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# Role of local protein synthesis in presynaptogenesis

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

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## Abbreviations

**5-FDU** 5-fluoro-2'-deoxyuridina

**AMPA**  $\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid

**Arc** Activity-regulated cytoskeleton-associated protein

**BDNF** Brain-derived neurotrophic factor

**BSA** Bovine Serum Albumin

**CA3** Cornu Ammonis region 3

**CAM** Cell-adhesion molecule

**CaMKII** Ca<sup>2+</sup>/calmodulin-dependent protein kinase II

**CNS** Central nervous system

**CPE** Cytoplasmic polyadenylation element

**CPEB1** Cytoplasmic polyadenylation-element-binding protein 1

**CREB** cAMP response element binding

**DCC** Deleted in colorectal carcinoma

**DIV** Days in vitro

**DRG** Dorsal root ganglion

**eIF** Eukaryotic translation initiation factor

**eIF4E-BP** Eukaryotic translation initiation factor 4E binding protein

**ELH** Egg-laying hormone

**EphA2** Ephrin type A receptor 2

**ERK** Extracellular signal-regulated kinase

**FBS** Fetal bovine serum

**FGF** Fibroblast growth factor

**FGF22** Fibroblast growth factor-22

**FGFR** Fibroblast growth factor receptor

**FMRP** Fragile-X mental retardation protein

**GFP** Green fluorescence protein

**Glu** Glutamate

**GTPase** Guanosine triphosphatase

**HEPES** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid



**hnRNP** Heterogeneous nuclear ribonucleoproteins  
**IRES** Internal ribosomal entry sites  
**KIF** Kinesin  
**LCM** Laser capture microdissection  
**Limk1** Lim-domain containing protein kinase 1  
**LTD** Long-term depression  
**LTF** Long-term facilitation  
**LTP** Long-term potentiation  
**MAPK** Mitogen-activated protein kinase  
**MAP2** Microtubule-associated protein 2  
**miRNAs** MicroRNAs  
**mTOR** Mammalian target of rapamycin  
**NGF** Nerve growth factor  
**NMDA** N-methyl-D-aspartate  
**NMJ** Neuromuscular junction  
**PBs** Processing bodies  
**PBS** Phosphate buffered saline  
**PACAP** Pituitary adenylate cyclase-activating peptide  
**PDL** Poly-D-lysine  
**PDMS** Poly-dimethylsiloxane  
**PNS** Peripheral nervous system  
**PSD-95** Postsynaptic density protein 95  
**PTV** Piccolo-Bassoon transport vesicle  
**RBP** mRNA-binding protein  
**RISC** RNA-induced silencing complex  
**RNP** Ribonucleotide protein  
**rRNA** Ribosomal RNA  
**SGs** Stress granules  
**Sema3A** Semaphoring-3A  
**SNAP-25** Synaptosomal-associated protein-25  
**SNARE** Soluble NSF attachment receptor

**SPRC** Synapse-associated polyribosome complexes  
**STAT3** Signal transducer and activator of transcription 3  
**STV** Synaptic vesicles transport particle  
**SV** Synaptic vesicle  
**SynCAM** Synaptic cell-adhesion molecule  
**TBS** Tris buffered saline  
**TBS-T** Tris-buffered saline with 0.1 % Tween 20  
**TrkB** Tyrosine-related kinase B  
**tRNA** Transfer RNA  
**SV2** Synaptic vesicle protein 2  
**UTR** Untranslated region  
**VAMP** Vesicle SNARE synaptobrevin  
**VGLUT1** Vesicular glutamate transporter 1  
**ZBP1** Zipcode-binding protein 1



## Abstract

Neurons are highly specialized cells that receive, process and transmit information to other cells. For many years, and until the discovery of polyribosomes in the base of dendritic spines, the predominant idea was that all proteins were translated in the cell body and then transported to the neurites. However, this canonical view has been challenged in the last decade and presently it is well established that local protein synthesis occurs in dendrites. Local translation in dendrites has been object of intense studies and is of particular importance in synaptic plasticity, namely during LTP and LTD. In contrast, axonal protein synthesis is still poorly understood. Surprisingly, recent studies identified a large number of mRNAs localized at distal axons and growth cones, suggesting that intra-axonal translation may play an important role in different steps of neuronal development. In line with these evidences, early studies in axons demonstrated the requirement of local translation during axon chemotrophic responses to guidance cues. Moreover, was demonstrated that local protein synthesis is required for other neurodevelopmental mechanisms, such as axonal outgrowth, axon responsiveness, neuronal survival and axon regeneration. Recent studies in the model organism *Aplysia* suggest that local translation might be important for synaptogenesis, as clustering of mRNAs and proteins at sites of synaptic contacts was observed. Interestingly, it was also observed in *Aplysia* that synapse formation induces the transcription of sensorin mRNA. However, sensorin has no mammalian homologue and the relevance of these observations to mammalian model systems remains elusive. In this work we investigated the requirement of local protein synthesis in presynaptic

differentiation. Using FGF22, a presynaptic organizing molecule, and a novel platform, a microfluidic chamber system which allows the physical separation of axons from cell bodies and dendrites, we were able to specifically manipulate axons without the cell body contribution. Using primary cultures of rat embryonic hippocampal neurons in microfluidic devices we specifically stimulated distal axons with FGF22 and determined the “hot spots” of axonal mRNA translation. For that we evaluated the levels of p-4E-BP1, a translation marker, and our results show that FGF22 induced an increase in intra-axonal translation. Moreover, when translational inhibitors were present in the axonal compartment, FGF22-induced presynaptic differentiation effect was abolished, demonstrating that FGF22-induced presynaptogenesis is dependent on local protein synthesis. Lastly, our data demonstrates that presynaptic differentiation induced by FGF22 has transcriptional-dependent component, since actinomycin D, a transcriptional inhibitor, partially blocked FGF22 presynaptogenic effect. These results unraveled a novel role for axonal protein synthesis in neuronal development and can potentially lead to the identification of new molecules involved in synapse formation.

**Key Words:** Microfluidic Devices, Local protein synthesis, Transcription, Presynaptogenesis, FGF22.

## Resumo

Os neurónios são células altamente especializadas que recebem, processam e transmitem informação para outras células. Durante muitos anos, e até à descoberta de poliribossomas na base das dendrites, a ideia predominante era que todas as proteínas eram traduzidas no corpo celular e depois transportadas para as neurites. Contudo, esta visão canónica tem sido posta em causa nas últimas décadas e actualmente está estabelecido que a síntese proteica local ocorre em dendrites. A tradução local em dendrites tem sido objecto de intensos estudos e é de particular importância na plasticidade sináptica, nomeadamente durante o LTP e o LTD. Por outro lado, a síntese proteica nos axónios é ainda pouco conhecida. Surpreendentemente, estudos recentes identificaram um grande número de mRNAs localizados em axónios distais e em cones de crescimento, sugerindo que a tradução intra-axonal pode ter um papel importante em diferentes fases do desenvolvimento neuronal. Em linha com estas evidências, estudos recentes em axónios demonstraram a necessidade da existência de tradução local durante as respostas quimiotróficas dos axónios a guidance cues. Além disto, foi demonstrado que a síntese proteica local é necessária para outros mecanismos do neurodesenvolvimento, tais como o crescimento axonal, a capacidade de resposta dos axónios, a sobrevivência neuronal e a regeneração axonal. Estudos recentes no organismo modelo *Aplysia* sugerem que a tradução local possa ser importante para a sinaptogénese, devido à observação de agregados de mRNAs e de proteínas em locais de contacto sináptico. Curiosamente, foi também observado em *Aplysia* que a formação sináptica induz a transcrição do mRNA

sensorina. Contudo, a sensorina não tem uma proteína homóloga nos mamíferos e a relevância destas observações para sistemas modelo de mamíferos continua por elucidar. Neste trabalho nós investigámos a necessidade de síntese proteica local na diferenciação pré-sináptica. Usando FGF22, uma molécula organizadora da pré-sinapse, e uma nova plataforma, as câmaras microfluidicas que permitem a separação física dos axónios dos corpos celulares e das dendrites, nós fomos capazes de manipular especificamente axónios sem a contribuição do corpo celular. Usando culturas primárias de neurónios do hipocampo de embriões de rato em câmaras microfluidicas nós estimulámos especificamente axónios distais com FGF22 e determinámos os “hot spots” da tradução axonal de mRNAs. Para isto, avaliámos os níveis de p-4E-BP1, um marcador de tradução, e os nossos resultados mostram que o FGF22 induziu um aumento na tradução intra-axonal. Além disto, quando inibidores da tradução estão presentes no compartimento axonal, a diferenciação pré-sináptica induzida por FGF22 é abolida, demonstrando que a presinaptogénese induzida por FGF22 é dependente da síntese proteica local. Por fim, os nossos resultados demonstram que a diferenciação pré-sináptica induzida pelo FGF22 tem uma componente dependente da transcrição, pois a actinomicina D, um inibidor da transcrição, bloqueia parcialmente o efeito presinaptogénico do FGF22. Estes resultados revelam um novo papel para a síntese proteica local no desenvolvimento neuronal e pode potencialmente levar à identificação de novas moléculas envolvidas na formação sináptica.

**Palavras-chave:** Câmaras microfluidicas, Síntese proteica local, Transcrição, Presinaptogénese, FGF22.

Chapter 1

Introduction





## **1.1. mRNA trafficking and regulation of local protein synthesis in neurons**

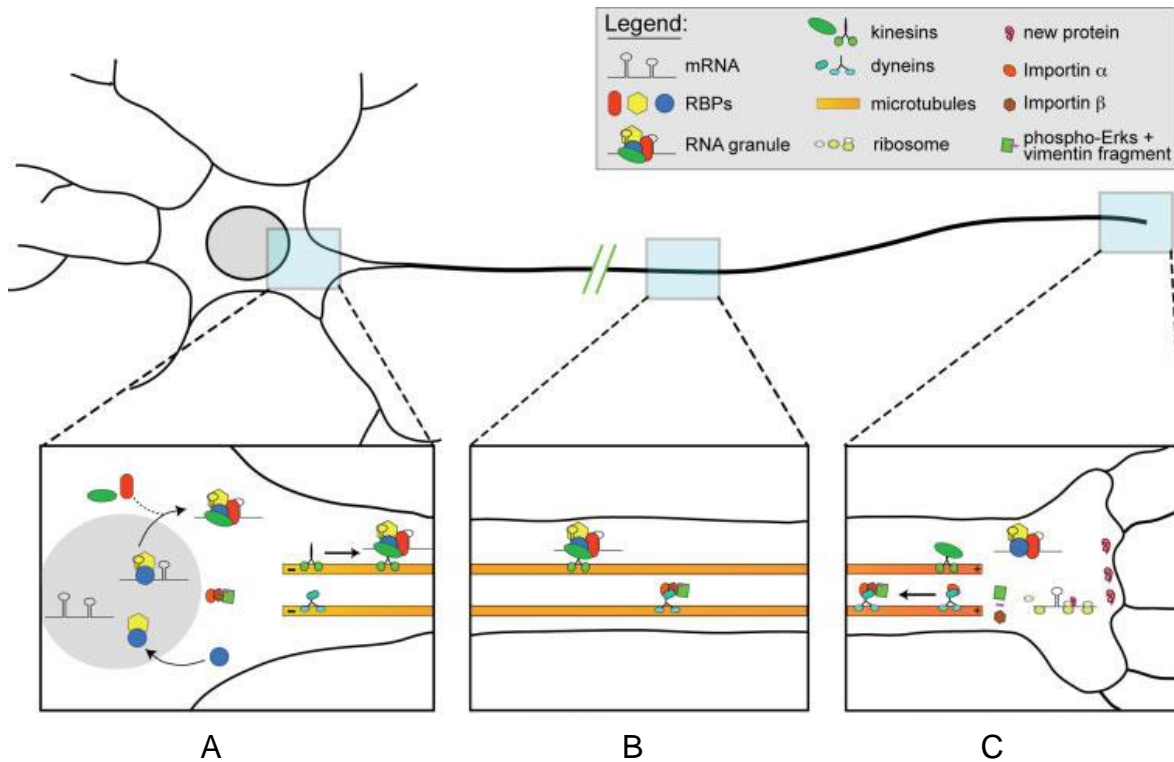
### **1.1.1. mRNA trafficking**

Neurons are a highly polarized cells with several dendrites and one axon extending from the cell body. During many years, and until the discovery of polyribosomes in the base of dendritic spines (Steward and Levy, 1982), the prevalent idea was that all proteins were translated in the cell body and then transported to the neurites. In the last years several studies proved the existence of localized protein synthesis in axons and dendrites (reviewed in Hirokawa, 2006; reviewed in Willis and Twiss, 2006). This mRNA localization and translation can influence synaptic plasticity, synapse formation, axon guidance and nerve regeneration (reviewed in Kiebler and Bassell, 2006).

Nowadays, it is widely accepted that localized mRNAs are transported in large ribonucleoprotein particles (RNPs), also called RNA-containing granules (reviewed in Kiebler and Bassell, 2006). In neurons there are at least three types of RNA-containing granules: ribonucleoprotein particles (RNPs), stress granules (SGs) and processing bodies (PBs). The contents of each one of these granules are different in terms of RNA binding proteins and mRNAs (reviewed in Sossin and DesGroseillers, 2006).

As shown in Figure 1, the formation of RNA-containing granules begins in the nucleus, where a mRNA-binding protein (RBP), which has the ability to inhibit translation, binds to a specific mRNA. Then the mRNA/RBP complex is packaged into the RNA-containing granules. The granules are then transported along the

microtubules by motor proteins (kinesin), until they reach their final localization in axons or dendrites, where local translation of the transported mRNAs will occur (reviewed in Wang et al., 2007).



**Figure 1 – Schematic representation of the different regulatory steps that regulate local translation – RNA granule formation (A), RNA transport (B) and local mRNA translation (C) (Adapted from Wang et al., 2007).**

$\beta$ -actin mRNA has been an object of intense studies in the recent past and is a good example of mRNA trafficking. In this case, the zip-code-binding protein 1 (ZBP1), a RBP family member, binds to  $\beta$ -actin mRNA in its 3' untranslated region (UTR) (Gu et al., 2002; Zhang et al., 2001). The binding of ZBP1 to  $\beta$ -actin mRNA

prevents the translation of this mRNA during its transport, by blocking translation at the initiation step (Huttelmaier et al., 2005). ZBP1 is also a key component for  $\beta$ -actin mRNA localization, since interfering with ZBP1's ability to bind the zip-code, the 3'UTR region of  $\beta$ -actin mRNA, or reducing the expression of ZBP1 protein in neurons leads to a reduction in  $\beta$ -actin mRNA localization in axons and dendrites (Eom et al., 2003; Zhang et al., 2001).

Previous studies demonstrated that RNA-containing granules are heterogeneous (Elvira et al., 2006; Jonson et al., 2007; Kanai et al., 2004; Krichevsky and Kosik, 2001; Mallardo et al., 2003). Hirokawa and colleagues took advantage of the interaction between RNA-containing granules with KIF5 and isolated transport RNPs from adult mouse brain. Two dendritic mRNAs were found in KIF5-granules, one for Arc (activity-regulated cytoskeleton-associated protein) and another for  $\alpha$ CaMKII ( $\alpha$ -subunit of calcium/calmodulin-dependent protein kinase II) (Kanai et al., 2004). Sossin and colleagues observed, in large granules, the actin-binding protein ZCBP and a number of other binding proteins that had not been identified in KIF5 granules such as IEB2 and -3, ELAVs, and G3BP1 and -2 (Elvira et al., 2006). Moreover, it was observed that these large granules were significantly enriched in ribosomes, while in KIF5 granules, the presence of ribosomes was reduced (Elvira et al., 2006). However, common components between the two granules were identified (Elvira et al., 2006). Some of these shared common components were heterogeneous nuclear ribonucleoproteins (hnRNPs), regulators of mRNA transport (Pur  $\alpha/\beta$ , Staufen), RNA-binding proteins implicated in mRNA stabilization (synaptotagmin-binding, cytoplasmic-RNA-interacting protein – SYNCRIP), RNA-binding proteins implicated in translation

(FMRP), RNA helicases (DEAD boxes 1, 3, and 5) (Elvira et al., 2006). Taken together, these results show that the RNA-containing granules are heterogeneous, but there are also some core components for RNA granules and RBPs that are added to the granules in different stages of the development (reviewed in Bramham and Wells, 2007).

### **1.1.2. Regulation of mRNA translation**

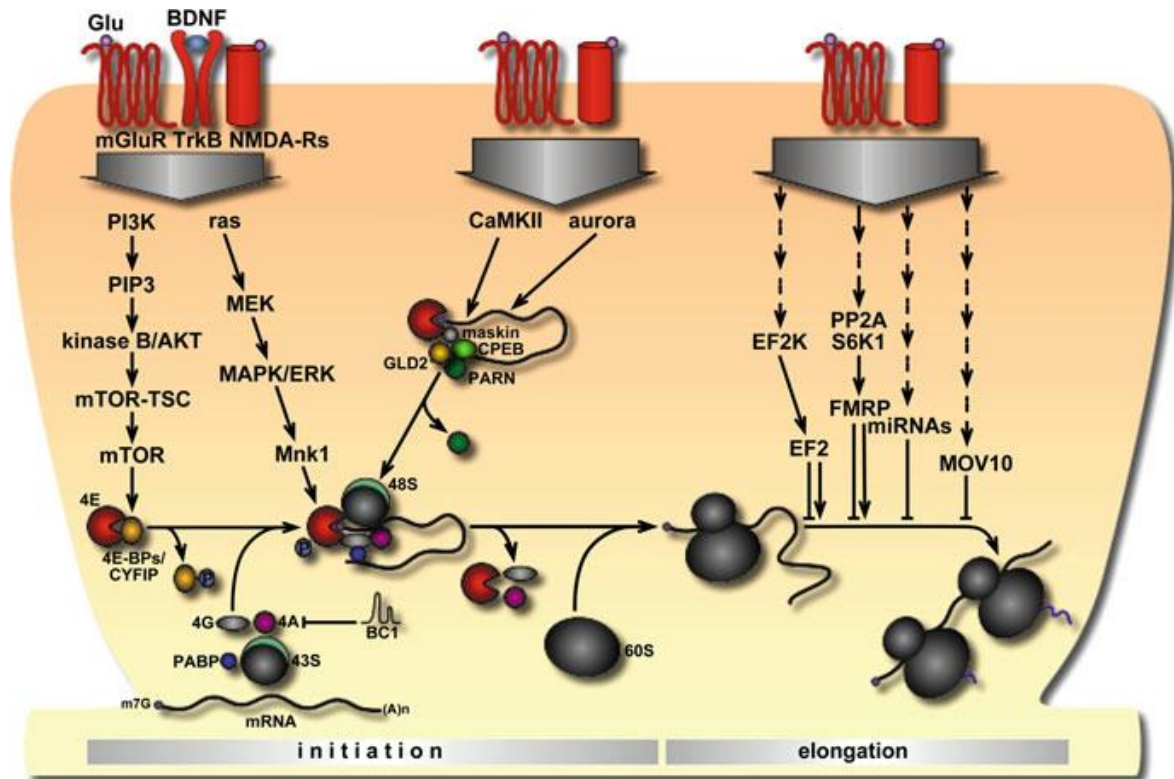
mRNA translation has three different phases – initiation, elongation and termination – and each one of these phases can be regulated, since that initiation is the rate-limiting event, which makes it the first target of regulation (reviewed in Kindler and Kreienkamp, 2012). Local protein synthesis may have different triggers, such as receptors for neurotransmitters, hormones, neurotrophins and extracellular matrix molecules. In the initiation, the control triggered by the transmembrane receptors has as main target the eukaryotic translation initiation factor 4E - eIF4E. The two receptor-coupled kinase pathways involved in this control are: extracellular signal-regulated kinase (ERK) signaling and mammalian target of rapamycin (mTOR) signaling. ERK signaling triggers the phosphorylation of eIF4E, whereas mTOR signaling triggers the phosphorylation of eIF4E-binding protein (eIF4E-BP) which causes the release of eIF4E (reviewed in Bramham and Wells, 2007). Once free, eIF4E binds to mRNAs 5' methylated GTP cap which enhances the translation (Pfeiffer and Huber, 2006). For example, the administration of Brain-derived neurotrophic factor (BDNF), which leads to long-

term potentiation (LTP), acts through the activation of ERK signaling which leads to an increase in eIF4E phosphorylation, that triggers  $\alpha$ CaMKII synthesis (Kanhema et al., 2006). Another example is the activation of NMDAR which leads to the mTOR-signaling, resulting in an increase of dendritic protein synthesis (Gong et al., 2006).

