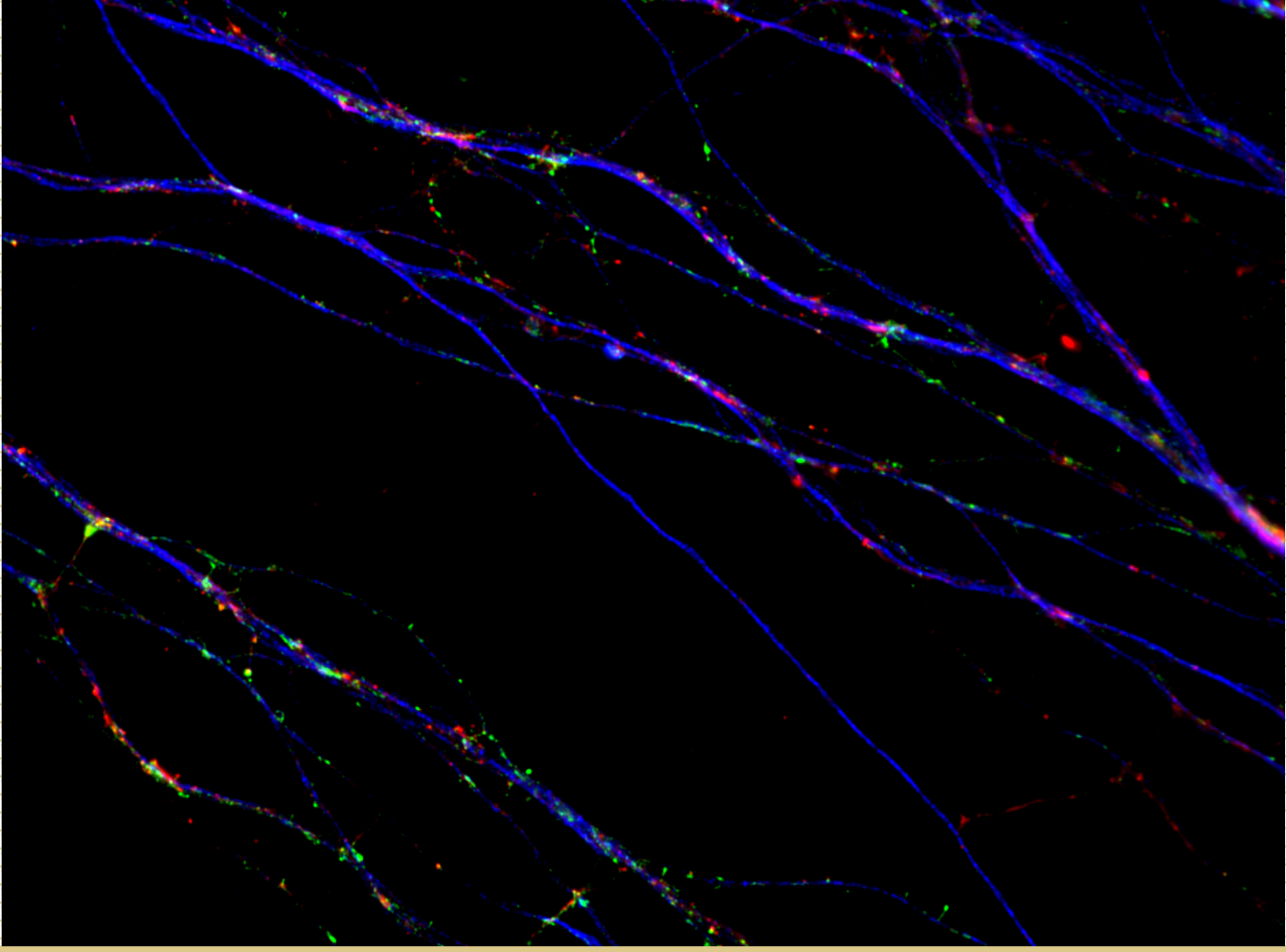

Joana Catarina Reis Pedro

# Intra-axonal translation of beta-actin mRNA underlies presynaptic differentiation

Tese de doutoramento em Biociências, especialização em Neurociências,
orientada pelo Doutor Ramiro Almeida e pela Professora Doutora Ana Luísa Monteiro de Carvalho,
apresentada ao Departamento Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.
Setembro de 2015

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Universidade de Coimbra

# Intra-axonal translation of *beta-actin* mRNA underlies presynaptic differentiation

# Tradução intra-axonal do mARN da *beta-actina* regula a diferenciação pré-sináptica

UNIVERSIDADE DE COIMBRA

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Tese apresentada ao Departamento de Ciências da Vida (DCV) e à Faculdade de Ciências e Tecnologia da Universidade de Coimbra (FCTUC), para prestação de provas de doutoramento em Biociências, especialização em Neurociências.

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In cover image, *in vitro* culture of axons stained for axonal marker, Neurofilament (blue); presynaptic vesicle 2 (green) and actin filaments (red).

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## Abbreviations:

| | |
|---|---|
| 3D | three dimensional |
| 4E-BPs | eIF4E-binding protein |
| AChE | Acetylcholine |
| AChR | Acetylcholine receptor |
| ADF | Actin depolymerizing factor |
| AIY | interneurons of *c.elegans* |
| Akt | Protein kinase B |
| ALCAM | Activated Leukocyte-cell adhesion molecule |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ATP | Adenosine triphosphatease |
| AZ | Active zone |
| BDNF | brain-derived neurotrophic factor |
| BF | Bright field |
| BHK-1 | Baby Hamster Kidney-1 cells |
| BSA | Bovine Serum Albumin |
| *C. elegans* | microorganism: *caenorhabditis elegans* |
| Ca2+ | Ion calcium |
| CaMKII | Ca2+/calmodulin-dependent protein kinase II |
| cAMP | cyclic Adenosine monophosphate |
| CAM | Cell adhesion molecule |
| CASK | Peripheral plasma membrane protein |
| CAZ | Cytomatrix active zone |
| cDNA | Complementary deoxyribonucleic acid |
| CEE | Chick embryo extracts |
| CEF | Chick embryo fibroblasts |
| CG | Ciliary Ganglia |
| CNS | Central Nervous System |
| CNTF | Ciliary neurotrophic factor |
| CPE | Cytoplasmic polyadenilation element |
| CPEBs | Cytoplasmic polyadenilation elemento-binding protein |
| CREB | cAMP responsive element-binding protein |
| Ctr | control |
| DCC | Deleted in colorectal receptor |
| DIGE | 2D-difference electrophoresis |
| DIV | Days *in vitro* |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DRGs | Dorsal Root Ganglia |
| EDTA | Ethylenediamine tetraacetic acid |
| EF1α | Elongation factor |
| EGFP | Enhancer Green Fluorescent Protein |
| eIF | Eukaryotic initiation factor |
| ELKS/CAST | Active zone-specific protein |

| | |
|---|---|
| EM | Electron Microscopy |
| EphA2 | Ephrin receptor |
| ERK | MAP Kinase |
| ES-MNs | Stem cells-derived motor neurons |
| F-actin | Filamentous actin |
| FBS | Fetal Bovine Serum |
| FGF | Fibroblast Growth Factor |
| FGFR | Fibroblast Growth Factor Receptor |
| FMRP | Fragile X Mental Retardation |
| G-actin | Monomeric actin |
| GDNF | Glia-Derived Neurotrophic Factor |
| GEF | Rac-guanoside nucleotide exchange factor |
| GFP | Green Fluorescent Protein |
| GS | Goat Serum |
| GTP | Guanosine triphosphate |
| HBSS | Hank's Balanced Salt Solution |
| hnRNP | Heterogeneous nuclear ribonucleoproteins |
| HS | Horse Serum |
| HSN | Hemaphrodite Sensory neurons |
| IGFII | Human insulin growth factor |
| IgSF-CAM | Immunoglobulin superfamily member-CAM |
| IRES | Internal Ribosomal entry site |
| JNK | c-Jun N-terminal kinase |
| KIF | Kinesin motor protein family |
| KO | Knock-out |
| KOR | Opioid receptor |
| LCM | Laser-capture microdissection |
| LRRTMs | Leucine-rich repeat transmembrane neuronal proteins |
| MAP | Microtubule-associated protein |
| MAPK | Mitogen-activated protein kinase |
| MBP | Maltose-binding protein |
| MEM | Minimum essential media |
| MKK | Map kinase kinase |
| MN | Motor Neuron |
| mRNAs | Messenger Ribonucleic Acid |
| RNP | Ribonucleo particles |
| MT | Microtubules |
| mTOR | mammalian target of rapamycin |
| N1E-115 cells | Neuroblastoma cell line |
| NES | Nuclear exprot signal |
| NF | Neurofilament |
| NGF | Neurotrophic Growth Factor |
| NLS | Nuclear localization signal |
| NMDA | N-methyl-D-aspartate receptor |
| NMJ | Neuromuscular junction |
| nt | nucleotide |

| | |
|---|---|
| NT3 | Neurotrophin 3 |
| O/N | Over Night |
| ORF | Open reading frame |
| PBS | Phosphate-buffered saline |
| PDL | Poly-D-Lysine |
| PDMS | Polydimethylsiloxane |
| Pen/Strep | Penicillin/Streptomycin |
| PI3K | Phosphoinositide 3-kinase |
| PNS | Peripheral Nervous System |
| PPVs | Membrane percursor vesicles |
| PSD95 | Postsynaptic density protein 95 |
| PSF | Point spread function |
| PTVs | Piccolo-Bassoon transport vesicles |
| PVDF | Polyvinylidene fluoride |
| RBPs | RNA-binding proteins |
| RGC | Retinal Ganglia cells |
| RhoA | Ras homolog protein - small GTPase protein |
| RNA | Ribonucleic acid |
| RNAi | Ribonucleic acid interference |
| ROI | Region of Interest |
| RP | Reserve pool |
| RPTPs | presynaptic type IIa receptor-type protein tyrosine phosphatases |
| RRMs | RNA recognition motif |
| rRNA | Ribosomal Ribonucleic acid |
| RRP | Ready releasable pool |
| RT | Room temperature |
| RT-PCR | Reverse Transcription polymerase chain reaction |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylmaide gel electrophoresis |
| siRNA | Small interference ribonucleic acid |
| SMA | Spinal Muscular Atrophy |
| SMN | Survival Motor Neuron |
| SN | Sensory Neuron |
| SNAP25 | Synaptosomal-associated protein 25 |
| SNARES | Soluble NSF attatchment receptor |
| STVs | Synaptic transporter vesicles |
| SV | Synaptic vesicle |
| SV2 | Synaptic vesicle 2 |
| TBS | Tris-buffered saline |
| tRNA | Transfer Ribonucleic acid |
| Tyr | Tyrosine aminoacid |
| UNC40/DCC | transmembranar receptor |
| UTR | Unstranslated region |
| UV | Ultra-violet |
| VAMP1/2 | Vesicle-associated membrane proteins 1/2 |

| | |
|---|---|
| VEGF | Vascular endothelial growth factor |
| VGAT | Vesicular GABA transporter |
| VGCCs | Voltage-gated calcium (Ca2+) channels |
| VGLUT | Vesicular glutamate transporter |
| ZBP1 | *Zipcode*-binding protein 1 |
| α-BTX | α-bungarotoxin |

## Resumo

As sinapses são a principal unidade do sistema nervoso. A sua formação resulta do contacto entre duas estruturas bem organizadas, os terminais pré- e pós-sinápticos. A formação do terminal pré-sináptico envolve a acumulação de material pré-sináptico no local de contacto, e depende de inúmeros eventos interrelacionados, os quais não são ainda bem conhecidos. Assim, é de extrema importância estudar os mecanismos intracelulares que coordenam a diferenciação das sinapses.

A tradução local do mARN é um mecanismo que regula processos neuronais. Os sinais extracelulares que chegam à célula activam a tradução de mARNs no axónio, originando novas proteínas neste compartimento celular. Consequentemente, estas proteínas participam em vários processos no desenvolvimento do axónio, favorecendo assim o comportamento autónomo do mesmo. A restrição na localização de mARNs é feita através de complexos macromoleculares que direccionam os transcritos desde o núcleo para as zonas distais do axónio. A coordenação temporal e espacial da tradução de mARNs confere aos axónios a extraordinária capacidade de se adaptar a circunstâncias distintas.

A síntese local de proteínas nos axónios tem sido intensamente estudada durante os processos de crescimento, condução e sobrevivência axonal. Neste trabalho observámos que a tradução intra-axonal de mARNs regula a formação do terminal pré-sináptico. Inicialmente, observámos que mecanismos de tradução são ativados em axónios isolados em resposta a moléculas sinápticas, como o FGF-22. Em seguida, verificámos que a acumulação de material pré-sináptico, induzida por FGF-22 ou esferas revestidas com polímeros de lisina, depende da tradução local de mARNs. Assim, este primeiro conjunto de resultados, demonstra que a diferenciação pré-sináptica depende da síntese local de proteínas em axónios.

Muitos estudos têm demonstrado que a organização do citoesqueleto regula a formação de novos terminais pré-sinápticos. Observámos que, após um estímulo sinaptogénico, a polimerização de actina aumenta nos axónios e que este processo é igualmente dependente de síntese local de proteínas. Isto mostra que os mecanismos de tradução de mARN estão envolvidos no desenvolvimento sináptico.

O axónio contém milhares de mARNs. O mARN da β-actina foi um dos primeiros transcritos encontrados em zonas distais e cones de crescimento axonais. Através de microscopia em células vivas, demonstrámos que um repórter de β-actina, é expresso em axónios após o estímulo sinaptogénico. Estes resultados indicam que o mARN da β-actina

é um importante regulador da formação do terminal pré-sináptico. Com o objetivo de compreender o papel da β-actina na formação sináptica usámos este mesmo repórter para bloquear o transporte anterógrado do mARN β-actina. A ausência do mARN da β-actina nos axónios levou à inibição da formação de novos locais pré-sinápticos e à polimerização de actina. Fizémos também bloqueio da tradução através de *siRNA* (do inglês *small interfering RNA*), após o que verificámos defeitos na formação do terminal pré-sináptico. Este conjunto de resultados evidencia que a localização e tradução do transcrito para a β-actina é um pré-requisito para a formação pré-sináptica.

Neste trabalho foram ainda desenvolvidas co-culturas entre neurónios e células musculares, com o intuito de estudar a importância da β-actina durante a formação da junção neuromuscular. Desta forma, verificámos que a junção neuromuscular foi negativamente afectada após o bloqueio do transporte do mRNA da β-actina para os axónios, o que demonstra a importância desta proteína durante o estabelecimento de novas sinapses. Em conclusão, o nosso estudo permitiu identificar um novo mecanismo regulatório da diferenciação pré-sináptica. Além disso, demonstrámos que o mARN da β-actina constitui um importante interveniente deste processo axonal, e identificámos um novo papel desta proteína durante o desenvolvimento do axónio.

**Palavras-chave:** tradução intra-axonal; pré-sinapse; mARN da β-actina;

## Abstract

Synapses are the principal unit of the nervous system. Synapse formation relies on the proper establishment of contacts between the pre- and the postsynaptic side. Presynaptic differentiation involves accumulation of presynaptic material at the contact site. Moreover, it depends on multiple interrelated steps, which are not entirely understood. As such, studying the intracellular mechanisms that govern synaptic differentiation is of utmost importance to better solve the glitches of the nervous system.

Axonal mRNA translation is a mechanism that allows local control of neuronal events. The autonomous axonal behavior is highly dependent on local protein synthesis since, depending on external cues axons respond by activating translation of specific mRNAs. Therefore, newly synthesized proteins participate in the molecular events required for axonal development. To be spatial restricted, mRNAs are transported from the nucleus to distal axons into molecular complexes that regulate the exact location of transcripts. The spatial and temporal regulation of mRNA translation confers axons an amazing capacity to adapt to distinct environments.

Local protein synthesis has been intensively studied during axon outgrowth, guidance and survival. In this work, we showed that local mRNA translation modulates presynaptic differentiation. We also observed that a synaptogenic factor, such as FGF-22, can activate translation mechanisms in isolated axons. In addition we observed that both FGF-22 and PDL-coated beads induce an increase of presynaptic clusters, a process dependent on local mRNA translation. Thus, axonal translation is required for presynaptic differentiation.

It is well-known that the rearrangement of the actin cytoskeleton supports the formation of newly presynaptic boutons. Interestingly, we observed that actin polymerization increases during FGF-22/bead-induced presynaptic differentiation. This event also required local protein synthesis, indicating that local mRNA mechanism underlies synaptic development.

Thousands of mRNAs are localized to axons. One of the first transcripts found in distal axons and growth cones was *β-actin* mRNA. Using live image approaches, we observed that a β-actin reporter is locally synthesized upon stimulation with FGF-22, indicating that the *β-actin* mRNA might act as a strong intervenient in presynaptic formation. To addressed this question we blocked the transport of endogenous *β-actin* mRNA to distal axons. This blockade prevented formation of new presynaptic clusters and

F-actin polymerization. In addition, we specifically down-regulated the axonal translation of *β-actin* mRNA using a siRNA approach. We observed defects in FGF-22-induced synaptic differentiation. Accordingly, we demonstrated that the localization and translation of *β-actin* mRNA is required to form presynaptic clusters.

In order to understand the role of local *β-actin* transcript during NMJ formation *in vitro*, we developed a novel neuron-muscle co-culture. Our results demonstrate that the mislocalization of the *β-actin* mRNA blocks the formation of neuromuscular junctions. Hence, axonal β-actin is required for the establishment of new synapses. In conclusion, this study determines a novel mechanism required for presynaptic differentiation. Also, we dissected a new role for *β-actin* transcript during axonal development, which supports the hypothesis that the *β-actin* mRNA plays an important role in modulating axonal differentiation.

**Keywords:** intra-axonal translation; presynaptic terminal; β-actin mRNA;

# CHAPTER I

## GENERAL INTRODUCTION

## 1. THE SYNAPSE

Synapses are "evolutionary old" structures that establish contact between a network of neurons. In synapses, a proper transmission of electrical and chemical signals between a signaling neuron (presynaptic terminal) and a receiving target cell (neuron or muscle - postsynaptic terminal) is central to define the neuronal circuitry.

During the early development of the nervous system, the bulk of synaptogenesis occurs upon an interaction between two opposed membranes from adjacent cells. In the central nervous system (CNS), the synapses are mainly formed by axon-dendrite contact[reviewed in 1] instead, in the peripheral nervous system (PNS), the synapses are formed for example by axon-muscle contact[reviewed in 2,3]. Neuromuscular junction (NMJ) is a synapse between a motor neuron and skeletal muscle fibers. This peripheral synapse is intensively studied in diverse organisms (mammalians, *C. elegans*, *Drosophila melanogaste*r and frog), because of its easily experimental accessed and its similarity with central synapses, NMJ has contributed to increase the knowledge about how synapses are formed.

The stabilization of intercellular contact and the assembly into functional synapses involves trans-synaptic connection, cytoskeleton rearrangements and recruitment of synaptic components forming the pre- and the postsynaptic terminal (see section 1.2). Briefly, in postsynaptic terminal occurs the recruitment of scaffold proteins of the postsynaptic density protein 95 (PSD95) family[4–6], N-methyl-D-aspartate receptor (NMDA)-type[7,8] and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors[8–11]. Other important proteins also present in postsynaptic membrane are calcium calmodulin-dependent protein kinase II (CaMKII)[12], which regulates trapping of local protein pools, and scaffold proteins as Homer1c and Shank2/3[4,13,14]. Two major components characterize the presynaptic terminal: the active zone (AZ) and neurotransmitter-containing presynaptic vesicles (SV) clusters[1,15]. The presynaptic terminal governs neuronal communication by calcium ($Ca^{2+}$)-triggered releasing neurotransmitters. At the synaptic cleft, neurotransmitters bind to specific postsynaptic receptors and initiate a signaling cascade that regulates neuronal processes. In axons, the precise presynaptic specializations are mandatory for proper synaptic signaling. Throughout this work we will explore in detail the multistep events occurring in the presynaptic site.

## 1.1 The presynaptic terminal

A typical presynaptic bouton is a highly organized structure composed by different pools of SVs and the active zone (AZ) (**Figure 1.1A**). The presynaptic components are well orchestrated in order to accomplish their purpose: chemical transmission.

Reconstructions of presynaptic organization, using a combination of freezing/freeze etching electron microscopy (EM)[16,17] and electron tomography techniques[18–22], revealed a clear picture of how SVs are organized at the presynaptic terminal (**Figure 1.1B-C**). Each SV (40-60nm in diameter) is connected to one or two neighboring vesicle by short filaments with 30-60nm in length[16,17,19]. These short filaments, termed connectors[20], link more than 80% of SVs. Therefore, connectors form vesicle clusters of various size (from few to >50 vesicles), building a dense meshwork of vesicles at the presynaptic terminal[19,20]. Interestingly, three dimensional (3D) architecture of the presynaptic matrix is consistent between species[19,20,22–24]. Synapsin I was identified as the molecular element of connectors[17], however, a recent study demonstrated that a triple knockout (3-KO) of the synapsin genes failed in complete disintegration of SVs clusters[19,25]. Nevertheless, synaptic transmission was altered in cultured neurons from the hippocampus of the 3-KO mice. In addition, synapsin I seems to have distinct presynaptic roles in excitatory and inhibitory synapses. Synapsin I is required to maintain vesicles at the reserve pool in excitatory synapses, however, in inhibitory synapses synapsin I regulates the size of readily releasable pool (RRP)[25]. Although the exact function of the connectors remains undisclosed, it is believed that they regulate SVs mobility[20].

Synaptic vesicles are linked with each other by connectors, furthermore docked vesicles are also tethered to the active zone membrane though other filaments of variable length, the tethers (**Figure 1.1C.2**)[16,17,20,25].

Synaptic vesicles are trafficking organelles that transport neurotransmitters. SVs membrane contains all molecular components necessary for the transport, docking, fusion and endocytosis. Numerous copies of exocytotic soluble NSF attatchment receptor (SNARES) (synaptobrevin/VAMP1/2, SNAP25, Syntaxin I), endocytosis-related proteins (Clathrin, AP1/2/3, dynamin), active zone proteins (Bassoon, piccolo, $Ca^{2+}$ channels), cytoskeleton proteins (actin, tubulin); synaptic vesicle proteins (Rab3, synapsin, Synaptotagmin 1, synaptophysin, synaptic vesicle 2 (SV2)), neurotransmitter transporters (VGlut1/2) and signaling proteins (CamKII) are found in the proteome of purified synaptic vesicles (**Figure 1.1D**)[26,27].

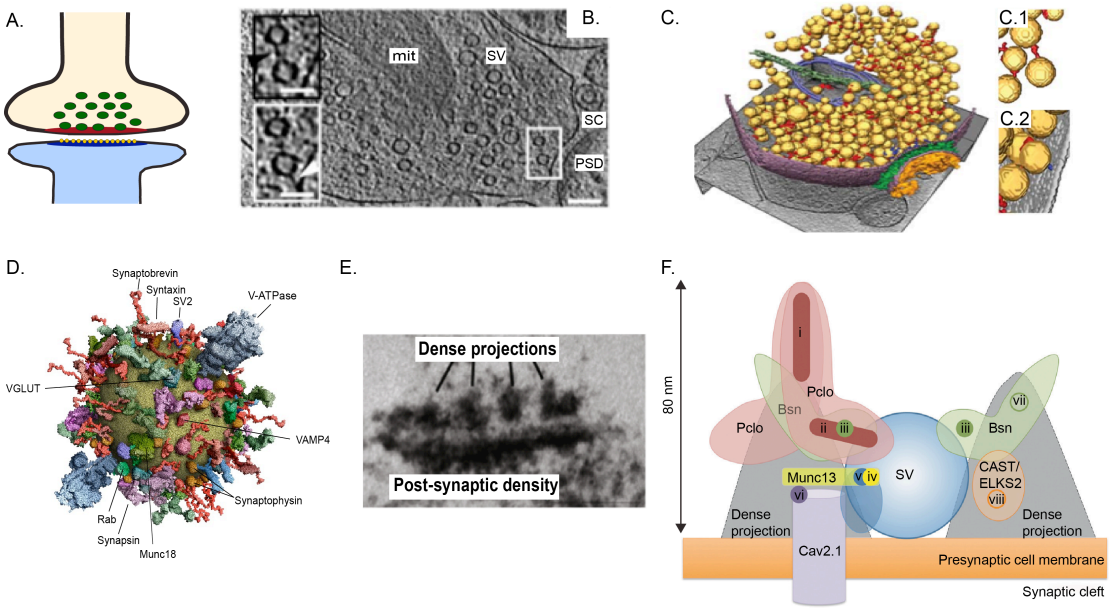



**Figure 1.1 - Components of presynaptic terminal: synaptic vesicles and the active zone.** (**A**) Schematic drawing of a synapse. Synaptic vesicles at the presynaptic terminal are represented as green circles and active zone as red lines. On the opposite side, is the postsynaptic terminal (blue) with membrane receptors (yellow) [Adapted from 28]. (**B-C**) Structural organization of synaptic vesicles within presynaptic terminal [from 29]. (B) Electron tomography of a synaptosome with synaptic vesicles (SV), mitochondrion (mit), synaptic cleft (SC) and postsynaptic density (PSD). Upper inset: Connectors linking two SVs (black arrow). Lower inset: Tethers linking one SV to the active zone (AZ) (white arrow). **(C)** 3D structure of synaptosome representing SVs (yellow), connectors (red), tethers (blue), mitochondrion (light blue), microtubule (dark green), synaptic cleft (green), plasma membrane (purple) and PSD (orange). (**C.1 and C.2**) High resolution of connectors (C.1) and thethers (C.2). (**D**) Molecular model of a synaptic vesicle [adapted from 27]. Some molecular componentes of SVs membrane are identified. (**E**) Electron micrograph of pre- and postsynaptic specializations [Adapted from 28]. (**F**) Structural organization of cytomatrix active zone (CAZ). Immunogold localization of epitopes against AZ proteins: N-terminal of Piccolo (i) extends the dense projection while the C-terminal (ii) directs towards SVs. C-terminal of Bassoon (iii) co-localizes with the C-terminal of piccolo. RIM and Mun13 connect with both C-terminal, Piccolo and Basson. CAST/ELK2 interacts with RIM, Munc13, Piccolo and Bassoon [from 30].

The active zone is seen under the microscope as a dark (electron-dense) area opposed to the postsynaptic membrane (**Figure 1.1E**). The active zone comprises a network of five evolutionarily conserved proteins, the Rab3-interactin molecule (RIM), Munc13, RIM-binding protein, Liprin-α/syd-2 (synapse defective 2) and ELKS/CAST/Bruchpilot (glutamic acid (E), leucine (L), lysine (K) and serine (S)-rich protein; CAZ-associated structural proteins)[31,32]. Additionally, Piccolo (Pclo) and Bassoon (Bsn), two large homologous proteins, are important organizers of the active zone. Proteins are located at a distance of 20-100 nm from the presynaptic membrane[33] and they form the core of the cytomatrix at the active zone (CAZ). Ultrastructure analysis of the active zone using immunogold-EM of fixed and stained samples shows a hexagonal grid of dense

projections intercalated with synaptic vesicles[34]. The RIM and Rab-3, which are essential for synaptic vesicle docking and priming[35–38], are close to the plasma membrane adjacent to SVs[34]. Aczonin/Piccolo, a multidomain scaffold molecule, displays its N-terminal spatial arranged parallel to the plasma membrane (20nm horizontal diameter). However, C-terminal of Aczonin/Piccolo that co-localizes with Bassoon, is horizontally directed to docked SVs (**Figure 1.1F**)[34].

The principal function of active zone is the establishment of the precisely opposition between the pre- and postsynaptic specializations via trans-synaptic connections, the recruitment of the voltage-gated $Ca^{2+}$ channels (VGCCs) to the presynaptic membrane and to support the docking and priming of synaptic vesicles and its subsequent exocytosis.

### 1.2 The presynaptic terminal differentiation

Presynaptic differentiation refers to changes experienced by the axon when it encounters an appropriate target to synapse. The presence of specific instructors at the pre- and postsynaptic membrane favors the contact and initiates a cascade of events that leads to recruitment of the SVs, the AZ proteins and organelles (mitochondrion) to the nascent synapse. Thus, axoplasma increases in volume and area giving rise to the presynaptic bouton.

#### Instructors for presynaptic terminal formation: Trans-synaptic components and soluble molecules

It is conceivable that synapse formation requires bidirectional transmission of synaptic signals across the synaptic cleft. An elegant coordination of trans-synaptic proteins in the pre- and postsynaptic membranes bridges that cleft. Throughout this subsection we will only focus on the trans-interaction that is relevant for presynaptic differentiation.

Trans-synaptic proteins make part of a large family of cell-adhesion molecules (CAMs) that are typically transmembrane receptors whose extracellular domains interact with other CAMs located at the opposite cell membrane. The heterophilic adhesion through neuroligins (postsynaptic) and neurexins (presynaptic) were the first pair to be identified as synaptic organizers[39,40]. The deletion of either neurexin or neuroligin negatively affects neurotransmitter release therefore impairing neuronal behavior[41–46]. Besides, the leucine-rich repeat transmembrane neuronal proteins (LRRTMs)[47–49] and the

complex of Cbln1-GluRδ2[50,51] were identified as others trans-synaptic partners for neurexin.

The mechanism by which neuroligin, LRRTMS and Cbln1-GluRδ2 induce presynaptic differentiation is through the accumulation of neurexin on the axon surface[40,47,49,51]. When neurexin aggregates at the presynaptic membrane it directly or indirectly interacts with diverse proteins responsible for the recruitment of presynaptic material or for the reorganization of active zone[52–55]. For example, neurexin interacts with peripheral plasma membrane protein CASK[53], which in turn, binds to protein 4.1 that regulates actin cytoskeleton[52]. Moreover, neurexin also binds to Mint1, which interacts with CASK and Munc18[54].

Studies using triple-knockout mice for alpha-neurexin revealed that absence of neurexin affects the coupling of functional $Ca^{2+}$ channels at the presynaptic membrane impairing neurotransmission[45,46]. In *Drosophila* NMJs, neurexin loss of function leads to a decrease in the number of synaptic boutons, it also changes active zone morphology which affects synaptic transmission[56,57]. Recently, a study using cultured cortical neurons demonstrated that accumulation of neurexin at presynaptic membrane creates spots of SVs at the synaptic site[58]. The number of SVs that forms the spots varies with concentration of neurexin at the presynaptic membrane. Indeed, besides adhesion functions, neurexin also defines the site where presynaptic material will assemble[58].

