinct morphology compared to other cell types in the body. Neurons are composed of a cell body (or soma), a single axon, and multiple branched dendrites, as illustrated in figure 1. The latter is responsible for receiving different stimuli and input from other cells, and then generates an action potential, which travels down the axons to cause the release of neurotransmitter in the synapse.

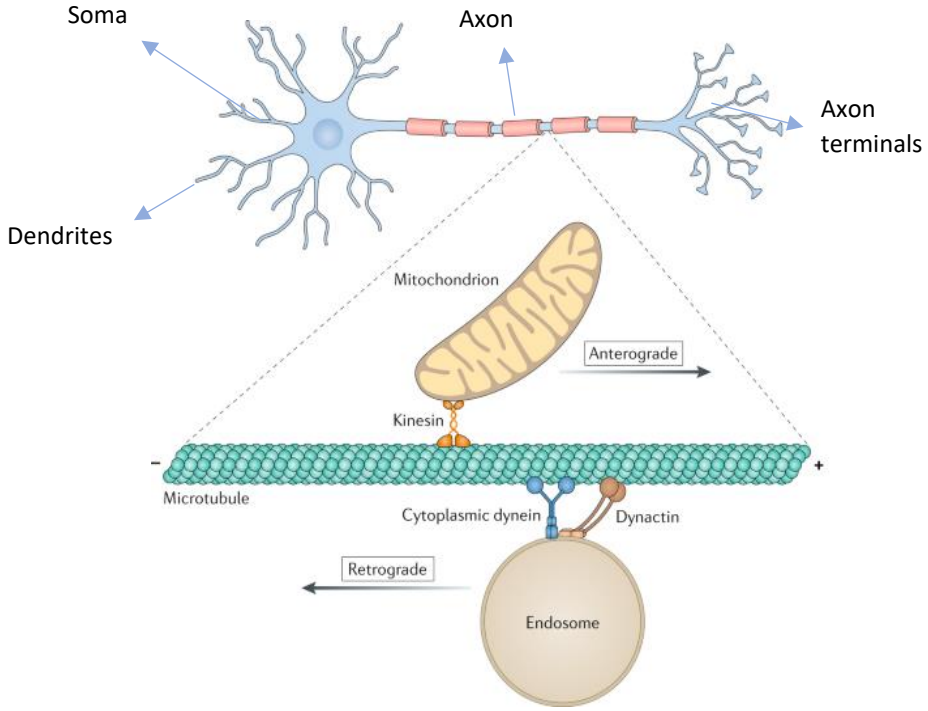


Figure 1 – Schematic representation of a neuron and its different constituents namely: dendrites, soma, axons and axonal terminals. Illustration of the axonal transport machinery. Adapted from: (Sleigh, Rossor, Fellows, Tosolini, & Schiavo, 2019)

During evolution, species developed into bigger animals, which would require long distant neurons for a proper nervous system function (Gonzalez & Couve, 2014). For example, as illustrated in figure 2, whales have incredibly large bodies and neurons that can reach up to 30 meters long. In these animals, axons can emanate towards other cells and grow hundreds of centimetres in length to reach their target. Not only the morphological complexity but also the neuronal plasticity made the long axonal length very challenging to respond to stimuli in a short period. Transport along the neurons, or axonal transport, can address the necessities mentioned above.

The cellular transport is defined by any dynamic intracellular movement of either molecules, organelles, vesicles, membrane components, or other substances, in order to transport and/ or arrange spatially the cellular constituents (Maday, Twelvetrees,

Moughamian, & Holzbaur, 2014). The prefabricated components in the soma are transported through the cytoskeleton, depending on the necessities of the cell on other sites. In this cellular mechanism, cytoskeletal filaments, such as microtubules, function as a rail, and the superfamily of motor proteins, dynein and kinesis, function as a train that transport the cargo. This bidirectional, ATP-dependent, process is defined by which motor protein is being used (figure 1). For instance, kinesins move along the growing plus end and mediate transport towards the axon – **anterograde transport**. On the other hand, dynein moves along the stable minus end and mediates transport towards the soma – **retrograde transport**. The anterograde transport is used by the cell to supply distant regions with essential molecules, such as neurotransmitters, vesicles, or mitochondria. On the opposite, the cell recurs to retrograde transport to send signals to the soma, to transport exogenous substances, or respond to nerve injury (George J Siegel et al.).

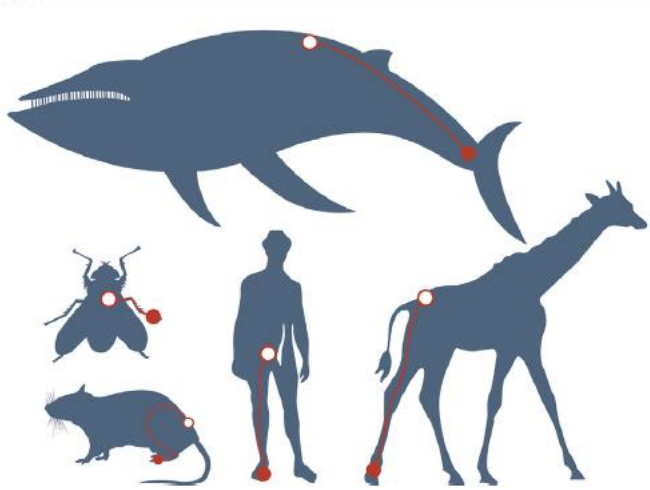


Figure 2 - Variation of axonal length within different animal species, ranging from few microns to meters. Giraffe, for example, has a laryngeal nerve that can measure up to 5 meters. Source:(Gonzalez & Couve, 2014)

For many years, axonal transport was thought to be the only pathway able to supply distant cellular regions. In this classical model, the axonal transport was divided into two subcategories, namely, fast and slow transport, with an average rate of 50-100 mm/day and 0.2-10 mm/day, respectively (Sleigh et al., 2019). Although both mechanisms are carried by the same motor proteins, the different velocities only depend on the stationary time of the cargo. For example, considering a one-meter-long neuron, the average time required for fast transport to the tip of the axon is estimated around 1 week. However, for the same length neuron, it would take about one year to reach the same location, in slow axonal transportation. Nonetheless, in some occasions slow transport can be preferable to the cell, since it is able to deliver up to three times more cargo than fast axonal transport.

Despite this oversimplified explanation of the transport process, axonal transport also includes modulators, several adapters, and regulatory proteins, which increases its complexity (Maday et al., 2014). As would be expected any dysregulation in one of these components might compromise the viable function of the cell to transport its cargo, and

therefore, may be related to pathological states. For instance, mutations in transport machinery can lead to neurological disorders. Several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis have been reported to have impairments in axonal trafficking (Roy, Zhang, Lee, & Trojanowski, 2005; Sleigh et al., 2019). In this type of pathogenesis, the neuronal dysfunction is caused by either accumulation of cargos or inability to traffic important molecules or cues. Discovering the underlying molecular mechanism could bring diversified targeted approaches to some of these diseases.

THE AXONAL PROTEOME

Neuroproteomics is the field that studies the neuronal proteome, which refers to the group of proteins produced in the nervous system. This pool of proteins is vital for normal structural and physio-morphological functions of neurons, and consequently to the nervous system.

Due to the extension of the neuronal cytoplasm and the spatial distance with the primary source of the proteins (the nucleus), the protein content changes within the different subcellular regions, creating localized proteomes. Thus, the local proteome refers to a subcellular spatial domain, such as dendrites or axons, where there is a distinct subset of proteins, which differs considerably from the global proteome. As such, the axonal proteome is defined by the entire repertoire of the present proteins, in the axons, at a given moment. This proteome repertoire can be either produced locally, in the axon, or in the cell soma and carried to different subcellular compartments. This pool of proteins is in a continuous and dynamic changing process according to the different cell internal activities, metabolism, and external stimuli. In a more detailed look, the proteome is maintained by different cellular mechanisms such as protein synthesis and protein degradation. In a non-pathological state, there is homeostasis between these two-key processes, the proteostasis (Yerbury, Farrawell, & McAlary, 2020). Protein synthesis is an anabolic process responsible for *de novo* production of proteins and mainly depends on transcription, translation, mRNA localization, alternative splicing, chaperones, and trafficking. On the opposite side, protein degradation also helps to regulate the proteome by removing proteins through processes as autophagy, ubiquitin-proteosome system, secretion, and mRNA decay.

It is important to note that the local proteome, and in particular the axonal proteome, depends not only on the double biosynthetic processes previously mentioned (axonal and somal), but also on the recycling of axonal components (eg.: synaptic vesicles) and endocytosis. All these sources directly impact and regulate the bulk of present proteins (Gonzalez & Couve, 2014; Sann, Wang, Brown, & Jin, 2009). In addition, has been recently a growing awareness of the importance of both glial cells and Schwann cells in the axonal proteome. Ultimately, these non-neuronal cells might contribute with several proteins and ribosomes, that are transferred to the axons (Farias, Sotelo, & Sotelo-Silveira, 2019). Thus,

to better mimicry the *in vivo* environments, in the future, these research models should include these support cells. However, throughout this work, we will only focus on proteins from axonal biosynthesis or proteins transported by axonal trafficking.

LOCAL PROTEIN SYNTHESIS

In the classic perspective, the soma was considered the only supplier of newly synthesized proteins (E. Kim & Jung, 2015; Satkauskas & Bagnard, 2007). However, as we saw before, neurons are highly polarized cells that can attain a very long length to reach their target, and axonal transport may not be completely efficient in these cases. Hence, to overcome this drawback, and to optimize a more rapid and autonomous response to chemotropic and environmental stimuli, local protein synthesis provides several advantages to the neurons over the transport of prefabricated proteins. In this mechanism, after transcription in the nucleus, the mRNA is transported throughout the cytoskeleton and translated into the axon. In addition, mRNAs can as well agglomerate in mRNA-ribonucleoprotein (mRNPs) vesicles and be stored for later translation (Figure 3). These mRNAs are tagged to a specific sub localization within the cell, where they are rapidly translated according to the cellular necessities. This cellular mechanism is directly involved in the renewal and transformation

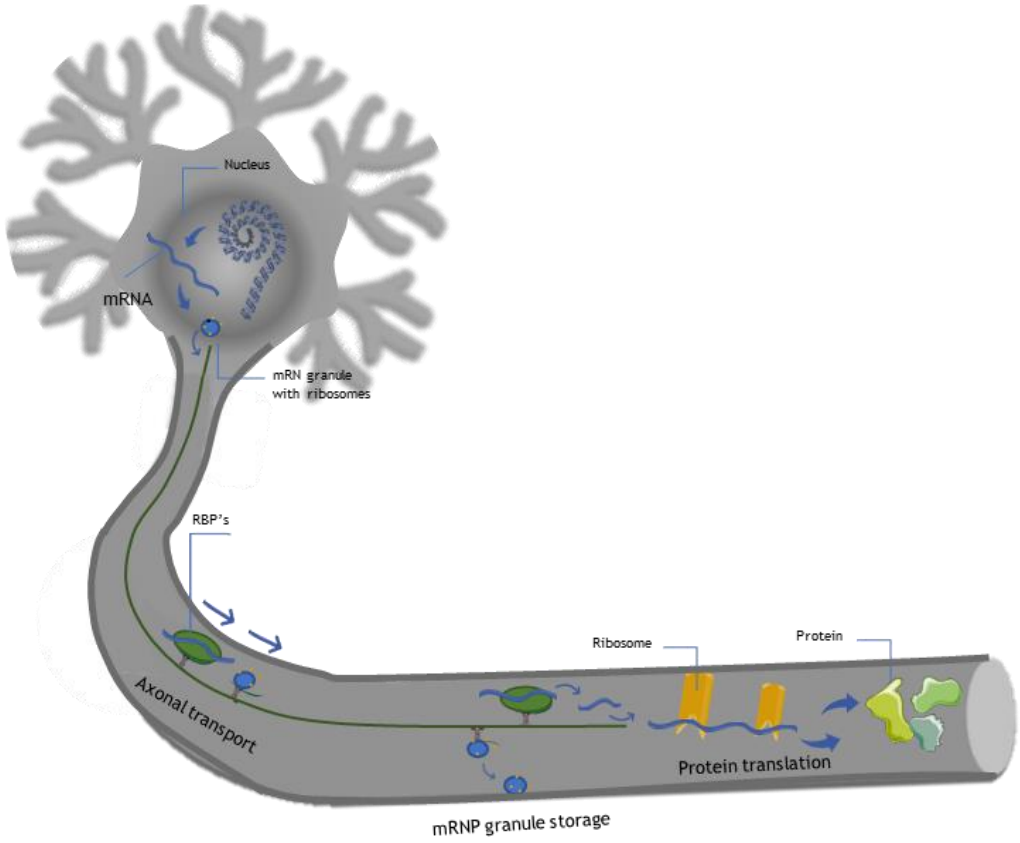


Figure 3 - Axonal protein synthesis illustration: mRNA is transcribed in the nucleus and can be either packed in mRNP granules with ribosomes or be transported with RBP. These molecules are transported using motor proteins of the cytoskeleton. In the axon, local translation of some mRNA can occur, while other are stored in the mRNP granules for later use.

of the axonal protein content, which allows the cell to control the local proteome by regulating where, when, and what proteins the cell needs. Thus, local protein synthesis is a vital mechanism in several crucial processes such as axon survival, regeneration, guidance, and elongation (E. Kim & Jung, 2015).

The geographic separation of neuronal processes provides several **advantages** over axonal transportation. For instance, this mechanism not only takes advantage of the cell's capacity of storage to stash silent forms of mRNA and translate them only when required but also this process becomes more economic for the cell, since from one single copy of mRNA it is possible to make as many copies as necessary of the same protein. Consequently, this is not only more efficient than transport and store proteins but also reduces the risk to accumulate deficient proteins in the axons. Another advantage to produce protein locally is retrograde signalling, since enables the axon to communicate with the rest of the cell by producing retrograde cues, such as CREB and STAT3, which regulate transcription in the nucleus (Deglincerti & Jaffrey, 2012). In addition, locally synthesized proteins may, in fact, acquire different properties compared to those produced in the soma, as they suffer different post-translation modification. For example, since newly synthesized proteins, such as β-actin, have a lack of post-translational modifications, the β-actin pool produced in the nucleus has a distinct pattern from their newly synthesized counterparts (Farias et al., 2019). Therefore, it has been suggested that newly synthesized β-actin has other proprieties since can nucleate polymerization more efficiently than β-actin transported from the soma. Thus, this mechanism can provide an additional layer of signalling information. Another advantage comes from the massive number of synapses that axonal arbors can get and the synaptic mitochondrial enrichment, where local translation can also help to maintain a healthy supply of mitochondria to avoid neurodegeneration. In support of nuclear-produced proteins, axonally synthesized inner and outer mitochondrial membrane proteins allow to face the high turnover rate and finetune the mitochondrial replacement (Spaulding & Burgess, 2017). Lastly, in case of axonal injury, due to aggression or exposure to different environmental stimuli, cells are able to respond independently of soma regulation mechanisms. Thus, with localized synthesis, proteins are available within adequate time frames at sites far away from the cell body, and able to respond to the diverse range of cell necessities.

Local protein synthesis also plays crucial **functions** in the cell during several important neurological events, such as axon elongation and pathfinding, participating in growth cone guidance and pre-synapse formation. Growth cones are motile structures from the nervous system, that guide the axons to their correct target. This structure is highly sensitive to guidance cues, which produce chemotropic responses in growth cones, and induces protein synthesis depending on whether they are repulsive or attractant. Some of the classical families of guidance cues are slits, netrins, ephrins, and semaphorins. For instance, when stimulated with attractant cues, such as BDNF, growth cones prompt local production of β-actin. On the opposite, repulsive cues promote the translation of proteins that disassemble the cytoskeleton (A. C. Lin & Holt, 2007). Although the polarity of these stimuli, and the opposite responses that they trigger, they work together causing an asymmetrical

translation triggering different synthesis pathways, as illustrated in figure 4. This modulatory mechanism allows the axon to fine-tune their responses to subtle environmental changes, during growth cone expansion, instead of changing the whole proteome. In fact, when inhibited, local translation impaired elongation of regenerating axons, and growth cones immediately lose their ability to turn into chemotropic cues (Campbell & Holt, 2001; Verma et al., 2005). Also, this mechanism becomes extremely crucial since the majority of growth cone proteins (actin, neurofilaments, and mitochondria) travel through slow anterograde transport.

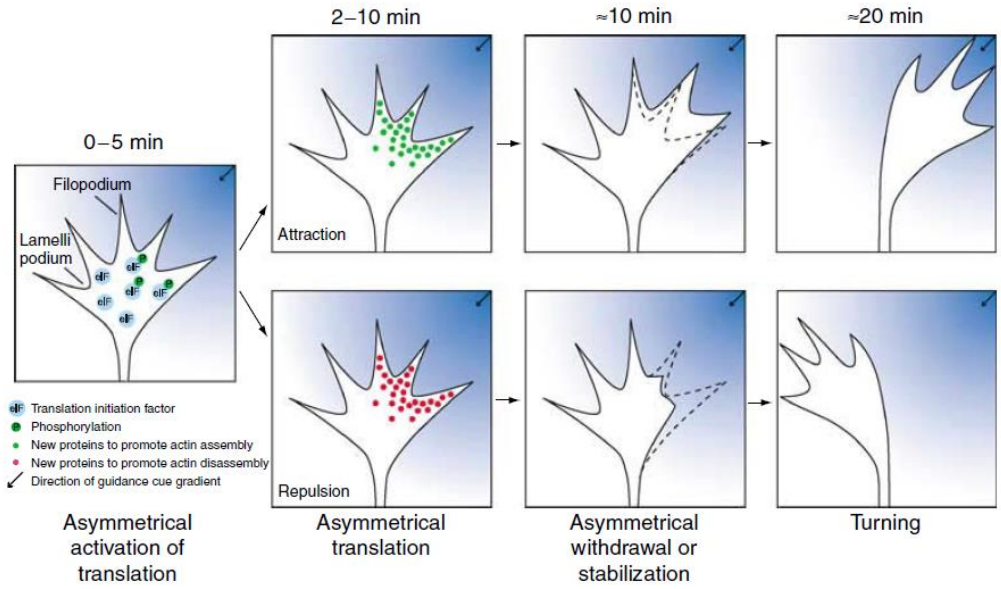


Figure 4 - Asymmetrical synthesis of cytoskeletal proteins in growth cones. Apadted from: (A. C. Lin & Holt, 2007)

Lastly, after arriving to its target, the local translation machinery starts to work on synapse formation, promoting the presynaptic growth and synapse stabilization (A. C. Lin & Holt, 2007). Similarly, to growth cones, the presence of specific mRNAs, active ribosomes, and RBP in pre-synapses confirms the undergoing local translation (Sasaki, 2020). The experimental detection of ribosomes using electron microscopy confirmed the presence of ribosomal protein in synaptosomes. Moreover, the identification of key players such as FMRP, which regulates local translation in synapses, helps understand the involvement of local translation in pre-synapse. Electron microscopy experiments have already identified the presence of FMRP granules in pre-synapse of hippocampal neurons and knock out of this gene led to upregulation of several gene expression levels (Sasaki, 2020).

Finally, even though the protein synthesis rate may be lower than that occurring in the cell body, the bulk of locally synthesized proteins might be over one order of magnitude than the soma, due to the long extension of the axon, as estimated by *Alvarez, et al.* (Alvarez & Benech, 1983).

EVIDENCE OF LOCAL PROTEIN SYNTHESIS

In 1960 Koenig and Koelle showed, for the first-time, evidence of resynthesis of acetylcholinesterase in axons of cat cholinergic neurons, in which this particular enzyme was irreversibly inactivated (E. Koenig & Koelle, 1960; Yoon, Zivraj, & Holt, 2009). This became the earliest evidence of local protein synthesis in axons. In the subsequent years, other historical marks about axonal protein synthesis emerged taking advantage of different labelling techniques and analytical methods (Brittis, Lu, & Flanagan, 2002; EDWARD KOENIG, 1997). Another major evidence was brought in 1969 when cultured squid giant axons, which were earlier separated from their soma, were able to incorporate radioactive amino acids into newly synthesized proteins (Giuditta, Dettbarn, & Brzin, 1968). Later in 1997, *Minnen et al.* demonstrated that isolated axons kept the capacity to translate injected foreign mRNA even without soma (MINNEN & BERGMAN, 1997).

Additionally, the observation of ribosomes in the initial segment of the axons brought insight into the presence of translation machinery in the axons. Further, electron microscopy and immunohistological studies allowed the detection of ribosomes in several types of axons of mammals (Jung, Yoon, & Holt, 2012). Although the earlier difficulty on detecting, the later confirmed existence of polysomes indicates the presence of actively translating ribosomes in the axon. Recently was confirmed, that their peripherical location along the axon, allows ribosomes to spatially isolate the translation processes to the sites where surface receptors receive the extrinsic cues.

Further, cytoskeleton proteins were among the first transcripts to be identified in axons. In fact, β-actin was amongst the first axonal mRNA to be detected. *Campenot et al*., who first develop a novel chamber for axon isolation, was able to recognize that both β-tubulin and β-actin mRNAs were among the most abundant translated proteins in axon (Eng, Lund, & Campenot, 1999). This finding quickly led to the assumption that local translation is a good candidate for how axons can quickly modulate their cytoskeleton. Ultimately, the observation of ribosomal RNAs, mRNAs, polysomes, and the presence of active puncta of the *de novo* synthesized proteins, finally made proof of an active local translation in mature neurons, concluding that local translation is directly involved in the modulation of the local proteome.

Along with the discovery of translation machinery in the axon, evidence of local protein synthesis during nervous system development and also during neuronal regeneration are among the most important findings in the field. As mentioned before, several important molecules that participate in growth cones have been reported. In fact, this involvement was first observed by the increased amount of β-actin after stimulation with neurotrophins (Spaulding & Burgess, 2017).

The findings that local translation was, in fact, part of the axonal development system, led to hypothesize that regenerative axons might depend on this mechanism as well. *In vitro* and *in vivo* experiments in adult rat dorsal root ganglion (DRG) cells and rat motor axons, respectively, confirmed the presence of translation machinery in the injury site. In fact,

during this injury condition, axons were able to increase their local translation and produce and secrete newly synthesized proteins (Spaulding & Burgess, 2017).

Recently, during the 'omics' era, several progresses have been made in terms of the axonal transcriptome and proteome, resulting in additional evidence of local translation. Consequently, there are already available several experimental evidence that provide a robust confirmation of the ability of local protein synthesis in axons. Thanks to advances and development of new methods that allowed the production of pure axonal preparations and techniques with higher sensitivity such as, mass spectrometry in proteomics, and RNA-seq in genomics, scientists were able to deeper characterize the axonal content. In addition, was possible to study alterations and variations in the gene expression during different conditions and pathological stages. Currently, about 20 papers have been published on the axonal transcriptome from different species, different types of cells, and physiological conditions (Farias et al., 2019). On the other hand, only 3 published papers on proteomics have reported the axonal proteome. As shown in table 1, the vast majority of the published articles report the axonal transcriptome, studied through mRNA expression. Since transcriptomics is a high sensitivity technique and has the capacity to amplify DNA molecules and sequence the entire molecule, this encourages most of the researchers to opt for this alternative. However, there is a poor correlation between the transcriptome and the proteome. For instance, high protein turnover, different half-lives, and post-transcription mechanisms make difficult to estimate the level of protein expression from the transcriptome. Hence, exploring proteomics approaches, such as mass spectrometry, to profile axonal protein expression, provides a better approximation to the real protein expression in the axon. Thus, a proteomic analysis can, as well, be a potential complementary approach to genetic analysis, since has the advantage of being more truthful to the cellular protein expression and cellular phenotype. There is still a lot of questions that remain to be answered, such as relative contribution of different neuronal compartments to the total proteome and how much of the local proteome actually comes from local translation.