Translation can also be initiated at internal ribosomal entry sites (IRES), a cap-independent translation. In this pathway, ribosomes are recruited to the mRNA through these IRES. IRES are RNA structural elements that bind ribosomes to the mRNA (reviewed in Fitzgerald and Semler, 2009). It appears that the inhibition of cap-dependent translation enhances IRES-mediated translation (reviewed in Bramham and Wells, 2007). Krushel and colleagues identified dendritic mRNAs which contain these IRES sequences, such as Arc,  $\alpha$ CaMKII, dendrin and microtubule-associated protein 2 (MAP2), suggesting that this mRNAs can be translated through an IRES sequence (Pinkstaff et al., 2001).

In elongation, the control of translation is mediated through eEF2 activity (Figure 2). When amino acids are added to the peptide chain, the eEF2, a GTP-binding protein, promotes the translocation of peptidyl-tRNAs from the A-site to the P-site on the ribosome. eEF2 kinase (eEF2K) phosphorylates eEF2, which in this phosphorylated form is unable to bind to the ribosome and as a consequence arrests elongation (reviewed in Bramham and Wells, 2007). However, Constantine-Paton and colleagues showed that eEF2 phosphorylation increases translation of  $\alpha$ CaMKII mRNAs in neurons, but suppresses global protein synthesis (Scheetz et al., 2000). Further studies must be executed to elucidate how the local translation of specific transcripts is sustained when eEF2 is phosphorylated (reviewed in

Bramham and Wells, 2007). Lastly, different release factors mediate the termination of translation at the stop codon (reviewed in Kindler and Kreienkamp, 2012).



**Figure 2 – Schematization of the main pathways regulating protein synthesis at synapses.** Different signaling pathways, that are activated through action of several receptors (mGluR, TrkB, NMDAR), regulate cap-dependent initiation and elongation (Adapted from Kindler and Kreienkamp, 2012).

Regulation of protein translation can be exerted through external stimuli. This mechanism seems to be mediated by RBPs that silence mRNA translation by binding to *cis*-elements present at the 3'UTR, and allows translation of a specific

mRNA (reviewed in Kindler et al., 2005). ZBP1, cytoplasmic polyadenylation-element-binding protein 1 (CPEB1) and Fragile X Mental Retardation Protein (FMRP) are particularly well studied and will be discussed in detail below.

As previously mentioned, ZBP1 binds to *β-actin* mRNA at the nucleus. ZBP1/*β-actin* mRNA is transported along the cytoplasm to their final target where ZBP1 can be phosphorylated by Src, upon an external stimuli. This phosphorylation triggers the dissociation of ZBP1 from *β-actin* mRNA and allows it to be translated (reviewed in Bramham and Wells, 2007).

There are some evidences pointing to the existence of dendritic mRNAs that are not translated until polyadenylation occurs in the 3'UTR tail when CPEB1 binds to the 3'UTR, anchors several proteins such as Gld2, a poly(A)-polymerase, which results in the polyadenylation of the target mRNA. Maskin, an eIF4E binding protein, symplekin, a scaffolding protein, and a deadenylase (poly(A)-specific ribonuclease (PARN)) (reviewed in Bramham and Wells, 2007). Initially, when CPEB1 binds to the mRNA, translation is inhibited by maskin which binds eIF4E. However aurora kinase A and  $\alpha$ CaMKII can phosphorylate CPEB1 and PARN is dissociated from the mRNA allowing polyadenylation to occurs. This polyadenylation triggers the dissociation between maskin and eIF4E which allows translation to be initiated (Costa-Mattioli et al., 2009; Wells, 2006). The CPEB-mediated translation can be triggered through the activation of NMDAR and mGluR (reviewed in Kindler et al., 2005).

The regulation of mRNA translation by FMRP is mediated by the activation of mGluR. The best characterized FMRP-mediated translation is the *FMRP* transcript itself. FMRP binds *fmrp* mRNA, and when mGluR is activated the *fmrp*



mRNA complex is disinhibited and this promotes an enhancement of protein synthesis (reviewed in Bramham and Wells, 2007).

MicroRNAs (miRNAs) are small RNA molecules encoded by the complex eukaryotic genomes, which negatively regulate the expression of genes (Tang et al., 2012). miRNAs have the ability to inhibit translation through their binding to mRNA at 3'UTR. One miRNA can bind to several mRNAs and each mRNA can be bound by several different miRNAs, which makes miRNAs potent regulators of translation (Schratt et al., 2006). These effects are mediated by a macromolecular complex designated RISC (RNA Induced Silencing Complex). This miRNA regulatory mechanism was demonstrated in dendrites, where miR-134 binds specifically to 3'UTR of *limk1* mRNA inhibiting the translation of Limk1 (Lim-domain containing protein kinase 1) mRNAs (Schratt et al., 2006). Greenberg and colleagues observed that miR-134 bound specifically to 3' UTR of *limk1* mRNA (Schratt et al. 2006). To demonstrate that miR-134 binds *limk1* mRNA, Schratt *et al.* fused the 3' UTR of *limk1* mRNA with the coding sequence of GFP and observed a poor GFP expression in dendrites. However when miR-134 binding site in the 3' UTR was mutated, GFP levels increased significantly in dendrites (Schratt et al., 2006). In addition, BDNF application relieved miR-134's translation repression of LimK1 (reviewed in Tai and Schuman, 2006). This data shows that miRNAs have a role in the control of local translation (reviewed in Kindler and Kreienkamp, 2012).

## **1.2. Local protein synthesis in dendrites**

### **1.2.1. Existence of translational machinery - mRNAs and ribosomes - in dendrites**

Nowadays it is widely accepted that mRNAs can be locally translated in dendrites (reviewed in Sutton and Schuman, 2006). The story of the discovery of local translation in dendrites began in 1982 with the discovery of polyribosomes at the base of dendritic spines by Steward and Levy, suggesting that some proteins could be produced locally (Steward and Levy, 1982). These polyribosomes were associated with cisterns from endoplasmatic reticulum forming synapse-associated polyribosome complexes (SPRC) (Steward and Schuman, 2001).

Local translation at dendrites is known to contribute to long-term synaptic plasticity (reviewed in Sutton and Schuman, 2006). In 1996, Kang and Schuman observed that when a protein synthesis inhibitor is administered, BDNF-induced synaptic plasticity does not occur. They also observed that isolating synapses from the cell body by severing, some of them maintained protein synthesis, what supported the idea of local translation in dendrites (Aakalu et al., 2001; Kang and Schuman, 1996). Other studies also showed that in isolated dendrites fragments and postsynaptic sites, occurs incorporation of radiolabeled amino acids into new proteins, which supports the idea of local translation in dendrites (reviewed in Sutton and Schuman, 2006). In 2001, Schuman and colleagues observed that BDNF induced protein synthesis of a GFP reporter in hippocampal neurons. They also showed that BDNF stimulates protein synthesis in transected neurites.

Together, these results unequivocally that protein synthesis occurs in dendrites (Aakalu et al., 2001).

Another important observation was done by Steward and colleagues that verified the transport of mRNAs to dendrites (Davis et al., 1987). In line with these evidences, Eberwine and colleagues amplified mRNAs in dendrites and identified mRNAs for ionotropic glutamate receptors and for proteins involved in modulating the translation of proteins (Miyashiro et al., 1994). However, the acceptance of these mRNAs as localized mRNAs were delayed until the appearance of high sensitivity *in situ* hybridization studies (reviewed in Martin and Zukin, 2006).

There might be several different mRNAs in dendrites, but just a few of them have been experimentally confirmed. Different neurons contain different mRNAs and some of them seems to be specific of some classes of neurons (reviewed in Bramham and Wells, 2007). Some of these mRNAs are localized within dendrites have: microtubule-associated protein 2 (MAP2), the  $\alpha$ -subunit of  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II ( $\alpha\text{CaMKII}$ ), brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated protein (Arc), tyrosine-related kinase B (TrkB) receptor, IP3 receptor, the NMDA receptor (NMDAR), the SH3 domain and ankyrin repeat protein (Shank1–3), FMR1, Jacob, dendrin and so many others (reviewed in Kindler and Kreienkamp, 2012; Martin and Zukin, 2006) (for details please see Table I).

**Table I - mRNAs localized within dendrites** (adapted from Kindler and Kreienkamp, 2012; and Steward and Schuman, 2001).

<b>mRNA</b>	<b>Class of Protein</b>	<b>Protein Function</b>
<b>MAP2</b>	Cytoskeletal	Microtubule-associated
<b><math>\alpha</math>CaMKII</b>	PSD protein	Multifunctional kinase Ca <sup>2+</sup> signaling
<b>BDNF</b>	Growth factor	Survival support Growth and differentiation of neurons and synapses
<b>Arc</b>	Cytoskeleton-associated	Actin-binding
<b>TrkB</b>	Integral membrane	Receptor of neurotrophines
<b>IP3 receptor</b>	Integral membrane (endoplasmatic reticulum)	Ca <sup>2+</sup> signaling
<b>Shank1–3</b>	PSD protein	Contribution to functional and morphological maturation of postsynaptic specializations
<b>NMDAR</b>	Integral membrane	Receptor of glutamate
<b>FMR1</b>	RNA-binding protein	Synaptic plasticity Translation regulator
<b>Jacob</b>	Caldendrin binding partner	Synaptic plasticity regulator
<b>Dendrin</b>	Putative membrane	Unknown

### 1.2.2. Function of local protein synthesis in dendrites

Currently it is known that local protein synthesis in dendrites is crucial for synaptic plasticity, namely during LTP and LTD (long-term depression) (Steward and Schuman, 2001).  $\alpha$ CaMKII is one of the four different chains that composes CaMKII, the others are  $\beta$ CaMKII,  $\gamma$ CaMKII and  $\delta$ CaMKII (Tighilet et al., 1998).  $\alpha$ CaMKII is found in high concentration in postsynaptic densities (PSDs) of excitatory synapses, what suggest a role in plasticity (reviewed in Kindler and Kreienkamp, 2012). In fact,  $\alpha$ CaMKII is necessary for hippocampal LTP and spatial learning (Elgersma et al., 2002). In 1999, Ouyang and colleagues provided evidence for local dendritic translation of  $\alpha$ CaMKII after high-frequency stimulation of hippocampal neurons and observed a significant increase in nonphosphorylated  $\alpha$ CaMKII, which was blocked in the presence of protein synthesis inhibitors suggesting that  $\alpha$ CaMKII was local synthesized within dendrites (Ouyang et al., 1999). In line with this, Mayford and colleagues produced a mutated form of  $\alpha$ CaMKII lacking its 3'UTR. This mutant displays a total loss of  $\alpha$ CaMKII mRNA within dendrites and a loss of 50% of the total  $\alpha$ CaMKII levels. The protein levels of  $\alpha$ CaMKII were very low in the PSD, what suggested that local translation in dendrites is crucial for postsynaptic targeting of  $\alpha$ CaMKII (Miller et al., 2002). In addition, they observed deficits in late phase LTP and in memory consolidation in mice with the mutated form of  $\alpha$ CaMKII (Miller et al., 2002). In line with these evidences, Steward concluded that low amounts of  $\alpha$ CaMKII in postsynaptic site caused deficits in behavior and reduction in L-LTP (late-LTP) and these observations are due to decreased local translation of  $\alpha$ CaMKII mRNA (Steward,

2002). Taking together, this data concludes that local translation of *αCaMKII* mRNA in dendrites is important for synaptic plasticity (reviewed in Kindler and Kreienkamp, 2012).

During LTP there is an alteration in the structure of spines, changing from a stubby to a mushroom-like structure, which are accompanied by an increase in PSD size, and these alterations are mediated by actin polymerization (Fukazawa et al., 2003; Harris et al., 2003; Matsuzaki et al., 2004). Inokuchi and colleagues showed that LTP induces an increase in actin polymerization, which in other words means an increase of F-actin in spines, which is accompanied by cofilin phosphorylation, a main regulator of actin dynamics, in spines (Fukazawa et al., 2003). Following LTP induction in synapses cofilin was phosphorylated and thus F-actin-disassemble mediated by cofilin was inhibited, allowing F-actin formation and stabilization and the consequent enlargement of spines (Rex et al., 2007).

Arc, whose mRNA was already identified in dendrites, is a very important protein for LTP consolidation, since Arc is essential for stabilization of F-actin (reviewed in Holt and Bullock, 2009). To test the function of local synthesis of Arc during LTP, Bramham and colleagues injected Arc antisense oligodeoxynucleotides in granule cell synapses after a LTP stimulus, and they observe a great decrease in dendritic *arc* mRNA and, consequently, in protein levels. Due to this decrease in Arc levels, there was a decrease in nascent F-actin at synapses. So, local synthesis of Arc is a key mechanism for LTP maintenance, since it connects gene expression and F-actin formation (Messaoudi et al., 2007).

Local protein synthesis is also important for LTD, and in this case is thought to be regulated by RNA-binding proteins such as CPEB1 and FMRP (Huber et al.,

2002; McEvoy et al., 2007). When a mutated form of CPEB1, able to bind the CPE region of an mRNA but unable to induce translation after a stimulus, is expressed in neurons, these neurons showed loss of LTD similar to a loss of LTD in the presence of protein synthesis inhibitor. So, local protein synthesis is important for LTD formation (McEvoy et al., 2007).

In turn, FMRP is important to limit the effects of LTD. FMRP, in normal conditions, acts as a negative regulator of protein synthesis and when it is not present occurs an enhancement of translation in dendrites, resulting in excess AMPAR internalization and exaggerated LTD (Bear et al., 2004). In line with these evidences, De Zeeuw and colleagues verified that when FMRP is knocked out or specifically deleted from Purkinje neurons, the LTD increases. This suggests that when FMRP is present in later phases of LTD, there is less local protein synthesis, which results in a decrease of LTD (Koekkoek et al., 2005). Recent evidences indicate that FMRP suppress translation of its target mRNAs (Darnell et al., 2011; Edbauer et al., 2010). Thus, FMRP mediates LTD through regulation of dendritic protein synthesis (Ronesi and Huber, 2008).

Recently another mRNA, *jacob*, was identified in dendrites (reviewed in Kindler and Kreienkamp, 2012). Jacob protein is a plasticity-related protein, which is associated with Caldendrin and also is greatly expressed in the limbic system and cerebral cortex (Dieterich et al., 2008). Activation of NMDARs, coupled with  $Ca^{2+}$ -influx, leads to cleavage of the N-terminal myristoyl membrane anchor of Jacob by the  $Ca^{2+}$ -dependent protease calpain. Once cleaved, Jacob goes to the nucleus through association with importin- $\alpha$ , where interferes with transcription leading to loss of synaptic plasticity. It was demonstrated that local translation of

*jacob* mRNA is necessary for an appropriate function of Jacob protein and consequently necessary for a proper regulation of synaptic plasticity (reviewed in Kindler and Kreienkamp, 2012).

The studies described above, showed that local protein synthesis in dendrites is a very important process for synaptic plasticity and in the last decades several other transcripts have been identified in dendrites (reviewed in Bramham and Wells, 2007). For example, local translation of dendritic *glur1* mRNA is important for synaptic strength and local synthesis of matrix metalloproteinase 9 (MMP-9) is significant for regulation of extracellular matrix and also for modulation of synaptic efficacy (for a detailed review see Bramham and Wells, 2007).

### **1.3. Local protein synthesis in axons**

#### **1.3.1. Existence of translational machinery - mRNAs and ribosomes - in axons**

Pioneer studies in the 1970s using electron microscopy identified translation machinery components in growth cones of developing axons from rat and rabbit neurons (Tennyson, 1970; Yamada et al., 1971; Zelena, 1970). Similar to dendrites, the general idea was that proteins were synthesized in the cell body and then transported to axons (reviewed in Piper and Holt, 2004). However, this transport could not explain the presence of proteins in axons, whose transport was considerably longer than the protein half-life (Varshavsky, 1996), which means that some proteins could not survive to the entire journey (reviewed in Alvarez et al.,



2000). So, the “cell body” theory had some weaknesses and neglected important electron microscopy observations (Tennyson, 1970; Yamada et al., 1971; Zelena, 1970).

