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Role of local protein synthesis in FGF22-induced presynaptic differentiation

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Abbreviations

5-FDU	5-fluoro-2'-deoxiuridina
ALS	Axonal localization element
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AP3	Adaptor complex 3
APS	Ammonium persulfate
AraC	Cytosine arabinose
Arc	Activity-regulated cytoskeleton-associated protein
BC1	Brain cytoplasmic 1
BNDF	Brain-derived neurotrophic factor
BSA	Bovine Serum Albumin
CA3	Cornu Ammonis region 3
CAM	Cell-adhesion molecule
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CASK	Calcium/calmodulin-dependent serine protein kinase
CAZ	Cytomatrix at the active zone
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CPE	Cytoplasmic polyadenylation element
CPEB	Cytoplasmic polyadenylation element binding protein
CPR-2	Conopressin receptor 2
CREB	cAMP response element binding
DCC	Deleted in colorectal carcinoma
DIV	Days *in vitro*
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTE	Dendritic targeting element
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
ECF	Enhanced chemifluorescence substrate
eIF	Eukaryotic translation initiation factor
eIF4EBP	Eukaryotic translation initiation factor 4E binding protein
ELH	Egg-laying hormone
EphA2	Ephrin type A receptor 2
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion 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
GABA	Gamma-aminobutyric acid
GFP	Green fluorescence protein
Glu	Glutamate
Grb7	Growth factor receptor-bound protein 7
GSK-3	Glycogen synthase kinase 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	High frequency stimulation
hnRNP	Heterogeneous nuclear ribonucleoproteins
IGF-II	Insulin-like growth factor
IRES	Internal ribosomal entry sites
KIF	Kinesin
LFS	Low-frequency stimulation
LTD	Long-term depression
LTF	Long-term facilitation
LTP	Long-term potentiation
MAP2	Microtubule associated protein-2
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium eagle
miRNAs	MicroRNAs
mTOR	Mammalian target of rapamycin
MW	Multi-well
Na_3VO_4	Sodium orthovanadate
NaF	Sodium fluoride
NGF	Nerve growth factor
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate
PACAP	Plasma cell induced ER protein 1
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
PDMS	Poly-dimethlysiloxane
PKMζ	Protein kinase M ζ
PSD-95	Postsynaptic density protein 95
PTV	Piccolo-Bassoon transport vesicle
PVDF	Polyvinylidene difluoride

RIM	Regulating synaptic membrane exocytosis
RISC	RNA-induced silencing complex
RNP	Ribonucleotide protein
SDS	Sodium dodecyl sulfate
Sema3A	Semaphoring-3A
SIRP	Signal regulatory proteins
SNAP-25	Synaptosomal-associated protein-25
SNARE	Soluble NSF attachment receptor
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
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGS	Tris-glycine-SDS buffer
TLS	Translocation in liposarcoma
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
TSP	Thrombospondin
TUJ1	Neuronal Class III β-tubulin
UTR	Untranslated region
VAMP	Vesicle SNARE synaptobrevin
VGLUT1	Vesicular glutamate transporter 1
WB	Western Blot
ZBP1	Zip-code binding protein 1
κor	κ-opioid receptor

Abstract

Neurons are highly complex and polarized cells with an incredible network of functionally active processes that extend outwards the cell body. Communication between neurons occurs at a specialized structure, the synapse, frequently distant from the soma, the neuron trophic center. For long, proteins were thought to be synthesized in the cell body and then guided by microtubule-mediated transport to specific sites in dendrites and axons. However, this model has been challenged by accumulating evidences suggesting local translation of specific mRNAs selectively localized to neurites.

Local protein synthesis in axons has not been object of intense studies in the past and its functional significance is not clear. It is believed to be essential for several neurodevelopmental events like axonal guidance, synapse formation, synaptic plasticity, axonal regeneration and retrograde signaling. However, the involved mechanisms are far from being understood.

In the present work we investigated the potential role of axonal translation in presynaptogenesis, the mechanism by which a functional presynaptic terminal is formed. During presynaptic differentiation, freely moving packets containing presynaptic material such as synaptic vesicles, vesicular and fusion proteins, recycling machinery and active zone components, accumulate at pre-defined sites along the axon shaft. Despite this, transition of nascent synapses to mature synapses also involves correct alignment between pre- and postsynaptic terminals, presynaptic growth and cytoskeletal restructuring. Lately, the required action of presynaptic organizing molecules, such as synaptic cell adhesion molecules (SynCAMs), fibroblast growth

factor-22 (FGF22), neuroligins, WNTs and thrombospondins, has been highlighted by several studies.

In this work we focus our attention on FGF22-induced presynaptic differentiation, a target-derived soluble molecule recently proven to promote synaptic vesicles clustering in central nervous system (CNS) synapses. This work comprises two distinct parts. Firstly, we stimulated primary cultures of rat embryo hippocampal neurons with recombinant human FGF22; assessment of synapsin clustering demonstrated an increase in both number and size of presynaptic sites. Secondly, we tested whether this presynaptogenic effect of FGF22 was dependent on axonal translated proteins. For that, we cultured dissociated hippocampal neurons in microfluidic devices capable of physically and fluidically isolating axons. We then stimulated axons, under localized protein synthesis inhibition, with FGF22. Our results clearly show an abrogation of FGF22-induced presynaptic assembly when protein synthesis inhibitors are added to the medium, proving that FGF22 depends on translation of axonal mRNAs to exert its function. We present the first evidence that, not only axonal translation has a role in presynaptic formation in a mammalian system, but also that presynaptic organizing molecules action might rely on newly and locally synthesized proteins.

Further studies will allow us to understand how FGF22 acts, revealing the intracellular events (signaling pathways, induction and regulation of local protein synthesis and mRNAs involved) that lead to the appropriate formation of presynaptic terminals.

Keywords: FGF22, BDNF, presynaptic differentiation, axonal protein synthesis, microfluidic devices.

Resumo

Os neurónios são células altamente complexas e polarizadas com uma incrível rede de prolongamentos funcionalmente activos que se estendem do corpo celular. A comunicação entre neurónios ocorre numa estrutura especializada, a sinapse, normalmente distante do centro trófico do neurónio, o corpo celular. Durante vários anos, assumiu-se que as proteínas eram sintetizadas no corpo celular e enviadas para locais específicos nas dendrites e axónios, através do transporte mediado pelos microtúbulos. Contudo, novas evidências têm desafiado este modelo ao demonstrarem a ocorrência de tradução local de mRNAs, selectivamente localizados nas neurites.

No passado, poucos estudos se têm focado na síntese proteica em axónios, e consequentemente, a sua relevância funcional não é clara. Actualmente, a tradução axonal parece ser essencial para vários eventos do desenvolvimento do sistema nervoso, como condução axonal, formação sináptica, plasticidade sináptica, regeneração axonal e sinalização retrógrada. Contudo, os mecanismos envolvidos estão longe de ser descortinados.

O presente trabalho tem como objectivo investigar o papel da tradução axonal na pré-sinaptogénese, o mecanismo pelo qual se forma um terminal pré-sináptico. Durante a diferenciação pré-sináptica, estruturas móveis contendo material pré-sináptico como vesículas sinápticas, proteínas vesiculares e de fusão, maquinaria de reciclagem de vesículas e componentes da zona activa, acumulam-se em locais pré-definidos ao longo do eixo axonal. Para além disso, a formação de sinapses maduras também requer um correcto alinhamento entre os terminais pré- e pós-sinápticos, crescimento pré-sináptico e remodelação do citosqueleto. Ultimamente, vários estudos têm salientado a

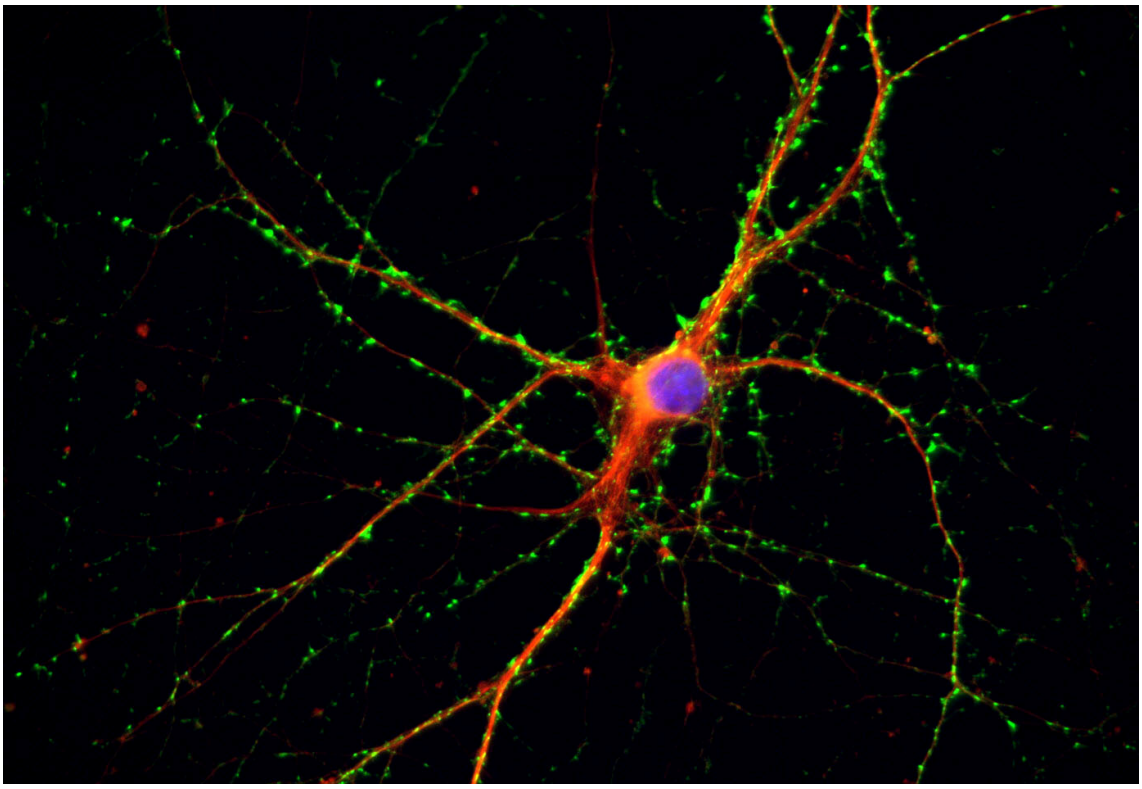
acção de moléculas organizadoras do terminal pré-sináptico, como SynCAMs, FGF22, neuroliguinas, WNTs e trombospondinas.

Neste trabalho, focámo-nos na diferenciação pré-sináptica induzida por FGF22, uma molécula solúvel secretada pelo terminal pós-sináptico alvo cuja acção indutora da agregação das vesículas sinápticas foi demonstrada recentemente em sinapses do sistema nervoso central. Este trabalho compreende duas partes distintas. Em primeiro lugar, estimulámos culturas primárias de neurónios de hipocampo de embrião de rato com FGF22 e avaliámos a agregação da sinapsina, observando-se um aumento no número e tamanho dos locais pré-sinápticos. Em segundo lugar, procurámos testar se este efeito presinaptogénico do FGF22 está dependente de proteínas traduzidas localmente. Para tal, plaqueámos neurónios de hipocampo em câmaras microfluídicas capazes de isolar, física e fluidicamente, axónios. De seguida, estimulámos axónios com FGF22, sob inibição local de síntese proteica, ou seja, apenas a nível axonal. Os nossos resultados mostram que na presença de inibidores da síntese proteica o efeito do FGF22 na formação sináptica é abolido, provando, deste modo, que o FGF22 depende da tradução de mRNAs nos axónios para exercer a sua função. Neste trabalho apresentamos a primeira evidência de que a tradução proteica axonal está envolvida na formação pré-sináptica em neurónios do sistema nervoso central de mamíferos. Por outro lado, demonstrámos pela primeira vez que moléculas organizadoras do terminal pré-sináptico requerem novas proteínas sintetizadas localmente.