Finally, chemotropic responses for growth cones; growth cone adaptation and gradient sensing; changes at intermediate targets; axonal elongation; synapse formation; transmitter biogenesis; cell survivance and axon maintenance; response to nerve injury and axon regeneration; and receptor expression, summarize all known functions of local protein synthesis in the axons (Jung et al., 2012)

Table 1 - Summary of both axonal transcriptome and proteome research articles. Adapted from (Farias et al., 2019)

Type of neuron	Method	No. of identified RNA	Reference
TRANSCIPTOMIC			
DRG injury conditioned (Rat)	Microarray	206	(Willis et al., 2007)
Cortical (Rat)	Microarray	Naïve~310 Regenerating~850	(Taylor et al., 2009)
Sympathetic neuron (Rat)	SAGE	~350	(Andreassi et al., 2010)
RGC (Xenopus -X-, Mouse-M)	Microarray	Stage 24: 171 Stage 32: 444 E16: 1800	(Zivraj et al., 2010)
DRG (Rat)	Microarray	E16: 2627 Adult: 2924	(Sahoo, Smith, Perrone-Bizzozero, & Twiss, 2018)
CA1 synaptic neuropil (Rat)	RNA-Seq	2550 (dendritic-axon)	(Cajigas et al., 2012)
Cortical (Mouse)	qPCR	105 miRNAs	(Sasaki, Gross, Xing, Goshima, & Bassell, 2014)
DRG (Mouse)	RNA-Seq	6118	(Minis et al., 2014)
Hippocampal (Rat)	RNA-Seq	775 mRNAs changes levels with Aβ1-42 treatment	(Baleriola et al., 2014)
Motorneuron (Mouse)	Microarray		(Saal, Briese, Kneitz, Glinka, & Sendtner, 2014)
Motorneuron (Mouse)	RNA-Seq	>11.000	(Briese et al., 2016)
N2A y CAD cells	RNA-Seq	778 in common	(Taliaferro et al., 2016)
Motorneuron (Mouse)	RNA-Seq	mRNAs: 1812	(Rotem et al., 2017)
hESC-neurons: glutamatergic	Microarray	3696 (highest expressed transcripts)	(Bigler, Kamande, Dumitru, Niedringhaus, & Taylor, 2017)
mESC: iNeurons	RNA-Seq	18111	(Zappulo et al., 2017)
iCell neurons: GABAergic and glutamatergic	RNA-Seq	~930	(Toth et al., 2018)
Gigant axon of Stellate cell	RNA-Seq	~8000	(Mathur et al., 2018)

(Squid)			
R cell (Drosophila)	T-TRAP (Rpl10)	9806	(K. X. Zhang, Tan, Pellegrini, Zipursky, & McEwen, 2016)
RGC (Mouse)	axon-TRAP (Rpl22)	E17.5 : 1783 P0.5 : 2117 P7.5 : 1419 Adult: 1217	(Shigeoka et al., 2016)
mESC: iNeurons (Mouse)	Ribo-Seq		(Zappulo et al., 2017)
Cortical (Mouse)	Synap-TRAP (Rpl10a)	1398	(Ouwenga et al., 2017)
mESC-derived motor neurons (mouse)	RNA-seq	~3500	(Nijssen, Aguila, Hoogstraaten, Kee, & Hedlund, 2018)
iPSC-derived human motor neuron (human)	RNA-seq	24,989	(Maciel et al., 2018)
Forebrain synaptosomes (mouse)	RNA-seq	~450	(Hafner, Donlin-Asp, Leitch, Herzog, & Schuman, 2019)
PROTEOMIC			
mESC: iNeurons		Proteome: 661 and Nascent proteome: 380	(Zappulo et al., 2017)
Cortical (Rat)		Proteome: 2548	(Chuang, King, Ho, Chien, & Chang, 2018)
RGC (Xenopus)		Proteome: > 1000 Nascent proteome: ~350 Nascent proteome in response to cues: 300	(Cagnetta, Frese, Shigeoka, Krijgsveld, & Holt, 2018)

REGULATION OF LOCAL PROTEIN SYNTHESIS

The basic ability of neurons to communicate through cell-cell interaction triggers distinct and diversified types of stimuli that depend on adjustments of local proteome along the neurons. This impressive capacity of neuronal processes to rapidly alter protein synthesis, relies on different factors that play key roles in its management. Axons in particular regulate their local translation depending on physiological circumstances such as synaptic plasticity and neuronal activity (Biever et al., 2019; E. Kim & Jung, 2015; Rodriguez, Czaplinski, Condeelis, & Singer, 2008).

However, it is important to consider that regarding the global axonal proteome, is still challenging to identify which proteins are regulated by local translation and which portion is transported from the soma.

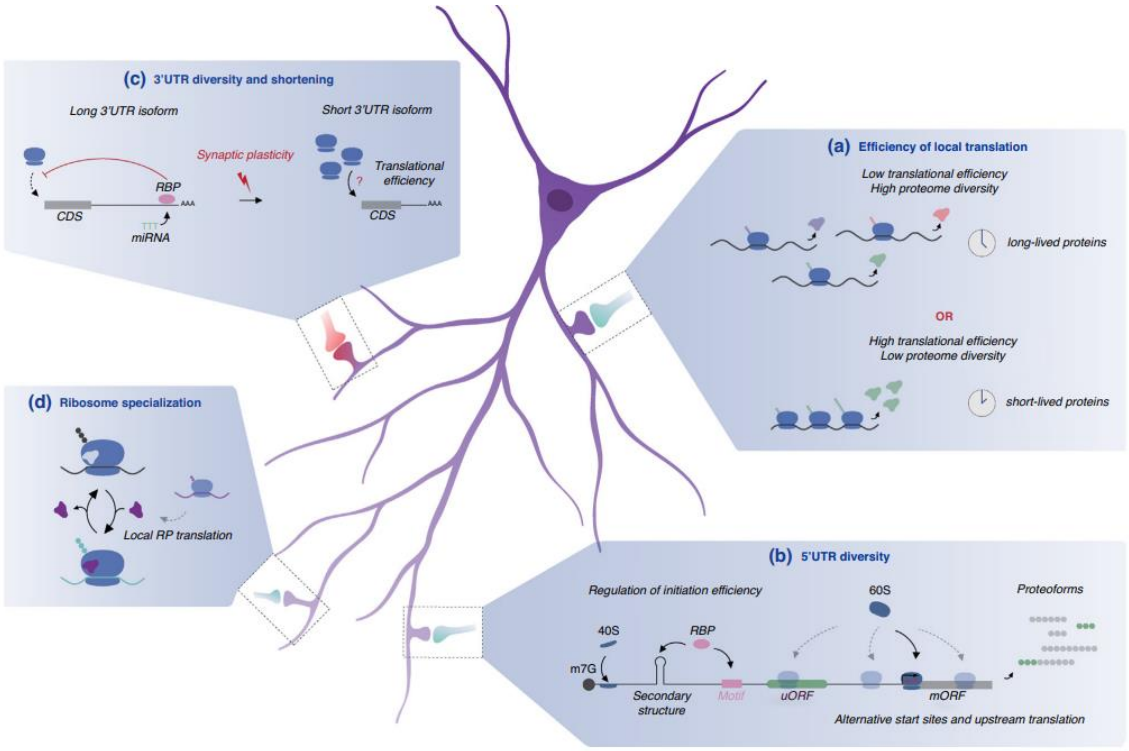


Figure 5 - Different regulatory mechanisms of the axonal proteome: (A)Translation of specific mRNAs can be enhanced by the number of ribosomes and the half-life of a protein, affecting the protein abundance. (B) 5'UTR regions have a major role in regulation of translation. Trans-acting factors directly interact with 5'UTR region where cis-elements are incorporated. For instance, alternative starting sites can lead to different proteins fabricated from one single mRNA molecule. (C) Similarly, 3'UTR regions serve as platform where different ribonucleoproteins and microRNA bind to regulate the translation. (D) Local translation of ribosomal proteins allows the cell to not only to control its local composition of ribosomes, but also to adapt the translation machinery for specific mRNA translation. This way, the cell is able to translate only specific transcript subsets in response to certain synaptic stimuli. Source: (Biever, Donlin-Asp, & Schuman, 2019)

Localized presence of ribosomes

Evidence of differential ribosome distribution and organization around the peripheral region of axons suggests that ribosomes interact with cell surface receptors, regulating local

translation (Tcherkezian, Brittis, Thomas, Roux, & Flanagan, 2010). Additionally, the association of ribosomes with the cytoskeleton provided control over where the proteins are being translated. Moreover, it has been hypothesized that axonal ribosomes can also be targeted for specific mRNAs, translating only specific proteins depending on certain stimuli. This fine-tune of ribosomes could actually create a heterogeneous ribosomal pool that promotes an individualized local protein synthesis (E. Kim & Jung, 2020). Finally, the presence of polyribosome complexes at synapse in response to synaptic activity in myelinated axons confirmed the subcellular organization of the translation machinery (Willis & Twiss, 2010).

Regulation of cytoskeleton

It has been widely accepted that cytoskeleton is a crucial player in temporal and spatial regulation of local translation (S. Kim & Coulombe, 2010; Willis & Twiss, 2010). Moreover, cytoskeleton helps to organize key components of the translation apparatus, such as polysomes (ribosomes complexes) and translation effectors (ex.: aminoacyl t-RNA, eEF1, and eIF4). For instance, the actin-eEF1 interaction plays a fundamental function in the initiation of protein synthesis, and disruption of this factor represses protein production (S. Kim & Coulombe, 2010). This eukaryotic elongation factor is capable of not only bind but also cross-link F-actin, and its monomeric GTPase is responsible for transfer the aminoacyl-tRNA onto the polysome. Additionally, substantial evidence showed that when suppressed, F-actin is incapable of support local translation and therefore occurs a reduction in the bulk proteome observed (S. Kim & Coulombe, 2010). Thus, cytoskeletal proteins have an active and crucial participation on regulation of the local proteome. Furthermore, distinct proteins are produced locally in response to specific stimulus, such as neurotrophins, which modulate the cytoskeleton by acting as depolymerizers or polymerizers of filamentous actin (Willis & Twiss, 2010).

Subcellular localization and storage of mRNA

The differences between the mRNA profiles in a cell usually allow a rough estimation and prediction about what proteins are being produced locally. However, it is important to consider that although these transcripts are situated in axons, they could be locally stored and thus not informative of the actual translation. mRNA storage enables the cell to control when and what proteins are necessary to translate at a given moment. Moreover, cells are also capable to define the subcellular domain that mRNAs are transported to and control their translation, responding more adequately to stimulus and necessities. This ability of cytoplasmic RNA localization is an evolutionarily conserved system that cells use to provides a spatial control over protein synthesis. mRNA localization functions as an important mechanism of spatial control of the proteome, as well as regulation of protein expression (Minis et al., 2014). Moreover, due to the polarity of nerve cells, this specific targeting is

particularly important to maintain the different functions and structures between dendrites and axons.

Generally, mRNAs can be tagged using Untranslated regions (UTRs), to address the molecule to different cellular compartments. Afterward, mRNAs can be either translated or saved in silent forms or stored in granules on axons. RNA-binding proteins (RBPs) also play an important role not only in controlling and regulating mRNA localization, stability, and metabolism but also in suppressing or activating the mRNA translation (Liu-Yesucevitz et al., 2011). These special proteins bind to specific regions of the mRNA and are capable to form RNA-protein complexes, which form granules and inhibit translation. Both granules and binding proteins make mRNA inaccessible to translational machinery and whenever necessary, the mRNA is picked from the granule-storge and rapidly translated to meet cellular demands. This storage became advantageous for the cell not only because it is less time-consuming but is also more economic and energetically favorable since it allows to make as many protein copies as needed from one single transcript. The first-ever reported mRNA sub-localization was first detected in dendritic synapses of spinal cord neurons, the presence of ribosomes associated with endoplasmic reticulum (BODIAN, 1964).

Several studies have been conducted using RNA crosslinking and immunoprecipitation assays to explore more details and functions about the mechanisms behind RBPs. Recently, *Zappulo et al.* have identified specific RBPs that play a critical function not only in regulating mRNA localization and its stability but also in regulating local translation (Zappulo et al., 2017). Generally, RBPs are not only able to bound to the mRNA and together attach to motor proteins, forming an mRNA granule or ribonucleoprotein particles (RNPs), but also regulate expression by either suppress the translation or promote translation upon activation.

mRNA tagging

The dynamic interaction of UTR, functions as another layer of spatio-temporal control of local translation. Not only these elements are important to target the subcellular domain, without changing the structure and function of the proteins they encode, Untranslated regions also regulate mRNA transcription by displaying different 5’ and 3’ UTR, which can oscillate between different isoforms (Minis et al., 2014). The 5’ UTR generally is where the process of translation begins by capturing the ribosome sub-units into the start sites. In this circumstance, ribosomes attached to the upstream open reading frame (uORF) repressing the downstream translation of the coding sequence. On the other hand, 3’UTR also has shown evidence of affecting the translation, in particular through trans-acting factors like RNA binding proteins (RBP) and miRNAs (Biever et al., 2019). In this way, cell can adjust the local translation by selective targeting competitive UTR isoforms between compartments.

Recently, it was also identified a regulatory function of poly-A tails on the UTR regions. It was observed that stable deadenylated mRNAs are usually silent and cytoplasmic polyadenylation can, in fact, reactivate expression (Weill, Belloc, Bava, & Mendez, 2012).

mRNA Inactivation

mRNA molecules usually are carried in silent forms during transportation through the cell. As we saw before, both cis- and trans-acting elements have a direct influence on protein expression. This translational repression usually occurs in RNA recognition motifs, where different classes of modulators bind, such as miRNAs (class of small non-coding RNAs in axons) and RNA binding proteins (Yoon et al., 2009). One of the most known examples occurs in the β-actin mRNA, where a zipcode-binding protein-1 (ZBP1), binds to represses mRNA translation (Ross, Oleynikov, Kislauskis, Taneja, & Singer, 1997).

Axonally synthesized proteins generate retrograde signals

After local production, the axonal proteins can travel to the soma, via retrograde transport. Subsequently, these proteins are capable of producing a retrograde signal that might affect the gene expression in the nucleus (Sahoo et al., 2018). For instance, the production of ATF4 in response to Aβ treated axons, can be transported retrogradely to the nucleus where will induce apoptosis (Baleriola et al., 2014). In 2008 was also reported that CREB was translated within axons and then transported back to the nucleus, where it can induce transcription of anti-apoptotic genes, leading to neuronal cell survival (J. Cox et al., 2008). Another similar example occurs when after an axonal injury, the axon begins to produce locally Kpnb1, a nuclear importin, which will induce an anterograde signal and modulate gene expression for support nerve regeneration (Perry et al., 2012). Moreover, hippocampal axon can synthesise HMGN5, a chromatin-interacting protein, which is capable of modulating the gene expression.

HOW AXONAL PROTEOME ASSAYS ARE PERFORMED

The recent advances of methodologies allowed researchers to greatly expand our understanding in molecular diversity within axons due to increased sensitivity and precision. Throughout the years, several methods have been developed to allow the screen of axonal molecules: either mRNA, in transcriptomics, or proteins, in proteomic assays. Generally, most of the characterization were made using transcriptome screens as we saw before. However, recently more proteomic studies have arisen. In either one of these analytical techniques, the bottleneck process in studying the localized protein synthesis is to obtain sufficient amounts and purity of axonal material. Extracting cellular material from the axons without getting contaminations from the cell body is very difficult and extremely important so that misidentifications can be prevented.

In order to experimentally prevent any trace of the cell body, there are several methods that allow a physical separation of these two cellular regions. These different approaches for neurobiology research consist in four main methods described in figure 6.

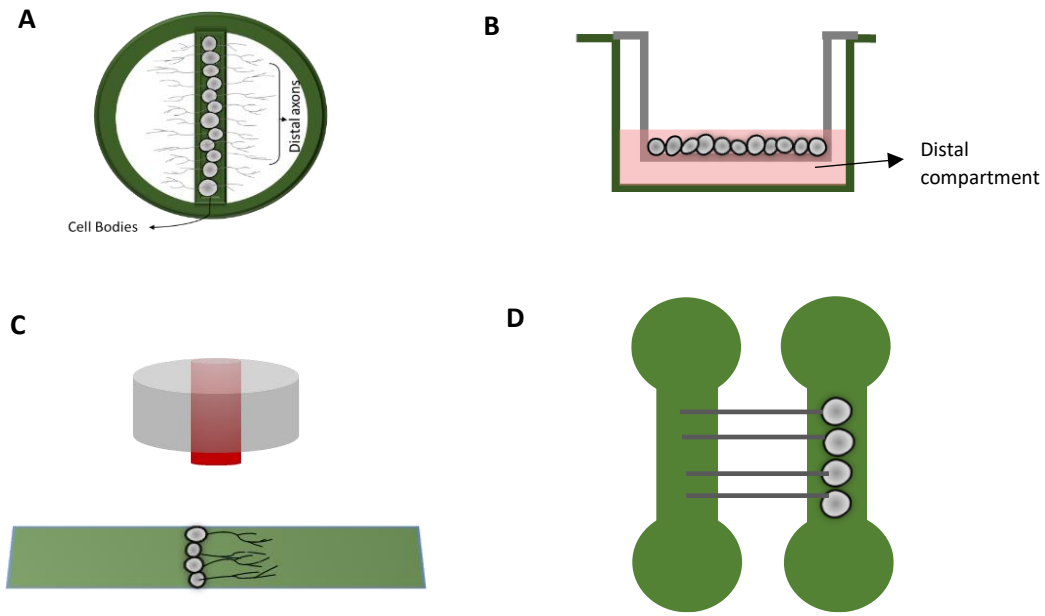


Figure 6 -Different methods for preparation pure axonal isolates: (A) Campenot chamber; (B) Modified Boyden chamber; (C) Laser-capture microdissection and (D) Microfluidic device

Campenot chamber

Campenot chambers were one of the first devices for cell culture used in neurobiology, capable of isolation of axons. Structurally, this chamber is composed of three compartments separated by Teflon barriers, figure 6-A. The neurons are plated in the central chamber and the axons grow towards the adjacent compartments. Due to the fluidic separation, it is possible to perform assays with different stimulations in the distal or proximal chamber. Although these chambers are simple to use, they are difficult to integrate in live cell imaging and the fluid isolation is not perfect (Jadhav, Wei, & Shi, 2016).

Modified Boyden chamber

The Boyden chamber was originally developed by Boyden for cell migration assays, particularly leukocyte chemotaxis (H. C. Chen, 2005). This two-compartment device is composed by a customizable porous cell culture insert, which divides the upper surface where are the neuronal cell bodies and the lower compartment where the axons grow – figure 6-B. In this technique, the neurons are plated in the upper chamber, on top of the insert, where the axons pass through the membrane porous and grow towards the distal chamber. The porous length can be adjustable so that only the axons can pass through the distal compartment. The membrane can be functionalized as well, to optimize and facilitate cellular growth. Although the simple steps and the good yield obtained, this chamber is not capable of fluid separation, and microscopy for evaluating axonal growth is difficult due to the porous insert.

Microfluidic device

Microfluidic devices are another recent strategy that uses small chambers with several sub-millimetric channels. These devices are usually fabricated using a technique called soft-lithography, and a polymer (generally polydimethylsiloxane -PDMS). PDMS is a transparent, biocompatible, and cheap polymer, that has been used for several and different purposes, in particular, neurobiology experiments. The culturing of neurons in microfluidic devices, allows excellent isolation due to the intricate design, using microgrooves, through which axons can grow, figure 6-D. In this technique, PDMS is poured into a master mold, and after the reticulation, the PDMS is hardened and a replica of the mold is made. Finally, a coverslip or a glass cover is treated with plasma, to allow the PDMS and glass bonding to close the microfluidic chip (Jadhav et al., 2016).

Although the great purity that this technique offers, microfluidic devices can be more expensive than other methods since it is very costly to fabricate customizable molds, with a design and grooves size that suits our experience. Also, it is important to have some expertise to work with these cell culture models since if the neurons are plated too distant from the microgrooves, the axons won´t grow to the adjacent compartment.

More recently other fabrication methods have been emerging and gaining great interest, namely 3D printing technologies like stereolithography that allows a more economic and versatile solution to this type of assays.

Laser-capture microdissection

Laser-capture microdissection (LCM) is another technique that allows exceptional isolation, with the highest purity, of either cell populations or selected regions from tissue, like axons (Espina et al., 2006). LCM allows observation of the sample under the microscope during the microdissection, having a direct visualization of the cells of interest. As such the cellular culture is pre-stained to mark the regions of interest. This technique usually requires different cellular staining methods and tissue preservation protocols to improve selectivity and precision. Afterward, the laser mounted in the microscope is used to cutting the region of interest.

Although the high precision and ability to perform with either fresh or fixed tissues, the LCM technique is still very expensive and requires a proper microscope and compatible slides. Additionally, this technique does not allow fluidic separation, thus secreted molecules from different cellular regions could contaminate the analysis.

PROTEOMICS TECHNOLOGIES (labelling methods and bioinformatic tools)

Generally, the proteome is the large study of proteins. This fast and powerful technique is capable to identify the whole proteome but also determine protein structure, and physiological roles and functions (Yu, Stewart, & Veenstra, 2010). Proteomics and, in

particular, mass spectrometry has been widely used as a biomedical technology in neuroscience research (Grant & Blackstock, 2001; Hosp & Mann, 2017). Each neuronal cell, namely neurons and glial cells, has different protein contents, which also changes with the different brain regions. These differential patterns of gene expression throughout the brain, adds another degree of complexity to the neuroproteome (Hosp & Mann, 2017; Tannu & Hemby, 2006). Thus, neurological samples, such as neuronal cells or Cerebrospinal fluid (CSF) have extremely complex protein constituents, which make them good candidates for MS analysis.

Briefly, proteomics relies on three basic processes: first it is applied a separation technique to divide and fractionate complex protein or peptide mixtures; then run a Mass Spectrometry (MS) analysis to acquire the data; and finally, bioinformatics to assemble the data. These technologies can be used in different scenarios, such as: determine where and when proteins are being synthesized; identify signalling and metabolic pathway; understand protein modifications, and how it affects cellular processes, and quantify production versus degradation rates. Overall, MS is a powerful high throughput technique, used to identify and quantify simple or complex mixtures, such as biological samples, and organic and inorganic chemical compounds (Büyükköroğlu, Dora, Özdemir, & Hızel, 2018; Perdomo et al., 2014).

There are different analytical methods available that can be used to investigate the proteome. Besides mass spectrometry, gel-based techniques such as differential in-gel electrophoresis (DIGE) are able to investigate proteomes in depth. Nevertheless, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) still is one of the most powerful

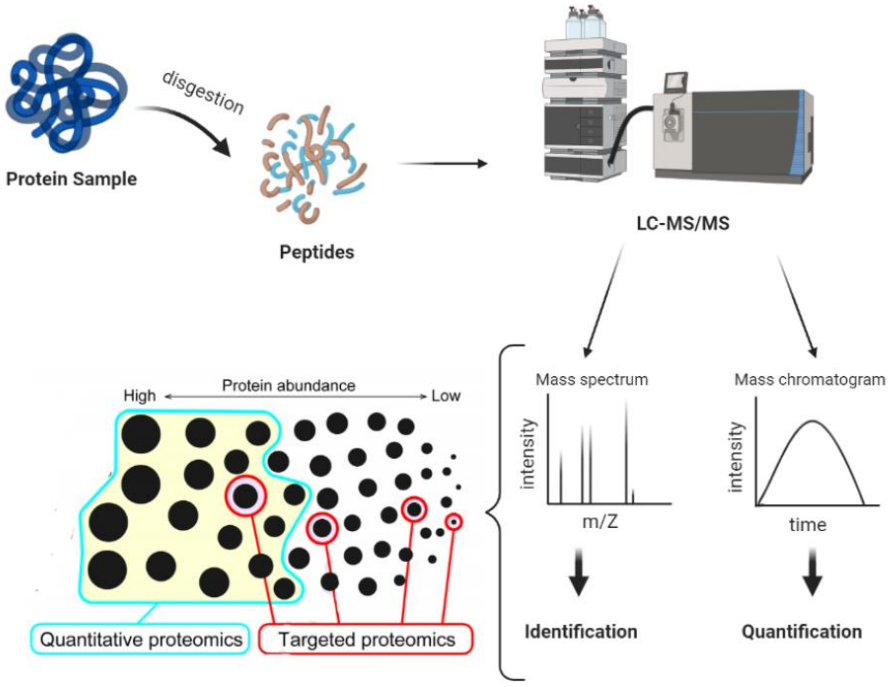


Figure 7 - Schematic overview of a LC-MS/MS workflow. First protein samples, from tissues or body fluids, are digested using trypsin, forming smaller peptides easier to process. Next, labeled or unlabelled peptides are separated in Liquid chromatography (LC) and identified and measured in the mass spectrometer (MS). Proteomics can be characterized by quantitative and targeted proteomics. In quantitative proteomics, proteins are identified and catalogued in order of their relative abundance, while in targeted proteomics only selected peptides before measurements are studied. Adapted from: (Masuda, Mori, Ito, & Ohtsuki, 2020)

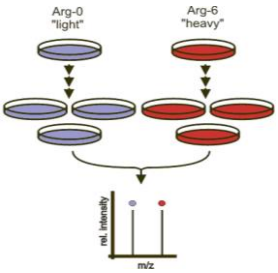
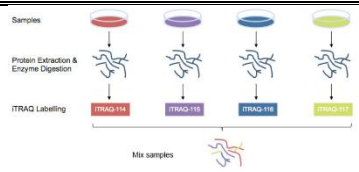
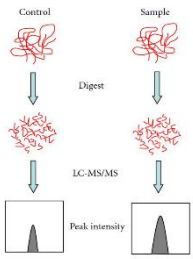
and used techniques in proteomic studies. LC-MS/MS, illustrated in figure 7, combines the separation capacity of liquid chromatography with sensitive mass analysis of mass spectrometry. Coupling on-line separation techniques like LC, previously to run the MS analysis, not only facilitate a faster and unambiguously identification but also is a key determinant for separate and divide complex mixtures of thousands of proteins (Yu et al., 2010).

