DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Ribosomal Regulation in Axonal Development

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Ramiro Almeida (Centro de Neurociências e Biologia Celular) e do Professor Doutor Carlos Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

Helena Sofia Caria Martins

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Abbreviations

5-FDU 5-fluoro-2'-deoxiuridina

AChR Acetylcholine receptor

ADF Actin depolymerizing factor

BNDF Brain-derived neurotrophic factor

BSA Bovine Serum Albumin

CDKN Cyclin-dependent kinase inhibitor

cDNA Complementary DNA

CEF Chicken embryo fibroblast

CNS Central nervous system

CNTF Ciliary neurotrophic factor

COXIV Cytochrome c oxidase IV

CPEB Cytoplasmic polyadenylation-element-binding protein

CPG15 Candidate plasticity gene 15

DCTN2 Dynactin subunit 2

DIV Days in vitro

DRG Dorsal root ganglion

dsRNA Double stranded RNA

eIF Eukaryotic translation initiation factor

EM Electron Microscopy

FBS Fetal bovine serum

FGF Fibroblast growth factor

FGF22 Fibroblast growth factor-22

FGFR Fibroblast growth factor receptor

GDNF Glial cell derived neurotrophic factor

GFP Green fluorescence protein

HBS HEPES Buffered Saline

hnRNP Heterogeneous nuclear ribonucleoproteins

hnRNP Heterogeneous nuclear ribonucleoproteins

KIF Kinesin

M-cell Mauthner Cell

MN Motor neurons

mRNA Messanger RNA

NMJ Neuromuscular junction

PABP Poly (A) binding protein

PBS Phosphate buffered saline

PDL Poly-D-lysine

PDMS Poly-dimethlysiloxane

PFA Paraformaldehyde

PGK Phosphoglycerate kinase

PNS Peripheral nervous system

RBP mRNA-binding protein

RNP Ribonucleotide protein

RRM RNA recognition motif

rRNA Ribosomal RNA

S.O.C Super Optimal broth with catabolite repression

Sema3A Semaphorin-3A

SMA Spinal muscular atrophy

SMN Survival of motor neurons

SN Sensory neurons

SNARE Soluble NSF attachment receptor

Src Proto-oncogene tyrosine-protein kinase

SV Synaptic vesicle

SV2 Synaptic vesicle protein 2

Syn Synapsin

TAF Trans-acting factor

tRNA Transfer RNA

UTR Untranslated region

VAMP Vesicle SNARE synaptobrevin

WPRE Woodchuck hepatitis post-transcriptional regulatory element

ZBP Zipcode binding protein

ZBP1 Zipcode-binding protein 1

Abstract

For many years, the predominant idea was that all proteins were translated in the cell body and then transported to their final destinations in dendrites and axons. However, the discovery of polyribosomes at the base of dendritic spines challenged this canonical view and the concept of local protein synthesis is now widely accepted. Axonal translation is a well-regulated process that dynamically changes during development. Developing axons actively translate proteins related to axon growth, elongation and pathfinding, consistent with the structural reorganization distinctive of this stage. In adult axons, both mRNAs and ribosomal RNA are more difficult to find. Evidences point towards a decrease of the translational machinery as axons mature. How this happens is currently not known. We hypothesize that the reason for this loss of ribosomes during neuronal maturation is synapse formation. Therefore, the aim of this study was to establish that mature neurons have decreased ribosomal levels and determine if synapse formation induces ribosomal loss from axons. We did so using FGF22, a presynaptic organizing molecule, and a novel platform, a microfluidic chamber system that allows the physical separation of axons from cell bodies and dendrites. We first confirmed the presence of ribosomal proteins and RNA in developing axons of spinal motor neurons and hippocampal neurons. Motor neurons stimulated with FGF22, a synaptogenic stimuli, presented a decrease in the number of ribosomes. This reduction was also confirmed in hippocampal neurons suggesting a common feature of the central nervous system and the peripheral nervous system. Our study fills an important gap in this field and identifies for the first time the molecular trigger that leads to the reduction of the translational machinery in axons.

Keywords: Local protein synthesis, ribosomes, synaptogenesis.

Resumo

Por muitos anos, a ideia predominante era que todas as proteínas seriam traduzidas no corpo celular e depois transportadas para os seus destinos finais em dendrites e axónios. No entanto, a descoberta de poliribossomas na base das espículas dendríticas levou ao aparecimento de vários estudos contratiando esta visão. O processo de tradução proteica nos axónios é um mecanismo bastante dinâmico e regulado que muda ao longo do desenvolvimento. Axónios em desenvolvimento traduzem proteínas relacionadas com crescimento, elongamento e quimiotaxia axonal, necessárias á reorganização estrutural característica deste estádio. mRNAs e RNA ribosomal são difíceis de identificar em axónios adultos. Estudos apontam para um decréscimo da maquinaria de tradução á medida que os axónios se desenvolvem, no entanto é desconhecida a razão deste fenómeno. Postulámos a hipótese que os ribossomas diminuem durante a maturação axonal devido à formação de sinapses. Assim, o objectivo deste estudo foi estabelecer que axónios adultos apresentam níveis reduzidos de ribossomas e determinar se a formação de sinapses induz esta diminuição. Para tal, usámos FGF22, uma molécula organizadora da pré-sinapse, e câmaras microfluídas, um sistema que permite a separação física entre axónios e o corpo celular e dendrites. Primeiro, confirmámos a presença de proteínas e RNA ribossomais em axónios em desenvolvimento de neurónios motores espinhais e neurónios de hipocampo. Neurónios motores estimulados com FGF22, um estímulo sinaptogénico, apresentam uma diminuição do número de ribossomas. Esta redução foi confirmada em neurónios do hipocampo indicando que esta é uma característica comum ao sistema nervoso central e periférico. O nosso estudo preenche uma importante lacuna e identifica pela primeira vez o inductor molecular que leva á redução da maquinaria de tradução em axónios.

Palavras-chave: Síntese local de proteínas, ribossomas, sinaptogénese.

Chapter 1 Introduction

1.1 Cell body, the "trophic center of the neuron"

In 1850, Augustus Waller hypothesized for the first time that the cell body was essential for the survival of the axon. By cutting the nerves of a frog's tongue Waller observed that the distal stump rapidly degenerated while the proximal one remained intact. Waller explained these observations inferring that nerve fibers were nurtured by the soma (Waller, 1850). The presence of machinery necessary for protein synthesis and posttranslational processing in neuronal cell bodies as well as the existence of selective axonal and dendritic transport mechanisms capable of delivering proteins anywhere within neurons led to a central tenet of neurobiology according to which proteins are exclusively synthesized in the soma and then transported to their final destinations.

However, behind the scenes, in the early 1980s, there already had been speculative ideas just waiting for new experimental techniques and new approaches of investigation to challenge this theory. After it was shown that polyribosomes (Steward and Fass, 1983)'(Steward and Levy, 1982) as well as mRNAs (Garner et al., 1988)'(Racca et al., 1997) localized preferentially under the base of dendritic spines, beneath postsynaptic sites, experimental evidences supporting the idea of compartmentalized synthesis of proteins in neurons started to build up. The concept of dendrites synthesizing new proteins was then launched and further studies indicated a vital role in protein turnover, regulation of activity and synaptic plasticity (Steward and Schuman, 2003).

On the other hand, protein synthesis seemed more and more unlikely in the axoplasm. The controversy surrounding this subject dates back to the 1970s when investigators failed to find translational machinery in the axons, neither ribosomes (PALAY and PALADE, 1955) nor mRNA (Lasek et al., 1973). If there were no RNA or ribosomes in

axons, then all axonal proteins would have to be synthesized in the soma and transported to the axon.

1.1.1 Slow axonal transport theory

The lack of evidences regarding the presence of ribosomes in axons together with transport mechanisms capable of supplying axonal proteins provided the answer that the investigators were looking for. The idea of slow axoplasmic transport adjusted perfectly to the currently accepted view that the cell body was the only source of neuronal proteins and, therefore, became itself the established conception of the axoplasm's maintenance.

Like other specializations of the neuron, the axon is dynamic, constantly changing and continuously being renewed. As a result, a supply line must be maintained in order to provide all the necessary materials for axonal function making the axonal transport mechanisms key in this dynamics.

Axonal transport, introduced by Weiss and Hiscoe (Weiss and Hiscoe, 1948), can be divided into two groups depending on the rate proteins are transported and the type of proteins transported. The slow axonal transport was reported for the first time in 1962, when Droz and Leblond took advantage of radioactive tracers like tritium labeled amino acids. Using high radioautographic resolution they were able to show that injected H3-amino acids were incorporated into neuronal proteins and transported along the nerve cell (Droz and Leblond, 1963). In the case of slow transport, cytoskeletal and cytosolic proteins are anterogradely transported at rates approximate 0.1-3 mm/day (Campenot and Eng, 2000).

Proteins associated with vesicles and membranous organelles are distributed by fast axonal transport, either anterogradely or retrogradely, at a rate of 50-200 mm/day (Campenot and Eng, 2000).

Although fast axonal transport could meet the needs of the terminals timely, the impasse reached regarded the slow axonal transport. At rates of 1mm/day, proteins would take years till they reach the terminal of a meter-long axon (Campenot and Eng, 2000). Given the half-lives of proteins, ranging from a few seconds to many days (Varshavsky, 1996), a fundamental question is raised immediately: Are axoplasmic proteins capable of lasting long enough to travel a meter or a few years long journey? How would then the proteins supposedly transported by slow transport be delivered to the axon? Beyond this obvious concern, investigators also questioned if the cell body was capable of supplying uniformly all the branches of the axon and if the proteins transported could suffer any kind of metabolic degradation along the way (Alvarez and Torres, 1985). Apart from the fact that the slow flow of radioactive proteins remained the only and best indirect evidence of the axonal flow theory, any of the subsequent reports were able to consistently answer the previous questions.

Accompanying the fall of the slow axonal transport model, the notion of proteins being translated in the axons gained credence and the evidences once overlooked concerning the presence of protein synthesis machinery were re-assessed.

1.2 Local protein synthesis and proposed functions

Although the theory of slow axonal transport did not meet all the investigators uncertainties, to prove that proteins were actually translated in the axon was a challenging task. Even if it was possible to isolate axonal proteins it would be difficult to determine their origin. They could be either axonal or glial or from other cell type, since the axons are not isolated in the nervous system but rather associated with other cell types.

The histoautoradiography technique proved to be very useful to overcome this difficulty since it allowed the cellular localization of radioactivity. Radioactive amino acids were added to Mauthner (M-) cell and after a period of incubation the investigators were able to see their incorporation by axonal proteins, meaning that local protein synthesis was occurring at some level in the M-cell axoplasm (Edström, 1966). The Mauthner cell is a vertebrate neuron present in the brain stem of many fishes and amphibians. It is constituted by two major dendrites and a crossed axon that descends to the spinal cord (Eaton et al., 2001). The same approach was also applied to mammalian nerves, confirming the incorporation of [^{3}H]-leucine in newly synthesized axonal proteins (Koenig, 1967). Moreover, this incorporation was sensitive to protein synthesis inhibitors, like cycloheximide (Alvarez and Zarour, 1983).

Later on, experiments performed mainly in the squid giant axon and *Aplysia* cultured neurons showed that invertebrate axons dispose of all the required machinery to locally translate proteins, including tRNA (Black and Lasek, 1977), rRNA (Martin et al., 1998),

aminoacyl-tRNA synthetases (Giuditta et al., 1977), polypeptide elongation factors (Giustetto et al., 2003) and mRNAs (Giuditta et al., 1977).

β-actin mRNA was one of the first mRNAs to be identified in growth cones and distal axons of developing cerebrocortical neurons. Using isoform-specific antibodies and transfection of epitope-tagged β-actin, investigators observed that β-actin protein was highly enriched within growth cones and filopodia of developing dendritic and axonal processes. Similar results were observed regarding β-actin mRNA. Importantly, mRNA localized to growth cones and processes in the form of granules. These granules co-localized with translation machinery, such as polyribosomes, suggesting that β-actin mRNA was locally translated. Also, β-actin mRNA was shown to associate with microtubules implying its transport to neuronal processes. From these experiments investigators concluded that β-actin mRNA granules are most likely transported along microtubules to distal processes where β-actin protein synthesis occurs (Bassell et al., 1998).

In an attempt to further analyze the populations of axonal mRNAs present in axons, Taylor and collegues used a novel system to separate axons from the cell body and dendrites of cortical neurons, the microfluidic chambers. Through this system, they were able to isolate axonal mRNAs and categorize them. Four main categories were identified: translation, mitochondrion, intracellular transport and cytoskeleton (Figure 1). Transcripts related to mitochondria lie beneath the energetic needs of the axon, for instance local protein synthesis and axon function, whereas the presence of cytoskeleton and intracellular transport transcripts suggest that protein synthesis in the axon is necessary to maintain these two functions.

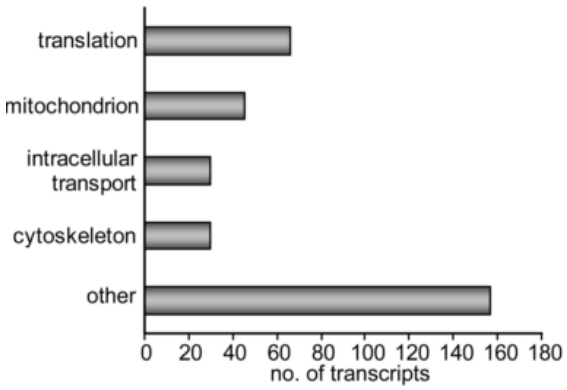


Figure 1: mRNA population of cortical axons. Axonal mRNA pool comprises transcripts mainly related to translation but also associated with mitochondria function, intracellular transport and cytoskeleton dynamics [Adapted from Taylor et al. 2009].

Importantly, transcripts related with translation constitute the most enriched category in the cortical axon, establishing that machinery necessary for protein synthesis is present in the axons thereby supporting a functional role for local translation (Taylor et al., 2009). Interestingly, transcripts for ribosomal proteins were also identified in this study. Moccia and colleagues had previously showed the presence of mRNAs encoding ribosomal proteins in Aplysia sensory neurites. During their experiments, mRNAs encoding for the ribosomal proteins L8, L18 and S6 were identified not only in the cell body but also throughout the neurites (Moccia et al., 2003). These results not only confirmed the existence of translational machinery in axons but also that mRNAs translated at the synapse may be responsible for an enhanced translational capacity of local ribosomes.

Notwithstanding the great improvements in the area, we cannot forget that both *Aplysia* and the squid giant axon do not share the same architecture as vertebrate neurons. They

possess only one type of process that although named as axon it receives and transmits information; therefore, it cannot accurately model what happens in vertebrate axons.

Local translation was much more complex to address in vertebrate axons. Electron Microscopy (EM) studies were not able to detect ribosomes and purification of the presynaptic terminal without contamination from other cell types and cellular debris continued to dare investigators. Regardless of these experimental difficulties, ribosomes, both in the initial segment of the axon (Steward and Ribak, 1986) and intermittently along the axon shaft (Koenig et al., 2000) along with tRNA (Koenig, 1979), initiation factors (Zheng et al., 2001) and mRNAs (Bassell et al., 1998) were shown to be targeted to vertebrate axons.

Once investigators seeded the concept of local protein synthesis, the challenge ahead was to unravel the diverse functional roles that newly synthesized proteins could undertake. Numerous lines of evidences point to a possible function of intra-axonal protein synthesis in several stages of neuronal development, including: growth cone collapse, axonal pathfinding, retrograde survival, synaptogenesis and synaptic plasticity (Yoon et al., 2009).

1.2.1 Axon Guidance

During brain development, growth cones guide growing axons to their correct post-synaptic targets. Growth cones are highly motile and sensitive structures present at the tip of the axon and specialized in the recognition of attractive or repulsive guidance cues present along the axonal pathway, including netrins, Slits, semaphorins, and ephrins (Dickson, 2002). They respond to attractive cues by steering towards the

attractive molecule and to repulsive cues steering away from the repulsive molecule. By doing so, they are able to make directional decisions that will lead the axon to its synaptic target.

The proper functioning of the neuronal circuitry and its intricate network of connections relies on a precise pattern that is achieved through the ability of axons to navigate and to locate their appropriate synaptic partners. This often means travelling distances that place the axon many centimeters away from the soma, which complicates the rapid supply of proteins to distal regions of the axons and a rapid and yet proper response of growth cones to external cues.

Fighting back this challenge, several studies demonstrated that local protein synthesis was in fact a mean to a rapid supply of axonal proteins. In fact, the earliest evidences of a functional role for local protein synthesis in axons came from studies of axon guidance. Primarily, in 1987, growth cones of *Xenopus laevis* retinal ganglion cells previously detached from their cell bodies were shown not only to survive but also to respond to guidance cues and to migrate (Harris et al., 1987), confirming that growth cones had a certain degree of independence from the cell body and possessed the required machinery was present in the axons. Later in 2001, Campbell and Holt demonstrated that the growth cone responded to guidance cues turning towards the attractive molecule Netrin-1 and turning away from the repulsive molecule Sema3A in a protein synthesis-dependent manner (Figure 2) (Campbell and Holt, 2001). These results confirmed that intra-axonal protein synthesis is required for the steering of growth cones, providing growth cone autonomy and allowing rapid regulation of axonal proteins.

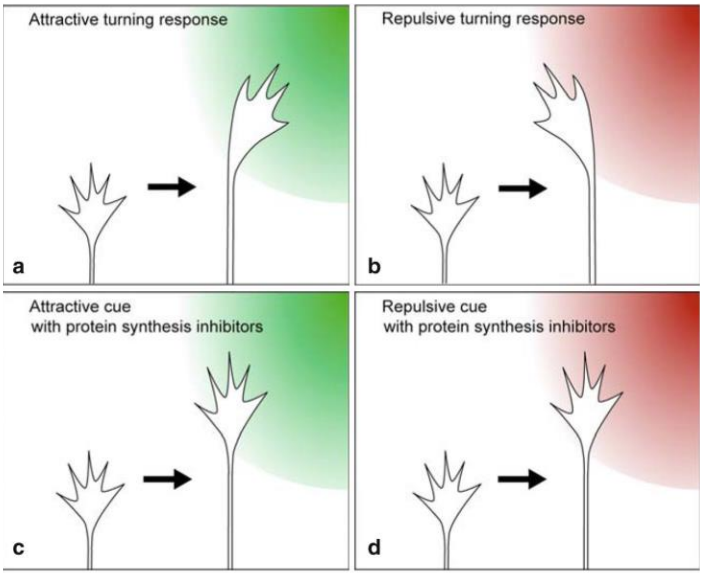


Figure 2: Local protein synthesis is required for axon pathfinding. Growth cones respond to guidance cues steering in direction of attractive molecules (a) or away from repulsive molecules (b). However, protein synthesis inhibitors blocked growth cone steering to guidance cues (c,d) [Adapted from yoon et al., 2009].