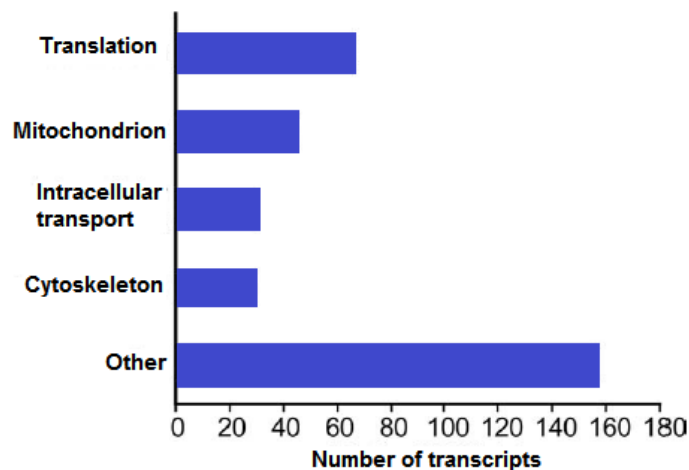
New observations emerged in this field which identified components of translation machinery in invertebrates' axons such as tRNA (Black and Lasek, 1977), rRNA (Martin et al., 1998), aminoacyl-tRNA synthetases (Giuditta et al., 2002), elongation factors (Giustetto et al., 2003) and mRNAs, particularly in growth cones (Bassell et al., 1994; reviewed in Jung et al., 2011; Kleiman et al., 1990), suggest the occurrence of local axonal translation (Giuditta et al., 2002). Axonal protein synthesis has been directly demonstrated in a study carried out by Syed and colleagues, that injected *elh* (egg-laying hormone) mRNA in isolated axons, and observed local protein synthesis (Van Minnen et al., 1997).

In addition, other studies identified ribosomes in different regions of vertebrates' axons (Bassell et al., 1998; Giuditta et al., 2002; Koenig et al., 2000; Steward and Ribak, 1986; Zheng et al., 2001), as well as tRNAs, initiating factors and several mRNAs, suggesting that local axonal translation occurs in vertebrates (reviewed in Satkauskas and Bagnard, 2007). In vertebrates' mature axons initial studies demonstrated the presence of mRNAs (Ressler et al., 1994; Trembleau et al., 1996; Vassar et al., 1994; Weiner et al., 1996), however ribosomes were not observed (Koenig et al., 2000). An exception to this observation was reported by Rajasekaran and colleagues, who detected ribosomes in axons of regenerating mature vertebrate neurons (Zheng et al., 2001). Thus, the translation machinery is present in axons and, more importantly, is functional (reviewed in Sinnamon and Czaplinski, 2011).

### 1.3.2. mRNAs that have been shown to be localized to axons

Recent studies, taking advantage of new and more sensitive techniques, identified a large number of mRNAs localized in axons (reviewed in Jung et al., 2011). Cotman and co-workers, using microfluidic chambers that allowed them to harvest purified axonal populations, extracted mRNA from axons of cortical and hippocampal (Taylor et al., 2009). Using microarray expression profiling, the investigators identified 308 mRNAs which they considered trustworthy localized to axons. Afterwards, they categorized these mRNAs and identified four main functional categories, which had a considerable number of transcripts (>20) (Taylor et al., 2009).

As described in Figure 3, the four main functional categories identified in axons were translation, mitochondrion, intracellular transport and cytoskeleton. However, 157 of the 304 mRNAs localized to axons were not included in these four main functional categories, and we can hypothesize that they might have equally important roles in axonal development (Taylor et al., 2009).



**Figure 3 – The four main functional categories of mRNA population localized to naive cortical axons** – translation, mitochondrion, intracellular transport and cytoskeleton (adapted from Taylor et al., 2009).

Translation was the functional category with the highest number of transcripts identified in axons. Most of these translation transcripts were ribosomal proteins mRNAs, but mRNAs of elongation factors were also identified in axons (Taylor et al., 2009).

The ribosomal proteins mRNAs identified in this study had similarities with the ribosomal proteins mRNAs present in axons of *Aplysia* (Moccia et al., 2003) and mammalian dorsal root ganglion (DRG) (van Niekerk et al., 2007; Willis et al., 2007), in which local axon translation is fairly well established. In addition, it was identified an elongation factor in cortical axons, the eukaryotic elongation factor 1a1 (Eef1a1). This elongation factor acts as a binder between ribosome and aminoacyl tRNA during peptide synthesis and, in *Aplysia* neurites, is necessary to maintain the long term facilitation (Giustetto et al., 2003). Thus, the presence of these translation mRNAs is important for local translation in axons, suggesting the presence of protein synthesis machinery at axons (Taylor et al., 2009).

Transcripts related to intracellular traffic were also enriched at axons. Investigators identified transcripts of microtubule-associated motors, namely two kinesin and two dynein transcripts. The presence of kinesin transcripts, kinesin family member 5B (kif5B) and kinesin light chain 1 (klc1), and dynein transcripts, cytoplasmic light chain 1 and 2a, imply that axons transport cargo and organelles

since these transcripts are involved in the transport of cargo and mitochondria inside of axons (Taylor et al., 2009).

Furthermore, cytoskeleton associated transcripts were also found enhanced in axons. The majority of the identified mRNAs in this study had been previously identified in non-CNS axons (Moccia et al., 2003; Willis et al., 2005). However, investigators also identified transcripts earlier unrevealed, such as *β-catenin* mRNA that provide a link between the synapse and actin cytoskeleton and is also essential for synaptic vesicle clustering on presynaptic terminals (Bamji et al., 2003).

In addition to transcripts related with translation, also cellular transport-, cytoskeleton- and mitochondria-related transcripts were enriched in axons. The presence of these transcripts, which are nuclei derived, at axons provides maintenance of mitochondria autonomy in these structures, since local translation of these mitochondria mRNAs supplies proteins are essential for local mitochondria regulation and preservation (Taylor et al., 2009).

Holt and co-workers identified distinct populations of mRNA (Zivraj et al., 2010). Using laser capture microdissection (LCM), the authors captured growth cones or distal axons of RGC *Xenopus laevis* neurons and performed two distinct microarray analyses. In this study, the investigators identified 5105 mRNAs in axons and 958 transcripts in growth cones (Zivraj et al., 2010). Furthermore, they also analyzed growth cones of mouse retinal neurons and identified the outstanding number of 2162 mRNAs. In the axon microarray, similar to the previous study (Taylor et al., 2009), the functional category with more transcripts in axons was translation. In growth cone analysis the major functional category was

also translation with 31% of the mRNAs identified, followed by metabolic/glycolytic transcripts (14%) and by cytoskeletal/motor (9%) (Zivraj et al., 2010). When comparing both axons and growth cones transcripts was observed that there were mRNAs present both in axons and growth cones, nevertheless were also detected 58 transcripts specifically enriched in growth cones (Zivraj et al., 2010), suggesting that the growth cones are a distinct subcellular compartment instead of a simple extension of the axon (reviewed in Jung et al., 2011).

Taking together, these results show that the majority of the axonal transcripts were associated with translation and molecular transport, suggesting that axons have the ability to maintain or increase the production of proteins through the synthesis of the local translation machinery (reviewed in Jung et al., 2011). These recent studies provide evidences that the number of mRNAs in axons is much higher than previously thought (reviewed in Jung et al., 2011).

### **1.3.3. Axonal protein synthesis**

#### **1.3.3.1. *Growth cone guidance***

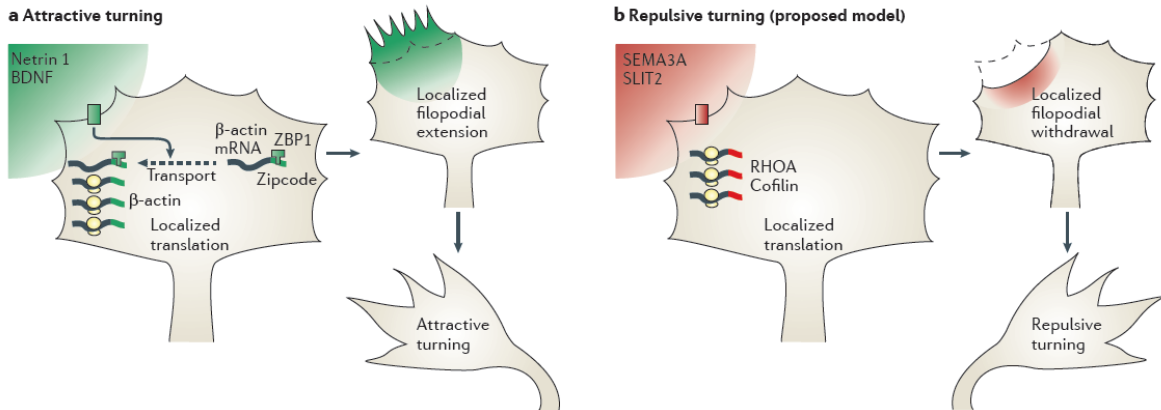
##### **1.3.3.1.1. Guidance cues**

In the developing nervous system, axons are guided by attractive and repulsive guidance cues to their targets. The growth cone is the structure that receives and translates these cues into turning responses and these might be relatively autonomous from cell body due to local translation within axons (reviewed in Lin and Holt, 2007).

An initial study tested the function of local axonal translation in axon chemotropic responses to an attractive (netrin-1) and to a repulsive cue (semaphorin 3A – Sema3A) (Campbell and Holt, 2001). Sema3A has the ability to induce repulsive turning of growth cones, but when protein synthesis inhibitors were administered to axons was observed the abolishment of growth cones repulsive turning (Campbell and Holt, 2001). Campbell and Holt also tested if protein synthesis was necessary for the growth cone turning in response to attractive cues, such as netrin-1. They verified that in the presence of protein synthesis inhibitors, growth cones were not attracted in direction to a netrin-1 source and showed a neutral turning response, meaning that the production of new proteins is essential for turning in response to attractive cues (Campbell and Holt, 2001). Thus, these guidance cues enhanced protein synthesis in growth cones of *Xenopus* RGCs neurons, but this enhancement was blocked by protein synthesis inhibitors, suggesting that protein synthesis is required for axon chemotropic responses (Campbell and Holt, 2001). Further, Campbell and Holt carried out experiments on severed axons to demonstrate if local axonal translation, and not translation in cell body, was necessary for the growth cone chemotropic responses (Campbell and Holt, 2001). The investigators observed that protein synthesis inhibitors abolished the growth cone responsiveness to guidance cues in severed axons, indicating that local proteins synthesis is required for axon chemotropic responses (Campbell and Holt, 2001).

A subsequent study demonstrated that dissimilar guidance cues trigger different signaling pathways (Campbell and Holt, 2003). In line with these evidences Jaffrey and co-workers demonstrated that netrin-1 induced a fast

trafficking of  $\beta$ -actin mRNA to the side of the growth cone closest to source of netrin-1, resulting in asymmetric  $\beta$ -actin synthesis (Figure 4a) (Leung et al., 2006; Yao et al., 2006). Moreover, it was demonstrated that Sema3A triggers translation of RhoA, a small guanosine triphosphatase (GTPase) that regulates the actin cytoskeleton, which in turn triggers growth cone collapse (Figure 4b) (Wu et al., 2005). Taking together these results, it is suggested that local axonal translation of specific mRNAs is required for growth cone collapse and turning (reviewed in Piper and Holt, 2004). Other studies also identified different guidance cues, such as Slit2 (Piper et al., 2006), pituitary adenylate cyclase-activating peptide (PACAP) (Guirland et al., 2003), BDNF (Yao et al., 2006), and Engrailed-2 (Brunet et al., 2005), which require local axonal translation to induce chemotropic responses (reviewed in Satkauskas and Bagnard, 2007). Slit2-induced growth cone collapse requires local protein synthesis and endocytosis. Moreover, Slit2, through activation of p38 and p42/p44 MAPK pathways, induced local translation of cofilin, which in the growth cone may mediate actin depolymerization and repulsion (Figure 4b) (Piper et al., 2006). In addition, Zheng and colleagues observed that BDNF, an attractive cue, induced local synthesis of  $\beta$ -actin in growth cones (Figure 4a), and inhibitors of local protein synthesis blocked the BDNF-induced attraction (Yao et al., 2006). Taking together, these results show that local protein synthesis in growth cones are required to exert appropriate guidance effects (reviewed in Satkauskas and Bagnard, 2007).



**Figure 4 – Schematic representation of growth cone turning controlled by translation of different mRNAs in response to attractive or repulsive cues (adapted from Jung et al., 2012)**

#### 1.3.3.1.2. Axonal responsiveness and outgrowth

Besides the fast local translation of cytoskeletal proteins for chemotropic responsiveness, axons also locally synthesize transmembrane and secreted proteins throughout the course of the axon growth in order to modulate responsiveness (reviewed in Lin and Holt, 2007). Axons use intermediate targets to modify their responsiveness to guidance cues, but this responsiveness alteration only occurs when axons pass through this intermediate targets (reviewed in Piper and Holt, 2004). For example, vertebrate commissural interneurons become sensitive to the repellent Slit and loose sensitivity to attractant netrin-1 after crossing the midline (Stein and Tessier-Lavigne, 2001). It was suggested that intermediate targets modify axon responsiveness to guidance cues through local axonal translation (reviewed in Lin and Holt, 2007). This role of local axonal



translation in axon responsiveness was first described by Flanagan and colleagues (Brittis et al., 2002). The investigators observed that when chick commissural axons cross the midline, occurs an upregulation of a reporter regulated by the EphA2 3'UTR in the distal part of the axons that had crossed the midline (Brittis et al., 2002). Although the presence of mRNAs in these axons had not been demonstrated, these results suggest that intermediate targets trigger local translation in axons (reviewed in Lin and Holt, 2007). Local axonal translation is also required for resensitization, which is important for axonal adaptation during alteration of guidance cues levels (reviewed in Lin and Holt, 2008).

A key factor for axonal pathfinding is axon growth rates and its regulation. The regulation of axon growth rates is very important for establishment of synapses and consequently for a proper formation of the nervous system (Hengst et al., 2009). Netrin-1 and nerve growth factor (NGF) are two molecules with the capacity to promote axonal elongation during the development of the nervous system (Macara et al., 2009) and it was previously described that netrin-1 and NGF induce local axonal translation (Campbell and Holt, 2001; Cox et al., 2008b).

A recent study demonstrated that NGF-stimulated axon elongation was abolished when axons were grown in the presence of protein synthesis inhibitors, suggesting that local axonal translation is required for stimulated outgrowth (Hengst et al., 2009). PAR complex has been found in growth cones of embryonic axons of hippocampal neurons and this complex specifies the axon from neurites during initial neural development (Shi et al., 2003). Jaffrey and colleagues identified *Par3* mRNA in developing axons (Hengst et al., 2009). Furthermore, the authors found that local translation of the PAR complex is required for netrin-1 and

NGF-stimulated axonal outgrowth, but not for basal outgrowth (Hengst et al., 2009). The authors observed that NGF increased PAR3 protein levels in isolated axons and this effect was totally blocked by a protein synthesis inhibitor (Hengst et al., 2009). Altogether, these results suggest that local axonal translation of *Par3* mRNA is a necessary step for NGF-stimulated axonal outgrowth (Hengst et al., 2009). In a recent study, Twiss and colleagues demonstrated that  $\beta$ -actin locally synthesized in rat DRG axons increased filopodia formation and branching of axons and the locally synthesized GAP-43 regulated axonal length (Donnelly et al., 2013).

#### **1.3.3.2. Local protein translation in synapse formation**

Synaptogenesis has different stages, being initiated by target selection, followed by synapse assembly and finally synapse maturation and stabilization (reviewed in Giagtzoglou et al., 2009). Local translation is a very important process for synapse formation (reviewed in Lin and Holt, 2008). This idea started when it was demonstrated the requirement of local protein synthesis for long-term synaptic plasticity at preexisting connections in invertebrates (Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 1997; Sherff and Carew, 1999). Kandel and colleagues demonstrated that when a single bifurcated *Aplysia* sensory neuron was cultured together with two spatially separated motor neurons, a single axonal branch could undergo long-term branch-specific facilitation after a serotonin stimuli and this facilitation was dependent on local protein synthesis (Martin et al., 1997).

Schacher and co-workers observed induction and local clustering of functional NMDA receptors followed by addition of AMPA receptors at postsynaptic sites close to *Aplysia* sensory neurons varicosities during synapse formation and maturation, suggesting that local protein synthesis is essential for the establishment of synaptic connections (Conrad et al., 1999). In a very ingenious experiment, Schacher and Wu showed that synapse formation required local protein synthesis in *Aplysia* (Schacher and Wu, 2002). To reach this conclusion, the authors removed the cell body from the presynaptic sensory neuron, the cell body from the postsynaptic motor neuron or both, and followed variations in synaptic efficacy and structure. Schacher and Wu observed that *de novo* synapse formation was blocked by protein synthesis inhibitors, indicating that local translation is necessary for a correct synapse formation (Schacher and Wu, 2002). In line with these evidences, Zhang and Poo observed that local protein synthesis in *Xenopus* was required for synaptic potentiation induced by localized BDNF (Zhang and Poo, 2002). In this study, the authors demonstrated that local exposure to a BDNF-coated bead enhanced neurotransmitter secretion at developing synapses, and this potentiation required presynaptic protein translation (Zhang and Poo, 2002). A recent study, using live cell microscopy and a photoconvertible fluorescent protein translational reporter, directly visualized local translation at synapses during long-term facilitation of *Aplysia* sensory-motor synapses (Wang et al., 2009). Translation of the reporter required multiple applications of serotonin, was spatially restricted to stimulated synapses, transcript- and stimulus-specific and occurred during long term facilitation but not during LTD of sensory-motor synapses. Altogether, these findings support the idea that different patterns of

activity stimulate discrete patterns of local protein synthesis that facilitate the induction of the appropriate plasticity mechanisms (Wang et al., 2009).

The contact of sensory neurons of *Aplysia* with specific targets initiating synapse formation leads to branch-specific changes in transport and stability of specific mRNAs and enhanced accumulation of some mRNAs (Hu et al., 2002; Schacher et al., 1999). One of the mRNAs identified was *syntaxin* mRNA, expression and distribution of this mRNA during synapse formation was observed through *in situ* hybridization. *Syntaxin* mRNA was uniformly distributed in the absence of any target or when sensory neurons interacted with a target that failed to initiate synapse formation, but in initial phases of synapse formation, *syntaxin* mRNA accumulated at axon hillock (Hu et al., 2003). In the same study it was also observed that *sensorin* mRNA accumulates at the axon hillock in the presence of an appropriate synaptic target (Hu et al., 2003). Consistent with this observation, Schacher and co-workers showed that sensorin peptide regulates the formation and stabilization of sensory-motor synapses (Hu et al., 2004).