Liquid chromatography (LC) is an analytical method for physical separation, in which a liquid mixture is distributed between two immiscible phases: stationary and mobile phase. First, the sample is diluted within the mobile phase, which is composed of a specific mixture of eluents, and then injected at high pressure in a column (stationary phase). The proprieties of both the stationary phase and the mobile phase affect the migration rates through the LC column, which causes the proteins to flow out at different times, separating them according to their affinity to the column. Both the stationary and mobile phases can be designed and customized according to the sample to improve extraction and sensibility. Thus, the LC is used before MS, to reduce the sample complexity by separating, previously digested, components of the sample and concentrate them before the MS analysis. After elution from the LC column, the effluent is directed to the mass spectrometer. The ionization creates charged particles that can be detected by different mass analysers. Thus, by applying an electrical or magnetic field under vacuum, the ions will be separated according to their mass-to-charge ratio that will be detected and displayed in a mass spectrum. In tandem mass spectrometry (MS/MS), the first mass analyser is used for ion separation, while the second is used for filtering the product ions generated by the fragmentation. Thus MS/MS has an overall enhanced sensitivity and is able to collect more information about the sample.

In quantitative proteomics, quantities of protein are measured, rather than just providing lists of proteins, enabling to estimate the amount of each molecule in a sample. The development of different techniques allowed an accurate quantification, even at low abundances and in complex samples. This allowed to yield information about the basal protein expression in different cells or tissues. Also, the quantification of the absolute abundance of proteins in clinical samples enables us to compare over-or under-expressed protein profiles in pathological states (Schubert, Rost, Collins, Rosenberger, & Aebersold, 2017).

The quantification in MS can fall under one of the three categories: **stable isotope-labelling method** (eg.: SILAC and iTRAC methods), **label-free methods** (eg.: IBAQ) and **selective reaction monitoring** [less used since one needs to know what to look for in advance and employ internal standards, and for that reason not discussed here] (E. Kim & Jung, 2015; Maier, Guell, & Serrano, 2009).

Table 2 - Summary of principal labelling methods for protein synthesis

Method/technique	Probe	Labelling	Scheme
SILAC (stable isotope labelling of amino acids in cell culture)	Labeled amino acids – stable isotope	Incorporates heavy isotopes into proteins during treatment, without additional purification steps	
iTRAQ (Isobaric tags for relative and absolute quantitation)	iTRAQ-labeled proteins	Incorporates one of the 4 iTRAQ tags	
Label-free method	None	None	

In the stable isotope-labelling method, proteins from different samples are labelled with isotope-labels during treatment (require live axons) and are detected in newly made proteins. In general, this method uses tagged amino acids that live axons can use as building blocks during protein synthesis. The **SILAC** (stable isotope labelling of amino acids in cell culture) method is largely used to characterize very complex samples by incorporating stable isotopes (eg.: H^3, C^{13}, N^{15}) in amino acids, such as lysine or arginine, that are integrated in the proteome during cell culture. This "heavy amino acid" gets stable incorporated in the proteome and causes a mass shift and produce different signal intensities in comparison with the cultured in "light amino acids", allowing a relative quantification (Mueller, Brusniak, Mani, & Aebersold, 2008). On the other hand, the **iTRAQ** (Isobaric tags for relative and absolute quantitation) method can be used to determine the amount of proteins from different sources in one single experiment. This technique uses different iTRAQ reagents with different masses, which bounds to the proteins present in the sample. Differentially labeled peptides appear as single peaks in MS scans, reducing the probability of pear overlapping (Chong, Gan, Pham, & Wright, 2006; Rauniyar & Yates, 2014). This quantitative proteomics method has a particular advantage in the field of clinical proteomics, due to the ability to analyse different samples when studying a specific disease or drug.

Regarding the label-free methods, these are based on the peak intensity from the MS spectra. In this method, after identifying the proteins and/or peptides in the mixture, the protein expression is estimated using the respective intensity, which corresponds to the maximum detector peak intensity. In an m/z plot, the area of the peak reflects the number of ions detected by the spectrometer. Even though the ion abundance cannot be used to calculate the peptide correlation, due to distinct ionization patterns between peptides, this measurement can be used to compare the differential expression (C. Chen, Hou, Tanner, & Cheng, 2020). Since label free experiments does not use any tag, the number of samples is not limited by the number of labels available and thus is less expensive.

Bioinformatic tools

The current progressive development of high-throughput techniques, such as gene expression micro-arrays, next-generation sequencing or mass spectrometry, has become extremely important not only in fundamental research but also during drug screening, generating large amounts of data. Thus, high-throughput screenings require appropriate data processing due to the challenging and massive outputs, which cannot be manually curated or analysed. Bioinformatics tools represent a compelling and robust strategy to manage/integrate large datasets and extract/analyse the relevant biological information(Gulcicek et al., 2005).

Bioinformatic tools are available as databases or softwares and are widely used not only to understand normal cell physiology but also to unravel pathological states and explore pharmacological therapies, for example, by studying drug toxicity or diseases biomarkers. These software were built to assist in a comprehensive analysis of proteomic and genomic studies by first identifying the genes or proteins present. Furthermore, these tools are also able to predict isoforms, interactions, networks, and molecular structure, among others. The databases are also crucial since it enables the careful storage of all the proteomic outputs, which can be later used for researchers to identify connections between their work and the existing knowledge.

Nowadays, there is a large spectrum of different types of software and databases available according to the subject and type of analysis intended. As such, they became indispensable in a proteomic study. These tools are designed to suit different research areas and the different types of analysis inherent to them. Therefore, several tools emerged as a solution to different scientific questions, creating different tools already specific to areas such as oncology or phylogenetic evolution, for example.

Broadly, there are some fundamental and essential databases such as Uniprot and Entrez gene (from NCBI) that provide information that is transversal to all studies, namely the coding genes of each protein, protein names and respective ID, functions, length, related diseases, among other features. UniProt stores the data in two categories: the **Uniprot/Swiss-Prot** database contains only curated information about reviewed proteins, this way achieving the minimal level of redundancy, and **UniProt/TrEMBL** database contains

information that is computationally annotated and not reviewed. UniProt also enables to compare protein sequences to investigate areas of homology. On the other hand, Entrez gene from NCBI, gathers all the knowledge and associations about genes and their sequences.

Both biological pathways and biological networks represent a framework to incorporate biological knowledge, as illustrated in figure 8. This information is usually gathered in databases such as KEGG and Reactome (curated pathway databases) and interactively integrated by software such as Metacore and Cytoscape. Biological pathways represent a series of causal interactions between molecules that lead to a certain product and are used to characterize cellular processes and biochemical interactions. The latter is usually illustrated by an interaction map that might form a cascade, like metabolic pathways or a signalling pathway. Kyoto Encyclopedia of Genes and Genomes (KEGG) is one of the most popular pathways databases, best known for its pathways diagrams (also known as maps) that usually represent metabolic and regulatory pathways. KEGG also describes a great part of genomic information as well as cellular process and human diseases.

On the other hand, biological networks connect the interacting constituents of a biological system. These networks are constituted by nodes (circles in figure 8 - B) and edges (lines in figure 8 - B). Nodes are the components that represent the basic units in the network while edges represent the different interactions that each unit has. These basic units can translate molecules, like proteins or genes, or even cells or neuronal networks, for example.

Cytoscape is an academic free software for biological interactions and pathways visualization. Cytoscape has a vast range of plugins, which add more features to this software, and new visualization options, namely new cluster layouts, graph analysis and enrichment analysis, among others. This type of softwares for biological networks are usually more adequate for visualizing all the different pathways associated within a work. In this way, analyse and understand the biological information such as identify crosstalk between pathways. STRING is an online platform capable of retrieve both known and predicted protein-protein interactions. This online platform also includes network enrichment statistics GO analysis, and Pfam and KEGG annotations.

Another important asset when performing a proteomic study is Gene Ontology analysis, which is used to describe the different roles of each protein or gene according to different GO terms, namely, biological process, molecular function and cellular compartment. The combination of all these different approaches help to construct a more reliable analysis. Overall, these tools are extremely useful to select research molecule candidates, drug targets or identify biomarkers.

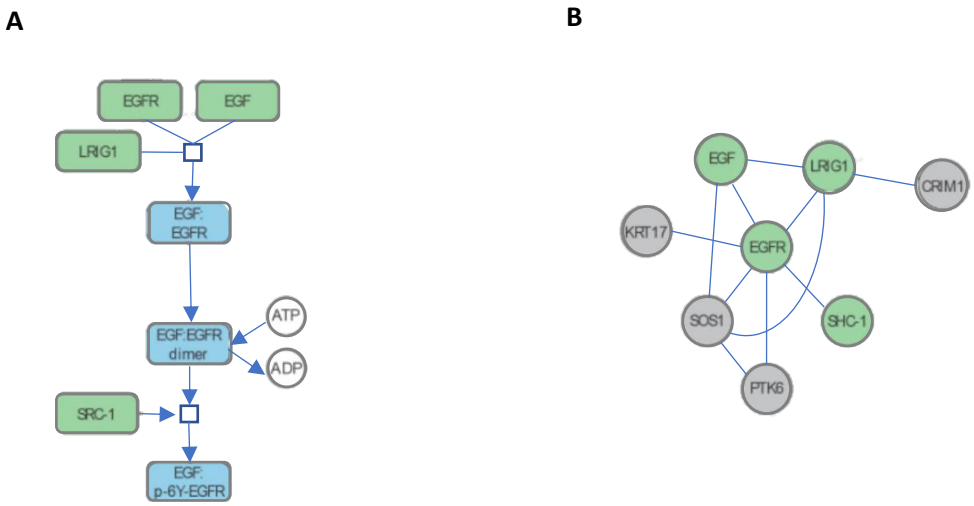


Figure 8 - Schematic representation of (A) Biological pathways, and (B) Biological networks, in which circle are designated nodes and lines are designated edges. Nodes represent components that are the basic units in the network and edges represent the different interactions that each unit has.

AIM OF THE WORK

The axon is a highly specialized cellular region of the neuron and has a dynamic and versatile cellular content according to its special needs and functions. A synergy of local translation and transported cargo allows the cell to control and localize subcellular contents, creating a distinct composition from the global neuronal proteome. Both these cellular processes are involved in various neurological events such as axoplasmic transport, axon elongation and pathfinding, growth cone guidance and pre-synapse formation. Characterize and understand the axonal proteome can help to elucidate the ongoing cellular processes and link our understanding of the protein content with the biology of the cell.

As previously stated, significant efforts have been made to unravel the components and mechanisms in axonal content. However, there is still a lack of proteomic studies that can provide valuable information about the axonal proteome, particularly covering the different subdomains of the distinct neuronal populations. This knowledge allows to characterize the normal physiology of specific axons, but also to identify new molecular targets to tackle neurological disorders.

Thus, in the present study we aim to determine and characterize the protein profile of pure axonal lysates of rat hippocampal neurons, grown in microfluidic chambers. As such, we will use different approaches and take advantage of the various available bioinformatics tools to extensively characterize the axonal proteome, while confronting findings with recent evidence. As follows, we will elucidate the presence and potential roles of local proteome in physiological and pathophysiological conditions. For this purpose, we will run an enrichment analysis and search for different neurological disorders fingerprints among the proteome, using several databases to create a disease-association profile, and ultimately identify possible future research candidates.

CHAPTER II: METHODS

I. PROTEOMIC ANALYSIS

i. After growing in microfluidic devices, pure axonal samples were reduced with dithiothreitol (27 nmols, 1 h, 37°C) and alkylated in the dark with iodoacetamide (54 nmol, 30 min, 25 ^{0}C).

i.1. The resulting protein extract was first diluted 1/3 with 200 mM NH_4HCO_3 and digested with 0.9 µg LysC (Wako, cat # 129-02541) overnight at 37 ^{0}C and then diluted ½ and digested with 0.9 µg of trypsin (Promega, cat # V5113) for eight hours at 37 ^{0}C.

i.2. The peptide mix was acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc) prior to LC-MS/MS analysis.

i.3. The peptide mixes were analysed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to an EasyLC (Thermo Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column at a flow rate of 1.5-2 µl/min using a wash-volume of 4 times the injection volume and were separated by reversed-phase chromatography using a 50-cm column with an inner diameter of 75 µm, packed with 2 µm C18 particles spectrometer (Thermo Scientific, San Jose, CA, USA). Chromatographic gradients started at 95% buffer A and 5% buffer B with a flow rate of 300 nl/min and gradually increased to 22% buffer B in 105 min and then to 35% buffer B in 15 min.

i.4. After each analysis, the column was washed for 10 min with 5% buffer A and 95% buffer B. Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile.

i.5. The mass spectrometer was operated in DDA mode and full MS scans with 1 micro scans at resolution of 120.000 were used over a mass range of m/z 350-1500 with detection in the Orbitrap. Auto gain control (AGC) was set to 2E5 and dynamic exclusion to 60 seconds.

i.6. In each cycle of DDA analysis, following each survey scan Top Speed ions with charged 2 to 7 above a threshold ion count of $1e^4$ were selected for fragmentation at normalized collision energy of 28%. Fragment ion spectra produced via high-energy collision dissociation (HCD) were acquired in the Ion Trap, AGC was set to 3e4, isolation window of 1.6 m/z and maximum injection time of 40 ms was used. All data were acquired with Xcalibur software v3.0.63.

II. PROTEIN IDENTIFICATION

ii. After laboratory experiments and mass spectrometry was carried out, raw data needed to be processed so that peptides could be assembled into proteins.

ii.1. We used two different search engines in this analysis, to take advantage of distinct approaches to attain the final proteome. MaxQuant (version 1.6.3.3) and Proteome Discovery software's were used separately for peptide identification and quantification.

ii.2. Raw data were subjected to database search against the Rattus norvegicus database (Swiss-Prot database, version 2020_05, 29.943 proteins), which included a list of common contaminants and all the corresponding decoy entries.

ii.3. Trypsin was chosen as enzyme and the maximum of miscleavages were set to two.

ii.4. Mode of decoy database was set to "randomize", searches were performed using a peptide tolerance of 7 ppm, a product ion tolerance of 0.5 Da.

ii.5. The resulting data files were filtered for False Discovery Rate (FDR) < 5 %.

ii.6. The iBAQ option was selected for quantification purposes.

ii.7. To remove contaminants and increase results confidence, all proteins matched to the reverse "REV_" and contaminants "CON_" databases were removed from the final proteome list.

ii.8. The minimum number of "Razor + unique peptides" identification required for each protein was set to two.

ii.9. This array of settings was employed in both softwares (MaxQuant e Proteome Discover).

III. UNIPROT COONVERSION

iii. To integrate the results of both programs in the same UniProt KB identifiers, which are readily recognized by the majority of software's, both datasets were uploaded in the "Retrieve/ID mapping" tool from UniProt.

iii.1. Each protein list was uploaded to provide original identifiers and converted to a final desired identifier, such as UniProt KB. Organism was set as Rattus norvegicus, and then the target list was downloaded.

iii.2. Using the same tool from Uniport, FASTA sequences of each protein were downloaded, for later use in the Secretome P assay.

iii.3. Human orthologues list was generated using the same tool by providing a gene name list as the original identifiers.

IV. VEEN DIAGRAMS

iv. A Venn diagram was generated using the jvenn tool in order to compare data obtained from both MaxQuant and Proteome Discovery, and visualize the results overlap of both datasets. (http://jvenn.toulouse.inra.fr/)

iv.1. For each condition, the list of proteins accession IDs of each dataset was added to the query. The program automatically built the diagram with a central section with the common proteins.

iv.2. To retrieve the proteins found in the overlap region, this particular region was selected, and the list was generated beneath the diagram.

iv.3. The output was downloaded in PNG image and CSV list.

V. SECRETOME ANALYSIS

v. Using the mammalian version of SecretomeP as a tool we were able to predict the secretion of each protein from the axonal proteome.

v.1. Fasta sequences of all proteins were downloaded from Uniprot and uploaded in SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) with the maximum of 100 sequences in each input.

v.2. To evaluate the results, each 'SecP score' valued above 0.5 was consider as possible secreted.

VI. STRING ANALYSIS

vi. To predict which biological processes and pathways are related to the proteins associated with the axonal proteome, a STRING analysis was carried out. (https://string-db.org/)

vi.1. In “multiple proteins” tab the protein list was uploaded, and the organism set as rattus norvegicus. A protein interaction network was displayed with the known associations and interactions.

vi.2. In data settings, the “network-edges meaning” was defined by evidence (different data sources help build the network), with medium confidence, and only query proteins interactions were showed.

vi.3. Using the option of “kmeans clustering”, from the “cluster” tab, we were able to set number of clusters for five clusters, that were displayed in the network with different colours.

vi.4. The main network statistics and the most relevant annotated biological processes were downloaded in a tsv. file from the “export” tab.

VII. GENE ONTOLOGY ANALYSIS

vii. Gene ontology (GO) analysis were performed by using the functional annotation plug-in ClueGO (version 2.5.7) and CluePedia (version 1.5.7) from the Cystoscape software. In this enrichment analysis the different proteins were assigned to different categories of GO terms based on their functional characteristics, helping to identify and aggregate proteins that share common functions or in the same pathway or network.

vii.1. Ontologies, pathways and annotation files, for rattus norvegicus, were updated on the 21st of June of 2020.

vii.2. Only significant biological pathways and processes with p-value ≥ 0.05 were showed, and “GO Term fusion” option was selected, to aggregate similar terms.

vii.3. Network specificity was set to medium, as default.

vii.4. The p-value for each identified GO term was calculated using the two-sided hypergeometric test with a Bonferroni step-down correlation.

vii.5. The GO enrichment analysis was carried out regard the three main categories of the terms: “biological process”, “molecular function”, and “cellular component”, using the same settings, described above, for each condition.

vii.6. For all the conditions, an enrichment CluePedia analysis was added, to increase the biological knowledge by looking for potentially associated to pathways, or other protein-protein interaction.

vii.7. All data results were finally exported as tables excel sheets and also pie-charts.

VIII. DISGENET ANALYSIS

viii. After processing and examine of the axonal proteome, a disease-association analysis was carried out to check if the proteins found were already associated to any disorders. We employed the DisGeNET database in our study, since is one of the largest publicly available collection of gene-diseases association. Therefore, this non-targeted analysis allowed to forecast the most abundant genes associated with different pathologies.

viii.1. The gene list associated to the axonal proteome was uploaded in the "search" tab of the website and with the default query on "genes".

viii.2. Summary of gene-disease association was downloaded in a xls. file and data were organized by "score gda", which spans from 0 to 1 depending on the strength of association.

viii.3. From all the associated diseases, the neurological disorders had particular interest in the context of this work. With that aim, results were manually curated and filtered by "neurologic" and "mental" related terms. From all the neurological diseases associated, only those with a "score-gda" above 0.1 was consider.

viii.4. The cytoscape (version 3.6.1) software was used to build a gene-disease association network and display relevant interactions between proteins, in a pathological context

viii.5. To build the network, the genes were defined as "source nodes" (green circles), the diseases names were defined as "target nodes" (double orange circle) and the score_gda was set as "interaction type" (purple triangle).

viii.6. From the network, were identified which nodes were exclusively related to each gene, and what genes were more prevalent and significantly associated with neurological pathologies among the proteomes.

viii.7. Since we were analysing several genes and several conditions, we used the "network analyser" tool from cytoscape. This tool was helpful to identify proteins that have multiple interactions with different pathologies, since it can generate nodes with different sizes proportionally to the amount of interactions.

viii.8. In the network analysis tool, the option "treat the network as undirected" was selected. Next, the "node size" and "node colour" were both defined by "betweenness Centrality", with low values corresponding to small sizes and bright

colours, respectively. Similarly, “edge size” and “edge colour” were defined by “edge betweenness” and low values correspond to small sizes and bright colours, respectively.

viii.9. The network was exported and saved as a pdf. file, to optimize quality and labels would be visible and clear.

IX. NEUROLOGICAL DATABASES ANALYSIS

ix. Finally, the axonal proteome was compared with some available neurological databases. Orthologues genes were compared against SFARI DB https://gene.sfari.org/ , visited at 26/08/20) for autism, CARPE DB (http://carpedb.ua.edu/ , visited at 26/08/20) for epilepsy and ALSoD (https://alsod.ac.uk/ visited at 29/09/20) for Amyotrophic Lateral Sclerosis. Common genes between the database and our results were reported as potential precursors for diseases and selected for further analysis.

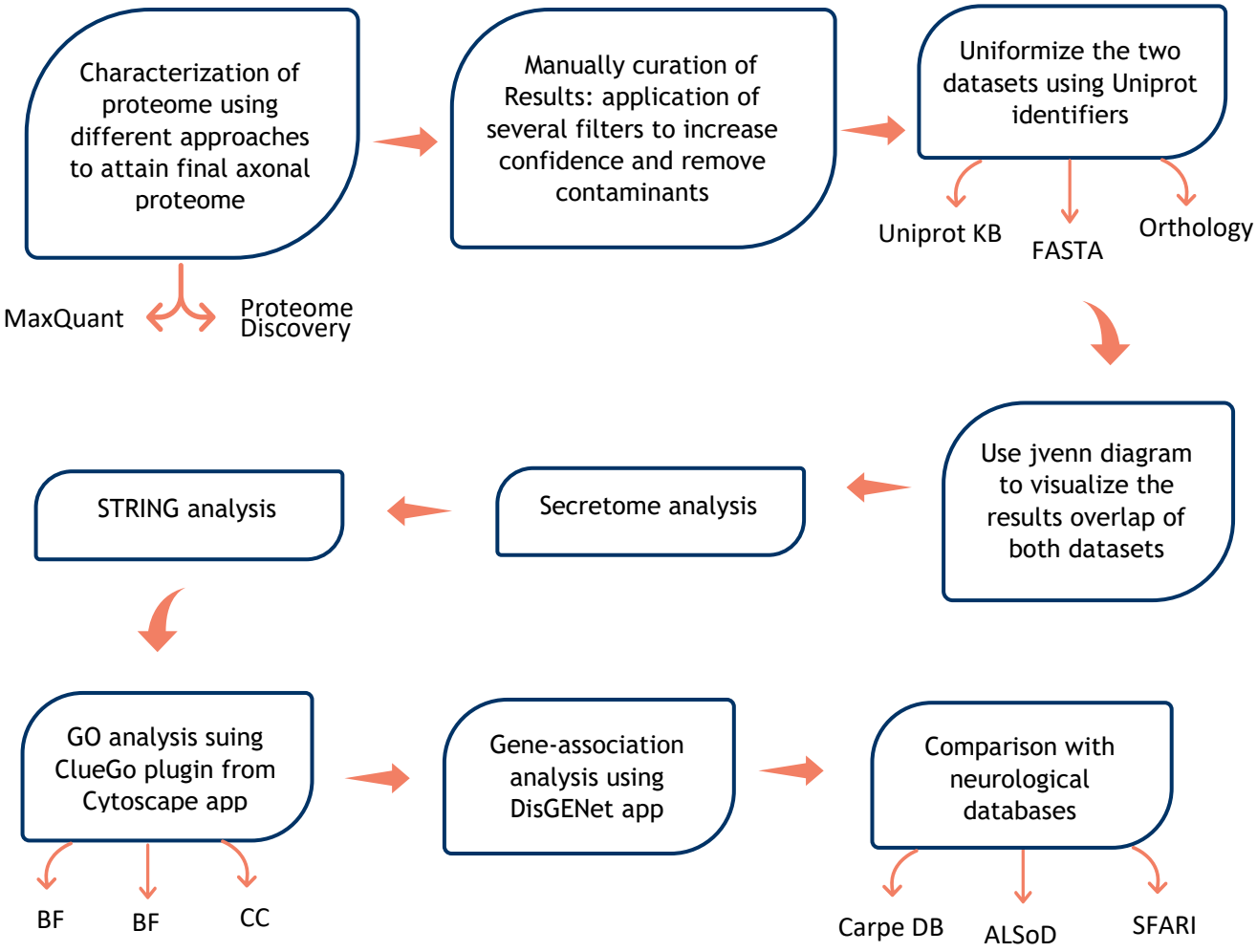


Figure 9 - Schematic representation of the experimental design used to unravel the axonal proteome

CHAPTER III: RESULTS AND DISCUSSION

PROTEOME ANALYSIS

Analysis of embryonic hippocampal rat neurons, that grew on microfluidic chambers, gave us access to pure axonal lysates that were then analysed in mass spectrometry (LC-MS/MS) to generate a robust and extensive data on axonal proteome. After collecting the mass spectrometry data, a proteomic systematic analysis was carried out, using bioinformatic tools to handle the large data sets of proteins. The identification and quantification of these proteins were carried out in two different proteomics search engines: MaxQuant™ and Proteome Discover™. The employment of these two different approaches in the study allowed a characterization improvement, by taking advantage of the different capabilities of each software, leading to a larger diversity, more complete, and richer analysis.