1.2.2 Synaptogenesis

Information flows from one neuron to another through synapses. There are different types of synapses. The neuromuscular junction (NMJ), where a motor neuron axon synapses with a muscle cell, continues to be the most widely studied synapse and whose formation we know best, mostly due to its easy manipulation and accessibility. Three cellular portions – motor neuron, muscle cell and Schwann cell, constitute the NMJ. The motor neuron comprises the presynaptic terminal, responsible to neurotransmitter release while the muscle cells embody the post-synaptic membrane where neurotransmitter receptors are concentrated (Figure 3). When the growth cone of developing neurons recognizes its correct post-synaptic target, contact between the two

cells is established and signals are exchanged in order to occur differentiation of the synaptic structures and synapse formation.

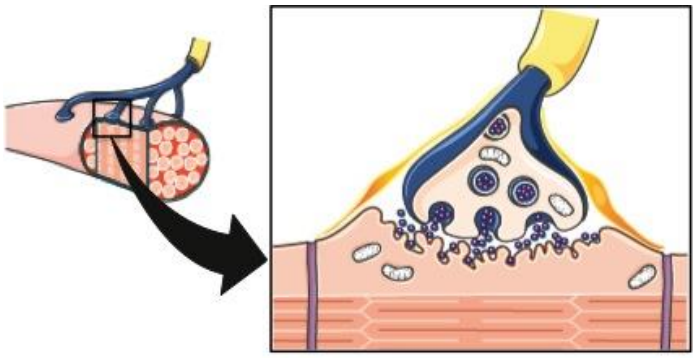


Figure 3: Synaptic elements of the neuromuscular junction. Neuromuscular synapses are composed of a motor neuron capped by a Schawann cell and a muscle cell. The nerve terminal is localized in shallow depressions of the muscle fiber membrane that is invaginated forming deep and regular folds, entitled post-junctional folds.

In mature muscle fibers, neurotransmitter receptors (AChR) reach a density of ≈1000/μm^2. Upon a synaptic stimulus, AChRs are redistributed and accumulate at the post-synaptic membrane, reaching densities of >10,000/μm^2. Clustering of neurotransmitter receptors at synaptic sites is a hallmark of post-synaptic differentiation. Also during this stage of development, post-synaptic density forms comprising major cytoskeleton reorganizations in order to support the high number of AChRs (Sanes and Lichtman, 1999) (Figure 4).

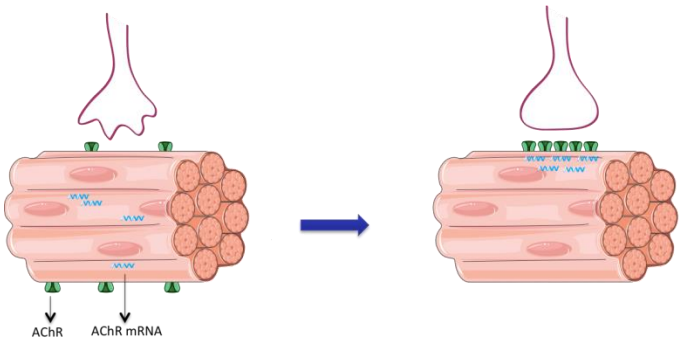


Figure 4: Post-synaptic differentiation. After recognition of the muscle fiber by the motor neuron axon, acetylcholine receptors (AChR) cluster in the postsynaptic membrane in the future synaptic site. mRNAs encoding different subunits of the AChR (AChR mRNA) also concentrates in the prospective synaptic site. The axon grows toward the muscle fiber further clustering the AChR receptors.

On the other hand, nerve terminals are situated where the muscle cell membrane invaginates into deep and regular folds, named post-junctional folds. During presynaptic differentiation, synaptic vesicles increase in number and become clustered where they will fuse with the presynaptic membrane, the active zone, in order to occur neurotransmitter release (Sanes and Lichtman, 1999). Also in this step of synaptogenesis, cytoskeleton reorganizes leading to polarization of the nerve terminal (Kelly and Zacks, 1969) and increases are observed in synaptic area and volume as well as in the frequency of spontaneous exocytosis (Dennis, 1981) (Figure 5). Neuromuscular synapses are initially very weak not only because of the unspecialized nature of the first contacts but also due to the low concentration of AChRs in the post-synaptic terminal and low neurotransmitter release from the presynaptic terminal (Kullberg et al., 1977).

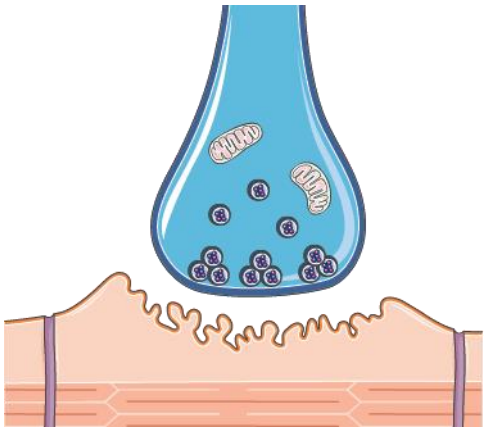


Figure 5: Presynaptic differentiation. Presynaptic axons increase in volume and area. Synaptic vesicles increase in number and cluster at the active zone of the presynaptic membrane. Active zones are organized at regular intervals and perfectly aligned with the mouths of the post-junctional folds.

Increasing evidences point towards a functional role of intra-axonal protein synthesis in the formation of new synaptic connections. In 2006, Lyles and collegues identified sensorin mRNA from a cDNA library generated from isolated processes of *Aplysia* Sensory Neurons (SNs). Sensorin is a SN-specific neurotransmitter previously shown to be involved in the regulation of neuromuscular synapse formation and stabilization. From their experiments, Lyles and colleagues observed that when SNs were isolated in culture, sensorin mRNA was diffusely distributed along the neurites. However, when *Aplysia* SNs were culture with target motor neurons (MNs), sensorin mRNA puncta co-localized with areas of high GFP-VAMP staining, a synaptic marker, and with sites were SNs contacted MNs, suggesting that sensorin mRNA was translocated and concentrated at synaptic sites. A similar result was obtained when investigators evaluated the distribution of sensorin protein, suggesting that sensorin mRNA was translated at synapses. Importantly, when sensorin mRNA was reduced using dsRNA

synapse formation was inhibited (Lyles et al., 2006) (Figure 6). These results strongly suggested that sensorin mRNA translation is required for synapse formation, confirming the role of local protein synthesis in synaptogenesis.

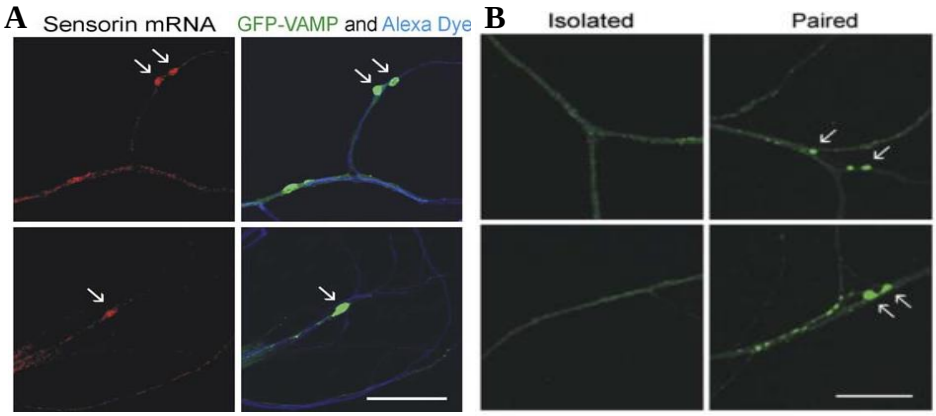


Figure 6: Local protein synthesis of sensorin mRNA in synapse formation. A) Sensorin mRNA localizes to synaptic sites, characterized by high GFP-VAMP (green) staining. B) Sensorin protein also localizes to synaptic varicosities, where contact between *Aplysia* sensory neurons and motor neurons is established [Adapted from Lyles et al., 2006].

1.2.2.1 FGF22, a presynaptic organizing molecule

Fibroblast growth factors (FGFs) are a family of proteins that play a role in diverse biological processes. The human family of FGFs comprises 22 members that can be divided into 7 subfamilies according to their phylogeny. These subfamilies were further grouped with respect to their mechanism of action: the intracellular *Fgf*11/12/13/14 subfamily, the hormone-like (endocrine) *Fgf*19/21/23 subfamily, and the canonical Fgf subfamily comprising the *Fgf*1/2/5, Fgf3/4/6, *Fgf*7/10/22, *Fgf*8/17/18, and *Fgf*9/16/20 subfamilies (Itoh and Ornitz, 2008). Among the functions controlled by FGFs in development, repair and metabolism we can list wound healing (Komi-Kuramochi et

al., 2005), bone formation (Lazarus et al., 2007), proliferation of gland epithelial cells (Steinberg et al., 2005) and morphogenesis of embryonic tissues (Revest et al., 2001). In the central nervous system, FGFs were also found to be key regulators. They play crucial roles in the induction and early patterning of the neuronal layers of the neural plate, in the development of the neuronal layers in the embryonic neocortex and in the developing spinal cord (Dono, 2003).

The FGF22 protein along with its related members FGF7 and FGF10 belong to the canonical subfamily. This means that in order to have biological activity they have to bind and activate FGF receptors (FGFRs). Canonical FGFs are extracellular proteins that bind to cell surface FGFRs inducing their dimerization and autophosphorylation of tyrosine residues. This activates signal transduction pathways, mainly RAS/MAP kinase and the phospholipase-C gamma pathways, that ultimately elicit a biological response (Itoh and Ornitz, 2008). FGFRs are encoded by 4 *fgfr* genes (*fgfr1-fgfr4*). Each FGFR is composed of an extracellular domain with three immonuglobulin domains, which contains the ligand bonding domain, a transmembrane domain and a split intracellular tyrosine kinase domain. Different FGFs bind to FGFRs with different degrees of specificity. FGF22 protein binds with high specificity to the FGFR2b isoform (Umemori et al., 2004).

Experiments carried out by Umemori and colleagues, demonstrated for the first time that this subfamily shared a presynaptic organizer activity and that FGF22 was the major active species. The ability of FGF22 to induce synaptogenesis was assessed in motor neurons stimulated with recombinant FGF22. Results showed clustering of synapsin and SV2 (Figure 7). Because synapsin and SV2 are components of synaptic

vesicles, clustering formation suggests presynaptic differentiation and ultimately synapse formation. Evidences are also given for a target-derived organizer role of FGF22 on presynaptic differentiation, i.e. FGF22 is released from the post-synaptic terminal, binds to FGFR2b in the presynaptic terminal and induces presynaptic differentiation on mossy fibers. These results show that FGF22 is an excellent tool to induce synaptogenesis (Umemori et al., 2004).

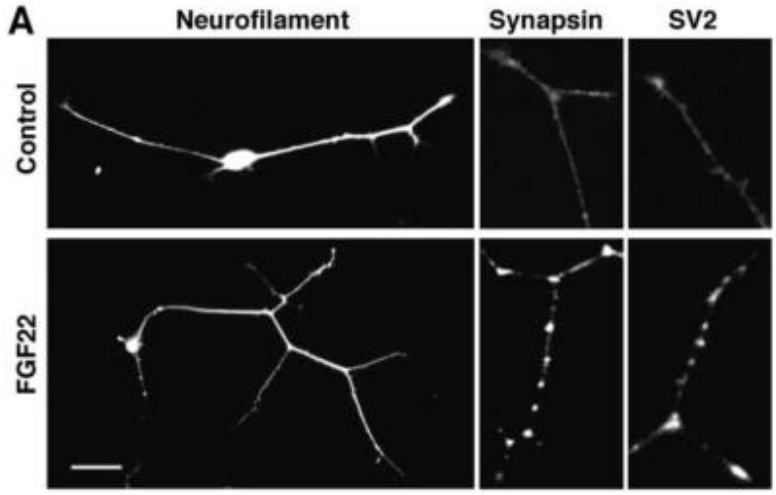


Figure 7: FGF22 induces presynaptic differentiation. FGF22 induced clustering of synapsin and SV2, synaptic vesicle components, and increased axon branching in cultures neurons [Adapted from Umemori et al., 2004].

1.3 Ribosomal and mRNA regulation

Despite all the previous evidences of the presence of ribosomes and mRNAs in axons, their regulation during neuronal development and more specifically during formation of new synaptic connections it still is poorly understood. Much of the current knowledge come from studies performed in dendrites. However, with regards to the subject of this seminar it is of great interest to focus the attention in axon.

1.3.1 mRNA and ribosome transport to axons

For highly polarized cells, as neurons, translation of localized mRNAs largely contribute to the functionality of a specific sub-cellular compartment and to spatially and temporally control and restrict protein synthesis. The presence of translational machinery, including ribosomal RNA and mRNAs, in distal axons imply their transport from the cell body, mostly because mRNAs are synthesized in the nucleus. Hence, investigators concentrated their efforts in unraveling the molecular mechanisms underlying axonal mRNA transport and translational control.

Targeting of mRNAs to axons starts right after its generation in the nucleus, when *trans*-acting factors, also called RNA binding proteins (RBPs), recognize specific *cis*-acting elements present in the transcript sequence. This interaction forms the ribonucleoprotein particles (RNPs) that assemble in RNA granules to travel long distances through axons till they reach their target destination, such as growth cones. RNA granules also associate with a variety of proteins, many of them still unknown, in order to control appropriate transport and translation of mRNA molecules.

Proteomic studies from developing brains unraveled some of these proteins (Figure 8). The large majority of proteins found in RNA granules are ribosomal proteins or ribosome-associated, such as transcription factors and eukaryotic initiation factors. RNA-binding proteins and cytoskeletal proteins were also relatively abundant. The RNA-binding proteins identified (hnRNP A1, hnRNP A2, and ZBP) as well as the transport motor protein dynein, were already known to be implicated in mRNA transport and expected in this proteomics study, since RNA particles are moving organelles (Elvira et al., 2006). Other RNA-binding proteins can also be important in

the regulation of translation while other proteins can help in the assembly of messenger ribonucleoprotein complexes into transport granules (Bassell and Kelic, 2004).

Currently, the most accepted view defends that mRNA transport is a "multi-step" process that begins with recognition of *cis*-acting factors within the mRNA sequence by RBPs, transport of RNA granules along cytoskeletal filaments through motor proteins along which translation is suppressed, ending with local translation at the final destination (Yoon et al., 2009).

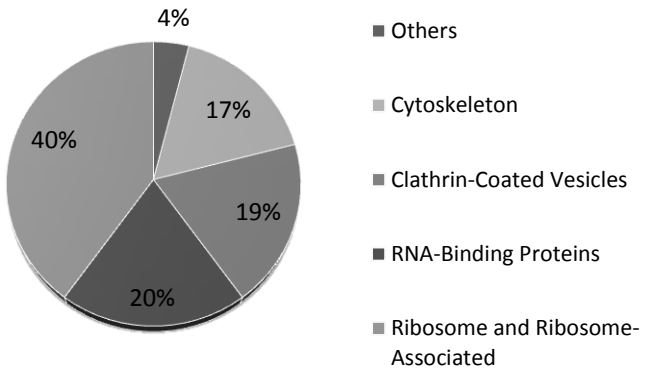


Figure 8: Protein composition of RNA granules. Proteomic analysis of RNA granules identified four major classes of proteins: ribosome and ribosome-associated (40%), RNA-binding proteins (20%), Cytoskeleton (17%) and Clathrin-Coated vesicles (19%) [Adapted from Elvira et al., 2006].

1.3.1.1 Cis-acting factors

Cis-acting elements consist of specific nucleotide sequences as well as secondary or tertiary stem-loop structures present in the 3' or 5' untranslated region (UTR) of the mRNA. Their basic function is to target mRNAs to a specific location in the cell. The

most studied *cis*-acting factor is referred to as "zipcode". It is a 54 nucleotide sequence present in the 3'UTR of the β-actin mRNA that specifically targets this mRNA to growth cones of developing neurons (Sotelo-Silveira et al., 2006; Yoon et al., 2009). β-actin RNA zipcode was the first localization element studied. In one of the earliest studies, using reporter plasmids expressing different elements of the 3'UTR of β-actin mRNA in chicken embryo fibroblasts (CEFs) Singer and co-workers mapped a 54-nucleotide zipcode region in the 3'UTR of β-actin just proximal to the coding region. To address its functional role, the investigators used phosphorothioate-modified oligonucleotides as antisense to inhibit zipcode function. Treatment with antisense oligonucleotides resulted in a decreased localization of β-actin mRNA to the cell periphery and also to severe alterations in cell shape, lamellipodia structure, and actin stress fiber organization. This data supported a role for β-actin RNA zipcode in localization of β-actin mRNA to cell periphery and subsequent translation in a compartmentalized manner, which may be involved in cell structure, polarity, and motility (Kislauskis et al., 1994). Another often referred *cis*-acting element, consists of 240 nucleotide AU-rich sequence present in the 3'UTR of tau mRNA that also localizes this mRNA to axons (Sotelo-Silveira et al., 2006; Yoon et al., 2009).

1.3.1.2 Trans-acting factors

On the other hand, *trans*-acting factors (TAF) are responsible for the recognition of specific sequences within the mRNA where location is encoded, i.e. the *cis*-acting factors. They play a dual function, supporting RNA transport but also repressing its translation till it reaches the final destination (Yoon et al., 2009).

The zipcode binding protein 1 (ZBP1) is an mRNA binding protein that binds β-actin mRNA through its zipcode and is necessary for proper mRNA localization. Because ZBP1 has a nuclear localization sequence, it binds newly generated β-actin mRNA in the nucleus and allows the export of the mRNP localization complex to the cytoplasm through its export sequence (Bassell and Kelic, 2004). In order to detect proteins with capacity to bind the zipcode, Singer and colleagues performed band-shift mobility assays, UV cross-linking, and affinity purification experiments using cell extracts prepared from CEFs. Results allowed identification of a 68kDa protein that interacted with the proximal 27 nucleotide of the β-actin mRNA zipcode, which they termed ZBP-1 (Ross et al., 1997). The transport of this mRNA mediated by ZBP-1 was further characterized in CEFs as well. ZBP1 protein contains two NH_2-terminal RNA recognition motifs (RRMs) and four COOH-terminal hnRNP K homology (KH) domains. ZPB1 third and fourth KH domains bind β-actin RNA zipcode and facilitate granule formation and cytoskeletal association. These results were in accordance with previous studies suggesting an involvement of the actin cytoskeleton in β-actin mRNA transport and anchoring in its final destination (Sundell and Singer, 1991).

The recognition of the zipcode by ZBP-1 was recently addressed. KH domains are arranged in an intra-molecular anti-parallel pseudodimer conformation with the canonical RNA-binding surfaces at opposite ends of the molecule, implying the looping of β-actin mRNA in order to bind both KH domains simultaneously (Chao et al., 2010). Following studies demonstrated a functional role for ZBP-1 interaction with zipcode in axon guidance. Using a photoconvertible translation reporter, netrin-1, an attractive guidance cue, was shown to stimulate local translation of β-actin mRNA in axonal growth cones (Leung et al., 2006). Importantly, local β-actin protein synthesis was

dependent on ZBP-1 binding to β-actin, zipcode (Welshhans and Bassell, 2011). Similar results were obtained for brain-derived neurotrophic factor (BDNF)-induced translation of β-actin mRNA. BDNF leads to ZBP-1 phosphorylation and consequent β-actin synthesis and growth cone turning (Yao et al., 2006; Sasaki et al., 2010). These studies not only provided evidences for a functional role of ZBP-1 in growth cone motility and axon guidance, but also in the spatiotemporal control of protein expression.