In line with these evidences, Martin and colleagues, used a sensory-motor culture model system and observed that in isolated sensory neurons, *sensorin* mRNA was diffusely distributed throughout the neurites. However, when sensory neurons were co-cultured together with their target motor neurons, occurred an accumulation of *sensorin* mRNA at sites of synaptic contact (Lyles et al., 2006). In addition, this effect was not observed when sensory neurons were cultured with a non-physiological target motor neuron, thus synapse formation might trigger *sensorin* mRNA localization (Lyles et al., 2006). Moreover, was observed that sensorin protein distribution in paired neurons was similar to the *sensorin* mRNA

pattern, suggesting that sensorin could be locally translated at sites of synaptic contact (Lyles et al., 2006). These observations suggested that *sensorin* mRNA localization and local axonal translation of sensorin are required for synapse formation. Consistent with this, knockdown of *sensorin* mRNA and protein synthesis inhibition blocked synapse formation (Lyles et al., 2006). Taking together, these data demonstrated that the formation of synapses between sensory and motor neurons induced re-localization of *sensorin* mRNA to the sites of newly formed synapses followed by local translation of this mRNA (Lyles et al., 2006).

A recent study demonstrated that protein translation could dynamically and locally regulate presynaptic activity and synapse stability (Sebeo et al., 2009). The authors observed that a short period of protein synthesis inhibition was sufficient to reduce the pool of vesicles available for release, thus reducing synaptic function and increasing synapse elimination (Sebeo et al., 2009).

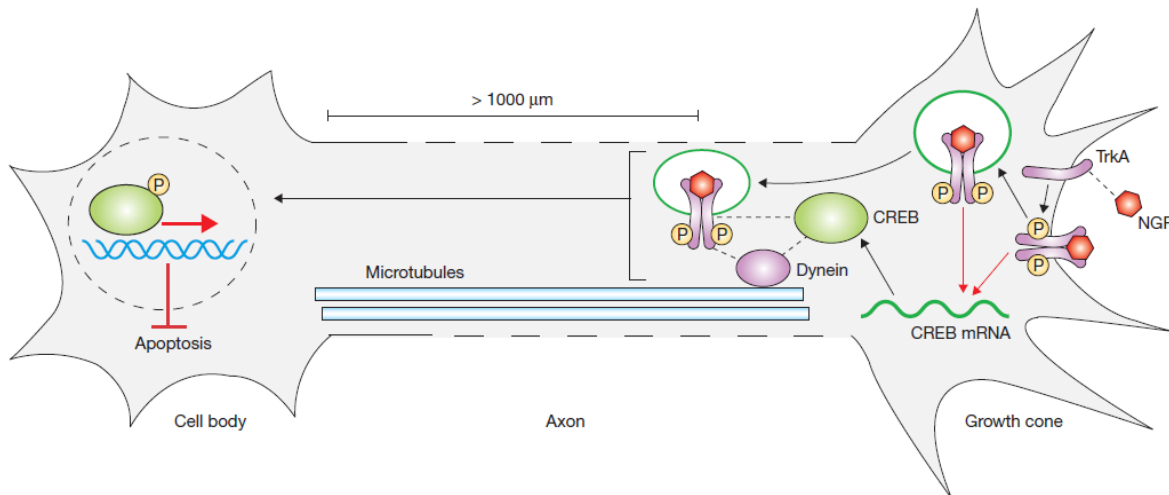
Together, all these studies suggest that local axonal translation might be required for proper synapse formation during vertebrate development.

### **1.3.3.3. Axonal regeneration**

#### **1.3.3.3.1. Local protein translation in retrograde signaling**

The neurotrophic hypothesis states that neurons compete for survival factors (e.g.: NGF), synthesized by target cells, which are detected by axon growth cones, resulting in a retrograde signal from the tip of the axon to the cell soma where transcription of pro-survival genes is activated (reviewed in Lin and Holt, 2008). A recent study demonstrated that mRNA encoding the cAMP-responsive

element (CRE)-binding protein (CREB) is present in axons, locally translated and then retrogradely transported to the cell soma in response to NGF, enhancing neuronal survival (Cox et al., 2008b). Axon-specific application of NGF increased translation (assessed by phosphorylation of 4EBP1) and activated a CREB-mediated survival pathway, which resulted in an increase of neuronal survival. This enhancement of neuronal survival was blocked by protein synthesis inhibitors and is dependent on a microtubule motor-dependent process (Cox et al., 2008b). A quite interesting observation reported in this study was the requirement of axonal CREB for phosphorylation (=activation) of CREB (pCREB) in the nucleus and also for CREB-mediated transcription, what suggests that axon CREB is the source of phosphorylated, and therefore active, CREB in the cell soma (Cox et al., 2008b; reviewed in Lin and Holt, 2008). Taken together, these results show that local CREB synthesis at axons is required for NGF-induced retrograde survival (Cox et al., 2008b). In a recent work in trigeminal ganglia, Ji and Jaffrey observed that SMAD1, 5 and 8 transcription factors are axonally translated in response to BDNF. SMAD1/5/8 translation is followed by the retrograde transport to the soma, together with BMP4 signaling endosomes that activate these transcription factors by phosphorylation (Ji and Jaffrey, 2012). Thus, target-derived factors control retrograde signaling through intra-axonal translation (Ji and Jaffrey, 2012).



**Figure 5 – Model of local translation and retrograde transport of CREB.** It was suggested a model in which NGF binding to TrkA triggered TrkA dimerization, autophosphorylation and endocytosis. Activated TrkA (pTrkA) induces local axonal translation of *CREB* mRNA. The NGF-pTrkA signaling endosomes are transported from the axon tip to the cell soma through motor protein dynein (Zweifel et al., 2005) in conjunction with axonal CREB. These NGF-pTrkA signaling endosomes are important for the activation of CREB signaling in the nucleus since it activates Erk5 and this kinase phosphorylates axonal CREB (Watson et al., 2001) that in the nucleus initiates the transcription of anti-apoptotic genes promoting neuronal survival (adapted from Cox et al., 2008b).

### 1.3.3.3.2. Regeneration

Nerve injury results in the loss of connection between the cell soma and the distal target. In these injuries axons are broken in two main fragments, the distal portion of the injured axon physically separates from the cell soma and undergoes *Wallerian degeneration* (reviewed in Willis and Twiss, 2006). The proximal portion,

which is still attached to the cell soma, initiates a growth program in order to restore any connectivity with target tissues (reviewed in Gummy et al., 2010). Growth cone re-formation, retraction, terminal enlargement formation and axon extension are required for the regeneration process, suggesting that there must be a protein turnover event (new proteins synthesized and old ones degraded) (reviewed in Gummy et al., 2010). Axon regeneration occurs in both peripheral nervous system (PNS) and central nervous system (CNS), however this regeneration is considerably more successful in PNS than in CNS (reviewed in Gummy et al., 2010). Consistent with the requirement of local protein synthesis for regeneration, the Fawcett group observed that in regenerating injured axons, occurs an enhancement of local protein synthesis. Blocking of local protein synthesis abolished regeneration, demonstrating the necessity of local axonal translation to regeneration (Verma et al., 2005). In line with these evidences, subsequent studies identified changes in mRNAs pools in injured comparatively to uninjured axons, namely an enhancement in axonal outgrowth-, targeting- and synapse formation-related transcripts (reviewed in Gummy et al., 2010). Localized synthesis of these mRNAs could provide to the axon a rapid source of cytoskeletal proteins essential to initiate the regenerative process (Wang et al., 2007). In 2001, Fainzilber group suggested that local axonal translation can be involved in retrograde signaling (Hanz et al., 2003). Injured DRG axons have higher levels of importin  $\beta$  protein and this is due to axonal translation of mRNA. The formation of a complex with a motor protein would then lead to the retrograde transport of signals that regulate nerve regeneration (Hanz et al., 2003). Moreover, another protein, axonally synthesized, was found upregulated after an injury stimulus - importin  $\beta$ 1, which regulates the



translocation of nuclear localization signal (NLS)-bearing proteins into the nucleus (Hanz et al., 2003). Vimentin, an intermediate filament, was also found enhanced after a nerve injury (Perlson et al., 2005). Fainzilber and colleagues observed that after being synthesized in response to an injured stimulus, vimentin undergoes proteolysis and then directly binds to importin  $\beta$ 1. Once formed, this complex vimentin/importin  $\beta$ 1 is retrogradely transported to the cell body together with phosphorylated ERK 1/2 (pErk) which may trigger a signaling function in the cell body (Perlson et al., 2005).

Another important observation was done by Fainzilber group, they demonstrated that nerve injury induced local translation of Ran-specific GTPase-activating protein (RANBP1) and importin- $\beta$ , which are important for Ran GTPase retrograde transport (Yudin et al., 2008). In a normal situation, Ran GTPase, a regulator of nuclear transport, is localized to axons in a GTP-bound state, preventing that cargo proteins link up with importin- $\alpha$ -dynein motor complex (Yudin et al., 2008). Ran GTPase bound to importin- $\alpha$ , forming the importin- $\alpha$ -importin- $\beta$ 1-dynein motor complex, when RANBP1 and importin- $\beta$  were synthesized after a nerve injury. When assembled, this motor bind and retrogradely transport transcription factor to the nucleus (Yudin et al., 2008). A recent study demonstrated that the signal transducer and activator of transcription 3 (STAT3), a transcription factor, is translated and phosphorylated in the injury site and then retrogradely transported to the nucleus. STAT3 was required to generate the injury signal (Ben-Yaakov et al., 2012).

As CNS neurons age, they lose the ability to regenerate, however, by increasing protein synthesis, CNS neurons might reestablish this capacity, suggesting that promoting protein synthesis might be an effective approach to improve neurons regenerative capacity, even in neurons which have lost that capacity (reviewed in Jung et al., 2012).

## **1.4. Synaptogenesis**

### **1.4.1. Synapse structure and function**

In the mammalian CNS, the majority of excitatory synapses are asymmetric, i.e., these synapses show a prominent postsynaptic membrane thickening; on the other hand, inhibitory synapses are symmetric, showing less prominent postsynaptic thickening (reviewed in Okabe, 2007).

The presynaptic compartment is a specialized region of the axons, which can exist in the ends or along the axon (varicosities), and is characterized by the existence of synaptic vesicles forming orderly clusters around a specialized region close to the membrane – the active zone (reviewed in Jin and Garner, 2008). Active zones are sites of synaptic vesicle docking and fusion and therefore sites of neurotransmitter release; moreover, active zones are juxtaposed against the postsynaptic density (reviewed in Zhai and Bellen, 2004). Biochemical purifications studies identified three major protein complexes in active zones (reviewed in Zhen and Jin, 2004). SNARE (soluble N-ethylmaleimide-sensitive component attachment protein receptor) complex is the elementary machinery required for vesicle docking

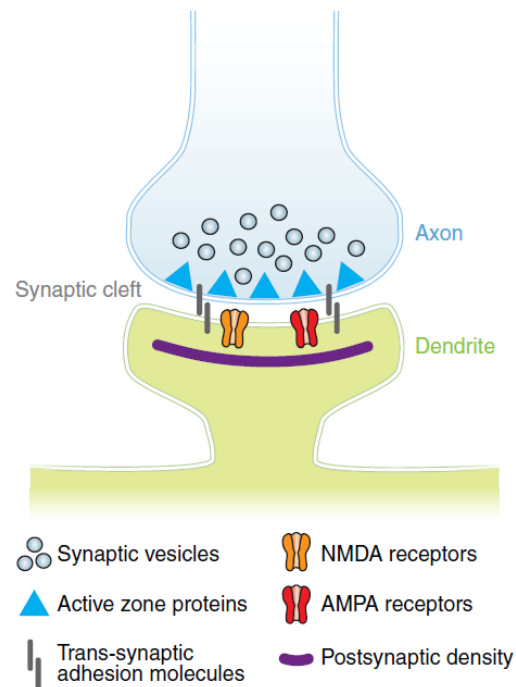
and fusion with the plasma membrane and includes syntaxin, synaptobrevin (VAMP), and synaptosomal-associated protein-25 (SNAP-25). Another complex identified interacts with the SNARE complex and regulates the exocytosis of vesicles and the major components of this complex are Munc18/UNC-18 (mammalian uncoordinated 18/uncoordinated-18), Munc13/UNC-13, and synaptotagmin (syt). The last complex identified might form a cytomatrix structure at the active zone that attaches vesicles and organizes the distribution of endocytosis and exocytosis machineries. This complex has a variety of components, such as Piccolo, Bassoon, Liprin/synapse defective-2 (SYD-2), Velis (vertebrate LIN-7 homolog), and Mints (Munc 18-interacting protein). These three major protein complexes of the active zone create a highly organized network allowing the correct arrangement of presynaptic terminals (reviewed in Zhen and Jin, 2004). In addition, there are trans-synaptic adhesion molecules localized in both pre- and postsynaptic membranes. These adhesion molecules bind to each other across the synaptic cleft and are required for regulation of the initial stages of synapse formation at CNS synapses and for modulating synaptic plasticity (reviewed in McAllister, 2007).

In the postsynaptic terminal, postsynaptic densities (PSDs) contain several molecules such as cytoskeletal proteins (actin, tubulin and neurofilament proteins), signaling molecules (calmodulin and CaMKII), membrane receptors (NR2A and NR2B subunits of the NMDA receptor, GluR1, 2, 3 and 4 of the AMPA receptor and eight different subtypes of mGluRs) and scaffolding proteins (PSD-95, Shank) (reviewed in Okabe, 2007). These glutamate receptors interact with a large number of scaffolding proteins, allowing their attachment with PSD. This interaction is

important for receptor trafficking, synaptic transmission, and synaptic plasticity (reviewed in McAllister, 2007). CaMKII is highly enriched in the PSD fraction and is one of the most abundant proteins in neurons (Kennedy et al., 1983). The content of CaMKII in PSD significantly increases after glutamate stimulation (Dosemeci et al., 2001) and it interacts with NR2B (Bayer et al., 2001) suggesting a role for CaMKII in synaptic plasticity (reviewed in Bourne and Harris, 2008). PSD-95 is a scaffolding protein enriched in the PSD, which interacts with cell-adhesion molecules and both NMDA and AMPA receptors allowing the cluster of these glutamate receptors (reviewed in Okabe, 2007). Recent studies demonstrated that PSD-95 not only forms structural a scaffold for anchoring AMPA receptors at the PSD, but also acts as signaling scaffold to link the intracellular signaling complex to the NMDA receptors, thus PSD-95 is a key component for synaptic plasticity (reviewed in Xu, 2011).

In addition to PSD, actin cytoskeleton is also important to the structure of postsynaptic compartments (reviewed in Bourne and Harris, 2008). Actin cytoskeleton is composed by filamentous actin (F-actin), which regulates spine morphology (Matus, 2000; Zito et al., 2004). Long-lasting LTP induces polymerization of F-actin and consequently spine enlargement; on the other hand, LTD induces F-actin depolymerization and spine shrinkage (reviewed in Bourne and Harris, 2008). Actin cytoskeleton interacts with PSD scaffolding proteins, suggesting that actin meshwork is important in the maintenance and remodeling of the PSD composition (Kuriu et al., 2006). Taken together these observations suggest that PSD and actin cytoskeleton are important structures for synaptic

plasticity ensuring an efficient synaptic transmission (reviewed in Bourne and Harris, 2008; Xu, 2011).



**Figure 6 – Schematic representation of a CNS glutamatergic synapse.** In the presynaptic site synaptic vesicles, which contain the neurotransmitter glutamate, localize closely to the active zone through a complex network of proteins. The postsynaptic site is characterized by the presence of glutamate receptors, namely AMPA receptors and NMDA receptors, which are in association with PSD proteins. These two structures are separated by the synaptic cleft, however trans-synaptic adhesion molecules provide a molecular connection between the pre- and postsynaptic membranes (adapted from McAllister, 2007).

Signal transduction in chemical synapses occurs at presynaptic active zones where the electrical signal is converted into a chemical signal. This chemical signal results in synaptic vesicle fusion and release of neurotransmitters into the synaptic cleft (Schikorski and Stevens, 1997). The released neurotransmitters, for example glutamate, cross the synaptic cleft and binds to glutamate receptors present in the PSD, resulting in pore opening (Kennedy, 2000). This opening leads to ion influx, local depolarization, and activation of voltage-gated ion channels as well as a number of signaling cascades, in other words, synaptic transmission takes place (reviewed in McAllister, 2007).

The neuromuscular junction (NMJ) is a specialized synapse, formed by a motor axon and a muscle fiber (Ruff, 2003). This specialized synapse is characterized by three main structural elements which are: i) the presynaptic region containing the nerve terminal that is capped by a terminal Schwann cell; ii) the postsynaptic surface which has junctional folds with the receptors for the neurotransmitters accumulated the crests; and iii) the synaptic basal lamina that occupies the synaptic cleft (reviewed in Hughes et al., 2006). The NMJ function is to transmit electrical impulses from the nerve terminal to the skeletal muscle via release of the neurotransmitter acetylcholine (ACh) (reviewed in Hughes et al., 2006).

These observations demonstrate that different synapses share the major structural elements (presynaptic region, synaptic cleft and postsynaptic region) and their function is to convert an electrical signal into a chemical signal, allowing a proper connection between the presynaptic and the postsynaptic cell (reviewed in Ziv and Garner, 2004).

#### **1.4.2. Axonal guidance**

During neuronal circuit formation axons extend to the appropriate targets through multiple guidance cues, which can be either attractive or repulsive, with short or long range action, forming a reproducible scaffold of nerves and tracts (reviewed in Raper and Mason, 2010). The axonal growth cone, a highly motile structure, shows different pathfinding responses to guidance molecules, such as collapse, retraction, bifurcation and attraction (reviewed in Wen and Zheng, 2006). The canonical families of guidance cues include, netrins, slits, semaphorins, and ephrins (Charron and Tessier-Lavigne, 2005; Dickson, 2002; Tessier-Lavigne and Goodman, 1996). However, there are also non-canonical guidance cues like morphogens and growth factors.

There are refined mechanisms employed by the growth cone that trigger distinct responses in the presence of different guidance molecules (reviewed in Wen and Zheng, 2006). One good example is the guidance of commissural axons during development. Commissural axons are initially lured to the midline by netrin (reviewed in Raper and Mason, 2010), once they reach and cross the midline, neurons lose their responsiveness to netrin, and become sensitive to slit being repelled away from the midline (reviewed in Wen and Zheng, 2006). This process is regulated by the Robo receptor family, when commissural axons approach to the midline they are insensitive to slits due to Robo-3 expression, which silences Robo-1 and Robo-2, the receptors for slits (Chen et al., 2008; Sabatier et al., 2004). After midline crossing, attraction mediated by netrin is silenced, to prevent re-crossing, and this is achieved by interaction of Robo with DTE, the receptor for

netrin-1, which blocks the signal transduction induced by netrin-1. Next midline repulsion is mediated by slit activation of the Robo receptor 1 and 2 (Stein and Tessier-Lavigne, 2001) and commissural axons are pushed away from the midline (reviewed in Raper and Mason, 2010). Thus, a complex interaction of different cues and receptors prevents premature repulsion and re-crossing of midline by commissural axons, permitting their projections to travel along the next intermediate path, the anterior–posterior track where they are guided by Wnt signals (reviewed in Wen and Zheng, 2006).