Notably, we were able to identify a total of 1694 peptides from the 584 proteins present in the sample, 470 of those proteins were identified by Proteome Discovery™ software and the remaining 114 belonged to MaxQuant software. From all proteins found in both softwares, 182 of those were common to both analysis (Figure 10). Several proteomics search engines are available, which apply different algorithms and may or may not lead to different outcomes. From the two that we have chosen, proteome discoverer™ software was clearly a more robust analysis tool, allowing a wide coverage and a more comprehensive protein identification and consequently lead to new biological insights. However, comparing with the other few proteomic studies available, summarized in table 1, revealed a very low yield. The number of identified proteins were lower than expected, possibly due to poor sample management. Although very efficient in the axonal isolation, the microfluidic chamber only allows the collection of low amounts of sample, which affects the number of proteins identified in the mass spectrometer. However, this novel technology has a good purity and great isolation capacity, confirming the adequacy of this method for axonal purification in future neurobiology studies.

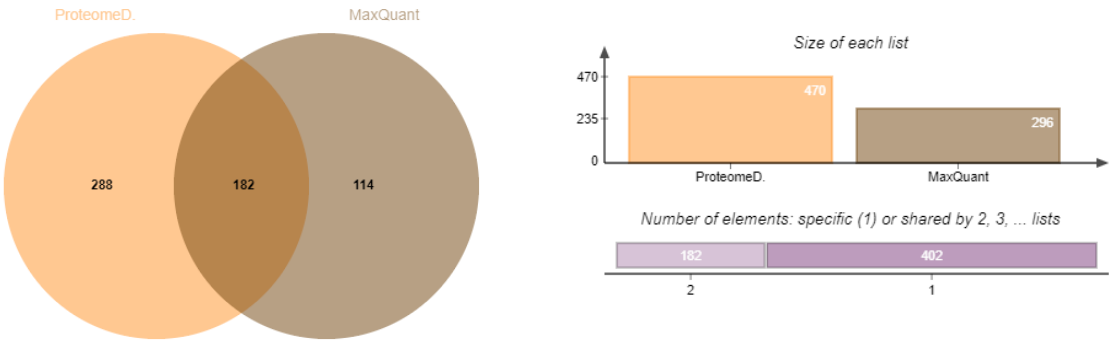


Figure 10 - Venn diagram comparing proteins datasets found in MaxQuant software (brown) and Proteome discover (orange). Number of proteins identified in hippocampal axon dataset.

Before further examination, several filters were applied to increase the general reliability of the found dataset. As such, only proteins with the minimum number of 2 peptides associated, and without contaminants were used to build the final proteome list, which formed the basis for all subsequent analysis.

Proteomics also provides a technological approach for systematic quantification of abundances in samples with several proteins. Protein quantification measures the abundance or concentration of a protein in a sample and reflects a dynamic balance among different processes and also the necessities of the cell. Moreover, the abundance is affected by mRNA translation mechanisms. Usually, protein concentration is erroneously considered proportional with the corresponding mRNA concentrations. However, the transcript abundances do not reflect the translation activity of the cell and therefore protein abundance provides a better approximation of the cellular expression (Vogel & Marcotte, 2012). In this study, quantification of proteins was achieved using the iBAQ value tool, of MaxQuant software, and values were then compared to correlate protein amounts. As illustrated in figure 11, the five most abundant proteins present in the sample, accordingly to iBAQ values, are: histone H4, histone H2B (type 1), beta-actin, alpha-tubulin 1 and alpha-tubulin 3, with the respective values $2{,}64E^{+08}$; $2{,}03E^{+08}$; $2{,}03E^{+08}$; $1{,}55E^{+08}$; $1{,}08E^{+08}$ and $1{,}08E^{+08}$.

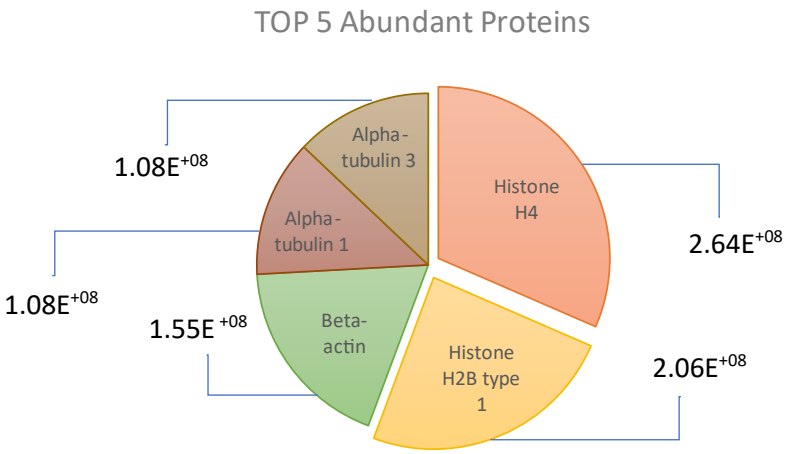


Figure 11 - Pie-chart of top 5 most abundant protein and respective IBAQ values

Histones are nuclear proteins that bind to DNA and are responsible for packing and unpacking nuclear chromatin, regulating gene expression depending on the cellular translation demands. Beyond histones and their other isoforms, other nuclear proteins such as nucleophosmin, lamin B2, and HMGB1, were found in the axonal proteome. Although nuclear proteins were already reported to be present in growth cones in the literature (Chuang et al., 2018; Deglincerti & Jaffrey, 2012; Estrada-Bernal et al., 2012; Zivraj et al., 2010) we do not know whether they play an active and unique role in the axon or if they maintain their native function. To our best knowledge, there isn't yet a

clear link between nuclear proteins and local translation in axons and, in particular with histones, which are the most abundant in our dataset. Nonetheless, they might be considered as “moonlighting” proteins (proteins that can perform more than one function), since they may be involved not only in remodulation of chromatin but also during the translation process. For example, very recently *Attar et al.* discovered a cupper reductase activity in histones of yeast, reinforcing that these ubiquitous proteins may still have many secret double functions that still are unknown (Attar et al., 2020). Hence, since histones and other nuclear proteins were highly present in the axonal proteome, it is reasonable to hypothesise that histones may play a different role in axons and may contribute to axonal growth and/or even regulate some processes or signalling in local translation. Another possible theory hypothesizes that these proteins may retain similar original functions and, after translated in axons, histones are then trafficked back to the nucleus where they can influence nuclear functions, acting as a retrograde signal. However, further testing is still required to unravel the molecular mechanisms involved.

Regarding to the other proteins, both actin and tubulin are constituents of the cytoskeleton, which helps to maintain and build the cell architecture, defining the cellular size and shape as well as the mechanical properties. They also participate in processes of cell organization, cytoplasm transport, and polarization. The cytoskeleton is primarily composed of microfilaments (made of actin), neurofilaments (named intermediate filaments in other cells), and microtubules (made of alpha and beta-tubulin). This network of interlinked proteins can build polymerized filaments that function as a “highway” in the cell to carry different cargos from the soma to the end of the axon. Both *Estrada-Bernal et al.* and *Chuang et al.* have reported a high abundance of cytoskeleton proteins in growth cone and rat cortical neurons proteomes, respectively (Chuang et al., 2018; Estrada-Bernal et al., 2012). As such, cytoskeleton proteins have an important and active role on axons, in particular actin, which is one of the main cytoskeletal components in growth cones and very enriched in synapses (Kevenaar & Hoogenraad, 2015).

Cytoskeleton plays a major role in axonal transport, which is responsible for the translocation of cellular cargos to distant regions in the cell, mediating the distribution of proteins, vesicles, organelles, and signalling molecules along the axon. This axoplasmic flow is made through the cytoskeleton and is a crucial process involved in several cellular mechanisms, such as neurodevelopment and local translation (S. Kim & Coulombe, 2010). Thus, this transport will affect some vital processes in the axons, such as elongation and polarization, among others (Kevenaar & Hoogenraad, 2015). Numerous constituents have been already identified in axonal transport, differing in their cargoes and the rate of transport. Additionally, the cytoskeleton also has an active role in regulation of local translation, since it mediates transportation of the translation machinery, in particular mRNA granules and polysomes (clustered ribosomes). However, to keep these molecules intact and inactive during translocation, several binding proteins are usually attached to the mRNA for translational repression and released later

to allow translation (Van Horck & Holt, 2008; Yoon et al., 2009). As such, the cytoskeleton proteins may have a positive effect on local translation by regulating the organization of the translational apparatus and also by maintaining the structural integrity and function of the protein synthetic machinery (S. Kim & Coulombe, 2010). For instance, according to *Chudinova et al.* depolymerization of both actin and microtubules (made of tubulin) actively decreased protein biosynthesis confirming a close interaction between the translation machinery and cytoskeleton (Chudinova & Nadezhdina, 2018; Van Horck & Holt, 2008).

α-Actinin was also present in our sample, as well as, other isoforms of tubulin, such as beta-tubulin 2B, beta-tubulin 4B, beta-tubulin 5, beta-tubulin 1B, beta-tubulin 3, alpha-tubulin 4A, and alpha-tubulin 1B. In this way, we conclude that from the eight known isomers of β-tubulin, five were present in this sample, and from the seven known α-tubulin isomers, 4 were present. Regarding actin, the iBAQ score of B-actin in our sample was ~2300 times higher than that of a-actinin. This result allows to hypothesize an ongoing growth process since β-actin distribution is highly enriched in axonal outgrowth processes (Micheva, Vallee, Beaulieu, Herman, & Leclerc, 1998; Moradi et al., 2017). Axonal growth cones are highly specialized regions that extend and guide the axonal growth. Together, these events, and in particular, axon branching processes exert a great demand for local protein synthesis in the cell, which would presumably be occurring during the experimentation procedures (Leung et al., 2018).

Additionally, there was also detection of other relevant proteins that showcase local translation activity. Several ribosomal proteins, initiation factors, elongation factors, RNA-binding proteins, Golgi apparatus proteins, and endoplasmic reticulum chaperones were detected in the proteome. This group of proteins belongs to different organelles, that are not typically expect in the axon, and neither are visible in axons through electric microscopy. However, similar to nuclear protein groups, the presence of Golgi apparatus and endoplasmic reticulum proteins, in the axon, have been described in the literature (Chuang et al., 2018; Horton & Ehlers, 2003; Merianda et al., 2009). Although these organelles both play important roles in modelling the protein structure, during biosynthesis, and also during folding and post-translation modifications, it is still unknown how axons fulfil these tasks.

GENE ONTOLOGY ANALYSIS

To perform a more detailed evaluation of the proteome and study the diversity of the present specimens, a Gene Ontology (GO) enrichment analysis was carried out. This strategy allows the identification of genes or proteins that are overrepresented in our axonal dataset of proteins, by grouping proteins that share common functions or the same pathway or network. Gene Ontology analysis defines the biological domains concerning three aspects: **Molecular Function** (describes the activities at a molecular level), **Cellular Component** (describes the relative localization on a cellular anatomy level), and **Biological Process** (describes the process/ activity carried out at a molecular level).

The biological process results, of the GO analysis, are shown both in table 3, which highlights the most significant terms according to the p-value, and in figure 12 with a pie-chart illustrating the abundance of the respective biological processes. The main prevailing biological functions, as illustrated in pie-chart of figure 12, were: "Axon development", "Establishment of cellular localization", "Response to organic cyclic compound"; "Carbohydrate catabolic process" and "Regulation of cellular component organization".

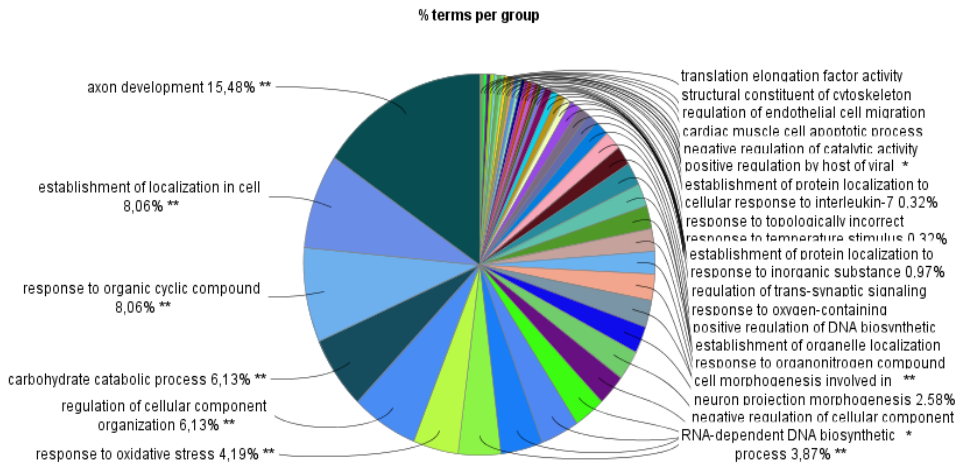


Figure 12 - Functional annotation (BIOLOGICAL PROCESS) analysis of the rat hippocampal axonal proteome (ClueGo)

Not surprisingly, "axon development" with 15,48%, was the most predominant biological function, which comprises processes like axonogenesis (eg.: APP, Kalrn, Rab10, Map1a, Myh10) and regeneration (eg.: GAP43, Ptn, LRP1, Map2k1). Both processes may involve protein expenditure and presumably require protein biosynthetic process at an axon level, contributing for the axonal proteome. On the other hand, "Establishment of localization in cell" (eg.: Actb, Arf5, Map2, VCP) is the second most

Table 3- Functional annotation (BIOLOGICAL PROCESS) analysis of the rat hippocampal axonal proteome (ClueGo)

BIOLOGICAL PROCESS	p-value	no. of molecules	% Associated Genes
Regulation of endothelial cell migration	0,05	11	6,51
Positive regulation of protein transport	0,05	18	4,60
Positive regulation of cellular component biogenesis	0,05	23	4,09
Release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	0,04	5	16,67
Release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	0,04	5	16,67
Regulation of RNA splicing	0,04	11	6,67
Memory	0,04	11	6,71
Cation channel activity	0,03	22	4,21
Positive regulation of DNA metabolic process	0,03	13	5,94
Positive regulation of DNA metabolic process	0,03	13	5,94
Glutathione metabolic process	0,03	7	10,94
Organelle transport along microtubule	0,03	8	9,41
Axo-dendritic transport	0,03	8	9,41
Response to topologically incorrect protein	0,03	10	7,52
Chromatin assembly	0,03	10	7,52
Chromatin assembly	0,03	10	7,52
Cardiac muscle cell apoptotic process	0,03	7	11,11
Negative regulation of cellular component movement	0,03	17	5,01
Negative regulation of cellular component movement	0,03	17	5,01
DNA biosynthetic process	0,02	12	6,56

predominant biological process in the axonal proteome, with 8%, and is associated with substance movement and cellular localization. This GO term is consistent with the results discussed above, particularly with cytoskeleton protein abundance, since they are involved in process of cell transportation and localization. Accordingly, in table 3, “Positive regulation of protein transport”, “Positive regulation of cellular component biogenesis”, “Regulation of RNA splicing”, “Organelle transport along microtubule”,

“Axo-dendritic transport”, “Response to topologically incorrect protein” are some of the most significant GO terms, that articulate the process of translation and protein transportation in axons.

Table 4 - Functional annotation (MOLECULAR FUNCTION) analysis of the rat hippocampal axonal proteome (ClueGo)

MOLECULAR FUNCTION	p-value	no. of molecules	% Associated Genes
Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0,05	7	5,15
Nucleotidyltransferase activity	0,04	8	4,60
Regulation of cation channel activity	0,04	8	4,37
Tubulin binding	0,03	15	4,05
Regulation of protein binding	0,03	11	4,78
Kinase regulator activity	0,03	8	4,04
Phosphatase binding	0,03	11	4,76
Positive regulation of protein binding	0,02	7	7,14
Regulation of transmembrane transporter activity	0,02	13	4,64
Calcium ion transmembrane transporter activity	0,02	11	5,07
Negative regulation of peptidase activity	0,02	13	4,55
Regulation of phosphatase activity	0,02	10	5,75
Voltage-gated ion channel activity	0,02	13	4,83
Translation factor activity, RNA binding	0,02	7	7,69
Gated channel activity	0,01	19	4,20
Positive regulation of transporter activity	0,01	9	6,82
Enzyme inhibitor activity	0,01	17	4,46
Ligand-gated ion channel activity	0,01	12	5,58
GDP binding	0,01	8	8,60
Cation channel activity	0,01	22	4,21

Molecular function analysis revealed that “disordered domain specific binding”, “peroxidase activity”, “nucleotide-binding”, “intramolecular oxidoreductase activity” and “dATP binding” are the most common activities among the proteome(Figure 13). In

general, this shows an abundant catalytic activity and binding activity in the proteome, in particular associated with RNA binding and cytoskeletal dynamics. Additionally, dATP binding activity, with 9,4%, is among the main molecular functions of the proteome, and is a crucial process in several mechanisms that participates in the axon, for example in chaperone machinery, assisting protein folding, and also cytoskeletal motor proteins, for cargo transportation (Clare & Saibil, 2013; Kevenaar & Hoogenraad, 2015).

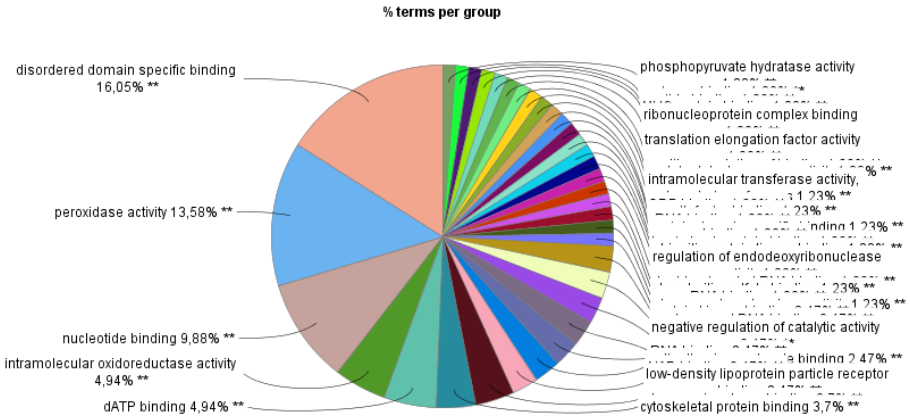


Figure 13 - Functional annotation (MOLECULAR FUNCTION) analysis of the rat hippocampal axonal proteome (ClueGo)

Concerning cellular compartment, the GO enrichment results showed that proteins were mainly located around the “cytoskeleton”, the “synapse”, “plasma membrane-bounded cell projection”, “ruffle” and “supramolecular fiber” (Figure 14). Not surprisingly the cytoskeleton (eg.: Actb, cct2, myh10, tuba1a), with 20,21%, was the most predominant cellular component, as expected by previous results and also since is highly enriched in the axonal proteome. This cellular component has an active and crucial role in axons, such as maintaining axonal integrity, promote axonal outgrowth, and axon guidance (Kevenaar & Hoogenraad, 2015). The cytoskeleton is also implicated in axonal transport, which is carried out by motor proteins such as kinesin, dynein, and myosin. According to our results, there was presence of both kinesins and dynein proteins in axonal proteome of rat hippocampal neurons. These molecules move specifically along the microtubules and have a movement in opposite directions: kinesins responsible for anterograde transport and on the other hand dynein is responsible for the retrograde transport (Kevenaar & Hoogenraad, 2015).

The cytoskeleton is not only responsible for transport but also provides structural support for highly specialized axonal structures such as presynaptic boutons. Actin, in particular, is a critical component for synapse formation and development. As the axon approaches and connects the post-synaptic site, it enlarges into a highly specialized structure, the synapse, also called presynaptic terminal. Taking into consideration the proximity and the crosstalk between these two structures, is coherent to propose that they might be intrinsically correlated, having an intimate relationship. For instance, axons produce and transport the proteins, membrane receptors, and neurotransmitters for the synaptic vesicles. According to our results, there was presence of presynaptic and synaptic formation-related proteins around the axon, in particular 94 proteins, among them Actb, Dbn1, and Myh10.

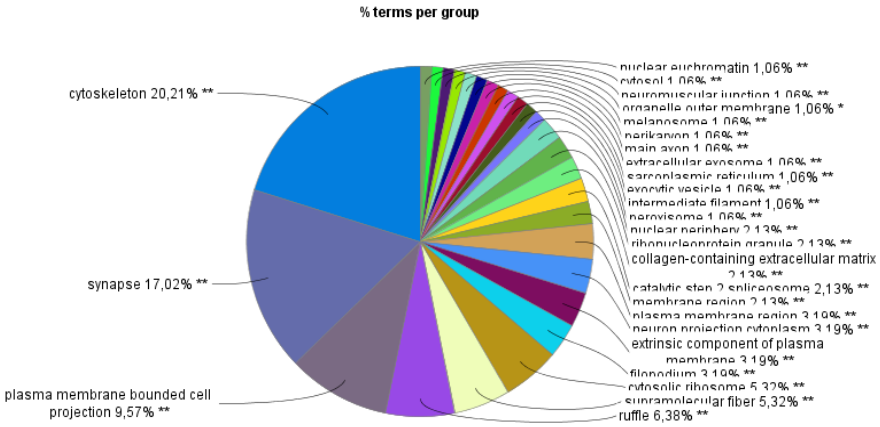


Figure 14 - Functional annotation (CELLULAR COMPARTMENT) analysis of the rat hippocampal axonal proteome (ClueGo)

In a deeper analysis it is possible to distinguish two main processes: one related with transportation ("extracellular exosome", "exocytic vesicle", "intermediate filament") and the other with translation ("nuclear euchromatin", "ribonucleoprotein granule", "cytoplasmatic ribonucleoprotein granule", "spliceosomal complex", "catalytic step 2 spliceosome").

Table 5 - Functional annotation (CELLULAR COMPARTMENT) analysis of the rat hippocampal axonal proteome (ClueGo)

CELLULAR COMPARTMENT	p-value	no. of molecules	% Associated Genes
Nuclear euchromatin	0,05	7	19,44
Cytosol	0,04	138	4,03
Melanosome	0,04	18	20,00
Perikaryon	0,03	10	5,10
Main axon	0,03	7	7,78
Extracellular exosome	0,03	9	10,47

Sarcoplasmic reticulum	0,03	8	11,59
Exocytic vesicle	0,03	12	4,32
Intermediate filament	0,03	10	7,19
Peroxisome	0,03	9	6,43
Neuromuscular junction	0,03	9	8,65
Organelle outer membrane	0,03	8	4,28
Nuclear periphery	0,02	14	11,38
Nuclear matrix	0,02	12	11,88
Ribonucleoprotein granule	0,02	16	6,87
Cytoplasmic ribonucleoprotein granule	0,02	14	6,31
Extracellular matrix	0,02	20	5,13
Collagen-containing extracellular matrix	0,02	17	7,20
Spliceosomal complex	0,02	10	5,49
Catalytic step 2 spliceosome	0,02	7	7,69

A complementary analysis was performed, searching for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, using STRING Database results (string-db.org). KEGG reports biological pathways that describe reaction cascades and biological processes among molecules, that result in a certain product or a change in the cell. Table 6 lists the top 20 pathways represented by the highest statistical significance. Among the others KEEGS pathways, “Carbon metabolism”, “Biosynthesis of amino acids”, “Metabolic pathways”, “Ribosome”, “Protein processing in endoplasmic reticulum”, and “Cysteine and methionine metabolism” have a particular interest in this study case since they participate in translation and protein biosynthesis. Specially, “Biosynthesis of amino acids” is the second most significantly enriched pathway related to the production of all non-essential amino acids in healthy axons, suggesting that the axon contains its own biosynthetic processes machinery for local translation.

Table 6 - Highest ranked KEGG Pathways in axonal proteome

TERM DESCRIPTION	OBSERVED GENE COUNT	FALSE DISCOVERY RATE
Carbon metabolism	24	5.65e-18
Biosynthesis of amino acids	19	1.22e-15
Glycolysis / Gluconeogenesis	17	1.04e-14
Alcoholism	18	6.75e-11
Systemic lupus erythematosus	15	1.67e-10
Necroptosis	17	9.34e-10
Metabolic pathways	48	2.28e-09
Ribosome	13	1.44e-06
Tight junction	14	1.44e-06
Pyruvate metabolism	8	1.58e-06
Protein processing in endoplasmic reticulum	14	1.58e-06
Phagosome	14	1.58e-06
Glucagon signaling pathway	11	1.58e-06
Fluid shear stress and atherosclerosis	13	1.58e-06
Gap junction	10	5.35e-06
Leukocyte transendothelial migration	11	8.29e-06
Alzheimer's disease	13	9.61e-06
Long-term potentiation	8	3.60e-05
Focal adhesion	13	3.78e-05
Cysteine and methionine metabolism	7	5.33e-05

Furthermore, KEGG enrichment analysis also showed an involvement between the axonal proteome and pathways related with neuro diseases such as “Alzheimer's disease” and “Systemic lupus erythematosus”, discussed in more detail below. In particular, dysregulation in local translation can either enhance the propagation of neurodegeneration or act as suppressor. Moreover, local protein synthesis has been gaining much attention in respect with its association with several neurological diseases, which will be explored below.