It is worth stating that the presynaptogenic effect of neurexin is intrinsically dependent on binding with its postsynaptic partner and other presynaptic proteins. In *Drosophila* NMJs, it was observed that SYD-1, which is a presynaptic protein from the active zone, binds to and immobilizes neurexin1 contributing to its clustering at the presynaptic terminal. In turn, settlement of nascent SYD-1 clusters is dependent on neuroligin-1. Syd-1, neurexin1 and neuroligin1 mutants have similar defects in neuromuscular junction formation[59]. This study indicates that a strictly cooperation between pre- and postsynaptic proteins are crucial for synaptic assembly.

The presynaptic type IIa receptor-type protein tyrosine phosphatases (RPTPs) are other hubs for synapse organization[60]. Studies in *Drosophila* and *C. elegans* demonstrated that RPTPs signaling regulates a downstream effector, the liprin-α[61,62], which in turn interacts with active zone proteins, the ELKS/Rab6-interactin/CAST [63] or RIM [28] to mediate presynaptic differentiation. Indeed, RPTPs act as a molecular link between extracellular signals and active zone assembly having a role in synapse formation[61].

Secreted molecules derived from the target postsynaptic site or neighboring astrocytes also instruct presynaptic differentiation. Glia-derived neurotrophic factor (GDNF) is a growth factor that promotes survival and neurite outgrowth[64]. In cultured hippocampal neurons binding between GNDF and its receptor, the GDFRα1, induces trans-homophilic interactions together with augment of clusters of synaptic vesicles (synaptophysin and synapsin I) and neurotransmitter transporters (VGlut1 and VGAT)[65]. Presynaptic differentiation in motor neurons is also regulated by muscle-released GDNF. Motor neurons express the receptor for GDNF and a conditional ablation of the receptor compromises the maturation of nerve terminals[66]. Reversely, muscle overexpression of this growth factor using Myo-Gdnf transgenic mice or Gdnf injection induces multiple muscle innervations[67,68]. Fibroblast growth factor-22 (FGF-22) and its relatives (FGF-7 and FGF-10) were isolated in a biochemical screen for proteins that induce vesicle clustering in motoneurons[69]. Cerebellar granule cells express FGF-22 that binds to FGF receptor 2 (FGFR2), which is expressed by pontine and vestibular neurons. FGF-22 induces glutamatergic synapses while FGF-7 is involved in GABAergic synaptogenesis[70], however, both FGF-22 and FGF-7 selectively induce synaptic vesicle clustering[69]. FGF-2 that belongs to the same family was also found to also induce presynaptic clustering[71].

**Recruitment of presynaptic vesicles to synaptic contacts**

At synaptic contact, the assembly of presynaptic terminal requires the recruitment of specialized active zones and SVs proteins. Soma-derived presynaptic material is assembled in the trans-Golgi compartment[72,73] into two distinct vesicular organelles[74–76]: the synaptic vesicle proteins transport vesicles (STVs), which incorporate the SV-associated proteins (synaptophysin, SV2, VGLUT1, Rab3a, synaptotagmin, synapsin, amphiphysin and VAMP2); and the Piccolo-Bassoon transport vesicles (PTVs), which are dense-core vesicles with approximately 80 nm in size containing active zone proteins, such as Piccolo, Bassoon, N-cadherin, syntaxin, synaptosomal-associated protein 25kDa (SNAP25), RIM, ELKS2/CAST, Munc13 and Munc18. The formation of PTVs is a multistep event and depends on the trans-Golgi network. Bassoon and Piccolo co-localizes with the Golgi apparatus at the hippocampal neurons and Bassoon undergoes trans-Golgi-dependent posttranslational modifications before its incorporation in the PTVs[72]. Interestingly, the biogenesis of active zone precursors is not exclusive from the trans-Golgi. It seems that during its travel from Golgi towards axon the PTVs suffer important changes in its composition. First, Piccolo/Basson/ELKS2/CAST forms one kind of PTVs,

while the Munc-13 was found in a distinct Golgi-derived transport vesicle. Later, during axonal transport both vesicles merge in one mature PTV. Additionally, RIMα, which is not associated to Golgi membrane, is though to also be added to PTVs throughout axonal transport[73].

Once assembled at the cell body, STVs and PTVs tethers to microtubule-associated motor cargos hence they are rapidly transported throughout the axon in both the anterograde and retrograde direction. Microtubules (MT) are disposed with their plus-end distally from soma and their minus-end directionally to the soma. Kinesin superfamily motors (KIF) are molecular cargos responsible for the anterograde transport (towards the MT plus-end) of STVs and PTVs[77–81]. However, PTVs and STVS selectively bind to different cargos, the former only binds to Kinesin1/KIF5[80,81] while the STVs bind to Kinesin1/KIF5 and also Kinesin3/KIF1A or KIF1B[77–79]. The anterograde transport of both STVs and PTVs is achieved by dynein, a MT-minus-end-directed motor protein[82,83].

The recruitment of STVs and PTVs occurs within minutes[9,75,84,85]. It seems that PTVs move faster, at a rate of 0.1-0.3 μm/sec than STVs that travel at a rate of 0.1-1.1 μm/sec[76,81]. However the question about which vesicles arrive first at the nascent synapse still in debate. The dominating idea is that PTVs arrive first than STVs[84]. However, a recent study performed in zebrafish demonstrated that STVs arrives first than PTVs [84]. Other study support another model where the recruitment of STVs and PTVs occurs simultaneously[86]. In this study was demonstrated that STVs and PTVs move and pause together at the same sites within the axon [86]. Given those different observations, it is suggested that STVs and PTVs might be recruited to nascent synapses without a specific order.

### 1.3 Neurotransmitter release

Neurotransmitter release occurs within milliseconds[87] and requires the fusion of synaptic vesicles with the plasma membrane. Synaptic vesicle exocytosis is restricted to the active zone, where a pool of vesicles, the RRP, is gathered ready to be released. The active zone lies at the interface between the presynaptic terminal and the synaptic cleft where diverse class of proteins are allocated forming the CAZ. Active zone proteins recruit VGCCs to the presynaptic membrane and support the dock and prime of SVs for exocytosis. The arrival of an action potential induces $Ca^{2+}$ influx, subsequently a small percentage of vesicles from the RRP fuse with the membrane and the neurotransmitters are release by $Ca^{2+}$-triggered synaptic vesicle exocytosis into the synaptic cleft. In order to

sustain continuous synaptic transmission, the RRP needs to be replenished with new SVs. Therefore, those new SVs arise from the reserve pool (RP) that is 150-200 nm away from the presynaptic membrane and it is devoted to refill the RRP upon its depletion. In turn, the RP needs to be repopulated with SVs to avoid its own exhaustion. This is accomplished by endocytosis of SVs that previously underwent exocytosis or some recycling vesicles are also shared between adjacent boutons[18,88,89]. Endocytosis takes place in the periactive zone, an axoplasma region surrounding active zone and enriched in components for endocytic pathway. Then, vesicles are converted to functional SVs and will replenish the reserve pool of vesicles to undergo a new exocytosis-endocytosis cycle (**Figure 1.2**). The SV recycling involves a retrieval of SV components from the plasma membrane into presynaptic space. This is achieved by a clathrin-mediated mechanism[90–92] or by a mechanism designated "kiss and run"[93–95].

Diverse proteins interact to regulate the steps behind neurotransmitter release. The fusion of presynaptic vesicle with the presynaptic membrane occurs through the trans-interaction of SNARES: the vesicular SNARE protein synaptobrevin/VAMP forms a SNARE/SM complex with plasma membrane SNARES syntaxin-1 and SNAP25. The formation of this complex is important to open the fusion pore thus regulating neurotransmitter release. Synaptotagmins is a $Ca^{2+}$ sensor that binds to the SNARE complex. An AZ protein complex constituted by RIM (Rab3-interactin molecule), RIM-BP and Munc13 acts in order to position the synaptic vesicle in close proximity with VGCCs. RIM binds to vesicular Rab proteins, such as small Rab3 and Rab27 GTP-binding proteins, to mediate docking of the vesicles. Concomitantly, RIM binds to the priming factor Munc13 to prime the SNARE/SM protein fusion machinery. An efficient neurotransmitter release also depends on the interaction between Munc13 and other active zone components such as Piccolo, Bassoon and CASTs [96]. Furthermore, RIM binds directly or indirectly to $Ca^{2+}$ channels promoting its recruitment to the priming and docking vesicle sites. The localization of the $Ca^{2+}$ channels in the vicinity of vesicles is of extreme importance to the rapidly $Ca^{2+}$-triggered exocytosis. Moreover, the number of docked vesicles and its probability of release define the efficacy of neurotransmitter release. Although the steps between exocytosis and endocytosis remains to be completely understood, it is clear that the trafficking cycle of vesicles is a requisite for a proper synaptic transmission [reviewed in 91,and 92].

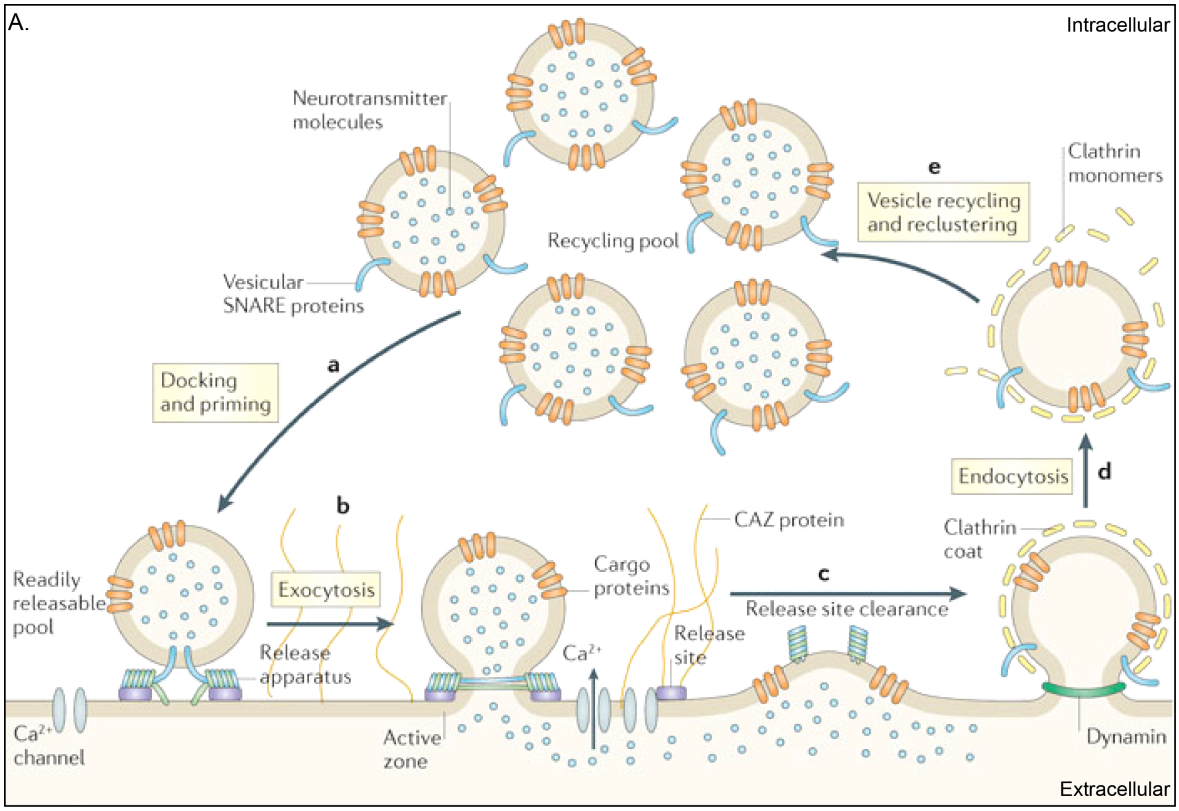



**Figure 1.2 – Synaptic vesicle cycle: priming, docking (a), exocytosis (b) and endocytosis (c-e).** The priming and docking of neurotransmitter-containing vesicles near the $Ca^{2+}$ channels, form the readly releasable pool of vesicles (RRP) **(a)**. Following $Ca^{2+}$ influx the RRP undergoes exocytosis **(b)** releasing its neurotransmitter to the synaptic cleft (extracellular). An intermediate step called *release site clearance* occurs between exocytosis and endocytosis **(c)**. The *release site clearance* is a mistery step but is thought to preserve the release sites for subsequente exocytic reactions. Clathrin-mediated endocytosis occurs at the periactive zone **(e)**. Retrieved vesicles are uncoated from clathrin and refilled with neurotransmitters **(d)**, then synaptic vesicles return to the reserve recycling pool or reserve pool (RP) where they undergo a new cycle of exocytosis (e) [from 97].

## 1.4. The F-actin networks in presynaptic differentiation

The building of a synapse is a highly complex event that involves a variety of cellular components and it requires coordination between diverse cellular processes. Actin cytoskeleton is a key piece behind all neurodevelopmental processes, including synaptogenesis. In neurons, the disruption of actin filaments (F-actin) by pharmacologic treatment results in the dispersal of synaptic vesicles[98]. This phenomenon occurs in early development of synaptogenesis as F-actin disruption in mature neurons is not effective for synaptic disintegration[98,99].

Actin is a major cytoskeleton element of the presynaptic terminal, forming an actin network close to the active zone[16,17]. The F-actin network takes diverse roles during presynaptic specializations. Upon cell-cell contact, CAMs instruct intracellular protein-

protein interaction that governs actin cytoskeleton remodeling, providing a scaffold for presynaptic components. In interneurons (AIY) of the nematode *C. elegans*, netrin signals through the transmembranar receptor UNC40/DCC to instruct synaptic vesicle clustering[100,101]. The UNC40/DCC receptor interacts with a Rac-guanoside nucleotide exchange factor (GEF), CED5, which, in turn, signals through CED10/Rac1 (a Rac GTPase) and Mig10 (cytoplasm adaptor molecule). Mig10 is present in presynaptic sites and co-localizes with F-actin filaments. A knock-down of Mig10 reduces actin concentrations at synapses, resulting in a decrease of the amount of Rab3-synaptic marker. However, no change was observed in the levels of the active zone proteins. Results indicate that Netrin-1 promotes presynaptic vesicle assembly, but not active zone remodeling, through CED10/Rac1/Mig10-mediated F-actin polymerization[101] (**Figure 1.3**). In cultured rat hippocampal neurons, the cadherin/β-catenin adhesion complex plays important role in clustering SVs at synapses by regulation of F-actin polymerization mediating activation of other GEF, the β-Pix (**Figure 1.3**)[102].

Active zone is also regulated by F-actin remodeling. Indeed, in *C. elegans* motor neurons (hermaphrodite specific neurons - HSN) it was identified one signaling pathway responsible for regulating F-actin-mediated active zone assembly. So, it was demonstrated that the adhesion molecule SYG-1 interacts with an intermediate molecule, the NAB1 to recruit and organize F-actin at presynaptic terminal. SYG1/NAB1/F-actin interaction recruits active zone organizers, the SYD-1 and the SYD-2, which are crucial for presynaptic development (**Figure 1.3**)[103]. Together, these studies reveal that organization of the presynaptic components is mediated by F-actin network. The referred studies also suggest that actin polymerization acts upstream from presynaptic terminal formation.

It has been demonstrated that active zone proteins, such as Piccolo, regulates the dynamic assembly of the presynaptic F-actin[15,104]. Piccolo is associated to actin polymerizing proteins, Damm1 (formin) and profilin, directing activity-dependent F-actin assembly at the presynaptic terminal[104]. Interestingly, F-actin assembly mediated by Piccolo increases neurotransmitter release (**Figure 1.3**)[105]. Actually, it had been previously suggested that F-actin modulates synapsin Ia dispersion and vesicle exocytosis [106]. Controversly, a recent study demonstrated that a double mutant for ADF/cofilin, an actin-depolymerizing factor, has defects in excitatory synapses due to impaired glutamate release[107]. It was observed that in the absence of ADF/cofilin the dynamics of actin is altered, resulting in defects in the organization, mobilization and exocytosis of synaptic vesicles[107]. Although the evidences show an important role of presynaptic actin in vesicle

exocytosis, the controversy over its mechanism of action remains. It is hypothesized the existence of a "dynamic actin cycle", which claims that both depolymerization and polymerization are synchronized in order to fulfill neurotransmitter release (**Figure 1.3**)[107].

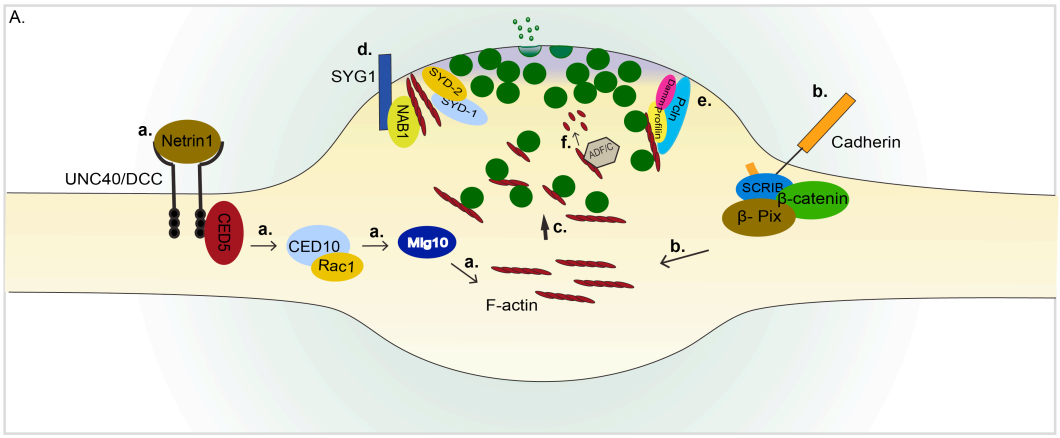



**Figure 1.3 – Actin in action in the presynaptic terminal. (a-d) Actin as a molecular scaffold during presynaptic differentiation.** (a and b) F-actin mediates presynaptic assembly. In *C. elegans* **(a)** and hippocampal neuron **(b)** trans-synaptic molecules activate and regulate rac-guanoside nucleotide exchange factors (CED5, CED10/Rac1 and β-Pix) regulating F-actin polymerization. **(c)** Consequently, F-actin is involved in presynaptic vesicle assembly by a non-specific mechanism. **(d)** F-actin mediates active zone assembly. In *C. elegans*, NAB1 interacts with the adhesion molecules SYG1 to recruit F-actin into presynaptic terminal. Indirectly binding between F-actin and SYG1 recruits molecular organizers of the active zone, SYD1 and SYD2. **(e-f)** Actin dynamics regulates synaptic vesicle mobility and exocytosis. (e) Actin-binding proteins Damm1 (pink) and profilin (yellow) associates with Piccolo to assembly F-actin at the presynaptic terminal. Here, F-actin controls the motility of synaptic vesicles from the RP to the RRP and regulates vesicle exocytosis. (f) Depolymerization of F-actin by ADF/cofilin is required to regulate synaptic mobility and exocytosis.

Another fragmentary and controversy evidences are those that claim for a role of actin in vesicle pool organization. Because actin was observed to co-localized with synapsin Ia connectors[17,108] it has been speculated that actin acts as a molecular scaffold for the reserve vesicle pool. Additionally, actin is indicated to be the transfer of vesicles between RP and RRP[109]. However, studies performed in mice central synapses and frog NMJ demonstrated opposite effects on vesicle mobility when axons were treated with actin depolymerizing agent. In cultured hippocampal neurons, F-actin disruption results in an augment of mobility of vesicles[110] while in frog NMJ, F-actin disruption did not change synaptic vesicle mobility[111].

Actin co-localizes with synaptic vesicles at the endocytosis regions[112,113] indicating a possible role of actin in endocytic pathway.

## 2. LOCAL mRNA TRANSLATION

Neurons are highly compartmentalized cells with specific functions in restricted subcellular domains, axons and dendrites. These local compartments have an autonomous competence for information processing.

The concept that mRNAs localized in discrete subcellular domains undergoes cell body-independent translation was a debated topic during decades. This discussion arose after Steward and Levy had identified, by electron microscopy, the presence of polyribosomes beneath the base of dendritic spines[114] and later *in situ* hybridization revealed the localization of mRNAs coding for MAP2[115] and the alpha subunit of CAMKIIα[116]. Metabolic labeling provided evidences that mRNAs were transported from cell bodies to neuritis[117] and synthetic activity occurs in dendrites isolated from cell bodies[118]. Indeed, these evidences showed that mRNAs were not exclusive from cell body, dismissing the central dogma that proteins were only synthesized in soma and transported to specific regions of the neuron. In dendrites, subsequent studies provide the definitive proof that local protein synthesis is a key mechanism for neuronal processes. These studies demonstrated that translation of specific mRNAs, localized at the synapse[119], were directly linked to synaptic plasticity[120–123].

In axons, although ribosomes were identified in the 70s by ultra structural studies[124], its hostile localization, close to the plasma membrane[125] and at the surface of the receptors[126], or even the lack of effective imaging techniques delayed the belief that mRNAs were also present in axons.

### 2.1 Local protein synthesis in axons

One of the first evidences for local protein synthesis in axons emerged in 1960 decade[127–130]. A study performed in cat cholinergic axons demonstrated that after irreversible inactivation of the acetylcholinesterase (AChE) in axons, the enzymatic activity was recovered in two hours [128]. The rapid recovery suggested an eventually local synthesis of AChE in axons. In order to clarify previously observations, the authors inactivated AChE in axotomized axons therefore they observed an increase in the levels of AChE in the distal portion of the axons. That increment in protein levels was abolished in the presence of protein synthesis inhibitor, puromycin, indicating the local synthesis of AChE[130]. Later, the incorporation of a radiolabelled aminoacid in axons proved the presence of axonal protein synthesis[129]. Subsequent studies used the metabolic labeling

technique to evidence that local protein synthesis occurs in the axoplasma of the squid giant axon and the Goldfish Mauther neurons[131–136].

Since that period, attention was directed to identify species of mRNAs in axons. In the squid giant axon, it was identified the presence of mRNAs coding for β-actin and β-tubulin[137], for kinesin[138] and squid enolase[139] whereas in Mauther axons it was mainly identified ribosomal RNAs[140]. In the CNS the first mRNAs identified were components of cytoskeleton, tau[141], β-actin[142] and β-tubulin[143]. Despite strong evidences for axonal protein synthesis, the skepticism relating to axonal RNA mechanism persisted for long time due to the lack of proofs for a functional role of axonal mRNA translation.

The acceptance of axonal protein synthesis emerged at the beginning of the XXI century when C. Holt and co-workers demonstrated that cue-induced directional steering was abolished by protein synthesis inhibitors[144]. Additionally, studies performed in adult neurons showed that axon regeneration occurring after an injury requires newly synthesized proteins[145,146]. To date several studies confirmed that axonal mRNA translation underlies directional turning, gradient sensing, growth cone adaptation[147–151], regeneration and maintenance[146,152–155]. Nowadays, it is well established that RNA translation provides an exceptionally adaptable mechanism during axonal development.

**Local protein synthesis for axon outgrowth/elongation**

Throughout the development of the nervous system, a neuron polarizes[156] forming a single and long axon that navigates in a path until it reaches a specific target. In an initial stage, the developing axon elongates and a highly motile structure, the growth cone, is formed at the tip of the axon. The leading growth cone detects instructive cues-induced signals[157] and reacts by profound morphological changes[158] that guides the axon in a specific direction or stops it for presynaptic terminal differentiation. Stimulus-induced changes in growing axon are often a result of regulated translation of localized mRNAs[144,149–151]. Indeed, synthesis of new proteins guarantees axonal autonomy and the capacity of the growth cone for pathfinding.

Ramón & Cajal defined the growth cone as a dynamic structure with ameboid-like movements that confer the axon the ability to go forward and back and overcome obstacles. The growth cone is enriched in cytoskeleton elements, F-actin and microtubules, that have a typical organization and its coordinately interaction guarantee growth cone motility[159]. Three distinct regions are identified in the growth cone: the periphery of the growth cone (P-region) contains long bundled actin filaments (F-actin bundles) and a

mesh-like branched F-actin networks forming filopodia and lamellipodia structures. The former functions for environmental sensing while the later is important for growth cone movement. The central domain (C-region) includes microtubules bundles that come from the axon shaft to support growth cone movement. The majority of microtubules stop in this region but a small number enter in the P-region and interact with actin filopodia to control motility. Then, the transition domain (or T-region) is the region at the interface between the P and C-domains. It is constituted by actomyosin contractile structures (actin arcs) positioned perpendicular to F-actin bundles likewise forming hemi-circumferential rings within the T-region. The actomyosin regulates the rearward flow of actin and regulates onward movements of microtubules (**Figure 1.4A**)[160].

Actin filaments and microtubules are in a constant rate of flux. Actin dynamic is regulated by actin-binding proteins that participate in a diverse set of actions: filament nucleation, capping, severing and crosslinking (**Figure 1.4B**)[158].

In the T-region, F-actin is severed and depolymerized by ADF/cofilin, an actin-associated protein that regulates actin dynamics. In the classic model, ADF/cofilin increases the rate of ADP-actin dissociation from the pointed end of actin filaments thus released actin monomers are transported to the leading edge for further actin polymerization. Simultaneously, ADF/cofilin break the F-actin in small actin filaments creating new barbed ends (or new nucleation sites) available for further polymerization so promoting new filaments assembly and membrane protrusions. Indeed, ADF/cofilin activation (increase dephosphorylation state) mediates Netrin1-induced growth cone attraction and neurotrophic growth factor (NGF)-induced growth cone protrusion by increasing free actin barbed ends and F-actin levels[161]. Controversly, activation of ADF/cofilin seems to facilitate Slit2-induced collapse[149]. The precise function of ADF/cofilin in neuronal growth cone is highly discussed however the main accepted idea is that an optimal ratio between phosphorylation and non-phosphorylation state of ADF/cofilin is critical to generate the dynamic turnover of actin cytoskeleton[159,162–164]. ADF/cofilin activity is regulated by several signaling pathways[165] and adaptors proteins, as 14-3-3 proteins[166]. Recently it was demonstrated that the transcript for 14-3-3/14-3-3ς isoform is present in the *Xenopus* retinal growth cones[163,167] and the localized protein regulates retinotectal projections by modulating ADF/cofilin activity[163]. Interestingly, inhibition of 14-3-3/14-3-3ς protein increases ADF/cofilin activity, decreases the axon elongation rate *in vivo* and *in vitro* and sensitizes the Slit2-induced collapse[163]. The local synthesis of 14-3-3/14-3-3ς protein induced by attractants or repellents was not addressed

but it is of extreme importance o understand the contribution of cue-induced mRNA translation in ADF/cofilin-regulated actin dynamics.

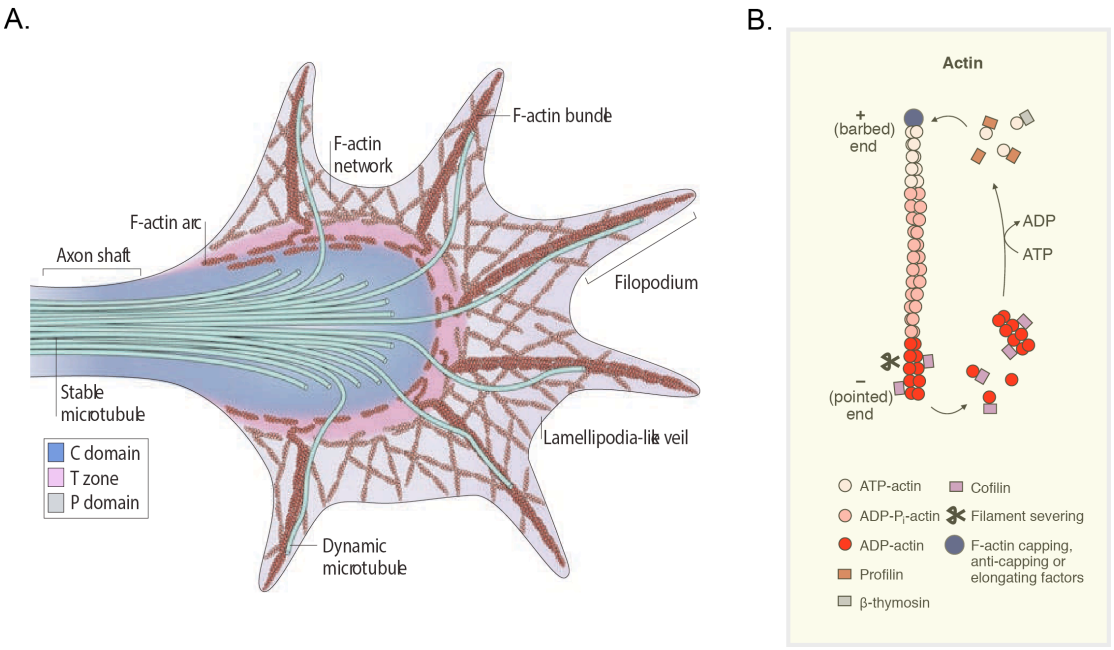



**Figure 1.4 – Growth cone structure and actin dynamics. (A)** Organization of the growth cone [obtained from 160]. For more details see text in subsection 2.1 "local protein synthesis for axon outgrowth/elongation". **(B)** Actin steady-state. An imbalance in actin steady-state mediates remodeling of cytoskeleton critical for growth cone motility. In the actin plus-end (or barbed-end) the binding of ATP-actin monomers is mediated by polymerizing factors (profilin). Capping proteins and sequestering proteins (β-thymosin) regulates the rate of actin polymerization. In the negative-end (or pointed-end), the hydrolysis of ATP detaches actin monomers from the filaments leading to the dissociation of F-actin. The depolymerization is mediated by depolymerizing agents such as cofilin (or ADF/cofilin). To complete the cycle, ADP-actin monomers are converted in ATP-actin monomers and recruited to maintain actin filaments[adapted from 159].

Microtubules dynamic is also regulated by microtubule-associated proteins (MAPs)[159], such as MAP1b which in its activated form stabilizes microtubules[168] and regulates microtubule-actin dynamics[169]. The MAP1b is present along the axon shaft and in growth cones[170] contributing for axon guidance and outgrowth[171,172]. Activation of MAP1b is regulated by a MAP kinase kinase (MKK7) for c-Jun N-terminal kinase (JNK). In neuronal differentiated neuroblastoma N1E-115 cells, the mRNA coding for MKK7 was localized in neuritis and growth cones[173]. The role of the newly synthesized protein for axon outgrowth was not addressed although, local translation of a Kaeda-MKK7 3'UTR reporter was observed[173]. This is a pioneer study that demonstrates a possible role of local mRNA translation in microtubules stability and axon outgrowth. Nevertheless, further evidences in primary neuronal cells are needed.