Experiências futuras permitir-nos-ão compreender o modo de acção do FGF22, ajudando-nos a revelar os eventos intracelulares (vias de sinalização, indução e regulação da síntese proteica local e mRNAs envolvidos) que conduzem à formação do terminal pré-sináptico.

Palavras-chave: FGF22, BDNF, diferenciação pré-sináptica, síntese proteica axonal, câmaras microfluídicas.

Chapter 1 Introduction



1.1. The cell body hypothesis vs local protein synthesis

The mammalian brain is characterized by a tremendous and complex interconnectivity of its neurons. The communication between neurons occurs at a specialized structure: the synapse, frequently far away from the cell body. The formation of this distal connections and their synaptic activity requires a specific set of proteins. According to this, a question has been puzzling neurobiologists since the 19th century: how do neurites acquire this indispensable set of proteins during development and functional maintenance?

After the discovery that axons degenerate when separated from their cell body (Waller, 1851) and that they directly grow out of the neuronal cell body (Harrison, 1910), the concept of the exclusive trophic role of the neuron soma arose, firstly stated by Ramón y Cajal in his "Neuron theory" (reviewed in López-Muñoz et al., 2006). In the following years, new findings concerning protein transport in neurons helped to solidify this theory – the "cell body hypothesis". In 1963, Droz and Leblond observed, by autoradiography, migration of proteins in axons of the central nervous system, and in 1980 the intracellular transport of proteins in neurons was already undoubtedly accepted (Grafstein and Foreman, 1980). Protein synthesis and posttranslational processing machinery was believed to be present in neuronal cell bodies (Einarson, 1933). It was thought that the protein building blocks of axons and dendrites were synthesized in the cell body and then transported to specific places within the neuron. However, this view was challenged by posterior accumulating evidences suggesting that translation of mRNAs occurs locally in dendrites. The discovery of polyribosomes under the base of dendritic spines in granule cells (Steward and Levy, 1982), along with the detection of specific mRNAs within dendrites, such as microtubule associated protein 2 (MAP2)

(Garner et al., 1988) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) alpha subunit (Burgin et al., 1990), first gave rise to the possibility that protein synthesis could occur at a synaptic level. Nowadays, local protein synthesis in dendrites is widely accepted and is postulated to provide the basic mechanisms underlying synaptic plasticity and regulation of synaptic activity (Skup, 2008).

On the contrary, the idea of axonal protein synthesis in vertebrates has remained controversial, despite the accumulating evidences for their occurrence in vertebrate axons (summarized in Giuditta et al., 2008). Early studies demonstrating absence of ribosomes in axons (Lasek et al., 1973) are now disbelieved by the discovery of intermittently localized ribosomes along the axon shaft (Koenig et al., 2000) and the detection of mRNAs in axons of vertebrate and invertebrate nerve cells (Mohr and Richter, 2000). In a different perspective, local protein synthesis in axons can explain theoretical inconsistencies inherent in the "cell body hypothesis". Firstly, there is a great discrepancy between proteins half-lives and the duration of slow axonal transport of cytoskeletal and cytosolic proteins, resulting in protein loss-of-function during their transport to the distal-most targets (Alvarez et al., 2000). Secondly, the exclusive somatic origin of axonal and presynaptic proteins fails to ensure the two-way signaling between axon and neuron soma that would be required for plastic events occurring at growth cones or presynaptic terminals. For example, Šatkauskas and Bagnard (2007) emphasized the role of growth cone protein synthesis in the acquisition of adaptative properties that allow growth cones to respond rapidly and autonomously (soma independently) to spatiotemporal regulation of growing processes.

Why some proteins must be synthesized locally remains mostly speculative, but some possible rationales had been pointed out by Lin and Holt (2008). For example, mRNAs have regulatory elements located in the 5' untranslated region (5'UTR) and

3'UTR that encode information regarding localization and activation, and therefore mRNAs are more easily regulated than the functional protein. Another advantage of translationally dormant mRNAs in relation to inactive proteins is the less space they require to be stored. Upon activation, a single mRNA serves as template for the synthesis of a huge amount of protein. Moreover, in addition to the obvious acquisition of independence and autonomy, the dendrites and axons are able to respond more promptly to extracellular stimuli, without having to wait for protein delivery from the cell body. Local protein synthesis is also characterized by a precise spatial and temporal regulation, meaning that instead of being constitutively synthesized, proteins are formed only where and when needed.

This emerging field is under intense investigation and axonal protein synthesis is gradually gaining acceptance. The mechanisms of mRNA transport, local regulation of translation and the roles of axonal protein synthesis will be discussed in later sections. We also include a small section concerning presynaptic differentiation and presynaptic organizing molecules.

1.2. mRNA trafficking and regulation of local protein synthesis in neurons

The mechanism by which specific mRNAs are trafficked and locally regulated is not yet fully characterized. The knowledge acquired until now resulted, almost completely, from investigation in dendrites. Supposing that the mechanisms underlying mRNA trafficking and regulation of local protein synthesis in axons might be similar to the ones in dendrites, in this section we describe these findings mainly in dendrites. However, there are accumulating evidences suggesting similar mechanisms to occur in axons, which will also be described along the text.

An important aspect of local protein synthesis within a microdomain of the neuron is the targeting and transport of mRNA to the appropriate compartment. This localized transport of mRNAs is essential for the establishment and maintenance of subcelular locations in charge of specific and unique functions (recall the functional heterogeneity within a neuron). Actually, neurons have developed a selective mechanism able to specifically sort mRNAs into various compartments. It must be emphasized the crucial effect of this sorting in the selection of mRNAs to dendrites and axons, which are functionally distinct, therefore requiring different mRNAs.

In general, mRNAs contain segments that codify information for specific functionality (such as subcellular targeting), denominated cis-acting elements. Active targeting involves their recognition by trans-acting RNA-binding factors, which then interact with motor proteins, thus promoting traveling of these newly formed ribonucleotide protein (RNP) complexes along cytoskeletal filaments (Kindler et al., 2005). This happens to be the case in neuronal cells and it is accepted that most mRNAs are transported into dendrites as part of large RNPs, commonly referred as RNA granules (for a review see Bramham and Wells, 2007).

Kosik and colleagues firstly observed endogenous RNA granules in dendrites using the fluorescence vital RNA dye SYTO14 (Knowles et al., 1996). Later, it was discovered that RNA granules include translationally silenced mRNAs and clusters of ribosomes, which upon stimulation (such as synaptic activation in the form of depolarization) move to the polysome fraction to be translated (Krichevsky and Kosik, 2001) (figure 1, step 5). Besides this activity-regulated translation, there seems to be an activity-regulated transport of RNA granules. Indeed, studies showed that neuronal activity, in the form of depolarization, N-methyl-D-aspartate (NMDA) activation or metabotropic glutamate receptor activation, promotes: an increase in the number of

dendritic localized αCaMKII mRNA-containing granules (Rook et al., 2000), an increase in the movement of zip-code binding protein 1 (ZBP1)-containing granules (Tiruchinapalli et al., 2003) or the localization of mRNAs encoding α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor subunits GluR1 and GluR2 into dendrites (Grooms et al., 2006), respectively. These results suggest that synaptic activity increases dendritic protein synthesis.

It should be noted that nuclear proteins (such as heterogeneous nuclear ribonucleoproteins (hnRNPs)) reside in RNA granules, suggesting that the formation of RNA granules begins in the nucleus. Once in the cytoplasm RNA granules are transported via microtubules to dendrites through interaction with the C-terminal tail of the motor protein kinesin5 (KIF5), whose overexpression enhances granules movement (Kanai et al., 2004). This microtubule mediated transport of mRNAs has been proved to occur in axons too: firstly, selective perturbation of the cytoskeleton revealed that the presence of axonal mRNA was dependent on microtubules (Olink-Coux et al., 1996, Muslimov et al., 2002) and secondly, HuD and kinesin KIF3A were identified as components of the tau RNP granules in neuronal axons and growth cones (Aronov et al., 2002). Another important contribution came from Tiedge and colleagues that suggested a two-step process in the delivery of brain cytoplasmic 1 (BC1) RNA to local axonal sites, a long-range axial transport along microtubules and a local radial transfer to cortical domains via actin filaments (Muslimov et al., 2002). In fact, the RNA binding protein translocation in liposarcoma (TLS) transport to dendrites was shown to be dependent on actin filaments (Fujii et al., 2005), what contributed to the speculation that myosin may promote the transport of the mRNAs within the spine (figure 1, step 2).

RNA binding proteins are key regulators of cellular functioning due to their ability to specifically bind to cis-acting elements in mRNAs and thereby regulate the

transport of the bound RNA. Dendritic targeting element (DTE), which is a cis-acting element that instructs mRNA transport to dendrites, was shown to be present in several mRNAs, such as CaMKII, Shank1, vasopressin and protein kinase M ζ (PKMζ) (Kindler et al., 2005). By analogy, axonal localization cis element (ALS) in the 3'UTR of tau mRNA are essential for the localization of this mRNA into developing axons (Behar et al., 1995).

Several RNA binding proteins have been reported to be involved in sorting and targeting of mRNAs to dendrites and axons (figure 1, step 1). For example, the cytoplasmic polyadenylation element binding protein (CPEB) binds to mRNAs and facilitates their transport to dendrites (Huang et al., 2003; Bramham and Wells, 2007). In axons, the requirement of a cytoplasmic polyadenylation element (CPE) sequence in 3'UTR for axonal translation of ephrin type A receptor 2 (EphA2) (Brittis et al., 2002), suggests a role for CPEB in regulating axonal mRNAs transport in a way similar to dendrites.

Another well studied examples are: the fragile-X mental retardation protein (FMRP) and ZBP1 (reviewed in Bramham and Wells, 2007; Wells, 2006). The activity and requirement of these mRNA binding proteins in axonal RNA targeting has also been suggested (Antar et al., 2006; Wu et al., 2005; Sotelo-Silveira et al., 2008; Zhang et al., 2001). On the other hand, in a functional level, the formation of an RNP complex between β-actin and ZBP1 seems to be required for growth cone response to netrin-1 (Leung et al., 2006) or brain-derived neurotrophic factor (BDNF) (Yao et al., 2006) gradient. Furthermore, FMRP is involved in protein synthesis-dependent growth cone collapse induced by semaphorin 3A (Sema3A) (Li et al., 2009).

In addition to specific mRNA transport, if a message is to have a local effect, translation during transport must be repressed, thus preventing delocalized synthesis *en*

route to its destination. Actually, the mRNAs being trafficked into dendrites have been shown to be in a translationally dormant state (figure 1, step 3).

Translational repression of mRNAs can be achieved by multiple mechanisms (reviewed in Sossin and DesGroseillers, 2006; Bramham and Wells, 2007): repression of translation initiation by eukaryotic translation initiation factor 4E binding protein (eIF4EBP) that prevents eIF4E binding to eIF4G and recruitment of the ribosome (Richter et al., 2005); cytoplasmic deadenylation, which is the removal of the poly(A) tail and thus reduction of the translatability capacity of the mRNA (de Moore et al., 2005); and maintainance of mRNAs in a translational dormant state by mRNA binding proteins, such as ZBP1, FMRP and CPEB (Huttelmaier et al., 2005; Li et al., 2001 and reviewed in Wells et al., 2006, respectively). Another common mechanism of protein synthesis regulation is the RNA interference pathway[1], in which microRNAs (miRNAs) retained within the RISC complex identify mRNAs to be cleaved or repressed translationally, rendering them incapable of protein synthesis (Mello and Conte, 2004). Several studies raise the possibility that miRNAs might function to maintain mRNAs in a translationally dormant state, and to thereby negatively regulate translation both in axons (Hengst et al., 2006; Murashov et al., 2007; Wu et al., 2005) and dendrites (Schratt et al., 2006).

In summary, the transport of mRNAs in a repressed state prevents ectopic expression. Furthermore, the maintenance of those repressed mRNAs at the destination site (for example a postsynaptic spine, a growth cone or an axonal varicosity) guarantees that translation only occurs upon certain stimulation and in a particular time.

[1] RNAi pathway uses small endogenous RNA molecules produced by Dicer, denominated microRNAs (miRNAs). These strands are incorporated in RNA-induced silencing complexes (RISCs), which, according to the complementarity between mRNA and miRNA, induce mRNA catalytic cleavage or translational repression (Mello and Conte, 2004).