As shown by previous results, cytoskeletal proteins, and proteins related with axonal and synaptic formation/development have been reported in all GO analysis, and thus is reasonable to assume that both processes are very active in the hippocampal axonal proteome. Additionally, the presence of translation machinery, such as ribosomes, and various biosynthetic related processes confirmed the presence of active local translation in axons. Therefore, these observations have been consistent, exhibiting the clear contribution of both axonal transportation and local protein synthesis in the axonal proteome. Finally, the consistent presence of cytoskeleton throughout the analysis may indicate that this cellular component has a vital role during cellular transport and also local translation, and consequently in maintaining a healthy and normal axonal function. Figure 15 provides an integrated view of the most enriched GO terms in the axonal proteome.

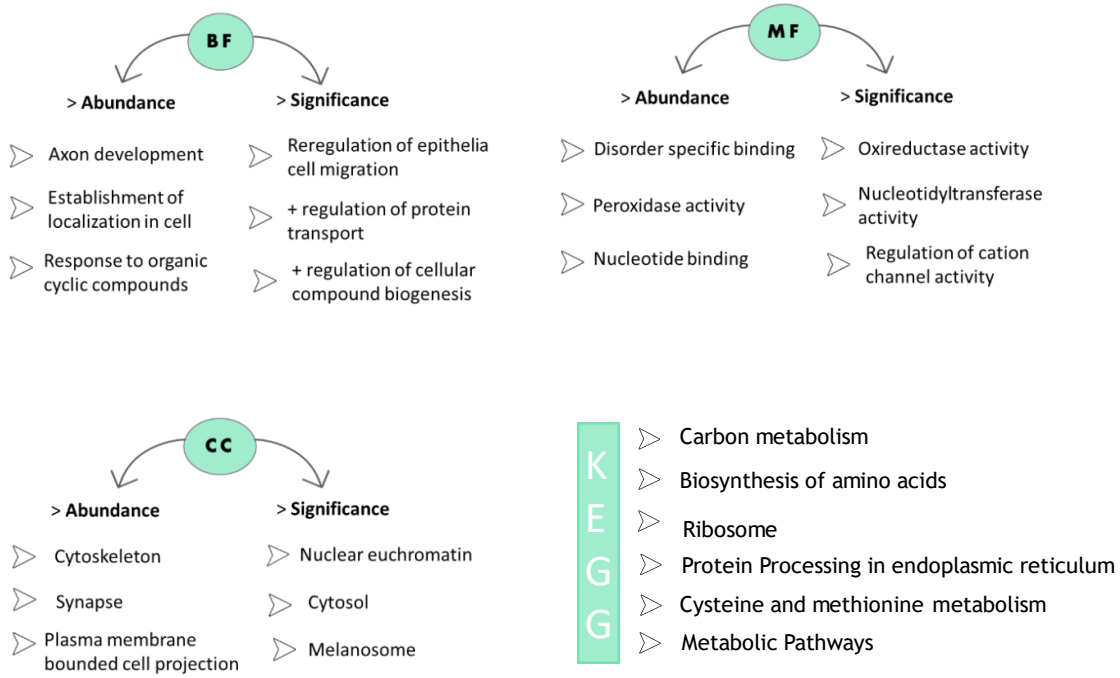


Figure 15 - Summary of the most enriched GO terms in the axonal proteome, regarding abundance and significance. Significant KEGG pathways that participate in translation and proteins synthesis.

PROTEOME VS TRANSCRIPTOME

Comparing the earlier results with previously reported transcriptome datasets makes possible to speculate about gene expression and infer the axonal necessities. Thus, gene expression comparison is used to understand and describe the normal cell function and ongoing cellular processes (eg.: responses to environmental cues), and also to help linking our understanding of gene expression and protein content to the biology of the cell. Correlation between the transcriptome and proteome may have crucial aspects, to understand what specific genes are being translated and also to clarifying what proteins or functions are conserved. In 2009, *Taylor et al*. reported for the first time a dataset of mRNAs, with approximately 300 transcripts in matured cortical axons of rat, grown in similar microfluidic chambers (Taylor et al., 2009). Overall, the number of transcripts was similar to the number of proteins discovered here, in hippocampal axons. However, we estimated that approximately only 20% of the proteome share mutual proteins, with no more than 61 proteins in common to the previous reports (Figure 16A). The 61 common proteins might be the ones that both types of axons are constitutively producing and thus have a conserved role in the cell. In addition, both studies reported, that both cortical and hippocampal neurons have an active translation machinery in axon, to synthesize proteins locally and fulfil the cellular necessities. Also, both gene ontology analysis were enriched for intracellular transport, translation, and cytoskeleton, including the motor proteins. This may suggest that the transcripts of cortical axons may correspond to a different subset of proteins, possible either other isoforms or proteins from the same family. Thus, we conclude that protein expression was significantly different between the two datasets, perhaps due to fundamental differences between the two types of axons, which may have different protein content according to the respective needs of each type of neuron. Accordingly, as shown previously by several studies, the protein content does not always reflect the transcript content and its expression in the cell due to the different cellular rates of mRNA and protein production, different degradation rates, regulation of translation, or even different aging or growth stages, which may reflect significant differences between the two (Bathke, Konzer, Remes, McIntosh, & Klug, 2019; Kumar et al., 2016; Ori et al., 2015). A weak correlation between mRNA and the respective protein has also been reported in the literature (Maier et al., 2009). This low correlation between expressions could possibly be due to either a high protein turnover, which means that the cell has a high capacity to produce more new proteins with a low degradation rate, or from cellular economy to synthesize proteins from a single mRNA transcript, justifying the lower concentration of the transcripts of each protein. The protein half-life has also a great impact in mRNA-protein translation and can be dependent on several factors such as protein stability and post-transcriptional modifications (Maier et al., 2009).

To investigate the biological relevance, we analysed the most enriched biological process of the common proteins between the transcriptome and proteome of rat axons (Figure 16). Both purine metabolism (eg.: Aldoa, Atp5f1b, Nme1), which is responsible for

synthesis and degradation of purine nucleotide, and cytoplasmatic translation (eg.: Eif4a2, Rpl18, Rpsa) are biosynthetic processes involved in local translation.

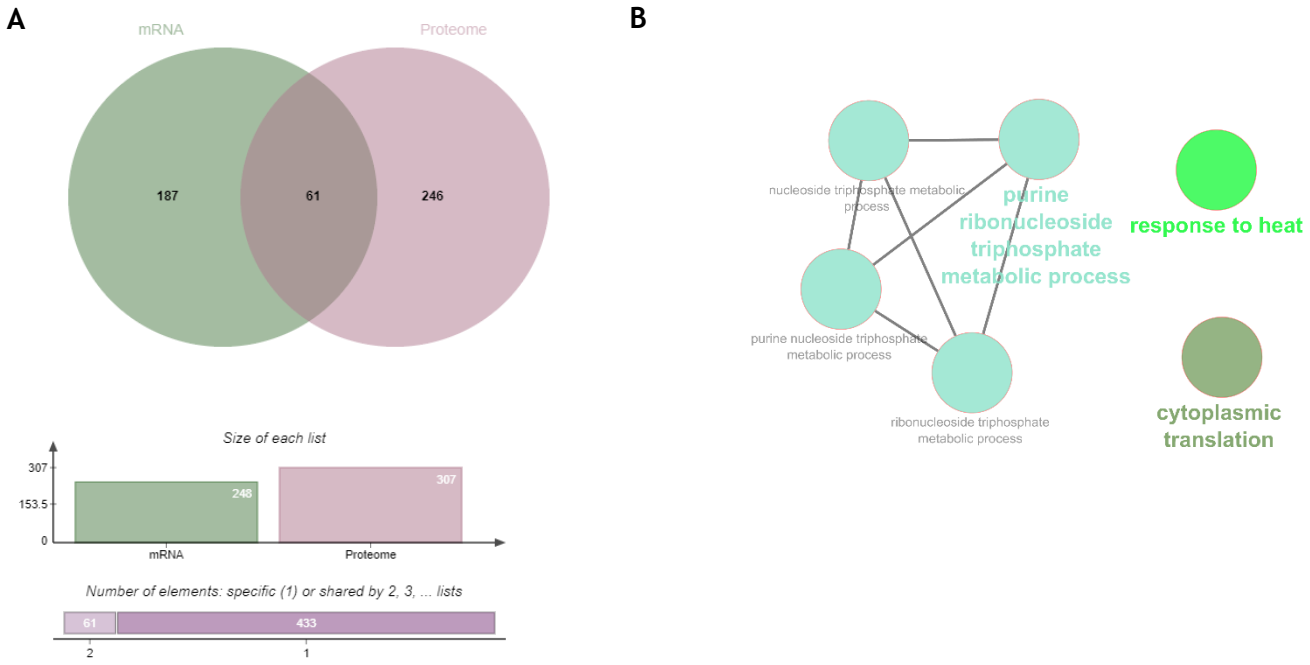


Figure 16 - Comparison of transcriptome and proteome of cortical and hippocampal axons, respectively. (A) Venn diagram comparing mRNA and protein content of each dataset; (B) GO analysis of common proteins between the two datasets.

DISEASE-ASSOCIATION ANALYSIS

Although neglected, axon pathologies can cause serious neurological diseases. In many neurological diseases, axonal morphological changes and functional dysregulations occur before symptom expression (Van Battum, Brignani, & Pasterkamp, 2015). Axons are unable to regenerate, have high metabolic activity, constant energy demands, and an exceptional morphological structure, which made them susceptible to transport defects and injuries (Coleman, 2002). Thusly, the implications of axonal-transport defects and local protein synthesis in neurodegenerative diseases are widespread in a variety of neurological diseases (Liu, Rizzo, & Puthanveettil, 2012; Sleigh et al., 2019). In order to identify the presence of biomarkers and other proteins involved in the metabolism of neurological disorders, we performed a protein-disease associations assay, within the bulk of axonal proteins here reported. This analysis could help characterize the involvement of the axonal proteome in neuro and mental illness. Additionally, by pinpointing protein clusters involved in neurological pathogenesis could lead to a therapeutic target in the future (Figure 17).

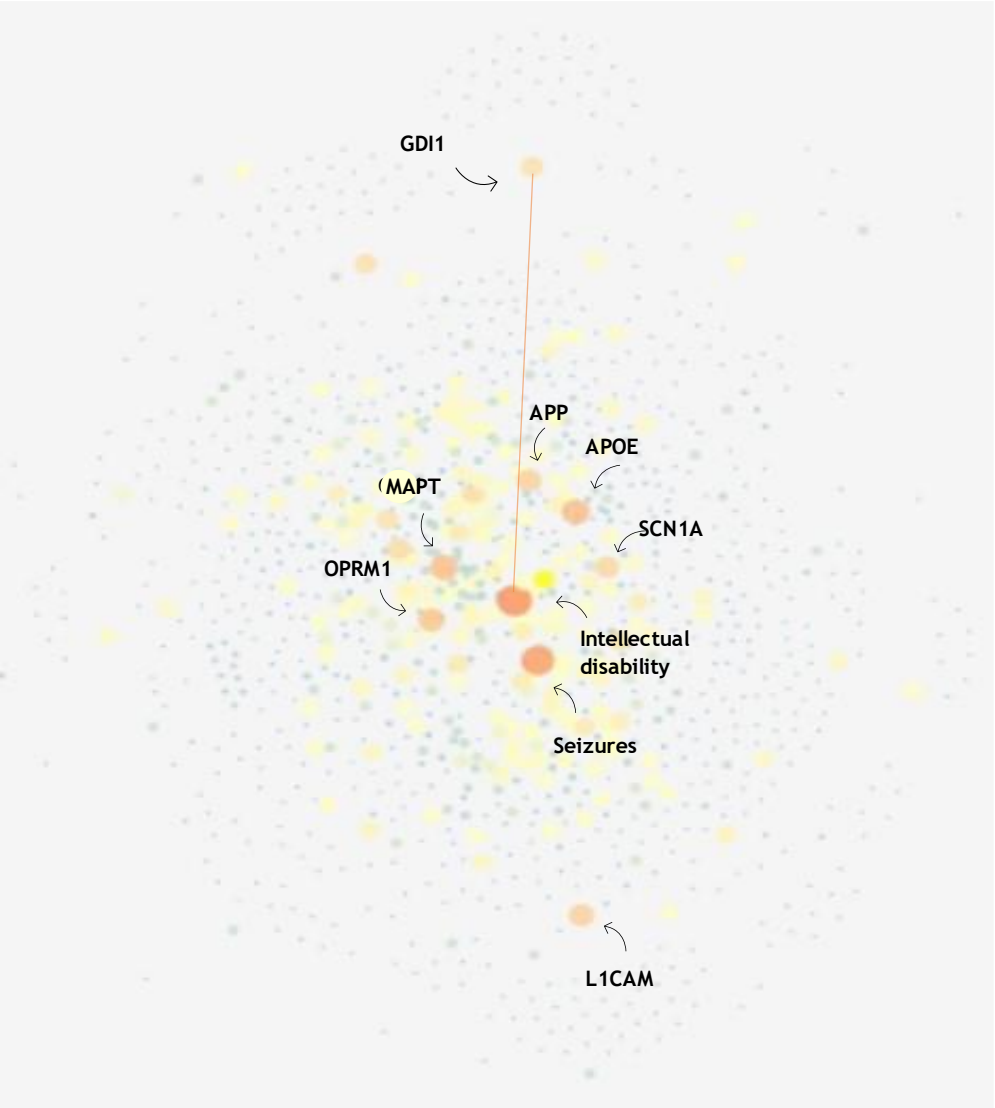


Figure 17 – Neurological Gene-disease association map. Shown are most relevant genes and diseases from the DisGeNET analysis.

Concerning biomedical research, disease analysis with comprehensive information about different illness and their associations became unbearable with the massive amount of data available. Specific databases, including those related to diseases, which collect data on known interactions between genes and diseases, play an important role in gathering this information and facilitate this type of analysis (Bianco et al., 2013). To conduct this study, we perform a disease-association analysis using different specific databases for neurological disease (CarpeDM, SFARI, and ALSos), and also a general human gene-disease association database, DisGeNET (www.disgenet.org). DisGeNET is a free online available platform containing a large public collection of gene-disease associations. As the DisGeNET database is not specific for neurological disorders, and to better estimate all the interactions and biomarkers for neurological disorders, we used less restricted version of the proteome, with fewer filters applied. Thus, by increasing the spectrum of the protein input, we will gain a better insight into the network of gene-disease association. In addition, the manually curated

output was analyzed within the GDA-score interval of 1 – 0.1, in order to visualize the most significant results. Score-GDA is one of the metrics used in the DisGeNET search engine and is based on the number of sources reporting the association.

Additionally, an orthology analysis was carried to convert rat genes, obtained in our data, into human genes. The orthologue prediction is crucial for future studies, in order to identify the genes to explore in a human cell model, and also to adjust our data to the available information in the majority of online platforms since human disease reports and databases are much more frequent. Genes descend from two different species and are called orthologues since they share a common ancestor from where they differentiated throughout a speciation event. Figure 19 illustrates the relation between these two phylogenetic groups, indicating that approximately half of the genes have a correspondent orthologue gene in humans.

The results confirm the presence of several relevant genes related to neurological diseases. DisGENET results are illustrated in figure 17 and show ten relevant disease-associated genes related to neurological pathologies: APP, APOE, CLU, ACTB, OPRM1, MAPT, SCN1A, L1CAM, GDI1, IGF1. These proteins reflect three main disorders: Alzheimer's disease, Parkinson's disease, mental retardation, and seizures.

a) APP, APOE, CLU, IGF1 and MAPT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive loss of memory and intellectual function. Alzheimer's is the most common cause of dementia and usually affects elder people and patients with a family history of the condition. In an AD brain, at a molecular level, is observed accumulation of protein aggregates, which form both senile plaques and Tau tangles.

APP (beta-amyloid precursor protein) and APOE (apolipoprotein E), are already well-documented proteins and implicated in Alzheimer's etiology, with accepted and known molecular mechanisms. Both proteins are significantly represented in the chart of figure 17. APP is a cellular transmembrane receptor that is cleaved by secretases to form several peptides. Briefly, when APP suffers mutations or miscleavages, it produces a larger and stickier fragment, which can aggregate and accumulate in the brain and form clumps, called amyloid plaques, that are very characteristic of Alzheimer's disease (Y. W. Zhang, Thompson, Zhang, & Xu, 2011). Amyloid plaques are primarily formed by β-amyloid precursor protein (β-APP), which is usually produced constitutively by neurons and functions as a biological marker of acute axonal damage.

Nevertheless, APP, APOE, and Clu are all implicated in Alzheimer's diseases and interact all together since APP is a precursor of Aβ, and APOE and Clu are directly involved in Aβ metabolism. Moreover, an increase in the mRNA of these two molecules was observed in hippocampal axons, after Aβ triggered its recruitment, as demonstrated by *Baleriola et al.* (Baleriola & Hengst, 2015). Thus, these results showed a selective recruitment of specific mRNAs into axons, and their local translation, in response to a local stimulus, demonstrating a clear association between AD pathogenesis and the axonal translation. Even though APOE

and Clu are mainly associated with the pathology, they are also constantly produced during non-pathological events, such as growth cones of intact axons and in regenerative process (Yin, Guo, He, Wang, & Sun, 2019). Although they are the most common proteins in Alzheimer's, their response to Aβ might have a neuroprotective effect, by neutralizing Aβ, rather than a neurotoxic effect, as *Baleriola et al.* proposed (Baleriola & Hengst, 2015). Thus, this network can serve as an example that axonal translation can, in fact, be considered as a target therapeutic pathway for neurodegenerative diseases. This concept can be very enlightening to learn how to distinguish mRNA translation events from pathogenic to restorative. Additionally, dynein, a retrograde motor protein, has been previously associated with senile plaques from AD and retrograde signalling (Baleriola et al., 2014). This type of linkage unravels the function of axonal transport during the pathogenesis of AD (Sun & Cavalli, 2010).

Despite being better known as a protein typically associated with Alzheimer's, APP has recently been reported to accumulate in other chronic neuropathologies such as multiple sclerosis, HTLV-I myelopathy, and HIV encephalitis, due to the inflammation of CNS (Mankowski, Queen, Tarwater, Fox, & Perry, 2002). Additionally, APP also has a strong correlation with dementia and seizures, as shown in figure 17. APP was previously documented as a constituent of the axonal proteome and is already described as a protein transported through the axon via anterograde transport (Chiba et al., 2014; Sherriff, Bridges, Gentleman, Sivaloganathan, & Wilson, 1994).

APOE is an apolipoprotein that participates in the normal lipid metabolism. Additionally, the neuron is capable of production and secretion of APOE in response to neuronal injury and for its own regeneration (Rozenbaum et al., 2018). APOE is also associated with schizophrenia and neurodegenerative diseases such as amyotrophic lateral sclerosis and multiple sclerosis. The presence of mRNA encoding for APOE in axons, suggests, together with our data, that APOE is locally synthesized in the axon (Yin et al., 2019).

CLU (clusterin) is an extracellular chaperone, which binds to misfolded proteins and turns into a soluble and stable aggregate. This chaperone acts in a cytoprotective manner, which is expressed during environmental stress and nerve injury, being able of cellular debris clearance (Gregory et al., 2017). Although it has been already reported nuclear translation of Clu (Prochnow et al., 2013), there is still lack of information on how is transported to the injury site or any association has been made to local translation yet.

IGF1 (Insulin-like growth factor 1) has also been involved in the regulation of Aβ metabolism (Werner & LeRoith, 2014). This growth factor protein is capable of modulating Aβ concentration not only regulating its clearance by carrier proteins such as CLU and TTR, but also participate in degradation of Aβ through extracellular proteases like insulin-degrading enzyme (IDE) (Bates et al., 2009). Thus, dysregulation or low values of IGF1 may lead to development of Alzheimer's phenotype.

Histologically, AD is also characterized by the accumulation of neurofibrillary tangles, caused by aggregation of Tau protein. **Mapt,** also known as Tau, is a microtubule-associated protein

that has an important role in assembly and stabilizing microtubules and regulating axonal transport. Besides, it is believed that Tau-pathologies, which cause microtubule dysfunction may promote neurodegeneration (X. Zhang et al., 2018). Tau has been associated with both Alzheimer's and Parkinson's disease. Hyperphosphorylation and misfolding of Tau are the main causes of propagation of neurodegeneration in Tau pathologies. Tau aggregates are characterized by their deposition in neurons, and the impairment they cause in the structure and function of microtubules, which lead to neuronal dysfunction and death observed in several diseases. Since hyperphosphorylation of Tau leads to an inability to bind to microtubules and eventually will compromise all axonal transport, due to impairment of microtubule function and loss of transport capacity of the axon, which APOE, Mapt, IGF1, and Park7 have been also related with Parkinson's disease (Castilla-Cortazar, Aguirre, Femat-Roldan, Martin-Estal, & Espinosa, 2020; Li, Luo, Liu, Fu, & Tang, 2018; Pascale et al., 2016; Sun & Cavalli, 2010). Parkinson's disease (PD) is a progressive neurodegenerative disease that affects dopaminergic neurons in the substantia nigra. Loss of these neurons leads to impairment of movement, that gradually causes stiffness and disability (Poewe et al., 2017). Dysfunction of axonal transport may contribute to the etiology of Parkinson's disease. For instance, cytoskeleton changes on dopaminergic neurons are common in PD patients, in particular dysregulation of actin.

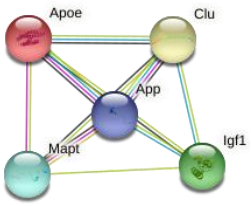


Figure 18 - STRING interaction network of the sub-group (APP, APOE, CLU, IGF1 and MAPT) of proteins identified in the DISGENET analysis. All proteins have multiple predicted interactions between them.

b) SCN1A and OPRM1

Both SCN1A, OPRM1 (Opioid Receptor Mu 1), and L1CAM have been associated with occurrence of seizure, as illustrated in figure 17. Seizures are characterized by bursts of electrical activity between two nerve cells in the brain and cause abnormal behaviors and muscle tone. Patients with recurring seizures are diagnosed with epilepsy. The axonal segment has been correlated with the occurrence of seizures, since it is where the excitatory and inhibitory integration takes place. Therefore, dysfunctions in the axons are a promising target for antiepileptic drugs, in the future.

Voltage-gated sodium channels are responsible for the initiation and propagation of action potential in excitable cells. **SCN1A** channels (Sodium Voltage-Gated Channel Alpha Subunit 1) are especially enriched in the initial portion of axons and alterations can cause hyperexcitability and seizure activity. Interestingly, local translation of several transmembrane proteins, namely SCN1A has been reported (Taylor et al., 2009). Dysfunctions in these proteins are highly associated with epilepsy, as exhibited in figure 17.

In addition, SCN1A function impairment is present in Dravet syndrome, in early-onset epilepsy but also in other neurologic disorders, such as autism and intellectual disability (Buffington & Rasband, 2011; Escayg & Goldin, 2010; Han et al., 2012). Variants in other familiar proteins, such as SCN9A (also present in the proteome) have been associated with epilepsy, and both SCN1A and SCN9A can suffer mutations in patients with febrile seizures and Dravet syndrome (Singh et al., 2009).

The opioid Receptor Mu 1 (**OPRM1**) has been already proposed to participate in several neurological disorders such as Alzheimer's diseases, schizophrenia, seizures and alcoholism dependence (Samochowiec et al., 2019). OPRM1 is responsible for the neuroexcitatory effect of morphine in the brain. Thus, a high concentration of morphine trigger spontaneous seizure activity. However, low concentrations actually demonstrated a preventive effect on seizures (Frost et al., 1988). This biphasic effect has a potential advantage for therapeutic purposes since the anticonvulsant effects of both peptide and nonpeptide opioids are mediated through mu opiate receptors. Additionally, *Frost et al*, showed an increased opiate receptor binding in the brain of patients with epilepsy (Frost et al., 1988).

c) L1CAM and GDI1

L1 cell adhesion molecule (**L1CAM**) is an axonal glycoprotein involved in the generation of transmembrane signals and the dynamics of cell adhesion. L1CAM is also implicated during the processes of nervous system development, where participates not only in neuronal migration, differentiation, and synaptogenesis but also in complex cognitive and memory processes (Marin et al., 2015). Mutations in L1CAM lead to L1 syndrome (also called L1CAM syndrome), which is a group of diseases that are characterized by neurological damage. Intellectual disability and seizures are one of the main clinical signs of the L1 syndrome. Additionally, *Patzke et al*. observed that defects in L1CAM cause a considerable reduction of axonal size and dendritic arborizations (Patzke, Acuna, Giam, Wernig, & Sudhof, 2016). Another study by *Nishimura et al.* showed a relation between L1CAM and actin, which can bind together and promote neurite elongation (Nishimura et al., 2003).