HuD is a TAF protein that binds to the *cis*-acting region of tau mRNA (Sotelo-Silveira et al., 2006) and also of Cpg15 mRNA. CPG15 protein promotes axonal and dendritic arbor growth as well as synapse maturation. Interestingly, survival of motor neuron (SMN) associates with this complex and probably regulates local stability and/or translation of cpg15 mRNA within the motor neuron axon and growth cone. Loss or mutation of the SMN gene that results in reduced SMN levels and a selective dysfunction of motor neurons causes Spinal Muscular Atrophy (SMA), an autosomal recessive neuromuscular disorder characterized by the degeneration of α motor neurons. Because CPG15 is an important downstream effector of SMN it may play a role in SMA disease by regulating axon extension and axon terminal differentiation and serve as a modifier gene of SMA pathology in humans (Akten et al., 2011).

1.3.1.3 Cytoskeletal elements and molecular motor proteins

The most likely mechanism for RNA granule transport suggests the recruitment of motor proteins by TAF and movement along cytoskeletal tracts. Although some evidences are already available supporting this hypothesis, the subject remains an open field full of numerous research possibilities. One of these studies, observed that the β-actin mRNA and ZBP1 RNP complex travelled along microfilaments in fibroblasts and along microtubules in neurons (Bassell et al., 1998). Also, RNA granules transporting

tau mRNA were shown to contain HuD and KIF3A, a subunit of motor protein Kinesin II, in neuronal P19 cells (Aronov et al., 2002). Other motor proteins have been proposed to be involved in RNA transport, including Kinesin I and II, and Myosin II and V (Sotelo-Silveira et al., 2006). In dendrites, both dynein and kinesin motor proteins were present in CPEB granules (Huang et al., 2003). Because kinesin family of motor proteins travels in the anterograde direction and dynein motor proteins travel retrogradelly, this result suggests a bidirectional movement of RNA particles. Yet there are no evidences regarding interactions between trans-acting factors and molecular motor proteins.

1.3.1.4 Translational repression

During transport, translation of mRNA is repressed to prevent ectopic expression. This repression can be achieved through translation repressors or as more recently shown by microRNAs. Translational repressors bind to targeted mRNAs and function mainly by inhibition of the initiation step of the translation process. Translational repressors can interfere with eIF4F complex preventing eIF4G binding and complex formation, which is an important step in translation initiation. ZBP1 for example, act as a translation repressor blocking 60S ribosomal subunit assembly. Another possible mechanism through which translation can be repressed consists in the modulation of poly(A)-tail length and PABP recruitment. Shortening of the poly(A)-tail length results in a repressed state of translation (Besse and Ephrussi, 2008).

Recently, microRNAs were shown to participate in the control of mRNA control. MicroRNAs are non-coding sequences of RNA with

approximately 22 nucleotides involved in several regulatory processes through their ability to target mRNAs for degradation or translational repression. The first known role for a microRNA in axons was described in the regulation of cytochrome c oxidase IV (COX IV) mRNA. The 3'UTR contains a binding site for microRNA-338, which results in a reduction of COX IV levels as well as a diminished mitochondrial function (Aschrafi et al., 2008).

1.3.1.5 Translational derepression

When mRNAs arrive to their target location translation has to be derepressed so protein synthesis can be initiated. Phosphorylation of translation repressors and competitive binding of pre-localized proteins are often referred as two mechanisms involved in translational derepression. In the already mentioned β-actin mRNA and ZBP1 RNP complex regulatory process, at the arrival of an RNA granule ZBP1 is phosphorylated by the Src kinase. Src kinase belongs to Src family of protein tyrosine kinases and is responsible for phosphorylation of the tyrosine residue 396 of ZBP1. This phosphorylation greatly decreases the binding affinity of ZBP1 for β-actin mRNA. As a result β-actin mRNA is released and translated in a spatially restricted manner (Besse and Ephrussi, 2008). The proposed model for mRNA transport and location is shown in Figure 9.

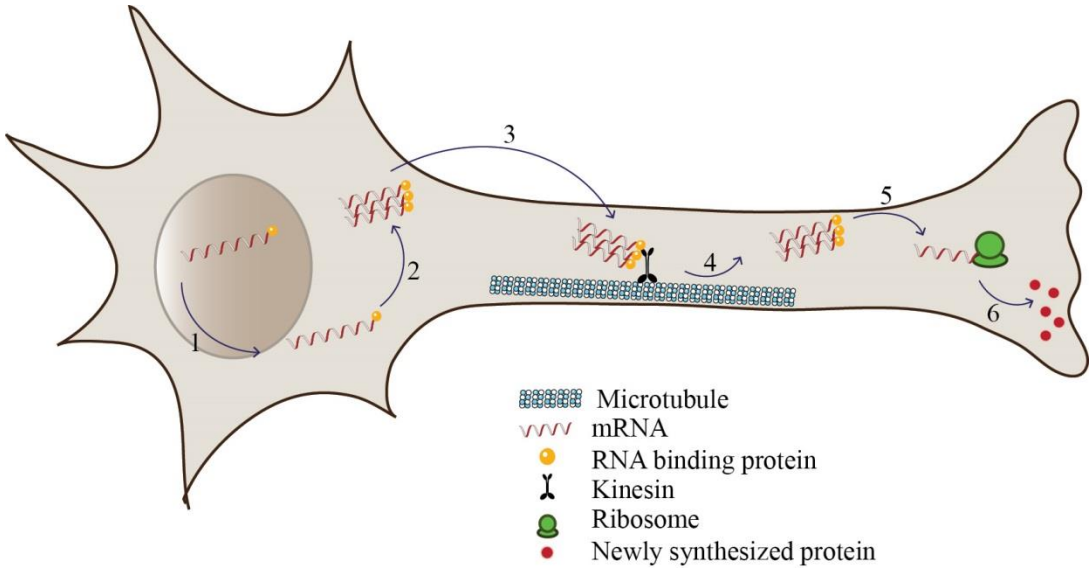


Figure 9: Proposed model for mRNA transport and location in neurons. Following transcription in the nucleus, trans-acting factors recognize cis-acting elements in the mRNA forming a ribonucleoprotein particle (RNP) complex. RNP is exported from the nucleus to the cytoplasm (1) and additional RNPs are recruited to form a RNA granule (2) that is anterogradely transported to the axon with the help of kinesin motor proteins (3). Arriving to the axon, RNA granule is anchored (4), translational machinery including ribosomes attaches to mRNAs (5) and translation is initiated (6) [Adapted from Bassell & Kelic, 2004].

1.3.2 Axonal mRNA pool changes with maturation

As previously shown in Figure 1, both mature and developing axons are provided of mRNAs that fit into four categories: protein synthesis, mitochondrion, intracellular transport and cytoskeleton. However, how this pool changes during the different stages of neuronal development was only recently uncovered and the regulatory processes that underlie such changes remain to be revealed.

An undoubtedly evidence that the mature pool of mRNAs differ from the mRNA pool present on developing axons was provided by Taylor and colleagues using CNS neurons cultured in microfluidic chambers. Actb and Syp mRNA levels were accessed by qPCR and results showed that Actb transcript levels decreased significantly from 7 to 13 days in culture whereas Syp transcript levels presented an increase during the same time interval (Taylor et al., 2009).

Beyond that, developing axons as well as mature axons contain exclusively localized transcripts as showed by Gumy and colleagues (Gumy et al., 2011). Developing axons are mainly enriched in mRNAs related to cytoskeleton and transport of vesicles/trafficking. In the first set are included mRNA transcripts for Tubulin, regulators of actin dynamics, microtubule associated proteins and microtubule stabilizing proteins. The second category comprises mostly molecular motor proteins, such as Kif5A and Dctn2. These localized transcripts are in accordance with the major cytoskeletal reorganizations that take place in the initial steps of neuronal development: axonal branching, turning, and pathfinding, growth cone collapse and extension (Gumy et al., 2011). Indeed, evidences exist proving that guidance cues elicit local translation of mRNAs enriched in growing axons of developing neurons. For example, Semaphorin 3A elicit local translation of RhoA mRNA resulting in growth cone collapse (Wu et al., 2005). Transcripts related to cell cycle, such as CDKN1B and CDKN1C mRNAs of the family of tumor suppressors Cip/Kip, Cyclin I and Cyclin D2 were also enriched in developing axons. All these transcripts share common roles in cytoskeleton regulation and axonal growth (Gumy et al., 2011).

Alternatively, mature axons have their own mRNA pool that is not detected in the previous analyzed developing axons. The identified mRNAs encode mostly inflammation and immune-related proteins with known functions, like cytokine-cytokine receptor interactions, Toll-like receptor signaling pathways and immune response/antigen presentation and processing (Gumy et al., 2011).

Importantly, the mature pool of mRNAs was shown to be reduced when compared to the immature mRNA pool. The same situation applies to ribosomal RNA as it shares a significant decrease in later stages of development. Kleiman and colleagues, in 1994, performed hybridization experiments with poly (A) RNA and rRNA probes and quantified both dendritic and axonal labeling. The results turned out to be surprising, rRNA levels presented a decrease from 82% of labeled axons in the first day of culture to 54% of labeling in the third day (Kleiman et al., 1994). By this time questions were firing endlessly regarding the mechanisms responsible for the disappearance of translational machinery and what could possible be the functional significance of local translation in adult vertebrate axons.

More than a decade later, more evidences came up confirming the accentuated decline of ribosomes and mRNAs as neurons develop. Besides the above described mRNAs that are unique to each of the development stages, looking at the transcripts common to both embryonic axons and mature axons, 1272 transcripts are enriched in earlier developmental stages whereas only 173 are enriched in matured ones (Gumy et al., 2011). Nevertheless, none of the previous questions were solved, leaving numerous possibilities of research wide open for the future.

1.3.3 Following Injury

The next question to be answered regarded how mRNA composition changed after axonal injury. Indeed, several evidences showed a functional role of local protein synthesis in regenerating axons. Conditioning by axonal crush, resulted in a mammalian dorsal root ganglia (DRG) axons vigorous regeneration, which is paralleled by an increased localized translation of the ribosomal protein L4 mRNA (Twiss et al., 2000). In a further attempt to better characterize local translation in regenerating DRG axons, Twiss and collegues extended their previous work and demonstrated that this regenerating axons were still capable of synthesizing proteins as showed by the presence of ribosomal proteins, translational initiation factors, and rRNA (Zheng et al., 2001).

Recent work characterized the axonal mRNA pool after axotomy and found that mRNAs related to axonal targeting, synaptogenesis and synaptic function "returned" to regenerating axons (Taylor et al., 2009). Investigators enclosed the identified transcripts into three major classes: cell differentiation, cell-cell signaling and secretion. Cell differentiation related transcripts, for examples ephrin B1 and ephrin A3, are of particular interest for the proper development of the nervous system. Also, ephrin signaling is an important known mechanism of axon guidance. Cell-cell signaling and secretion comprised mRNAs associated with synaptogenesis and neurotransmitter release, for example mRNAs encoding for the synaptic vesicle component synapsin, neurexin-3 a mRNA important for synaptic function, frequenin homolog and Rims1 responsible for exocytosis at the synapse were up-regulated in axons after axonal severing (Taylor et al., 2009).

Mitochondrial, cytoskeletal and intracellular transport-related transcripts were also altered following injury. Within mitochondrial-related mRNAs, ATP synthase transcripts and cytochrome c oxidase subunit transcripts were down-regulated whereas genes related with electron transport such as mitofusin 1 and 2 were enriched in regenerating axons. These results point to an alteration in mitochondrial function rather than a reduced or complete elimination of energy production by mitochondria. Actin and microtubule transcripts are among the cytoskeleton mRNAs with reduced localization following injury while intermediate filament protein mRNA is increased. There was also a change in intracellular transport-related mRNAs localization, with vesicle trafficking transcripts altered the most (Taylor et al., 2009).

Concluding, axonal injury induces altered intracellular transport and mRNA location with the purpose of supporting axon regeneration, growth cone and axon pathfinding, as well as, the formation of new synaptic connections.

Chapter 2 Objectives

The aim of this study is to identify the molecular trigger that leads to decreased ribosomal levels in mature axons. It is well established that the levels of mRNA decline during development and are significantly reduced in mature axons. Ribosomes however are not so well described in adult vertebrate axons. In early stages of neuronal development a significant number of mRNAs encoding cytoskeletal proteins such as β-actin, β-tubulin, ADF, RhoA, and cofilin were shown to be present and locally translated in axonal processes (Hengst and Jaffrey, 2007). Such observations are consistent with neuronal needs, as they project their axons through long distances. For example the process of finding the post-synaptic target and establish new synaptic connections requires a rapid and extensive reorganization of the growth cone into a functional presynaptic terminal, the conventional anterograde protein transport is unlikely to support such an event. Interestingly, as neurons develop and transform into a more static and differentiated structure the translational capacity of axons seems to change and the number of poly (A) mRNAs significantly decrease (Bassel et al., 1994). Although previous studies demonstrated the presence of ribosomal RNA (Piper and Holt, 2004) and ribosomes (Bunge, 1973) in vertebrate axons little evidences have been shown so far concerning the presence of ribosomes in mature axons. The existence of such a gap in the field led us to explore in further detail how ribosomes decline during development and what is the molecular trigger responsible for this decrease.

mRNA and ribosomal decline seems to be coincidence with the time point where axons mature to form functional synapses. Therefore, we propose that the observed decrease in the translational capacity of axons may be due to synapse formation. To accomplish our goal we will first demonstrate that ribosomes are indeed present in developing axons and secondly exploit if FGF22, a known trigger of pre-synaptic differentiation

(Umemori et al., 2004), controls the mRNA and ribosomal decline. We will assess the number of ribosomes with and without the FGF22 synaptogenic stimulus by immunolabeling cultured neurons for ribosomal proteins S6 and P0, and ribosomal RNA 5.8S. Finally, we propose to look at ribosome levels when FGF22 is applied specifically to axons, by means of a microfluidic chamber, mimicking the physiological environment found *in vivo*.

Chapter 3 Materials and Methods

3.1 Reagents

Bovine Serum Albumin (BSA), poly-D-lysine (PDL), Minimum essential medium eagle (MEM), 5-fluoro-2'-deoxiuridina (5-FDU), paraformaldehyde, deoxiribonuclease, L-15 medium and Opti Prep Density Gradient Medium were purchased from Sigma-Aldrich (Sintra, Portugal). MEM-Non essential amino acids Neurobasal medium, penicillin-streptomycin, B27 supplement, sodium pyruvate, fetal bovine serum (FBS), trypsin, glutamine, 2-mercaptoethanol, Hank's Balanced Salt Solution (HBSS) and Horse Serum were purchased from GIBCOTM Invitrogen Corporation (Carlsbad, California, USA). Mouse laminin I was obtained from Cultrex®, as part of Trevigen, Inc (Helgerman Court, Gaithersburg, USA). Mounting media ProLong Gold antifade reagent with DAPI was obtained from Molecular Probes®, as part of Invitrogen Life Technologies (Eugene, Oregon, USA). Recombinant human fibroblast growth factor-22 (FGF22) was purchased from R&D Systems, Inc (Minneapolis, USA). Glial derived neurotrophic factor (GDNF), Ciliary neurotrophic factor (CNTF) and Brain derived neurotrophic factor (BDNF) were purchased from PeproTech Inc. (Rocky Hill, USA). Pierce Immunostain Enhancer was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). In this study we used the following antibodies: anti-Neurofilament (Chicken), anti-Synapsin I (Rabbit), Anti-SV2 (Mouse), anti-Ribosomal protein S6 (Rabbit), anti-Ribosomal protein P0 (Human), anti-Ribosomal RNA 5.8S (Y10B) (Mouse).

3.2 Neuronal Cultures

3.2.1 Glass coverslip treatment

Glass coverslips (Corning No.1 24 mm × 40 mm) were treated with nitric acid 65% for 24h under constant agitation and then washed 5 times with mQ H_2O for 30min each. After that, coverslips were rinsed twice in pure ethanol and then dried for approximately 20 min at 50ºC. Sterilization was guaranteed with a 15min ultraviolet radiation exposure.

3.2.2 Preparation of microfluidic devices

The microfluidic devices consist of a molded PDMS (polydimethylsiloxane) chamber assembled in a glass coverslip. Microfluidic chambers were always assembled in the plating day. Previously cleaned and sterile glass coverslips were placed in a 10cm dish, coated with 0.1mg/ml PDL overnight at 37ºC and washed 3 times with mQ H_2O. Afterwards coverslips were air-dried, the chambers were assembled on top of them and 3 μg/ml of laminin (prepared in neurobasal media) was added to the reservoirs making sure the microgrooves were completely filled. Laminin was washed out with plain neurobasal medium after 2 hours of incubation at 37ºC.

3.2.3 Primary cultures of embryonic spinal motor neurons

The purification method of spinal motor neurons consider four fundamental steps [Henderson]: (i) dissection of embryonic spinal cord of E14.5 Wistar rats; (ii) preparation of cellular suspensions from dissociated spinal cords; (iii) optiprep density gradient centrifugation; and (iv) culture of motor neurons. Whole embryonic spinal cords were dissected and separated from their meninges and the dorsal half of the cord

was discarded. After dissection, spinal cords were cut in approximately 15 fragments and incubated for approximately 8 min at 37ºC with trypsin (0.025% w/v) in Hank's balanced salt solution (HBSS) (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0,001% phenol red). Immediately after trypsin incubation, the fragments were transferred to the dissociation solution (L-15 medium plus 0.4% BSA (w/v) and 0.1 mg/ml deoxyribonuclease) and agitated by hand until tissue fragments were disaggregated. Fragments were then mechanically dissociated twice for 2 min, with a long fire-polished Pasteur pipette, to prevent cell destruction and allowed to settle. The supernatant was collected to a separate tube. The remaining spinal cord fragments were mechanically dissociated again for eight times with a long fire-polished Pasteur pipette, allowed to settle for 2 min and the supernatant was collected and pooled to the same tube. This step was repeated 2 more times. Afterwards, a 1.5 ml 4% BSA (w/v) cushion was prepared on the bottom of the cell suspension using a long Pasteur pipette and cells were centrifuged (5 min, 470 g at room temperature). The supernatant containing trypsin and debris was discarded and the pelleted cells were re-suspended 6 times with a long fire-polished Pasteur pipette in 2 ml of incomplete medium (neurobasal medium supplemented with 2% B27, 2% Horse Serum, 0.5 mM glutamine, 25 μM glutamate, 0.025 mM 2-mercaptoethanol and 1x penicillin-streptomycin solution).

To perform the OptiPrep density gradient centrifugation a 1.5 ml cushion of 6.5% OptiPrep solution (w/v in L-15 medium) was prepared disposing it into the bottom of the tube (one tube per three to four spinal cord equivalents) using a long Pasteur pipette in order to create a sharp interface. Because OptiPrep has a high density, it will create a density barrier and motor neurons will float at the interface facilitating their

fractionation. Cells were then centrifuged (15 min, 830 g at room temperature) resulting in a pellet at the bottom of the tube (small cells, corresponding mainly to sensory neurons) and a turbid band at the medium-Optiprep interface (motor neurons). This band was collected, diluted up to 10 ml to lower the density of OptiPrep and centrifuged (5 min, 470 g at room temperature) through a 1.5 ml 4% BSA (w/v) cushion. Cells were re-suspended in 100µl of complete medium (incomplete medium supplemented with 0.1 ng/ml GDNF, 0.5 ng/ml CNTF, 1ng/ml BDNF). All centrifugations were carried with no acceleration and no break to reduce the vibration.