Upon arrival of a synaptic stimulation, translation of localized mRNAs is induced, and so de-repression of dormant mRNAs must take place (figure 1, step 4).

Regulation of local protein synthesis may occur at the level of translation initiation. As seen previously, eIF4E and eIF4EBP are regulators of translation initiation known to function in dendrites and axons. Evidences in axons attribute great importance to this kind of de-repression in guidance cues-regulated translation of localized mRNAs. It was reported that netrin-1 and Sema3A induce phosphorylation of eIF4EBP via mitogen-activated protein kinases (MAPK) and mammalian target of rapamycin (mTOR) (Campbell and Holt, 2001, 2003). Moreover, guidance cues activate eIF4E by phosphorylation via MAPKs (Campbell and Holt 2003; Piper et al., 2006). Another mechanism for specifically regulating translation initiation of dendritic mRNAs uses internal ribosomal entry sites (IRES). Internal initiation of translation occurs in five dendritically localized mRNAs, such as activity-regulated cytoskeleton-associated protein (Arc), αCaMKII and BC1 (Pinkstaff et al., 2001).

The control of local protein synthesis discussed so far involves mRNA translational machinery, and so it is not mRNA specific. The regulation of local protein synthesis in an mRNA specific manner results from RNA binding proteins, the principal examples being ZBP1, FMRP and CPEB (Wells, 2006).

Firstly, translational dormant β-actin, due to ZBP1 binding, arrives at the base of spines. In these spots, ZBP1 on the β-actin mRNA is released through protein kinase Src-dependent phosphorylation of ZBP1 whose affinity for β-actin mRNA decreases and consequently promotes β-actin protein synthesis (Huttelmaier et al., 2005). This kind of local regulation of translation was also detected in axons by Zheng and colleagues, who reported that ZBP1-mediated localization of β-actin mRNA and its translation are essential for bidirectional turning (Yao et al., 2006).

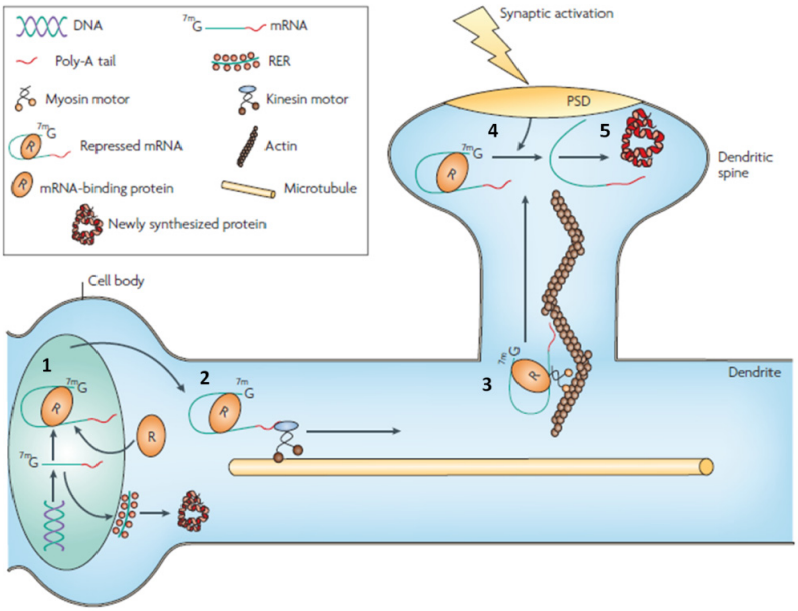


Figure 1 - Proposed model for mRNA translation in neuronal dendrites. In the nucleus, an mRNA binding protein (R) binds to a specific mRNA (1). Binding of other proteins will eventually form a RNA granule with motor proteins for translocation along cytoskeletal elements (kinesin for microtubules and myosin for actin filaments) (2). The mRNA bound to mRNA binding proteins in the granule is translationally silenced during transport (3). Following synaptic activation, de-repression of translation (4) promotes movements of the mRNAs to polysome particles and local dendritic translation (5) (Adapted from Bramham and Wells, 2007).

Secondly, once repressed mRNAs bound to CPEB get to sites of translation, CPEB is phosphorylated by Aurora A kinase. This phosphorylation converts CPEB into a translation activator that recruits an atypical poly(A) polymerase Gld-2, and thereby elongates the poly(A) tail of the mRNA it binds. The maskin-eIF4E association is disrupted after poly(A) tail elongation, therefore allowing the binding of eIF4G and translation (reviewed in Wells, 2006). Indeed, NMDA activation in synaptosomes increases Aurora activity, CPEB phosphorylation and, as a result, polyadenylation of

αCaMKII mRNA (Huang et al., 2003). Thirdly, FMRP is also thought to regulate mRNA translation in dendrites, however the precise mechanism is not yet known.

Another example of a RNA-binding protein regulating local translation arises from axons, where growth factor receptor-bound protein 7 (Grb7) was demonstrated to repress κ-opioid receptor mRNA translation by binding to its 5'UTR (Tsai et al., 2007). Upon stimulation by netrin-1, focal adhesion kinase (FAK) phosphorylates Grb7, which then releases κor mRNA allowing κor translation (Tsai et al., 2007).

Considering all the described findings in this section, Bramham and Wells (2007) proposed a model for mRNA translation in dendrites (figure 1). According to it, mRNAs bind to mRNA binding proteins in the nucleus and then they are transported, in a translational repressed state, to dendritic spines. Upon synaptic activity, de-repression of dormant mRNAs present in localized RNA granules is induced, and so, translation is activated. It can be hypothesized that a similar model might work for axons as well, once several evidences point to identical regulatory mechanisms.

1.3. Local protein synthesis in dendrites

Many dendritic proteins are specifically localized at spines, the postsynaptic terminals that protrude from dendritic shafts, where they are known to determine structure and function. Nowadays, new proteins synthesis in dendrites, especially at synaptic sites, is regarded as a specific supply of these proteins and is accepted to be involved in the regulation of synaptic activity, providing the basic mechanism of fast changes in the strength of neuronal connections.

Nowadays, it is widely accepted that protein synthesis can occur in neuronal dendrites. Not only polyribosomes where detected in dendrites (Steward and Levy,

1982), but also incubation of isolated dendrites with radiolabeled aminoacids, with or without transfection with mRNAs, resulted in a strong labeling of proteins, indication that local protein synthesis in dendrites actually takes place (Torre and Steward, 1992; Crino and Eberwine, 1996). Furthermore, using radioactive uridine precursors, Steward and colleagues demonstrated that mRNAs were transported into dendrites of cultured hippocampal neurons (Davis et al., 1987). Recently, dendritic 'hot spots' of translation, regions where translation seems to occur repeatedly over time, were observed by transfecting dendrites with mRNA (Eberwine et al., 2001). Nowadays, these dendritic 'hot spots' are known to be located at the base of spines.

In the past years, the hypothesis of dendritic protein synthesis has received further support in the identification of hundreds of mRNAs localized in dendrites. Some examples are: structural proteins (MAP2 and Arc), enzymes (αCaMKII), growth factors (BDNF and NT3), growth factor receptors (TrkA and TrkB), ligand-gated ion channels (gamma-aminobutyric acid (GABA) and NMDA receptor subunits and glycine receptor α subunit), voltage-gated ion channels (calcium channels) and transcription factors (cAMP response element binding protein (CREB)) (reviewed in Steward and Schuman, 2003).

Local protein synthesis in dendrites is believed to play a decisive role in synaptic plasticity, induction of both late long-term potentiation (LTP) (Bramham, 2008) and late long-term depression (LTD) (Bramham and Wells, 2007), in synaptic scaling[2] and synaptic tagging[3] (topics reviewed in Skup, 2008; Barrett and Eberwine, 2008, respectively).

[2] Synaptic scaling is a form of homeostatic plasticity that scales synaptic strengths up or down to compensate for prolonged changes in activity (Turrigiano, 2008).
[3] Synaptic tagging refers to the idea that the induction of LTP is associated with the setting of a 'synaptic tag' at activated synapses, which sequesters the relevant proteins to establish late LTP, by stabilizing temporary synaptic changes and so extend their persistence (Frey and Morris, 1998).

It has also been proposed a role for dendritic translation in synaptogenesis. First evidence came from findings by Burry (1985), who reported an essential role for dendritic protein synthesis in synapse formation. Later on, accumulation of ribosomes in dendrites during synaptogenesis (Steward and Falk, 1986; McCarthy, 2003) and posterior reduction with synapse maturation (Steward and Falk, 1986) strengthened this idea. More recently, acethylcoline receptor was shown to be synthesized in the postsynaptic muscle during neuromuscular differentiation (Hall and Sanes, 1993). Lack of novel evidences may be due to difficulty in isolating dendrites and in assessing postsynaptic differentiation.

1.4. Local protein synthesis in axons

A growing body of evidence supports the idea that local translation in axons, as it was seen for the postsynaptic terminal, is important for the development and plasticity of neural circuits. This section considers the direct evidence that proteins can be synthesized in axons and its possible functional significance.

1.4.1 - Early evidences from invertebrates

Although being a controversial area, protein synthesis in invertebrate axons is already well accepted, in part due to the presence of translation machinery (Black and Lasek, 1977; Lasek et al., 1973; Giuditta et al., 1977; Giuditta et al., 1980; Martin et al., 1998; van Minnen and Syed, 2001) and several mRNAs, such as β-actin and β-tubulin (Kaplan et al., 1992), kinesin (Gioio et al., 1994), enolase (Chun et al., 1995) and neurofilament proteins (Giuditta et al., 1991), among many others.

The capacity for translation in truncated neurites and growth cones was demonstrated by Kater and colleagues through the incorporation of radiolabeled amino acids into unspecific synthesized proteins in a soma-independent manner (Davis et al., 1992). Later on, new studies demonstrated the occurrence of *de novo* protein synthesis in isolated axons by the synthesis of a foreign mRNA encoding the peptide egg-laying hormone (ELH) (van Minnen et al., 1997), or the synthesis and functional integration of the membrane-bound conopressin receptor 2 (CPR-2) from axonally injected mRNA (Spencer et al., 2000). These results show that the axon possesses the machinery to produce a functional and appropriately trafficked membrane protein.

1.4.2 - Emerging evidences in vertebrates

As for invertebrates, emerging evidences prove the existence of local protein synthesis in vertebrate axons, despite initial failure in detecting ribosomes in these processes (Lasek et al., 1973).