Dissociation Inhibitor 1 (**GDI1**) regulates GDP/GTP reactions and has a major role in mental disorders. This protein has been implicated in several endo/exocytic pathways, in particular, when mutated, GDI1 causes impairment of synaptic vesicles exocytosis. As illustrated in figure 17, GDI1 has a strong relation with mental retardation. The lack of this protein has shown a direct effect in short—term memory formation, which also causes behavioral alterations (D'Adamo et al., 1998). *D'Adamo et al.* showed impairment of working memories due to defects in forming short-term memories (D'Adamo et al., 2002). Mental retardation is a common neurological disorder caused by either genetic factors or environmental factors and causes impairment in the development of intellectual and learning abilities (D'Adamo et al., 1998).

d) ACTB

β-actin or **ActB** is one of the six actin proteins that form the microfilaments. This cytoskeleton protein is involved in cell motility, structure, and integrity. In particular, β-actin has a special role in regions of motile cytoplasm, such as axons and growth cones affecting the rate and direction of outgrowth (H. L. Zhang, Singer, & Bassell, 1999). Thus, the differential subcellular localization of β-actin could determine local polymerization and therefore outgrowth processes. *Z hang, et al*. have identified a regulatory effect of neurotrophins in localization of β-actin mRNAs granules on growth cones (H. L. Zhang et al., 1999). β-actin mRNA was actually one of the earliest transcripts to be discovered involved in axonal local protein synthesis and participating in neuronal development and synaptic plasticity (Bassell et al., 1998; Matthews, Eastwood, & Harrison, 2012; Spaulding & Burgess, 2017). Nevertheless, according to *J. Donnelly et al*., only a small percentage of β-actin of the axon is actually locally synthesized there, being the majority translated in the cell body and then transported (Donnelly et al., 2013). However, β-actin might be involved in the reorganization and formation of the cytoskeleton around growing cones during development, explaining the cellular necessity of local translation. β-actin has also been implicated in several neurological disorders such as downregulation in schizophrenia, as showed in the interaction map of figure 17, and autism spectrum disorders, as discussed later (Yan, Kim, Datta, Lewis, & Soderling, 2016).

Overall, these results, demonstrate clear implications of axonal transport and local protein synthesis within neurodegeneration. As described before, dysregulation of axonal synthesis pathways, play critical roles behind some neurodegenerative disease, as we could confirm by the implication of some of the proteome proteins in different neuronal disorders. However, further testing involving immunohistochemistry and protein synthesis suppressors would be required to better understand and characterize the role of these proteins in the local proteome.

Moreover, analysing separately the protein content of specific cellular compartments, such as axons, has some advantages compared to the global proteome. Examining the pathway's disruption or alterations in protein content can bring insight into novel synthesis modulators or molecular targets to actively reverse the defects in some of these neurodegenerative disorders. Also, connecting the etiology of neurological disorders with protein clusters can improve the current knowledge and, in the future, help identify new therapeutic targets. Finally, as shown in the previous results, due to their extensive presence in axons and involvement in important axonal processes described before, neuronal microtubules are good candidates for a non-specific approach to reverse transport anomalies in several neurodegenerative diseases. Moreover, there are already few published studies showing a positive effect of microtubule stabilizers in different neurological disorders, like Alzheimer's disease, Parkinson's disease, epilepsy, autism, and

Amyotrophic lateral sclerosis (Brunden, Lee, Smith, Trojanowski, & Ballatore, 2017; Kleele et al., 2014; Kovalevich et al., 2016; Sleigh et al., 2019).

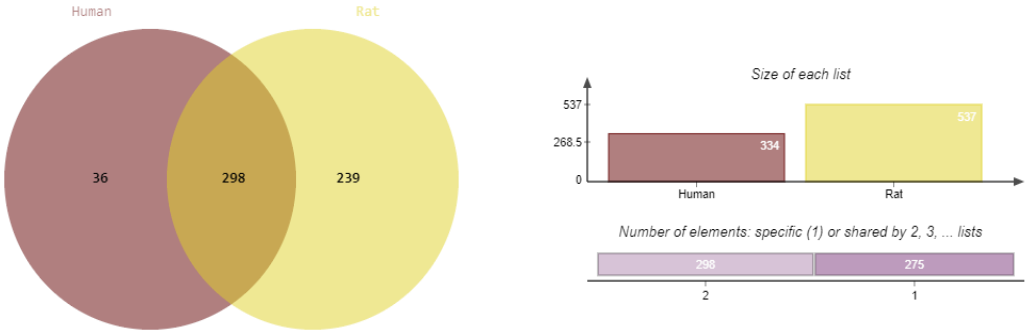


Figure 19 - Venn diagram comparing orthologs genes from Rattus norvegicus (yellow) and Homo Sapiens (red)

SECRETOME

Secretome is the bulk of proteins that are produced and delivered to the extracellular space, via secretory pathways. SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) is an online platform that generates predictions of protein secretion in eukaryotes, using the protein sequence in FASTA format. The secretion of proteins is involved in several pathological pathways, particularly in neuropsychiatric disorders (W. J. Lin & Salton, 2013). From the proteins mentioned above, SCN1A, SCN9A, OPRM1, GDI1, and ActB are predicted as not secreted, as we can confirm from our results. These results were expected since SCN1A, SCN9A and OPRM1 are membrane proteins while ActB is part of the cytoskeleton. On the other hand, APP, APOE, Clu, Mapt, and L1CAM have been already reported as secreted, and in some cases, are already linked to secretory pathways on neurodegenerative diseases (Fernandez, Hamby, McReynolds, & Ray, 2019; Gregory et al., 2017; Santiago-Medina, Gregus, Nichol, O'Toole, & Gomez, 2015; M. Zhang et al., 2020). Moreover, protein secretion can be implicated in secretory pathways of neurodegenerative diseases, due to not only protein aggregation but also protein misfolding. These mechanisms participate in the course of transmission between neurons of pathogenic proteins and neurodegeneration, as occurs in Alzheimer's disease with Aβ peptides secretion in endosomes or in Parkinson's disease with the secretion of TAU in exosomes (Lim & Lee, 2017).

According to our results, 118 proteins found in the axonal proteome of hippocampal neurons are possibly secreted. These protein hold great potential to be further investigated and explore their role in important physiological processes or neuronal degeneration, as well as be considered as possible blocking targets in future therapy approaches. However, the effect of axonal secretome is still poorly understood from the normal and healthy point of

view, much less in a neuropathological context. To our best knowledge, there has not been any report of the axonal secretome dataset nor any association with neurological diseases.

Take as an example multiple sclerosis, which is a common neurological disease characterized by neuron inflammation, with progressive loss of myelin sheath and consequent nerve fiver destruction. Despite the importance of myelination and the pathological consequences of demyelination in diseases like multiple sclerosis, the axonal secretome might trigger signaling mechanisms or bias responses during the re-myelination process. For instance, TGF-β, which is a multifunctional cytokine, involved during different physiological events such as proliferation, differentiation, growth and involved in activation of growth factors like the interferon-δ (Makwana et al., 2007). Due to the high involvement in the immune system, this cytokine has been implicated in the modulation of several immune cell populations, including the CNS resident microglia. In normal conditions, TGF-β is an important endogenous factor for adequate microglia function and maturation (Zoller et al., 2018). During pathological conditions, this cytokine has a potent immunoregulatory role which can impair some processes and may be involved in pathogenesis of several neurodegenerative diseases (Tesseur & Wyss-Coray, 2006). TGF-β mRNA has a high overexpression in the CSF of MS patients, comparing to other neurological diseases (Soderstrom et al., 1995). However, the crosstalk between the CNS cells via TGF-β secretion and subsequent effects is not completely understood at the moment. In particular, the bidirectional crosstalk, axon-microglia and microglia-axon, exchange signals that can play important roles during the pathogenesis of neurodegenerative diseases.

According to SecretomeP analysis, TGF-β is predictable secreted in the analysed axonal proteome from hippocampal neurons. In addition, to our best knowledge, until now there has not been reported the axonal secretion of TGF-β. Thus, we speculate that when there is aggression during an MS relapse, TGF-β might be overexpressed and secreted by the axon, suppressing the normal function of the microglia. Therefore, these immune resident cells would not be able to clear the debris from cell destruction, neither promote remyelination, entering in a conflicting effect.

Nevertheless, validation is still needed to confirm the presence and secretion of this molecule on the axon. Immunocytochemistry, mRNA sequencing or FISH techniques could be used to validate this claim. These assays will confirm the presence of the protein but also screen for the occurrence of TGF-β mRNA, which will inform us about the possible local translation in the axon. Further studies should also include pathological assays to confirm the involvement of TGF-β in multiple sclerosis pathogenesis. For this type of assays, organ-on-chip could be the fittest models to explore, since it can generate a three-dimensional co-culture, grown with controlled settings and different layers of complexity, mimicking the natural biological environment. Additionally, this system allows the integration in a multiple organ system, the body-on-chip, that can be very useful to study the neuro-enteric interaction, which may be crucial in several neurological diseases, including multiple sclerosis. In particular, there is a high TGF-β expression in the gut, where our immune system is also turned on, having a great potential to be studied.

Therefore, understand the effect of axonal secretome during pathological states would be a crucial step for unravelling any important mechanism between the axon and the pathogenesis of important neurological disorders.

DISEASE DATABASES

In order to corroborate and reinforce previous data and explore new pathways or clusters and identify protein interactions, we next analysed the presence of proteins of the axonal proteome within the most prevalent neurological disorders, by comparing our data with the available diseases databases. Although information is widely available online nowadays, there is still a lack of more databases to gather and report the available information in some of these diseases, such as Parkinson, Alzheimer, Multiple Sclerosis, Huntington's disease, Lupus among others. In particular, Lupus erythematosus databases were not yet existent and for that reason, we did not explore as suggested by our KEGG analysis and proposed above.

Carpedb is an online database with genetic information about epilepsy. Epilepsy is a neurological disorder characterized by complex chemical changes that occur in neurons, causing an imbalance between excitatory and inhibitory activity. Patients with epilepsy express unprovoked seizures that may have no diagnosed cause. Using the genetic database, CarpeDb (http://carpedb.ua.edu/) we were able to identify 15 proteins linked to epilepsy (Figure 20A). Some of these axonal proteins are responsible for generating and maintaining the action potential, and when dysregulated, might impair excitatory proteins, which leads to an epileptic event. In fact, Pernice *et al*. had recently presented evidence that local translation might be implicated in epilepsy, describing the relevance of RBPs for control of local translation of mRNA involved in the occurrence of seizures *(Pernice, Schieweck, Kiebler, & Popper, 2016).* RNA-binding proteins (RBP) are responsible for mRNA transport, stability, and translation, and thus can directly shape and modulate the local proteome. Mutations in RBP has been previouly proposed to be involved in epilepsy (Zhu, Han, Blendy, & Porter, 2012). Since in epileptic disorder there is a synaptic excitatatory/inhibitory ratio imbalance, and aberrant axon translation may underly the disease. In particular, some regulators of synaptic excitability, such as ion channels, as well as other epilepsy targets can be upregulated by RBP, which control local expression of neuronal hyperexcitability in epilepsy. Thus, increased excitability may lead to neuronal firing that eventually results in seizures. For instance, throughout the process of mRNA distribution and expression, the RBP can regulate protein synthesis of known epilepsy targets, such as ion channels, like SCN5A and CamKII, or other involved proteins like GAP-43 (Tiruchinapalli, Caron, & Keene, 2008; Tiruchinapalli, Ehlers, & Keene, 2008). GAP-43, is a crucial component of the axon and presynaptic terminal since is intrinsically involved during the process of neuroplasticity, axonal growth, and synaptogenesis, with a production decline observed after. Yet, has been observed a new

increase of expression in hippocampal granule cells after a seizure. As observed by Yamanouchi et al. the production of GAP-43 occurs in the cell soma and is then transported through fast axonal flow to axon (Yamanouchi et al., 2000; Ying et al., 2014). Therefore, the presence of GAP-43 suggests that the sample analyzed was taken during an active process of axonal growth.

Ultimately, increased local translation of ion channels and receptors and later misregulation of synaptic excitability may represent not only a risk factor for epilepsy but also a possible category for targeted therapeutics.

SFARI is an annotated list of genes generated by research studies, which provides autism gene candidates to explore future medical approaches. Autism Spectrum Disorder (ASD) is a neurological disease, characterized by neurodevelopmental abnormalities, caused by genetic and environmental factors. Autistic patients express a clear deficit of social interaction or communication and repetitive behaviours, and are accompanied by several comorbidities, including intellectual disability, anxiety, hyperactivity, and epilepsy. The prevalence of autism has been rapidly increasing, emerging a concern about a possible "epidemic" of autism (Chiarotti & Venerosi, 2020). The analysis of the axonal proteome of hippocampal rat neurons reveals several expressed proteins linked with autism spectrum disorder (Figure 20B). The analysis was carried out using the SFARI gene database (https://gene.sfari.org/). One of the many genes of interest identified in our screen is Insulin-like growth factor 1. IGF1 is an FDA approved drug, that has been showing a great potential in the pharmacological treatment of ASD. This molecule has a great capacity to promote neuronal cell survival, synapse maturation and synapse plasticity due to its ability to cross the brain blood barrier. Notably, IGF1 has lately been explored in neuronal models of Rett Syndrome and children with Rett Syndrome, suggesting a promising potential treatment of ASD with improvements in the core symptoms of ASD (Xu, Cao, Zhang, & Cheadle, 2018). Additionally, IGF1 was able to reverse synaptic and behavioural deficits.

Although great advances have been achieved in research, the autism pathogenesis is still not completely described, since there is a large heterogenicity in causes' of ASD. However, impaired synaptic transmission, defective axonal transport, which will decrease the number of synaptic buttons formed and their maturation, and deficiencies in cytoskeletal support of axon/neuron structures have a relevant connection in autism spectrum disorder pathogenesis (Abraham et al., 2019; Xiong et al., 2020). Additionally, ASD patients also have an under-expression of axo-guidance proteins and receptors (Sleigh et al., 2019). This class of proteins is usually responsible for axonal growth and development although, when dysregulated, are capable of triggering defects in neuronal networks. Since in ASD occur a deficient modulation of connectivity between different brain regions, axon-growth and guidance proteins may play a major role during the pathogenesis. Thusly, axonal development might be involved during pathogenesis events in autism, namely by local translation processes. Building knowledge can lead to the identification of molecular pathways with possible therapeutic potential.

ALSoD (https://alsod.ac.uk/) is an online bioinformatics repository of the annotated Amyotrophic Lateral Sclerosis (ALS) genes. ALS is a progressive neurodegenerative disease that affects motor neurons, which are responsible for controlling voluntary muscle movement. The death or degeneration of this type of neurons leads to a severe disability of the patient, since the brain loses control of voluntary movement function and ultimately leads to respiratory failure that causes death within a few years. Although progress has been made to determine the genetic basis of amyotrophic lateral sclerosis, the mechanism of how genetic changes cause the disease is incompletely understood. According to our results, there was presence of twelve proteins associated with ALS, namely: Pfn1, Hnrnpa1, Apoe, Mapt, Prph, Tuba4a, Crym, Hnrnpa2b1, Vcp, Dync1h1, Lmnb1, and Matr3 (Figure 20C). SOD1 was one of the first genes linked to ALS and the only used in disease models. More recently, the list of genetic causes expanded, and other genetic alterations associated with ALS have been explored, in particular, VCP, Tuba4a, and PFN, which are among the most reported (Nijssen et al., 2018). Overall, most of the known ALS pathological associated proteins seem to be related with nucleo-cytoplasmatic mRNA transport (Barton, Gregory, Chandran, & Turner, 2019). Moreover, this pathology was already linked with several cellular dysfunctions, in particular, protein aggregation and impairment in axonal local translation (Nijssen et al., 2018). In fact, axonal transport dysregulation is actually considered one of the initial pathological mechanisms in ALS (Khalil, Morderer, Price, Liu, & Rossoll, 2018). Motor neurons possess axons that can extend to long lengths, so any axonal transport or translation dysfunctions can be detrimental to the cell viability. Impairment in RNA trafficking and axonal transport leads to cellular dysfunction that is observed in both ALS patients and ALS mouse models (Millecamps & Julien, 2013). *Yasuda et al.* proposed that local RNA translation disruption plays a central role in the ALS pathogenesis, in particular with a feedback effect in the disease's progression (Yasuda & Mili, 2016). The same author also highlights the role played by RBPs aggregates in the pathogenesis of ALS. Additionally, *Perlson et al*. observed a shift of retrograde signals in patients with ALS, in particular, dynein transported cargo started to promote death signals instead of the usual survival singling (Perlson et al., 2009).

Intra-axonal translation might be linked to possible pathogenic effects. For instance, epilepsy, ALS, Alzheimer, fragile X syndrome and ASD are some of the most prevalent diseases that have shown a relation with defects in either axonal protein synthesis or axonal transportation (Cagnetta et al., 2018; Fernandez-Moya, Bauer, & Kiebler, 2014; Sleigh et al., 2019; Van Battum et al., 2015).

Ultimately, local protein synthesis can be manipulated in different therapeutic approaches, in order to neutralize and decrease the neurotoxicity and neurodegeneration effect. Silent RNA (sRNA or siRNA) is a prominent genetic technology capable of interfering with protein expression. siRNA can be employed in axons to selectively deplete specific transcripts and since the mRNA pool in the axon is lower than in the soma, the side effects and the off-target probability are significantly reduced. Another class of molecules that are promising therapeutic targets for several neuropathologies are axon-guidance proteins (Van Battum et al., 2015).

As demonstrated in a study by *Baleriola et al.*, siRNA is an effective therapeutic approach and can be considered in future interventions to prevent the spread neurodegeneration (Baleriola et al., 2014).

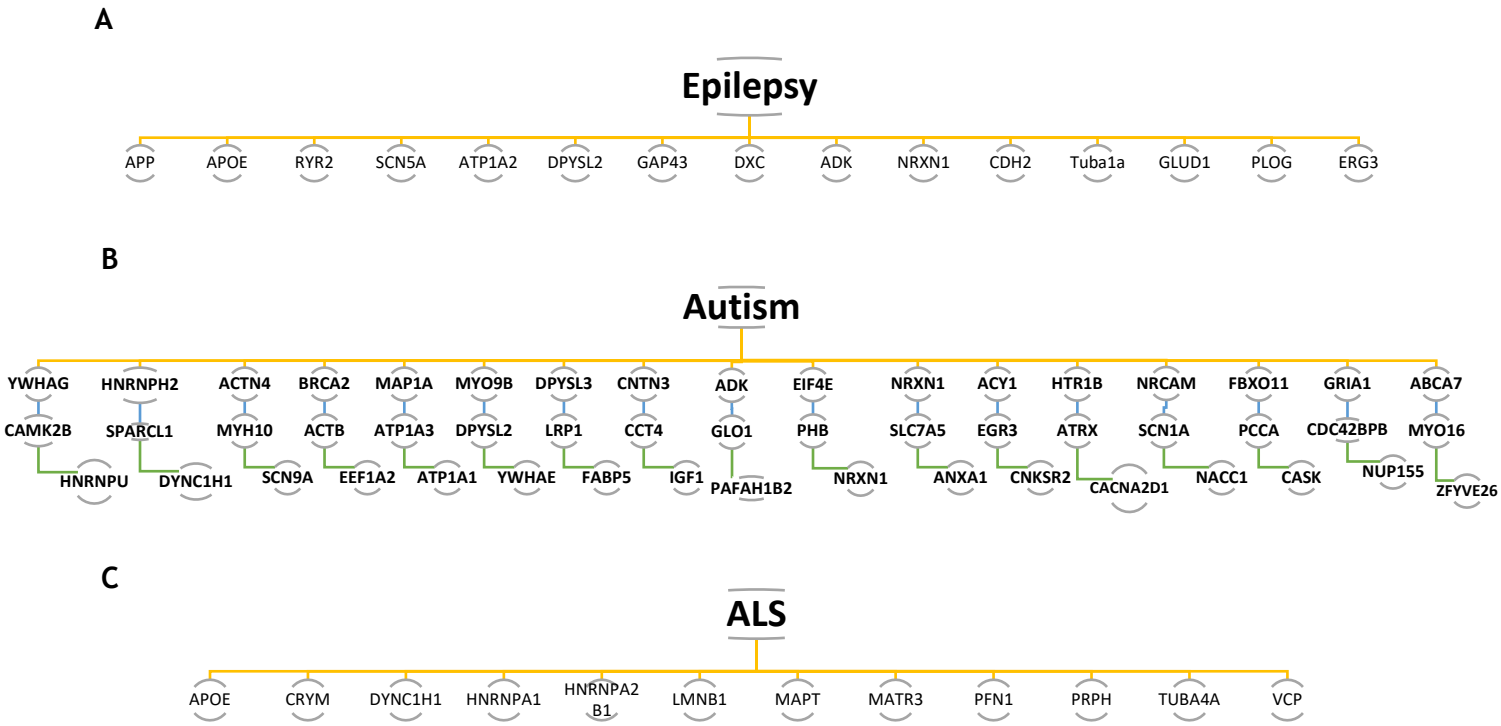


Figure 20 - (A) Genes associated with epipelsy - CarpeDB; (B) Genes associated with autism - SFARI DB and (C) Genes associated with Amyotrophic Lateral sclerosis (ALS) – ALSoD. All lines represent proteins found both in our proteomic dataset and the respective database.

SYNAPSE SUB-ANALYSIS

Synapses are communication structures that allow the axon to pass the electrochemical signal to the dendrites of the next neuron. Synaptic modulation is intrinsically related with adjustment of the neuronal network, specifically by long-term potentiation (LTP) and long-term depression (LTD). These cellular processes are the basic units of cognitive development, learning and memory. Impairment in some of these cellular processes may hinder mental illness (Zukin, Richter, & Bagni, 2009). Table 7 summarizes the subgroup of the synaptogenic proteins and synapse organization proteins present within the axonal proteome here reported. Some of these synaptogenic proteins were earlier mentioned, namely Actb, Gap43, Mapt, Apoe, and APP. Dysfunction of proteins involved in synapse development and maturation can lead to synaptopathies, that may include in some of the aforementioned disorders like Alzheimer's disease, Autism spectrum disorders, and epilepsy (Lepeta et al., 2016). Nevertheless, due to the difficulty of detecting defects in such small subcellular compartments, the implications of dysregulated local translation inside presynaptic domains has been difficult to address.

Local protein synthesis plays a major role during synaptic plasticity. For instance, since activation of synapse can trigger a different set of signal cascades, which involves different molecules and receptors, suggesting that a distinct protein pool is required. Local protein synthesis can holistically supply the synapse, to provide proper plasticity. Moreover, the synapse can also regulate the translation in neurons depending on synaptic activity, through several synaptic stimuli such as BDNF. Thus, local protein synthesis is capable not only of producing local changes in synaptic efficacy, but also promote synaptogenesis (A. C. Lin & Holt, 2008).

Table 8 - Synapse sub-analysis: group of Synaptogenic and synapse organization present in the axonal proteome. Some proteins are listed in multiple categories. (cytoscape)

Synaptogenic proteins 	Igf1; Actb; Apoe; App; Cdh2; Crmp1; Cdc42; Dbn1; Dcx; Gap43; Kalrn; Lrp1; Map1b; Map2; Myh10; Ncam1; Slit1; Stmn1; Uchl1; Mmp2; Mapt; Ptn; Clstn1; Clstn2; Cnksr2; Farp1; Il1rap; Marcks; Ncam; Nrxn1; Pfn1; Sparc; Sparcl1; VCP; L1Cam; Igf1, Ncam1; LANP
Synapse Organization 	Actb, Actn1, Apoe, App, Arf4, Cdh2, Cfl1, Clstn1, Clstn2, Cnksr2, Cntn2, Dbn1, Farp1, Gap43, Hspa8, Il1rap, Kalrn, Map1b, Mapt, Marcks, Myh10, Ncan, Nrxn1, Pfn1, Ptn, Slit1, Sparc, Sparcl1, Tuba1a, Tuba1b, Tubb5, Vcp, Ywhaz

CHAPTER VI: CONCLUSIONS AND FUTURE PERSPECTIVES

An extensive analysis was conducted from the mass spectrometry raw data, reviling a diverse set of proteins within the sample. Proteome Discoverer™ software made the biggest contribution for the total proteome, allowing a wide coverage and a more comprehensive protein identification. Since this was the first time characterizing pure axonal lysate samples, we used two different software to process the mass spectrometer raw data, allowing us to maximize the spectrum of identified proteins. Notably, we were able to identify 314 proteins in the hippocampal rat axonal proteome, that were assigned to different biological relevant categories. Although very efficient in the axonal isolation, the microfluidic chamber has had a clear low yield, affecting the amount of sample used for MS and thus the proteins identified. However, this novel technology showed a good purity and great isolation capacity, confirming the adequacy of this method in future neurobiology assays.

The findings discussed throughout this work, confirmed a clear enrichment and high abundance of cytoskeletal proteins, suggesting a link with both axonal translation and axonal transport, which helps to regulate and influence the axonal proteome. As previously suggested, these proteins gather great potential for non-specific therapeutic approaches in order to reverse transport anomalies in neurodegenerative diseases. Furthermore, all GO analysis showed enrichment of cytoskeletal proteins and axonal and synaptic formation/development proteins, concluding that both have important roles in the hippocampal axonal proteome. Additionally, to these proteins, various biosynthetic processes and the presence of several proteins that make up the translation apparatus, such as ribosomes, initiation factors, and RNA-binding proteins, allowed us to conclude that local protein synthesis was very active in the axon.