When the growth cone detects a substrate on the adjacent cell (or matrix), adhesion molecules interact to link the cell to the external environment thus forming trans-interactions (see section 1.2). At intracellular levels (axonal compartment) CAMs form a complex called molecular "clutch", which consists in the coupling of CAMs with actin that undergoes retrograde flow. This interaction creates a mechanical force that impulses the actin movement towards the adhesion region instead backward[160]. An appropriate regulation in the clutch strength is required to axon attaches at contact sites, explores it and easily detaches in the case the synapse is not established. Thus a combination of intracellular mechanisms, like endocytosis, recycle events and recently discovered local protein synthesis[174] adjusts the concentration of CAMs localized at cell surface contributing to a proper axon behavior[175]. The activated leucocyte cell adhesion molecule (ALCAM) is an immunoglobulin superfamily member (IgSF-CAM) that interacts homophyllically with itself[176] or heterophyllically with other IgSF-CAM (i.e CAML1)[177]. During cell-cell contact, the ALCAM is present at the cell surface and then undergoes endocytosis for degradation[178]. The reduced levels of ALCAM are compensated by local synthesis of new molecules. Indeed, the mRNA coding for ALCAM was localized in axons and growth cones from retinal ganglia cells (RGC)[174]. Moreover, analysis of its 3'UTR sequences revealed the presence of a cytoplasmic polyadenylation element (CEP) that tightly regulates the mRNA translation[174]. In developing retinal axons the ALCAM acts in order to regulate axon growth[179,180] and this type of axons preferentially navigate in ALCAM-coated substrate, both *in vivo* and *in vitro*[180]. It was shown that local ALCAM translation is important to favor axon outgrowth in ALCAM/laminin substrate[174]. Another CAM locally synthesized in axons is the *Xenopus* homolog Pcdh7, the NF-Protocadherin (member of cadherin family)[181]. In this study it was observed that Sema3A-neuropilin signaling activates local synthesis of the protocadherin which positively regulates axon pathfinding[181]. These studies show that local translation regulates growth cone adhesion.

Axon outgrowth requires an expansion of the plasma membrane. The nascent membrane is built with phospholipids synthesized in cell body and transported in plasma membrane precursor vesicles (PPVs) to the growth cone. Then, PPVs are tethered to the plasma membrane by the exocyst, an effector that subsequently supports the exocytosis of PPVs. The exocyst is a complex constituted by eight proteins (Sec3, Sec5, Exo3/Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) whose assembly is support by the small GTP-binding proteins[182]. TC10 is one small cdc42-like GTPase involved in the transport of the Exo3, Sec8 and Exo70 to the plasma membrane to form the exocyst[183]. Moreover, TC10 is

found in injury and naïve cortical axons[184] and it plays a role during injured sensory axons by promoting axon elongation[185]. Recently, mRNA coding for TC10 was localized in axons from dorsal root ganglia (DRG) neurons. NGF-induced local translation of TC10 is required for axon growth and membrane expansion[186]. Additionally, local synthesis of TC10 is mediated by Akt-Rheb-mTOR signaling pathway which in turn is triggered by cue-induced phosphatidylinositol 3-kinase (PI3K) signaling[186]. During axon outgrowth, this signaling pathway also mediates synthesis of the Par3 complex[186] a cytoskeleton element required for axon elongation[187]. All together, these evidences demonstrated that cue-induced axon outgrowth simultaneously activates translation of several transcripts coding for different types of proteins (an exocyst complex-associated protein and a cytoskeleton protein) both of which are important for a proper axon elongation.

Considerable amounts of cytoskeleton proteins are needed for rapid motility. Given the long distance and the slow rate of protein transport between soma and the tips of the axons the neuron requires local regulated mRNA translation for a correct supply of proteins to growth cone. In fact, the majority of axonal *β-actin* mRNA is anterogradely transport from the cell body into axons. Singer and co-workers (1999) showed that *β-actin* mRNA co-localizes with components of the polyribosomes complex and its localization in developing axons was increased by the presence of BNDF and NT3[188]. Local axonal translation of *β-actin* mRNA is important for cytoskeleton dynamics and consequently axon outgrowth. RhoA, a small guanosine triphosphatase (GTPase) that regulates the actin cytoskeleton is also localized in developing axons and growth cones from cultured DRGs[150].

**Local protein synthesis for growth cone guidance**

An old challenge in neurobiology is to understand how neurons 'wire up' to form functional neural circuits. Guidance cues have been identified as specific proteins that cooperatively instruct the axon through their pathway until it reaches a synaptic target[189]. The cues guide the axon in an activity-independent manner and they may act at long or short distance to mediate attraction or repulsive guidance.

Four major families of guidance cues are netrins, semaphorins, slits and ephrins furthermore certain morphogens (hedgehog and Wts) and growth factors (Neurotrophins, FGFs, brain-derived neurotrophic factor (BDNF)) have also been implicated in neuronal guidance[190].

Guidance cues rarely act by themselves being important the existence of various cues for axon guidance decisions. The trajectory of the commissural neurons is a beautiful example of how axons are driven by a cooperative function of different cues (**Figure 1.5**). During development, commissural axons grow ventrally from dorsal spinal cord to the floor plate (midline) guided by chemoattractants, the netrin-1[191–193], the sonic hedgehog (shh)[194,195] and the vascular endothelial growth factor (VEGF)[196]. The mechanisms underlying the interaction of the three cues to axon guidance are not known, however it is speculated that they act in an additive manner by activating the same signaling pathway. For example, Src family kinases are involved in the attractive turning responses to netrin-1[197,198], shh[199], and VEGF[196]. In parallel, each cue might regulate particular pathways in order to coordinate different axonal processes. In this case, netrin-1 attracts the commissural axons but it also induces their growth[187] by regulating local synthesis of proteins[126]. Netrin-1 binds to the deleted in colorectal cancer (DCC) receptor[200], in which translation components are associated[126]. Activation of DCC by netrin-1 promotes dissociation of those components, as a consequence local translation of mRNAs initiates giving rise to new proteins[126]. In cultured commissural axons, Netrin-1/DCC complex activates signaling pathways that trigger translation of mRNAs coding for PAR complex, a cytoskeleton regulator, involved in axon outgrowth (**Figure 1.5a and a.1**)[187].

After arriving to the midline, axons lose sensitivity to attractant cues and react to chemorepulsive cues, the Slits1-3 and ephrins, which prevent axons to recross the midline but allow them to orderly growth along the longitudinal axis (**Figure 1.5b**). The Slit receptors, Robo1 and Robo2, are expressed in the commissural axons. Its activation silences the DCC receptor[201,202] and prevents axons to cross back[202]. Robo3 has two isoforms, Robo3.1 and Robo3.2, obtained after alternative splicing of its pre-mRNA. Robo3.1 and Robo3.2 are also present in commissural axons and they sequentially act with opposed effects in axon guidance: Robo3.1 is present on the pre-crossing axons and favors midline crossing by suppressing slit repulsion mediated by Robo1 and Robo2 (**Figure 1.5a.1**)[203]; whereas Robo3.2 supports slit repulsion[203,204] and directs axons in a diagonal trajectory after they cross the midline. The spatiotemporal regulation of the expression of Robo3 isoforms is crucial for a correct axon guidance[203]. That regulation is mediated by local synthesis of Robo3.2 once axons reach the midline (**Figure 1.5b.1**). Indeed, this isoform is locally synthesized in response to a signal provided by the floor plate[204]. Interestingly, the expression of Robo3.2 activates a feedback mechanism that prevents an overexpression of Robo3.2 preventing an abnormal axonal guidance post-crossing[204].

Accordingly, the changes in axonal responsiveness need a rapid increase in receptors availability for receiving and transmitting the signals from guidance cues. Thus, local axonal translation could provide a suitable mechanism to coordinate the local expression of receptors essential for a rapid axon response. In line with this idea, EphA2 receptor is also locally synthesized in growth cones of commissural axons as they cross the midline in the developing spinal cord (**Figure 1.5b.1**)[205].

Post-crossing commissural axons also gain sensitivity to Sema3F (**Figure 1.5c**). This guidance cue is expressed in the grey matter and it acts in order to constrain axon trajectory to a permissive passage along the longitudinal tract. In mouse embryonic stem cell-derived spinal motor neurons (ES-MNs), Sema3F causes axon repulsion in a manner dependent on local protein synthesis[206]. However, this effect has not been investigated in commissural axons.

Neuronal circuit relies on the ability of nervous system to project their axons with high precision to appropriate targets. A combination of guidance molecules with highly regulated mechanisms prompts a correct neuron connection. Nevertheless, there is some controversy regarding to the intracellular pathways triggered by guidance cues, including cue-induced local protein synthesis. Several studies established that local protein synthesis is required for attraction and repulsion of growth cone during axon guidance[144,149,150,207], however these studies were challenged by Roche and colleagues (2009), who claim that local protein synthesis was not required either for ephrinA2, slit3 or Sema3A-induced growth cone collapsed or NGF-induced growth cone protrusions[208]. In this study the authors used DRGs, retinal and sympathetic neurons from embryonic chick while other studies used retinal neurons from *Xenopus laevis*[144,149]. The difference in species would explain the differences in guidance cue response. However, results obtained in DRGs from embryonic mice were not in accordance with the previous report that used the same type of neurons[150]. Another plausible explanation for the conflicting studies was the variation of *in vitro* metabolic rates between laboratories. However, subsequent studies demonstrated that dependence of axon guidance in local protein synthesis varies according to cue concentration. In chick DRGs neurons[209] or in ES-MNs[206], low doses (<500-650ng/mL) of semaphorins, Sema 3A[206,209] and Sema3F[206], induces growth cone collapse in a manner dependent on local protein synthesis. Contrary, response to high doses (>650ng/mL) of that guidance cue is protein synthesis independent. At high levels of Sema3a, an eventual reorganization in the complexes neuropilin2/L1[210] and neuropilin2/plexinA[211] trigger a prevailing activation of the protein synthesis-independent pathway[209]. Therefore neuronal

growth cones adjust to a chemical concentration by combining local protein synthesis with dissimilar intracellular mechanisms.

Indeed, during axon pathfinding the growth cone adapts to the heterogeneous environment by resetting (desensitization phase) and recovering (resensitization phase) its sensitivity to some signals[148,212]. In order to understand the cellular mechanism of this adaptation process, cultures of retinal explants from *Xenopus laevis* were pretreated with low doses of Netrin (1 min.) or Sema3A (2 min.), which caused a rapid desensitization in the growth cone and a decrease of growth cone collapse. After 10min incubation with high doses of Netrin-1 and Sema3A the growth cone recovered its ability to respond to guidance cues. The resensitization effect was blocked by cycloheximide indicating that this phase, but not desensitization, is dependent on protein synthesis[212]. Previously, it had been demonstrated that chemotactic guidance of growth cones in a netrin-1 or BDNF gradient undergoes an adaptation induced by mitogen-activated protein kinase (MAPK)-dependent local protein synthesis[148]. So, mRNA translation facilitates growth cone guidance in a peculiar environment towards a specific target.

The class of proteins locally synthesized during axon guidance is of extreme importance. Growth cone motility occurs after drastic morphological changes experienced by the axon, which are mediated by cytoskeleton remodeling. Therefore, mRNAs of cytoskeleton regulators are key targets for cue-induced translation. In fact, Sema3a-induced collapse is mediated by local translation of RhoA[150], a small GTPase that regulates actin dynamic[213]. In this study, *in situ* hybridization reveals the presence of RhoA transcript in axons and growth cones from rat DRGs. Sema3a induces synthesis of RhoA in axons and inhibition of RhoA mRNA translation by protein synthesis inhibitors or small interference RNA (siRNA) knock-down prevents Sema3a-induced collapse indicating that local synthesis of RhoA is required for Sema3a-mediated collapse[150]. Later, a study performed in cultured *Xenopus* retinal neurons demonstrated that Slit2 activates MAPK pathway leading to local translation of cofilin, an actin-depolymerizing protein. The increase in Slit2-induced cofilin levels leads to a reduction in F-actin present in growth cone filopodia thus to their collapse and repulsion[149]. So, cue-induced growth cone retraction mediates synthesis of proteins involved in the disruption of actin filaments. On the other hand, attractive cues induce synthesis of proteins required for actin polymerization, such as β-actin[147,151].

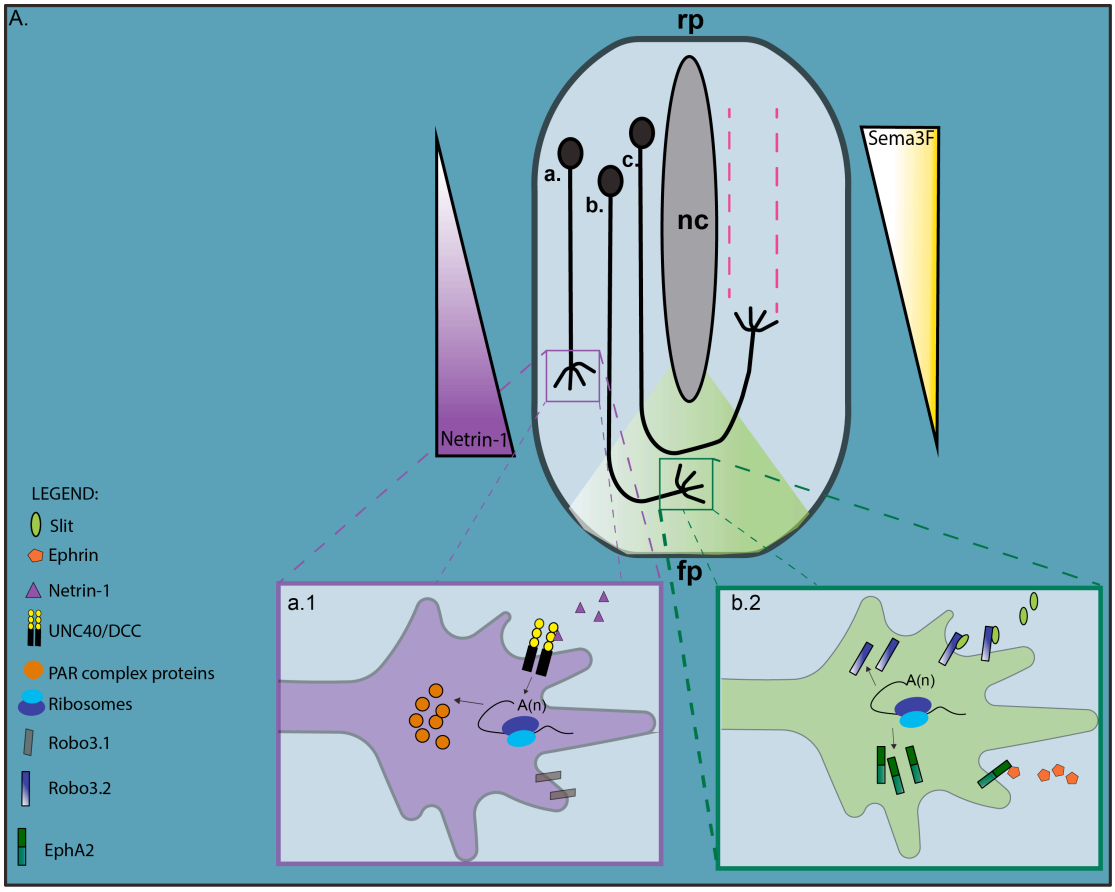



**Figure 1.5 – Guidance of commissural axons requires local mRNA translation.** During the development of the spinal cord, commissural axons extend from the roof plate (**rp**) to the floor plate (**fp**). **(a)** The axon extension is guided by an increasing gradient of Netrin-1 (represented in purple). **(a.1)** Axons are sensitized by Netrin-1 that activates the DCC receptor and initiate translation of proteins from the PAR complex. Thus, axon guidance and outgrowth are regulated by local *par3* mRNA translation that in turn is mediated by Netrin1 signalling. The localization of Robo3.1 receptors prevents repulsion from slit-1 so it supports axon guidance. **(b)** As axons cross the midline, they lose the sensitivity to the attractant Netrin-1 and acquire sensitivity to repellent molecules such as slits and ephrins (green gradient). **(b.1)** This change in axon responsiveness occurs due to up-regulation of the Robo3.2 splicing isoform receptor (slits) and EphA1 receptor (Ephrin). The newly synthesized receptors allow axons to follow a new trajectory preventing them to re-cross the midline. **(c)** Sema3F is expressed in the grey matter (yellow gradient) causing repulsion in post-commissural axons in order to maintain the axon in its correct trajectory (dashed red lines). In stem cell-derived motor neurons, Sema3F induces repulsion in a manner dependent on local protein synthesis, however in commissural axons that dependence is not known. During axon guidance, the local mRNA translation steers axons in their correct pathway. (rp – roof plate; fp-floor plate; nc – notochord).

A new model for local translation in response to guidance cues defined as the "differential translation model" claims that attractive and repulsive cues induce asymmetrical translation of proteins, which in turn build up or down the cytoskeleton, respectively. Two parallel studies supporting this model demonstrated that Netrin-1 and BDNF induce asymmetric β-actin protein synthesis in *Xenopus laevis* retinal growth cones and the increased levels of β-actin protein were localized on the nearest side of the gradient

of Netrin-1[147,151]. Local β-actin synthesis was dependent on $Ca^{2+}$ and it has a crucial contribution to the directional motility of growth cone during guidance[151]. Thus, asymmetric protein translation may also be a mechanism that permits directional movement.

**Local protein synthesis for presynaptic differentiation**

Axons navigate towards a stereotyped target in order to form a functional synapse. Synaptogenesis is highly regulated by diverse signaling events including mRNA localization and translation. In *Aplysia,* the connection between sensory neurons (SNs) and the L7 motor neuron (L7-MN) requires local protein synthesis[214]. In this study, the cell body from SN was removed and synaptic efficacy between SNs and L7–MN was evaluated in the presence and absence of protein synthesis inhibitors. Observations revealed that the increase in the number of varicosities in the SNs-contacting L7–MN was dependent on protein synthesis[214]. Thus axons have the ability to regulate synaptic efficacy independently from the cell body. Moreover, the synaptic contact induces accumulation and stability of specific mRNAs (sensorin and β-actin mRNA) in the sensory neuritis[215]. Sensorin is a neuropeptide that regulates presynaptic growth and stabilization and its mRNA was found to be distributed throughout neuritis of isolated sensory cells[216]. Upon contact between the presynaptic sensory neuron (SN) and the postsynaptic motor neuron (MN), *sensorin* mRNA relocates to the sites of newly formed synapses [216]. The redistribution pattern of both mRNA and protein is similar during synapse formation raising the possibility that sensorin protein increase at synapses as a result of local translation.

BDNF is a neurotrophin involved in axon guidance and outgrowth it regulates synapse function in a manner dependent on local protein synthesis. In *Xenopus* nerve-muscle co-cultures, the contact of the presynaptic axon with BDNF-coated beads potentiated secretion of neurotransmitters at synapses. This potentiation effect of BDNF requires not only the expected persistent increase of intracellular $Ca^{2+}$ but also a continuous protein synthesis within the presynaptic axon[217]. Thus, local protein synthesis plays a role in neurotrophin-dependent modulation of synapses. A study performed in *Drosophila* reported that a transcript coding for the human insulin growth factor (IGF-II) ortholog (Imp) is required in the presynaptic membrane for the formation of the neuromuscular junction[218]. Imp belongs to the RNA-binding proteins (RBPs) family and regulates the localization, translation and stability of several mRNAs[219,220]. The presence

of Imp at presynaptic synapses suggests the specific localization of transcripts in this region and possibly its translation during synapse formation. Taken together these studies suggested a role of local protein synthesis in the formation of synapses in non-mammalian organisms.

Using rat hippocampal cultures from postnatal embryos, Schuman and co-workers (2013) observed that accumulation of β-catenin in axons was dependent on local mRNA translation. Mature hippocampal neurons were incubated with protein synthesis inhibitor, cyclohexamine, resulting in decreasing of β-catenin levels in presynaptic terminals[221]. Consequently, release of neurotransmitters for synaptic cleft is negatively affected. The β-catenin is present in developing and mature neurons[184,222] and it is involved in the assembly of synaptic vesicles pool[223]. Taylor et al. (2013) established that axonal protein synthesis plays a role in hippocampal synapses by controlling vesicle release dynamics.

**Local protein synthesis for axon regeneration**

Neurons experience a balance between axon survival and regeneration in order to maintain its integrity and functionality. Several processes contribute for the refinement of neuronal network, including cue-induced retrograde signaling, cytoskeleton remodeling and mitochondrial functions. In the last decade, evidences demonstrated a cooperatively role of local mRNA translation for axon survival and regeneration.

The embryological studies performed by Vitor Hamburger and Rita Levi-Montalcini (nobel prize in Physiology or Medicine in 1986) revealed the amazing finding that NGF is the factor required for neuronal survival. This cue contributes to axonal survival by regulating transcription through retrograde signals transported from axons to nucleus[224]. NGF-mediated axon survival is dependent on local protein synthesis[225]. Interestingly, in isolated axons, the authors identified the presence of the mRNA coding for cAMP responsive element-binding protein (CREB), a transcription factor. Furthermore, application of NGF in axons induces local synthesis of CREB that is retrogradely transported by a microtubule motor-dependent mechanism. It was demonstrated that neuronal survival mediated by NGF-induced transcription requires the axonal CREB[225].

Mitochondria functions are important to supply energy to axons in order to maintain axonal integrity. A dysfunction in mitochondria metabolism is associated with neurological disorders and axon degeneration[226]. Recently, it was demonstrated that axonal synthesis of a mitochondria-associated protein, the laminB2 (LB2), is important to maintain mitochondrial health[152]. In *Xenopus* retinal ganglia cells (RGC), a proteomic

screen in cue-stimulated axons reveals the presence of laminB2[152]. LaminB2 is normally associated to nuclear membrane[227] so this intriguingly finding tempted the authors to explore about the role of axonal laminB2. They found that LaminB2 is locally translated in axons, where it associates to the membrane of mitochondria in order to preserve mitochondria structure. Indeed, axonal injection of morpholinos against *lb2* mRNA altered the structure of mitochondria to an elongated form. Additionally, when local synthesis of LB2 was abolished mitochondria membrane potential was compromised and axonal degeneration occurred[152]. These results reveal that axonal LB2 is important to axonal maintenance by guaranteeing the integrity of mitochondria thus allowing an efficient energy production for axon survival.

Axons from the PNS have the ability to regenerate. The regeneration process is very rapidly occurring within a day and it requires a rapidly remodeling and formation of a new growth cone[228]. For that, a constant supply of diverse proteins (cytoskeleton, membranous and cytoplasmic elements) is crucial at the tip of the axon to support the regenerative process. Local protein synthesis has a crucial role for axon regeneration[155]. Indeed, injured sensory neurons (DRGs) contain components for translation machinery, including ribosomal proteins, translation initiation factors and ribosomal RNA (rRNA)[146]. Upon axonal injury the retrograde injury signaling is mediated by local synthesis of certain components that are part of the retrograde complex[229]. Importin β is a soluble transport factor that contains a nuclear localization signal (NLS) important for the translocation of injury signals into the nucleus[230]. Phosphorylation of MAP kinases, ERK1 and ERK2, which occurs at the lesion site, is an important signal to initiate the axon regeneration[231]. Upon sciatic nerve injury, phosphorylated ERKs binds to importin β, which is locally synthesized after sciatic nerve lesion[230]. The complex is retrogradely translocated in a manner dependent on dyneins[230]. The presence of *importin β* mRNA in isolated DRGs axons[230] is mediated by its 3'UTR[154]. In an importin β null mice, the axonal knock-out of importin β alters transcription responses and delay regeneration process in vivo[154] indicating a role of mRNA mechanism for axonal regeneration.

### 2.2. Regulation of mRNA transport, localization and translation

Subcellular targeting of mRNA and its on-site translation is an efficient mechanism that provides spatial and temporal gene expression control within subcellular domains. So, it is imperative that mRNAs are accurately addressed to their final destination. Targeting of mRNAs to subcellular domains involve multi steps. In the nucleus, the mRNA

associates with RBPs forming a RNA-protein complex. Then, in cytoplasm, this complex assembles in granular-like particles (ribonucleoprotein particles – RNPs)[232,233]. The RNPs transport the mRNA from soma to distal cellular regions in a microtubule-based mechanism[234]. Additionally the encapsulation of mRNA in granules silences mRNA translation throughout its journey. At their destination, granules form an incredible outpost of mRNAs that are synchronously translated in response to external stimuli.

The mRNA-RBPs complex is formed after the recognition of *cis*-acting elements present in the transcripts by *trans*-acting factors in RBPs. The *cis*-acting elements are short mRNA sequences mainly present in the 3'UTR of diverse mRNAs. One of the first elements identified was the *zipcode* sequence present in the *β-actin* mRNA with 54 nucleotide (nt) in length[235,236]. The "*zipcode* signal" is recognized by *zipcode*-binding protein 1 (ZBP1) and subsequently transported to distal regions of axons and dendrites[237] (more details in next subsection "Local regulation of β-actin mRNA translation"). The 3'UTR of *tau* mRNA has an axon-localization signal with 240 base pairs (H fragment)[238], which is recognized by HuD, an RBP that mediates anterograde transported of *tau* mRNA along the axons[239,240]. Other RBPs and their binding site include the cytoplasmatic polyadenylation element-binding proteins (CPEBs) that recognize specific *cis*-elements CEP[241,242]. The 3'UTR of the *CamKII* mRNA contains a long element (94 nt) that is recognized by CPEB therefore mediating dendritic targeting[243]. However, the *CamKII* mRNA also contains a G-quartet structure as a signaling for dendritic mRNA targeting[244]. Indeed, some RBPs, such as FMRP[245] and Staufen[246], recognize secondary structures present in their target mRNAs. In *Drosophila*, the 3'UTR of *bicoid* mRNAs contains stem-loop structures important to mediate the localization of *bicoid* mRNAs in oocytes[247]. The interaction between RBP and its specific *cis*-elements dictates where distinct mRNAs will be localized.

At subcellular domains, the enclosed mRNAs are anchored to the plasma membrane. In dendrites the Arc mRNA is anchored underneath dendritic spines indicating a micro-domain for synaptic-specific mRNA translation[248]. However, the general mechanism underlying mRNA anchorage in neurons is undefined. Studies in the bud tip of yeast and in oocytes from *Xenopus* or *Drosophila* show a possible role for actin- and microtubule-dependent mRNA granules anchoring[249–251]. Additionally, in chick fibroblasts it was demonstrated that the complex formed between F-actin and the translation elongation factor (EF1α) acts as a scaffold for *β-actin* mRNA anchoring[252]. Moreover, in

*D. melanogaster*, it was demonstrated that the molecular motor dynein is not an exclusively transporter element and it also functions as an anchor protein[253].

Upon its localization the mRNA is not immediately translated. Anchored mRNAs are associated with translation inhibitors[248] indicating that localized transcripts are maintained in its repressor state until receiving a particular stimuli. Indeed, the RNPs granules contain translation regulators (repressors or activators), small ribosomes and poly(A)-binding proteins[232,233] that contribute to the mRNA translation repression during mRNA trafficking and regulate its local translation. As an example, during synaptic plasticity, neuronal depolarization "disintegrates" the complex mRNA-Staufen and releases the RNAs involved in plasticity from their "enclosure". As a consequence, mRNA translation increases at the synaptic site[232]. Recently, it was defined that mRNAs molecules are in a masked stated (protected in granules), which are unmasked for translation[254]. In this study the authors used single-molecule *in situ* hybridization combined with high resolution imaging techniques to visualize a single molecule of *β-actin* mRNA and the ribosome dynamics in dendrites. During long-term potentiation, it was observed a transient increase in the levels of both, mRNA and ribosomes, which correlates with the increase of *β-actin* synthesis.

Another mechanism to regulate local translation is the direct phosphorylation of RBPs. An mRNA-binding protein, the Grb7, acts as an intermediate between netrin-1 signals and local synthesis of the opioid receptor (KOR)[255]. This receptor interacts with opioid drugs, regulating pain sensation. Its mRNA is transported and locally synthesized in primary sensorial neurons[256]. The Grb7 binds to the 5'UTR of the kor mRNA promoting its local repression, which is release through Netrin-1-induced Grb7 phosphorylation [255].

Eukaryotic transcribed mRNAs posses a 5'-end cap structure where two macromolecular complexes, the eIF4F and the 43S complex, bind in order to regulate translation initiation[257,258]. The two complexes work in parallel: first the heteromeric complex eIF4F unwinds the mRNA secondary structure to facilitate the binding of 43S complex. The eIF4F complex is composed by the scaffold protein eIF4G, the helicase eIF4A and the cap-binding protein eIF4E. The association of eIF4E with eIF4G is the rate-limiting step for translation initiation[259] since eIF4E also has a competitive binding site for the eIF4E-binding proteins (4E-BPs), a translation repressor, [259]. The scaffold of eIF4E by 4E-BPs prevents the formation of the eIF4F complex inhibiting translation initiation. This inhibition is reverted by hyper-phosphorylation of the 4E-BP that releases eIF4E to bind to eIF4G and form the final initiator complex. The phosphorylation of 4E-BPs is regulated by

the mammalian target of rapamycin (mTOR), which is a serine-threonine protein kinase of the PI3K-related kinase family[260]. The mTOR also phosphorylates others kinases (S6 kinase; eEF2K) that regulate translation initiation and elongation[260]. Thus, the mTOR-signaling pathway is a foremost regulator of local protein synthesis. Additionally, local translation is also regulated in a cap-independent manner, some mRNAs lack the cap structure but it contains an internal ribosomal entry (IRES)[261] that recruits the 40S ribosomal subunit regulating translation initiation[262]. Although some dendritic mRNAs contain IRES, the IRES-mediated translation mechanism is poorly understood.

Another regulator of the local mRNA translation is the CPEB, an RBP that control translation by regulating the length of the polyA tail of the target mRNAs. A good example is, the EphA2 receptor that contains CPE elements and its local translation is regulated by CPEB [205].