Firstly, different papers demonstrate the presence of components of the protein synthesis machinery: ribosomes in developing axons (Tennyson, 1970); ribosomal RNA and tRNA in Mauhtner axon (Koenig, 1979); identification of polysomal aggregates stained by YOYO-1 and antiribosomal antibodies in Mauhtner axon (Koenig and Martin, 1996) and in rabbit and rat root fibers (Koenig et al., 2000); identification of initiation factors (Zheng et al., 2001) and 7S RNA, a key component of the signal recognition particle (Walter and Johnson, 1994). The detection of several mRNAs in mature (adult) and developing axons (embryonic or newborn) also constitutes a precious evidence for axonal translation (see Table 1) – the role of their local translation will be highlighted in the next sections. It should be noted the prevalence of mRNAs coding

cytoskeletal proteins and regulators (*), evidencing a role in cytoskeleton dynamics during axonal translation. Another class of proteins well represented is characterized by

Table I – Examples of mRNAs located in axons of vertebrate nerve cells (adapted from Piper, 2004 and Mohr and Richter, 2000).

mRNA	Species	Tissue	Age	References
Vasopressin	Rat	Hypothalamus	Embryonic	Mohr et al., 1991
Tau (MT-associated protein) *	Rat	Cortex	Embryonic	Litman et al., 1993
β-actin *	Chick	Sympathetic neurons	Embryonic	Olink-Coux and Hollenbeck, 1996;
	Rat	Cortex		Bassel et al., 1998
	Xenopus	Spinal neurons		Yao et al., 2006
ADF (actin depolymerizing factor) *	Chick	Sympathetic neurons	Embryonic	Lee and Hollenbeck, 2003
CGRP (calcitonin gene-related peptide)	Rat	Olfactory bulbs Sensory neurons	Embryonic	Denis-Donini et al., 1998; Toth et al., 2009
EphB2	Chick	Retina	Embryonic	Brittis et al., 2002
N-CAM	Chick	Retina	Embryonic	Brittis et al., 2002
SCG10 (an actin binding protein) *	Rat	Cerebellum	Embryonic	Hannan et al., 1996
Tm-5 tropomyosin isoform *	Rat	Spinal cord, dorsal root ganglion (DRG) neurons	Embryonic	Hannan et al., 1995
CREB	Rat	DRG neurons	Embryonic	Cox et al., 2008
RhoA *	Rat	DRG neurons	Embryonic	Wu et al., 2005
κ-opioid receptor	Mouse	DRG neurons	Embryonic	Tsai et al., 2006
Cofilin *	*Xenopus*	Retina	Embryonic	Piper et al., 2006
Synaptophysin	Rat	Cortex, hippocampus	Embryonic	Taylor et al., 2005
β-tubulin *	Rat	Sympathetic neurons	Newborn	Eng et al., 1999
BC1 (brain specific transcript of RNA polymerase III)	Rat Goldfish	Hypothalamus Mauthner neurons	Adult	Tiedge et al., 1993; Muslimov et al., 2002
Dynorphin	Rat	Hypothalamus	Adult	Mohr and Richter, 1992
Galanin	Rat	Hypothalamus	Adult	Landry and Hoekfelt, 1998
Neurofilament L *	Rat	Hypothalamus Sciatic nerve	Adult	Mohr and Richter, 1992; Sotelo-Silveira et al., 2000
Oxytocin	Rat	Hypothalamus	Adult	Mohr et al., 1991; Jirikowski et al., 1990
Olfactory marker protein	Rat	Olfactory bulb	Adult	Wensley et al., 1995
Olfactory receptors	Rat	Olfactory bulb	Adult	Ressler et al., 1994
Purkinje cell protein-2/L7	Mouse	Cerebellum	Adult	Wanner et al., 1997
Neurofilament M *	Goldfish	Mauthner neurons	Adult	Weiner et al., 1996
VR1 (vanilloid receptor 1)	Rat	Sensory neurons	Adult	Tohda et al., 2001
β-importin	Rat	Sciatic nerve	Adult	Hanz et al., 2003

* Cytoskeletal proteins and cytoskeleton regulators

their involvement in neuron activity and includes peptide hormones (vasopressin and calcitonin gene-related peptide (CGRP)), the opioid peptide dynorphin and its receptor, the neuropeptide galanin, the neurotransmitter oxytocin and the ion selective channel vanilloid receptor. Thus, a role for axonal translation in synaptic function can also be hypothesized.

Secondly, experiments using radiolabeled amino acids (Edström, 1966; Alvarez and Chen, 1972; Koenig, 1967) revealed protein synthesis-dependent incorporation of labeled metabolic precursors into dissected axons. An improvement in this basic methodology for investigating protein synthesis consists in the extraction and SDS-PAGE analysis of radiolabeled axonal proteins (exclusively synthesized in axons once cell bodies had been removed). The use of this technique led to the identification of actin, tubulin (Koenig, 1991; Eng et al., 1999) and neurofilament proteins (Koenig, 1991) as the principal locally synthesized proteins.

To sum up, the presence of mRNAs and protein synthetic machinery, the incorporation of amino acids and production of a specific set of proteins in isolated axons firmly demonstrate that axonal translation in vertebrates is not a fictitious process and that it must be associated with a functional role.

1.5. Role of local protein synthesis in axons

1.5.1. Developing axons

During development of the nervous system, axons pass through distinct stages, characterized by profound morphological and functional changes. In a first stage, the developing axons seek for a synaptic target. In order to achieve this objective,

developing axons undergo elongation and possess at their tips an actin-rich growth cone, which is a highly motile structure. During axon navigation, the growth cone senses the environment by detecting extracellular guidance cues and morphologically responding to them (Farrar and Spencer, 2008), a process that promotes axonal pathfinding. In a final stage, axon navigation halts in an area where connection to the target postsynaptic terminal must occur. In this moment, the growth cone undergoes a profound transition from a highly motile structure to a functional presynaptic terminal, and by the end of synaptogenesis, a functional synaptic connection is established.

An important issue that has received considerable attention is the level of dependence of the axon tip on the cell body during both axon guidance and synaptogenesis. As a matter of fact, protein translation at axon tips offers a higher degree of autonomy during axon development.

1.5.1.1. Axonal protein synthesis in axon guidance

During development and navigation toward target cells, the growth cone senses spatially and temporally distributed guidance cues and subsequently steers the axon in the appropriate direction (Tessier-Lavigne and Goodman, 1996). These extracellular guidance cues, including netrins, Slits, semaphorins and ephrins, can either attract or repel growth cones or even induce their branching or collapse (Tessier-Lavigne and Goodman, 1996). Guidance cues act by binding to surface receptors in growth cones: deleted in colorectal carcinoma (DCC) and UNC-5 receptors for netrins (Culotti and Merz, 1998; Keleman and Dickson, 2001), Robo receptors for Slits (Kidd et al., 1999), multimeric receptor complexes with plexin protein for semaphorins (Tamagnone et al., 1999) and Eph family of receptor tyrosine kinases for ephrins (Drescher et al., 1997)

(reviewed in Dickson, 2002). The activation of these surface receptors elicits localized intracellular signaling events, which ultimately control cytoskeletal activities to steer the growth cone, such as reorganization of actin filaments or stabilization of microtubules.

Growth cone responses to guidance cues have been proposed to be dependent on local protein synthesis, what could explain specific protein requirements, altered responsiveness along the way and specificity of an axon's trajectory. For example, growth cone turning is caused by extension on one side and collapse on the other, and is triggered by local attractive and repulsive cues. Initial experiments revealed that *in vitro* chemotropic responses to netrin-1 and Sema3A (attractive or repulsive and collapse, respectively) of isolated axons were dependent on local protein synthesis (Campbell and Holt, 2001). This study also demonstrated that Sema3A and netrin-1 trigger a burst of protein synthesis within the growth cone. Later on, new studies identified other guidance cues-induced chemotropic responses dependent on local axonal translation: plasma cell induced ER protein 1 (PACAP)-induced growth cone attraction (Guirland et al., 2003), Engrailed-2-induced turning responses (Brunet et al., 2005), Slit-2-induced collapse and repulsion (Piper et al., 2006) and BDNF-induced attraction (Yao et al., 2006).

Considering that guidance cues ultimately modulate cytoskeletal activities in growth cones, it is reasonable to assume that activation of their receptors leads to local translation of cytoskeletal proteins or regulators. In fact, an attractive gradient of netrin-1 or BDNF induces β-actin synthesis in growth cones (Leung et al., 2006; Yao et al., 2006); Slit2 causes a protein synthesis dependent increase in the actin-depolymerizing protein cofilin (Piper et al., 2006) and Sema3A induces intra-axonal translation of RhoA mRNA, which is a critical upstream regulator of the cytoskeleton and whose activation leads to growth cone collapse (Wu, et al., 2005).

Based in these evidences, the "differential translation model" has emerged, postulating that attractive and repulsive cues induce asymmetrical translation of proteins that ultimately promotes assembly or disassembly of the cytoskeleton, respectively (Lin and Holt, 2008) (see Figure 2). However, a recent study contradicts these findings stating that protein synthesis inhibition does not alter ephrin-A2, slit-3 and Sema3A-induced growth cone collapse and loss of actin filaments; neither NGF nor neurotrophin-3-induced growth cone protrusion and increased actin filaments (Roche et al., 2009).

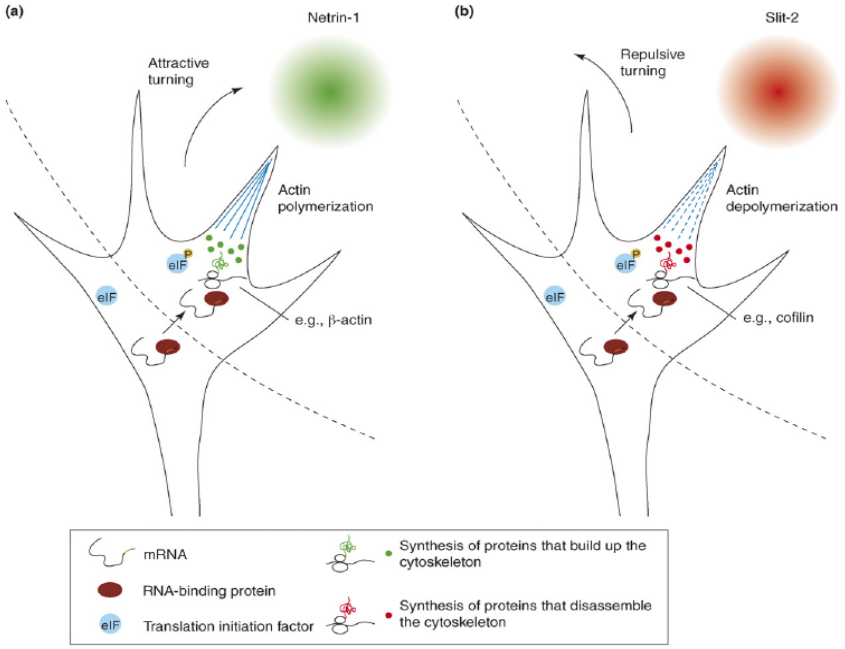


Figure 2 - The "differential translation" model for local translation in growth cones. This model states that (a) a gradient of an attractive guidance cue induces local translation of mRNAs (such as β-actin) that cause actin polymerization and thus attractive turning; on the other hand (b) a gradient of a repulsive guidance cue induces local translation of mRNAs (such as cofilin) that cause actin depolymerization and consequently repulsive turning (Adapted from Lin and Holt, 2008).

In their navigation, axons reach intermediate targets, and once they pass through them, they must change their responsiveness to the guidance cues. For example, as the vertebrate commissural axons cross the floor plate they lose sensitivity to the midline attractant, netrin, and acquire sensitivity to Slit and semaphorin repellents (Stein and Tessier-Lavigne 2001). Recently, it has been proposed that local translation may also mediate changes in axonal responsiveness. Flanagan and colleagues revealed a mechanism that could promote local synthesis of receptors for guidance cues. Focusing on EphA2 receptor and elaborating a construct made up of 3'UTR of EphA2 and green fluorescence protein (GFP), they observed that the fusion protein was expressed at high levels in the distal segments of the axons that had passed the midline (Brittis et al., 2002). This result suggests that signals from an intermediate target trigger the upregulation of a different set of receptors that are necessary for guiding the axon to the next intermediate point.

Desensitization and resensitization are mechanisms through which axons alter their sensitivity to external guidance cues. These mechanisms of regulation of growth cone behavior and responsiveness are dependent on local protein synthesis, since it was demonstrated that resensitization to netrin-1, BDNF or Sema3A requires axonal translation (Ming et al., 2002; Piper et al., 2005). The regained sensitivity to guidance cues is due to a recovery of functional surface receptors. Considering that axons have the ability to synthesize and translocate a functional surface receptor (Spencer et al., 2000), it can be suggested that local protein synthesis dependent-resensitization results from guidance cue receptor synthesis and local insertion into the plasma membrane. In fact, axons can promote synthesis and functional membrane integration of the κ-opioid receptor in response to netrin-1 (Tsai et al., 2006).

1.5.1.2. Axonal protein synthesis in synaptogenesis

Synaptogenesis is a process involving the formation of a neurotransmitter release site in the presynaptic neuron precisely aligned with a receptive field at the postsynaptic neuron. During development of the nervous system, synaptogenesis requires that a number of changes occur at the site of contact between pre- and postsynaptic neuron. These changes are mediated by the insertion of precursor vesicles containing many preassembled active zone molecules and synaptic vesicles components into the presynaptic terminal and by a sequential recruitment of molecules to the postsynaptic specialization.