The secretome analysis also revealed 118 possible secreted proteins from the global proteome. As previously stated, there is still a lack of secretome studies in healthy and unhealthy axons, which could help elucidate their function. This group of proteins may be implicated not only in important physiological process but also in pathological conditions. Describing what proteins are being secreted in hippocampal axons can function as baseline for future reference.

Ultimately, the association of neurodegenerative diseases with defects in both local protein synthesis and axonal transport suggests that these mechanisms have a major role in the normal and healthy maintenance of axons. As discussed earlier, some of the most common neurological disorders, such as autism spectrum disorder, amyotrophic lateral sclerosis, Alzheimer's disease, among others, present serious dysregulations in either axonal transport or axonal translation. In particular, from the admissible databases for specific neuro-diseases, we conclude an enrichment of biomarkers for autism. Unfortunately, there was a lack of several proteomic databases for disease-associated gene encoding proteins, which have countless advantages if well explored. These databases gather great potential to expand our knowledge and bring insight into novel therapeutic targets. Finally, the proteome

analysis here reported may be useful for future comparisons and contribute to further studies.

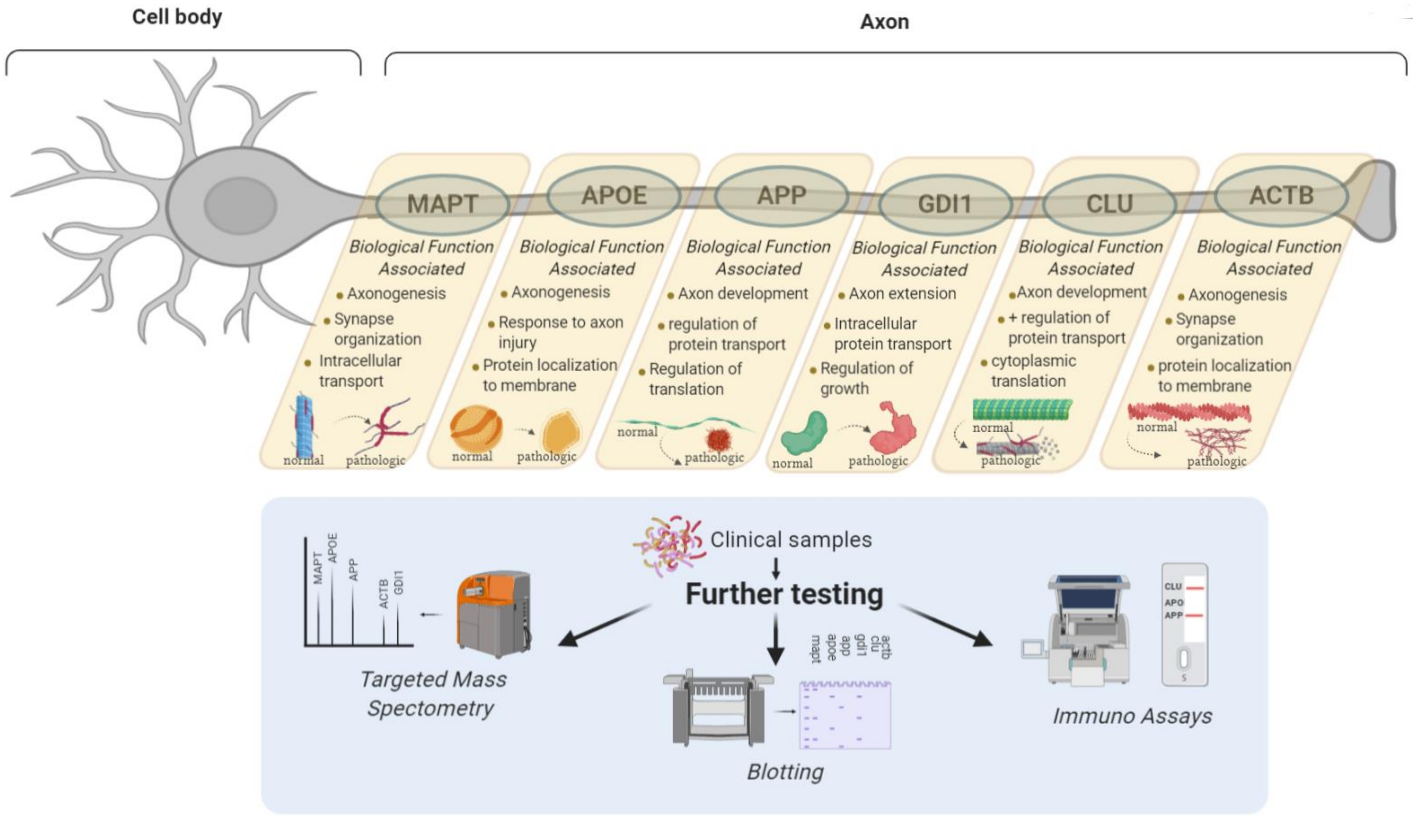


Figure 21 - Panel of proteins enriched in the axonal proteome with high biological and pathological relevance. These proteins may be eligible as future candidates in clinical settings, using different analytical techniques for their tracking.

Overall, Mapt, Apoe, App, Gdi1, Clu and Actb are a proposed panel of proteins enriched in the hippocampal axonal proteome with high biological and pathological relevance, as illustrated in figure 21. This group of candidates can be potentially explored in a diverse range of physiological conditions, and different types of samples, in order to eventually achieving clinical translation. Making use of different techniques such as blotting, immune assays or targeted mass spectrometry will allow not only to compare protein expression but also to easily track these proteins in clinical samples. These and other cytoskeleton proteins gather several translational opportunities that can be explored in the future.

Further validation of these results is still necessary not only to understand the underlying macromolecular mechanisms and functions but also to unravel the source of synthesis of some proteins and their subcellular location. Using diversified techniques such as FISH, siRNA, other label methods and live imaging, would be possible to explore how different stimulus triggers or inhibits the expression of specific proteins or even if whether a particular protein is locally translated in the axon. Further studies are still required, for example, unravel the function of nuclear proteins in the axons and distinguish the pool of proteins locally produced from those transported from the soma. In the present work synaptic proteins were reported during GO analysis. Thus, immunolabeling and microscopy

might still be needed to further investigate these proteins localization and their local function.

Hopefully, identifying defects during axonal transportation or detect over- or under-expression of protein, would help find new therapeutically candidates to actively reverse the defects or modulate the synthesis in pathological states. Additionally, describing a new pathological pathway could also have an enormous impact on discovering new pharmacological approaches, for blocking specific protein synthesis using, for example, siRNA and avoiding unnecessary protein translation. For instance, since axonal translation has such an active role during nerve injure and regeneration, by identifying produced or secreted proteins or neurotrophic factors, could have a huge impact in several neurological conditions.

Finally, it is important to raise attention to the variability and unpredictability of the axonal proteome depending on age, development stage, type of neurons, and physiological condition, leading to the hypothesis that the proteome of *in vivo* axons will significantly differ. Thus, it is necessary to explore new approaches and methods, such as TRAP-seq technique, which allows scientists to observe axonal protein synthesis *in vivo*. Besides, since non-neuronal cells, such as glial cells, may also contribute to the axonal proteome, to have a better mimicry of the *in vivo* biological conditions, it is important to develop cellular models that best represent physiological conditions. Organs on chips is an upcoming technology, with great potential to integrate multi-cellular cultures into neurobiology studies and face some of these hurdles mentioned before.

CHAPTER VII: REFERENCES

Abraham, J. R., Szoko, N., Barnard, J., Rubin, R. A., Schlatzer, D., Lundberg, K., . . . Natowicz, M. R. (2019). Proteomic Investigations of Autism Brain Identify Known and Novel Pathogenetic Processes. *Sci Rep, 9*(1), 13118. doi:10.1038/s41598-019-49533-y

Alvarez, J., & Benech, C. R. (1983). Axoplasmic incorporation of amino acids in a myelinated fiber exceeds that of its soma: A radioautographic study. *Experimental Neurology, 82*(1), 25-42. doi:10.1016/0014-4886(83)90240-6

Andreassi, C., Zimmermann, C., Mitter, R., Fusco, S., De Vita, S., Saiardi, A., & Riccio, A. (2010). An NGF-responsive element targets myo-inositol monophosphatase-1 mRNA to sympathetic neuron axons. *Nat Neurosci, 13*(3), 291-301. doi:10.1038/nn.2486

Attar, N., Campos, O. A., Vogelauer, M., Cheng, C., Xue, Y., Schmollinger, S., . . . Kurdistani, S. K. (2020). The histone H3-H4 tetramer is a copper reductase enzyme. *Science, 369*(6499), 59-64. doi:10.1126/science.aba8740

Baleriola, J., & Hengst, U. (2015). Targeting axonal protein synthesis in neuroregeneration and degeneration. *Neurotherapeutics, 12*(1), 57-65. doi:10.1007/s13311-014-0308-8

Baleriola, J., Walker, C. A., Jean, Y. Y., Crary, J. F., Troy, C. M., Nagy, P. L., & Hengst, U. (2014). Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. *Cell, 158*(5), 1159-1172. doi:10.1016/j.cell.2014.07.001

Barton, S. K., Gregory, J. M., Chandran, S., & Turner, B. J. (2019). Could an Impairment in Local Translation of mRNAs in Glia be Contributing to Pathogenesis in ALS? *Front Mol Neurosci, 12*, 124. doi:10.3389/fnmol.2019.00124

Bassell, G. J., Zhang, H., Byrd, A. L., Femino, A. M., Singer, R. H., Taneja, K. L., . . . Kosik, K. S. (1998). Sorting of β-Actin mRNA and Protein to Neurites and Growth Cones in Culture. *The Journal of Neuroscience, 18*(1), 251-265. doi:10.1523/jneurosci.18-01-00251.1998

Bates, K. A., Verdile, G., Li, Q. X., Ames, D., Hudson, P., Masters, C. L., & Martins, R. N. (2009). Clearance mechanisms of Alzheimer's amyloid-beta peptide: implications for therapeutic design and diagnostic tests. *Mol Psychiatry, 14*(5), 469-486. doi:10.1038/mp.2008.96

Bathke, J., Konzer, A., Remes, B., McIntosh, M., & Klug, G. (2019). Comparative analyses of the variation of the transcriptome and proteome of Rhodobacter sphaeroides throughout growth. *BMC Genomics, 20*(1), 358. doi:10.1186/s12864-019-5749-3

Bianco, A. M., Marcuzzi, A., Zanin, V., Girardelli, M., Vuch, J., & Crovella, S. (2013). Database tools in genetic diseases research. *Genomics, 101*(2), 75-85. doi:10.1016/j.ygeno.2012.11.001

Biever, A., Donlin-Asp, P. G., & Schuman, E. M. (2019). Local translation in neuronal processes. *Curr Opin Neurobiol, 57*, 141-148. doi:10.1016/j.conb.2019.02.008

Bigler, R. L., Kamande, J. W., Dumitru, R., Niedringhaus, M., & Taylor, A. M. (2017). Messenger RNAs localized to distal projections of human stem cell derived neurons. *Sci Rep, 7*(1), 611. doi:10.1038/s41598-017-00676-w

BODIAN, D. (1964). <A SUGGESTIVE RELATIONSHIP OF NERVE CELL RNA WITH SPECIFIC SYNAPTIC SITES.pdf>. *53*.

Briese, M., Saal, L., Appenzeller, S., Moradi, M., Baluapuri, A., & Sendtner, M. (2016). Whole transcriptome profiling reveals the RNA content of motor axons. *Nucleic Acids Res, 44*(4), e33. doi:10.1093/nar/gkv1027

Brittis, P. A., Lu, Q., & Flanagan, J. G. (2002). Axonal Protein Synthesis Provides a Mechanism for Localized Regulation at an Intermediate Target. *Cell, 110*(2), 223-235. doi:10.1016/s0092-8674(02)00813-9

Brunden, K. R., Lee, V. M., Smith, A. B., 3rd, Trojanowski, J. Q., & Ballatore, C. (2017). Altered microtubule dynamics in neurodegenerative disease: Therapeutic potential of microtubule-stabilizing drugs. *Neurobiol Dis, 105*, 328-335. doi:10.1016/j.nbd.2016.12.021

Buffington, S. A., & Rasband, M. N. (2011). The axon initial segment in nervous system disease and injury. *Eur J Neurosci, 34*(10), 1609-1619. doi:10.1111/j.1460-9568.2011.07875.x

Büyükköroğlu, G., Dora, D. D., Özdemir, F., & Hızel, C. (2018). Techniques for Protein Analysis. 317-351. doi:10.1016/b978-0-12-804659-3.00015-4

Cagnetta, R., Frese, C. K., Shigeoka, T., Krijgsveld, J., & Holt, C. E. (2018). Rapid Cue-Specific Remodeling of the Nascent Axonal Proteome. *Neuron, 99*(1), 29-46 e24. doi:10.1016/j.neuron.2018.06.004

Cajigas, I. J., Tushev, G., Will, T. J., tom Dieck, S., Fuerst, N., & Schuman, E. M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron, 74*(3), 453-466. doi:10.1016/j.neuron.2012.02.036

Campbell, D. S., & Holt, C. E. (2001). Chemotropic Responses of Retinal Growth Cones Mediated by Rapid Local Protein Synthesis and Degradation. *Neuron, 32*(6), 1013-1026. doi:10.1016/s0896-6273(01)00551-7

Castilla-Cortazar, I., Aguirre, G. A., Femat-Roldan, G., Martin-Estal, I., & Espinosa, L. (2020). Is insulin-like growth factor-1 involved in Parkinson's disease development? *J Transl Med, 18*(1), 70. doi:10.1186/s12967-020-02223-0

Chen, C., Hou, J., Tanner, J. J., & Cheng, J. (2020). Bioinformatics Methods for Mass Spectrometry-Based Proteomics Data Analysis. *Int J Mol Sci, 21*(8). doi:10.3390/ijms21082873

Chen, H. C. (2005). Boyden chamber assay. *Methods Mol Biol, 294*, 15-22. doi:10.1385/1-59259-860-9:015

Chiarotti, F., & Venerosi, A. (2020). Epidemiology of Autism Spectrum Disorders: A Review of Worldwide Prevalence Estimates Since 2014. *Brain Sci, 10*(5). doi:10.3390/brainsci10050274

Chiba, K., Araseki, M., Nozawa, K., Furukori, K., Araki, Y., Matsushima, T., . . . Suzuki, T. (2014). Quantitative analysis of APP axonal transport in neurons: role of JIP1 in enhanced APP anterograde transport. *Mol Biol Cell, 25*(22), 3569-3580. doi:10.1091/mbc.E14-06-1111

Chong, P. K., Gan, C. S., Pham, T. K., & Wright, P. C. (2006). Isobaric tags for relative and absolute quantitation (iTRAQ) reproducibility: Implication of multiple injections. *J Proteome Res, 5*(5), 1232-1240. doi:10.1021/pr060018u

Chuang, C. F., King, C. E., Ho, B. W., Chien, K. Y., & Chang, Y. C. (2018). Unbiased Proteomic Study of the Axons of Cultured Rat Cortical Neurons. *J Proteome Res, 17*(5), 1953-1966. doi:10.1021/acs.jproteome.8b00069

Chudinova, E. M., & Nadezhdina, E. S. (2018). Interactions between the Translation Machinery and Microtubules. *Biochemistry (Mosc), 83*(Suppl 1), S176-S189. doi:10.1134/S0006297918140146

Clare, D. K., & Saibil, H. R. (2013). ATP-driven molecular chaperone machines. *Biopolymers, 99*(11), 846-859. doi:10.1002/bip.22361

Coleman, M. (2002). Axon pathology in neurological disease: a neglected therapeutic target. *Trends in Neurosciences, 25*(10), 532-537. doi:10.1016/s0166-2236(02)02255-5

D'Adamo, P., Menegon, A., Lo Nigro, C., Grasso, M., Gulisano, M., Tamanini, F., . . . Toniolo, D. (1998). Mutations in GDI1 are responsible for X-linked non-specific mental retardation. *Nat Genet, 19*(2), 134-139. doi:10.1038/487

D'Adamo, P., Welzl, H., Papadimitriou, S., Raffaele di Barletta, M., Tiveron, C., Tatangelo, L., . . . Toniolo, D. (2002). Deletion of the mental retardation gene Gdi1 impairs associative memory and alters social behavior in mice. *Hum Mol Genet, 11*(21), 2567-2580. doi:10.1093/hmg/11.21.2567

Deglincerti, A., & Jaffrey, S. R. (2012). Insights into the roles of local translation from the axonal transcriptome. *Open Biol, 2*(6), 120079. doi:10.1098/rsob.120079

Donnelly, C. J., Park, M., Spillane, M., Yoo, S., Pacheco, A., Gomes, C., . . . Twiss, J. L. (2013). Axonally synthesized beta-actin and GAP-43 proteins support distinct modes of axonal growth. *J Neurosci, 33*(8), 3311-3322. doi:10.1523/JNEUROSCI.1722-12.2013

Eng, H., Lund, K., & Campenot, R. B. (1999). Synthesis of β-Tubulin, Actin, and Other Proteins in Axons of Sympathetic Neurons in Compartmented Cultures. *The Journal of Neuroscience, 19*(1), 1-9. doi:10.1523/jneurosci.19-01-00001.1999

Escayg, A., & Goldin, A. L. (2010). Sodium channel SCN1A and epilepsy: mutations and mechanisms. *Epilepsia, 51*(9), 1650-1658. doi:10.1111/j.1528-1167.2010.02640.x

Espina, V., Wulfkuhle, J. D., Calvert, V. S., VanMeter, A., Zhou, W., Coukos, G., . . . Liotta, L. A. (2006). Laser-capture microdissection. *Nat Protoc, 1*(2), 586-603. doi:10.1038/nprot.2006.85

Estrada-Bernal, A., Sanford, S. D., Sosa, L. J., Simon, G. C., Hansen, K. C., & Pfenninger, K. H. (2012). Functional complexity of the axonal growth cone: a proteomic analysis. *PLoS One, 7*(2), e31858. doi:10.1371/journal.pone.0031858

Farias, J., Sotelo, J. R., & Sotelo-Silveira, J. (2019). Toward Axonal System Biology: Genome Wide Views of Local mRNA Translation. *Proteomics, 19*(16), e1900054. doi:10.1002/pmic.201900054

Fernandez-Moya, S. M., Bauer, K. E., & Kiebler, M. A. (2014). Meet the players: local translation at the synapse. *Front Mol Neurosci, 7*, 84. doi:10.3389/fnmol.2014.00084

Fernandez, C. G., Hamby, M. E., McReynolds, M. L., & Ray, W. J. (2019). The Role of APOE4 in Disrupting the Homeostatic Functions of Astrocytes and Microglia in Aging and Alzheimer's Disease. *Front Aging Neurosci, 11*, 14. doi:10.3389/fnagi.2019.00014

Frost, J. J., Mayberg, H. S., Fisher, R. S., Douglass, K. H., Dannals, R. F., Links, J. M., . . . et al. (1988). Mu-opiate receptors measured by positron emission tomography are increased in temporal lobe epilepsy. *Ann Neurol, 23*(3), 231-237. doi:10.1002/ana.410230304

George J Siegel, Editor-in-Chief, Agranoff, B. W., Albers, R. W., Fisher, S. K., & Uhler, M. D. *Basic Neurochemistry: Molecular, Cellular and Medical Aspects* (6th ed.).

Giuditta, A., Dettbarn, W. D., & Brzin, M. (1968). Protein synthesis in the isolated giant axon of the squid. *Proc Natl Acad Sci U S A, 59*(4), 1284-1287. doi:10.1073/pnas.59.4.1284

Gonzalez, C., & Couve, A. (2014). The axonal endoplasmic reticulum and protein trafficking: Cellular bootlegging south of the soma. *Semin Cell Dev Biol, 27*, 23-31. doi:10.1016/j.semcdb.2013.12.004

Grant, S. G. N., & Blackstock, W. P. (2001). Proteomics in Neuroscience: From Protein to Network. *The Journal of Neuroscience, 21*(21), 8315-8318. doi:10.1523/jneurosci.21-21-08315.2001

Gregory, J. M., Whiten, D. R., Brown, R. A., Barros, T. P., Kumita, J. R., Yerbury, J. J., . . . Wilson, M. R. (2017). Clusterin protects neurons against intracellular proteotoxicity. *Acta Neuropathol Commun, 5*(1), 81. doi:10.1186/s40478-017-0481-1

Gulcicek, E. E., Colangelo, C. M., McMurray, W., Stone, K., Williams, K., Wu, T., . . . Wu, B. (2005). Proteomics and the analysis of proteomic data: an overview of current protein-profiling technologies. *Curr Protoc Bioinformatics, Chapter 13*, Unit 13 11. doi:10.1002/0471250953.bi1301s10

Hafner, A. S., Donlin-Asp, P. G., Leitch, B., Herzog, E., & Schuman, E. M. (2019). Local protein synthesis is a ubiquitous feature of neuronal pre- and postsynaptic compartments. *Science, 364*(6441). doi:10.1126/science.aau3644

Han, S., Tai, C., Westenbroek, R. E., Yu, F. H., Cheah, C. S., Potter, G. B., . . . Catterall, W. A. (2012). Autistic-like behaviour in Scn1a+/- mice and rescue by enhanced GABA-mediated neurotransmission. *Nature, 489*(7416), 385-390. doi:10.1038/nature11356

Horton, A. C., & Ehlers, M. D. (2003). Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J Neurosci, 23*(15), 6188-6199. doi:10.1523/jneurosci.23-15-06188.2003

Hosp, F., & Mann, M. (2017). A Primer on Concepts and Applications of Proteomics in Neuroscience. *Neuron, 96*(3), 558-571. doi:10.1016/j.neuron.2017.09.025

Jadhav, A. D., Wei, L., & Shi, P. (2016). Compartmentalized Platforms for Neuro-Pharmacological Research. *Curr Neuropharmacol, 14*(1), 72-86. doi:10.2174/1570159x13666150516000957

Jung, H., Yoon, B. C., & Holt, C. E. (2012). Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. *Nat Rev Neurosci, 13*(5), 308-324. doi:10.1038/nrn3210

Kevenaar, J. T., & Hoogenraad, C. C. (2015). The axonal cytoskeleton: from organization to function. *Front Mol Neurosci, 8*, 44. doi:10.3389/fnmol.2015.00044

Khalil, B., Morderer, D., Price, P. L., Liu, F., & Rossoll, W. (2018). mRNP assembly, axonal transport, and local translation in neurodegenerative diseases. *Brain Res, 1693*(Pt A), 75-91. doi:10.1016/j.brainres.2018.02.018

Kim, E., & Jung, H. (2015). Local protein synthesis in neuronal axons: why and how we study. *BMB Rep, 48*(3), 139-146. doi:10.5483/bmbrep.2015.48.3.010

Kim, E., & Jung, H. (2020). Local mRNA translation in long-term maintenance of axon health and function. *Curr Opin Neurobiol, 63*, 15-22. doi:10.1016/j.conb.2020.01.006

Kim, S., & Coulombe, P. A. (2010). Emerging role for the cytoskeleton as an organizer and regulator of translation. *Nat Rev Mol Cell Biol, 11*(1), 75-81. doi:10.1038/nrm2818

Kleele, T., Marinkovic, P., Williams, P. R., Stern, S., Weigand, E. E., Engerer, P., . . . Misgeld, T. (2014). An assay to image neuronal microtubule dynamics in mice. *Nat Commun, 5*, 4827. doi:10.1038/ncomms5827

KOENIG, E. (1997). <SYNTHETIC MECHANISMS IN THE AXON—IV. IN VITRO INCORPORATION OF [3H]PRECURSORS INTO AXONAL PROTEIN AND RNA.pdf>. *Journal of Neurochemistry, 14*, pp. 437 to 446. .