Regulated-local mRNA translation supplies axons with necessary proteins for neuronal processes. As it was described, translation regulation is very complex, requiring an incredible coordination between different signaling pathways, proteins and instructing signals. However, one puzzling aspect of local protein synthesis is how intracellularly axon governs the translation of only a subset of transcripts. Another intriguing question is how levels of newly synthetized proteins are regulated in order to circumvent their overload accumulation at the subcellular domains. It is known that axons contain not only elements of the translation machinery but also components of the protein degradation machinery, the ubiquitin-proteasome system – UPS [144]. Moreover, some studies reported a link between local protein synthesis and UPS-mediated local degradation[144,155,263]. Nevertheless, the coordinate mechanism and signals that are important to regulate local synthesis versus local degradation is still an open question.

**Intra-axonal regulation of β-actin mRNA translation**

Axonal β-actin mRNA translation is important for diverse events in axonal development. The *β-actin* mRNA has a 3'UTR with *cis*-acting sequences important for RNA localization, stabilization and translation[236]. Seminal studies using chick fibroblasts identified one of the first localization elements in the 3'UTR of *β-actin* mRNA, composed by a 54 nucleotide-long sequence[235]. The 54 nt segment is the "*zipcode signal*" and it is located proximal to the coding region. Nearby, there is an homologous but less active 43 nt segment[235]. The mRNA must form a 180 degrees loop in order for ZBP1 to bind forming the complex[264].

ZBP1 is a 68KDa protein that makes part of a highly conserved family of RBPs. ZBP1 has a RNA binding domain consisting on two RNA recognition motifs (RRMs) and four hnRNP (heterogeneous nuclear ribonucleoproteins) K homology (KH) domains, and a REV-like nuclear export signal (NES)[237]. This protein associates to *β-actin* mRNA contributing to its cytoplasmatic localization[265] and translation[266]. In the perinuclear region[267], the two C-terminal KH domains of the ZBP1 (KH3 and KH4) recognize a bipartite RNA element located within the first 28 nucleotide of the "*zipcode*" sequence of the *β-actin* mRNA[237]. Biochemistry analysis revealed that the KH4 domain binds to the 5' element (CGGAC) and the KH3 binds to a variable 3' element (C/A – CA – C/U) [268]. After complex formation, it is transported from the nucleus to cell periphery where translation occurs. During ZBP1-mRNA complex translocation to the cytoplasm the mRNA must be silenced in order to prevent translation until it reaches its target. ZBP1 is responsible for preventing the assembly of 80S ribosome on the *β-actin* transcript thus blocking translation initiation. As ZBP1-mRNA complex reaches its destination a key tyrosine residue of ZBP1 (Tyr396) is phosphorylated by the protein kinase Src. The complex is disassembled and *β-actin* mRNA translation occurs [266].

In axons, ZBP1 regulates the *β-actin* mRNA localization to growth cones where stimulus-induced local translation of this mRNA leads to growth cone navigation[188,269], turning[270] and guidance[271]. It was observed that a disruption of ZBP1 impairs the precise localization of the transcript and, as a consequence, growth cone motility is altered[272]. Recently, morphological and electrophysiological analysis revealed that low levels of ZBP1 compromises the PNS axonal regeneration[273].

*β-actin* mRNA is not exclusively transported by ZBP1. The hnRNP-R, an RBP that belongs to the hnRNP family, contains two recognition motifs (RRM1 and RRM2) that interact with the 3'UTR of the *β-actin* mRNA[274]. In *C. elegans*, neuronal knock-down of hnRNP-R reduces the *β-actin* mRNA levels in growth cone filopodia but not its global levels indicating that hnRNP-R mediates axonal transport of *β-actin* mRNA[274]. The interaction between hnRNP-R with *β-actin* mRNA depends on the spinal muscular motor (SMN) protein[275], which acts in the nucleus assembling the RNP complexes. Both, hnRNP-R and SMN regulates localization of *β-actin* mRNA in presynaptic terminals of motor neurons[276]. Therefore, deficits in hnRNP-R and SMN reduce axonal *β-actin* mRNA levels. Consequently, the normal recruitment of $Ca^{2+}$ channels is reduced thus compromising neuromuscular junctions[274,277]. Indeed, deficits in NMJ is an hallmark of

spinal muscular atrophy (SMA), a motor neuron disease caused by the dysfunction of SMN[278].

*β-actin* mRNA is an interesting example of the neuronal complexity. In the nucleus, *β-actin* mRNA associates with different RBPs, being guided to specific sites in the axon (presynaptic terminals, filopodia or the regeneration regions). Therefore, a tightly regulated translation allows synthesis of axonal β-actin with space and time precision, governing its physiologic role during axon outgrowth, regeneration and synapse specializations. Singer and co-workers have made important contributions to understand the dynamics of *β-actin* mRNA inside the cell. Using transgenic mice that express fluorescent *β-actin* mRNA[279,280] they investigated mRNA motility. They observed that in soma and proximal dendrites *β-actin* mRNA-transport granules are enriched with several copies of *β-actin* mRNA, however, as granules travel towards the distal part of the dendrite, only a single copy of that mRNA is identified in granules[280]. Additionally, upon neuronal depolarization with potassium chloride (KCl), each RNP particles containing multiple *β-actin* mRNAs (merge event) splits into spots containing one single molecule of *β-actin* mRNA (split events). The split event favors the released state of mRNA possibly to facilitate local translation. Although, this study demonstrates the behavior of only one type of mRNA the new observations might be extremely valuable to understand RNA trafficking and localization.

### 2.3 Methods to study axonal mRNA translation

One initial challenged to study local events in neuronal processes was to isolate axons or dendrites from their soma to further obtain pure axonal or dendritic material. A drawback of pioneer studies was the possible contamination with material from the cell body. One of the first techniques was to surgically sever the soma from its processes[121,123,144]. Another tool is to use a substrate with specific pore sizes that allow processes (axons and dendrites) but not cell bodies to penetrate into a new compartment[118,146]. Campenot chambers emerged to specifically isolate axonal processes[281]. This tri-compartment chamber consists in a teflon insert attached to the coated dish with grooves that communicate between compartments. Cells are cultured in the middle compartment, where cell bodies and dendrites are restricted while axons develop through the adjacent compartments. Demanded challenge in these devices was to maintain a proper seal between the teflon insert and the dish, mixture of media between compartments was recurrent, which prevented axonal isolation. Recently, Jeon and colleagues developed the microfluidic chambers, a polydimethylsiloxane (PDMS) device

where axonal processes are fluidically and physically isolated from the cell body[282]. Another strategy to collect axonal preparations is the laser-capture microdissection (LCM). Visualizing neurons under the microscope it is possible to select and dissect a portion of the axon obtaining pure axonal material. Using LCM is also possible to distinguish between different parts of the axon, for example axon shaft versus growth cone[167].

One important challenge in studying local mRNA translation is to monitor, visualize and identify the newly synthetized proteins. The use of radiolabelled aminoacids was helpfully to create the initial idea of local protein synthesis[131–136], however its low sensitivity was an disadvantage. So, other techniques using non-canonical tagged amino acids (BONCAT, FUNCAT or NCAT) have been developed. These amino acids cross the cell membrane and are incorporated into nascent polypeptide chain by a process dependent on the tRNA synthetases thus the labeled proteins are part of the pool of newly synthesized proteins and easily identified. These techniques have been combined with 2D-difference electrophoresis (DIGE) to compare proteomes of different compartments [152].

In addition, fluorescent molecules such as green fluorescent protein (GFP) or photo-switchable Kaede, fused with consensus/regulatory sequences of the target mRNA (i.e 3'UTR or zipcode) have been used to address the local protein synthesis[123,147,150,187,205].

Since RNA mechanism is now accepted to be an important regulator of axonal processes, future technological advances are of extreme importance to unravel the numerous questions that remain to be clarified.

## 3. OBJECTIVES OF THE PRESENT WORK

The objective of this work was to determine the role of local proteins synthesis in presynaptic differentiation.

Local mRNA translation is a mechanism that regulates the spatial and temporal accumulation of proteins in axons. A multitude of axonal mRNAs has been identified by genomic analysis. In addition, biochemical studies have revealing distinctive mRNAs domains that regulate its axonal transport and translation. Moreover, functional studies have uncovered the role of many locally synthesized proteins during axon development. So, in the last two decades, these studies contributed to establish the concept that axonal mRNA translation governs autonomous axonal behavior.

During presynaptic differentiation the axon forms specialized structures responsible to orchestrate a series of events that culminate in transmission of neuronal information.

The formation of the presynaptic terminal is complex and involves interaction between a set of molecular components, each one has a specific function however all of them need to interact with its partners to achieve a properly synaptic terminal structure. F-actin network is involved in all presynaptic differentiation steps acting as structural scaffold or governing synaptic vesicles motility.

In the first part, we addressed the role of local protein synthesis in presynaptic terminal formation and investigate whether local synthesis of *β-actin* mRNA is activated during presynaptic differentiation. For that we used FGF-22, a soluble presynaptic organizing molecule, to induce presynaptic differentiation in fluidically isolated axons. The differentiation of nerve terminals was measured by clustering of the synaptic vesicle protein SV2 along the axon shaft. Additionally, when protein synthesis is inhibited in axons, SV2 clustering is reduced to basal levels. Moreover, we show that FGF-22 induces an increase in the phosphorylation of 4E-BP1, which is a hallmark for protein synthesis.

In addition, FGF-22 increases axonal translation of a fluorescent β-actin reporter indicating a role for axonal β-actin in presynaptic differentiation. Lastly, we explored the dependence of cytoskeleton remodeling on local protein synthesis. Early in presynaptic differentiation (induced by FGF-22), the formation of filamentous actin increases at SV clusters. FGF-22-induced F-actin polymerization is abolished by protein synthesis inhibitors, consequently there is a reduction in presynaptic clusters. All together the results indicate that local mRNA translation is required for synapse formation and axonal β-actin might be required for cytoskeleton remodeling underlying “presynaptogenesis”.

In the second part of this work, we addressed the functional and physiologic role of axonal β-actin during presynaptic differentiation. For that purpose we used fluorescent reporters and RNA interference strategies to alter the levels of axonal *β-actin* mRNA in primary cultured neurons then we evaluated the consequences of that in presynaptic cluster formation and F-actin formation. First, transducing CG neurons with sindbis virus expressing the β-actin reporter prevented axonal localization of *β-actin* mRNA, which abolished FGF-22-induced SV2 clusters and F-actin polymerization. These results were corroborated after by knocking down axonal *β-actin* mRNA. In order to understand the physiologic role of axonal β-actin in synapse formation, we use neuronal-muscle co-cultures to mimic the physiological environment. We observed that mislocalization of axonal *β-actin* mRNA affects neuromuscular junction formation. Overall, localization and translation of *β-actin* mRNA is required for formation of the presynaptic terminal.

# CHAPTER II

## EXPERIMENTAL PROCEDURES

## 2.1 MATERIALS AND METHODS

### Microfluidic chambers for cell cultures

Poly(dimethylsiloxane) **(**PDMS) microfluidic chambers were prepared as described previously[282]. In detail, a pre-polymer mixture of Sylgard 148 Silicone elastomer (Dow Corning) were added to a master mold (kindly provided by Dr. Noo Li Jeon, from Institute of Advanced Machinery and Design, Seoul National University). Replica-molded pieces of PMDS were peeled away from the master and cleaned with 3M Scotch Brand 471 tape. Then, PDMS devices were sterilized with 75% ethanol, air-dried in sterile conditions and cleaned and sealed to substrate-coated glass coverslips (Assistent, Karl Hecht). Sealed chambers create a microenviroment were two symmetric compartments are separated by a set of microgrooves. Neurons are plated in one compartment and axons growth through the microgrooves to the opposite compartment (**Figure 3.1**), thus being physically isolated from cell body. Coverslips were coated with 0.1mg/mL poly-L-lysine (cultrex), O/N at 37ºC. After microfluidic device assemble, coverslips for neuronal cultures were coated with 10µg/mL laminin (mouse) (Cultrex) for 2h, at 37ºC, washed with neurobasal media and neurons plated at the desired density

### Primary neuronal culture

Ciliary ganglia (CG) neurons cultures were prepared as previously described[283]. Ciliary ganglia, from 7-day-old chick embryos (E7), were dissociated by incubating the ganglia neurons in 0.1% trypsin (Gibco) in HBSS media for 20min at 37ºC, washed in fresh medium containing 10% of horse serum (HS) and 2% of fetal bovine serum (FBS) to stop trypsin activity. Finally, cells were dispersed by trituration through a fire-polished Pasteur pipet. This procedure yields between 5000-10000 cells per ganglion. Cells were allowed to attach for 45min-1h before adding cell medium. For live imaging experiment, CG neurons were plated in Poly-D-Lysine (PDL)-coated live imaging plates (diameter of 35cm$^2$). Cell growth medium consist of neurobasal media (Life Technologies) with 10% horse serum (HS) (Gibco), 2% fetal bovine serum (FBS) (Gibco), 1x B27 (Gibco), 2mM glutamine (Gibco), 12.5U/mL penicillin and 12.5ug/mL streptomycin (Gibco) supplemented with 5ng/mL ciliary neurotrophic factor (cntf) (Peprotech) and 5ng/mL glial cell-derived neurotrophic factor (gdnf) (Peprotech). The 5-fluoro-2'-deoxyuridine (5-FDU) ($10^{-5}$M) was added to the culture media to inhibit growth of non-neuronal cells. When explants were used, each ganglion was placed in one compartment of microfluidic

chambers. Cells were maintained in a humidified incubator with 5% $CO_2$ at 37ºC. After 2-3 days in vitro (DIV) axonal processes are observed in axonal compartment.

**Muscle culture**:

Chick embryo skeletal muscle culture was adapted from Fischbach, 1972[284]. At embryonic day 10 (E10), chick was sacrificed by cutting the head. The body was placed in ice-cold Hank's solution (HBSS) media with its pectoral region exposed for muscle dissection. Tissue was cut in slight pieces and incubated with 0.25% trypsin (Gibco) in HBSS solution, for 30min at 37ºC. Cells were dissociated, filtered using a 100 µm filter and submitted to pre-plating for 30-45 min in order to reduce fibroblast contamination. Afterwards, cells were plated in the axonal compartment in growth media, which consists in Minimum essential media (MEM) (Sigma) with 10% HS (Gibco), 5-10% chick embryo extract (CEE) (home-made) and 12.5 U/mL penicillin and 12.5 U/mL streptomycin. At DIV3, 10µM of AraC is added in fresh fusion media, which consists in normal growth media with 2% of CEE.

**Isolation of fibroblasts from Chick embryos**

Chick embryo fibroblast (CEF) isolation was adapted from Goldman A.[285]. Fibroblasts-like cells are derived from 10 day-old embryos. After removing the embryo from its eggshells, the head was removed from the body, which, in turn, was placed in a sterile petri dish. Then, the tissue was finely minced with sterile scissors, rinsed in PBS and incubated with 0.25% trypsin (Gibco) diluted in HBSS, at 37ºC for 15min. Dissociated tissue was removed and trypsin activity was inactivated using 10% of FBS. Trypsinization step was repeated until all remaining tissue was dissociated. Dissociated cells were washed in PBS and resuspended in complete medium, Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 2mM glutamine, 10% FBS and 12.5 U/mL penicillin and 12.5 U/mL streptomycin. Cells were plated in 100 mm-dish and they were incubated at 37ºC in a humidified 5% $CO_2$ incubator. When cells reached confluence, culture media was removed, cells were washed with an EDTA (Ethylenediamine tetraacetic acid) solution (0.53mM EDTA at pH=8), followed by 0.05% trypsin in PBS. Cell were then centrifuged and re-suspended in fresh media and re-plated in a new 100 mm-dish.

**Generation of pSindbis vector and viral particles**

The pSinRep5-myrEGFPd$_2$3'UTR$^{\beta\text{-actin}}$ vector was generated from the pSinRep5-myrEGFPd$_1^{3'\text{UTR }\beta\text{-actin}}$, kindly offered by Dr. Samie Jaffrey (Weill Medical College of

Cornell University). The $d_2$ domain was inserted (in replacement of the $d_1$ domain) by PCR at the C-terminus of EGFP to generate the EGFP$d_2$ variant, and the resulting fragment was inserted at the Mlu-SphI sites generating the pSinRep5-myrEGFP$d_2$ vector. The 3'UTR of *Homo sapiens* β-actin (GenBank Accession Number: BC001301.1) and *Gallus gallus* H1 Histone (GenBank Accession Number: NM_001044673) were amplified by reverse transcriptase polymerase chain reaction (RT-PCR). Then, inserted into the SphI-ApaI sites to generate a construct expressing a myristoylation consensus sequence and a destabilized EGFP with the 3'UTR of β-actin (pSinRep5-myrEGFP$d_2$3'UTR$^{\beta\text{-actin}}$) or the 3'UTR of Histone (pSinRep5-myrEGFP$d_2$3'UTR$^{Hist}$). In order to generate the viral genome, pSinRep5-myrEGFP$d_2$ constructs and the helper plasmid, DH26S, were linearized with Xho I and the DNA was *in vitro* transcribed using the mMESSAGE mMACHINE SP6 kit (Life Technonlogies). Pseudoviruses particles were prepared according to the manufacturer's instructions (Life Technologies) using baby hamster kidney 1 (BHK-1) cells. In sum, BHK-1 cells were electroporated with 12µg DH26S and 12µg of the desired pSinRep construct. The cell medium was harvested 36h after transfection, centrifuged and viral particles were purified from supernatant, which was centrifuged at 60000 x g for 2h20min at 15ºC. Viral particles were resuspended in PBS with 0.1% bovine serum albumin (BSA) and stored at -80ºC. The virus titer was determined in CG neuronal cultures and a volume that infects 85% of cells was used for subsequent experiments.

**siRNA transfection**

To knock-down axonal β-actin, it was used RNA interference (RNAi) against *Gallus gallus* β-actin (GenBank Accession Bank: NM_205518.1). Two different Stealth RNAi ™ siRNA were designed using the BLOCK-iT™ RNAi designer from Invitrogen and purchased from Life Technologies. One siRNA sequence targets the open reading frame (ORF): 5'-CCGTAAGGATCTGTATGCCAACACA-3' (siRNA1); whereas the other siRNA binds to 5'-untranslated region (UTR) of *Gallus gallus β-actin* mRNA: 5'-CACAGCCAGCCATGGATGATGATAT-3' (siRNA2). At DIV3, axons were transfected using Lipofectamine RNAimax (Life Technologies) according to the manufacturer's instructions. For transfection in 24 multi-well plates (validation by real time PCR), 45000 cells of CGs were transfected for 48h. A control non-targeting siRNA, which is a siRNA sequence that do not target any gene product, was acquired from Life Technologies. To evaluate axonal efficiency a siRNA conjugated with Alexa555 fluorophore (modified accell non-targeting siRNA) was purchased from Thermo Scientific.

**Microfluidic chambers assays**:

For all experimental approaches, except co-cultures, conditional media from somal and axonal compartment were changed to starvation media 6h before stimulus or drug treatment. Starvation media consists in non-supplemented growth media without HS and FBS (neurobasal media (Life Technologies) with 1x B27 (Gibco), 2mM glutamine (Gibco) and 12.5U/mL penicillin and 12.5ug/mL streptomycin (Gibco).

To induce presynaptic differentiation: 2nM recombinant-human FGF-22, (R&D) or *E. Coli*-derived recombinant MBP-His-FGF-22, 6nM, was added into the axonal side. 0.1% of Bovine serum albumin (BSA) dissolved in PBS or purified maltose-binding protein (MBP) were used as vehicle (Ctr). MBP-His-FGF-22 was used in the siRNA experimental approach.

Stimulus with CNTF: 10ng/mL of CNTF (Peprotech) were added to axonal compartment for 2h and phosphorylation of 4EBP1 was monitorized.

Inhibition of protein synthesis: protein synthesis inhibitors, 2μM anisomycin (Calbiochem) and 2μM emetine dihydrochloride hydrate (Sigma), were added in the axonal side 20-30min before FGF-22 or PDL-beads.

Cytoskeleton disruption: 100nM of *in solution* Latrunculin-A (Calbiochem) was added to axons 20min before stimulus with FGF-22 for a maximum of 8h of incubation. Dimethyl sulfoxide (DMSO) was used as vehicle (Ctr).

Viral infection in microfluidic devices: at DIV2-3 cell bodies were infected with Sindbis virus, pSinRep5-myrEGFPd$_2$3'UTR$^{\beta\text{-actin}}$ or pSinRep5-myrEGFPd$_2$3'UTR$^{Hist}$ for at least 20hours. Then, FGF-22 was added to axonal compartment for 14h for presynaptic differentiation. In co-cultures assays, neurons (cell bodies) were infected for 20h-24h following cell fixation. At the end of each experimental approach, cells were fixed and immunocytochemistry was performed.

**PDL-beads**

PDL-beads were prepared as described by Taylor et al. 2013[221]. Briefly, two drops of aliphatic amine latex beads were added to 0.5mL of PDL and incubated for 30min-2h, at 37ºC with shaking. Then, PDL-beads were washed three times with sterile mQH$_2$O and diluted in 0.5mL of neurobasal media (Invitrogen). 30μL of PDL-beads suspension were added to axonal compartment for the indicated period of time.

**Immunocytochemistry**

Neurons plated in microfluidic chambers were fixed in 4% paraformaldehyde (in PBS with 4% sucrose), for 10min at RT. For F-actin immunolabeling paraformaldehyde solution was prepared in cytoskeleton buffer (60mM PIPES, 27mM HEPES, 10mM EGTA, 4mM $MgSO_4$ and 4% sucrose). After 3 washes with Tris-buffer saline (TBS) cells were permeabilized in TBS with 0.25% Triton X-100 for 5min at RT. Nonspecific labeling was blocked by pre-incubation in 5% normal goat serum (GS) (Gibco) and 5% normal FBS in TBS for 30min at RT. Preparations were incubated with the mix of primary antibodies in blocking buffer at 4°C overnight. Then, washed three times in TBS and incubated with the mix of secondary antibodies diluted in blocking buffer, 1h at room temperature. After two washes with TBS with 0.1% Triton X-100 and one with TBS, microfluidic devices were removed from coverslips; coverslips were rinsed in water and mounted in prolong mounting media with or without DAPI (Invitrogen). Synaptic vesicles were identified using antibodies against SV2 (mouse, DSHB) and axons were identified using chicken anti-neurofilament M (Chemicon). Rabbit anti-p-4E-BP1 (Ser65/Thr79) (Santa Cruz Biotech) was used for local translation reporter. In some cases, tubulin III (mouse, Sigma-Aldrich) was used as neuronal marker (indicated in legend as Tuj1). To identify dendrites, cells were immunostained with chicken anti-MAP2 (Chemicon). F-actin was visualized using Acti-stain 555 phalloidin (Cytoskeleton). α-bungarotoxin AlexaFluor 647 (invitrogen) was used to stain acetylcholine receptors (AChR). AlexaFluor secondary antibodies conjugated to fluorophores with 488nm, 568nm and 647nm (Invitrogen) and AMCA were used.

**RNA extraction and reverse transcription-PCR**

At DIV7, axonal compartments were examined for the presence of cell bodies to confirm complete separation between cell bodies and axons. RNA extracts from individual compartments, somal and axonal, were obtained from six microfluidic devices using Trizol reagent and following manufactures' instructions. cDNA was transcribed using the Superscript III Kit (Invitrogen) and amplified using platinum PCR supermix (Invitrogen). Results were visualized in a 1% agarose gel.

**RNA extraction and real-time PCR**

Total RNA was extracted from 90000 cells with Trizol reagent (Invitrogen), following the manufacturer's specifications. The total amount of RNA was quantified by optical density

at 260nm and the quality evaluated using the Experion system (100-120V, #700-701, BioRad). For first strand cDNA synthesis, 0.5ug of total RNA and iScript cDNA synthesis kit (BioRad) were used. For quantitative gene expression analysis, 20µL of reaction were prepared with 2µL of 1:100 diluted cDNA, 10uL of 2x EvaGreen master mix (Bio-Rad) and o.5µL of specific primers to a final concentration of 250nM. β-actin specific primers were 1 – fwd: 5'-ATGAAGCCCAGAGCAAAAGA-3' and rev: 5'GGGGTGTTGAAGGTCTCAAA-3' an 2 – fwd: 5′-CTCCCTGATGGTCAGGTCAC-3' and rev: 5'-ATGCCAGGGTACATTGTGGT-3'. The fluorescence signal was measured after each elongation step of the PCR reaction in the iQ5 Multicolor Real-Time PCR detection System (BioRad). Histone was used as control, the primers used were fwd: 5'-ACGGAGAGCCTGGTCCTATC-3' and rev: 5'- GTTGTGGCCCACCTTGTAGT-3'. The levels of expression for each condition were determined and related to the level of expression of control condition (siRNA neg).

**WesternBlot**

Chick embryo fibroblasts cells were treated with mitomycin and transfected with siRNA for 96h. Cells were lysed in RIPA lysis buffer (150mM NaCl, 50mM Tris-HCl (pH=7.4), 5mM EGTA, 1% Triton, 0.5% deoxycholate and 0.1% SDS, pH=7.5, supplemented with 50mM NaF, 1.5mM $Na_3VO_4$, 0.1mM PMSF and 1µg/mL CLAP). Protein concentration was determined by the BCA method (ThermoScientific). Samples were denatured with 5x denaturating buffer (250mM Tris, ph=6.8, 10% sodium dodecyl sulfate (SDS), 1% (v/v) β-mercaptoethanol, 10% glycerol, 3mM Na3VO4, and 0.25% bromophenol blue) for 5min at 95ºC. Cell lysates were analyzed by SDS-PAGE in 12% polyacrylamide gels and electro-transferred to a polyvinylidene difluoride (PVDF) membrane at 40V, overnight. Membranes were blocked with 3% (w/v) BSA solubilized in Tris-buffer saline (137mM NaCl and 20mM Tris-HCl, ph=7.6) containing 0.1% (v/v) Tween 20 (TBS-T) and blotted with mouse anti-β-actin (Abcam). Mouse anti-β-Tubulin (Sigma) was used as loading control. Membranes were incubated 1h, at RT, with alkaline phosphatase secondary antibodies (anti-mouse or anti-rabbit from Jackson immunoresearch). Membranes were then incubated with a chemifluorescence substrate (Amersham) and scanned with the Storm 860 scanner (Amersham Biosciences). Blot quantification was performed using ImageJ software.

**Live-cell imaging**

At DIV2/3, neurons were infected with Sindbis viral construct pSInREPmyr-d2EGFP3'UTR$^{\beta actin}$ for 30-36h. At DIV3/4, cell culture medium was first replaced for HEPES-based imaging medium (119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2.2H_2O$, 2 mM $MgCl_2.6H_2O$, 30 mM glucose, 20 mM HEPES, pH 7.4) and allowed to recover for 10min. Global stimulus with FGF-22, in presence or absence of anysomicin, were applied for 1h. Imaging was performed using an inverted microscope Zeiss Axiovert Observed Z1, and an AxioCamHRm camera. Several x,y fixed positions with GFP-positive isolated axons were selected and images were acquired both before (0 h) and 1 h after FGF-22 stimulus. Acquisition of images was performed using an EC Plan-Neofluor 40x oil objective (numerical aperture 1.3). For each position, a z-stack of 9 slices interspaced by 0.31 μm was acquired both at 0h and 1h.

Image acquisition: each stack of images was three dimensionally deconvoluted using Diffraction point spread function (PSF) 3D and Iterative Deconvolve 3D plug-ins in Fiji software, and later collapsed to a single image using the maximal intensity projection. Images were converted to 8-bit images and background signal was subtracted by adjusting the minimum grey value. Correction for the x,y drift was accomplished by running the Stackreg plug-in in Fiji software for each pair of images (0 h and 1 h images). For quantification purposes, images were subtracted using the image calculator tool in Fiji, so that the new and lost myr-EGFP signal along axons throughout the experiment is obtained (subtraction of 0 h image to 1 h image and the reverse operation results in new and lost signal images, respectively). The final images show a punctuated pattern of myr-EGFP expression along axons that is coincident with the sites of higher expression of the reporter. Quantification of puncta was performed using particle analysis in Fiji and the number of new and lost myr-EGFP puncta was calculated. Per axon, the gain in number of myr-EGFP puncta (number of new - number of lost puncta) per length was calculated.

**Microscopy**

Fluorescence images of fixed preparations were acquired from the axonal compartment (unless indicated) using an inverted microscope Zeiss Axiovert Observed Z1, an AxioCamHRm camera and a plan-Apochromat 63x oil objective (numerical aperture 1.4). For PDL-beads, images with z-stack (7 slices spaced by 0.230 μm) were acquired. Co-cultures images were acquired in z-stacks (9 slices interspaced by 0.230 μm) using a plan-Apochromat 63x oil objective (numerical aperture 1.4). Contiguous images, of somal and

axonal compartment, were acquired using a plan-Apochromat 20x oil objective and the tile option.

**Image processing and analysis**

Fluorescence images from fixed preparations were quantified using NIH software Image J. For all analysis the quantifications were performed in a blind manner. For puncta analysis (number, fluorescence and size of puncta), images were converted to 8-bit depth. Images without bundle axons were chosen; in order to eliminate background, images were subjected to a user-defined intensity threshold. Then, number and size of particles (circularity 0.00-1.00), along axon shaft, were quantified using the *analysis of particles* tool. The length of axons were calculated using the plugin skeletonize 2D/3D. For contact-induced presynaptic differentiation or contact-induced F-actin polymerization: bright field (BF) images were used to locate beads in contact with axons and to define regions of interested (ROIs) (On-bead). At the same axon, ROIs without bead-contact was defined and labeled Off-bead. Quantification of mean pixel values of synaptic vesicles and F-actin were recorded in and out regions that contact with beads. For co-localization analysis: we used *JaCop* plugin from imageJ to analyze co-localization-based object (2D) as previously described[286]. The analysis was performed based on geometrical centres-particles coincidence, where the percentage of SV2 geometrical-centre closer to F-actin particles was evaluated. For analysis of p-4E-BP1 fluorescence intensity, integrated intensity corrected to background was quantified and then it was related to total axonal area. All values were normalized to control condition and presented as percentage of control. For co-culture experiments, each stack of images was deconvoluted using Hyugens software and maximum intensity projection was performed using ImageJ software. The number and size of SV2 puncta per axon length was calculated, as previously described, along axons contacting with muscle fibers. The percentage area of α-BTX that contains SV2 particles was quantified and results were presented as % of α-BTX occupancy.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism. When more than two conditions were analyzed, statistical significance was determined using one-way ANOVA and Bonferroni multiple-comparisons *post hoc* test. T-test was used to compare differences between two conditions. For p-4E-BP1 analysis a two-way ANOVA and Dunnet's test was performed.