Local translation in axons was also demonstrated to be important in synaptogenesis. Early evidence came from findings that interaction of a sensory neuron growth cone with a specific postsynaptic target regulates the local export and accumulation of sensorin A mRNA in sensory neurons (Schaher et al., 1999; Lyles et al., 2006; Hu et al., 2002), and that the release of this peptide promotes the formation of sensory-motor synapses (Hu et al., 2004). These results suggest that branch specific targeting of mRNA encoding synapse-related molecules may contribute to the formation of specific synapses through local protein synthesis. Later on, Schacher and Wu concluded that protein synthesis, at or near synaptic sites, is required for the establishment of new synaptic connections in the absence of cell bodies (Schacher and Wu, 2002). Indeed, Martin and colleagues suggested that local protein synthesis of sensorin mRNA in the presynaptic terminal is required for synapse formation, since knockdown of sensorin mRNA abolishes synapse formation (Lyles et al., 2006).

Neurotrophins (BDNF and NT-3) produce a long-lasting enhancement of synaptic transmission in developing axons (Kang and Schuman, 1995) and this capacity

seems to require axonal translation (Kang and Schuman, 1996). Indeed, BDNF-induced potentiation of transmitter secretion at developing synapses requires constitutive presynaptic protein translation (Zhang and Poo, 2002). Local protein synthesis thus plays a role in neurotrophin-dependent modulation of developing synapses.

Another paper demonstrates that in *Drosophila* the insulin-like growth factor (IGF-II) mRNA binding protein Imp is required presynaptically for the formation of neuromuscular junctions (Boylan et al., 2008). Being an mRNA binding protein, that regulate mRNA transport and local translation, Imp may enhance axonal translation of specific proteins (its own mRNAs targets) necessary for synapse formation. Recently, Benson and colleagues observed that a local gradient of anisomycin applied to presynaptic terminals on dendrites rapidly reduces the pool of vesicles available for fusion, while terminals on nearby cell bodies lying outside the gradient are unaffected (Sebeo et al., 2009).

Although these are preliminary evidences, it can be postulated that local protein synthesis in axons has a relevant role in the presynaptic alterations that occur during synaptogenesis. A curtained description of presynaptic differentiation will be addressed later.

1.5.2. Mature axons

Following the establishment of synaptic connections, the mature axons exert their function in the nervous system as synaptic information conductors. Comparing to developing axons, mature axons are more stable structures and metabolically less active. Axonal maturation represents the switch from an actively translating axon to an axon with reduced translation capability, as a result of the disappearance of axonal

polyribosomes and mRNAs. Thus, the axonal synthesis of proteins in mature mammalian axons is controversial even though several mRNAs (see Table I) and ribosomes were detected in adult mammalian axons (Koenig et al., 2000). Moreover, studies demonstrating the participation of axonal translation in events occurring in mature axons, such as axon regeneration (reviewed in Willis and Twiss, 2006) and synaptic plasticity (Martin, 2004), are gaining acceptance.

1.5.2.1. Axonal protein synthesis in axon regeneration

During regeneration, in order to restore the connectivity with target tissues, the cut end of the axon must be remodeled and initiate a growth program to reform a new growth cone. This process has been shown to occur in isolated axons, which could regenerate new growth cones after injury, suggesting autonomous capacity to synthesize molecules needed for regeneration (Shaw and Bray, 1977).

Studies of regeneration in adult sensory axons demonstrated the functional importance of local intra-axonal protein synthesis in growth cone initiation after axotomy. The neurite regeneration of PC12 cells was shown to be dependent on the translation of the mRNA encoding the ribosomal L4 protein (Twiss et al., 2000). Furthermore, blocking synthesis in regenerating sensory axons causes a rapid retraction of their growth cones when separated from the cell body (Zheng et al., 2001) and reduce the proportion of transected axons able to reform growth cones (Verma et al., 2005). Twiss and colleagues showed that cytoskeletal, chaperone, metabolic and anti-oxidant proteins are synthesized by regenerating sensory axons (Willis et al., 2005). Theoretically, this huge set of proteins provides the axon with cytoskeletal elements to reform the growth cone, and provides autonomy in responding to environmental cues.

Amazingly, Toth and colleagues (2009) demonstrated that a specific protein, calcitonin gene-related peptide (CGRP), is synthesized during regeneration and its mRNA is transported to regenerating axons (Toth et al., 2009).

Retrograde signaling is also important for axonal regeneration, as the cell body has to be updated on the injured status of the axon to modulate a program of repair (Hanz and Fainzilber, 2006). The involvement of local translation in retrograde signaling was highlighted by the axonal synthesis of importin-β (a protein that transports nuclear localization signal (NLS) bearing proteins to the nucleus) after nerve lesion and its retrograde transport by the motor protein dynein (Hanz et al., 2003). The activated MAP kinase (Erk 1/2) has been proposed to be transported by importins via vimentin binding after nerve injury (Perlson et al., 2005), thus promoting changes in the cell body that support regeneration. Indeed, a detailed model of axonal retrograde signaling after nerve lesion regulated by RanGTPase and importin-β was proposed (Yudin et al., 2008). Another study identified a signaling role for axonally derived CREB in mediating the response to the neurotrophin nerve growth factor (NGF) (Cox et al., 2008). These studies raise the possibility that newly synthesized transcription factors can be retrogradely transported on microtubules to the cell body where they influence transcription of genes for axonal repair.

1.5.2.2. Axonal protein synthesis in synaptic plasticity

Synaptic plasticity refers to the capacity of neurons to modulate the strength of their synaptic connections. Synaptic plasticity contributes to a variety of physiological processes in the adult brain, including memory, learning and age-related memory loss. It can exist in two distinct forms: high frequency stimulation (HFS) triggers a long-lasting

enhancement of synaptic strength, known as long-term potentiation (LTP) (Bliss and Collingridge, 1993), whereas prolonged low-frequency stimulation (LFS) results in a long-lasting decrease in synaptic strength, termed long-term depression (LTD) (Mulkey and Malenka, 1992).

Succinctly, the increased synaptic strength that occurs during LTP should arise from an increase in neurotransmitter release from the presynaptic terminal, or from an increase in postsynaptic terminal responsiveness to the neurotransmitter, or both. It is widely accepted that translation of dendritically localized mRNAs is required in long-term synaptic plasticity through regulation of actin dynamics, which ultimately leads to insertion of additional AMPA receptors in the postsynaptic membrane (Bramham, 2008). In a more functional level, it is believed that presynaptic LTP is triggered by a tetanus-induced rise in presynaptic Ca^{2+} that results in the activation of a Ca^{2+}-calmodulin-dependent adenylyl cyclase, which in turn causes an increase in presynaptic cAMP levels and activation of protein kinase A (Huang et al., 1994). Protein kinase A can initiate intracellular signaling cascades that, by activating mechanisms perhaps involving Rab3A and its interacting protein, regulating synaptic membrane exocytosis (RIM1α), cause a long-lasting enhancement of synaptic vesicle fusion and neurotransmitter release (Castillo et al., 1997; Castillo et al., 2002). The development of new probes designed to directly assay synaptic vesicle cycling undoubtedly revealed the existence of presynaptic LTP in the form of accelerated synaptic vesicle release (reviewed in Blundon and Zakharenko, 2008).

Accumulating evidences support a role for axonal protein synthesis in synaptic plasticity. Presynaptic protein synthesis is required for long-term facilitation (LTF) of *Aplysia* (Martin et al., 1997) and crayfish (Beaumont et al., 2001) sensory to motor neurons synapses. Moreover, this facilitation depends on CREB-mediated transcription

(Martin et al., 1997), a transcription factor later proven to be translated in axons and to mediate retrograde signaling (Cox et al., 2008). Possibly, once in the nucleus CREB induces specific changes in gene expression and once the newly-synthesized proteins are transported to the presynaptic terminal, they may support the sustained vesicle release in LTP (for example through the replenishment of vesicle components). It was also discovered that serotonin-induced presynaptic translation of CPEB is necessary for LTF stabilization and persistence (Si et al., 2003; Miniaci et al., 2008). Another study reported the occurrence of presynaptic synthesis of sensorin in LTF of *Aplysia* sensory neurons, and also showed that secreted sensorin leads to phosphorylation and translocation of MAPK into the nuclei of sensory neurons (Hu et al., 2007).

The reversal of synaptic strength from the potentiated state (LTP) to pre-LTP levels has been termed as depontentiation (Huang and Hsu, 2001). Surprisingly, the synaptic resistance to depotentiation is dependent on presynaptic protein synthesis (Huang and Hsu, 2004), meaning that axonal translation is necessary for the maintenance and stability of long-term information storage.

On the other hand, presynaptic protein synthesis was also demonstrated to be required for striatal LTD in response to retrograde endocannabinoid signaling caused by HFS (Yin et al., 2006), however the precise mechanism is far from being unmasked.

1.6. Balancing local protein synthesis and local protein degradation

The advantage conferred by local protein synthesis is the ability to regulate protein composition, in a specific manner, in different local domains. Regulation of protein composition is the result of an interplay between protein synthesis and degradation, being obvious to assume that there are mechanisms mediating local protein

degradation as well, possibly with outstanding importance for neuronal function. In fact, recent evidences support this idea (for a review Steward and Schuman, 2003; Hedge, 2004; Gumy et al., 2009). Indeed, the detection of ubiquitinated proteins in adult rat forebrain synaptic fractions (Chapman et al., 1994) and the detection of the machinery responsible for ubiquitin-dependent degradation, such as ubiquitin, ubiquitin-activating enzyme E1 and proteasome subunits in retinal growth cones (Campbell and Holt, 2001) and in presynaptic terminals (Speese et al., 2003), suggest that both the attachment of ubiquitin to substrate proteins and their subsequent degradation may occur locally. It is important to notice that local protein degradation is critical in neuronal functions already proved to be dependent upon local protein synthesis, and thus revealing a possible balance between these mechanisms in neuronal activities occurring far from the cell body.

Campbell and Holt observed that proteasome inhibitors prevented netrin-1-induced chemotropic responses and that these guidance cues elicit rises in ubiquitin-protein conjugates on growth cones (Campbell and Holt, 2001). In addition, loss of function mutations of the ubiquitin-activating enzyme (E1) or proteasome subunits in neurons of *Drosophila* mushroom bodies block axon pruning[4] (Watts et al., 2003). These findings suggest that protein degradation is important for both axon guidance and pruning.

As it is described in the section 1.5.2.1., regeneration of axons requires the re-creation of the growth cone. Besides local protein synthesis, local degradation has gained attention in the past years. In regenerating sciatic nerves, an increase in ubiquitinated proteins was observed (Jack et al., 1992). On the other hand, Fawcett and colleagues reported that application of proteasome inhibitors resulted in a reduction in

[4] Axon pruning is widely used for the refinement of neural circuits, and consists in the development of exuberant axonal branches, followed by a selective pruning of a subset of these branches.

the proportion of transected axons to regenerate growth cones (Verma et al., 2005). Recently, it was observed that mammalian CNS axons after a stretch injury accumulate ubiquitin associated with cytoskeleton elements (Staal et al., 2009), suggesting UPS-dependent reorganization of the cytoskeleton in axons after injury.

With respect to synaptic plasticity, several studies indicate that the ubiquitin-proteasome pathway plays a role in long-term and short-term effects. For example, the presynaptic protein DUNC-13 (a synaptic vesicle priming protein) is ubiquitinated and degraded by the proteasome in *Drosophila* neuromuscular synapse (Speese et al., 2003), and so acute inhibition of proteasome activity promotes rapid strengthening of neurotransmition (Speese et al., 2003). Two other synaptic vesicle proteins, synaptophysin and syntaxin have been shown to be substrates for ubiquitin-proteasome-mediated degradation (Wheeler et al., 2002). On the other synaptic side, it has been shown that ubiquitin-proteasome pathway is involved in receptor internalization, modulating synaptic transmission through endocytosis of AMPA receptor subunits (Patrick et al., 2003). In addition to the neurotransmitter receptors, proteins of the postsynaptic density are substrates of the ubiquitin-proteasome degradation (Schwartz, 2003) in an activity-dependent manner (Ehlers, 2003).