Purified motor neurons were cultured in 24 multi-well plates (MW24) and microfluidic chambers. After a 2-hour incubation period at 37ºC, saturating humidity, and 5% CO_2, complete medium was added to microfluidic chambers. At DIV 2, 5-FDU (10 µM) was added to ensure culture purity.

3.2.4 Primary cultures of embryonic hippocampal neurons

Primary cultures of rat hippocampal neurons were prepared from the hippocampus of E17-18 Wistar rat embryos. After dissection, hippocampi were treated for 15 min at 37ºC with trypsin (0.045%) and deoxyribonuclease (0.01% v/v) in Hank's balanced salt solution (HBSS) (5.36 mM KCl, 0.44 mM KH2PO4, 137 mM NaCl, 4.16 mM NaHCO3, 0.34 mM Na2HPO4.2H2O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0,001% phenol red). After centrifugation for 1 min at 1000 rpm to deposit the hippocampi, Hank's solution with trypsin was removed and the hippocampi were washed with plating medium containing 10% FBS to stop trypsin activity. In order to obtain a homogeneous cell suspension the hippocampi were mechanically dissociated with a Pasteur pipette. Cells were then plated in microfluidic chambers coated with PDL (0.1 mg/ml) and laminin (2 µg/ml). After 4 h incubation at 37ºC, the plating medium

was removed and replaced for culture medium (Neurobasal medium supplemented with 2% B27, 25 μM glutamate, 0.5 mM glutamine and 1:400 penicillin-streptomycin).
At DIV 3/4, 5-FDU was added to the cultures at a final concentration of 10 μM.

3.4 Induction of synaptogenesis in the presynaptic compartment

To induce synaptogenesis in our neuronal cultures, primary cultures at DIV 4 (motor neurons) and DIV7 (hippocampal neurons) were stimulated with FGF22 for 14h at 37ºC in conditioned medium. Recombinant human FGF22 was used at 2nM in both MW24 and microfluidic chambers.

In microfluidic chambers, a minimal volume difference between the somal compartment and the axonal compartment (≈25μl) was maintained during the incubation to prevent the diffusion of the applied factor from the axonal to the somal side. The slightly higher volume on the somal side will cause a slow net flow of liquid from the somal to the axonal compartment and not the other direction.

3.4.1 Immunocytochemistry

Due to the sensibility of the axons, after stimulation, cells were pre-fixed for 5 minutes in 1% paraformaldehyde (in PBS with 1% sucrose) at 37ºC to a more gentle approach. Cells were then fixed for 10 minutes with 4% paraformaldehyde (in PBS with 4% sucrose) at 37ºC, washed three times for 5 minutes each with ice-cold PBS and permeabilized by adding PBS with 0.25% Triton X-100 for 5 minutes at room temperature. Next, cells were washed once with PBS followed by blockade of non-specific binding with 3% BSA (in PBS) for 30 minutes at room temperature. Primary

antibodies were diluted in 3% BSA (in PBS) and incubated overnight at 4ºC. After overnight incubation, primary antibodies were washed out with PBS, three times, 5 minutes each, and cells were incubated for 1h with secondary antibodies diluted in 3% BSA, at room temperature. Finally, cells were washed two times in PBS with 0.1% Triton X-100 and once with PBS, 5 minutes each. Coverslips were then rinsed once with mQ H_2O and mounted in prolong mounting media with DAPI. When using microfluidic devices, the PDMS mold was carefully disassembled before this step.

3.4.2 Fluorescence microscopy and quantification

Fluorescent images were taken using an inverted microscope Zeiss Axiovert 200, an AxioCam HRm camera and ZEN 2011 software. Images were acquired with a Plan-Neofluar 63× oil objective (numerical aperture 1.4) and exposure times conserved in single experiments. In microfluidic chambers images were acquired from the axonal compartment. Synapsin, SV2, S6, P0 and Y10B number of puncta per axon length were quantified with Image J 1.47n.

3.5 eGFP-L10a gene insertion in a lentiviral vector using the In-Fusion® HD Cloning Kit

The aim of this task was to clone the eGFP-L10a gene that was already inserted in the Syn-DsRed-Syn- eGFP-L10a cloning vector into the pRRLSIN.cPPt.PGK-GFP.WPRE cloning vector. For that purpose we used the *In-Fusion® HD Cloning Kit* (clontech), which is a fast and efficient strategy designed for cloning of one or more fragments of DNA into any vector. The basic principle behind the strategy is the recognition of a 15 bp overlap at the ends of DNA fragments, e.g. PCR-generated sequences and linearized

vectors, by the In-Fusion Enzyme that will then fuse the fragments together. This 15 bp overlap was engineered designing primers for amplification of the desired sequences.

The protocol used for In-Fusion® HD Cloning Kit consist of six steps: (i) selection of the base vector and linearization of the vector by restriction enzyme digestion; (ii) amplification of the gene of interest by PCR; (iii) spin-column purification of the PCR product; (iv) verification of the correct PCR amplification; (v) DNA purification from agarose gel; (iv) In-Fusion cloning reaction; (vi) transformation of competent cells.

3.5.1 Constructs

3.5.1.1 pRRLSIN.cPPt.PGK-GFP.WPRE cloning vector

The pRRLSIN.cPPt.PGK-GFP.WPRE cloning vector (*Appendix I*) is a mammalian expression, lentiviral vector. It contains a phosphoglycerate kinase (PGK) promoter that drives the expression of the green fluorescent protein (GFP) and a safety modified woodchuck hepatitis post-transcriptional regulatory element (WPRE). This pRRL vector contains an ampicillin resistance cassette. It also contains several restriction endonuclease recognition sites; of particular interest for this work are the recognition sites of *BamHI* and *SalI.*

3.5.1.2 Syn-DsRed-Syn- eGFP-L10a cloning vector

The Syn-DsRed-Syn- eGFP-L10a cloning vector (*Appendix II*) is a dual promoter lentiviral vector. GFP was previously replaced in our lab by eGFP-L10a using a similar cloning strategy. It contains two synapsin promoters (Syn) in order to express two exogenous cDNAs specifically in neurons; one directs the expression of the green

fluorescent protein GFP while the other drives the expression of the red fluorescent protein DsRed. This vector also contains two safety modified woodchuck hepatitis post-transcriptional regulatory element (WPRE), one downstream of each SYN promoter, and several restriction endonuclease recognition sites.

3.5.2 Restriction enzyme digestion

Restriction enzyme digestion was carried out to generate the linearized pRRL vector. The total reaction mixture was set up according to the Table X and the incubated at 37ºC for 2 hours.

Table I: Restriction Enzyme Reaction Components

Components	
Plasmid DNA	10 μL
10X Restriction Buffer	2 μL
Restriction Enzyme	1 μL
mQ H_2O	Up to 20 μL

3.5.3 Amplification of the eGFP-L10a gene by PCR

For amplification of the eGFP-L10a gene, PCR primers were designed with 15 bp extensions (5') complementary to the ends of the linearized vector. The total reaction mixture of 50 μL was set up according to the Table X.

Table II: PCR Amplification Reaction Components

Components	
NZYTech 2x Green Master Mix	25 μL
Forward Primer	2.5 μL
Reverse Primer	2.5 μL
DNA template (100 ng/μL minimum)	1 μL
Nuclease-free water	Up to 50 μL

The PCR Amplification program started with an initial 95ºC denaturation of 30 sec, followed by 30 sec of annealing at 55ºC, 72ºC elongation for 1 kb/min (depending on the size of the amplification product); one more elongation step at 72ºC for 5 min. Finally, the resulting samples were cooled at 4ºC and then stored at -20ºC.

3.5.4 Spin-column purification of the PCR product

The *QIAquick PCR Purification Kit* (QIAGEN) and protocol were used for PCR products purification according to the manufacturer's instructions.

3.5.5 Agarose Gel Electrophoresis

DNA samples were resolved by electrophoretic separation in order to purify the fragments of interest, namely eGFP-L10a and linear pRRL vector. For this purpose, DNA samples previously mixed with *5x GelPilot loading dye* (1x final concentration) and *1Kb Plus DNA ladder* were applied on a 1% (w/v) agarose gel in 1x TAE buffer, ran at 100 V between 30 – 45 min. DNA fragments were visualized on a UV Trans Illuminator [GelDoc^{TM} (BIO RAD)] and photographed.

3.5.6 DNA purification from agarose gel

The *QIAquick Gel Extraction Kit* (QIAGEN) and protocol were used for DNA extraction and purification from agarose gels according to the manufacturer's instructions

3.5.7 In-Fusion cloning reaction

The In-Fusion cloning reaction was set up according to the Table IV. The reaction mixture was incubated for 15 min at 50°C and then placed on ice.

Table III: In-Fusion Cloning Reaction Components

Components	
5X In-Fusion HD Enzyme Premix	2 μL
Linearized vector	100 ng
Insert	100 ng
mQ H_2O	Up to 10 μL

3.5.8 Transformation of competent cells.

For this cloning procedure we used the *Eschericia coli* (*E. coli*) strain DH5α (Invitrogen). We added 5 μL of In-Fusion cloning reaction to 50 μL of *E. coli* DH5α competent cells and mixed gently. Cells were incubated on ice for 30 min, heat-shocked at 42ºC for 45 sec and returned on ice for 2 min. Afterwards, 150 μL of Super Optimal broth with Catabolite repression (S.O.C) was added in order to obtain maximal transformation efficiency and cells were then incubated at 37ºC for 1 hour under constant agitation (225 rpm). After the incubation period the volume of transformation

mix was split in half and spread onto LB-agar plates containing the appropriate selection antibiotic. The plates were incubated overnight at 37ºC.

3.6 Isolation of plasmid DNA

3.6.1 Small-scale isolation of plasmid DNA

The *QIAprep Spin Miniprep kit* (QIAGEN) and protocol were used for small-scale plasmid DNA purification according to the manufacturer's instructions.

3.6.2 Medium-scale isolation of plasmid DNA

The *Pure Link^{TM} HiPure Plasmid Midiprep kit* (Invitrogen) and protocol were used for medium-scale plasmid DNA purification according to the manufacturer's instructions.

3.7 Calcium-Phosphate transfection

HEK 293T cells were transfected in order to create lentivirus expressing the plasmid containing the eGFP-L10a gene of interest. For that purpose, the day before transfection cells grown in 10 cm petri dishes were split so that in the transfection day they were 80% confluent. In the transfection day, 30 min – 3 hours before transfection, 6 mL of fresh medium (DMEM 7777 supplemented with 10% FBS and 1:100 penicillin-streptomycin) were added to the cells. To a solution of 2x HEPES buffered saline (HBS) (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4) pH 7.05 was added drop-wise a solution of $CaCl_2$ and DNA (Helper plasmids: 10 μg pLP1, 5 μg pLP2 and 6 μg pLP-VSVG; and 20 μg of the plasmid containing the gene of interest). The solution of 2x HBS was constantly mixed while adding the second solution. The mixture was

incubated at room temperature for 30 min and mixed by vortex every 5 min. After the incubation period, the resulting mixture was added dropwise to the cells. 5h after transfection the medium was changed and the cells were maintained at 37ºC, saturating humidity and 5% CO_2. 24h post-transfection the medium was collected and kept at 4ºC while 6 mL fresh medium was added to the cells. This was repeated at 48h post-transfection. The collected medium containing the lentivirus was then filtered using a cellulose acetate 0.45 μL syringe filter (Frilabo) and centrifuged at 22 000 rpm for 140 min at 22ºC (Beckman Coulter, Optima^{TM} L-100 XP ultracentrifuge). The supernatant was discarded, and 200 μL of 1% BSA (prepared in PBS) were added. The solution was kept rotating for 40 min and afterwards shortly centrifuged. The virus containing solution was then stored at -80ºC.

3.8 Statistical Analysis

Graphs and statistical analysis were performed in Graph Pad Prism 5 software. Statistical significance was assessed by unpaired t-test or one-way analysis of variance (ANOVA).

Chapter 4 Results and Discussion

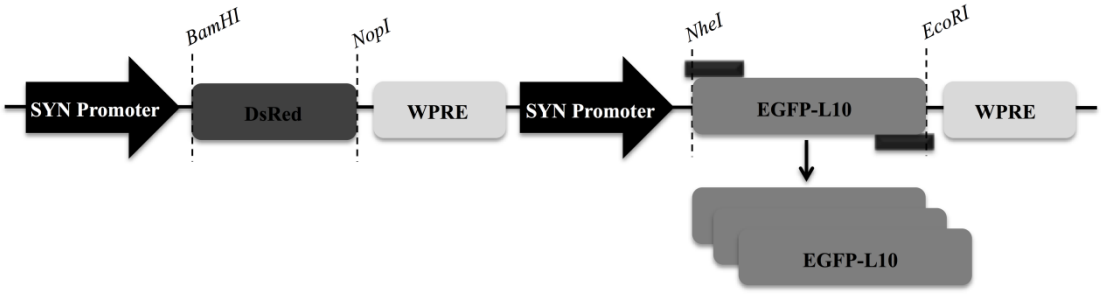
4.1 eGFP-L10a gene insertion into a lentiviral vector using the In-Fusion® HD Cloning Kit

The main objective of this study was to unravel why are ribosomes decreased in later stages of axonal development. For that purpose, it was an absolute requirement to identify ribosomes and quantify its axonal levels through fluorescence microscopy. To do this we labelled endogenous ribosomes with specific antibodies in fixed neuronal cultures. In addition it would be important to track ribosomes in living neurons and to assess their dynamics during neuronal development To accomplish this goal we developed a ribosomal reporter which consists of the L10a ribosomal protein fused to eGFP cloned in the pRRLSIN.cPPt.PGK-GFP.WPRE lentiviral vector. This construct will allow us to explore through live cell imaging studies the intricate processes that may govern the decrease of ribosomes in mature neurons.

Ribosomes are cellular organelles responsible for protein synthesis. In eukaryotes, ribosomes consist of a small 40S subunit and a large 60S subunit. They are composed of four RNA species and approximately 80 structurally distinct proteins that play a crucial role in their activity. The ribosomal protein L10a is part of the L10E family of ribosomal proteins and is a component of the 60S ribosomal subunit. It is essential for the joining of the 40S and 60S subunits and an excellent candidate to track ribosomes (Nguyen et al., 1998). We subcloned eGFP-L10a from SYN-DsRed-SYN-eGFP-L10a vector, previously generated in our lab, into the pRRLSIN.cPPt.PGK-GFP.WPRE lentiviral vector (Figure 10).

1. Linearization of the pRRLSIN.cPPt.PGK-GFP.WPRE vector by restriction enzyme digestion

2. PCR amplification of the eGFP-L10a gene



3. Cloning of EGFP-L10 into pRRLSIN.cPPt.PGK.WPRE linear vector

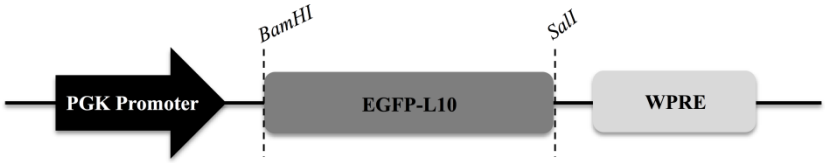


Figure 10: Cloning strategy scheme. eGFP-L10a was cloned into pRRLSIN.cPPt.PGK-GFP.WPRE vector using the *BamHI/SalI* cloning sites.

The pRRLSIN.cPPt.PGK-GFP.WPRE vector was digested with *BamHI* and *SalI* endonucleases in order to to remove the eGFP sequence. The digested fragments were validated through agarose gel electrophoresis (Figure 11). As expected, restriction enzyme digestion resulted in two fragments that correspond to the linearized vector and to the eGFP insert (Figure 11, lane 3). The upper band corresponds to the linearized pRRLSIN.cPPt.PGK.WPRE vector (6668 bp) while the lower band corresponds to the GFP fragment (716 bp).

We used recombination-based cloning to insert eGFP-L10a into the destination vector, which consists on the recognition of a 15 bp overlap at the ends of DNA fragments, e.g.

PCR-generated sequences and linearized vectors, by a recombinase enzyme that will then fuse the fragments together. This 15 bp overlap at the end of the eGFP-L10a gene was engineered by PCR amplification designing primers with sequence homology to the destination vector. The primers were as follows: primer 1: CTCCCCAGGGGGATCCGCCACCATGGACTACAAGGACGATGAC and primer 2: GAGGTTGATTGTCGACGGAGCGTCCTAATACAGACGCTGG. The 15 bp overlap is underlined. PCR amplification was confirmed by agarose gel electrophoresis (Figure 11). The SYN-DsRed-SYN-eGFP-L10a cloning vector has 8841 bp and is represented as a single band in the lane 2 of the gel. Amplification by PCR of eGFP-L10a fragment (1442 bp) resulted in a single band in lane 4, confirming the correct amplification of eGFP-L10a.

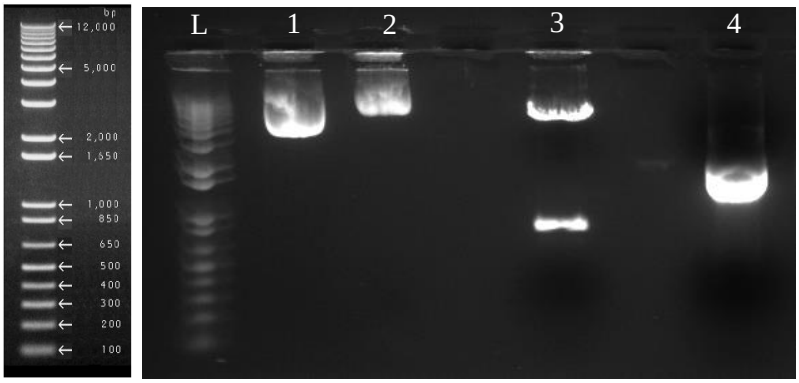


Figure 11: Validation of the pRRLSIN.cPPt.PGK-GFP.WPRE vector linearization and eGFP-L10a gene PCR amplification product. Digestion with *BamHI* and *SalI* generated a linearized pRRL cloning vector. L: 1 Kb DNA Ladder; Lane 1: uncut pRRL cloning vector (7384 bp); Lane 4: digested pRRL cloning vector. Two bands are represented: linearized pRRL vector (6668 bp) and GFP fragment (716 bp). eGFP-L10a fragment was amplified by PCR. Lane 2: dual promoter SYN-DsRed-SYN-eGFP-L10a (8845 bp); Lane 4: eGFP-L10a fragment (1442 bp).

After confirming the correct pRRLSIN.cPPt.PGK.WPRE vector linearization and eGFP-L10a fragment amplification, a ligation reaction was performed and the resulting DNA plasmid was transformed in *E. coli* DH5α competent cells. Positive clones were selected in an LB plaque supplemented with Ampicillin.