# CHAPTER III

## LOCAL mRNA TRANSLATION REGULATES PRESYNAPTIC DIFFERENTIATION: β-ACTIN mRNA AS A KEY TARGET

Results in this chapter are being submitted as: Pedro, J.R.; Pinto, M. J.; Costa, R.O.; Alves, P.L.; Ryu, H.R.; Noo L.J.; Jaffrey, S.R.; Almeida, R. D.; Intra-axonal translation of beta-actin mRNA is required for presynaptic differentiation. (Except for latrunculin-A experiments)

Joana R. Pedro performed all experiments except for figure 3.09 (performed by Rui O. Costa) and figure 3.13 (performed by Maria Joana Pinto).

## 3.1 ABSTRACT

Intra-axonal translation of mRNAs is a mechanism by which neurons are able to supply subcellular compartments with newly synthesized proteins. In axons, it has been described that local translation regulates axonal guidance, growth cone collapse and neurotransmitter release dynamics. However, evidences for the requirement of local mRNA translation in presynaptic differentiation are still missing. Here, we show that local protein synthesis is required for FGF-22-induced presynaptic differentiation. Using chick ciliary ganglia neurons we observed that FGF-22, a presynaptic organizing molecule, induces the differentiation of nerve terminals in isolated axons as measured by clustering of SV2, a hallmark of synapse formation. Additionally, when protein synthesis is inhibited specifically in axons, SV2 clusters are reduced to basal levels. We also observed that local protein synthesis regulates FGF-22-induced F-actin polymerization. Live cell imaging revealed that *β-actin* mRNA reporter is locally synthesized in axons upon FGF-22 stimulus. Together these results demonstrate that axonal translation is required for presynaptic differentiation and that *β–actin* mRNA is a key modulator of this process.

## 3.2 INTRODUCTION

Intra-axonal translation ensures the fine control needed for coordinated events during axonal development. For example during axon guidance, attractive and repulsive cues define the axonal route by regulating synthesis of specific transmembranar receptors[204,205]. Then, axon elongation requires interaction between CAMs from axon and its surrounding environment. Local protein synthesis underlies a proper pathfinding by regulating the amount of CAMs present at the axonal membrane surface[174]. Moreover, local mRNA translation also controls synthesis of regulators of the exocyst complex which are important to induce plasma membrane expansion[186]. In neuronal cell death, the axon integrates developmental cues by activating local synthesis of pro-survival factors[225]. Therefore, translation of axonal transcripts originates new on-site proteins required to regulate axonal and even neuronal fate in response to external stimuli.

During synapse formation, the axoplasma suffers incredible structural alterations to form the presynaptic terminal. The formation of specialized molecular complexes occurs in few minutes upon synaptogenic cues[39,40,50,51,59,211] with the recruitment of the SVs and AZ proteins to the site of contact[9,75,84–86]. As a result a presynaptic terminal bouton is formed where SVs accumulate at a proteinaceous active zone[31–34]. This multistep event requires a rapid supply of proteins and its origin is not entirely known.

As axons contain mRNAs related to synaptic function[167,287] its plausible to believe that proteins involved in presynaptic formation could emerge from prompt translation of these localized mRNAs. Indeed, studies in *Aplysia* showed that the mRNA coding for sensorin, a neuropeptide that regulate presynaptic terminal growth, is distributed throughout sensory neurons and it is localized to sites of newly formed synapses[216]. Recently, a study performed in rat hippocampal neuronal cultures demonstrated that treatment with the protein synthesis inhibitor cyclohexamine, decreases β-catenin accumulation in presynaptic terminals, contributing to alterations in vesicle release dynamics[221]. However, the role of local protein synthesis during presynaptic differentiation is unknown.

In this work, we address the role of intra-axonal translation for presynaptic differentiation. We observed that, in isolated axons FGF-22, a target-derived presynaptic organized molecule, and bead-induced synapse formation promotes synaptic vesicle clustering, which was abolished in the presence of protein synthesis inhibitors. In addition, we report that formation of new F-actin clusters, occurring during presynaptic

differentiation also required local protein synthesis. Moreover, new F-actin clusters co-localize with presynaptic boutons. In live cells, a β-actin reporter is locally synthesized in axons upon FGF-22 stimulation, an event that is prevented in the presence of protein synthesis inhibitors. These data indicates that local protein synthesis is required for presynaptic differentiation and that *β-actin* mRNA is a likely player in this process.

## 3.3 RESULTS

### 3.3.1 Presynaptic differentiation is dependent on local protein synthesis

In this study we used neurons from parasympathetic nervous system obtained from embryonic chick ciliary ganglion (CG)[288,289]. These neurons are located in the posterior region of the eye and closely to the oculomotor nerve, from which it receives preganglionic fibers from the Edinger-Westphal nucleus in the midbrain. Then, CGs conducts the postganglionic fibers through the ciliary and choroid nerves to innervate the intrinsic eye muscle where they establish cholinergic synapses on eye muscles[290,291]. Ciliary ganglia neurons are able to grow unusually rapidly on a laminin-based substratum[292,293] and extend their process in 2-3 days *in vitro* (DIV)[294,295]. We chose this model because: 1) CG neurons share morphological and biochemical characteristics and have a similar behavior *in vitro* with spinal motor neurons; 2) unlike motor neurons they grow consistently in microfluidic chambers; 3) they were described to form neuromuscular junctions in vitro[296,297].

Microfluidic devices are novel tools to study axonal processes. These devices are able to fluidically and physically separate axons from their cell body and, as a consequence it is possible to address soma-independent axonal behavior[282]. In order to obtained isolated axons we plated chick embryo CG neurons in microfluidic devices (**Figure 3.1A-D**). Microfluidic devices have two symmetrical compartments, here designated as somal and axonal compartment, separated by a set of microgrooves (**Figure 3.1A**)[282]. Compartments have a height of 100 μm while microgrooves only a 3 μm height (**Figure 3.1B**)[282]. This specific property of microgrooves prevents cell bodies from crossing to the adjacent compartment thus allowing a physical isolation between soma and axons. Moreover, the reduced height of microgrooves restraints the rate of flux in both compartments guaranteeing fluidic isolation[282].

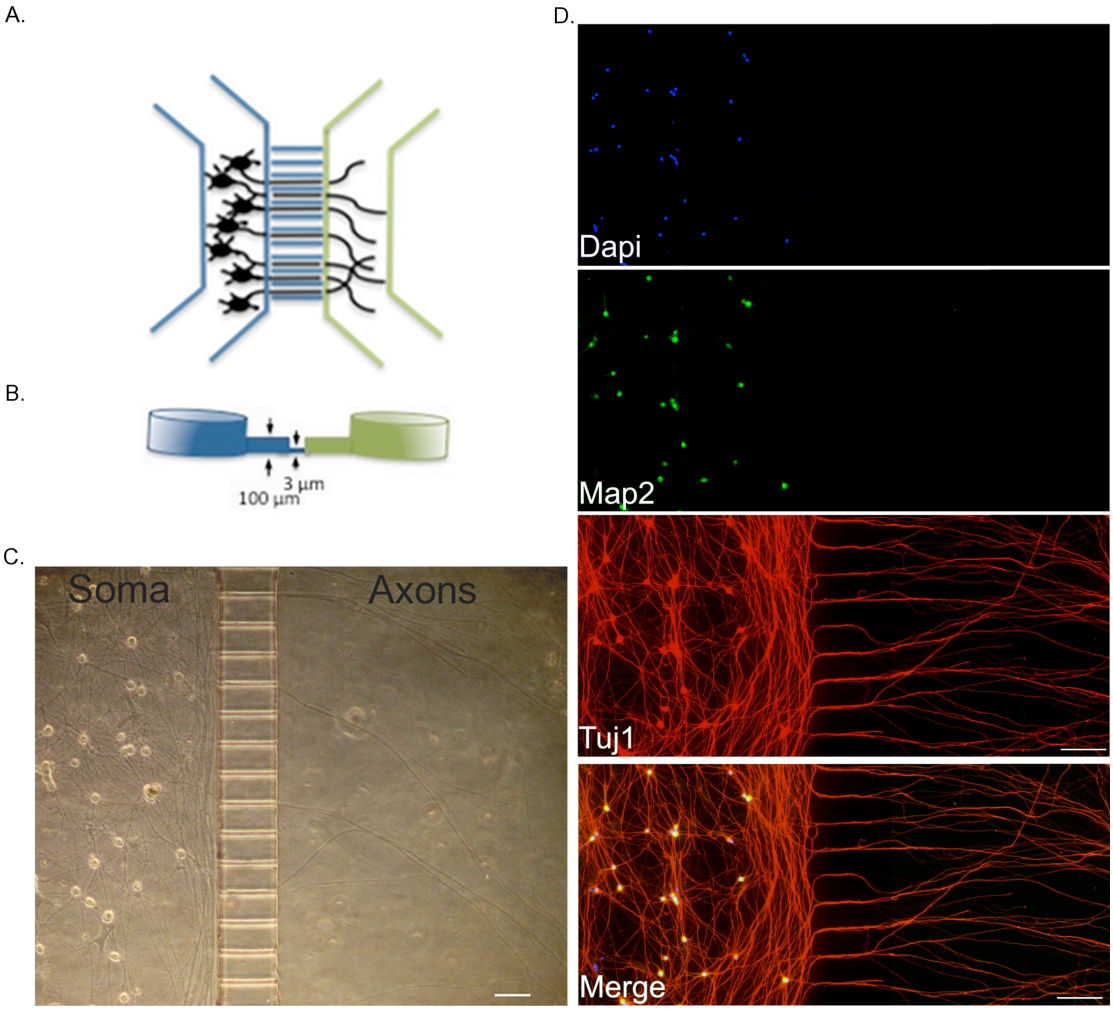



**Figure 3.1 - Axonal isolation in microfluidic devices**. **(A, B)** Schematic illustration of microfluidic devices. **(A)** Upper view representation shows two symmetric compartments (1.5 mm wide, 7 mm long) separated by a set of microgrooves with 150 µm in length and 10 µm wide. Cells are plated in somal compartment and axons grow through microgrooves to opposite symmetric side (axonal compartment). **(B)** Lateral view of microfluidic devices shows that microgrooves have an extremely small height (3 µm). When a little volume difference is maintained between the two compartments a high fluidic resistance guarantees fluidic isolation for at least 20 h between microenvironments[282]. **(C, D)** Ciliary ganglia neurons are able to grow in microfluidic devices. **(C)** Ciliary ganglia neurons were cultured in somal compartment and axonal development was monitored daily. At DIV3, axons elongate along microgrooves reaching the axonal compartment. Image was taken using a Canon Power Shot G10 digital camera associated to an Axiovert 40C microscope (Zeiss) with a 20x objective. The scale bar is 100 µm. **(D)** At DIV3, primary cultures of ciliary ganglia neurons were immunostained for Tuj1 (red), an axonal marker, and MAP2 (green), a dendritic marker. The image shows that axons cross into the axonal side throughout 150 µm microgrooves, cell bodies (DAPI, blue) and dendrites (green) are restricted to the somal side. Contiguous images were taken from a random area of the microfluidic chamber using an AxioObserver Z1 fluorescent microscope with a PlanApochromat 20x objective. The scale bar is 100 µm.

CG neurons were plated in the somal compartments and axons extended through the 150 µm microgrooves into the axonal compartment (**Figure 3.1C and D**). Phase contrast images show that no cell bodies are found in the axonal compartment (**Figure**

**3.1C**). In addition, neurons were immunostained with antibodies against the MAP2 (dendritic marker), Tuj-1 (or tubulin III, axonal marker) and DAPI (nuclear marker). At DIV3, we confirmed that long axons extended in axonal compartment while the dendrites and cell bodies were restricted to the somal compartment (**Figure 3.1D**). Hence, our results confirm that microfluidic chambers allow physical isolation of neuronal subcompartments.

Using microfluidic devices it is possible to apply a specific cue only in the axonal side and evaluate cue-stimulated axonal events, but also to identify with certainty the axonal localization of specific mRNAs and proteins. FGF-22 is a presynaptic organizing molecule described to induce synaptic vesicle aggregation in motor[298] and hippocampal neurons[69]. FGF-22 is target-derived molecule highly expressed in cerebellar granule cells and muscle and it binds to fibroblast growth factor receptor 2 isoform b (FGFR2b). Previous studies demonstrated that loss of FGFR2 impairs presynaptic differentiation *in vivo*[69,298]. We first assessed localization of FGFR2 in chick ciliary ganglion axons. FGFR2 is highly expressed in the cell body and it is also localized to axons, showing a punctuated distribution (**Figure 3.2**). Together these observations suggest that FGF-22-FGFR2 signaling pathways also regulate presynaptic formation in parasympathetic neurons, and that this system is an appropriate model to study axonal-specific events.

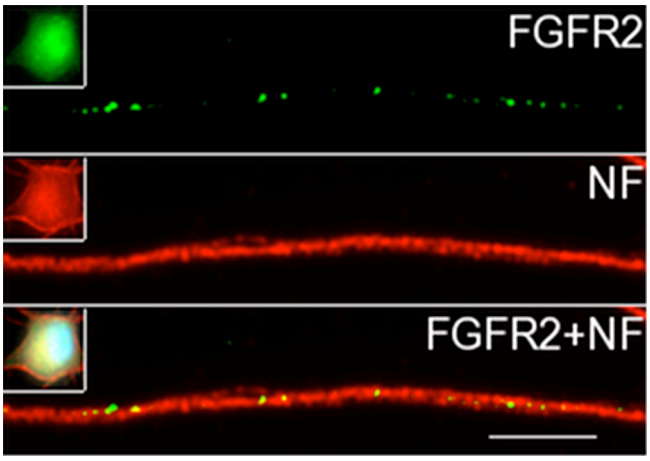



**Figure 3.2 – Localization of FGFR2 in ciliary ganglia axons.** To evaluate the presence of FGFR2 in primary cultures of ciliary ganglia neurons cells were cultured until DIV3 and then immunostained for FGFR2 (green) and neurofilament (NF, red). FGFR2 is expressed in cell body (inset) with a uniform pattern, while in axons FGFR2 was observed in a discrete punctuated pattern. Scale bar is 5µm.

We next asked if FGF-22 could induce presynaptic differentiation when applied specifically to isolated axons. To determine the best concentration to induce presynaptic differentiation we treated isolated axons with BSA (Ctr) or FGF-22 for the indicated time

points (**Figure 3.3 A**). FGF-22-stimulated cells were fixed at the indicated time and immunostaining against synaptic vesicle 2 (SV2) and axonal marker (NF) (**Figure 3.3 A**). Four different parameters were measured: number of SV2 particles, total SV2 puncta area, perimeter and intensity per axonal length. We observed that FGF-22 increased SV2-enriched aggregates by 5 hours of FGF-22 treatment until a maximum of 14 hours (**Figure 3.3 B-E**), indicating that local application of FGF-22 induces presynaptic differentiation.

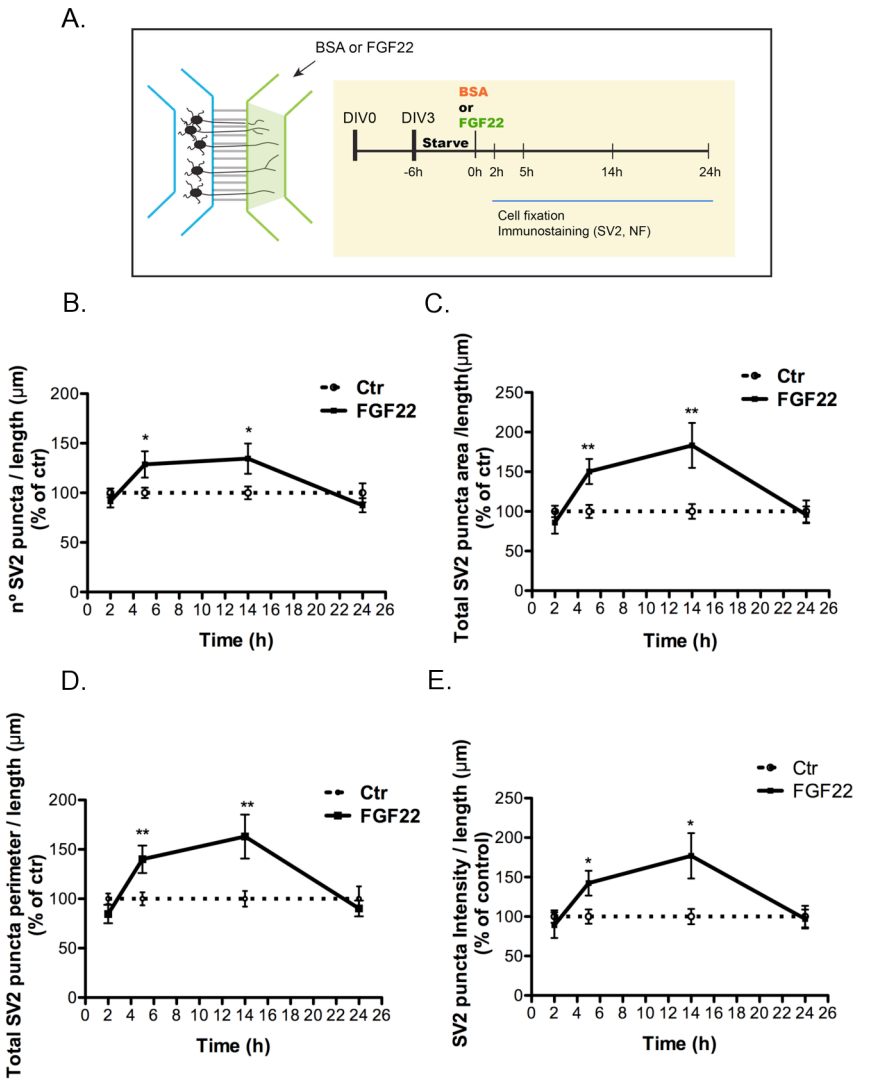



**Figure 3.3 - Axonal application of FGF-22 induces formation of SV2 clusters. (A)** Schematic illustration of the experimental design. Ciliary ganglia neurons cultured in microfluidic chambers have their axons physical isolated from cell bodies. At DIV3, neuronal cells were maintained in starving media (media without growth factors, B27 and FBS) for 6h. Then, vehicle (BSA), indicated as Ctr, or FGF-22 (2nM) were applied in axonal compartment for 2h, 5h, 14h and 24h. Presynaptic differentiation was assessed by immunostaining cells against SV2, a presynaptic marker. Axons were identified using an antibody against neurofilament (NF) (images not shown). **(B-E)** Quantification of SV2 clusters in axons. Analysis of SV2 clusters formation results in four different informative parameters: number of SV2 puncta per length **(B)**, total SV2 particle area per length **(C)**, total SV2 particle perimeter per length **(D)** and total SV2 puncta

intensity per length **(E)**. All those parameters demonstrate that FGF-22 is able to induce clusters of SV2 after 5h incubation. Moreover, its presynaptic effect slightly increases until 14h of stimulus. After that time point, it is observed a reduction in SV2 clusters suggesting that FGF-22 receptors are saturated and axons loss their ability to react to FGF-22. Bars represent the mean ± SEM of 40 images from randomly selected areas of at least two independent experiments. * represents $p<0.05$ and ** represents $p<0.01$ by paired t-test.

Considering that local mRNA translation is a potential regulator of axonal events, we then sought to evaluate whether FGF-22 activates translation pathways along axons. For that purpose, we analyzed the phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which is a marker for cap-dependent translation initiation[147,299]. Axons were stimulated with FGF-22 for different periods of time and immunostained against phospho-4E-BP1 (p-4E-BP1) (**Figure 3.4 A**). We observed that, within 2h, FGF-22 increases the integrated intensity of p4EBP1 per axonal area (**Figure 3.4 B**), indicating that FGF-22 triggers local protein synthesis by activation of a translation regulator. Accordingly, ciliary-neurotrophic factor (CNTF) a survival and differentiation factor for ciliary ganglia neurons also induced an increase in the integrated intensity of p-4E-BP1 per axonal area (**Figure 3.4 C**).

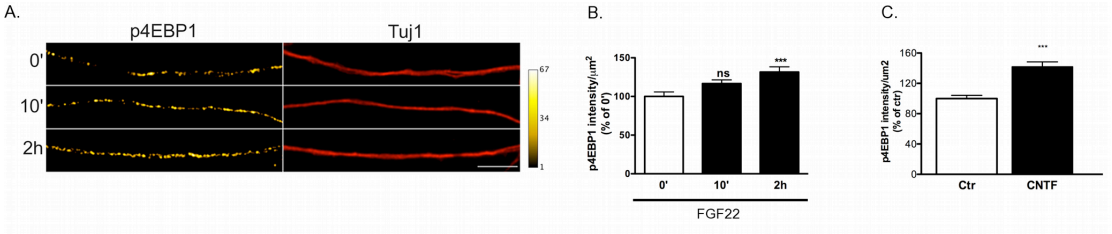
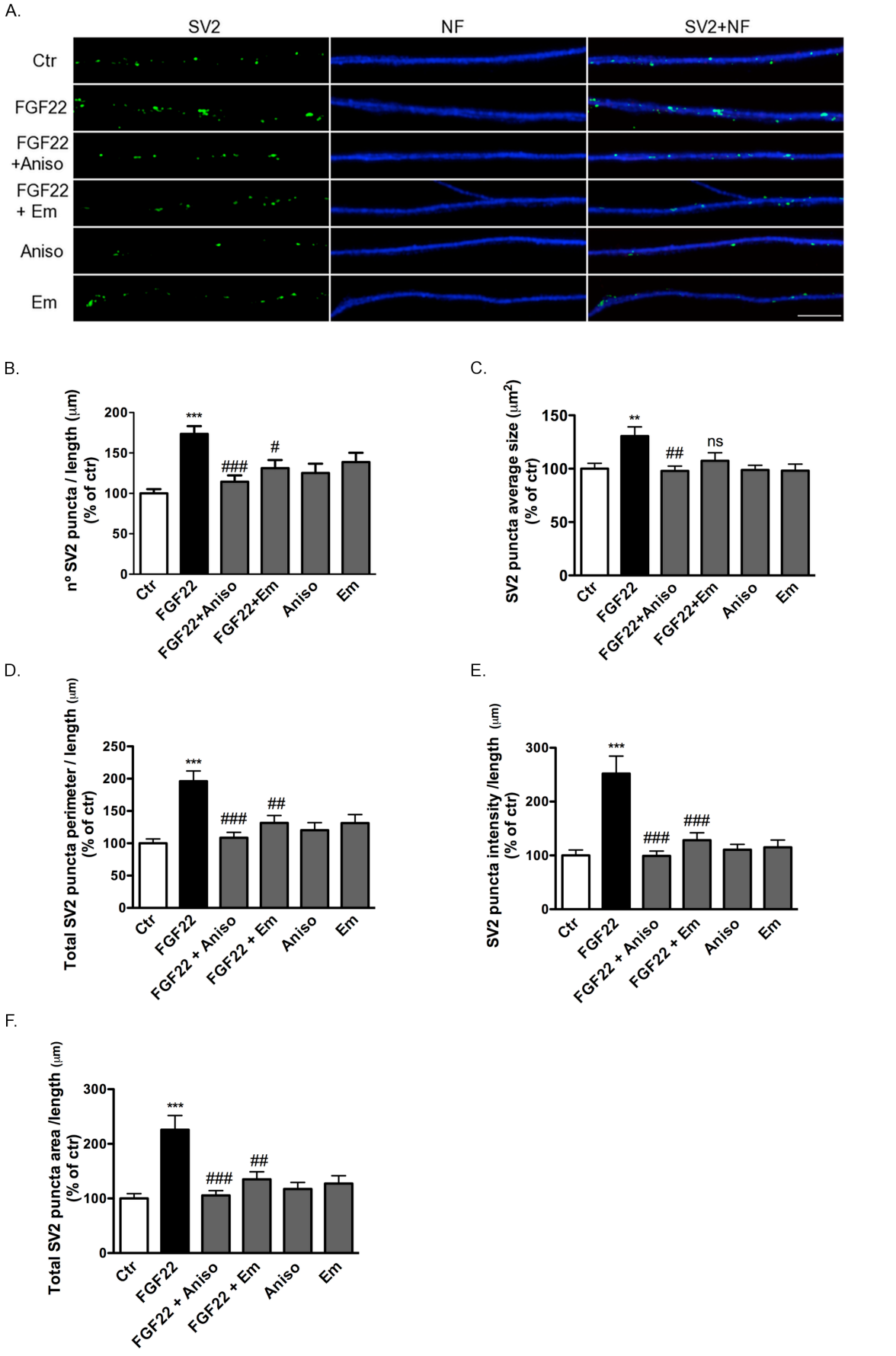



**Figure 3.4 - FGF-22 activates local translation mechanisms. (A and B)** Effect of FGF-22 in axonal translation initiation. (A) Ciliary ganglia neurons were stimulated at DIV3-4 with FGF-22 (2 nM) for the indicated time points. 4EBP1 phosphorylation (p4EBP1) was evaluated by immunocytochemistry using an anti-p4EBP1 antibody (yellow lookup table) and axons were immunostained using an anti-Tuj1 antibody (red). Scale bar is 5 µm. (B) Quantification of integrated intensity of p4EBP1/axonal area ($\mu m^2$). Results show that p4EBP1 levels increase after 2h of FGF-22 stimulation, demonstrating that intra-axonal translation occurs after axonal-specific application of FGF-22. Fluorescence intensity was measured using Image J 1.45e software. Bars represent the mean ±SEM of 20 images from randomly selected areas of 4 independent experiments. *** Represents $p<0.001$ by one-way analysis of variance using Dunnett's multicomparison test when compared to 0'. **(C)** CNTF induces intra-axonal translation. CNTF was applied to starved cells for 2h and integrated intensity of p-4E-BP1 per axonal area ($\mu m^2$) were analyzed. Results show that p-4E-BP1 levels increase after 2h of CNTF stimulation, demonstrating that intra-axonal translation occurs after axonal-specific application with this survival growth factor. Fluorescence intensity was measured using Image J 1.45e software. Bars represent the mean ±SEM of 50-60 images from randomly selected areas of 3 independent experiments. *** Represents $p<0.001$ by unpaired t-test.

Therefore, we asked if local mRNA-regulated translation is important for presynaptic differentiation. To this end, FGF-22 was added to the axonal compartment for 14h in the presence or absence of the protein synthesis inhibitors, anisomycin (aniso) and emetine (em). Then, synaptic formation was assessed by analyzing the formation of SV2 clusters (**Figure 3.5 A**). We observed that FGF-22 led to a significant increase in the number and size of SV2 particles along the axons (**Figure 3.5 B and C**). Additionally, three other parameters, SV2 puncta intensity, total SV2 perimeter and total area per length, were analyzed and significant differences were found between control and FGF-22-stimulated axons (**Figure 3.5 D-F**). Conversely, anisomycin and emetine, which were incubated 30min before FGF-22, prevented the augment in the number of SV2 puncta as well as the augment in SV2 intensity, total perimeter and total area (**Figure 3.5 B, D, E and F**); thus demonstrating that formation of new SV2 clusters induced by FGF-22 was blocked in the presence of protein synthesis inhibitors. Likewise, the effect of FGF-22 on presynaptic puncta size was also abolished by anisomycin (**Figure 3.5 C**). Although not statistically significant, emetine shows a similar tend (**Fig. 3.5 C**). These observations indicate that local mRNA translation is required for FGF-22-induced presynaptic differentiation.

Synapse formation relies on the release of target-derived molecules and on subsequently axo-dendritic contact. In order to confirm the previous results and to mimic synaptic contact, we applied PDL-coated beads (PDL-beads)[85,221] to the axonal compartment. PDL-beads induce adhesion to axonal processes and have been shown to induce accumulation of various presynaptic proteins, including N-cadherin, synaptophysin[85] and bassoon[85,221]. After axon-bead contact we observed that SV2 accumulates in the vicinity of beads as indicated by an increase in SV2 fluorescence intensity (**Figure 3.6 A and B**). In addition, contact-induced SV2 clustering was blocked in the presence of anisomycin or emetine (**Fig. 3.6 A, C and D**). This set of data shows that axon-bead contact induces an augment in presynaptic clusters, which is blocked after protein synthesis inhibition. The PDL-coated beads mimic, with spatial and temporal control, the axo-dendritic synapses. Thus, in addition to target-derived molecules, contact-induced presynaptic differentiation also requires local protein synthesis. Overall, these results indicate that intra-axonal translation is activated and required during presynaptic differentiation.