1.7. Presynaptic differentiation

Following axon outgrowth and arrival at the target area, the growth cone suffers tremendous morphological and functional alterations as it differentiates into a presynaptic terminal. In a first stage, the growth cone loses its highly motile properties: axon navigation slows down and the growth cone flattens (Yoshihara et al., 1997; Jontes et al., 2000), probably due to an increase in axonal membrane adhesiveness and

alteration of axonal cytoskeleton dynamics. It is interesting to point out the possible involvement of axonal translation in mediating these alterations, since several axonal localized mRNAs code for cytoskeleton proteins and regulators of cytoskeleton dynamics (see table 1).

The initial formation of axonal varicosities, which resemble immature presynaptic terminals with neurotransmitter containing vesicles, is accompanied by axonal branching (Alsina et al., 2001). Within minutes, a functional synapse (with a relatively immobile axon and a fully functional neurotransmitter-releasing presynaptic terminal) is formed, suggesting that axons can autonomously form a synaptic contact, and do not depend on soma-trafficked proteins.

Presynaptic formation involves coordinated action of several inter-dependent events: precise alignment between postsynaptic density and the presynaptic active zone; clustering and maturation of synaptic vesicles (SVs); establishment of the active zone; presynaptic growth; cytoskeletal restructuring and assembly of vesicle recycling machinery.

Synaptic vesicle genesis, including maturation and clustering, is crucial for presynaptic formation. In a functional presynaptic site, synaptic vesicles are clustered into two distinct pools: the reserve pool and the readily releasable pool, that allow for their rapid availability in case of stimulation (Dillon and Goda, 2005). These pools are maintained by actin filaments by forming a physical barrier to prevent vesicles dispersion (Dillon and Goda, 2005), and therefore the formation of an actin network during presynaptic differentiation is absolutely crucial for synaptic vesicles clustering. Indeed, it has been proposed that formation of actin networks beneath nascent synaptic sites leads to passive accumulation of mobile synaptic vesicles transport particles (STVs) (McAllister, 2007), which are vesicular structures carrying several SV-

associated proteins and other proteins critical for exo- and endocytosis (Ahmari et al., 2000 and Zhai et al., 2001).

Other proteins are involved in synaptic vesicles biogenesis and clustering. For example, the adaptor complex 3 (AP3), which associates with endosomes, is believed to regulate SV maturation at nascent synapses: mutant mice lacking AP3 exhibit abnormal SVs of GABA-inhibitory synapses (Nakatsu et al., 2004) and mistargeting of several SV membrane proteins (Salazar et al., 2004a,b). Another example is the exocyst, a protein complex composed of eight proteins, believed to be involved in targeting secretory vesicles to specific domains in the plasma membrane. Results showed that exocyst subunit mutants fail to maintain golgi-derived immature synaptic vesicles at the tips of axons (Murthy et al., 2003; Mehta et al., 2005). On the other hand, growth factor signaling is also involved in the modulation of synaptic vesicles clustering. Wnt7 interaction with its receptor, Frizzled, prevents β-catenin degradation and promotes phosphorylation of the microtubule-associated protein MAP1B leading to microtubule stabilization (Krylova et al., 2000; Ciani et al., 2004), thus contributing to axonal branching and recruitment of presynaptic vesicles (Hall et al., 2000) (figure 3, right image). Another example is the fibroblast growth factors (FGFs)-induced synaptic vesicle clustering through activation of FGF receptors in the presynaptic membrane (Dai and Peng, 1995).

In order to guarantee the perfect alignment between pre- and postsynaptic sides, trans-synaptic interactions are maintained by neuronal adhesion molecules that bind cytoplasmic scaffolding proteins. The neuroligin-neurexin complex (figure 3, left image), which directly bridges the synaptic cleft, induces the nucleation of calcium/calmodulin-dependent serine protein kinase (CASK) in the presynaptic site (Dean et al., 2003) and postsynaptic density protein 95 (PSD-95) in the postsynaptic site

(Graf et al., 2004). Through protein-protein interactions other proteins are recruited to CASK, which promotes synaptic vesicles clustering and recruitment of active zone components to that spot. On the presynaptic neuron, PSD-95 binds to NMDA receptors (Graf et al., 2004), contributing to the precise alignment between the active zone and the postsynaptic density. Another excellent example is the recruitment of the NMDA subunit NR1 to sites precisely aligned with presynaptic active zone by the ephrinB/ EphB2 complex (Dalva et al., 2000).

The active zone, term coined by Counteaux and Pecot-Dechavassine, defines the area of the presynaptic plasma membrane where regulated tethering, docking and fusion of the SVs takes place (Couteaux, 1963). It is designed to guarantee a controlled neurotransmitter release, and it must be juxtaposed to the postsynaptic density (Dresbach et al., 2006), in order to promote synaptic transmission efficacy.

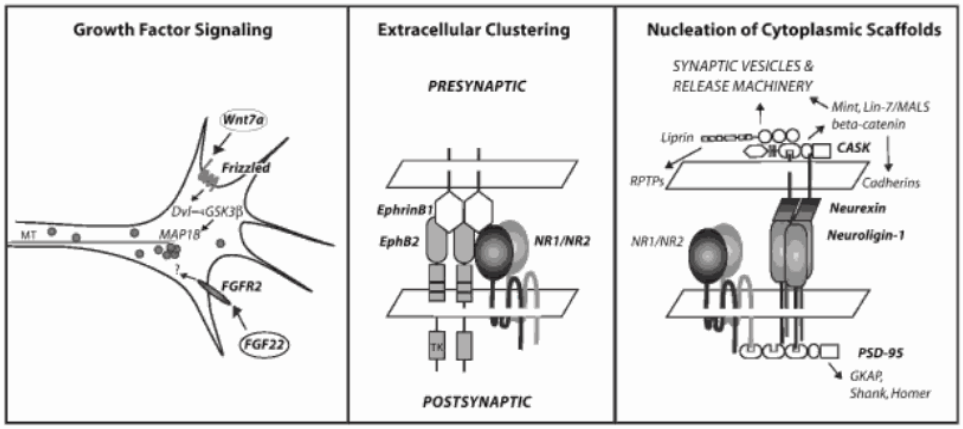


Figure 3 - Three general mechanisms that contribute to the differentiation and functional organization of synaptic membranes (Adapted from Zhen, 2007).

Synaptic vesicle fusion involves the soluble NSF attachment receptor (SNARE) complex, formed between the vesicle SNARE synaptobrevin (VAMP) and the plasma membrane SNAREs syntaxin and synaptosomal-associated protein-25

(SNAP-25) (Murthy and De Camilli, 2003; Rizo and Sudhof, 2002). The formation of this complex is believed to provide the driving force for fusion (Chen and Scheller, 2001; Jahn et al., 2003) and therefore these proteins must be located in the active zone. Even though this is actually true (Garcia et al., 1995), syntaxin and SNAP-25 also locate in other regions of neuronal plasma membrane (Garcia et al., 1995), showing the need for a specific machinery that restricts neurotransmitter release to active zones. In fact, the cytomatrix at the active zone (CAZ) is an electron-dense structure of specialized proteins that controls and promotes neurotransmitter release. CAZ is composed of several proteins: Munc13 confers fusion competence to docked SVs (Rosenmund et al., 2003; Varoqueaux et al., 2002); RIM is necessary in normal SV exocytosis and in synaptic plasticity (Kaeser and Sudhof, 2005; Calakos et al., 2004); CAST/ERC, Bassoon, Piccolo and liprin are scaffolding proteins or adaptors capable of homomeric interactions with other CAZ proteins, or the synaptic plasma membrane components of the synaptic cytoskeleton and synaptic vesicle release machinery (Fenster et al., 2000; Ohtsuka et al., 2002). This capability suggests that the presynaptic molecular scaffolds must exert spatial constraints on the distribution of synaptic vesicles and on the connection of SVs with the plasma membrane.

It has been shown that functional active zones can form within 30-60 minutes after initial axodendritic contact. In cultured hippocampal neurons most identified active zone proteins are preassembled and packaged in cytoplasmic transport vesicles, derived from the trans-Golgi network, that are delivered to the nascent synaptic sites (Ahmari et al., 2000; Shapira et al., 2003; Zhai et al., 2001). These vesicles are termed Piccolo-Bassoon transport vesicles (PTVs) and contain, besides Piccolo and Bassoon, SNAREs syntaxin and SNAP25, RIM1, Munc13, N-type voltage-gated calcium channels and Munc18 (Shapira et al., 2003; Zhai et al., 2001). The active zone transport vesicle

hypothesis (Dresbach et al., 2006) speculates that PTVs (modular units of active zone material) are completely packed in the cell body and trafficked via microtubule-based transport to nascent presynaptic sites, where fusion results in active zone components delivery.

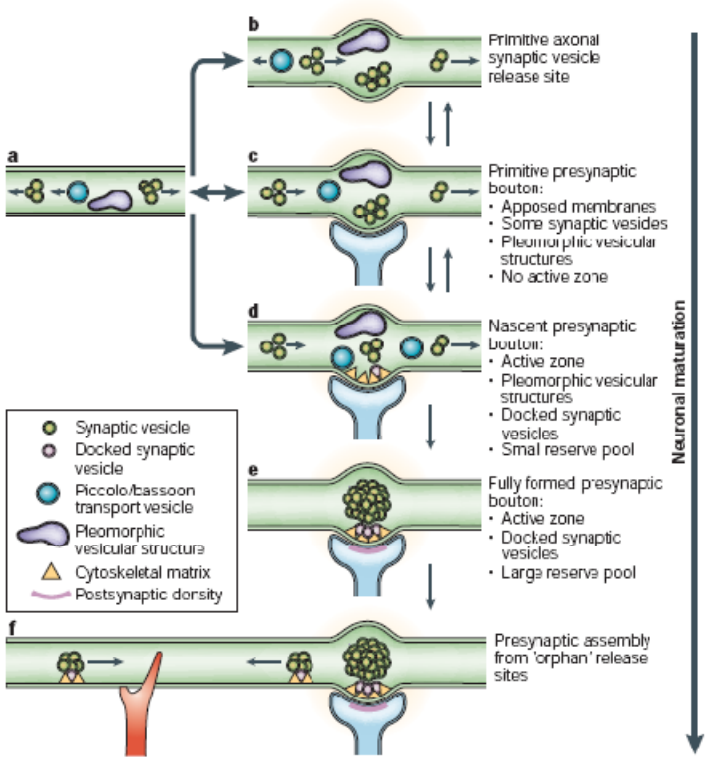


Figure 4 –Presynaptic assembly by modular transport vesicles. (a) Mobile packets of vesicles or vesicular material and active zone components are highly mobile within the developin axon. The vesicular packets containing active zone proteins are frequently associated, but not in the same package, with synaptic vesicles packets (Tao-Cheng, 2007). Packets move intermittently in both directions, with random freezing moments, they can split into smaller packets or merge into larger ones. (b) Even without a postsynaptic target, axons are capable of inducing assembly of transport packets, thus creating immature presynaptic terminals (Prokop et al., 1996). (c) Contact with dendrites leads to rapid recruitment of more STVs and PTVs, consequently stabilizing those sites. Pleiomorphic vesicular structures (unknown function) are present at newly-forming synapses during the

assembly of presynaptic boutons (Ahmari et al., 2000). (d) Fusion of PTVs with presynaptic membrane leads to formation of the active zone (Dresbach et al., 2006). (e) Within days, a totally mature presynaptic boutton is formed with a large reserve pool of SVs and a ready releasable pool (Dillon and Goda, 2005). (f) Portions of existing mature terminals can split off and mobilize to form new immature presynaptic sites (Krueger et al., 2003) (Adapted from Ziv and Garner, 2004).

Presynaptic differentiation is a highly complex event, and accounts for the involvement of various cellular events. Besides the described mechanisms, several others contribute to the formation of a properly organized and functional presynaptic terminal. A simple model for presynaptic assembly has been proposed (figure 4). Succinctly, it states that presynaptic components are constitutively generated in the cell body and then packed into vesicular intermediates in the trans-Golgi network (Jin and Garner, 2008). Freely-moving packets along the immature axon (McAllister, 2007), such as STVs and PTVs (Zhai et al., 2001 and Sabo et al., 2006, respectively), are transported and assembled in nascent synaptic sites upon contact between presynaptic axon and postsynaptic target (Jin and Garner, 2008; Ziv and Garner, 2004).