A good example for axon guidance importance in neuronal development is Horizontal Gaze Palsy with Progressive Scoliosis (HGPPS) which is resulted from mutations in the *ROBO3* gene (reviewed in Engle, 2010). In HGPPS, defects in the hindbrain axon guidance result in a deficient formation of the neuronal circuits (reviewed in Raper and Mason, 2010).

#### **1.4.3. Presynaptic differentiation**

Synapse formation is initiated by contacts between appropriate synaptic partner cells or by target-secreted molecules, which lead to intracellular assembly of the active zone, recruitment of synaptic vesicles beneath the presynaptic membrane, PSD formation and recruitment of neurotransmitter receptors to the postsynaptic membrane (reviewed in Fox and Umemori, 2006; reviewed in Williams et al., 2010).

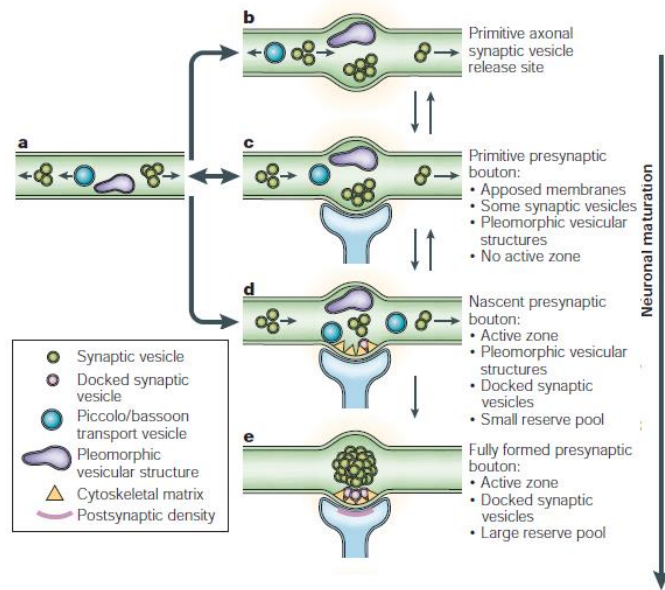


Before and during synaptogenesis, presynaptic proteins are transported in multi-molecular complexes (reviewed in Ziv and Garner, 2004), namely PTVs (piccolo-bassoon transport vesicles) and STVs (synaptic vesicle protein transport vesicles) (Sabo et al., 2006; Zhai et al., 2001). PTVs transport the active zone proteins piccolo and bassoon in addition to other proteins that mediate synaptic vesicle exocytosis, including Munc13, Munc18, syntaxin, and snap25 (Zhai et al., 2001). These transport vesicles move rapidly within the axon and can be transported in both directions in small or large clusters (Shapira et al., 2003). In addition, the recruitment of 2-5 PTVs is sufficient for the formation of new presynaptic sites (Bresler et al., 2004). In turn, STVs transport synaptic vesicle proteins and also proteins required for exo- and endocytosis (Ahmari and Smith, 2002; Sabo et al., 2006; Zhai et al., 2001). Moreover, STVs are greatly mobile within the axons as well as anterograde and retrogradely transported (reviewed in McAllister, 2007). Several studies demonstrated that STVs undergo depolarization-dependent cycling before contact with postsynaptic cells, and this STV cycling occurs within filopodia of growth cones and also along the axon shaft, suggesting that before synaptogenesis all regions of the axons are capable of releasing the STVs content (reviewed in Ziv and Garner, 2004). Taking together, these observations suggest that the core components of the presynapse are pre-assembled, i.e., before synaptogenesis the active zone is already formed and synaptic vesicles are attached at the membrane, allowing a rapid synapse formation (reviewed in McAllister, 2007).

Numerous contacts between the presynaptic axon and the postsynaptic dendrite could be conceivable. These axodendritic contacts can be originated by

both filopodia from axonal growth cones (Meyer and Smith, 2006; Washbourne and McAllister, 2002) or from dendritic growth cones (Sabo et al., 2006). In another example, filopodia from axons or dendrites form *en passant* synapses, triggering synapse formation (reviewed in McAllister, 2007). Another hypothesis is the formation of a contact between an axon and the dendritic shaft, resulting in the formation of a synapse (Friedman et al., 2000; Gerrow et al., 2006; Washbourne and McAllister, 2002).

During initial stages of neuronal differentiation, as axons are growing *en route* to their targets, there is an intrinsic capacity for rudimentary synaptic vesicle recycling at axonal growth cones and along the axonal plasma membrane (Figure 7a, b) (reviewed in Ziv and Garner, 2004). When contact between the dendrites and axons occurs, there is clustering of PTVs and STVs at contact sites (Figure 7c). These contacts can break up, resulting in the dispersion of PTVs and STVs, or can develop into functional presynaptic boutons, probably due to PTVs fusion with the presynaptic membrane and subsequent recruitment of synaptic vesicles at contact sites (Figure 7d). Gradually, these newly formed synapses create large reserve pools of synaptic vesicles, obtaining the structural and functional features of mature synapses (Figure 7e) (reviewed in Ziv and Garner, 2004).



**Figure 7 – Schematic model of presynaptic differentiation.** There appear to be multiple mechanisms for the presynaptic differentiation, which occur over several timescales. In neuronal development, the axon contains different mobile packets containing the precursors for the proper formation of the presynaptic compartment (a). Along immature axons there is the formation of primitive sites of synaptic vesicle recycling (b). Axon contacts with proper targets trigger the accumulation of mobile packets at these contact sites, resulting in the formation of primitive presynaptic boutons (c). These primitive presynaptic boutons can fall apart (b) or form nascent presynaptic boutons (d). In these nascent presynaptic boutons there is a fusion of PTVs, forming an active zone, and a recruitment of synaptic vesicles (d). As neurons mature, new synapses expand their reserve pools of synaptic vesicles, and present structural and functional characteristics of mature synapses (e) (adapted from Ziv and Garner, 2004).

#### **1.4.3.1. FGF-22**

Presynaptic differentiation requires target-derived factors which organize this differentiation (Umemori et al., 2004). Several groups found different presynaptic organizing molecules, for example: membrane-associated adhesion and signaling molecules (neuroligin, SynCAM, and Eph kinases), extracellular matrix components (laminin  $\beta$ 2), secreted differentiation factors (members of the Wnt and fibroblast growth factor (FGF) families), and cholesterol (Fox et al., 2007). FGFs are a family of 22 intercellular signaling molecules with a family of four transmembrane receptor tyrosine kinases (reviewed in Mason, 2007). These FGF receptors contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. In addition, the extracellular ligand binding domain of the FGF receptors contain two or three Ig-like domains. The Ig-like domain III undergoes alternative RNA splicing resulting in two different versions of Ig-like domain III in FGFRs 1, 2 and 3. This alternative splicing results in a different ligand binding specificity (Zhang et al., 2006). FGFs are implicated in a wide range of processes, such as cell proliferation, migration, differentiation, tissue repair, response to injury, axon guidance and synaptic formation (reviewed in Mason, 2007). FGF-22 is part of a subfamily that also contains FGF-7 and FGF-10, sharing close structural and functional homology with these molecules. Actually, these three molecules signal through the same FGF receptor, FGFR2b, an alternatively spliced product of the FGFR2 gene (Ornitz et al., 1996). In addition, FGF-22 also binds to FGFR1b but with low efficacy (reviewed in Mason, 2007).

Sanes and colleagues demonstrated that the FGF-7 family (FGF-22, FGF-10 and FGF-7) triggered the clustering of synaptic vesicles and neurite branching (Umemori et al., 2004). *In vivo*, FGF-22 should be secreted by the postsynaptic cell resulting in the presynaptic differentiation (reviewed in Fox and Umemori, 2006). To address this hypothesis, Sanes and colleagues studied the mossy fiber–cerebellar granular cell synapse, where postsynaptic neurons only express FGF-22 (but not FGF-7 and -10) and presynaptic neurons express FGFR2 (Umemori et al., 2004). FGF-22 and FGFR2 are highly expressed at the mossy fiber–cerebellar granular cell synapses, mainly during the onset of synapse formation. However, after synaptogenesis, the levels of FGF-22 and FGFR2 decreased significantly, suggesting that FGF-22 affects most likely the synaptic development of mossy fibers (Umemori et al., 2004). Sanes and co-workers demonstrated that FGF-22 was required for presynaptic differentiation *in vivo*, and for that these investigators injected a recombinant and soluble fragment of the extracellular domain of FGFR2b (FGFR2bAP), which binds and neutralize FGF-22, into the lateral ventricle of the brain of wild-type mice. They observed that mice with FGFR2bAP, i.e. with FGF-22 neutralized, showed a decrease in synaptic vesicle immunolabeling in the cerebellum. Moreover, mice that received an injection of FGFR2cAP, a recombinant fragment that does not neutralize FGF-22, had no defects in synapse formation (Umemori et al., 2004). In line with these evidences, Sanes and colleagues conditionally inactivated FGFR2 gene, and observed that these mice showed a reduction both in synaptic vesicle and active zone number, and thus demonstrated that FGFR2b is required in presynaptic differentiation (Umemori et al., 2004). Taking together, these results demonstrate that FGF-22

has the ability to induce synaptogenesis both *in vitro* and *in vivo*, in cerebellar mossy fibers (Umemori et al., 2004).

A subsequent study tested the ability of FGF-22 to mediate the formation of synaptic varicosities at NMJs formed *in vitro*. Using embryonic spinal motor neurons and the muscle C2 myogenic cell line they observed that FGFR2 is expressed in the motor neurons while FGF-22 is secreted by the muscle (Fox et al., 2007). Furthermore, the investigators observed that mice with FGF-22 neutralized with FGFR2bAP exhibited a decreased incidence of varicosities at neurite-myotube contacts, suggesting that FGF-22 is a target-derived factor of presynaptic differentiation at the NMJ (Fox et al., 2007). Moreover, the investigators observed that knockout mice for FGFR2 died before the birth, however, when they used a conditionally inactivated FGFR2 observed that NMJ are properly formed but synaptic vesicles were less concentrated at synaptic sites, and intriguingly at P7 there was a recovery of synaptic vesicles concentration at synaptic sites (Fox et al., 2007). Together, these results suggest that FGF-22 signaling is necessary for clustering of synaptic vesicles at embryonic stages, but other mechanisms can compensate the loss of FGF22 at later development stages (Fox et al., 2007).

In a recent report, Terauchi *et al.* identified *fgf22* and *fgf7* mRNA and protein highly expressed in CA3 pyramidal neurons. In addition, they also identified the mRNAs for FGFR2 expressed throughout the hippocampus (Terauchi et al., 2010). The investigators demonstrated that FGF-22 acted as target-derived presynaptic organizer, promoting the organization of excitatory presynaptic terminals while FGF7 is responsible for the differentiation of inhibitory synapses in the

hippocampus *in vivo* (Terauchi et al., 2010). More recently, Fox and co-workers found that FGF-22 was enriched in the dorsal lateral geniculate nucleus (dLGN) (Singh et al., 2012). The investigators used targeted mouse mutants lacking FGF-22 and observed that synapse formation and maturation of retinogeniculate was impaired in the absence of this target-derived presynaptic organizer, suggesting that FGF-22 is also required for a proper development of retinogeniculate synapses (Singh et al., 2012).

In summary, FGF-22 is a target-derived molecule, which released from the postsynaptic cell to induce differentiation of the presynaptic cell. Moreover, FGF-22 and FGFR2b, its cognate receptor, are present both in CNS and PNS where they regulate the formation of distinct synapses and neuronal circuits (Fox et al., 2007; Fox and Umemori, 2006; Singh et al., 2012; Terauchi et al., 2010; Umemori et al., 2004).

#### **1.4.4. Postsynaptic differentiation**

Postsynaptic differentiation is well established in glutamatergic synapses (reviewed in McAllister, 2007). In these glutamatergic postsynaptic sites, NMDARs are transported in mobile packets that move in both directions within dendrites, similar to presynaptic STVs (Washbourne et al., 2004; Washbourne and McAllister, 2002). NMDARs mobile packets, also transport a scaffolding molecule, SAP-102, and an exocytic protein, Sec 8 (Sans et al., 2003; Washbourne et al., 2004). In addition, there are subsets of NMDARs mobile packets that also transport

AMPA receptors (Washbourne and McAllister, 2002). Intriguingly, when NMDAR mobile packets stop during their trafficking along the axon, cycling of NMDARs with the plasma membrane is observed (Washbourne et al., 2004), suggesting that NMDAR mobile packets are sensitive to glutamate during their transport (reviewed in McAllister, 2007). Moreover, complexes of scaffolding proteins (including PSD-95, Shank, and GKAP) are also transported within dendrites in multi-molecular mobile packets (Gerrow et al., 2006).

In early studies, it was reported that presynaptic differentiation occurred before postsynaptic differentiation (Friedman et al., 2000). PSD-95 might be the first component transported to postsynaptic sites, followed by AMPARs and NMDARs (Friedman et al., 2000). Intriguingly, recent studies reported a different situation, where postsynaptic differentiation occurred simultaneously with presynaptic differentiation (reviewed in McAllister, 2007). In these studies, the first components that were localized in nascent synapses were NMDARs, trailed by PSD-95, with AMPA receptors being recruited with a slower rate (Washbourne and McAllister, 2002). These observations suggest that the different time and orders of recruitment of synaptic proteins can be result of different types of synapses being formed or the different ages of the neurons examined (reviewed in McAllister, 2007).



Chapter 2

Materials and Methods



## **2.1 Reagents**

Bovine Serum Albumin (BSA), poly-D-lysine (PDL), Minimum essential medium eagle (MEM), 5-fluoro-2'-deoxyuridina (5-FDU), paraformaldehyde, emetine dihydrochloride hydrate were purchased from Sigma-Aldrich (Sintra, Portugal). MEM-Non essential amino acids Neurobasal medium, penicillin-streptomycin, B27 supplement, sodium pyruvate, fetal bovine serum (FBS), trypsin and glutamine were purchased from GIBCO™ Invitrogen Corporation (Carlsbad, California, USA). Mouse laminin I was obtained from Cultrex®, as part of Trevigen, Inc (Helgerman Court, Gaithersburg, USA). Anisomycin and the transcription inhibitor actinomycin D were obtained from Calbiochem®, a division of Merck KGaA (Darmstadt, Germany). Mounting media ProLong Gold antifade reagent with DAPI was obtained from Molecular Probes®, as part of Invitrogen Life Technologies (Eugene, Oregon, USA). Recombinant human fibroblast growth factor-22 (FGF22) was purchased from R&D Systems, Inc (Minneapolis, USA). Recombinant Murine  $\beta$ -NGF was purchased from PeproTech Inc. (Rocky Hill, USA). Pierce Immunostain Enhancer was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

### **2.1.1. Antibodies**

Anti- $\beta$ -tubulin I mouse monoclonal antibody (T7816) was acquired from Sigma Aldrich (Saint Louis, Missouri, USA). Anti-synapsin I rabbit polyclonal antibody (AB1543), Anti-Vesicular Glutamate Transporter 1 (guinea pig) (AB5905)

were obtained from Millipore (Billerica, Massachusetts, USA). Anti-SV2 (mouse) was acquired from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA). Anti-4E-BP1 (mouse) and anti-p-4E-BP1 (Ser65/Thr70) (rabbit) were acquired from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). The secondary antibodies used for immunocytochemistry were acquired from Molecular Probes®, as part of Invitrogen Life Technologies (Eugene, Oregon, USA).

## **2.2. Hippocampal neurons**

### **2.2.1. Preparation of microfluidic devices**

The microfluidic devices, used for the hippocampal cell cultures, consist of a molded PDMS (polydimethylsiloxane) chamber placed against a glass coverslip (Corning No.1 24 mm × 40 mm). The PDMS devices were kindly fabricated and offered by Noo Li Jeon (School of Mechanical & Aerospace Engineering, Seoul National University, Seoul 151-472, Korea). The coverslips were cleaned with nitric acid 65% for, at least, 24h and then rinsed with water before a wash with pure ethanol. After that, the glass slides were dried at 50°C (15-20 min). Before the plating step, glass slides were coated with 0.1 mg/ml PDL overnight at 37°C and then washed with sterile mQ H<sub>2</sub>O. The microfluidic chambers were rinsed with filtered 75% ethanol, dried and assembled with the glass slides. Finally, the devices were coated with laminin in neurobasal plain media for to 2h at 37°C and washed once with plating media (minimum essential medium eagle (MEM)

supplemented with 0.026 M NaHCO<sub>3</sub>, 3.5 g/L glucose (to achieve a final concentration of 0.025 M), 1 mM sodium pyruvate and 10% FBS).

### **2.2.2. Culture of rat embryonic hippocampal neurons**

Primary cultures of rat hippocampal neurons were prepared from the hippocampus of E17-18 Wistar rat embryos. After dissection, hippocampi were treated for 15 min at 37°C with trypsin (0.045%) and deoxyribonuclease (0.01% v/v) in Hank's balanced salt solution (HBSS) (5.36 mM KCl, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 4.16 mM NaHCO<sub>3</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0,001% phenol red). After centrifugation, 1 min at 1000 rpm, to deposit the hippocampi, Hank's solution with trypsin was removed and the hippocampi were washed with plating medium containing 10% FBS to stop trypsin activity. In order to obtain a homogeneous cell suspension the hippocampi were mechanically dissociated with a P1000-pipette and then with a Pasteur pipette.  $7 \times 10^5$  cells were then added to the somal side of microfluidic chambers coated with PDL (0.1 mg/ml) and laminin (2 µg/ml). (see microfluidic chamber scheme, figure 8). After 2 h incubation at 37°C, the plating medium was removed and replaced for culture medium (Neurobasal medium supplemented with 2% B27, 25 µM glutamate, 0.5 mM glutamine and 1:400 penicillin/streptomycin). In the microfluidic chambers, the reservoirs of the axonal compartment were filled with glutamate-free culture medium (Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine and penicillin-streptomycin), in order to reduce the

exposure to high glutamate concentrations that could induce excitotoxicity in growing axons. At DIV 3/4, 50  $\mu$ l of 5-FDU in glutamate-free culture medium was added to the cultures at a final concentration of 10  $\mu$ M.