**(Figure 3.5 – Legend on the next page)**

**Figure 3.5 – Local protein synthesis in axons is required for FGF-22-induced presynaptic differentiation. (A)** Effect of protein synthesis inhibitors in presynaptic assembly. Ciliary ganglia neurons were cultured in microfluidic devices and at DIV3 axons were stimulated with either vehicle (BSA), referred as Ctr, or FGF-22 (2 nM), a presynaptic organizing molecule, for 14h. 2 µM anisomycin (aniso) and 2 µM emetine (em), two distinct protein synthesis inhibitors, were added to the axonal compartment 20 min prior to FGF-22 stimulation. The formation of synaptic clusters was assessed by immunocytochemistry using an antibody against SV2 (green), a synaptic vesicle marker; axons were detected using an antibody against neurofilament (NF, blue). Scale bar, 5 µm. **(B, C)** Quantification of SV2 puncta number per axonal length (B), SV2 average size (C), total SV2 puncta area (D), SV2 puncta intensity (E) and total SV2 puncta perimeter (F) per axon length. FGF-22 stimulation increased the number, intensity and size of particles. Moreover, emetine and anisomycin blocked the increase induced by FGF-22. Similar results were observed for all evaluated parameters. Exception was for SV2 average size in axons treated with emetine, here no significant differences were found. Results indicate that local protein synthesis is required for presynaptic differentiation. Bars represent the mean ± SEM of 40 images from randomly selected areas of at least four independent experiments. *** represents $p<0.001$ and ** represents $p<0.01$ by one-way analysis of variance using Bonferroni's post test when compared to Ctr; ### represents $p<0.001$, ## represents $p<0.01$ and # represents $p<0.05$ by one-way analysis of variance using Bonferroni's post test when compared to FGF-22. ns is non-statistically significant relative to FGF-22. (Image on previous page)

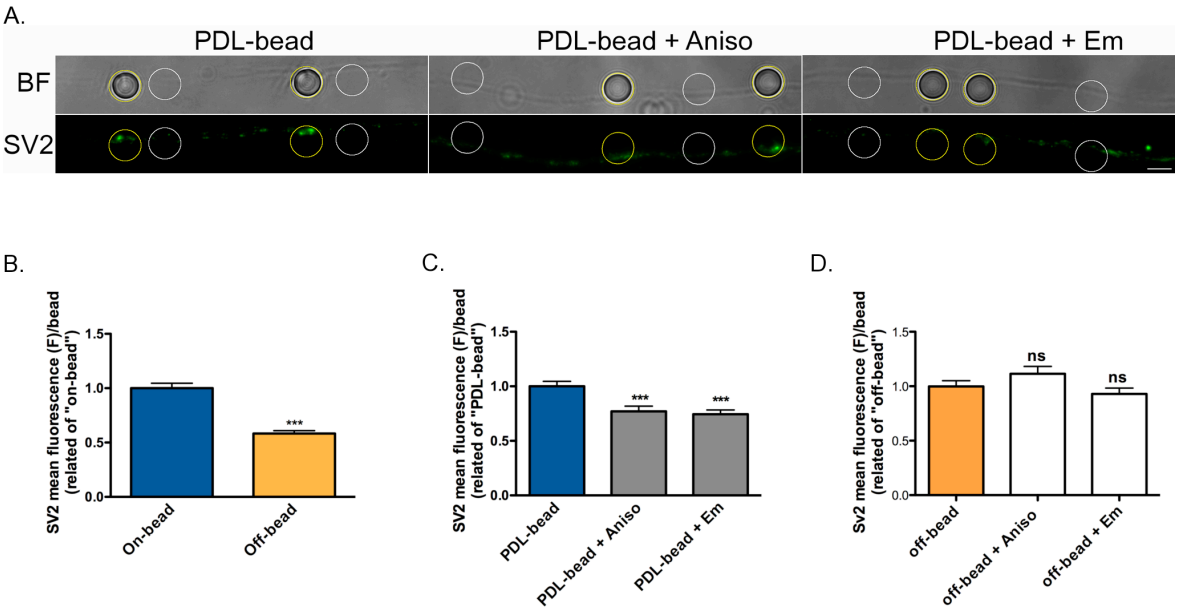



**Figure 3.6 – Contact-induced presynaptic differentiation is dependent on local protein synthesis. (A)** Effect of protein synthesis inhibitors in presynaptic assembly upon axon-bead contact. Axons were exposed to PDL-coated beads, which induce formation of presynaptic terminals[85,221]. Bright field (BF) images show regions of axon-bead contact. Presynaptic assembly was evaluated 14h after beads addition by measuring SV2 (green) intensity within defined regions of interest (ROI) that encompass the bead (On-bead, yellow circle); nearby axonal segments with no beads were used as control and defined using an equal ROI (Off-bead, white circle). 2 µM anisomycin (aniso) and 2 µM emetine (em), were added where indicated to the axonal compartment 20 min prior to the addition of beads. Scale bar is 5µm. (**B**) Assessment of bead-induced presynaptic differentiation. Quantification of SV2 mean fluorescence On-bead vs. Off-bead shows a significant increase in areas of axon-bead contact, indicating that PDL-beads induce presynaptic differentiation in ciliary ganglion axons. Mean pixel values were normalized to the On-coated bead condition. Bars represent ± SEM of at least 100 beads from three independent experiments. *** represents $p<0.001$ by paired t-test analysis. **(C)** Effect of

protein synthesis inhibitors in bead-induced presynaptic assembly. Accumulation of SV2 upon axon-bead contact is blocked by anisomycin (aniso) or emetine (em). Results demonstrate that clustering of presynaptic vesicle requires mRNA local translation. Mean pixel values were normalized to the PDL-coated bead condition. Bars represent ± SEM of at least 100 beads from three independent experiments. *** represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to PDL-bead. **(D)** Protein synthesis dependence of bead-induced presynaptic assembly is contact specific. Assembly of presynaptic clusters was evaluated in axonal regions with no bead contact (Off-bead, white circle). Protein synthesis inhibitors have no effect on SV2 intensity values at basal levels. Results indicate the specificity of local protein synthesis during contact-induced presynaptic assemble. Bars represent ± SEM of at least 100 beads from three independent experiments. ns is non-statistically significant.

### 3.3.2 Local protein synthesis underlies formation of actin filaments in presynaptic formation

Synapse formation is dependent on actin cytoskeleton and its degree of dependence changes with the type of synapses[98,99]. We next asked if local remodeling of the actin cystoskeleton is required for presynaptic differentiation. To that purpose we used a pharmacological approach making use of latrunculin-A. This drug binds to the G-actin monomers and prevents F-actin polymerization. It is also reversible and when removed the actin monomer is free for polymerization[300]. First, we evaluated the local effect of latrunculin-A in the axonal cytoskeleton. For that, we treated axons with DMSO (Ctr) and latrunculin-A, for 5 hours. Then, the levels of axonal F-actin were detected and analyzed by fluorophore-tagged phalloidin (**Figure 3.7 A**). The analysis of three different parameters, the number and total area of F-actin per axonal length and the average size of F-actin, revealed that 5h of treatment causes a significant reduction in the number and total area of F-actin per axonal length (**Figure 3.7 B and C**). F-actin average size showed a tendency to decrease but was not statistically significant when compared to control (Ctr) (**Figure 3.7 D**).

We next evaluated whether formation of presynaptic formation is dependent on cytoskeleton remodeling. Axons were stimulated with FGF-22 in the presence or absence of latrunculin-A (using the appropriate solvents as vehicle (Ctr)). The drug was incubated in axonal compartment during the first 8 hours of FGF-22 stimulus and then removed to prevent toxicity. After 14 hours of stimulation with FGF-22, cells were immunostaining against SV2, NF and F-actin (**Figure 3.8 A**). Quantitative analysis demonstrated that early alterations in actin cytoskeleton during FGF-22-induced presynaptic differentiation affects the number and total area of SV2 clusters per axonal length without affecting SV2 average size (**Figure 3.8 B-D**). Unexpectedly, the average size of SV2 particles increases in axons treated only with latrunculin-A (**Figure 3.8 D**) suggesting that parallel FGF-22-

independent mechanisms regulate actin polymerization to compensate for the initial F-actin disruption. Overall the results indicate that the F-actin network regulates the formation of new presynaptic sites.

Interestingly, axons treated with latrunculin-A did not experience drastic reduction in F-actin levels when compared with non-treated axons. In fact, in drug-treated axons the levels of F-actin tend to increase when compared to the control condition (Ctr) (**Figure 3.8 E-G**). A possible explanation for this effect is the reversible effect of latrunculin-A. In the last 6h of the experimental protocol the drug was removed from media so the actin monomers previously blocked by latrunculin-A are now available to initiate actin polymerization. Importantly, this "delayed" polymerization is not sufficient to re-establish the number of SV2 clusters induced by FGF-22 (**Figure 3.8 B and C**). These results indicate that the early polymerization of actin filaments is an important step for FGF-22-induced presynaptic differentiation.

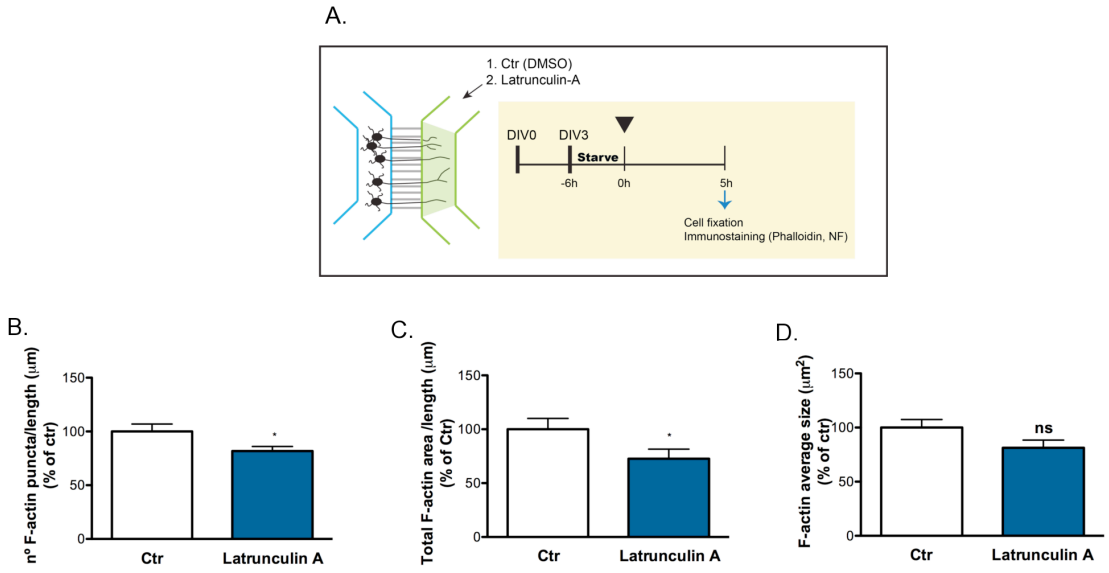



**Figure 3.7 – Local application of latrunculin-A disrupt F-actin filaments in ciliary ganglion axons. (A)** Schematic representation of the experimental procedure. Starved cells were incubated with vehicle (DMSO), indicated as Ctr, or latrunculin-A (latrunculin A), for 5h, a drug that prevents F-actin polymerization. **(B, C, D)** Quantification of F-actin number per axonal length (B), total F-actin area per axon length (C) and F-actin average size (D). Latrunculin-A treatment reduces number and size of F-actin per axon length but do not affect F-actin average size. Results suggest that latrunculin-A treatment prevents polymerization of new F-actin filaments without altering the size of pre-existing actin filaments. Bars represent the mean ± SEM of 30-40 randomly selected images of at least three independent experiments. * represents $p<0.05$ by unpaired t-test analysis. ns is non-statistically significant.

We next asked whether, early in synaptic development, FGF-22 activates actin polymerization in a manner dependent on local mRNA translation. In order to address this question, axons were incubated with FGF-22 in the presence or absence of anisomycin and then immunostaining against actin filaments. In the first 5 hours of FGF-22 stimulation it was detected significant changes in F-actin polymerization. Local application of FGF-22 increases F-actin puncta number per axonal length but not its average size indicating that new actin filaments are being formed during FGF-22-induced presynaptic differentiation (**Figure 3.9 A-C**). Thus, FGF-22-mediated F-actin formation is regulated by local protein synthesis.

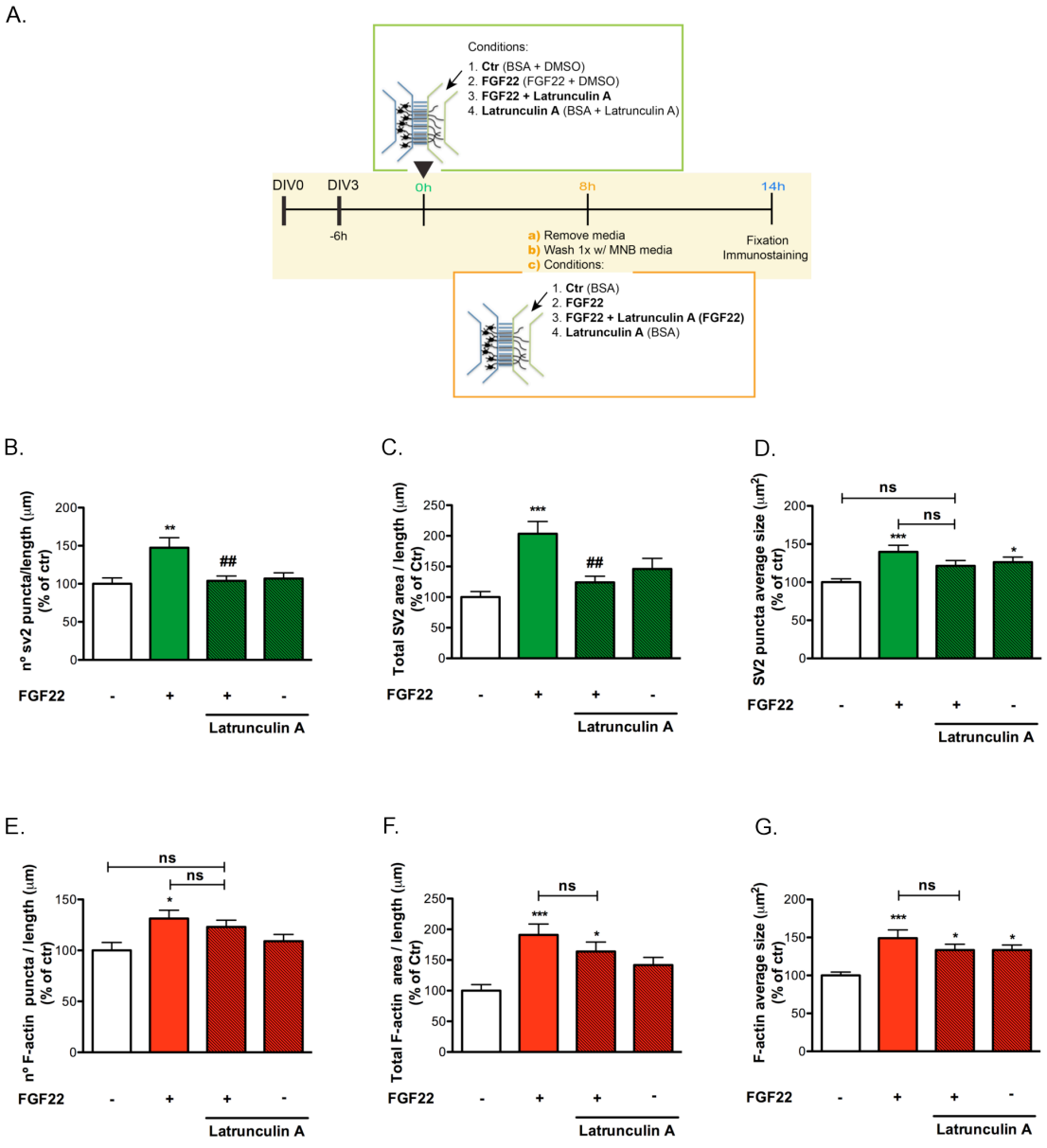



**Figure 3.8 – (legend on next page)**

**Figure 3.8 – Initial disruption of F-actin negatively affects formation of SV2 clusters. (A)** Schematic representation of axonal treatment with latrunculin-A and FGF-22. Starved cells were incubated with vehicle (BSA + DMSO), indicated as Ctr, or FGF-22 (FGF-22+DMSO) for 14h. During the first 8h, axons were simultaneously treated with latrunculin-A. Since this drug induces cell toxicity, after a 8h incubation period media with lantrunculin-A was removed and fresh media containing BSA or FGF-22 was added until 14h, cells were then fixed and labeled for F-actin and immunostained against SV2 (synaptic marker) and NF. **(B-D)** Quantification of SV2 number (B), total SV2 area per axon length(C) and SV2 average size (D). Treatment with latrunculin-A prevents formation of FGF-22-induced presynaptic clusters indicated by reduction in SV2 puncta number and area per length. No statistical difference was observed in SV2 average size Bars represent the mean ± SEM of 40 randomly selected images of 4 independent experiments. * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to Ctr and $^{\#\#}$ represents $p<0.01$ by one-way analysis of variance using Bonferroni's post test when compared to FGF-22. ns is non-statistically significant. **(E-G)** Quantification of F-actin puncta number (E), total F-actin area per axon length (F) and F-actin average size (G). Latrunculin-A has no effect of FGF-22-induced F-actin puncta, presumably due to the reversible nature of the drug. Bars represent the mean ± SEM of 40 randomly selected images of 4 independent experiments. * represents $p<0.05$ and *** represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to Ctr. ns is non-statistically significant. (Figure on previous page)

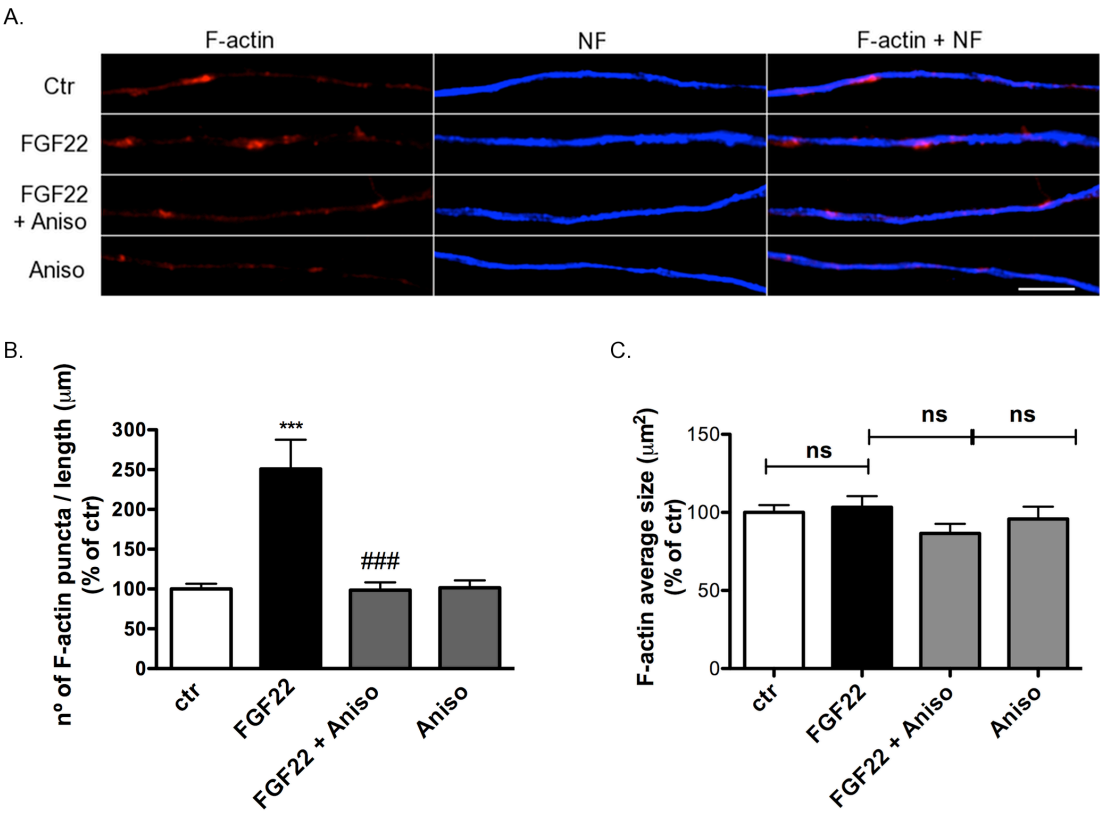



**Figure 3.9 – FGF-22-induced F-actin polymerization is dependent on local protein synthesis. (A)** FGF-22 induces the formation of F-actin clusters in axons. Ciliary ganglia axons were stimulated with either vehicle (BSA), indicated as Ctr, and FGF-22 (2 nM) for 5h. Protein synthesis inhibitor, 2 μM anisomycin (aniso), were pre-incubated 20min before addition of FGF-22. The polymerization of β-actin (F-actin) was assessed by fluorescence microscopy using the actin-stain phalloidin 555 (red). Neurofilament (NF) was used as axonal marker (blue). Scale bar is 5μm. **(B, C)** Quantification of F-actin puncta number per axon length (B) and F-actin average size (C). Axonal application of FGF-22 increased the number and size of F-actin puncta, which was blocked in the presence of anisomycin. The results demonstrate that

formation of new F-actin filaments is dependent on axonal translation. Bars represent the mean ± SEM of 40 randomly selected images of at least four independent experiments. *** represents p<0.001 by one-way analysis of variance using Bonferroni's post test when compared to Ctr and ### represents p<0.001 by one-way analysis of variance using Bonferroni's post test when compared to FGF-22.

Additionally, specific axon contact with PDL-coated beads also demonstrated similar results (**Figure 3.10 A-D**). These results suggest that mRNA translation regulates appearance of new F-actin spots during presynaptic differentiation.

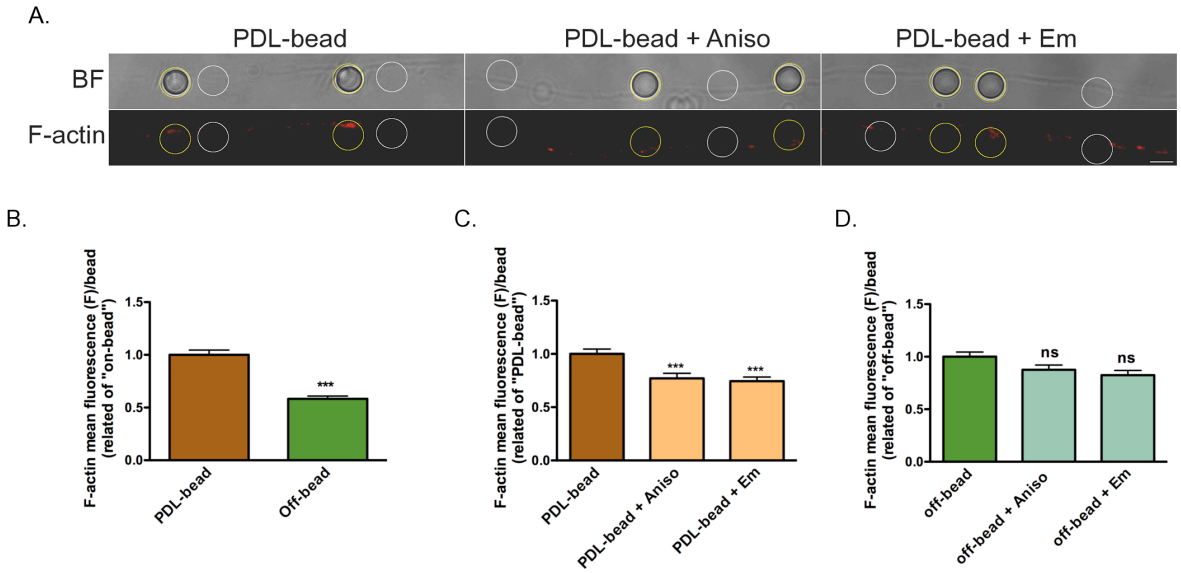



**Figure 3.10 – F-actin clusters are formed upon axon-bead contact, in a manner dependent on local protein synthesis. (A)** Effect of local translation in contact-induced F-actin formation. Axons were exposed to PDL-coated beads in presence or absence of protein synthesis inhibitors, as previously described in Figure 3.6. F-actin was measured within defined regions of interest (ROI) that encompass the bead (On-bead, yellow circle) and nearby axonal segments with no beads (Off-bead, white circle) (upper panel). The polymerization of β-actin (F-actin) was assessed by fluorescence microscopy using the actin-stain phalloidin 555 (red) (lower panel). Scale bar is 5µm. **(B-D)** Quantification of F-actin mean fluorescence within bead. Analysis comparing On-bead vs Off-bead shows that F-actin accumulates within bead-ROI (p<0.0001, paired t-test) (B). However, the protein synthesis inhibitors anisomycin (aniso, 2µM) and emetine (em, 2µM) block F-actin accumulation in bead-induced synapse formation (C) but not in off-bead areas (D). Contact-induced F-actin formation is dependent on local protein synthesis. Bars represent ± SEM of at least 100 beads from three independent experiments. *** represents p<0.001 by one-way analysis of variance using Bonferroni's post test when compared to PDL-bead. ns is non-statistically significant.

The simultaneous increase in F-actin puncta and SV2 clustering raises the possibility that presynaptic differentiation might rely on local F-actin polymerization. To analyze the spatial distribution of new F-actin puncta relatively to presynaptic boutons we performed co-localization analysis. We observed matched co-localization of FGF-22-

induced F-actin puncta with new presynaptic sites (**Figure 3.11 A and B).** We further observed that when local protein synthesis was blocked co-localization was lost (**Figure 3.11 B**). Altogether, these results show that F-actin polymerization occurs in the vicinity of synaptic vesicle clustering and establish a new link between axonal translation and formation of an F-actin network at new presynaptic sites.

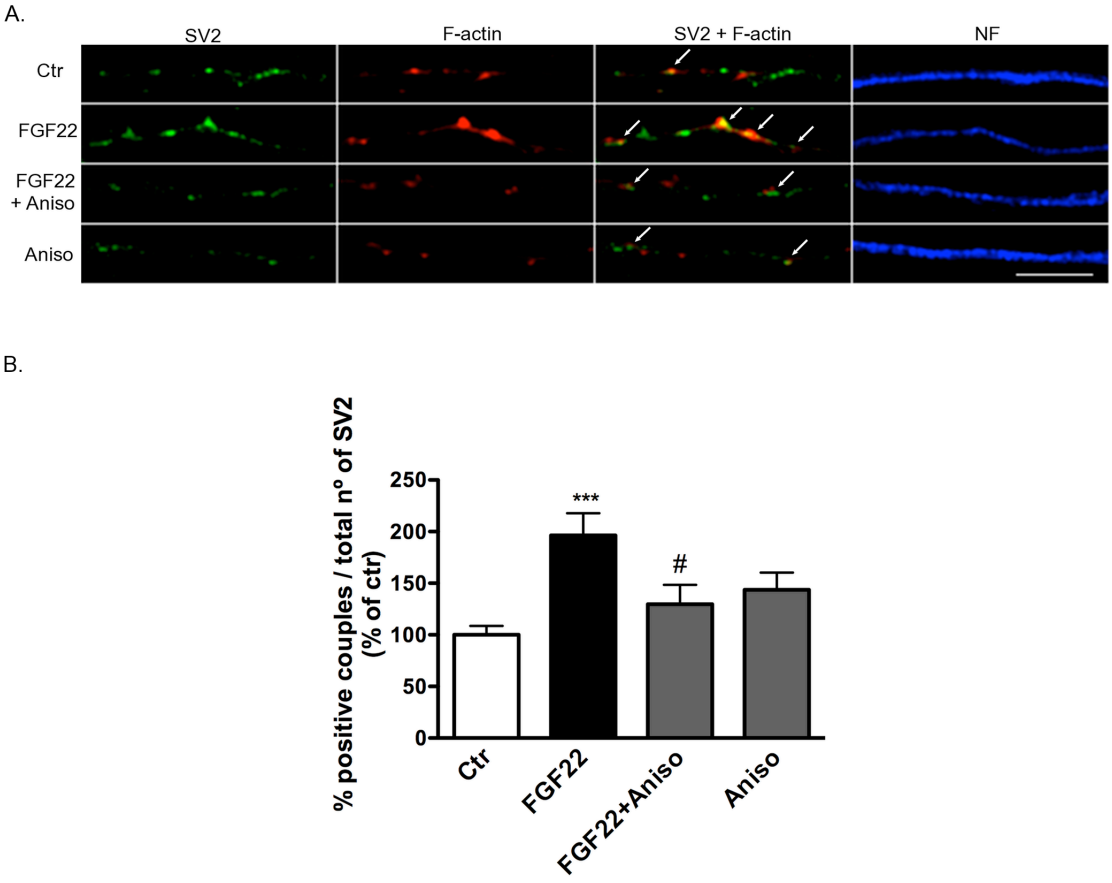



**Figure 3.11 – F-actin accumulation occurs at synaptic sites. (A)** F-actin puncta co-localizes with presynaptic clusters. Ciliary ganglion axons were grown on microfluidic chambers and axons stimulated with either BSA (Ctr) or FGF-22 (2 nM) for 5h. Anisomycin (aniso, 2 µM), was pre-incubated 20min before FGF-22 addition. The polymerization of β-actin (F-actin) was assessed using the actin-stain phalloidin 555 (red) and presynaptic assembly using an anti-SV2 antibody (green). Axons were labeled with anti-NF (blue). White arrows in the merged image indicate the co-localization between F-actin and SV2. Scale bar is 5µm. **(B)** Quantification analysis of F-actin particles coincident with SV2 clusters. F-actin particles (red) increase nearby synaptic clusters (SV2, green) after FGF-22 stimulation. This event is blocked when local protein synthesis is inhibited by anisomycin (Aniso). These observations indicate that FGF-22-induced presynaptic differentiation recruits F-actin to synaptic sites. More importantly, this process is dependent on local protein synthesis since anisomycin blocked this effect. Results are expressed as the percentage of positive couples (SV2+F-actin) divided by the overall of SV2 particles. Bars represent the mean ± SEM of 40 randomly selected images of at least four independent experiments. *** represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to Ctr and # represents $p<0.05$ by one-way analysis of variance using Bonferroni's post test when compared to FGF-22. Co-localization was performed using the plugin JACoP from ImageJ.

### 3.3.3 *β-actin* mRNA is locally translated in axons during presynaptic differentiation

*β-actin* mRNA was one of the first mRNAs identified in distal axons[142,188]. Because it codes for a cytoskeleton protein its local translation is relevant for axon processes like outgrowth and guidance[147,151]. In line with these evidences we found *β-actin* mRNA in axons of CG neurons. Reverse transcription polymerase chain reaction (RT-PCR) from axonal and somal RNA extracts revealed the presence of *β-actin* mRNA in soma and axons. *Histone* mRNA, which is restricted to the cell body, was used as a negative control (**Figure 3.12**).

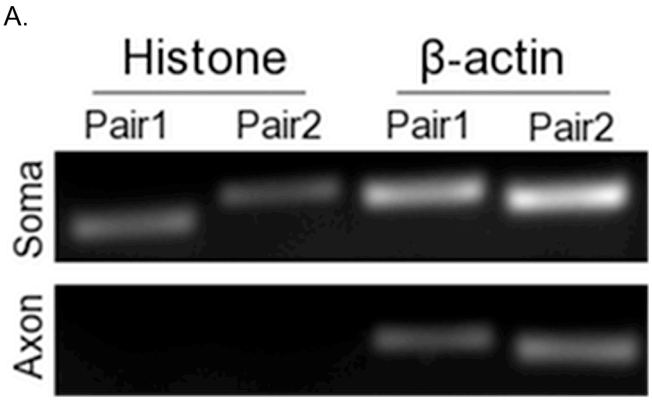



**Figure 3.12 – *β-actin* mRNA is present in axons of ciliary ganglia neurons.** *β-actin* mRNA was shown to be localized in distal axons of different neuronal populations. To confirm the presence of *β-actin* mRNA in parasympathetic neurons (ciliary ganglia) reverse transcription-PCR (RT-PCR) was performed. Neurons were grown for 7 days in microfluidic chambers and then the RNA from the Somal and Axonal side was extracted using Trizol. cDNA was generated and RT-PCR with two distinct primer pairs for both Histone and *β-actin* was performed. Somal vs axonal preparations reveals the presence of *β-actin* mRNA in the somal and axonal side. As expected Histone mRNA (negative control) was only observed in the somal side demonstrating the purity of the axonal preparation. Taken together this results show that *β-actin* mRNA is present in distal axons of ciliary ganglia neurons.