Koenig, E., & Koelle, G. B. (1960). Acetylcholinesterase regeneration in peripheral nerve after irreversible inactivation. *Science, 132*(3435), 1249-1250. doi:10.1126/science.132.3435.1249

Kovalevich, J., Cornec, A. S., Yao, Y., James, M., Crowe, A., Lee, V. M., . . . Brunden, K. R. (2016). Characterization of Brain-Penetrant Pyrimidine-Containing Molecules with Differential Microtubule-Stabilizing Activities Developed as Potential Therapeutic Agents for Alzheimer's Disease and Related Tauopathies. *J Pharmacol Exp Ther, 357*(2), 432-450. doi:10.1124/jpet.115.231175

Kumar, D., Bansal, G., Narang, A., Basak, T., Abbas, T., & Dash, D. (2016). Integrating transcriptome and proteome profiling: Strategies and applications. *Proteomics, 16*(19), 2533-2544. doi:10.1002/pmic.201600140

Lepeta, K., Lourenco, M. V., Schweitzer, B. C., Martino Adami, P. V., Banerjee, P., Catuara-Solarz, S., . . . Seidenbecher, C. (2016). Synaptopathies: synaptic dysfunction in neurological disorders - A review from students to students. *J Neurochem, 138*(6), 785-805. doi:10.1111/jnc.13713

Leung, K. M., Lu, B., Wong, H. H., Lin, J. Q., Turner-Bridger, B., & Holt, C. E. (2018). Cue-Polarized Transport of beta-actin mRNA Depends on 3'UTR and Microtubules in Live Growth Cones. *Front Cell Neurosci, 12*, 300. doi:10.3389/fncel.2018.00300

Li, J., Luo, J., Liu, L., Fu, H., & Tang, L. (2018). The genetic association between apolipoprotein E gene polymorphism and Parkinson disease: A meta-Analysis of 47 studies. *Medicine (Baltimore), 97*(43), e12884. doi:10.1097/MD.0000000000012884

Lim, Y. J., & Lee, S. J. (2017). Are exosomes the vehicle for protein aggregate propagation in neurodegenerative diseases? *Acta Neuropathol Commun, 5*(1), 64. doi:10.1186/s40478-017-0467-z

Lin, A. C., & Holt, C. E. (2007). Local translation and directional steering in axons. *EMBO J, 26*(16), 3729-3736. doi:10.1038/sj.emboj.7601808

Lin, A. C., & Holt, C. E. (2008). Function and regulation of local axonal translation. *Curr Opin Neurobiol, 18*(1), 60-68. doi:10.1016/j.conb.2008.05.004

Lin, W. J., & Salton, S. R. (2013). The regulated secretory pathway and human disease: insights from gene variants and single nucleotide polymorphisms. *Front Endocrinol (Lausanne), 4*, 96. doi:10.3389/fendo.2013.00096

Liu-Yesucevitz, L., Bassell, G. J., Gitler, A. D., Hart, A. C., Klann, E., Richter, J. D., . . . Wolozin, B. (2011). Local RNA translation at the synapse and in disease. *J Neurosci, 31*(45), 16086-16093. doi:10.1523/JNEUROSCI.4105-11.2011

Liu, X. A., Rizzo, V., & Puthanveettil, S. V. (2012). Pathologies of Axonal Transport in Neurodegenerative Diseases. *Transl Neurosci, 3*(4), 355-372. doi:10.2478/s13380-012-0044-7

Maciel, R., Bis, D. M., Rebelo, A. P., Saghira, C., Zuchner, S., & Saporta, M. A. (2018). The human motor neuron axonal transcriptome is enriched for transcripts related to mitochondrial function and microtubule-based axonal transport. *Exp Neurol, 307*, 155-163. doi:10.1016/j.expneurol.2018.06.008

Maday, S., Twelvetrees, A. E., Moughamian, A. J., & Holzbaur, E. L. (2014). Axonal transport: cargo-specific mechanisms of motility and regulation. *Neuron, 84*(2), 292-309. doi:10.1016/j.neuron.2014.10.019

Maier, T., Guell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Lett, 583*(24), 3966-3973. doi:10.1016/j.febslet.2009.10.036

Makwana, M., Jones, L. L., Cuthill, D., Heuer, H., Bohatschek, M., Hristova, M., . . . Raivich, G. (2007). Endogenous transforming growth factor beta 1 suppresses inflammation and promotes survival in adult CNS. *J Neurosci, 27*(42), 11201-11213. doi:10.1523/JNEUROSCI.2255-07.2007

Mankowski, J. L., Queen, S. E., Tarwater, P. M., Fox, K. J., & Perry, V. H. (2002). Accumulation of beta-amyloid precursor protein in axons correlates with CNS expression of SIV gp41. *J Neuropathol Exp Neurol, 61*(1), 85-90. doi:10.1093/jnen/61.1.85

Marin, R., Ley-Martos, M., Gutierrez, G., Rodriguez-Sanchez, F., Arroyo, D., & Mora-Lopez, F. (2015). Three cases with L1 syndrome and two novel mutations in the L1CAM gene. *Eur J Pediatr, 174*(11), 1541-1544. doi:10.1007/s00431-015-2560-2

Masuda, T., Mori, A., Ito, S., & Ohtsuki, S. (2020). Quantitative and targeted proteomics-based identification and validation of drug efficacy biomarkers. *Drug Metab Pharmacokinet*, 100361. doi:10.1016/j.dmpk.2020.09.006

Mathur, C., Johnson, K. R., Tong, B. A., Miranda, P., Srikumar, D., Basilio, D., . . . Holmgren, M. (2018). Demonstration of ion channel synthesis by isolated squid giant axon provides functional evidence for localized axonal membrane protein translation. *Sci Rep, 8*(1), 2207. doi:10.1038/s41598-018-20684-8

Matthews, P. R., Eastwood, S. L., & Harrison, P. J. (2012). Reduced myelin basic protein and actin-related gene expression in visual cortex in schizophrenia. *PLoS One, 7*(6), e38211. doi:10.1371/journal.pone.0038211

Merianda, T. T., Lin, A. C., Lam, J. S., Vuppalanchi, D., Willis, D. E., Karin, N., . . . Twiss, J. L. (2009). A functional equivalent of endoplasmic reticulum and Golgi in axons for secretion of locally synthesized proteins. *Mol Cell Neurosci, 40*(2), 128-142. doi:10.1016/j.mcn.2008.09.008

Micheva, K. D., Vallee, A., Beaulieu, C., Herman, I. M., & Leclerc, N. (1998). beta-Actin is confined to structures having high capacity of remodelling in developing and adult rat cerebellum. *Eur J Neurosci, 10*(12), 3785-3798. doi:10.1046/j.1460-9568.1998.00391.x

Millecamps, S., & Julien, J. P. (2013). Axonal transport deficits and neurodegenerative diseases. *Nat Rev Neurosci, 14*(3), 161-176. doi:10.1038/nrn3380

Minis, A., Dahary, D., Manor, O., Leshkowitz, D., Pilpel, Y., & Yaron, A. (2014). Subcellular transcriptomics-dissection of the mRNA composition in the axonal compartment of sensory neurons. *Dev Neurobiol, 74*(3), 365-381. doi:10.1002/dneu.22140

MINNEN, J. V., & BERGMAN, J. J. (1997). DE NOVO PROTEIN SYNTHESIS IN ISOLATED AXONS OF IDENTIFIED NEURONS. *Neuroscience, 80*(1), 1-7. doi:10.1016/s0306-4522(97)00137-1

Moradi, M., Sivadasan, R., Saal, L., Luningschror, P., Dombert, B., Rathod, R. J., . . . Sendtner, M. (2017). Differential roles of alpha-, beta-, and gamma-actin in axon growth and collateral branch formation in motoneurons. *J Cell Biol, 216*(3), 793-814. doi:10.1083/jcb.201604117

Mueller, L. N., Brusniak, M. Y., Mani, D. R., & Aebersold, R. (2008). An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. *J Proteome Res, 7*(1), 51-61. doi:10.1021/pr700758r

Nijssen, J., Aguila, J., Hoogstraaten, R., Kee, N., & Hedlund, E. (2018). Axon-Seq Decodes the Motor Axon Transcriptome and Its Modulation in Response to ALS. *Stem Cell Reports, 11*(6), 1565-1578. doi:10.1016/j.stemcr.2018.11.005

Nishimura, K., Yoshihara, F., Tojima, T., Ooashi, N., Yoon, W., Mikoshiba, K., . . . Kamiguchi, H. (2003). L1-dependent neuritogenesis involves ankyrinB that mediates L1-CAM coupling with retrograde actin flow. *J Cell Biol, 163*(5), 1077-1088. doi:10.1083/jcb.200303060

Ori, A., Toyama, B. H., Harris, M. S., Bock, T., Iskar, M., Bork, P., . . . Beck, M. (2015). Integrated Transcriptome and Proteome Analyses Reveal Organ-Specific Proteome Deterioration in Old Rats. *Cell Syst, 1*(3), 224-237. doi:10.1016/j.cels.2015.08.012

Ouwenga, R., Lake, A. M., O'Brien, D., Mogha, A., Dani, A., & Dougherty, J. D. (2017). Transcriptomic Analysis of Ribosome-Bound mRNA in Cortical Neurites In Vivo. *J Neurosci, 37*(36), 8688-8705. doi:10.1523/JNEUROSCI.3044-16.2017

Pascale, E., Di Battista, M. E., Rubino, A., Purcaro, C., Valente, M., Fattapposta, F., . . . Meco, G. (2016). Genetic Architecture of MAPT Gene Region in Parkinson Disease Subtypes. *Front Cell Neurosci, 10*, 96. doi:10.3389/fncel.2016.00096

Patzke, C., Acuna, C., Giam, L. R., Wernig, M., & Sudhof, T. C. (2016). Conditional deletion of L1CAM in human neurons impairs both axonal and dendritic arborization and action potential generation. *J Exp Med, 213*(4), 499-515. doi:10.1084/jem.20150951

Perdomo, D., Ait-Ammar, N., Syan, S., Sachse, M., Jhingan, G. D., & Guillen, N. (2014). Data set for the proteomics analysis of the endomembrane system from the unicellular Entamoeba histolytica. *Data Brief, 1*, 29-36. doi:10.1016/j.dib.2014.08.007

Perlson, E., Jeong, G. B., Ross, J. L., Dixit, R., Wallace, K. E., Kalb, R. G., & Holzbaur, E. L. (2009). A switch in retrograde signaling from survival to stress in rapid-onset neurodegeneration. *J Neurosci, 29*(31), 9903-9917. doi:10.1523/JNEUROSCI.0813-09.2009

Pernice, H. F., Schieweck, R., Kiebler, M. A., & Popper, B. (2016). mTOR and MAPK: from localized translation control to epilepsy. *BMC Neurosci, 17*(1), 73. doi:10.1186/s12868-016-0308-1

Perry, R. B., Doron-Mandel, E., Iavnilovitch, E., Rishal, I., Dagan, S. Y., Tsoory, M., . . . Fainzilber, M. (2012). Subcellular knockout of importin beta1 perturbs axonal retrograde signaling. *Neuron, 75*(2), 294-305. doi:10.1016/j.neuron.2012.05.033

Poewe, W., Seppi, K., Tanner, C. M., Halliday, G. M., Brundin, P., Volkmann, J., . . . Lang, A. E. (2017). Parkinson disease. *Nat Rev Dis Primers, 3*, 17013. doi:10.1038/nrdp.2017.13

Prochnow, H., Gollan, R., Rohne, P., Hassemer, M., Koch-Brandt, C., & Baiersdorfer, M. (2013). Non-secreted clusterin isoforms are translated in rare amounts from distinct human mRNA variants and do not affect Bax-mediated apoptosis or the NF-kappaB signaling pathway. *PLoS One, 8*(9), e75303. doi:10.1371/journal.pone.0075303

Rauniyar, N., & Yates, J. R., 3rd. (2014). Isobaric labeling-based relative quantification in shotgun proteomics. *J Proteome Res, 13*(12), 5293-5309. doi:10.1021/pr500880b

Rodriguez, A. J., Czaplinski, K., Condeelis, J. S., & Singer, R. H. (2008). Mechanisms and cellular roles of local protein synthesis in mammalian cells. *Curr Opin Cell Biol, 20*(2), 144-149. doi:10.1016/j.ceb.2008.02.004

Ross, A. F., Oleynikov, Y., Kislauskis, E. H., Taneja, K. L., & Singer, R. H. (1997). Characterization of a beta-actin mRNA zipcode-binding protein. *Mol Cell Biol, 17*(4), 2158-2165. doi:10.1128/mcb.17.4.2158

Rotem, N., Magen, I., Ionescu, A., Gershoni-Emek, N., Altman, T., Costa, C. J., . . . Perlson, E. (2017). ALS Along the Axons - Expression of Coding and Noncoding RNA Differs in Axons of ALS models. *Sci Rep, 7*, 44500. doi:10.1038/srep44500

Roy, S., Zhang, B., Lee, V. M., & Trojanowski, J. Q. (2005). Axonal transport defects: a common theme in neurodegenerative diseases. *Acta Neuropathol, 109*(1), 5-13. doi:10.1007/s00401-004-0952-x

Rozenbaum, M., Rajman, M., Rishal, I., Koppel, I., Koley, S., Medzihradszky, K. F., . . . Fainzilber, M. (2018). Translatome Regulation in Neuronal Injury and Axon Regrowth. *eNeuro, 5*(2). doi:10.1523/ENEURO.0276-17.2018

Saal, L., Briese, M., Kneitz, S., Glinka, M., & Sendtner, M. (2014). Subcellular transcriptome alterations in a cell culture model of spinal muscular atrophy point to widespread defects in

axonal growth and presynaptic differentiation. *RNA, 20*(11), 1789-1802. doi:10.1261/rna.047373.114

Sahoo, P. K., Smith, D. S., Perrone-Bizzozero, N., & Twiss, J. L. (2018). Axonal mRNA transport and translation at a glance. *J Cell Sci, 131*(8). doi:10.1242/jcs.196808

Samochowiec, A., Samochowiec, J., Pelka-Wysiecka, J., Kucharska-Mazur, J., Grochans, E., Jablonski, M., . . . Grzywacz, A. (2019). The role of OPRM1 polymorphism in the etiology of alcoholism. *Adv Clin Exp Med, 28*(2), 199-202. doi:10.17219/acem/78592

Sann, S., Wang, Z., Brown, H., & Jin, Y. (2009). Roles of endosomal trafficking in neurite outgrowth and guidance. *Trends Cell Biol, 19*(7), 317-324. doi:10.1016/j.tcb.2009.05.001

Santiago-Medina, M., Gregus, K. A., Nichol, R. H., O'Toole, S. M., & Gomez, T. M. (2015). Regulation of ECM degradation and axon guidance by growth cone invadosomes. *Development, 142*(3), 486-496. doi:10.1242/dev.108266

Sasaki, Y. (2020). Local Translation in Growth Cones and Presynapses, Two Axonal Compartments for Local Neuronal Functions. *Biomolecules, 10*(5). doi:10.3390/biom10050668

Sasaki, Y., Gross, C., Xing, L., Goshima, Y., & Bassell, G. J. (2014). Identification of axon-enriched microRNAs localized to growth cones of cortical neurons. *Dev Neurobiol, 74*(3), 397-406. doi:10.1002/dneu.22113

Satkauskas, S., & Bagnard, D. (2007). Local protein synthesis in axonal growth cones: what is next? *Cell Adh Migr, 1*(4), 179-184. doi:10.4161/cam.1.4.5561

Schubert, O. T., Rost, H. L., Collins, B. C., Rosenberger, G., & Aebersold, R. (2017). Quantitative proteomics: challenges and opportunities in basic and applied research. *Nat Protoc, 12*(7), 1289-1294. doi:10.1038/nprot.2017.040

Sherriff, F. E., Bridges, L. R., Gentleman, S. M., Sivaloganathan, S., & Wilson, S. (1994). Markers of axonal injury in post mortem human brain. *Acta Neuropathol, 88*(5), 433-439. doi:10.1007/BF00389495

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, J. Q., . . . Holt, C. E. (2016). Dynamic Axonal Translation in Developing and Mature Visual Circuits. *Cell, 166*(1), 181-192. doi:10.1016/j.cell.2016.05.029

Singh, N. A., Pappas, C., Dahle, E. J., Claes, L. R., Pruess, T. H., De Jonghe, P., . . . Leppert, M. F. (2009). A role of SCN9A in human epilepsies, as a cause of febrile seizures and as a potential modifier of Dravet syndrome. *PLoS Genet, 5*(9), e1000649. doi:10.1371/journal.pgen.1000649

Sleigh, J. N., Rossor, A. M., Fellows, A. D., Tosolini, A. P., & Schiavo, G. (2019). Axonal transport and neurological disease. *Nat Rev Neurol, 15*(12), 691-703. doi:10.1038/s41582-019-0257-2

Soderstrom, M., Hillert, J., Link, J., Navikas, V., Fredrikson, S., & Link, H. (1995). Expression of IFN-gamma, IL-4, and TGF-beta in multiple sclerosis in relation to HLA-Dw2 phenotype and stage of disease. *Mult Scler, 1*(3), 173-180. doi:10.1177/135245859500100308

Spaulding, E. L., & Burgess, R. W. (2017). Accumulating Evidence for Axonal Translation in Neuronal Homeostasis. *Front Neurosci, 11*, 312. doi:10.3389/fnins.2017.00312

Sun, F., & Cavalli, V. (2010). Neuroproteomics approaches to decipher neuronal regeneration and degeneration. *Mol Cell Proteomics, 9*(5), 963-975. doi:10.1074/mcp.R900003-MCP200

Taliaferro, J. M., Vidaki, M., Oliveira, R., Olson, S., Zhan, L., Saxena, T., . . . Burge, C. B. (2016). Distal Alternative Last Exons Localize mRNAs to Neural Projections. *Mol Cell, 61*(6), 821-833. doi:10.1016/j.molcel.2016.01.020

Tannu, N. S., & Hemby, S. E. (2006). Methods for proteomics in neuroscience. *Progress in Brain Research, 158*, 41-82. doi:10.1016/s0079-6123(06)58003-3

Taylor, A. M., Berchtold, N. C., Perreau, V. M., Tu, C. H., Li Jeon, N., & Cotman, C. W. (2009). Axonal mRNA in uninjured and regenerating cortical mammalian axons. *J Neurosci, 29*(15), 4697-4707. doi:10.1523/JNEUROSCI.6130-08.2009

Tcherkezian, J., Brittis, P. A., Thomas, F., Roux, P. P., & Flanagan, J. G. (2010). Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation. *Cell, 141*(4), 632-644. doi:10.1016/j.cell.2010.04.008

Tesseur, I., & Wyss-Coray, T. (2006). A role for TGF-beta signaling in neurodegeneration: evidence from genetically engineered models. *Curr Alzheimer Res, 3*(5), 505-513. doi:10.2174/156720506779025297

Tiruchinapalli, D. M., Caron, M. G., & Keene, J. D. (2008). Activity-dependent expression of ELAV/Hu RBPs and neuronal mRNAs in seizure and cocaine brain. *J Neurochem, 107*(6), 1529-1543. doi:10.1111/j.1471-4159.2008.05718.x

Tiruchinapalli, D. M., Ehlers, M. D., & Keene, J. D. (2008). Activity-dependent expression of RNA binding protein HuD and its association with mRNAs in neurons. *RNA Biol, 5*(3), 157-168. doi:10.4161/rna.5.3.6782

Toth, E. N., Lohith, A., Mondal, M., Guo, J., Fukamizu, A., & Pourmand, N. (2018). Single-cell nanobiopsy reveals compartmentalization of mRNAs within neuronal cells. *J Biol Chem, 293*(13), 4940-4951. doi:10.1074/jbc.M117.800763

Van Battum, E. Y., Brignani, S., & Pasterkamp, R. J. (2015). Axon guidance proteins in neurological disorders. *The Lancet Neurology, 14*(5), 532-546. doi:10.1016/s1474-4422(14)70257-1

Van Horck, F. P., & Holt, C. E. (2008). A cytoskeletal platform for local translation in axons. *Sci Signal, 1*(8), pe11. doi:10.1126/stke.18pe11

Verma, P., Chierzi, S., Codd, A. M., Campbell, D. S., Meyer, R. L., Holt, C. E., & Fawcett, J. W. (2005). Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J Neurosci, 25*(2), 331-342. doi:10.1523/JNEUROSCI.3073-04.2005

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet, 13*(4), 227-232. doi:10.1038/nrg3185

Weill, L., Belloc, E., Bava, F. A., & Mendez, R. (2012). Translational control by changes in poly(A) tail length: recycling mRNAs. *Nat Struct Mol Biol, 19*(6), 577-585. doi:10.1038/nsmb.2311

Werner, H., & LeRoith, D. (2014). Insulin and insulin-like growth factor receptors in the brain: physiological and pathological aspects. *Eur Neuropsychopharmacol, 24*(12), 1947-1953. doi:10.1016/j.euroneuro.2014.01.020

Willis, D. E., & Twiss, J. L. (2010). Regulation of protein levels in subcellular domains through mRNA transport and localized translation. *Mol Cell Proteomics, 9*(5), 952-962. doi:10.1074/mcp.R900005-MCP200

Willis, D. E., van Niekerk, E. A., Sasaki, Y., Mesngon, M., Merianda, T. T., Williams, G. G., . . . Twiss, J. L. (2007). Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J Cell Biol, 178*(6), 965-980. doi:10.1083/jcb.200703209

Xiong, G. J., Cheng, X. T., Sun, T., Xie, Y., Huang, N., Li, S., . . . Sheng, Z. H. (2020). Defects in syntabulin-mediated synaptic cargo transport associate with autism-like synaptic dysfunction and social behavioral traits. *Mol Psychiatry*. doi:10.1038/s41380-020-0713-9

Xu, C., Cao, H., Zhang, F., & Cheadle, C. (2018). Comprehensive literature data-mining analysis reveals a broad genetic network functionally associated with autism spectrum disorder. *Int J Mol Med, 42*(5), 2353-2362. doi:10.3892/ijmm.2018.3845

Yan, Z., Kim, E., Datta, D., Lewis, D. A., & Soderling, S. H. (2016). Synaptic Actin Dysregulation, a Convergent Mechanism of Mental Disorders? *J Neurosci, 36*(45), 11411-11417. doi:10.1523/JNEUROSCI.2360-16.2016

Yasuda, K., & Mili, S. (2016). Dysregulated axonal RNA translation in amyotrophic lateral sclerosis. *Wiley Interdiscip Rev RNA, 7*(5), 589-603. doi:10.1002/wrna.1352

Yerbury, J. J., Farrawell, N. E., & McAlary, L. (2020). Proteome Homeostasis Dysfunction: A Unifying Principle in ALS Pathogenesis. *Trends Neurosci, 43*(5), 274-284. doi:10.1016/j.tins.2020.03.002

Yin, C., Guo, Z. D., He, Z. Z., Wang, Z. Y., & Sun, X. C. (2019). Apolipoprotein E Affects In Vitro Axonal Growth and Regeneration via the MAPK Signaling Pathway. *Cell Transplant, 28*(6), 691-703. doi:10.1177/0963689718808736

Yoon, B. C., Zivraj, K. H., & Holt, C. E. (2009). Local translation and mRNA trafficking in axon pathfinding. *Results Probl Cell Differ, 48*, 269-288. doi:10.1007/400_2009_5

Yu, L.-R., Stewart, N. A., & Veenstra, T. D. (2010). Proteomics. 89-96. doi:10.1016/b978-0-12-374934-5.00008-8

Zappulo, A., van den Bruck, D., Ciolli Mattioli, C., Franke, V., Imami, K., McShane, E., . . . Chekulaeva, M. (2017). RNA localization is a key determinant of neurite-enriched proteome. *Nat Commun, 8*(1), 583. doi:10.1038/s41467-017-00690-6

Zhang, H. L., Singer, R. H., & Bassell, G. J. (1999). Neurotrophin regulation of beta-actin mRNA and protein localization within growth cones. *J Cell Biol, 147*(1), 59-70. doi:10.1083/jcb.147.1.59

Zhang, K. X., Tan, L., Pellegrini, M., Zipursky, S. L., & McEwen, J. M. (2016). Rapid Changes in the Translatome during the Conversion of Growth Cones to Synaptic Terminals. *Cell Rep, 14*(5), 1258-1271. doi:10.1016/j.celrep.2015.12.102

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., . . . Ge, L. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell, 181*(3), 637-652 e615. doi:10.1016/j.cell.2020.03.031

Zhang, X., Gao, F., Wang, D., Li, C., Fu, Y., He, W., & Zhang, J. (2018). Tau Pathology in Parkinson's Disease. *Front Neurol, 9*, 809. doi:10.3389/fneur.2018.00809

Zhang, Y. W., Thompson, R., Zhang, H., & Xu, H. (2011). APP processing in Alzheimer's disease. *Mol Brain, 4*, 3. doi:10.1186/1756-6606-4-3

Zhu, X., Han, X., Blendy, J. A., & Porter, B. E. (2012). Decreased CREB levels suppress epilepsy. *Neurobiol Dis, 45*(1), 253-263. doi:10.1016/j.nbd.2011.08.009

Zivraj, K. H., Tung, Y. C., Piper, M., Gumy, L., Fawcett, J. W., Yeo, G. S., & Holt, C. E. (2010). Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. *J Neurosci, 30*(46), 15464-15478. doi:10.1523/JNEUROSCI.1800-10.2010

Zoller, T., Schneider, A., Kleimeyer, C., Masuda, T., Potru, P. S., Pfeifer, D., . . . Spittau, B. (2018). Silencing of TGFbeta signalling in microglia results in impaired homeostasis. *Nat Commun, 9*(1), 4011. doi:10.1038/s41467-018-06224-y

Zukin, R. S., Richter, J. D., & Bagni, C. (2009). Signals, synapses, and synthesis: how new proteins control plasticity. *Front Neural Circuits, 3*, 14. doi:10.3389/neuro.04.014.2009