1.7.1. Presynaptic organizing molecules

Presynaptic differentiation is known to depend on presynaptogenic factors, commonly referred to as presynaptic organizing molecules. Axon itself has the capacity to initialize the recruitment of presynaptic contents (Prokop et al., 1996), however, a mature and functional presynaptic terminal will only form after postsynaptic contact (Ziv and Garner, 2004). This observation has led scientists to speculate that probably

factors from the postsynaptic side would interact with the presynaptic membrane, thus affecting and promoting presynaptic differentiation. In fact, postsynaptic-derived soluble and membrane proteins and glia-derived molecules are known to be fundamental for presynaptic differentiation (Fox et al., 2006). We will briefly review the presynaptic organizing function of those factors at synapses of the central nervous system.

Neuroligin, a postsynaptic transmembrane protein that binds across the synaptic cleft to the presynaptic cell surface receptor β-neurexin (reviewed in Craig and Kang, 2007), is able to induce formation of presynaptic specializations. Preliminary data arose from co-cultures with neuroligin-transfected non neuronal cells and basal pontine neurons. In these cultures, neuroligin 1 and 2 showed a remarkable capacity to induce synaptic vesicles clustering along overlying axons (Scheifelle et al., 2000 and Fu et al., 2003). Later on, Dean and colleagues (2003) concluded that neuroligin effect on synaptic vesicles distribution is mediated by clustering of β-neurexin in the presynaptic membrane. Moreover, this clustering seemed to promote the formation of functional active zones as assessed by the ability of synaptic vesicles at these sites to recycle the styryl dye FM1-43 (Dean et al., 2003). Further published data helped to solidify the idea that neuroligin heterophilically binds presynaptic neurexin to form functional presynaptic terminals (Graf et al., 2004; Levinson et al., 2005; Prange et al., 2004; Sara et al., 2005; Chih et al.,2005). Interestingly, the β-neurexin/neuroligin complex is bi-directionally active regarding that presynaptic β-neurexin is also capable of triggering assembly of postsynaptic receptors through neuroligins (Nam and Chen, 2005 and Sudhof, 2008) (see also figure 3 right image). Against all odds, recent data contradicts the proposed role of neuroligins in the initial establishment of synapses. Using knockout mice for neuroligin 1, 2 and 3, different research groups observed an impaired synaptic

signaling and transmission, but not a decrease in synapse formation (Varoqueaux et al., 2006; Chubykin et al., 2007; Tabuchi et al., 2007), thus concluding that neuroligins must regulate the proper maturation of synapses. Further experiments should be performed to clarify neuroligin's function.

Synaptic cell-adhesion molecule (SynCAM) is a another cell surface adhesion molecule capable of inducing pre-synaptic differentiation. SynCAM, a transmembrane protein containing three Ig-domains and an intracellular C-terminal PDZ-binding motif, is a homophilic calcium-independent cell-adhesion molecule (CAM) expressed on both sides of the synapse (Biederer et al., 2002). Early studies revealed that contact with SynCAM induce the formation and assemble of presynaptic terminals capable of both spontaneous and evoked neurotransmitter release (Biederer et al., 2002; Sara et al., 2005), thus suggesting that these terminals are functionally active. Although being unusual, heterophilic adhesion between the various SynCAM isoforms can occur (Shingai et al., 2003) and, in fact, the transynaptic SynCAM1/2 complex promotes organization of presynaptic terminals and enhanced neurotransmitter release (Fogel et al., 2007). Another interesting feature of SynCAM is the fact that, after axotomy, SynCAM mRNA levels are elevated during restoration of central synapses on motoneurons, thus revealing their possible involvement in the re-formation of synaptic connections in the adult brain (Zelano et al., 2009). In a way similar to neuroligins, SynCAM is believed to trigger active zone formation (it binds CASK and contains PDZ binding motifs – Biederer et al., 2002), however the precise mechanism is far from being uncovered.

WNT family, composed of postsynaptic-secreted glycoproteins, is known to induce growth cone remodeling that would promote synaptic differentiation. Indeed, exposure of cerebellar granule cells or pontine mossy fibers to Wnt-7a induces

spreading of the axonal growth cone (Lucas and Salinas, 1997) and clustering of synapsin I (Hall et al., 2000). Similarly, Wnt-3 was shown to enhance clustering of synapsin I in spinal sensory neurons (Krylova et al., 2002). Wnt-7a acts by inactivating glycogen synthase kinase 3 (GSK-3) (Hall et al., 2000), which ultimately controls the phosphorylation of MAP-1B and thereby the stability of microtubules in the growth cone (Lucas et al., 1998) (see figure 3 left image and descriptive text). Recently, Frizzled-5 was reported to be the specific receptor that mediates the synaptogenic effect of Wnt-7a (Sahones et al., 2010). In a different perspective, Wnts in the developing hippocampus were shown to promote, not only pro-synaptogenic, but also anti-synaptogenic effects (Davis et al., 2008). This differential synaptogenic activity was proposed to result from activation of different signaling pathways downstream Frizzled receptors (Davis et al., 2008). Analogously, Wnts were shown to be required for correct synapse formation and target recognition in neuromuscular junction, both as pro- (Packard et al., 2002) and antisynaptogenic molecules (Inaki et al., 2007; Klassen and Shen, 2007).

FGFs constitute a large family of polypeptide growth factors implicated in the regulation of a wide range of processes. Their role in presynaptic rearrangement was early highlighted by Dai and Peng (1995), who clearly showed clustering of synaptic vesicles, as evidenced by anti-synaptotagmin labeling and electron microscopy, at the sites of contact between FGFs-coated beads and *Xenopus* spinal cord neurites. Later on, FGF22 and related family members FGF7 and FGF10 were identified as target secreted proteins capable of promoting synaptic vesicles clustering in axons of young cerebellum neurons (Umemori et al., 2004). They proposed that FGF22 was secreted by postsynaptic granule cells and activated fibroblast growth factor receptor 2 (FGFR2) located at the presynaptic terminals of mossy fibers, thus leading to presynaptic

organization and synaptic vesicles clustering. Furthermore, expression levels of FGF22 and FGFR2 correlated temporally with synaptogenesis (Umemori et al., 2004). Recently, using knockout mices for FGF22 and FGF7, Umemori and colleagues revealed that FGF22 and FGF7 promote the organization of excitatory and inhibitory presynaptic terminals, respectively, on nerve terminals of Cornu Ammonis region 3 (CA3) pyramidal neurons (Terauchi et al., 2010). Similarly to Sanes and colleagues, they concluded that FGF22/7 were expressed and secreted by postsynaptic CA3 pyramidal neurons at early stages of synapse formation (Terauchi et al., 2010). Additionally, presynaptic differentiation at embryonic neuromuscular junctions was shown to depend on FGFs signaling through FGFR2b receptor (Fox et al., 2007). Secreted FGFs signal to target cells by binding and activating cell-surface tyrosine kinase FGF receptors (FGFRs). Activated FGFRs signal through different intracellular signaling pathways (Böttcher and Niehrs, 2005), as it is shown in figure 5.

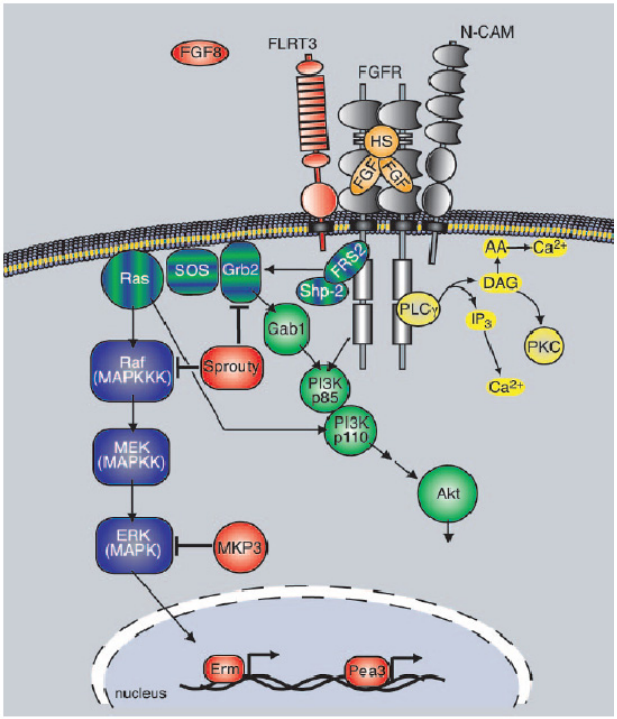


Figure 5 - Intracellular signaling pathways activated through FGFRs.

Upon ligand binding FGFRs dimerize and undergo autophosphorylation and

activation of intra-cellular tyrosine kinase domains. This leads to tyrosine phosphorylation of docking proteins, such as FRS2, which recruits the protein tyrosine phosphatase Shp2 and the adaptor protein Grb2, consequently promoting formation of Grb2/SOS complexes and activation of the Ras/MAPK signaling pathway. FGFRs can activate PI3kinase/Akt pathway by three ways: indirectly binding of Gab1 to FRS2 via Grb2 resulting in PI3K tyrosine phosphorylation; direct binding of PI3K-regulatory subunit p85 to FGFR; and activation of PI3K-catalytic subunit by activated Ras. FGFRs also activate PLCγ/Ca^{2+} pathway (Adapted from Böttcher and Niehrs, 2005).

Another class of secreted presynaptic organizing molecules recently discovered is signal regulatory proteins (SIRPS) (Umemori and Sanes, 2008). The extracellular domain of SIRP-α, purified from mouse brain, as well as close relatives SIRP-β and SIRP-γ, were demonstrated to induce clustering of synapsin-positive aggregates (Umemori and Sanes, 2008).

Secreted molecules from neighboring astrocytes also play a role in synapse formation. Indeed, cultured retinal ganglion cells establish few synaptic contacts unless they are co-cultured with astrocytes (Pfrieger and Barres, 1997; Nagler et al., 2001; Ullian et al., 2001), thus pointing to a requirement for soluble glial-derived signals. The first glia-derived molecule to be discovered was cholesterol, shown to be bound to apolipoprotein E and to promote synaptogenesis in the central nervous system (Mauch et al., 2001). Later on, Pfrieger and colleagues emphasized this idea by demonstrating that glia-derived cholesterol complexed to apolipoprotein E-containing lipoproteins formed numerous and efficient synaptic connections (Goritz et al., 2005). Recently, thrombospondins (TSPs), extracellular matrix molecules secreted by astrocytes, were

demonstrated to be necessary and sufficient for synapse formation both *in vitro* and *in vivo* (Christopherson et al., 2005). This synaptogenic effect of TSPs seems to be specific for glutamatergic synapses, since no change is registered when the secreted molecule is applied to GABAergic synapses (Hughes et al., 2010). Recently, scientists identified the neuronal thrombospondin receptor involved in CNS synapse formation as alpha2delta-1, which was shown to be required postsynaptically for thrombospondin- and astrocyte-induced synapse formation *in vivo* (Eroglu et al., 2009). Interestingly, a link between glia-derived factors and adhesion molecules in induction of synapse formation was revealed by Xu and colleagues (2010), who reported that TSP1 increase the speed of synapse formation in young hippocampal neurons through interaction with neuroligin 1. This work wonderfully reveals that presynaptic organizers must work in synchrony to consummate their duties. Concluding, astrocytes are active participants in CNS synaptogenesis, and it is now understandable why throughout the CNS the main phase of synaptogenesis starts instantaneously after glia differentiation.