### **2.3. Stimulation and protein synthesis inhibition**

In order to observe the presynaptogenic effect of FGF22 on hippocampal neurons, recombinant human FGF22 was added only to the axonal compartment. A minimal difference between the somal and the axonal compartment was maintained during the stimulus time allowing a fluidic isolation between the two compartments and, this way, preventing the diffusion of the applied factors from the axonal to the somal side, or vice-versa.

The protein synthesis inhibitors and the transcription inhibitors were applied to the axonal compartment or to the somal compartment, depending on the set of experiments performed. The inhibitors were incubated for 15 minutes at 37°C before FGF22, so that the drugs could enter the axons prior to FGF22 stimulation. The protein synthesis inhibitors used were emetine and anisomycin at 10  $\mu$ M and the transcription inhibitor used was actinomycin D at 1,5  $\mu$ M.

#### **2.3.1. Immunocytochemistry**

After stimulation hippocampal, cells were subjected to a pre-fixation of 5 min in 1% paraformaldehyde (in PBS with 4% sucrose), thereby reducing the aggressive effect of paraformaldehyde to the axons. Then the cells were fixed in 4%

paraformaldehyde for 10 min at room temperature, washed three times with tris-buffered saline (TBS) and then permeabilized using TBS with 0.25% Triton X-100 for 5 min at room temperature. Next the cells were washed once with TBS followed by a wash in PBS with 3% BSA, to block non-specific binding, for 30 min at room temperature. Primary antibodies were incubated overnight at 4°C in PBS with 3% BSA. After incubation with the primary antibodies, the preparations were washed three times, 5 min each, with TBS to remove primary antibodies, then incubated with secondary antibodies in PBS with 3% BSA for 1 h at room temperature, washed again twice, 5 min each, in TBS with 0.1% Triton X-100 and then a 5min wash in TBS. After that, the glass slides were rinsed with mQ H<sub>2</sub>O and mounted in prolong mounting media. All the immunocytochemistry steps were performed with the microfluidic devices assembled, only after the last wash the PDMS mold was carefully removed from the coverslip. The preparations were cured overnight at 4°C protected from light, sealed with nailpolish and kept at 4°C until microscopy analysis.

### **2.3.2. Fluorescence microscopy and quantification**

Fluorescent images were taken using an inverted Zeiss Axiovert 200 microscope, an AxioCam HRm camera, AxioVision 4.8 and ZEN 2011 software. Images were acquired with a Plan-Neofluar 63x oil objective (numerical aperture 1.3) and exposure times conserved in single experiments. In microfluidic chambers images were taken from the axonal side, and to be more accurate we selected

regions equally distant from the microgrooves' entrance (approximately 300-400  $\mu\text{m}$ ).

Synapsin, SV2 and VGlut clustering was measured with Image J 1.46 software. Axons were randomly chosen from exported 8-bit images and their lengths determined. It should be noted that selected axons had similar thickness and appearance, and that fragmented, bead-bearing axons or terminal regions were rejected. Moreover, this selection was carried out in  $\beta$ -tubulin or Tau images, without observation of any puncta pattern. Images were thresholded (synapsin, SV2 and VGlut threshold values conserved in single experiments) and the number of synapsin, SV2 and VGlut puncta were quantified. Per condition, 25 or 30 axons were analyzed in each experiment. Per each selected axon we determined number of synapsin, SV2 or VGlut puncta/ $\mu\text{m}$ . The values obtained per axon were normalized against the control mean of that single experiment. The number of experiments and the number of axons analyzed are indicated in the graphs.

## **2.4. Statistical analysis**

Results are presented as normalized means  $\pm$  SEM of the number of axons indicated. Graphs and statistical analysis were performed in Graph Pad Prism 5 software. Statistical significance was assessed by unpaired t-test or one-way ANOVA analysis followed by the Bonferroni's post test or Dunnet's post test.



Chapter 3

Results and Discussion



### **3.1. FGF22-induced presynaptic differentiation and axonal translation**

#### **3.1.1. Hippocampal cultures in microfluidic chambers**

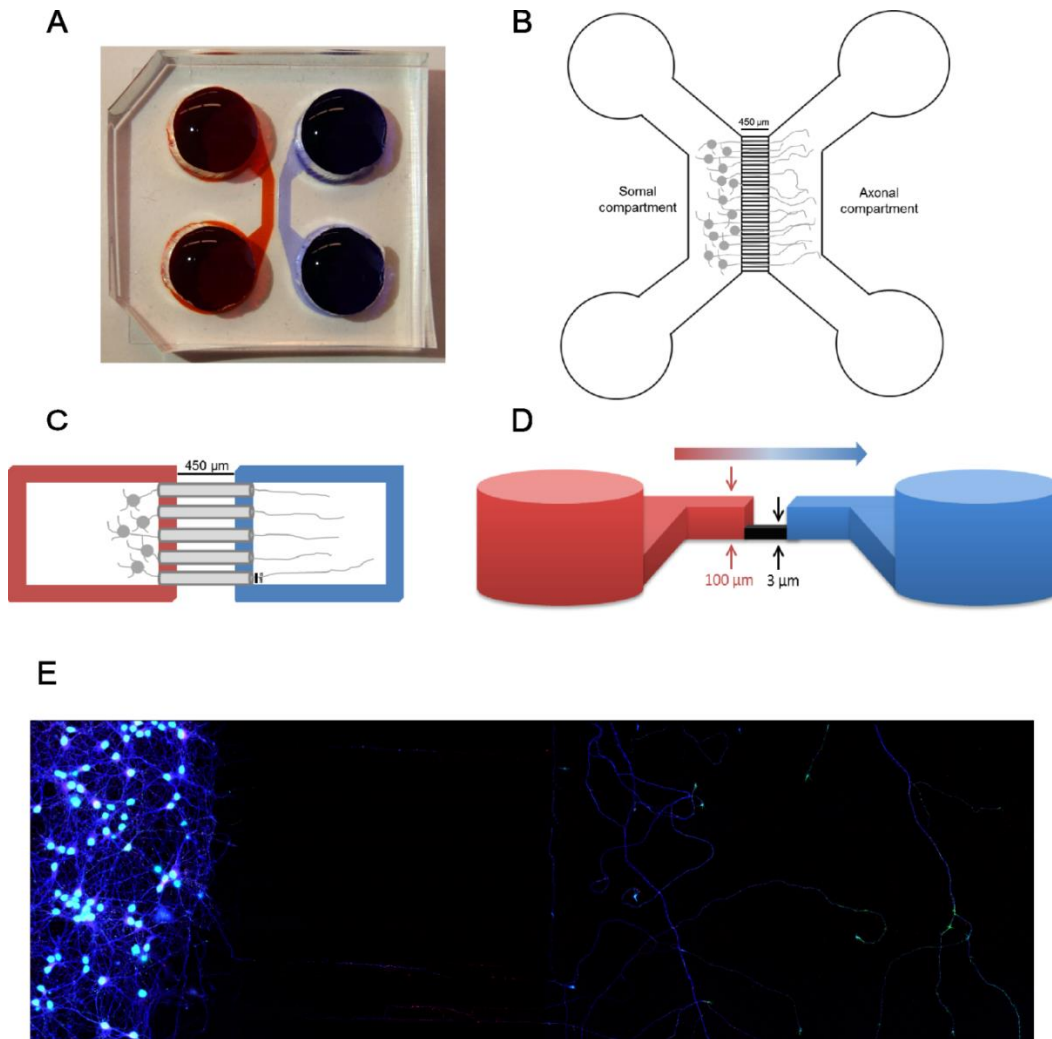
As previously mentioned, the purpose of this study was to determine if local protein synthesis is required for presynaptogenesis. However, the study of axonal events has been held back by the lack of reliable tools to specifically manipulate axons. To address this issue, we used a novel platform, a microfluidic chamber system which allows a fluidic isolation and physical separation of axons from cell bodies and dendrites (Taylor et al., 2005b)

Microfluidic chambers already have been used in studies focusing on axon injury and regeneration (Taylor et al., 2005b), identification of mRNAs in axons (Taylor et al., 2009), axonal elongation (Hengst et al., 2009), synapse-to-nucleus signaling (Taylor et al., 2010), axonal navigation and network formation (Millet et al., 2010), dendrite-to-nucleus signaling (Cohen et al., 2011) and synaptic vesicle dynamics (Taylor et al., 2013).

These microfluidic devices are composed of a molded PDMS piece placed against a glass coverslip (Figure 8A). The devices used in this work have two compartments, the somal and the axonal, which are connected through a set of small channels, the microgrooves (Figure 8B). This structure confines cell bodies in the somal compartment but allows axons to growth through the microgrooves into the axonal compartment. The compartments, each measuring 1.5 mm wide, 7 mm long and 100  $\mu$ m height, have at the ends two reservoirs that store culture medium. A constant flow between reservoirs should be maintained so that the

compartments have a continual renew of medium, allowing a proper nutrient and gas exchange. Due to the higher growth rate and length of axons when compared to dendrites, only axons cross the microgrooves into the axonal compartment, since dendrites are not long enough to go through the 450  $\mu\text{m}$  long microgrooves (Figure 8C). Besides physical isolation, microfluidic devices also feature fluidic isolation that is accomplished by a minimal volume difference ( $\sim 25 \mu\text{l}$ ) between the somal and the axonal compartment. This slight volume difference accompanied by the high fluidic resistance of the microgrooves, with 10  $\mu\text{m}$  wide and 3  $\mu\text{m}$  height, allows the fluidic isolation of the axonal compartment (Figure 8D).

Our first aim was to successfully grow rat embryonic hippocampal neurons in microfluidic chambers. For that we allowed the neurons to develop for 7 days on the microfluidic chambers, at which point the neurons were fixed and immunostained for  $\beta$ -tubuline (blue), synapsin (green), VGlut (red) and DNA (turquoise). Contiguous images were taken from a random area of the microfluidic device to obtain a representative image of the cultures (Figure 8E). On the axonal compartment (right side) we can observe only isolated axons, while the cell bodies and dendrites are restricted to the somal compartment (left side) due to the physical barrier provided by the microgrooves. So, with this culture platform we can isolate pure axonal populations, eliminating the contribution of cell bodies or dendrites, making it an ideal tool to study presynaptic differentiation.



**Figure 8 - Hippocampal neurons grown in microfluidic devices.** (A)

These small systems (20 mm × 25 mm) consist of a molded PDMS chamber placed against a glass coverslip. (B,C) The microfluidic device consist of a somal compartment (red) and an axonal compartment (blue), each 1.5 mm wide, 7 mm long, which are separated by microgrooves (450 μm long, 10 μm wide). Hippocampal neurons are plated in the somal compartment and between days 3-4 the axons pass through the microgrooves into the axonal compartment. (D) Side view of the microfluidic chambers. The height difference between microgrooves

(3  $\mu\text{m}$ ) and compartments (100  $\mu\text{m}$ ) combined with a minimal volume difference between the two sides ( $\sim 25 \mu\text{l}$ ) leads to a fluidic isolation between the two compartments for 20 hours (Taylor et al., 2005a). Primary cultures of hippocampal neurons, at DIV7, were immunostained for  $\beta$ -tubuline (blue), synapsin (green), VGlut (red) and DNA (turquoise). Contiguous images were taken from a random area of the microfluidic chamber using a Zeiss Axiovert 200 fluorescent microscope with a 40 $\times$  objective and assembled into a single image using the ZEN 2011 software.

### **3.1.2. FGF22-induced presynaptogenesis**

FGFs are a family of twenty-two molecules that are grouped in seven subgroups according with their evolutionary distance (Itoh, 2007). FGF22, as previously mentioned, belongs to a subfamily of presynaptogenic molecules, which can induce the differentiation of the presynaptic terminal. It was previously shown that FGF22 induced presynaptic differentiation in the cerebellum and in the neuromuscular junction, through the activation of FGFR2b (Umemori et al., 2004). In line with these observations it was demonstrated in our group that FGF22 is able to induce presynaptic differentiation in ciliary ganglia neurons (unpublished data). In order to determine the lowest concentration of FGF22 that induces the highest presynaptic differentiation we performed a dose-response curve at different stimulation time-points. For that, primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers until DIV6, a developmental stage prior to naturally occurring synaptogenesis in culture. The axonal compartment was

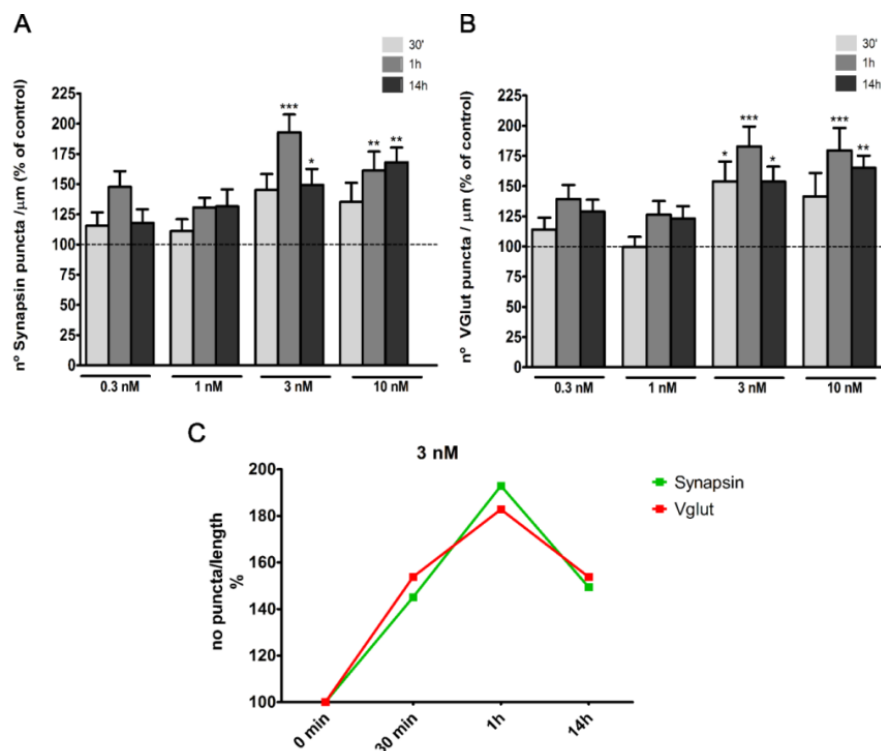
stimulated with 0.3, 1, 3 or 10 nM of FGF22 for 30 minutes, 1 hour or 14 hours. A minute volume difference (~ 25  $\mu$ l) was maintained to guarantee a complete fluidic isolation between the somal and axonal compartment and prevent diffusion of FGF22. Cultures were then fixed and immunostained against synapsin, VGlut and  $\beta$ -tubulin. Synapsin and VGlut are proteins associated to synaptic vesicles. Synapsin is a neuron-specific synaptic vesicle-associated phosphoprotein which is involved in the regulation of neurotransmitter release due to its capacity to dissociates and reassociates from synaptic vesicles in response to depolarization (Chi et al., 2001; Greengard et al., 1993; Llinas et al., 1985), and vesicular glutamate transporter 1 (VGlut1) is responsible for transporting glutamate into synaptic vesicles (Takamori et al., 2000) and is currently used as a tool to assess glutamate vesicles clustering in presynaptic differentiation studies (Terauchi et al., 2010). Thus, these proteins are used as hallmarks of presynaptic differentiation (Fox et al., 2007; Umemori et al., 2004).

The results obtained show that 1h stimulation with 3 nM of FGF22 induces the highest level of synapsin (192.91%,  $p < 0.05$ ) and VGlut (182.77%,  $p < 0.05$ ) puncta number per axonal length (Figures 9A, B). Clusters of synaptic vesicle accumulation are representatives of mature and functional presynaptic terminals. At the three different stimulation times (30min, 1h and 14h), FGF22-induced presynaptic differentiation seems to follow a similar pattern of dose-response, i.e., stimulation with 0.3 and 1 nM of FGF22 has a not significant effect on synaptic vesicles clustering, but upon stimulation with 3 nM of recombinant human FGF22 there is a significant increase in the clustering of both synapsin and VGlut. In addition, with 10 nM of FGF22 there was also an increase in synaptic vesicles

clustering (Figures 9A, B). This observation led us to conclude that FGF22-induced presynaptic differentiation is dose-dependent.

Interestingly, our results demonstrate a decrease in synaptic vesicle clustering, yet not significant, between the 1h and 14h stimuli with both 3 nM and 10 nM of FGF22. This suggests that, between 1h and 14h, might occur synapse elimination (reviewed in Fox and Umemori, 2006), which could explain the observed reduction in the number of synapses that are formed initially upon a stimulus with FGF22. However, at the moment it remains elusive what are the mechanisms responsible for this elimination.

To sum up, our observations demonstrate that the highest peak of FGF22-induced presynaptic differentiation is obtained with 3 nM of FGF22 and 1h stimulation (Figure 9A), suggesting that when FGF22 is acting locally a short time period is enough to induce the presynaptic differentiation.





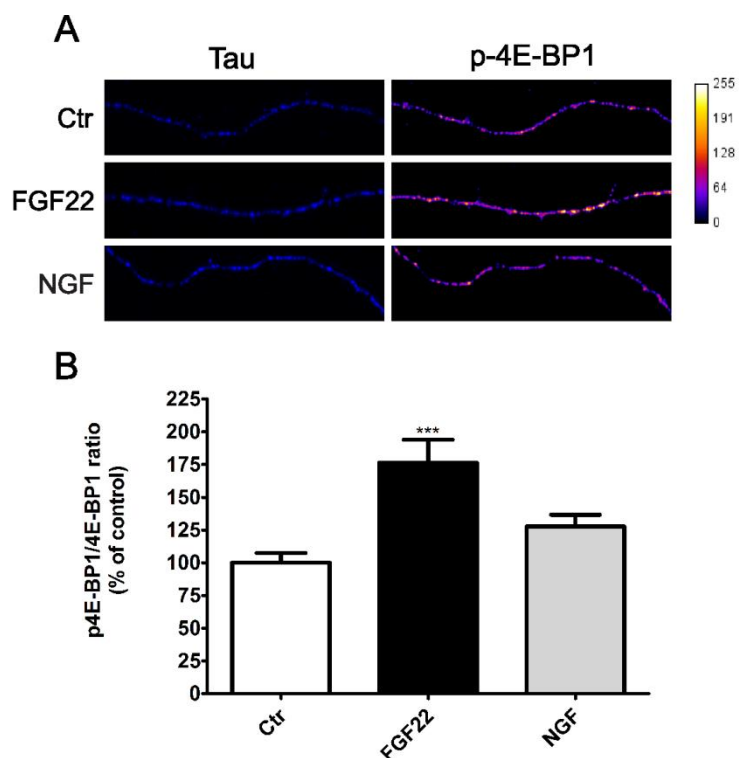
**Figure 9 - FGF22-induced presynaptogenic effect is dose-dependent.** (A, B) Primary cultures of rat embryonic hippocampal neurons were stimulated at DIV6 for 30 minutes, 1 hour and 14 hours at 37°C in conditioned medium with increasing doses of recombinant human FGF22 (0.3, 1, 3 and 10 nM). Immunostaining for synapsin, VGlut and  $\beta$ -tubulin was performed and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (C) The results show that at 1h stimulation with 3nM of FGF22 induces a maximum increase in both synapsin and Vglut clustering. The number of synapsin puncta/axon length and VGlut puncta/axon length were measured with Image J 1.46 software in randomly selected axons (30 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean  $\pm$  SEM of 90 axons of three independent experiments (\*\*\*) represents  $p < 0.001$ , \*\* and \* represents  $p < 0,05$  by ANOVA using Dunnett's post test when compared to control).