The plasmid DNA of eleven clones was isolated and digested with *BamHI* and *SalI*. The digested fragments were run in an agarose gel to identify the positive clones (Figure 12). The expected a positive result consists of two bands, an upper band corresponding to the linearized pRRLSIN.cPPt.PGK.WPRE vector (6668 bp) and a lower band corresponding to the eGFP-10a fragment (1442 bp). Therefore, clones 2, 8, 10 and correspond to negative clones, we selected clone 5 for sequencing validation. Sequencing results indicated that the construct contained the insert and the plasmid DNA vector in the correct orientation and without mutations of any kind.

We next generated lentivirus with the pRRLSIN.cPPt.PGK.eGFP-L10a.WPRE construct in order to exogenously express the L10a ribosomal protein fused to eGFP in our model system.

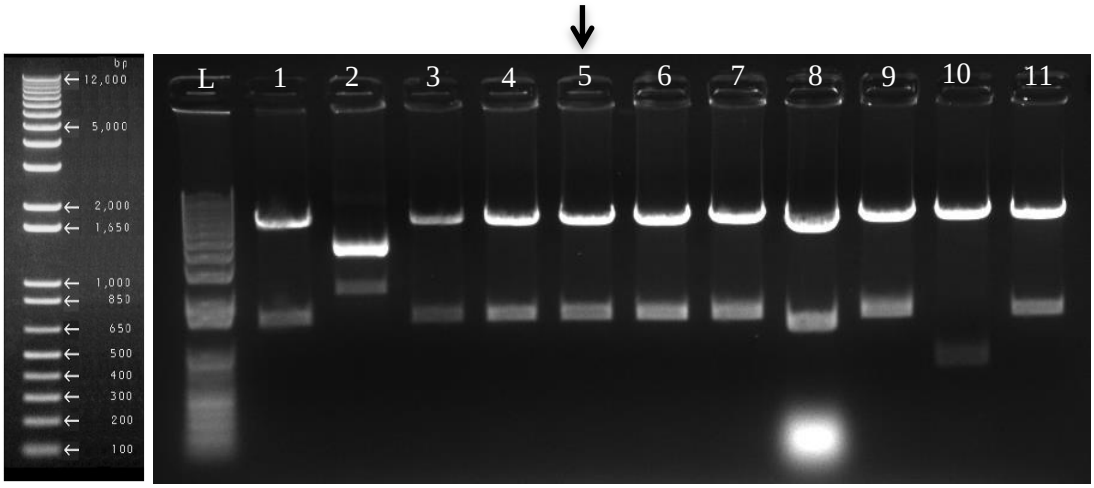


Figure 12: Identification of pRRLSIN.cPPt.PGK-eGFP-L10a.WPRE positive clones by restriction enzyme digestion. DNA of the selected clones was digested with *BamHI* and *SalI* endonucleases to confirm the presence of eGFP-L10a (1442 bp) and the linearized pRRL vector (6668 bp). L: 1 Kb DNA Ladder. The arrow indicates the clone selected for sequencing.

4.2 Ribosomes are present in developing axons

Although discouraged for decades, the idea of protein synthesis taking place in axons has accumulated significant evidences. Nerve cells benefit greatly from the advantages offered by this mechanism as a means to achieve precise temporal and spatial control of protein expression. In developing axons, the presence of both ribosomes and mRNA is well documented. rRNA and poly(A) mRNA were found in distal axons and growth cones of developing cortical and hippocampal neurons (Bassel et al., 1994), indicating that in this developmental stage neurons need proteins to be synthesized locally in axons. After being untangled the existence of axonal protein synthesis, the next question raised relates to the function of these locally translated proteins in axonal development. Interesting insight came from axonal pathfinding studies where mRNAs encoding cytoskeletal protein belonging to all three classes of actin microfilament, microtubule, and intermediate filament as well as their associated proteins are present in axons. For

example *β-actin*, *β-tubulin*, *ADF*, *RhoA*, and *cofilin* mRNAs were shown to be locally translated in axons of developing vertebrate neurons in response to guidance cues (Hengst and Jaffrey, 2007).

In this study we started by testing if ribosomes are present in developing axons of vertebrate neurons. To visualize these organelles we labeled ribosomes using antibodies against ribosomal proteins S6 and P0, and ribosomal RNA 5.8S (Y10B). Ribosomal protein S6 is one of the 33 proteins that compose the small 40S ribosomal subunit. It belongs to the S6E family of ribosomal proteins and it is essential for the translation initiation step of protein synthesis because directly interacts with the m7GpppG 5′-cap-binding complex (Hutchinson et al., 2011). On the other hand, ribosomal protein P0 is a component of the 60S ribosomal subunit and belongs to the L10P family of ribosomal proteins. This protein exists in complex with other two ribosomal proteins P1 and P2 and together they are responsible of recruiting translation factors to the ribosome (Uchiumi and Kominami, 1997). Lastly, ribosomal RNA 5.8S (Y10B) is part of the large subunit of the ribosome and it is thought to have an active role in the elongation step of protein synthesis and in the translocation of ribosomes (Abou Elela and Nazar, 1997). To detect axons we used an antibody against neurofilament, this protein is a neuron specific intermediate filament that is incorporated along the axon during axon growth. Also, neurofilament is the building block of the axonal cytoskeleton, is particularly abundant and, therefore, is an excellent neuronal marker.

We started by analyzing the presence of ribosomes in developing axons of rat spinal motor neurons (peripheral nervous system). The results showed a punctuated pattern along the axon for ribosomal proteins S6 (Figure 13A) and P0 (Figure 13B), and also

ribosomal RNA 5.8S (Figure 13C), which is in agreement with the expected distribution of ribosomes (Figure 13). These results demonstrate that developing axons of the peripheral nervous system, specifically spinal motor neurons (MN), contain ribosomes, in agreement with previous reports (Pannese and Ledda, 1991).

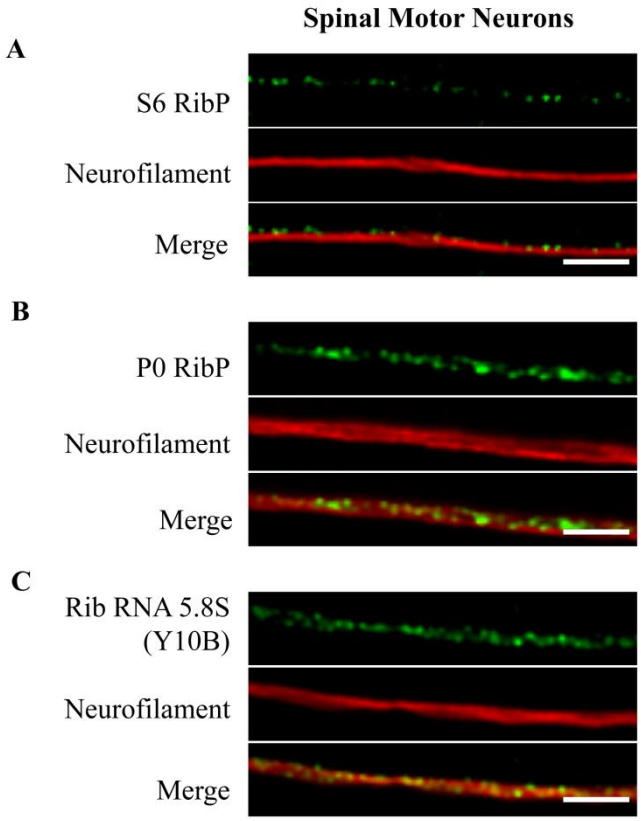


Figure 13: Ribosomes are present in developing axons of rat spinal motor neurons. Rat spinal motor neurons (MN) were cultured for 4 days and then fixed in 4% PFA and immunostained for S6 (A) and P0 (B) ribosomal proteins, ribosomal RNA 5.8S (Y10B) (C) and neurofilament. The results show that developing axons of the peripheral nervous system contain ribosomes and ribosomal RNA. Scale bar 2.5µm.

In rat hippocampal neurons (central nervous system) the same strategy also revealed, the presence of ribosomes in developing axons (Figure 14). Immunostaining against ribosomal proteins S6 (Figure 14A) and P0 (Figure 14B), and ribosomal RNA 5.8S

(Figure 14C) showed the same punctuated pattern as observed for motor neurons, characteristic of ribosomes distribution.

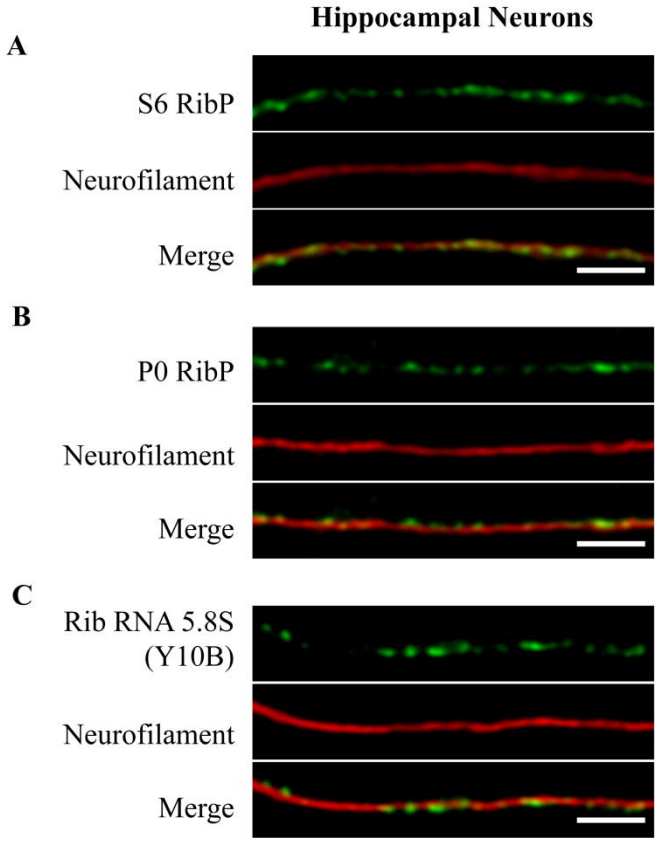


Figure 14: Ribosomes are present in developing axons of rat hippocampal neurons. Rat hippocampal neurons were cultured for 7 days and then fixed in 4% PFA and immunostained for S6 (A) and P0 (B) ribosomal proteins, ribosomal RNA 5.8S (Y10B) (C) and neurofilament. The results show that ribosomes are present in the central nervous system indicating that developing axons contain ribosomes and ribosomal RNA. Scale bar 2.5μm.

Together the results from developing rat spinal motor neurons and rat hippocampal neurons indicate that ribosomes exist in axons from both peripheral and central nervous system. Actually, mRNAs coding for components of the translational machinery, like ribosomal proteins, is one of the most enriched category in axons suggesting that local protein synthesis has itself a role in the increase of the translational capacity of axons (Moccia et al., 2003).

We next asked if exogenously expressed ribosomes would behave in a similar maner to the endogenous ones. For that purpose we expressed the ribosomal reporter generated previously in developing neurons (Figure 10). This reporter consists of the L10a ribosomal subunit fused to the C-terminal of eGFP (Figure 15A). Spinal motor neurons and hippocampal neurons were cultured for 4 and 7 days, respectively, and infected with eGFP-L10a lentivirus. We stained ribosomal protein L10a with an antibody specific for GFP and we used β3-tubulin as a neuronal marker. Tubulin is the basic building block of microtubules, and β3-tubulin is specifically expressed in neurons. In line with our previous results, eGFP-L10a expression resulted in a punctuated pattern characteristic of ribosomes and similar to the one obtained with the endogenous ribosomal proteins S6 and P0 as well as ribosomal RNA 5.8S (Figure 15B). Importantly, this result confirms that eGFP-L10a is a well-suited reporter to track ribosomes in future live cell imaging experiments.

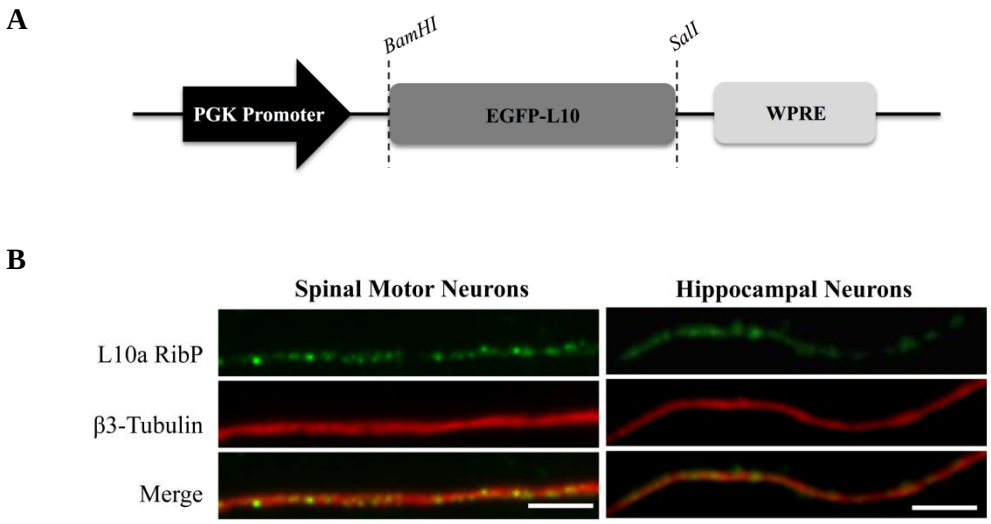


Figure 15: eGFP-L10a has a similar distribution to endogenous ribosomes. (A) pRRLSIN.cPPt.PGK.eGFP-L10a.WPRE ribosomal reporter scheme. (B) Rat spinal motor neurons (MN), and rat hippocampal neurons were cultured for 4 to 7 days, respectively, infected with eGFP-L10a for 60h and then fixed in 4% PFA and immunostained against GFP and β3-tubulin. The results show that exogenous ribosomes can be expressed and detected both in the peripheral nervous system (spinal motor neurons) and central nervous system (hippocampal neurons). Scale bar 2.5μm.

In opposition to developing neurons, there is considerable debate regarding the presence of ribosomes in mature neurons. Evidences for the existence and functional significance of ribosomes and local protein synthesis are scarce and controversial. Hypothalamic magnocellular neurons, sensory neurons projecting to the olfactory bulb, and the goldfish Mauthner mature neuron are the few examples of mature vertebrate nerve cells that contain mRNAs (Piper and Holt, 2004). Vasopressin and oxytocin encoding mRNAs were found in the axons of magnocellular mature neurons while the mRNAs encoding the olfactory marker protein and olfactory receptors were found in axons of sensory neurons projecting to the olfactory bulb (Hengst and Jaffrey, 2007). Both

studies suggest a role for axonal protein synthesis, helping neuropeptides to reach axonal terminals and directing expression of specific receptors to these terminals. Ribosomes and rRNA were only found in the goldfish Mauthner axon. Koenig and Martin used electron spectroscopic imaging to identify domains in Mauthner neurons axoplasm containing ribosomal RNA (Koenig and Martin, 1996). Fluorescent ribosomal RNA signal was identified in plaque like structures distributed in the cortical zone of the axon, which the authors attribute to a polyribosome-populated domain (Koenig and Martin, 1996). However, the lack of additional evidences for the existence of translational machinery in other types of adult axons generated a number of doubts that weaken the possibility of a functional role for ribosomes in later stages of neuronal development. Moreover, subsequent studies suggest that the translational capacity of axons changes and declines throughout development (Bassel et al., 1994; Gumy et al., 2011).

4.3 FGF22 induces differentiation of the presynaptic terminal

In light of our hypothesis, which proposes synapse formation as the trigger for the decrease in mRNA and ribosomal levels, it was it was crucial to have the ability to induce presynaptic differentiation. Importantly, we needed to have control of this process both spatially and temporally. To do so we used FGF22, a target-derived presynaptic organizing molecule, which was recently demonstrated by Umemori and collegues (Umemori et al., 2004) as an organizer of presynaptic activity.

The formation of a functional presynaptic terminal begins with the targeting of synaptic vesicles (SVs) that store neurotransmitters to the sites where new synapses will be formed. The clustering of SVs in these specific locations establish active zones where

SVs full of neurotransmitters are docked and undergo exocytosis upon Ca^{2+} influx that follows the arrival of an action potential. Both synapsin and SV2 are present in the membrane of these vesicles. Synapsins is a family of phosphoproteins specifically expressed in neurons that localizes to the cytoplasmic surface of SVs. They have been shown to be important players in the docking of SVs once they interact with actin-based cytoskeleton (Fornasiero et al., 2010). SV2 is a membrane glycoprotein and one of the first proteins to be localized to SVs. This protein is crucial in vesicle fusion and exocytosis of neurotransmitters triggered by calcium (Crowder et al., 1999).

Taking the previous considerations into account we decided to test the presynaptic organizing effect of FGF22 in our neuronal system. For this purpose we used rat spinal motor neurons in culture Stimulation of motor neurons resulted in a significant increase in the number of synapsin puncta per axon length (136.37%, $p<0.0011$) (Figure 16B). This increase is better visualized in the magnified axonal segment in Figure 16A. Synapsin is arranged in distinct puncta along the axonal shaft, indicating clustering of synaptic vesicles and the formation of presynaptic sites.

Results obtained were very similar when, under the same experimental conditons, we immunolabeled the cultured neurons for SV2 and neurofilament. FGF22 stimulation resulted in a statistical significant increase in the number of SV2 puncta per axon length (136.76%, $p<0.0004$) (Figure 17B). We observed the same punctuated distribution of SV2 protein throughout the axonal shaft, which is shown in the magnified axonal segment in Figure 17A, once again indicating the aggregation of synaptic vesicles.

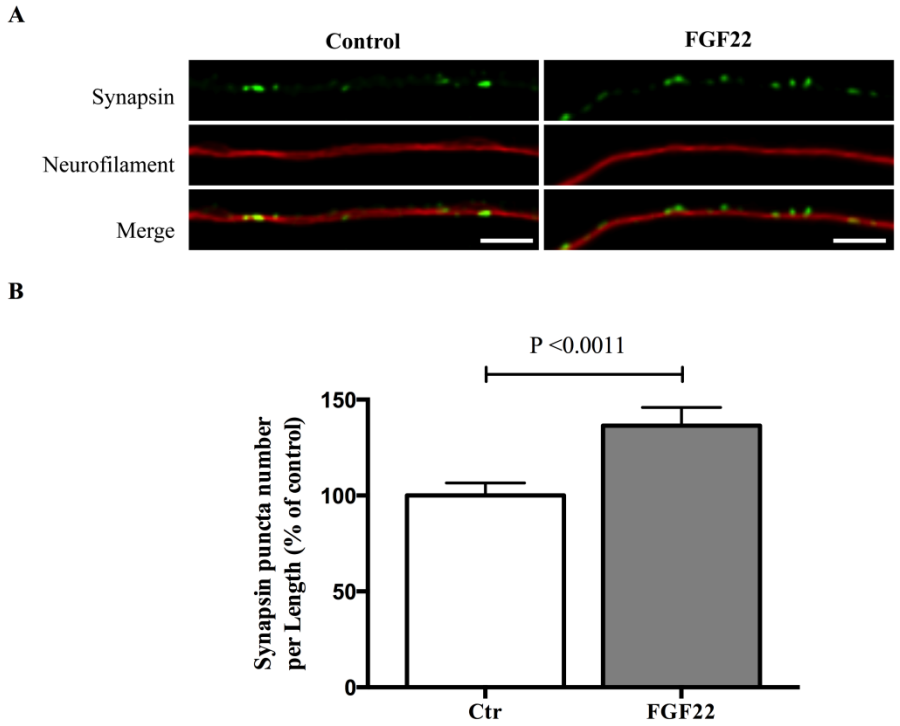


Figure 16: FGF22 induces clustering of synapsin in spinal motor neurons. (A) Primary cultures of rat spinal motor neurons were stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for synapsin (green) and neurofilament (red). Representative images of axonal segments show a clear FGF22-induced increase in the clustering of synaptic vesicles. Scale bar 2.5µm. (B) The number of synapsin puncta per axon length increases 36.37% in a statistically significant manner (136.37%, $p<0.0011$ by unpaired t-test). For each independent experiment, results were normalized to the control mean of each experiment. . Bars represent the mean ± SEM of at least ten independent experiments.