The actin cytoskeleton is a key component at the presynaptic terminal. Interesting, we previously observed that local protein synthesis regulates actin polymerization during FGF-22-induced presynaptic differentiation. So, we wondered whether translation of *β-actin* mRNA is activated upon a stimulus-inducing presynaptic differentiation. To explore this question we used a β-actin reporter assay which consists of a myristoylated and destabilized form of EGFP fused to the 3'UTR of β-actin, thus working as a β-actin reporter (EGFP-3'UTR$^{\beta\text{-actin}}$)[150] (**Figure 3.13 A**). The 3'UTR of β-actin contains an axonal localization signal, the *zipcode* sequence[235,301] which is responsible for mRNA transport

from the cell bodies to axons. Once in axons, synthesized EGFP-3'UTR$^{\beta\text{-actin}}$ is immediately anchors to the membrane and has a half-life of 2h, and so, its fluorescence signal is a direct temporal and spatial marker for β-actin 3'UTR-driven local protein synthesis events. A live imaging approach revealed an increase in EGFP-3'UTR$^{\beta\text{-actin}}$ puncta along axons upon global cell contact with FGF-22 (**Figure 3.13 B and C**). Moreover, blockage of protein synthesis, by anisomycin, prevented EGFP-3'UTR$^{\beta\text{-actin}}$ puncta formation (**Figure 3.13 B and C**). The augment in EGFP-3'UTR$^{\beta\text{-actin}}$ puncta along axons represents the translation of endogeneous *β-actin* mRNA, which is blocked by application of protein synthesis inhibitors. Thus, the presynaptogenic molecule FGF-22 activates local protein synthesis of a β-actin reporter, in a manner dependent on protein synthesis.

## 3.4 DISCUSSION

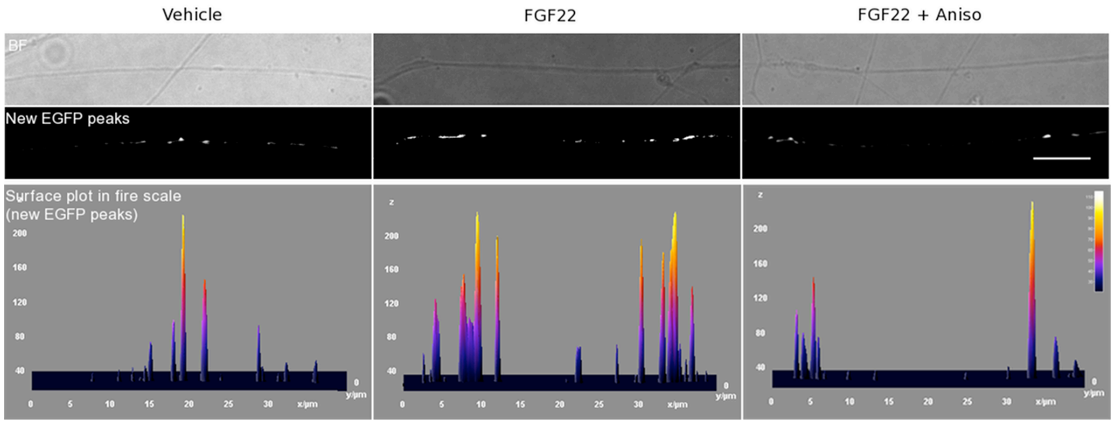
Synaptogenesis is a complex process that prompts signaling events for an accurate and functional neuron communication. Our study shows that FGF-22 regulates presynaptic differentiation and the actin cytoskeleton through local *β-actin* mRNA translation. FGF-22, a presynaptic organizing molecule, triggers signaling pathways that activate protein synthesis in axons, as indicated by an increase in p-4E-BP1 in FGF-22-stimulated axons. During development, axons rapidly respond to guidance cues by activating intra-axonal translation events that in turn support turning or outgrowth[144,148,149,151,187,302]. In our study, we observed that local protein synthesis is required for FGF-22-induced formation of new presynaptic boutons. In addition, similar results were observed upon axon contact with PDL-beads. Using this approach we observed, at axon-bead contact, an increase in synaptic clusters that was dependent on local protein synthesis. Overall, results suggest a role of local mRNA translation during presynaptic differentiation.

Studies performed in *Aplysia* demonstrated that synapse formation regulate the distribution of specific mRNAs[215] and the mRNA coding for sensorin, a neurotransmitter with neurotrophin-like properties, was localized to the sites of newly formed synapses[216]. Moreover, in *Xenopus* it was shown that the contact between presynaptic axon and BDNF-coated beads increases secretion of neurotransmitters at synapses and it requires synthesis of new proteins[217]. Recently, Schuman and co-workers (2013) established the importance of axonal β-catenin for vesicle release dynamics, proposing local translation as a propulsive mechanism for the regulation of synaptic function[221]. However, until now, no

study revealed a link between regulated local mRNA translation and presynaptic differentiation in mammalian systems. Our results demonstrate that mRNA translation is a key regulator mechanism for presynaptic differentiation.

A.


B.




C.

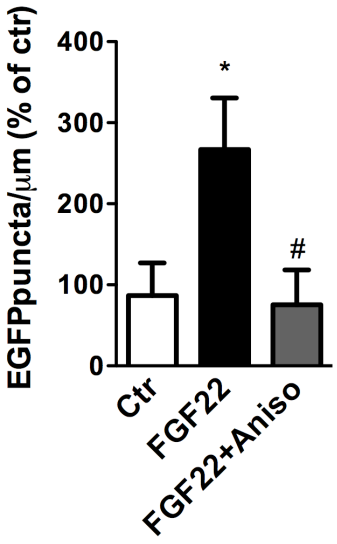



**Figure 3.13 - FGF-22 activates local synthesis of a β-actin reporter.** (**A**) Scheme of the pSinrep β-actin reporter construct. This reporter express a destabilized (d2) form of EGFP fused to the 3'UTR of β-actin that gets anchored to the plasma membrane at the site of translation due to a C-terminal myristoylation sequence (myr). The 3'UTR of β-actin contains a *zipcode* sequence described to be responsible for axonal localization of the mRNA[235,236] (**B**) Effect of FGF-22 in intra-axonal β-actin reporter translation. At DIV2, neurons were infected for 30-36h with the Sindbis virus containing the reporter construct described in (A). FGF-22 (2 nM) was applied to ciliary ganglia neurons for 1h. Images from distal

axons were acquired before (0h) and 1h after FGF-22 addition. Randomly selected axons were visualized in phase images (top panels) and new myr-EGFP puncta was observed by widefield fluorescence microscospy (middle panels). The bottom panel represents a fire-scale surface plot of new myr-EGFP signal. FGF-22 increases the number of new sites of translation of the β-actin reporter in axons and this effect is blocked in the presence of anisomycin (aniso, 2 μM). BSA was used as vehicle (Ctr). Scale bar is 10μm. (**C**) Quantification of EGFP puncta per axonal length. The increase in myr-EGFP puncta number was determined in individual axons and plotted. Anisomycin completely reverts the increase in myr-EGFP puncta along axons induced by FGF-22, indicating that local translation of a β-actin reporter is dependent on local protein synthesis. These results also suggest that endogenous a β-actin is locally translated in response to a synaptogenic cue. Bars represent the mean ± SEM of 75-90 axons per condition of three independent experiments. * and # represents $p<0.01$ by ANOVA followed by Bonferroni's post test when compared to Ctr and FGF-22, respectively.

Among events that control presynaptic terminal formation is the remodeling of cytoskeleton. At presynaptic terminals, F-actin network acts as a scaffold for developing synapses[103,105], it recruits and concentrates presynaptic vesicles at new presynaptic sites[100,102]. Moreover F-actin filaments are involved in synaptic vesicle accumulation that will originate the readily releasable pool[104,105]. In this study, we observed that recruitment of new synaptic vesicles is highly dependent on F-actin network corroborating the idea that actin cytoskeleton is a key organizer of the presynaptic terminal. However, how F-actin polymerization is regulated is not entirely understood. Here, we showed that local mRNA translation mediates FGF-22/bead-induced F-actin polymerization. Additionally, new actin filaments increase at the synaptic sites indicating an interaction between actin polymerization and presynaptic assembly. These results indicate an intracellular mechanism responsible for regulating cytoskeleton remodeling at the presynaptic terminal.

In neurons, the γ- and β-actin are the main components of actin cytoskeleton. However, the *β-actin* mRNA is localized in cell bodies and axons while the γ-actin is exclusive to the cell body[142] thus, in axons the actin cytoskeleton is singularly formed by β-actin monomers. We found that a β-actin reporter is locally synthesized in axons after FGF-22 stimulation. Therefore, we hypothesized that newly synthesized β-actin increases concentration of actin monomers promoting F-actin assembly. Accordingly, we suggest that local translation of *β-actin* mRNA underlines formation of F-actin required for presynaptic differentiation.

In conclusion, in this chapter we demonstrated that formation of presynaptic terminal boutons requires local mRNA translation and that β-actin mRNA is likely the mRNA target since it is an essential piece to built actin network at presynaptic boutons. Thus, presynaptic formation and cytoskeleton remodeling are intrinsically regulated by local mRNA translation.

# CHAPTER IV

## FUNCTIONAL ROLE OF AXONAL β-ACTIN IN PRESYNAPTIC ESTABLISHMENT

Results in this chapter are being submitted as: Pedro, J.R.; Pinto, M. J.; Costa, R.O.; Alves, P.L.; Ryu, H.R.; Noo L.J.; Jaffrey, S.R.; Almeida, R. D.; Intra-axonal translation of beta-actin mRNA is required for presynaptic differentiation.

Joana R. Pedro performed knock-down assays. Maria Joana Pinto performed figure 4.1. Rui O. Costa and Joana R. Pedro performed co-cultures experiment.

## 4.1 ABSTRACT

Local translation of *β-actin* mRNAs has been described in recent years, with evidence for local translation in axonal outgrowth, turning and guidance. β-actin is a cytoskeleton proteins and its local synthesis mediates cytoskeleton remodeling, which in turn is required for axonal processes. We previously identified the *β-actin* mRNA as a target for local translation occurring during presynaptic differentiation, however the role of its coding protein at synapses is not known. Using a reporter for the *β-actin* mRNA and RNA interference strategy we demonstrate that localization and translation of that mRNA is required for SV2 clusters formation and polymerization of actin. Moreover, mislocalization of the *β-actin* mRNA in axons compromises *in vitro* neuron-muscular junctions as evidenced by a reduction in the number of SV2 present at the synaptic contact.

Overall these results demonstrate that local synthesis of β-actin regulates presynaptic differentiation.

## 4.2 INTRODUCTION

Axonal mRNAs and its regulated translation confer to axons an autonomous ability to respond directionally to extrinsic signals. Indeed, local protein synthesis regulates cue-induced directional steering[144], axon turning[148,149,151], axon outgrowth[187,302], axon regeneration[146,154,155,225] and presynaptic release dynamics[221].

Axonal mRNA species represent a variety of protein families. Genome analysis of immature and mature axons has revealed the presence of a wide range of transcripts coding for cytoskeleton, presynaptic and translation machinery proteins[167,184,287]. One of such transcripts is *β-actin* mRNA, which is highly enriched within axonal growth cones[147,303,304]. Localization and translation of *β-actin* mRNA are regulated by RBPs that recognize isoform-specific 3'-untranslated (3'UTR) sequences[235,236]. ZBP1, zipcode-binding protein[237], and SMN/hnRNPR[274] are two RBPs with recognition sites for *β-actin* mRNA.

The *β-actin* mRNA is localized to the leading edge of the growth cone where actin polymerization is actively promoting directional movements. In fact, a gradient of netrin-1 or BDNF induces asymmetric localization and translation of *β-actin* mRNA on the gradient near side[147,151]. Consequently, occurs an augment of β-actin levels leading to actin cytoskeleton assembly, which supports Netrin-1/BDNF-induced directional motility of axons[147] and defines cue-induced axonal turning[151]. Therefore, spatial localization and translation of *β-actin* mRNA plays an important role in axonal outgrowth, turning and guidance.

The formation of an efficient presynaptic terminal is dependent on cytoskeleton remodeling. F-actin network supports structural organization of the active zone[103] and synaptic vesicle cluster[100–102] likewise coordinates vesicle mobility to exocytosis[105]. These studies have identifying molecular regulators of actin assembly however it is not understood how actin monomers are supplied to the presynaptic site in order to favor actin polymerization.

In the presynaptic terminal, the β-actin is a major constituent of the F-actin network. Moreover, in chapter III we demonstrated that *β-actin* mRNA is locally synthesized in axons upon a presynaptogenic stimulus. Hence, in this chapter we address the role of localized and translated *β-actin* mRNA for presynaptic bouton formation. We show that a mislocalization of *β-actin* mRNA affects FGF-22-induced F-actin polymerization and presynaptic differentiation. Moreover, inhibition of local β-actin synthesis by RNA interference negatively affects presynaptic clusters formation. Finally,

axonal delocalization of β-actin mRNA in an *in-vitro* neuron-muscular co-culture highlights a physiological role for β-actin synthesis in presynaptic formation.

## 4.3 RESULTS

### 4.3.1 Axonal *β-actin* mRNA is a prerequisite for F-actin polymerization and presynaptic differentiation

Previous results (Chapter III) suggest that local β-actin synthesis might regulate F-actin polymerization and, as a consequence, presynaptic differentiation. To further test this link, we investigated whether mislocalization of *β-actin* mRNA to axons affects actin remodeling and synapse formation. In these experiments, we used the β-actin reporter described previously (**Figure 3.13A**) as a dominant negative. Cells were cultured in microfluidic chambers and infected with pSindbis virus in the somal compartment. We hypothesized that overexpression of the reporter abolishes anterograde transport of endogenous *β-actin* mRNA. The 3'UTR of β-actin present in the reporter competes with endogenous β-actin mRNA for association with mRNA-binding proteins[123,269]. Consequently, reduced levels of endogenous β-actin mRNA will be transported to distal axons and growth cones. After 20h of viral infection, FGF-22 was applied to axons for 14h. Then, presynaptic puncta and F-actin clusters were assessed by immunocytochemistry (**Figure 4.1A**). Our results, revealed that, in axons lacking endogenous *β-actin* mRNA, FGF-22 was unable to induce changes in F-actin number and puncta size (**Figure 4.1B and C**). These results indicate that axonal localization of *β-actin* transcript is required for FGF-22-induced actin polymerization. To determine the effect of *β-actin* mRNA mislocalization in synapse formation, we analyzed SV2 clusters under the same condition described above. Indeed, similar to F-actin clusters, mislocalization of endogenous *β-actin* mRNA prevents augment of both number and SV2 puncta size (**Figure 4.1D and E)** indicating that FGF-22-induced presynaptic assembly is dependent on spatial localization of *β-actin* mRNA.

In conclusion, axonal *β-actin* mRNA is required for FGF-22-induced F-actin polymerization and presynaptic differentiation.

### 4.3.2 Local *β-actin* mRNA translation is required for presynaptic differentiation

Since F-actin polymerization and presynaptic differentiation depend on axonal localization of *β-actin* mRNA, we asked if locally translation of this transcript governs

synapse formation. For that purpose, we specifically silenced *β-actin* mRNA in axons using two different small interference RNAs (siRNAs) that target specific sequences in the open reading frame (ORF) (siRNA1) and in the 5'UTR (siRNA2) of chick *β-actin* transcript. In chick embryo fibloblasts (CEF) siRNA 1 and 2 reduce β-actin levels by 69% and 15%, respectively (**Figure 4.2 A and B**).

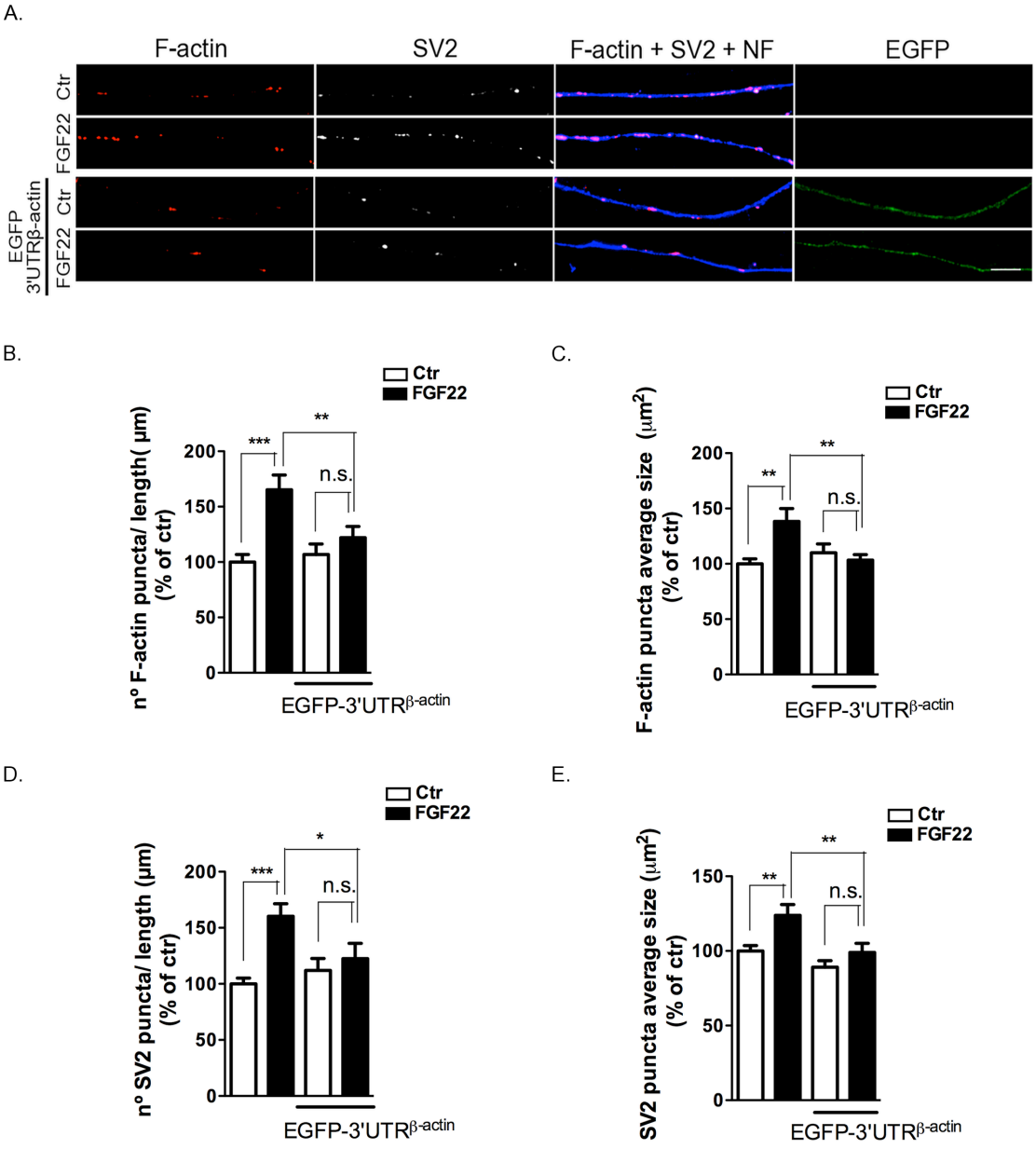



**Figure 4.1 – Mislocalization of *β-actin* mRNA abolishes FGF-22-induced actin polymerization and presynaptic differentiation. (A)** Role of axonal *β-actin* mRNA in actin polymerization and SV2 clustering. Neurons were cultured in microfluidic devices. At DIV2 cells were infected (somal compartment only) with pSindbis virus expressing the β-actin reporter (see **Figure 3.13A**), for 20h. At DIV3, axons were stimulated with FGF-22 (2 nM) for 14h. Presynaptic differentiation was assessed by immunocytochemistry using an antibody against SV2 (white) and β-actin polymerization was evaluated using the actin stain phalloidin 555 (red). EGFP (green) represents infected axons, as expected no GFP

signal is identified in non-infected neurons. BSA was used as vehicle. Scale bar is 5 µm. **(B, C)** Quantification of F-actin puncta number per axon length (B) and F-actin puncta size (C). β-actin reporter prevents the transport and axonal localization of endogenous *β-actin* mRNA in axons preventing FGF-22-induced F-actin formation. Bars represent the mean ± SEM of 20 axons per condition of three independent experiments. *** represents $p<0.001$, ** represents $p<0.01$ by ANOVA followed by Bonferroni's post test. ns is non-statistically significant. **(D, E)** Quantification of SV2 puncta number per axon length (D) and SV2 puncta size (E). The absence of *β-actin* mRNA in axons abolished synaptic vesicle clustering. Therefore, localized endogenous *β-actin* mRNA is required for FGF-22-induced presynaptic differentiation. Bars represent the mean ± SEM of 20 axons per condition of three independent experiments. *** represents $p<0.001$, ** represents $p<0.01$ and * represents $p<0.05$ by ANOVA followed by Bonferroni's post test. ns is non-statistically significant.

To further confirm these results we analyzed mRNA expression by real-time PCR. Ciliary ganglia neurons grown in multi-well plates were transfected with the indicated siRNAs and *β-actin* mRNA levels determined using two distinct sets of primers. Both siRNA 1 and 2 are able to reduce in more than 50% the abundance of *β-actin* mRNA in ciliary ganglia neurons (**Figure 4.2 C**).

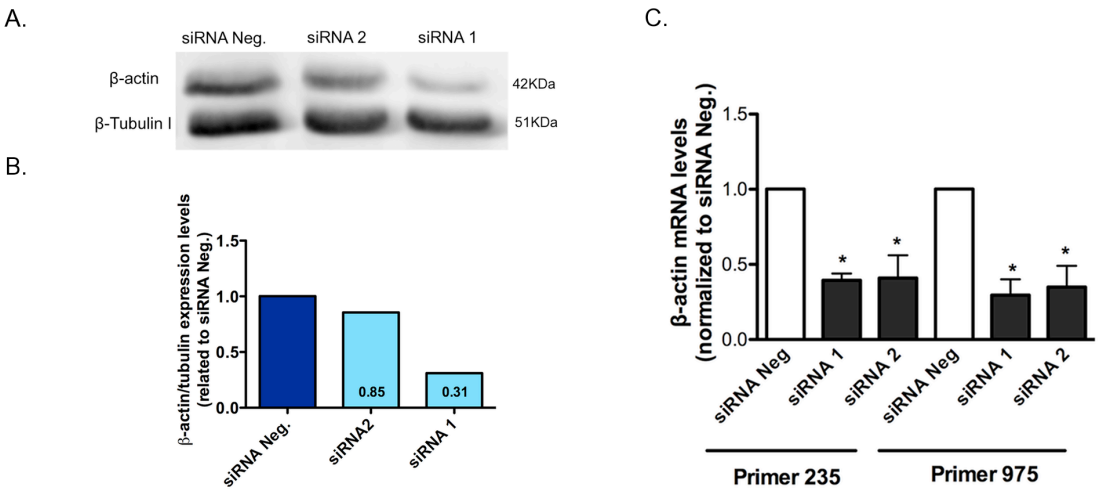



**Figure 4.2 – Knock-down of the *β-actin* mRNA by RNA interference**. **(A, B)** Efficient β-actin protein knock-down. Chick embryo fibroblasts (CEF) were transfected with selected siRNAs against the ORF (siRNA1) and 5'UTR (siRNA2) of chick *β-actin* mRNA for 96h. A non-targeting siRNA sequence was used as control (siRNA Neg.). (A) The levels of β-actin were evaluated by western blot using β-tubulin I as a loading control. (B) Quantification of western blot shows a reduction on the levels of β-actin in both siRNA1 and siRNA2 transfected cells, however, siRNA 1 shows higher knock-down efficiency. Results were obtained from one individual experiment. **(C)** Efficient *β-actin* mRNA knock-down. Ciliary ganglia neurons were transfected with specific siRNAs against the ORF (siRNA1) and 5'UTR (siRNA2) of chick *β-actin* mRNA for 48h. Then, *β-actin* mRNA levels were assessed by real-time PCR, using two different primer sets. Analysis of gene expression shows that levels of *β-actin* mRNA decrease significantly when cells were transfected with both siRNAs, demonstrating an efficient *β-actin* mRNA downregulation. Results are presented as the mean value ± SEM compared to control (siRNA Neg.) of two independent experiments. * represents $p<0.05$ by one-way analysis of variance using Bonferroni's post test when compared to siRNA Neg.

To evaluate the efficiency of transfection specifically in axons grown in microfluidic chambers we used a fluorophore-conjugated siRNA (siRNA555). RNA-silencing complexes are naturally present in axons and insertion of siRNA in the axonal compartment of microfluidic chambers results in down-regulation of target genes[305]. All axons exhibited fluorescent signal in a punctuated form demonstrating that transfection of siRNA555 in the axonal compartment was highly efficient (**Figure 4.3**).

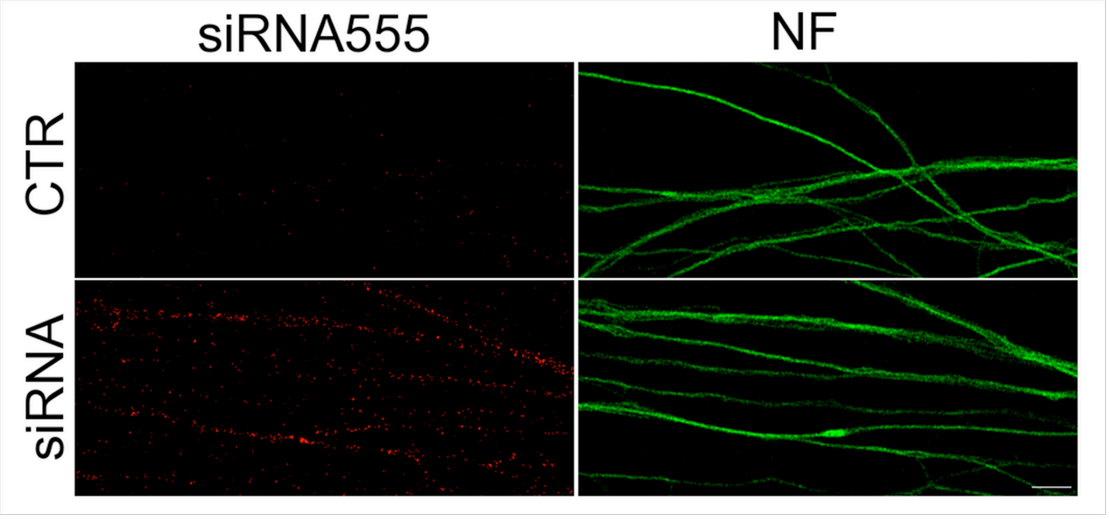



**Fig.4.3 - High siRNA axonal transfection efficiency.** At DIV3, axons were transfected with a fluorophore conjugated siRNA (siRNA555), for 14h. Cells were immunostained for NF and images from the axonal compartment were acquired in a confocal point-scanning microscope LSM 710, using a PlanApochromat 63x objective. For control conditions the siRNA was removed from the transfection solution. Representative images demonstrate that siRNA 555 was incorporated by the majority of axons present in axonal compartment. More 3 images from axonal compartment were acquired to confirm observations. Scale bar is 5 µm.

To determine the requirement of axonal *β-actin* mRNA translation for FGF-22-induced presynaptic differentiation we performed axonal-specific mRNA knock-down. Axons were transfected with a non-targeting siRNA (siRNA neg.), siRNA1 and siRNA2 and SV2 and F-actin clusters were analyzed in distal axons (**Figure 4.4 A**). We observed that knock-down of *β-actin* mRNA abolished FGF-22-induced increase in SV2 clusters as showed by a reduced number of SV2 particles per axon length and SV2 average size (**Figure 4.4 B and C**). Notably, that effect was only seen in conditions stimulated with FGF-22 (black bars **Figure 4.4 B and C**) but not in basal condition (white bars **Figure 4.4 B and C**). In addition, depletion of locally synthesized β-actin prevents FGF-22-induced F-actin polymerization as observed by a reduction in the number and total area of F-actin per axon length (black bars **Figure 4.4 D and E**). Moreover, the basal number and area of

F-actin were not altered upon blockage of axonal β-actin synthesis (white bars **Figure 4.4 D and E**) indicating that FGF-22-induced F-actin polymerization requires synthesis of newly actin monomers. Overall, these results demonstrate that *β-actin* mRNA translation is required for presynaptic differentiation.