### **3.1.3. Local protein synthesis induced by FGF22**

We next address the possibility of FGF22 induces/regulates local axonal mRNA translation. Recent studies demonstrated that protein synthesis occurs at synapses (Kar et al., 2013; Lyles et al., 2006; Sebeo et al., 2009; Taylor et al., 2013), suggesting that local axonal translation might be required for the synapse formation. In order to demonstrate that FGF22 induces intra-axonal protein synthesis, we evaluated the phosphorylation levels of 4E-BP1 after FGF22

stimulation. Phosphorylated 4E-BP1 is commonly used as a translational reporter (Cox et al., 2008a). A specific stimulus, such as growth factors, induces the phosphorylation of mammalian target of rapamycin (mTOR) which trigger the phosphorylation and inactivation of 4E-BP1. 4E-BP1 phosphorylation is followed by the release of the translation factor eIF4E (Pfeiffer and Huber, 2006). Therefore, the ratio p-4E-BP1/4E-BP1 is an excellent indicator of translation initiation.

The results show that a stimulus with FGF22 is able to induce a significant increase in the p-4E-BP1/4E-BP1 ratio (176.23%,  $p < 0.05$ ), indicating an increase in intra-axonal translation (Figure 10A, B). As expected, 1h stimulation with NGF did not affect the levels of p-4E-BP1 (127.7%,  $p < 0.05$ ) demonstrating that the increase in protein synthesis observed upon FGF22 stimulation is specific to this presynaptic organizer. NGF was used as a negative control due to the absence of trkA receptors in hippocampus (Ip et al., 1993). Thus, these results led us to conclude that FGF22 is able to induce local protein synthesis in axons.



**Figure 10 - FGF22 induces intra-axonal protein synthesis.** (A) Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic devices for 6 days. Axons present in the axonal compartment were then stimulated with 3 nM of FGF22 or 100 ng/ml of NGF, for 1h at 37°C. Immunostaining for 4E-BP1, p-4E-BP1 (firescale) and Tau (blue) was performed and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (B) The intensity levels of both p-4E-BP1 and total 4E-BP1 per axon area were measured with Image J 1.46 software in randomly selected axons (30 axons per experiment). The results demonstrate that locally applied FGF22 induces an augment in the ratio p-4E-BP1/4E-BP1, indicating an increase in local axonal translation. Values were normalized to the control mean of each experiment. Bars represent the mean  $\pm$  SEM of a minimum of 60 axons from two independent experiments (\*\*\*) represents

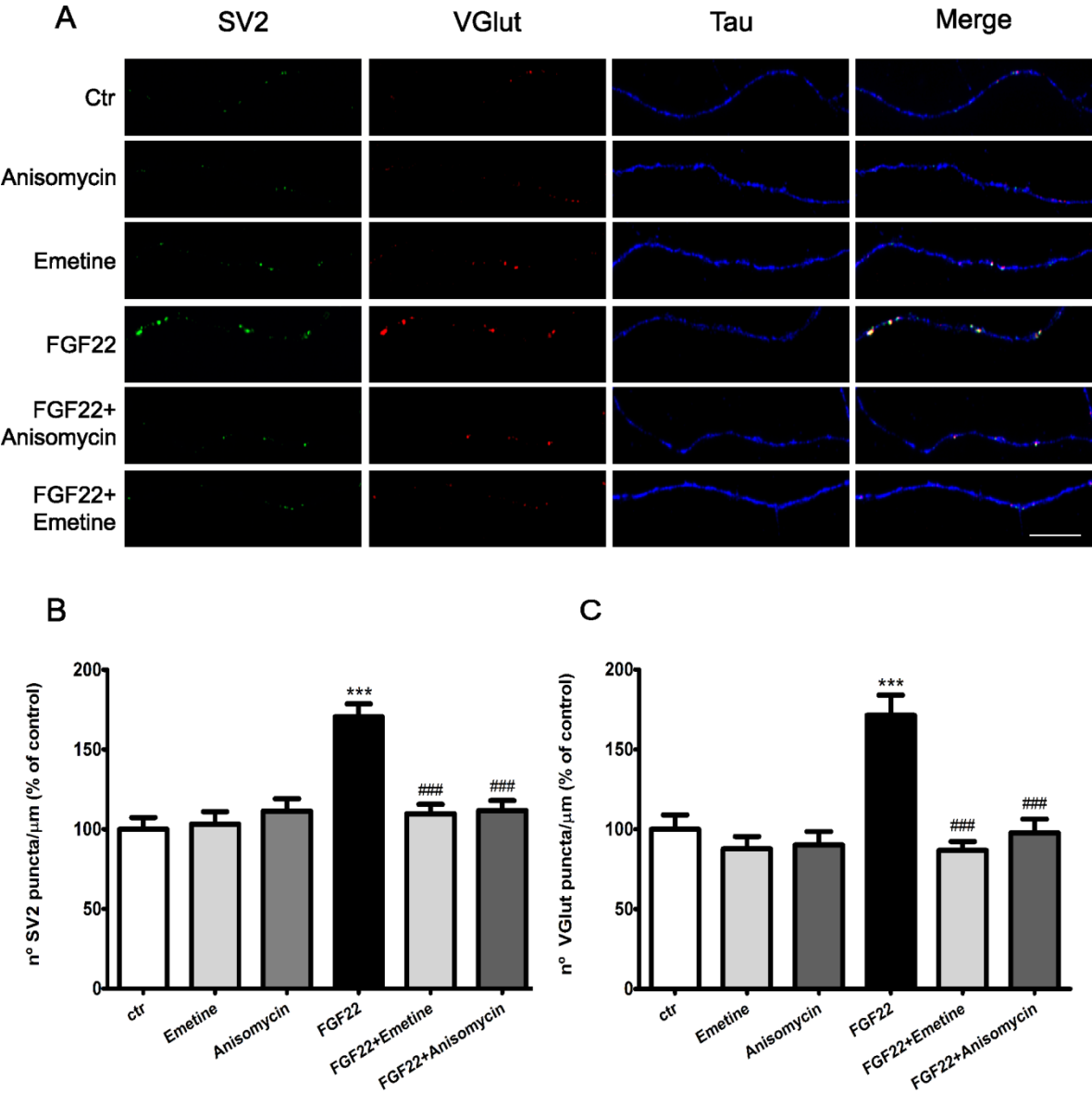
p<0.05 by ANOVA using Bonferroni's post test when compared to control).

#### **3.1.4. FGF22-induced presynaptic differentiation is dependent on axonal mRNA translation**

In line with the previous results we wanted to assess the role of local protein synthesis in FGF22-induced presynaptogenesis. Considering that FGF22 induces presynaptic differentiation and local axonal translation, as previously presented, we hypothesized that presynaptic formation depends on local protein synthesis.

To address this question the axonal compartment was stimulated for 1h with 3 nM of FGF22 in the presence or the absence of protein synthesis inhibitors (emetine or anisomycin, 10  $\mu$ M). Both emetine and anisomycin were pre-incubated for 15 minutes prior to FGF22 stimulation, allowing the drugs to penetrate the cells. The results demonstrate that when the axons were locally stimulated with FGF22 in the presence of the protein synthesis inhibitors, FGF22-induced presynaptogenesis was abolished (Figure 11A). Emetine decreased SV2, a vesicle transmembrane transporter (Feany et al., 1992), and VGlut puncta number to 109.71% (p<0.001) and 86.82% (p<0.001) respectively, and anisomycin reduces SV2 and VGlut number to 111.67% (p<0.001) and 97.74% (p<0.001) respectively (Figure 11B, C). We chose mechanistically distinct inhibitors, whereas emetine binds to the 40S ribosomal subunit blocking mRNA translation, anisomycin blocks protein synthesis binding to the 60S subunit. Therefore, the observed loss of

FGF22-induced presynaptic differentiation is not caused by a non-specific effect of the inhibitors but is in fact dependent on local protein synthesis. Taken together, our results demonstrate that local axonal translation regulates FGF22-induced presynaptic differentiation in hippocampal neurons.



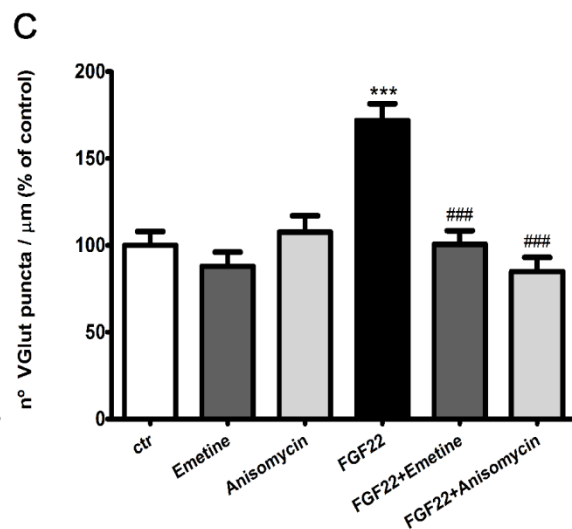
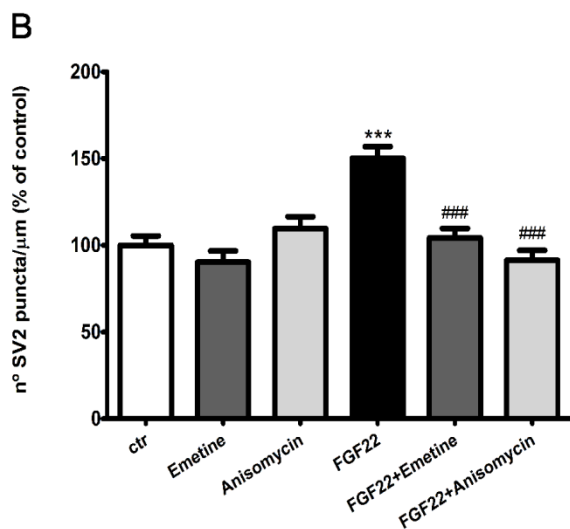
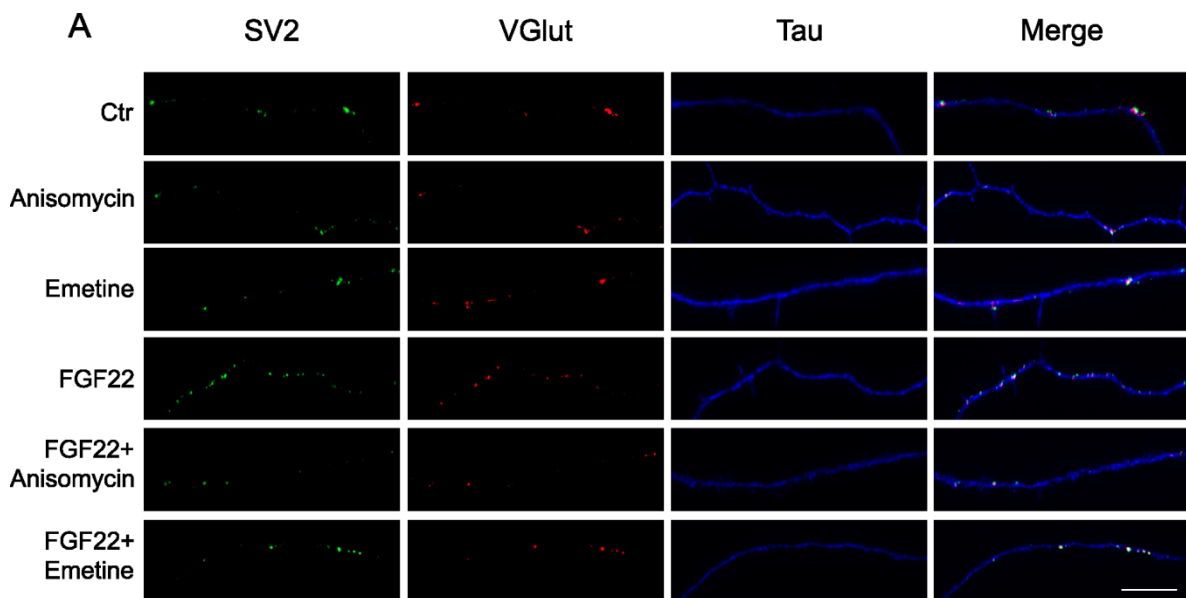
**Figure 11 - Intra-axonal protein synthesis is required for short-term presynaptogenesis.** (A) Primary cultures of rat embryonic hippocampal

neurons were grown in microfluidic device for 6 days. Axons present in the axonal compartment were then stimulated with 3 nM of FGF22 in the presence or absence of emetine or anisomycin, two distinct protein synthesis inhibitors, for 1 h at 37°C. Immunostaining for SV2 (green), VGlut (red) and Tau (blue) was performed and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (B, C) The number of SV2 puncta/axon length and VGlut puncta/axon length was measured with Image J 1.46 software in randomly selected axons (30 axons per experiment). The results demonstrate that when protein synthesis is locally inhibited with emetine or anisomycin, there is a significant reduction in FGF22-induced SV2 and VGlut clustering. Values were normalized to the control mean of each experiment. Bars represent the mean  $\pm$  SEM of a minimum of 90 axons from at least three independent experiments (\*\*\*) represents  $p < 0.001$  by ANOVA using Bonferroni's post test when compared to control; ### represents  $p < 0.001$  by ANOVA using Bonferroni's post test when compared to FGF22). The scale bar is 10  $\mu$ m.

As shown above (Figure 9), either 1h- or 14h-stimulus with FGF22 (3 nM) induces the formation of new synapses, however, some of these synapses were maintained, whereas others were lost. In addition, protein synthesis inhibition abolished the presynaptic differentiation induced by 1h stimulus of FGF22 (Figure 11). So, to confirm that local protein synthesis is also required for presynaptic differentiation induced by a longer FGF22 stimulus, i.e., the "stable pool" of

synapses, we used the same experimental strategy described above with a 14h stimulus with FGF22 (3 nM).

As expected, the results show that protein synthesis inhibition abrogate the presynaptic differentiation induced by FGF22 (Figure 12A). Emetine decreased SV2 and VGlut puncta number to 104.27% ( $p < 0.001$ ) and 100.71% ( $p < 0.001$ ) respectively, and anisomycin reduces SV2 and VGlut number to 91.31% ( $p < 0.001$ ) and 84.89% ( $p < 0.001$ ) respectively (Figure 12B, C).



**Figure 12 - Local mRNA translational is required for FGF22-induced presynaptogenesis.** (A) Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic devices until DIV7. Axons present in the axonal compartment were then stimulated with 3 nM of FGF22 in the presence or absence of emetine or anisomycin, for 14 h at 37°C. Immunostaining for SV2 (green), VGlut (red) and Tau (blue) was performed and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (B, C) The number of SV2 puncta/axon length and VGlut puncta/axon length was measured with Image J 1.46 software in randomly selected axons (30 axons per experiment). The results show that when protein synthesis is locally inhibited with emetine or anisomycin, there is a significant reduction in FGF22-induced SV2 and VGlut clustering. Values were normalized to the control mean of each experiment. Bars represent the mean  $\pm$  SEM of 90 axons from at least three independent experiments (\*\*\*) represents  $p < 0.001$  by ANOVA using Bonferroni's post test when compared to control; ### represents  $p < 0.001$  by ANOVA using Bonferroni's post test when compared to FGF22). The scale bar is 10  $\mu$ m.

### **3.2. FGF22-induced presynaptogenesis has a transcription-dependent component**

Our results clearly show that intra-axonal translation is required to FGF22-induced presynaptic differentiation. Consequently, we may speculate that FGF22

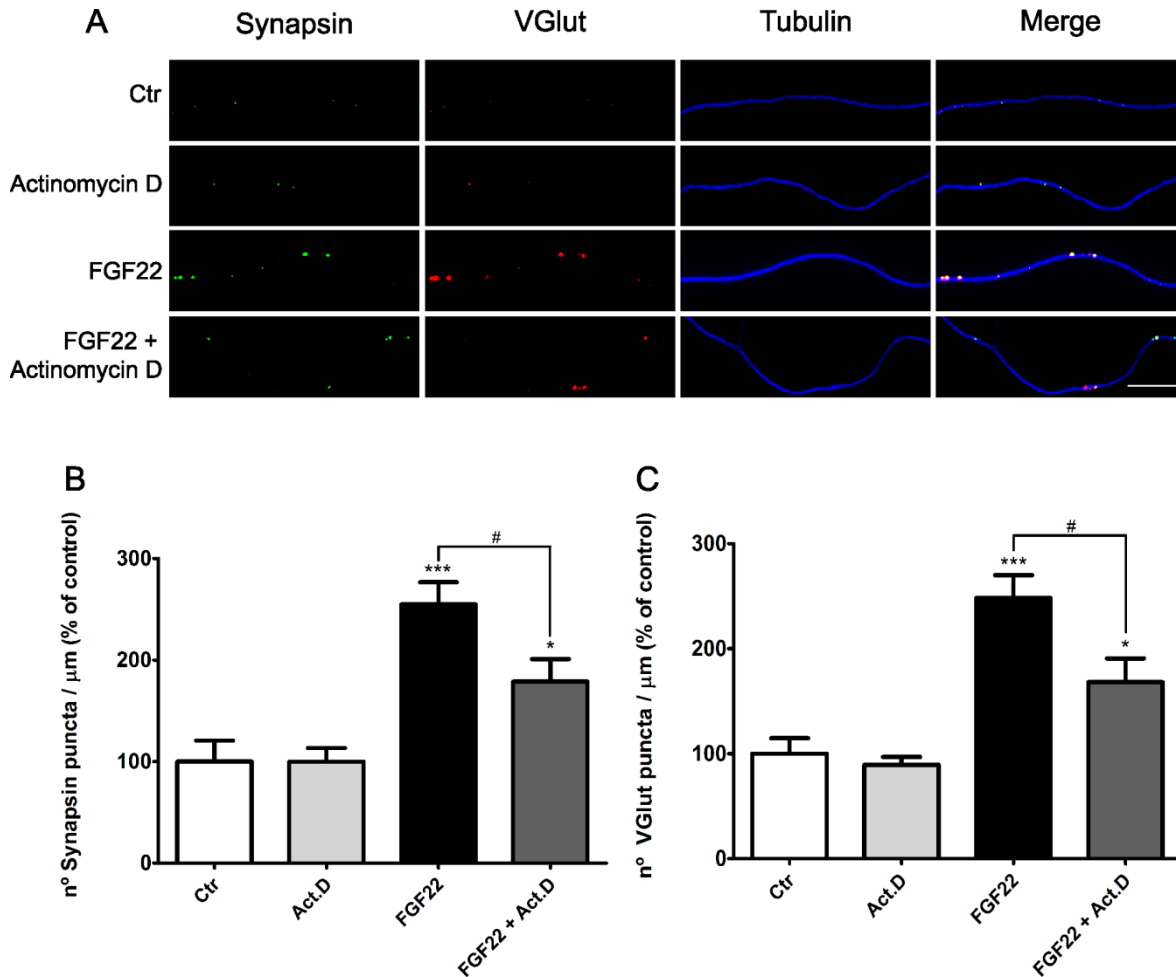


can regulate the targeting of mRNAs to presynaptic sites where it induces their translation. Actually, in *Aplysia* sensory neurons it was described that sensorin mRNA is targeted to presynaptic sites upon synaptic contact (Lyles et al., 2006). Also, in hippocampal neurons  $\beta$ -catenin is locally translated in axons contacting with poly-L-lysine coated beads and this is required for synaptic vesicle dynamics (Taylor et al. 2013). In accordance with these studies and with our results we could speculate that just after the synaptic contact, FGF22-FGFR2 signaling may induce the targeting of mRNA to presynaptic sites.