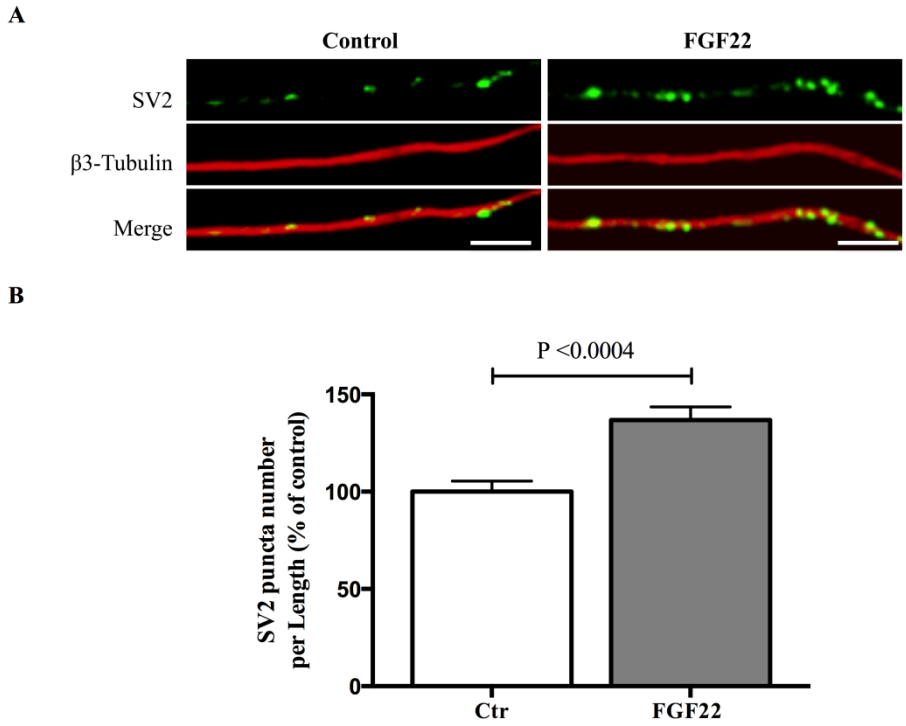


Figure 17: FGF22 induces clustering of SV2 in spinal motor neurons. (A) Primary cultures of rat spinal motor neurons were stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for SV2 (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced increase in the clustering of synaptic vesicles. Scale bar 2.5µm. (B) The number of SV2 puncta per axon length increases 36.76% in a statistically significant manner (136.76%, $p<0.0004$ by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of five independent experiments.

These results clearly demonstrated that a FGF22 stimulus is able to induce presynaptic differentiation of cultured motor neurons. Both synapsin and SV2 puncta were increased along the axons, indicating an effect on the distribution and clustering of synaptic vesicles, characteristic of synapse formation. Concluding, we were able to

establish a functional, reliable and reproducible assay to induce synaptogenesis in our *in vitro* model system.

4.3.1 FGF22 induces differentiation of the presynaptic terminal when applied locally

In the previous section we successfully demonstrated that FGF has a synaptogenic effect when applied globally to primary motor neuron cultures. However, our main goal is to understand the process of ribosomal decrease in axons, for that purpose we need to exclude any contribution of the cell body. Although in the previous set of experiments we focused only at the distal axons, we cannot completely rule out a possible contribution from the soma or dendrites. We used a novel platform, a microfluidic chamber system, which allows fluidic isolation and physical separation of axons from cell bodies and dendrites. Microfluidic devices have been described to be useful tools in the study of axon injury and regeneration (Taylor et al., 2005), localization and identification of mRNAs in axons (Taylor et al., 2005), synapse-to-nucleus signaling (Taylor et al., 2010), intracellular pH regulation in neuronal soma and neurites (Vitzthum et al., 2010), and axonal navigation and network formation (Millet et al., 2010).

The microfluidic chambers used in our lab are composed of a molded PDMS piece bearing desired surface embossed designs. Each individual PDMS piece or microfluidic chamber is placed against a properly coated-glass coverslip where neurons adhere. The microfluidic chambers used in this work have two compartments connected by a set of channels called microgrooves that separate the somal compartment from the axonal compartment. This structural property physically confine cell bodies in the somal

compartment, while allowing extending axons to penetrate though the microgrooves into the axonal compartment. Each compartment, measuring 1.5 mm wide, 7 mm long and 100 μm height, has two reservoirs at both edges to store culture medium (Figure 18A). Because microgrooves measure only 450 μm long, 10 μm wide and 3 μm height cell bodies cannot pass through such narrow channels. Dendrites, which are smaller than axons and grow at slower rates, don't reach the axonal compartment. In addition to physically isolated axons, microfluidic chambers also provide fluidic isolation that is accomplished by a minimal volume difference between the somal and the axonal compartment. This slight volume difference accompanied by the high fluidic resistance of the microgrooves, allows the fluidic isolation of the axonal compartment (Figure 18B). In this study we successfully cultured primary rat spinal motor neurons in microfluidic chambers (Figure 18C). Motor neurons grew, developed and established complex networks of axonal processes. Axons were able to cross the long microgrooves covering the entire surface of the compartment. We also observed the formation of bundles indicating that the axons are healthy.

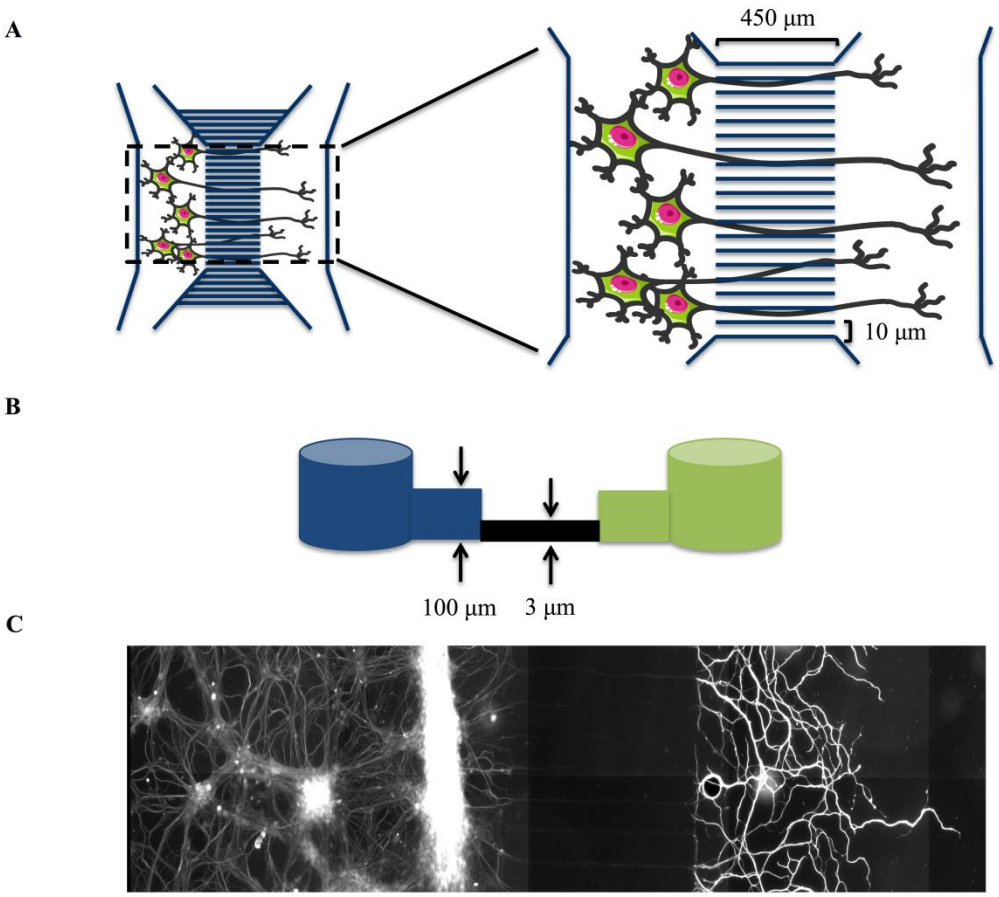


Figure 18: Spinal Motor Neurons grown in microfluidic chambers. (A) A microfluidic chamber (20 mm x 25 mm) consists of a molded PDMS chamber placed against a glass coverslip. It is composed by a somal compartment and an axonal compartment with 1.5 mm wide and 7 mm long each. Both compartments are separated by microgrooves (450 µm long, 10 µm wide). (B) The height difference between microgrooves (3 µm) and compartments (100 µm) combined with a minimal volume difference between the two sides leads to a fluidic isolation between the two compartments. (C) Representative image of primary spinal motor neurons were cultured in the microfluidic chambers. The neurons were immunolabledel, at DIV 4, for neurofilament (white).

To determine if FGF22 is capable of inducing synaptogenesis when applied only to axons only we cultured primary spinal motor neurons in microfluidic chambers. At DIV 4 neurons were stimulated with FGF22 and then fixed and immunolabeled for SV2 and neurofilament (Figure 19A). The results obtained show a significant increase in the number of SV2 puncta per axon length (188.07%, $p<0.0001$) (Figure 19B). This increase is better visualized in the magnified axonal segment in Figure 19A. Similar to what happened when FGF22 was applied globally, SV2 is arranged in distinct puncta along the axonal shaft, indicating clustering of synaptic vesicles. Moreover, when axons were locally stimulated we also observed a greater increase in SV2 puncta intensity (194.16%, $p<0.0001$) and area (191.54%, $p<0.0001$) per length.

To sum up, FGF22 induces differentiation of the presynaptic terminal as determined by the clustering of synaptic vesicles, a hallmark of synapse formation. This was demonstrated when FGF22 is globally applied but also when axons are specifically stimulated with this growth factor, mimicking the physiological events that take place in the organism, and is of particular importance in the context of nervous system development.

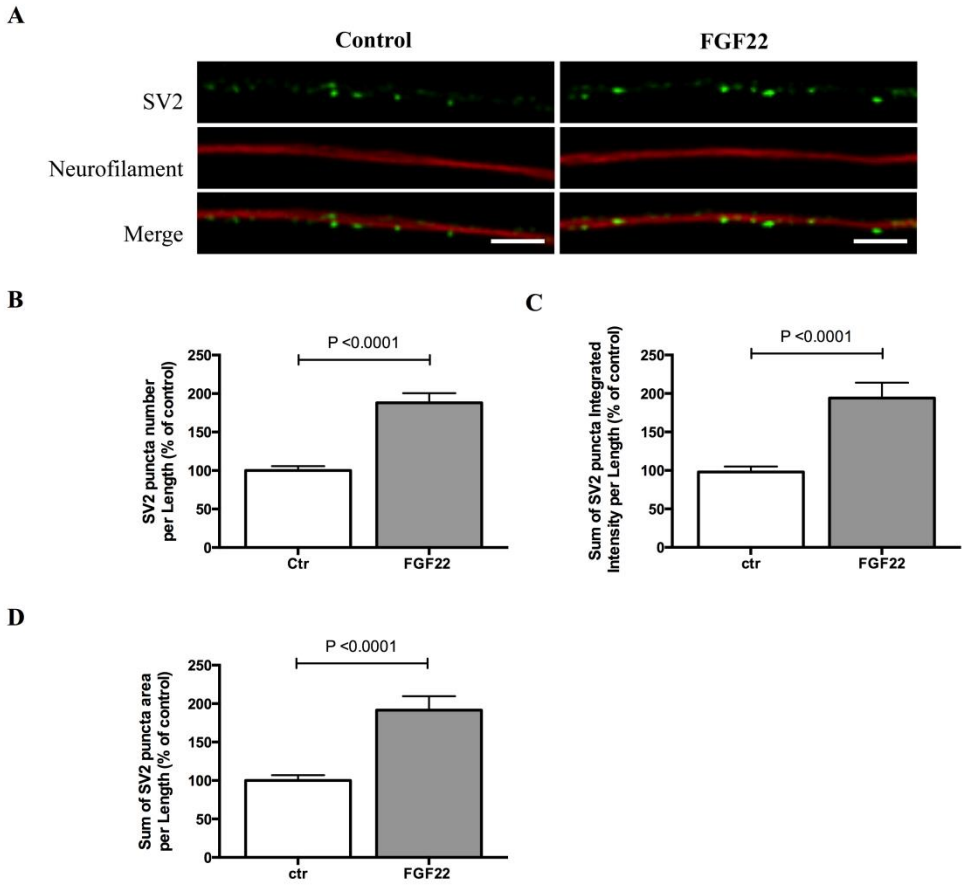


Figure 19: Local application of FGF22 to axons induces presynaptic differentiation. (A) Primary spinal motor neurons were cultured in microfluidic chambers and the axonal compartment was stimulated, at DIV 4, for 14 hours at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Neurons were fixed and immunostained for SV2 and neurofilament. Representative images of axon segments reveal a clear difference in SV2 clustering when the axonal compartment is stimulated FGF22. (B) The number of SV2 puncta per axon length increased significantly after FGF22 stimulation when compared to the control condition (188.07%, $p<0.0001$ by unpaired t-test). (C) SV2 puncta intensity (194.16%, $p<0.0001$) and (D) area (191.54%, $p<0.0001$) per length also increased. For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of three independent.

4.4 Ribosomes decrease upon FGF22 stimulation

For a long time electron microscopy studies performed in mature vertebrate neurons repeatedly reported the lack of ribosomes in axonal processes. It was later found that the translational capacity of axons changes throughout development, and it is reduced in later stages of development (Hengst and Jaffrey, 2007). Although adult invertebrate axons appear to contain mRNA, such observation cannot be extrapolated to vertebrate neurons. They present a single type of neurites instead of two functionally distinct processes, like axons and dendrites (Mohr and Richter, 2000). For this reason the presence of mRNA in mature invertebrate neurons should be interpreted with caution when trying to understand the functional role of local translation in adult vertebrate axons.

Corroborating the decline of local translation during development, Bassel and colleagues confirmed a transient nature of both ribosomes and mRNA. While trying to identify the cytoskeletal components that anchor mRNAs in neurons he faced with a far most interesting discovery. Using high resolution *in situ* hybridization, they detected poly (A) mRNAs throughout cerebrocortical axons during development. However, soon after 4 days in culture only about 10% of the axonal processes presented poly (A) mRNAs, the majority of transcripts were confined to the soma and dendrites (Bassel et al., 1994). This study was conclusive in the demonstration that the translational capacity of axons changes during maturation and that mRNA levels are significantly reduced in adult axons. However, neither the signal that triggers this decline nor the mechanism behind it is currently known.

To become fully matured, growing axons have to encounter the correct postsynaptic target and transit from a highly motile and plastic structure to a more static one. The axon then differentiates into a functional presynaptic terminal capable of forming new synapses with its postsynaptic partner. Because this stage of axonal maturation is coincident with the time point where axons partially loose their capacity to translate new proteins we hypothesize that synapse formation may be the trigger of this event.

In the previous section we established a functional, reliable and reproducible system to induce presynaptic differentiation, both globally and locally at axons, through FGF22 stimulation. We now aim to clarify if the number of ribosomes decreases when axons are stimulated with FGF22. To accomplish our objective rat spinal motor neurons were stimulated with FGF22 and the level of ribosomal proteins S6 and P0, ribosomal RNA 5.8S assessed by immunofluorescence. Presynaptic differentiation induced by FGF22 was accompanied by a significant decrease in the number of ribosomal protein S6 (69.07%, $p<0.0001$) (Figure 20B) and P0 (70.06%, $p<0.0001$) (Figure 21B) puncta per axon length; and in the number of ribosomal RNA 5.8S (72.64%, $p<0.0001$) (Figure 22B) puncta per axon length. The puncta area also suffered a significant decrease for both ribosomal proteins S6 (68.96%, $p<0.0083$) (Figure 20C) and P0 (45.15%, $p<0.0001$) (Figure 21C) and ribosomal RNA 5.8S (80.80, n.s.) (Figure 22C) as well. Representative images show the decrease of ribosomal proteins S6 (Figure 20A) and P0 (Figure 21A), and ribosomal RNA (Figure 22A along the axonal shaft. These results are the first demonstration that ribosomal decrease may be triggered by synapse formation. By reporting the disappearance of ribosomes from mature axons we confirm that mature axons have less translational capacity, in opposition to early developmental stages.

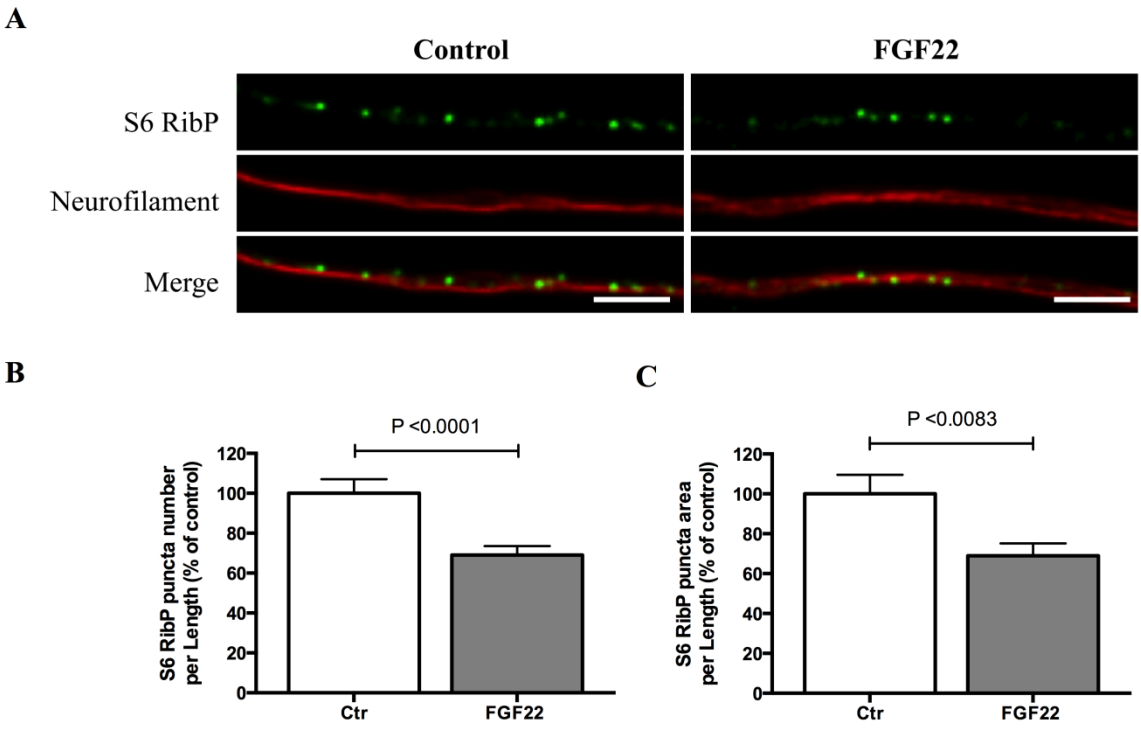


Figure 20: FGF22 induces a decrease in the 40S subunit ribosomal protein S6. (A) Primary cultures of rat spinal motor neurons were stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal protein S6 (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5µm. (B) The number of ribosomal protein S6 puncta per axon length decreases approximately 30% in a statistically significant manner (69.07%, $p<0.0001$ by unpaired t-test). (C) The S6 puncta area per axon length significantly decreased (68.96%, $p<0.0083$ by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of five independent experiments.