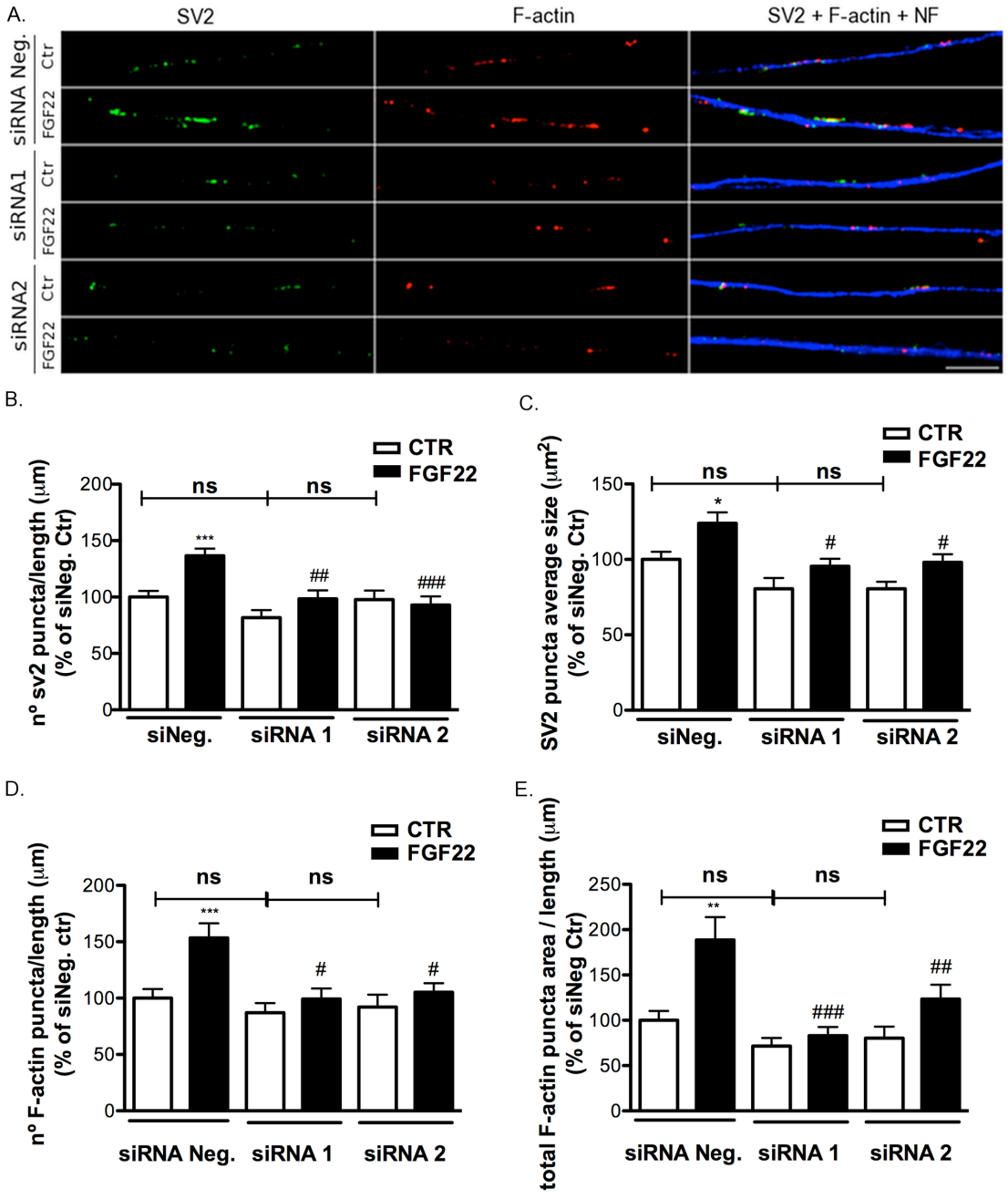



**Figure 4.4 – Specific down-regulation of axonal *β-actin* mRNA prevents presynaptic differentiation. (A)** Axonal *β-actin* mRNA is required for presynaptic differentiation. Axons from ciliary ganglia neurons were transfected with two different siRNAs, a non-targeting siRNA sequence was used as control (siRNA Neg.). Axons were stimulated with either BSA (Ctr) or FGF-22 (14h) and the formation of synaptic clusters was assessed by immunocytochemistry using an antibody against SV2 (green), a synaptic vesicle marker; F-actin filaments were identified using actin-stain phalloidin

555 (red). Axons were detected using an antibody against neurofilament (NF, blue). Scale bar is 5 μm. **(B, C)** Quantification of SV2 puncta number per axon length (B) and SV2 average size (C). Axonal β-actin knock-down by both siRNA1 and siRNA2 blocked the augment in the number and average size of synaptic clusters induced by FGF-22, indicating that loss of axonal β-actin prevents FGF-22-induced presynaptic differentiation. Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of 20 images of at least three independent experiments. * represents $p<0.05$ and *** represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to siRNA Neg control. # represents $p<0.05$, ## represents $p<0.01$ and ### represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to siRNA Neg. FGF-22. *ns* is non-statistically significant. **(D, E)** Quantification of number of F-actin filaments (D) and F-actin total area per axon length (E). Down-regulation of *β-actin* mRNA translation prevents augment in the number and size of F-actin induced by FGF-22. Results suggest that FGF-22-induced β-actin synthesis contributes for the formation of new F-actin filaments. Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of 20 images of at least three independent experiments. ** represents $p<0.01$ and *** represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to siRNA Neg control. # represents $p<0.05$, ## represents $p<0.01$ and ### represents $p<0.001$ by one-way analysis of variance using Bonferroni's post test when compared to siRNA Neg. FGF-22. *ns* is non-statistically significant.

### 4.3.3 Mislocalization of axonal β-actin mRNA precludes NMJ formation

To further confirm our results we developed a new nerve-muscle co-culture system, to reproduce the physiological events that occur during development. In this system only the distal axons contact with skeletal muscle fibers, their biological postsynaptic target (**Figure 4.5A**). In microfluidic chambers cell bodies and dendrites are restricted to the somal compartment thus ensuring the spatial distribution that occurs during the period of synaptogenesis (**Figure 4.5B**). Neuromuscular junctions are characterized by the clustering of acetylcholine receptors (AChRs) on the postsynaptic site and the clustering of synaptic vesicles (SVs) on the nerve terminal[306]. So, in order to assess synapse formation, co-cultures were immuno-labeled against SV2 and NF and stained with fluorophore coupled α-bungarotoxin (α-BTX) to label AchRs (**Figure 4.5C**). Co-localization of SV2 and α-BTX signals is observed throughout the culture (**inset in Figure 4.5C**) demonstrating the existence of neuromuscular synapses in this co-culture and the usefulness of the system.

We next asked if neuromuscular synapses require spatial localization of axonal *β-actin* mRNA. In these experiments, ciliary ganglia neurons were infected with a Sindbis virus expressing EGFP-3'UTR$^{\beta\text{-actin}}$ (**Figure 4.6A**). EGFP-3'UTR$^{Hist}$ was used as a control since histone mRNA is restricted to the cell body so its 3'UTR does not compete with *β-actin* mRNA-binding proteins allowing normal transport of endogenous *β-actin* mRNA to axons. Thus, EGFP3'UTR$^{histone}$ mRNA remains restricted to cell body and no EGFP

staining along axons is observed (**Figure 4.6B**). In contrast, the EGFP3'UTR$^{\beta\text{-actin}}$ mRNA contains *zipcode* sequences responsible for axonal translocation of this mRNA, as shown by robust EGFP puncta staining (**Figure 4.6B**). In this circumstance endogenous *β-actin* mRNA competes with EGFP3'UTR$^{\beta\text{-actin}}$ for axonal transport, becoming virtually restricted to the cell body.

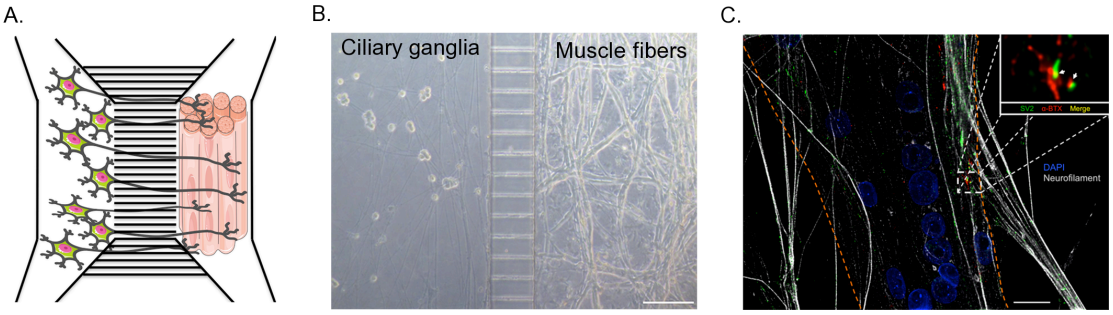



**Figure 4.5 – A novel nerve-muscle co-culture for studying neuromuscular junction formation. (A, B)** Schematic representation **(A)** and representative image (B) of mature co-culture grown in microfluidic chambers. **(A)** Ciliary ganglia neurons and chick pectoral muscle cells were cultured in separated compartments of microfluidic chambers. During development axons extend to muscle compartment forming *en passant* neuromuscular junctions. **(B)** Representative image shows a complete separation between neuronal cell bodies and muscle fibers. More importantly, myoblasts differentiate throughout the channel into contractile muscle fibers. Image was acquired using a Canon Power Shot G10 digital camera associated to an Axiovert 40C microscope (Zeiss) with a 20x objective. Scale bar is 150 µm. **(C)** Nerve-muscle contact. Co-cultures were immunostained against SV2 (green) and Neurofilament (white) and labeled with α-bungarotoxin (α-BTX, red), which binds with high affinity to the α-subunit of the nicotinic acetylcholine receptor (AChR) present on the postsynaptic regions. Synaptic contacts represented by co-localization between SV2 and AChR are depicted in the inset box, where white arrows point the co-localization sites (yellow). Dapi (blue) was used to show the muscular syncytium, resultant of multiple cell fusion. Muscle multinucleated fiber are delineate in dashed orange. Scale bar is 100 µm.

Subsequently, we analyzed how formation of neuromuscular synapses was affected by mislocalization of endogenous *β-actin* mRNA (soma expression of EGFP-3'UTR $^{\beta\text{-actin}}$ mRNA) in comparison to the control condition (soma expression of EGFP-3'UTR $^{\text{Histone}}$ mRNA). For this purpose, we quantified SV2 puncta number along axons contacting muscle fibers (**Figure 4.6C and C'**) and fractional area occupancy of α-BTX (**Figure 4.6D**). We observed that, axonal depletion of endogenous axonal *β-actin* mRNA reduces formation of neuromuscular synapses as indicated by a decline in the number of SV2 particles along axons in contact with muscle fibers (**Figure 4.6C**), with no changes in SV2 particles size (**Figure 4.6C'**). Furthermore, the percentage of α-BTX occupied by SV2 particles was diminished in the absence of endogenous *β-actin* mRNA (**Figure 4.6D**). These results indicate that upon nerve-muscle contact, presynaptic differentiation requires

localized synthesis of β-actin. In conclusion, these set of results demonstrate that axonal translation of *β-actin* mRNA is essential for the establishment of neuromuscular synapses.

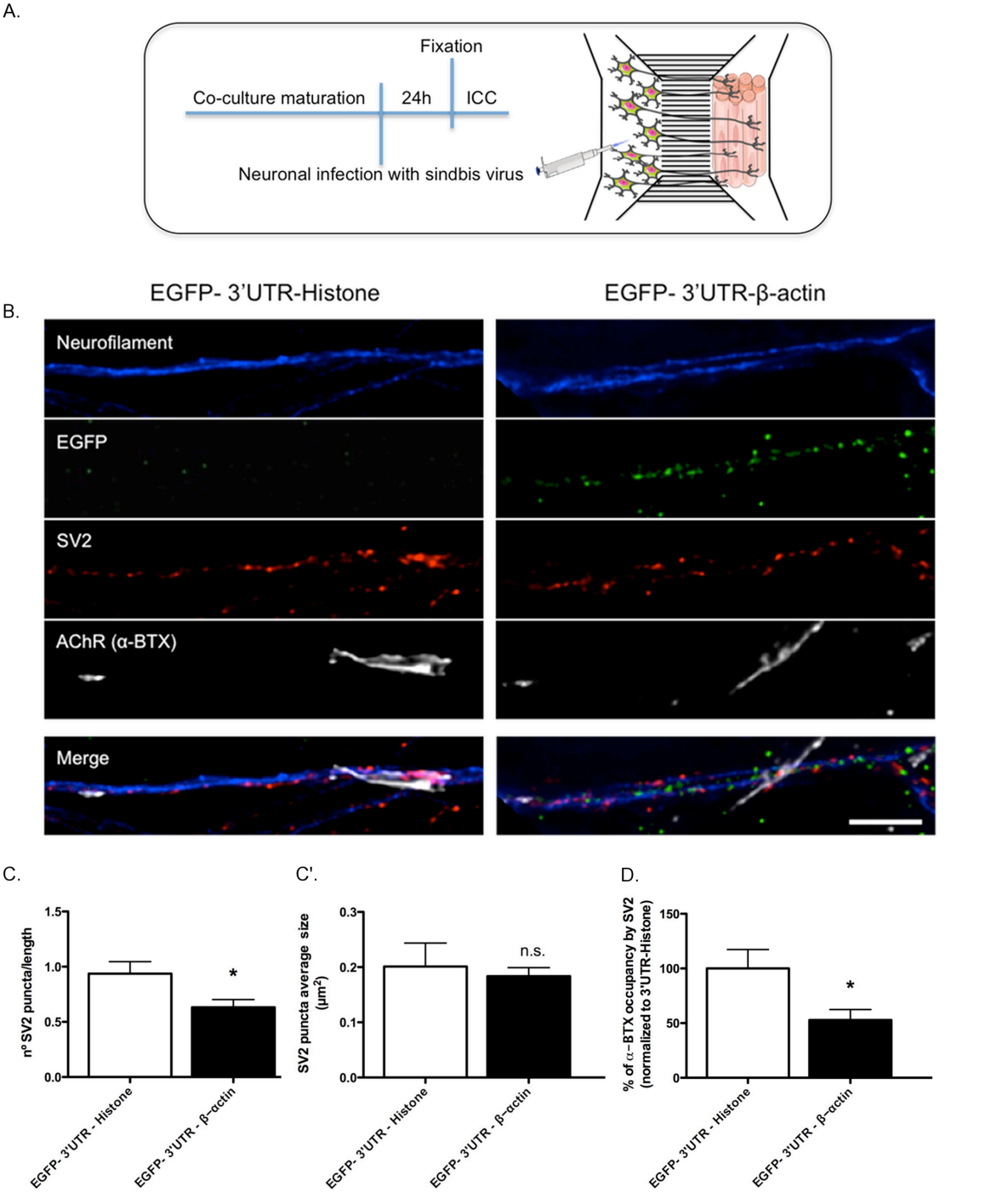



**Figure 4.6 – Neuromuscular junction formation is compromised in absence of *β-actin* mRNA. (A)** Schematic representation of the experimental approach. After co-cultured maturation**,** neurons were infected with pSindbis virus for 24h according to the diagram shown. Viral infection was restricted to the neuronal compartment. **(B)** Physiologic role of axonal *β-actin* mRNA in neuromuscular synapses. Representative images of axons infected with pSinRepmyrd$_2$EGFP3'UTR$^{histone}$ (control condition) or pSinRepmyrd$_2$EGFP3'UTR$^{β\text{-}actin}$ in somal side, for 24h.With the Histone reporter, SV2 (red) accumulated in axons upon axon-muscle contact. However, synaptic contacts were disrupted

(lower SV2 and AChR co-localization) when the β-actin reporter was expressed, which is a consequence of axonal *β-actin* mRNA absence. NF (blue) was used as axonal marker. Scale bar is 5μm. (**C, C' and D**) Endogenous *β-actin* mRNA is required for synapse formation. Analysis of SV2 puncta number (C), SV2 puncta average size (C') and α–BTX occupancy by SV2 (D), in axons contacting muscular fibers. Absence of axonal endogenous *β-actin* mRNA led to a reduction in the number of SV2 puncta, however the SV2 cluster size remained unaltered (C'). Analysis of the fractional area occupancy of α-BTX (α-BTX area occupied by SV2) shows that absence of axonal endogenous *β-actin* mRNA led to a reduction in co-localization between pre- and postsynaptic markers (D), which is a consequence of non-formation of neuromuscular junctions. Bars represent the mean ± SEM of 25-36 muscular fibers, containing axons on top, of three independent experiments. *Represents $p<0.05$ by student's t-test when compared to control condition. ns is non-statistically significant.

## 4.4 DISCUSSION

Our results demonstrate a novel role for intra-axonal protein synthesis in presynaptic differentiation. We find that a β-actin reporter is locally synthesized in axons after FGF-22 stimulation and that actin polymerization is regulated by local protein synthesis (Chapter III). β-actin is a cytoskeletal protein locally synthesized in growth cones[188] with particular significance in the process of axonal outgrowth and guidance[149,151]. These two parallel studies demonstrate that Netrin-1 and BDNF induce asymmetric synthesis of β-actin, which in turn has a crucial contribution to the directional motility of the growth cone during axon guidance[149,151]. Here, we demonstrate, for the first time, that axonal localization of β-actin regulates presynaptic formation. Our studies demonstrate that mislocalization of *β-actin* mRNA to axons prevented FGF-22-induced actin polymerization and, simultaneously, FGF-22-induced synaptic vesicles accumulation. Overexpression of the 3'UTR of *β-actin* mRNA abolished translocation of endogenous β-actin mRNA from soma to axons[269]. However, we cannot exclude the presence of low levels of endogenous *β-actin* mRNA in axons. To circumvent this possible limitation we targeted axonal *β-actin* mRNA through small interference RNA. Silencing of *β-actin* mRNA down-regulated β-actin and, as expected, prevented formation of F-actin polymerization, as consequence it abolished new presynaptic clusters induced by FGF-22. Thus, both localization and translation of axonal *β-actin* mRNA regulates cytoskeleton remodeling, which is required to build new synapses.

Thereafter, we established an *in vitro* co-culture between chick neurons and chick skeletal muscle and we showed that neuromuscular junctions were disrupted when *β-actin* mRNA was delocalized to axons, indicating that axonal targeting of this mRNA is required for an accurate NMJ formation. Thus, mRNA translation supports presynaptic

differentiation by regulating actin cytoskeleton remodeling. Interestingly, in *Drosophila melanogaster* IGFII-binding protein (IMP), the ortholog of chicken *zipcode*-binding protein, is associated with the 3'UTRs of transcripts involved in cytoskeleton remodeling and low levels of Imp in *drosophila* oocytes reduces actin cytoskeleton dynamics. Moreover, genetic mutations causing Imp loss-of-function reduces the number of synaptic boutons at *drosophila* NMJ[218,307]. This study is indicative that localization of mRNAs coding for cytoskeleton proteins is important for formation of new synaptic sites thus corroborating our observations. To the extent of our knowledge, this is the first study in vertebrates that demonstrates the role of a specific mRNA in presynaptic bouton formation.

A possible future approach could focus on monitoring, in real time, accumulation of GFP-tagged β-actin. We expected to see an accumulation of GFP upon synaptic contact, indicating that local translation of β-actin coincides with new presynaptic bouton formation. As conclusion, we identified a novel role for local protein synthesis in axons and demonstrated that spatial regulation of *β-actin* mRNA translation is required for presynaptic formation.

# CHAPTER V

## GENERAL CONCLUSION AND FUTURE DIRECTIONS

In this work we addressed the requirement of local mRNA translation for presynaptic differentiation.

Axonal localization of specific mRNAs creates an incredible genomic outpost that through regulated translation provides an efficient way for coordinate axonal processes. Axonal translation regulates synthesis of new proteins with spatiotemporal precision so proteins are synthesized only where and when they are needed.

The presynaptic terminal is an incredible modification of the axoplasm that occurs within a few minutes. One main player in presynaptic differentiation is the F-actin network and its disruption negatively affects synapses[98,99].

Here, we demonstrated that axonal synthesis of β-actin underlies local F-actin polymerization and synaptic vesicle clustering. We demonstrate that local *β-actin* mRNA translation is required for F-actin remodeling and presynaptic differentiation. We found that: i) axons respond to synaptogenic stimulus by activating local translation pathways ; ii) One of the mRNAs locally translated is *β-actin* ; iii) Axonal localization and on-site translation of *β-actin* mRNA are required for F-actin remodeling and synaptic clusters formation; iv) formation of neuromuscular synapses is dependent on targeting of *β-actin* mRNA to distal axons.

**Intra-axonal translation regulates presynaptic differentiation**

Studies performed in different organisms have elucidated how the synapse is formed. The coordination of diverse classes of synaptogenic molecules gives rise to a highly differentiated axonal region, the presynaptic bouton. However, much less is known about the intracellular signaling pathways that orchestrate this process, and the complete understanding of those processes has been a challenge in the Neuroscience field.

In this study we show that stimulus-induced presynaptic differentiation depends on local translation mechanisms. We show that a *β-actin* mRNA reporter is translated in axons when cells are stimulated with the synaptogenic stimuli. *β-actin* mRNA was one of the first mRNAs found in distal axons[142]. Moreover, local synthesis of β-actin regulates axonal processes requiring cytoskeleton remodeling like growth cone turning and axon growth[147,151], making *β-actin* mRNA an ideal candidate to test.

Others mRNAs coding for regulators of actin polymerization, such as WAVE, cortactin and Arp2 have been localized in axons[308]. NGF-induced axonal translation of those transcripts regulates formation of new actin patches and consequently axonal

branching[308]. The Arp2/3 complex is constituted by seven proteins subunits that nucleate actin assembly promoting actin polymerization[309], the WAVE is an activator of the Arp2/3 complex while the cortactin stabilizes the complex. In *drosophila* NMJ the presynaptic Arp2 and Arp3 are required for the formation and integrity of the synaptic boutons[310]. So, we could envision that those proteins in conjunction with β-actin may contribute to regulate F-actin network in the presynaptic terminal. Therefore, it would be interesting to determine whether FGF-22 activates synthesis of others regulators of the actin cytoskeleton.

In addition, the "actin dynamic cycle"[107] proposes that presynaptic formation requires continuously actin polymerization and depolymerization. So it is reasonable to speculate that actin severing proteins might be locally translated upon stimulus-induced presynaptic differentiation. Therefore, a genomic or proteomic study using stimulated versus non-stimulated axons would be useful to identify others proteins involved in FGF-22-induced presynaptic differentiation.

In the field of local mRNA translation several questions remain to be answered. At the molecular level we still do not understand how axons regulate the diverse axonal pool of mRNAs and how they initiate the translation of only a specific class of mRNAs at a particular moment. Although hundreds of mRNAs have been identified in axons[167,184,287] its physiologic role is still to be determined.

### *β-actin* mRNA regulates F-actin network at presynaptic terminals

In the lamellipodia of the mammary adenocarcinoma cells, the actin polymerization rate corresponds to the addition of $3.6 \times 10^6$ actin monomers per minute[311]. However, in the cytoplasm of the CEF only 6.5% of that value is synthesized ($2.34 \times 10^5$ actin monomers per minute)[312]. So it is unlikely that such low percentage contributes to form the new actin filaments, unless *β-actin* mRNA is accumulated and restricted to specific spots. In chick embryo fibroblasts it is thought that newly synthesized β-actin contributes to increase the nucleation site therefore increasing polarization of actin filaments that underlies the cell protrusion[313]. Based on this observation, we hypothesize that synaptic contact triggers local translation of *β-actin* mRNA contributing to on-site boost actin monomers, therefore increasing actin polymerization rate. Hence, we propose a model (**Figure 5.1**) where upon contact with a postsynaptic target, signaling pathways that control translation initiation are activated in axons. Subsequently, newly synthesized proteins emerge in those regions. β-actin is locally synthesized and it contributes to increased availability of actin monomers

leading to F-actin polymerization and ultimately recruitment and clustering of presynaptic components.

Newly synthesized proteins are assumed to have different properties from those of the soma-derived pool[257]. However, this is still speculative and we do not understand the relative contribution of axonal versus somal synthesized β-actin in axonal development. In order to address this question we propose to use a β-actin dual-color system where β-actin containing the full length 3'UTR will be fused to EGFP and β-actin without the "zipcode region" will be fused to mCherry. By studying the distribution of EGFP and mCherry across the developing neuron we will be able to evaluate the subcellular localization of the two types of actin. From this set of experiments we aim to understand when and how the pool of *β-actin* mRNA found in axons is recruited, and how this event contributes to neuronal development.

**The relevance of axonally localized β-actin in disease**

Axonal synthesis of β-actin is of utmost importance to remodel the cytoskeleton, which is required in axon outgrowth, elongation and guidance. In our study we demonstrate that synaptic differentiation, another axonal event dependent on dramatic structural rearrangements is also dependent on axonal *β-actin* mRNA translation. Merely by disrupting the transport of the endogenous *β-actin* mRNA we observed dramatic deficits in neuromuscular synapses.

Until now, the involvement of axonal *β-actin* mRNA at the presynapses is only being speculated in the SMA, a neuromuscular disease characterized by degeneration of motor neurons with progressive skeletal atrophy. This autosomal recessive disease is caused by genetic defects in the survival motor neuron gene 1 (*SMN1*), which codes for the SMN protein[314,315]. Although playing an important role in the biogenesis of small nuclear ribonucleoprotein particle (snRNPs) complexes[316–318] SMN also associates with the RBPs HuD[319,320], IMP1[321] and hnRNP-R[274,275,277] to transport diverse mRNAs into distal parts of the axons. The localization and translation of *β-actin* mRNA in cultured motor neurons is regulated by SMN/hnRNP-R complex[274,277] and its mislocalization after knocking down hnRNP-R is thought to cause deficits in axon outgrowth and induce irregular branching[274]. It is speculated that absence of axonal β-actin affects firstly axonal branching which, in turn, causes muscle innervation deficits. However, *in vivo* studies using a SMA mouse model observed no changes in the development and outgrowth of motor neurons. But, at embryonic stages, SMA mice showed loss of synaptic occupation by motor neurons[322].

Recently, the SMN/hnRNP-R complex was identified in the neuromuscular endplates of embryonic and postnatal mice, suggesting *β-actin* mRNA to be located at the NMJ[276], which is in accordance with our results.

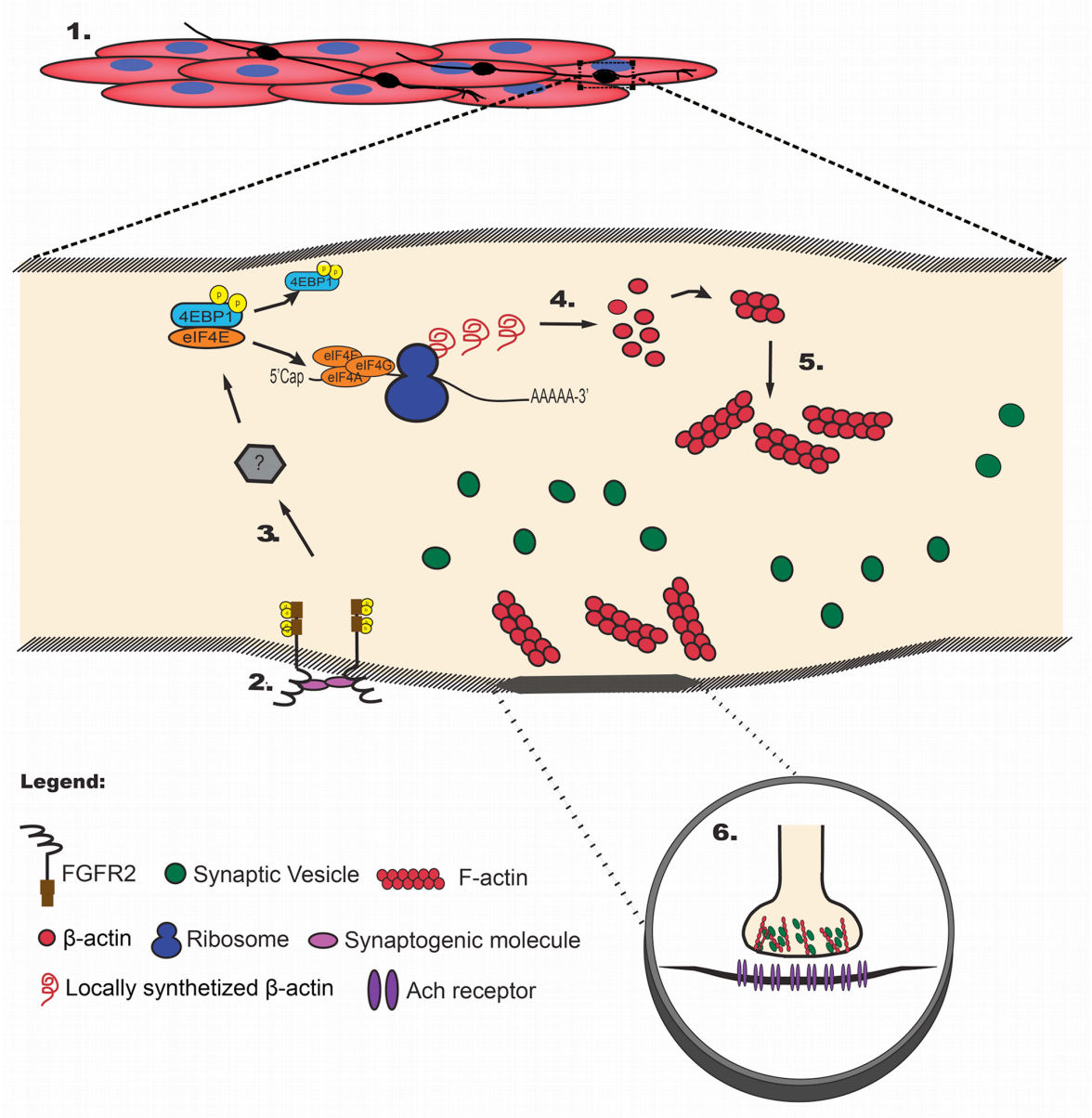



**Figure 5.1 – Local translation of *β-actin* mRNA mediates presynaptic differentiation.** (1.) An efficient contact between axon and muscle fiber initiates formation of *en passant* synapses (here, represented as black puncta along the axon). (2.) As the axon innervates the target tissues, synaptogenic molecules (e.g. FGF22) are released from the postsynaptic cell binding to specific receptors present on the presynaptic membrane. (3.) These receptors (e.g. FGFR2) activate a cascade of events that lead to phosphorylation of 4EBP1. Phospho-4EBP1 removes the inhibition of the eukaryotic translation initiation factor-4F (eIF4F) complex and cap-dependent local mRNA translation of β-actin is initiated. (4.) Newly synthesized β-actin contributes to an increase in F-actin polymerization in the axonal shaft. (5.) F-actin filaments accumulate and originate new presynaptic boutons where synaptic vesicles will cluster. Locally synthesized proteins are assumed to have different properties from the soma-derived pool[257]. Thus new F-actin filaments may associate and capture synaptic vesicle protein transport vesicles (STVs) leading to protein deposition in the contact site. (6.) Subsequently, a new presynaptic terminal is formed. This model demonstrates that, during synaptic contact, synthesis of new β-actin is required for the remodeling of the distal axon (or growth cone) into a new presynaptic site.

How do neurons regulate the localization of *β-actin* mRNA upon synaptic contact? SMN binds to different RBPs responsible to transport and localize *β-actin* mRNA to distal axons. Besides hnRNP-R SMN also binds to IMP1, a mammalian ortholog of the chicken ZBP1[321]. Considering these evidences, one interesting future approach would consist in down regulating neuronal SMN, hnRNP-R or IMP1 to understand which RBP is the main responsible for the localization and translation of *β-actin* mRNA at presynaptic terminal. We believe that identifying the RBPs and the signaling pathways that control localization and translation of *β-actin* mRNA at synapses would move us one step closer to design new therapeutic strategies for SMA.

In this study we have highlighted that intra-axonal translation of *β-actin* mRNA is required for presynaptic differentiation. We hypothesize that local protein synthesis of β-actin is required for cytoskeleton remodeling, leading to the structural organization required to form a new presynaptic bouton.

# CHAPTER VI

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