We next decided to address the role of DNA transcription in presynaptic differentiation. Previous studies have demonstrated that mRNAs are transported into axons through the action of RBPs (Donnelly et al., 2011; Eom et al., 2003; Zhang et al., 2001). Furthermore, Martin and collaborators observed in *Aplysia* that synapse formation induces the transcription of a specific mRNA (sensorin mRNA) (Lyles et al., 2006). In line with these observations and considering the presynaptic effect of FGF22, we next asked if DNA transcription has a role in FGF22-induced presynaptic differentiation. The axonal compartment of hippocampal cultures was subjected to a 14h stimulation with FGF22, while transcription inhibitor, actinomycin D (1.5  $\mu$ M), was present in the somal compartment to block DNA transcription.

The results demonstrate that when actinomycin D is administered to the somal compartment, FGF22-induced presynaptic differentiation is reduced (Figure 13A). Actinomycin D significantly reduces the number of synapsin (178.79%,  $p < 0.05$ ) and VGlut (168.07%,  $p < 0.05$ ) puncta number per axon length comparatively to FGF22 stimulation (Figure 13B, C). However, there is still an

increase in presynaptic differentiation when compared to control. It is also important to notice that 14h incubation with the inhibitor alone has no effect on the number of synapsin and VGlut puncta. These results show that FGF22-induced presynaptogenesis has a transcription-dependent component.

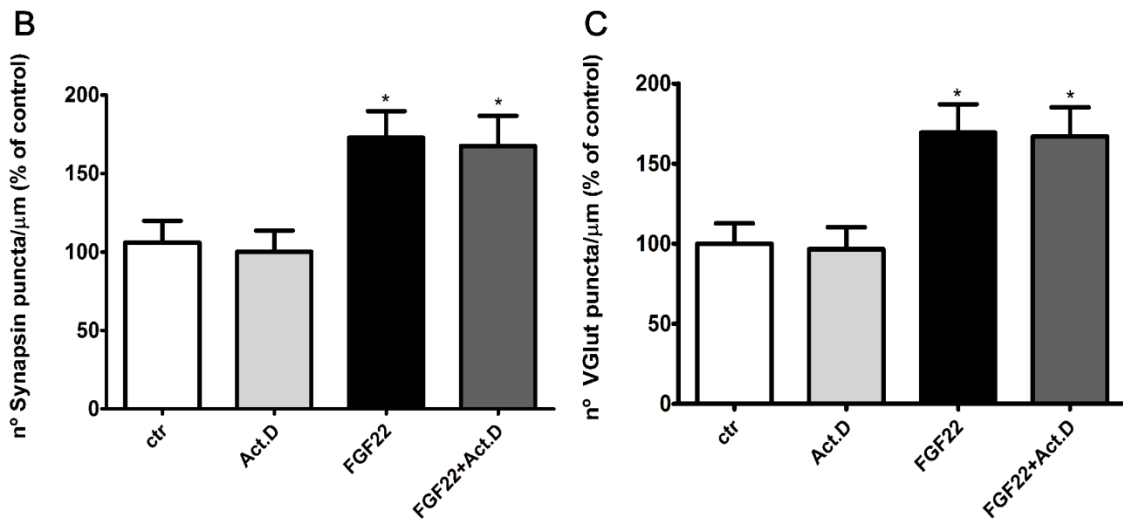
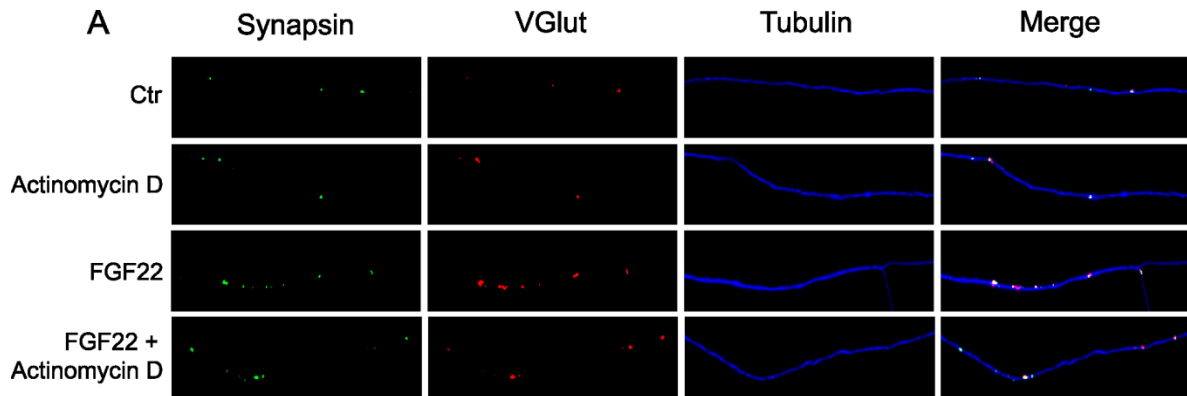


**Figure 13 - FGF22-induced presynaptic differentiation has a transcriptional component.** (A) Primary cultures of rat embryonic hippocampal neurons in microfluidic devices were grown until DIV7. Axons present in the axonal compartment were then stimulated with 10 nM of FGF22 in the presence or absence of actinomycin D, a transcriptional inhibitor, for 14 h at 37°C. Immunostaining for synapsin (green), VGlut (red) and  $\beta$ -tubulin (blue) was performed and images

were taken to the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (B, C) The number of synapsin puncta/axon length and VGlut puncta/axon length was measured with Image J 1.46 software in randomly selected axons (30 axons per experiment). The results show that when transcription is inhibited with actinomycin D, there is a partial reduction on FGF22-induced synapsin and VGlut clustering. Values were normalized to the control mean of each experiment. Bars represent the mean  $\pm$  SEM of 90 axons from three independent experiments (\*\*\*) represents  $p < 0.001$  by ANOVA using Bonferroni's post test when compared to control). The scale bar is 10  $\mu\text{m}$ .

To confirm that the actinomycin D effect was due in fact to the transcription inhibition in the somal compartment and not to a non-specific effect caused by the drug, we added actinomycin D to the axonal compartment only.

The results clearly show that the administration of actinomycin D in the axonal compartment, in the presence of FGF22, does not reduce FGF22-induced presynaptic differentiation (Figure 14A). The number of synapsin (167.43%,  $p < 0.05$ ) and VGlut (166.90%,  $p < 0.05$ ) puncta number per axon length in FGF22 and actinomycin D treated axons, when compared to control, is similar to FGF22 stimulated axons (Figure 14B, C). These results demonstrate that, when applied to the axonal compartment, actinomycin D has no effect on presynaptogenesis induced by FGF22, and that the reduction in presynaptic differentiation observed in Figure 13 was indeed due to DNA transcription inhibition.



**Figure 14 - DNA transcription inhibition in microfluidic chambers is specific.**

(A) Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers for 7 days. Axons present in the axonal compartment were then stimulated with 10 nM of FGF22 in the presence or absence of actinomycin D, applied to the axonal compartment, for 14 h at 37°C. Immunostaining for synapsin (green), VGlut (red) and  $\beta$ -tubulin (blue) was performed and images were taken to the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (B, C) The number of synapsin puncta/axon length and VGlut puncta/axon length was measured with Image J 1.46 software in randomly selected axons (30 axons per experiment). The results show

that actinomycin D when added to the axonal side has no effect on FGF22-induced synapsin and VGlut clustering. Values were normalized to the control mean of each experiment. Bars represent the mean  $\pm$  SEM of 90 axons from three independent experiments (\*\*\*) represents  $p < 0.001$  by ANOVA using Bonferroni's post test when compared to control). The scale bar is 10  $\mu\text{m}$ .

Our results suggest that FGF22 induces the transcription and, possibly, targeting of mRNAs to presynaptic sites, where they might be translated and contribute to presynaptogenesis. Indeed, it was described that in *Aplysia* sensory neurons transcription is required for synapse formation (Lyles et al., 2006). However, even in the presence of transcription inhibitors synaptic vesicle clustering was still observed, suggesting that translation of pre-existing mRNAs in axons is sufficient to induce presynaptic differentiation. Taking together, these observations indicate that presynaptic differentiation induced by FGF22 has a translational and a transcriptional component. mRNAs present at axons might be recruited to presynaptic sites, upon a FGF22 stimulus, where they would be translated and contribute to presynaptic differentiation. In addition, FGF22 might induce transcription and recruitment of mRNAs to presynaptic sites increasing the pool of mRNAs, which could enhance the levels of newly synthesized protein(s) promoting a proper synaptic formation and, possibly maintenance.

Chapter 4

Closing Remarks



#### **4. Conclusion and Future Perspectives**

In this study we demonstrate that FGF22 acting locally can induce differentiation of the presynaptic terminal. Moreover, FGF22 induces phosphorylation of 4E-BP1, a translational reporter, indicating that local mRNA translation is initiated upon stimulation with this growth factor. Our data also demonstrates that intra-axonal protein synthesis is required for presynaptogenesis and that this in part mediated by new gene transcription

First, we proposed to identify the optimal conditions (time and concentration), where FGF22 is able to induce presynaptic assembly in rat embryonic hippocampal neurons. We showed that 1h stimuli with 3 nM of FGF22 induces the maximum synaptic vesicle clustering, and by consequence presynaptic differentiation. These results are consistent with previous observations by Sanes and colleagues, who showed that 2 nM induces maximal effect of FGF22 in chick motoneurons, and with data from our group which demonstrates that a concentration equal or higher to 2 nM induces maximal activation of FGFR2b. In addition, we also demonstrated that FGF22-induced presynaptogenesis is dose-dependent.

Previous studies have demonstrated that local axonal translation is required in different axonal functions (Brittis et al., 2002; Campbell and Holt, 2001; Cox et al., 2008a; Hengst et al., 2009; Zhang and Poo, 2002). Furthermore, several mRNAs were detected in isolated axons (Taylor et al., 2009; Zivraj et al., 2010), but no role in synaptic formation has been described to date. We observed that FGF22, a presynaptic organizer, induces an increase in intra-axonal translation,

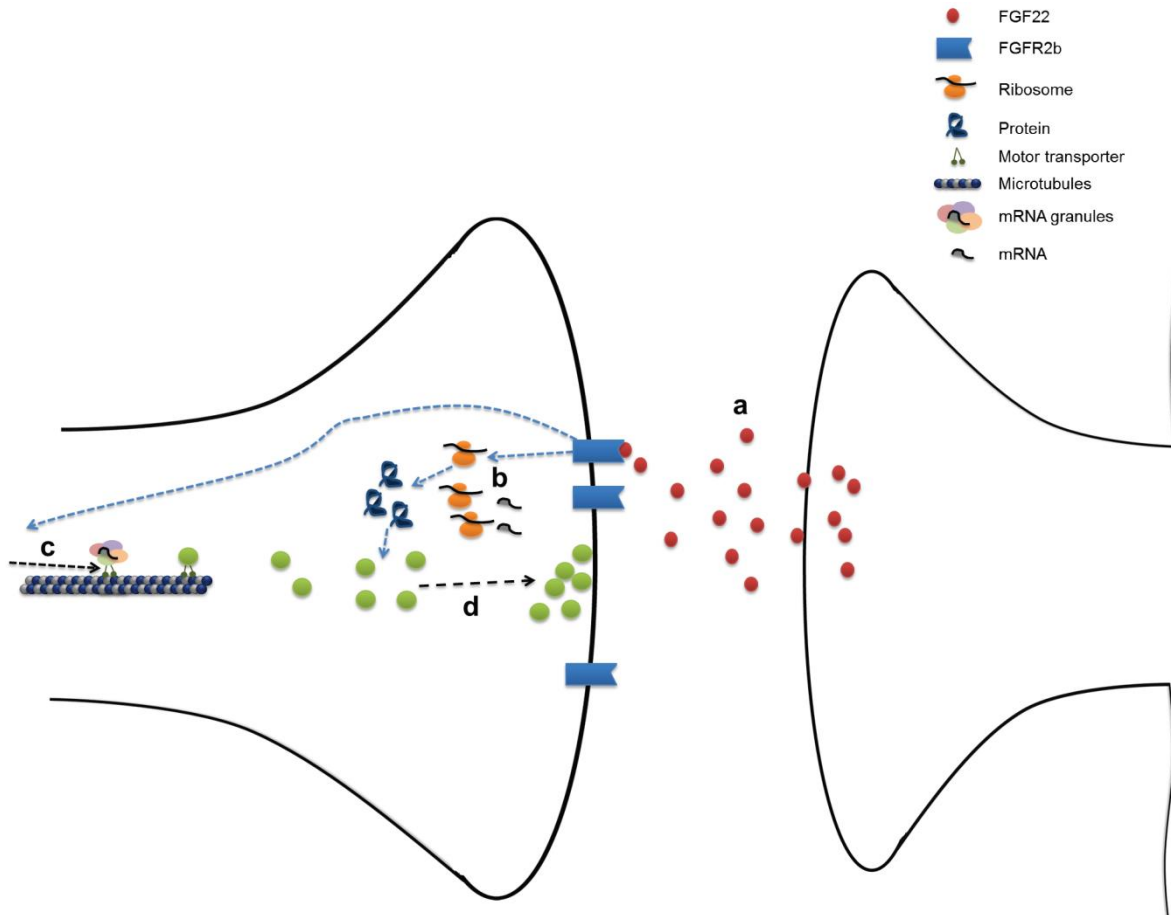


which suggested a link between local protein synthesis and presynaptic assembly. We found that newly synthesized proteins are required for FGF22-induced presynaptic differentiation. These observations are in agreement with recent evidences that support a role for intra-axonal translation in synaptic formation (Lyles et al., 2006; Schacher and Wu, 2002; Sebeo et al., 2009; Zhang and Poo, 2002).

Lastly, we aimed to determine the dependence of FGF22-induced presynaptic assembly on newly transcribed mRNAs. As local axonal translation occurs at presynaptic sites, mRNAs should be present or targeted to these sites. This idea led us to speculate that FGF22 induces a retrograde signal to the soma, through a yet unknown signaling transduction cascade. . The observation that actinomycin D, a transcription inhibitor, partially blocks FGF22-induced presynaptic effect raises the interesting possibility that FGF22 induces the transcription of new mRNAs, which will then associate to RNA granules and transported to the synaptic sites where they are going to be translated and participate in presynaptic formation.

Based on the established knowledge and on our data, we hypothesized a model where the postsynaptic cell releases FGF22, which will bind to FGFR2b, its cognate receptor, present in the presynaptic membrane (Figure 15a), leading to the activation of downstream signaling pathways that will induce translation of localized mRNAs (Figure 15b). These signaling pathways will induce de-repression of local translation, through the phosphorylation of 4E-BP1, and possibly the translocation of mRNAs to presynaptic sites. Furthermore, our results suggest that FGFR2b signaling pathway induces a retrograde signaling to the nucleus, where

new transcripts will be synthesized, assembled in RNA granules and transported to presynaptic sites providing a pool of mRNAs necessary for synaptic assembly (Figure 15c), in addition to the mRNAs previous present at the axons. These newly synthesized proteins will induce clustering of synaptic vesicles, triggering presynaptic differentiation (Figure 15d).



**Figure 15 - A model for FGF22-induced presynaptic differentiation.**

Activation of FGFR2b by target-derived FGF22 **(a)** induces local mRNA translation and **(b)** transcription and targeting of mRNAs to synapses where they will be translated **(c)**. Newly synthesized proteins required for synaptic vesicles clustering **(d)**.

It would be interesting to uncover which are the mRNAs that are locally translated upon a FGF22 stimulus. Using the microfluidic devices, we could purify distal axons and growth cones and screen for the proteins that are upregulated in these preparations using mass spectrometry. After the identification of the upregulated proteins in axons upon a FGF22 stimulus, we would validate the positive hits using the presynaptic assay described in this thesis and determine the specific role of these proteins in presynaptic differentiation.

In this work we showed that FGF22-induced presynaptogenesis is translation- and transcription-dependent.

With the demonstration of the role of newly synthesized proteins in presynaptic assembly induced by FGF22, would be interesting to uncover which are the specific proteins that are upregulated upon a FGF22 stimulus and their role in presynaptogenesis. To this purpose, I propose to use microfluidic devices and purify distal axons and growth cones, with or without stimulation with FGF22, and screen for the proteins present in these preparations using mass spectrometry. After the identification of the upregulated proteins in axons upon a FGF22 stimulus, we would search for the proteins that could be involved in presynaptogenesis and ask for their role in this process.

Would be also interesting observe, through live imaging, the transcription and transport of specific mRNAs upon a FGF22 stimulus. These mRNAs would be those corresponding to the upregulated proteins identified by mass spectrometry.

Chapter 5

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