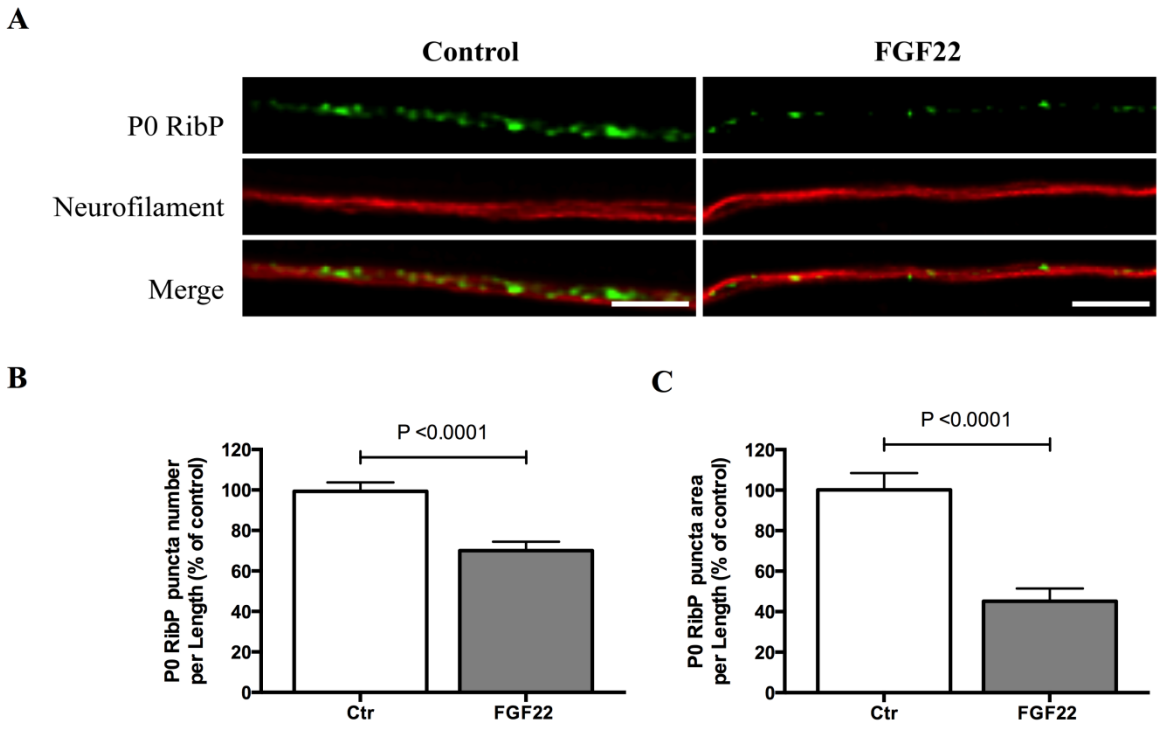


Figure 21: FGF22 induces a decrease in the 60S subunit ribosomal protein P0. (A) Primary cultures of rat spinal motor neurons were stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal protein P0 (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5µm. (B) The number of ribosomal protein P0 puncta per axon length decreases 30% in a statistically significant manner (70.06%, $p<0.0001$ by unpaired t-test). (C) The P0 puncta area per axon length significantly decreased (45.15%, $p<0.0001$ by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of three independent experiments.

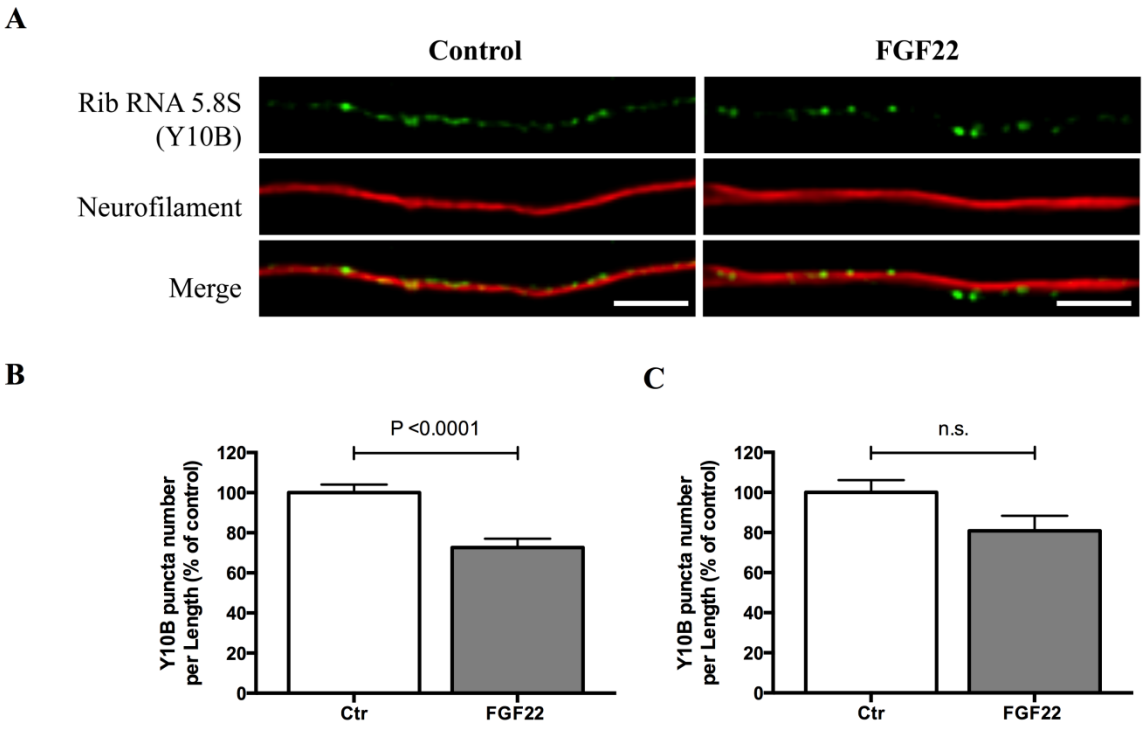


Figure 22: FGF22 induces a decrease in the ribosomal RNA content. (A) Primary cultures of rat spinal motor neurons were stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal RNA 5.8S (Y10B) (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5µm. (B) The number of ribosomal RNA 5.8S puncta per axon length decreases approximately 30% in a statistically significant manner (72.64%, $p<0.0001$ by unpaired t-test). (C) The ribosomal RNA 5.8S puncta area per axon length decreased (80.80%, n.s. by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of five independent experiments.

4.4.1 A synaptogenic stimuli induces ribosomal decrease in distal axons

Previously we described the reduction of ribosomes from adult axons when cells are globally stimulated with FGF22. To elucidate if this event also happens when the axon alone receives a synaptogenic stimulus we explored our hypothesis using the microfluidic chamber system described before (Figure 18).

Rat spinal motor neurons were cultured in microfluidic chambers and the axonal compartment was stimulated with FGF22. The levels of ribosomal protein S6 and ribosomal RNA 5.8S were assessed by immunofluorescence. Presynaptic differentiation induced by FGF22 was accompanied by a significant decrease in the number of ribosomal protein S6 puncta per axon length (80.32%, $p<0.0001$) (Figure 23B) and in the number of ribosomal RNA 5.8S (57.16%, $p<0.0001$) (Figure 24B) puncta per axon length. We also observed a reduction in the puncta area of ribosomal protein S6 (65.14%, $p<0.0001$) (Figure 23C) and ribosomal RNA 5.8S (33.47%, $p<0.0001$) (Figure 24C). Magnified axonal segments show for both ribosomal proteins S6 and ribosomal RNA the decrease of ribosomes along the axonal shaft, Figure 23A and 24A, respectively. Using an in vitro tool that closely recapitulates the physiological environment we demonstrated that mature axons have less ribosomes

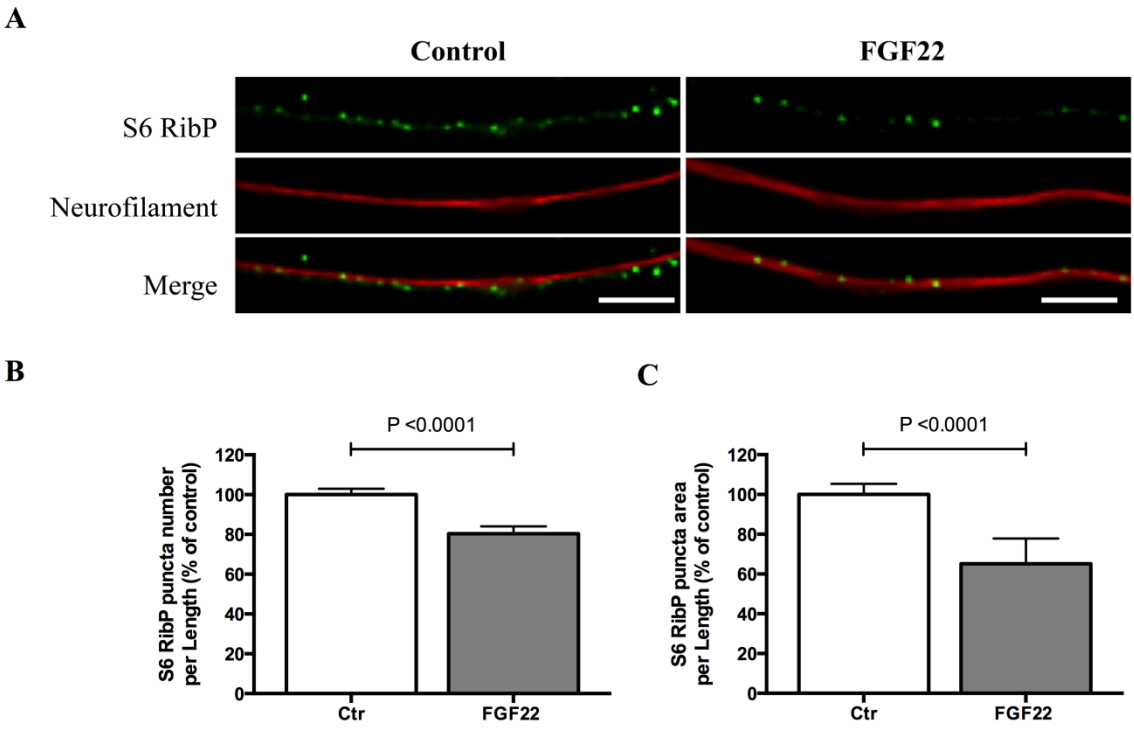


Figure 23: Axon-specific stimulation induces a decrease in the 40S subunit ribosomal protein S6. (A) Rat spinal motor neurons were cultured in microfluidic chambers and the axonal compartment stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal protein S6 (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5μm. (B) The number of ribosomal protein S6 puncta per axon length decreases approximately 20% in a statistically significant manner (80.32%, $p<0.0001$ by unpaired t-test). (C) The S6 puncta area per axon length was also reduced (65.14%, $p<0.0001$ by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of 5 independent experiments.

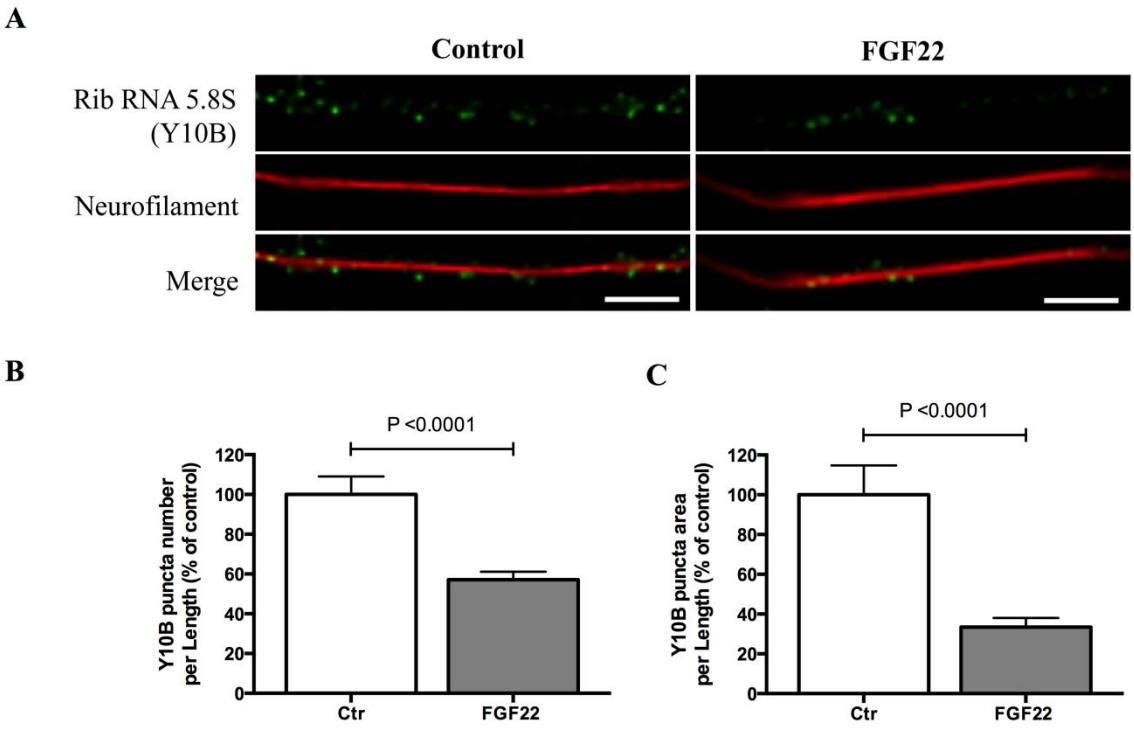


Figure 24: Axon-specific stimulation induces a decrease in the ribosomal RNA content. (A) Rat spinal motor neurons were cultured un microfluidic chambers and the axonal compartment stimulated at DIV4 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal RNA 5.8S (Y10B) (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5µm. (B) The number of ribosomal RNA 5.8S puncta per axon length decreases approximately 40% in a statistically significant manner (57.16%, $p<0.0001$ by unpaired t-test). (C) The ribosomal RNA 5.8S puncta area per axon length was also reduced (33.47%, $p<0.0001$ by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of 3 independent experiments.

4.5 Ribosomes decrease upon FGF stimulation in hippocampal neurons

In hippocampal neurons, the presence of mRNAs and ribosomal RNA was also reported by *in situ* hybridization studies. In early 90's Kleiman and colleagues described the existence of poly (A) RNAs and ribosomal RNA in both developing dendrites and axons of hippocampal neurons. Importantly, they characterized the subcellular compartmentation of RNAs during neuronal development. Poly (A) RNAs and ribosomal RNA levels increased over time in dendrites while in axons their overall levels diminished. At DIV 10, labeling of poly (A) RNA and ribosomal RNA in axons had almost disappeared (Kleiman et al., 1994).

In this section we aimed at localizing ribosomes in cultured hippocampal neurons and address if their levels also decrease in mature axons as we reported with PNS neurons. First, we asked if FGF22 also induced presynaptic differentiation in hippocampal neurons. Cells were cultured in microfluidic chambers and at DIV 7 axons were stimulated with FGF22. The results obtained clearly show an increase in the number of synapsin puncta (128.12%, $p<0,0090$) (figure 25) per axon length. The increase observed in the number of synapsin puncta indicates that FGF22-induced clustering of synaptic vesicles, a hallmark of synapse formation, is observed in both CNS and PNS neuronal populations.

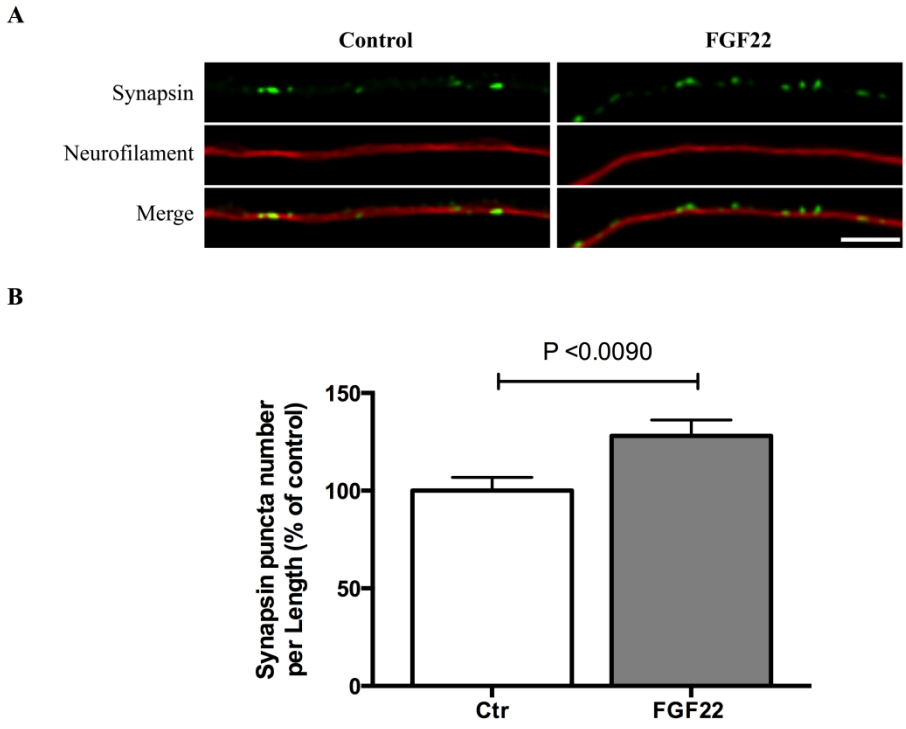


Figure 25: FGF22 induces presynaptic differentiation in hippocampal neurons. Primary cultures of rat hippocampal neurons were stimulated at DIV7 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2 nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for synapsin (green) and neurofilament (red). Representative images of axonal segments show a clear FGF22-induced increase in the clustering of synaptic vesicles. Scale bar 2.5µm. (B) The number of synapsin puncta per axon length increases approximately 30% in a statistically significant manner (128.12%, p<0,0090 by unpaired t-test). For each independent experiment, results were normalized to the mean of control. Bars represent the mean ± SEM of 6 independent experiments.

To extend our study to hippocampal neurons we assessed the levels of ribosomal protein P0 and ribosomal RNA 5.8S in axons stimulated with FGF22. Results showed a statistically significant decrease of approximately 15% in the number of ribosomal protein P0 (85.74%, p<0.0015) (Figure 26) and approximately 23% in the number

ribosomal RNA puncta (77.51%, $p<0.0173$) (Figure 27) per axon length. This result was also observed regarding puncta area per axon length of ribosomal protein P0 (55.61%, $p<0.0001$) (Figure 26C) and RNA 5.8S (85.16%, n.s.) (Figure 27C). Consistent with our previous observations in spinal motor neurons, FGF22 stimulus induced presynaptic differentiation, which correlates with a reduction of ribosomes from distal axons of hippocampal neurons as well.

In conclusion, axonal maturation is characterized by a reduction in the axonal levels of ribosomes. According to our results, FGF22 induces the formation of new synapses when applied specifically to axons. Moreover, FGF22 promotes axonal maturation, and as a consequence, the disappearance of ribosomes and ribosomal RNA. Importantly, our observations led us to conclude this neuronal feature is transversal to both the central nervous system (hippocampal neurons) and the peripheral nervous system (spinal motor neurons).

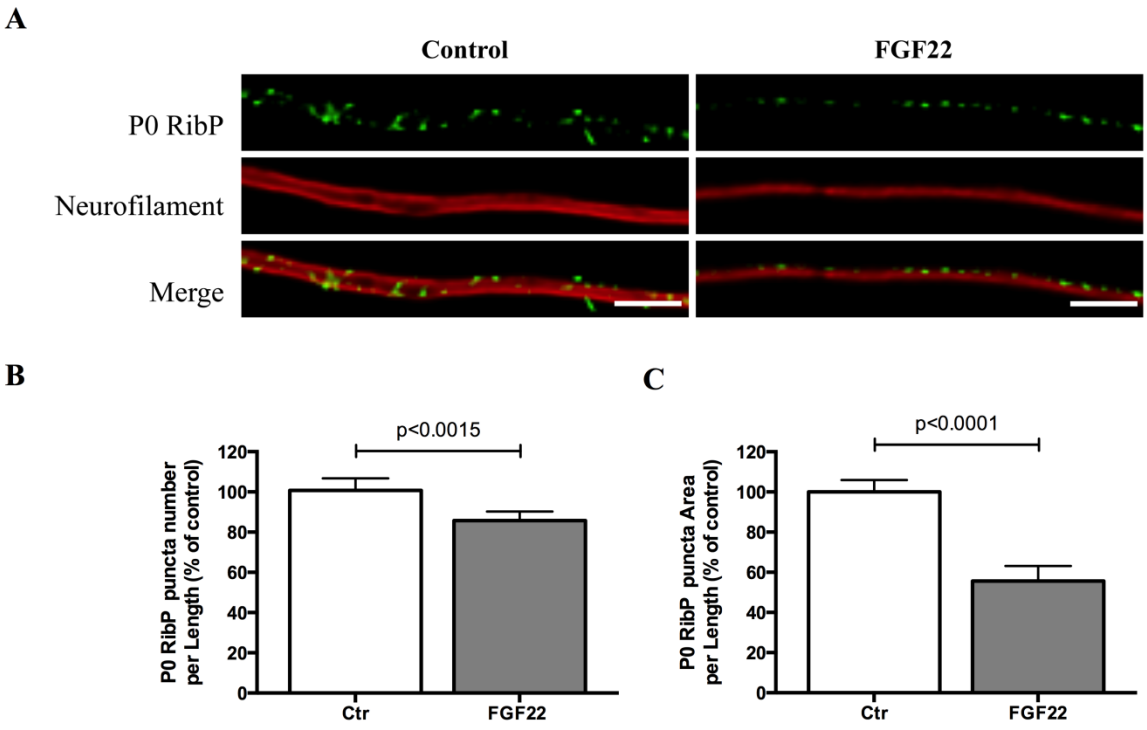


Figure 26: FGF22-induced presynaptic differentiation is accompanied by a ribosomal decrease. Primary cultures of rat hippocampal neurons were stimulated at DIV7 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal protein P0 (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5µm. (B) The number of ribosomal protein P0 puncta per axon length decreases approximately 15% in a statistically significant manner (85.74%, $p<0.0015$ by unpaired t-test). (C) Ribosomal protein P0 puncta area per axon length was also reduced (55.61%, $p<0.0001$ by unpaired t-test). For each independent experiment, results were normalized to the mean of control Bars represent the mean ± SEM of 3 independent experiments.

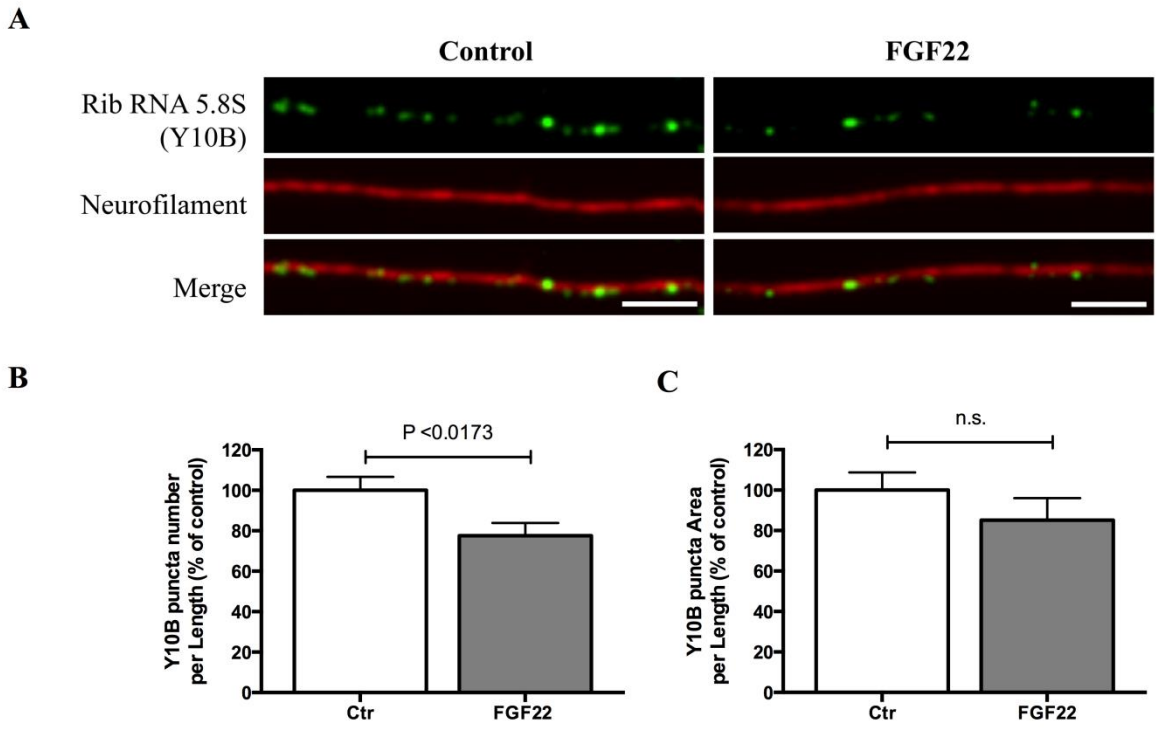


Figure 27: FGF22-induced presynaptic differentiation is accompanied by a ribosomal decrease. Primary cultures of rat hippocampal neurons were stimulated at DIV7 for 14 h at 37ºC in conditioned medium with either vehicle (control) or 2nM FGF22. Cultured neurons were then fixed in 4% PFA and stained for ribosomal RNA 5.8S (Y10B) (green) and neurofilament (red). Representative images of axonal segments reveal a clear FGF22-induced decrease in the number of ribosomes. Scale bar 2.5µm. (B) The number of ribosomal RNA 5.8S puncta per axon length decreases approximately 23% in a statistically significant manner (77.51%, $p<0.0173$ by unpaired t-test). (C) Ribosomal RNA 5.8S puncta area per axon length was also reduced (85.16%, n.s. by unpaired t-test). For each independent experiment, results were normalized to the mean of control Bars represent the mean ± SEM of 3 independent experiments.

Chapter 5 Closing Remarks

5.1 Conclusions

Previous reports have demonstrated the presence of ribosomal RNA and mRNA in developing neurons (Bassel et al., 1994) suggesting that local protein synthesis is required and crucial in early stages of the development. Firstly, we demonstrated that, in normal developmental conditions, ribosomal proteins S6 and P0 and ribosomal RNA 5.8S are present in developing axons of both spinal motor neurons (PNS) and hippocampal neurons (CNS), as observed by the distinct puncta distributed throughout the axonal shaft. Secondly, we established an effective system to induce the presynaptic differentiation of spinal motor neurons in culture. FGF22, when applied both globally and locally, led to the increase in the number of synapses, as indicated by the augment of synapsin and SV2 clusters. This increased number suggests clustering of neurotransmitter-containing vesicles in active zones where newly synapses begin to form. Lastly using FGF22, as a presynaptic differentiation stimulus, we investigated the loss of ribosomes in mature axons of spinal motor neurons. After FGF22 stimulation we observed approximately a 30% decrease in the puncta number of ribosomal proteins S6 and P0, and ribosomal RNA 5.8S along the axons.

The present work strengthens our hypothesis that synapse formation is the trigger for the removal of ribosomes from axons. Developing axons have the translational machinery to synthesize proteins, however when the growing axon encounters the correct post-synaptic partner, the formation a new synaptic contact triggers the disappearance of ribosomes causing adult axons to have reduced levels of ribosomes and mRNA. Importantly, our results do not exclude the possibility of adult axons having the ability to translate proteins, since we do not observe a complete absence of ribosomes in mature axons, suggesting that in adulthood axons might maintain at least

partially the capability of translating proteins. Interestingly, a recent report showed that the pool of mRNAs change between early developmental stages and the adult stage (Gumy et al., 2011). Developing axons have mRNAs related to protein synthesis, mitochondria and cytoskeleton, while mature axons present mRNAs related to inflammation and immune response that are not present in developing axons..

5.2 Future Perspectives

In this study we investigated how ribosomes are regulated during neuronal development. In the future, it would be important to corroborate our results with a different approach that could resemble with higher accuracy what happens *in vivo*. We propose to quantify ribosomal proteins S6 and P0 as well as ribosomal RNA 5.8S in a motor neuron-muscle co-culture. This model consists of embryonic motor neurons cultured on top of a skeletal muscular layer, allowing growing axons to contact and establish synapses with muscle cells. This system would allow us to study with a high degree of control the formation of the neuromuscular junction and how this impacts ribosomal decrease in axons. We are currently optimizing this co-cultures system, and we expect the loss of ribosomes when axons and muscle establish functional neuromuscular synapses.

Our results indicate that FGF22-induced presynaptic differentiation is the signal required for ribosomal disappearance from axons. However, we did not assess the mechanisms by which ribosomes are removed from axons upon maturation. It would be interesting to use the eGFP-L10a ribosomal reporter (Figure 15) in live cell imaging experiments to track ribosomes. Using this approach we could test if ribosomes are being retrogradely transported by motor proteins or if they are being locally degraded

by the ubiquitin proteasome system (UPS).

After axonal injury many mRNAs related to axonal targeting, synaptogenesis and synaptic function return to regenerating axons (Taylor et al., 2009). Interestingly, a previous study described the use of the existing ribosomes in axons to translate a virus-delivered RNA inducing axonal protein synthesis and growth of injured axons (Heintz et al., 2013). It would be interesting to manipulate the axonal content of mRNAs and ribosomes in cases of trauma and disease and determine if that would increase the translational capacity of the axons and as a consequence the rate of regeneration.

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Appendix I

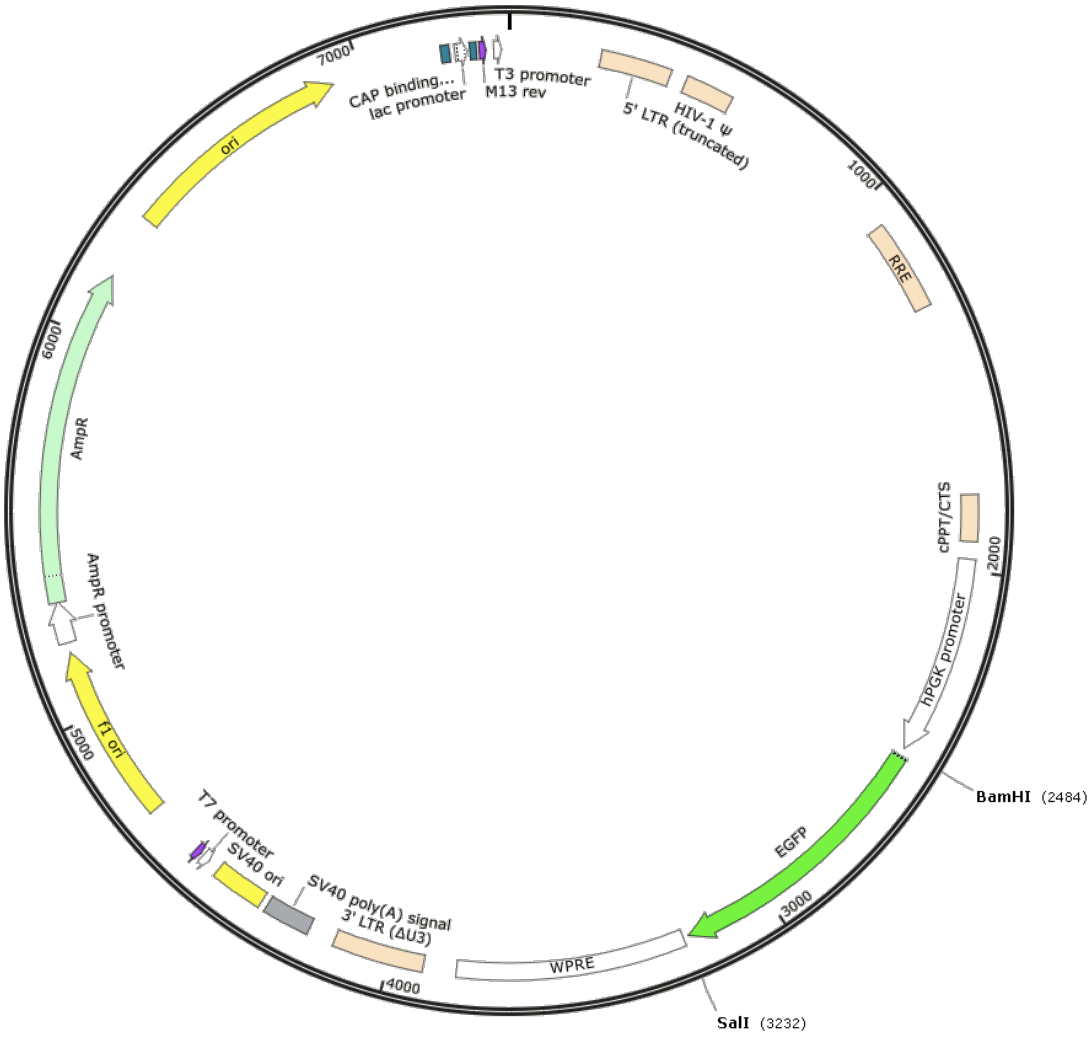

Figure 28: pRRLSIN.cPPt.PGK-GFP.WPRE cloning vector map. Representation of the linearized pRRL cloning vector used in this work. The recognition sites of *BamHI* and *SalI* restriction endonucleases are marked.

Appendix II

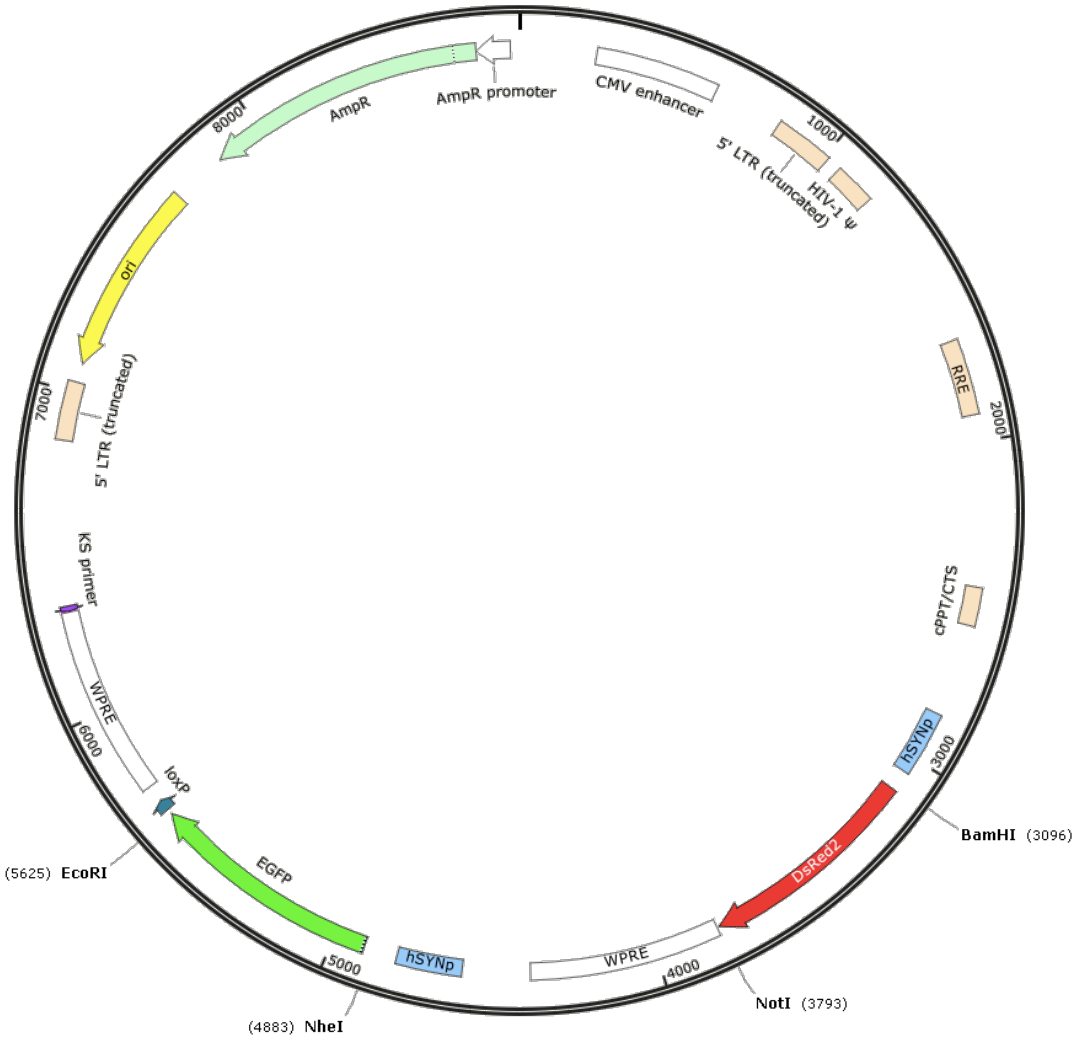

Figure 29: Syn-DsRed-Syn-GFP cloning vector map. Representation of the dual promoter vector used in this work. The recognition sites of *BamHI*, *NotI, Nhe I* and *EcoRI* restriction endonucleases are marked.