1.8. Objectives

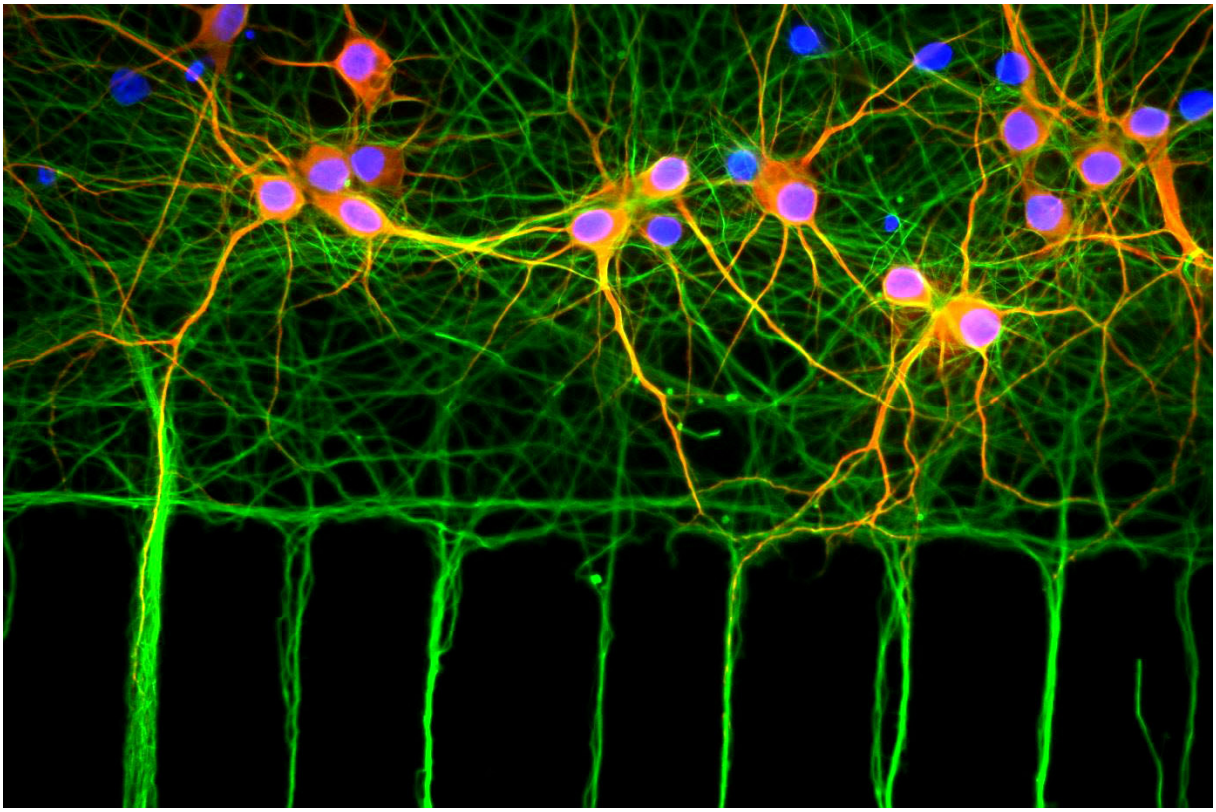
The objective of this work is to establish the role of axonal protein synthesis in FGF22-induced presynaptic differentiation, the mechanism by which a functional presynaptic terminal is formed. To our knowledge, no attemps were made to determine how inhibition of axonal translation will affect or impair the formation of the presynaptic terminal.

Neuronal mechanisms like axon guidance, synaptic plasticity, axonal regeneration and retrograde signaling all require intra-axonal protein synthesis. Moreover, recent data have shown that synapse formation also relies on proteins

axonally synthesized (Sebeo et al., 2009, Schacher and Wu, 2002, Lyles et al., 2006), but strong evidences are still lacking. The majority of the work published until now addressed this question by looking into the changes in potentiation of synaptic transmission in developing synapses by inhibition of protein synthesis (Zhang and Poo, 2002) or by specifically knocking-down an mRNA localized to neurites of *Aplysia* sensory neurons (Lyles et al., 2006). However, we currently do not know how local protein synthesis regulates presynaptogenesis.

In order to accomplish our goal, we first aim at characterizing FGF22 presynaptogenic effect on rat embryo hippocampal neurons. This factor was already reported to trigger presynaptic differentiation (Umemori et al., 2004; Terauchi et al., 2010). We will assess synaptic vesicles clustering by immunolabeling cultured neurons for synapsin, a membrane protein localized to the cytosolic side of SVs. Then, by using microfluidic chambers and taking advantage of their fluidic properties, we propose to look into the presynaptogenic effect of FGF22 when applied to axons. Having established an *in vitro* model of local induction of presynaptic differentiation by FGF22, we will then add protein synthesis inhibitors to isolated axons and evaluate the dependence on axonal protein synthesis of a developing axon during differentiation of the presynaptic terminal.

Chapter 2 Materials and Methods



2.1. Reagents

Bovine Serum Albumin (BSA), deoxyribonuclease I from bovine pancreas, cytosine arabinose (AraC), poly-D-lysine (PDL), Dulbecco's modified Eagle's medium (DMEM), Minimum essential medium eagle (MEM), 5-fluoro-2'-deoxiuridina (5-FDU), paraformaldehyde, L-glutamic acid, N,N,N',N'-Tetramethylethylenediamine (TEMED), ammonium persulfate (APS), the protein synthesis inhibitor emetine dihydrochloride hydrate and the phosphatases inhibitor sodium orthovanadate were all purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). MEM-Non essential amino acids Neurobasal medium, penicillin-streptomycin, B27 supplement, sodium pyruvate, fetal bovine serum (FBS), trypsin, glutamine and goat serum were purchased from GIBCO^{TM} Invitrogen Corporation (Carlsbad, California, USA). Mouse laminin I was obtained from Cultrex®, as part of Trevigen, Inc (Helgerman Court, Gaithersburg, USA). The protein synthesis inhibitor anisomycin was 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). Restore western blot stripping buffer was purchased from Thermo Fisher Scientific (Rockford, Illinois, USA). Sodium fluoride (NaF) was purchased from Merck (Darmstadt, Germany). Complete protease inhibitor cocktail tablets was obtained from Roche Applied Science (Carnaxide, Portugal). Enhanced chemifluorescence (ECF) was obtained from GE Healthcare (Uppsala, Sweden). Recombinant human fibroblast growth factor-22 (FGF22) was purchased from R&D Systems, Inc (Minneapolis, USA). Recombinant human brain-derived neurotrophic factor (BNDF) was from Peprotech (Rocky Hill, New Jersey, USA). All other reagents of high degree of purity were from Sigma (Saint Louis,

Missouri, USA), from Merck (Darmstadt, Germany), Panreac Quimica Sau (Castellar del Vallès, Barcelona, Spain), Fisher Scientific (Rockford, Illinois, USA) and from Bio-Rad Laboratories, Inc (Amadora, Portugal).

Stock solutions of FGF22 and BNDF were made in phosphate buffered saline (PBS) with 0.1% BSA. Stock solution of orthovanadate was made in dimethyl sulfoxide (DMSO). All other reagents were kept in aqueous stock.

2.1.1. Antibodies

Anti-p44/42 MAPK (Erk1/2) polyclonal antibody (rabbit) (9102) and anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) monoclonal antibody (rabbit) (4377) used for western blots were both purchased from Cell Signaling Technology **(**Danvers, Massachusetts, USA). The secondary anti-rabbit IgG alkaline phosphatase linked whole antibody (from goat) (NIF1317) was obtained from GE Healthcare (Uppsala, Sweden). The antibodies used for immunocytochemistry are listed below. Anti-β-tubulin I monoclonal antibody (mouse) (T7816) and anti-MAP2 (2a + 2b) monoclonal antibody (mouse) (M1406) were acquired from Sigma Aldrich (Saint Louis, Missouri, USA). Anti-synpasin I polyclonal antibody (rabbit) (AB1543) was obtained from Millipore (Billerica, Massachusetts, USA). Anti-neuronal Class III β-tubulin (TUJ1) monoclonal antibody (mouse) (MMS-435P) was obtained from Covance (Emeryville, California, USA). The secondary antibodies used for immunocytochemistry, TexasRed goat anti-mouse IgG (T862) and AlexaFluor 488 goat anti-rabbit IgG (A-11008) were both 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 device for neuron cell culture consists of a molded PDMS (poly-dimethlysiloxane) chamber placed against a glass coverslip. The glass slides (Corning No.1 24 mm × 40 mm) were cleaned with nitric acid 65% for 24 h in constant agitation and then washed 5 times 30 min each with mQ H_2O. The glass slides were rinsed twice in pure ethanol and then placed in the oven at 50^oC until they were dry (approximately 15-20 min). Exposure to ultraviolet radiation for 10 min guaranteed the sterilization of the glass slides, which were then maintained sterile for neuron culture.

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 PDMS mold (containing several chambers) was cut in individual pieces each bearing a single PDMS molded chamber. We punched holes in the place of the media reservoirs with a sharpened rounded tool and we cleaned each chamber with 3M Scotch Brand 471 tape to lift off any remaining debris. The chambers were rinsed once with filtered 75% ethanol. The following steps were carried out in sterile conditions.

The chambers were always constructed in the plating day. Glass slides placed in a 10cm dish were coated with 0.1 mg/ml PDL overnight at 37^oC and then washed 3 times with sterile mQ H_2O (1 hour in the last wash). As soon as the glass slides were dry, the microfluidic chambers were assembled on top of the PDL coated slides, and 2 μg/ml of laminin in neurobasal plain media was added to the medium reservoirs making sure that the microgrooves connecting the compartments were totally filled. After an incubation of at least 2h at 37^oC the chambers were washed once with plating media (minimum

essential medium eagle (MEM) supplemented with 0.026 M $NaHCO_3$, 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 embryonic rat 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_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0,001% phenol red). After centrifugation for 1 min at 1000 rpm to deposit the hippocampi, Hank's solution with trypsin was removed and the hippocampi were washed with plating medium containing 10% FBS to stop trysin activity. In order to obtain a homogeneous cell suspension the hippocampi were mechanically dissociated with a P1000-pipette and then with a Pasteur pipette. Cells were then plated in multi-wells and microfluidic chambers coated with PDL (0.1 mg/ml) and laminin (2 µg/ml). In microfluidic chambers, 10 µl of a 4×10^6 cells/ml cell suspension were added to the somal side (see microfluidic chamber scheme, figure 9). In order to create pseudo-explants of hippocampal neurons, 50 µl of a 2×10^5 cells/ml cell suspension (approximately 10000 cells) were plated inside a cloning cylinder (6 mm diameter × 8 mm length) placed in the middle of each coverslip-containing well of a 24 multi-well plate. For the preparation of hippocampal neurons extracts, neurons were plated at a density of 3.5×10^5 cells/ml in 12 multi-well plates. After 4 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, Ara C or 5-FDU (according to lab availability) was added to the cultures at a final concentration of 1.825 μg/ml or 10 μM, respectively. The volume of medium added in each type of culture depended on the medium evaporation and on the final volume used at DIV0.

To determine the percentage of viable cells in microfluidic devices and in 24 multi-well plates, we counted the number of apoptotic nuclei present in a total of 400-500 cells per independent experiments from cultures at DIV8-7, respectively.

2.3. Stimulation and total extracts preparation

Total extracts of 293T cells and hippocampal neurons after FGF22 stimulation were prepared for Western Blot (WB) analysis of Erk1/2 phosphorylation. Total cell extracts of hippocampal neurons were prepared at DIV8. Cells were stimulated with increasing doses of FGF22 (as indicated in the results section) for 5 min (293T cells) or 10 min (hippocampal primary cultures). To reduce the basal levels of Erk phosphorylation, 293T cells were subjected to a starvation period of 3 h in DMEM with 0.5% BSA prior to FGF22 stimulation. Cells were washed once with cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then ice-cold denaturing lysis buffer (0.125 mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 10% glycerol)

supplemented immediately before use with proteases inhibitors cocktail and phosphatases inhibitors (1.5 mM sodium orthovanadate (Na_3VO_4) and 50 mM sodium fluoride (NaF)) was added to the cells, which were then scraped. The lysates obtained were sonicated. Depending on the lysates volume, the sonication was performed in an ultrasonic bath for 15 min or using an ultrasonic probe with 2 pulses of 15 sec each (30 sec on ice between sonication pulses). Samples were denaturated at 95°C for 5 min and then left on ice for at least 5 min before electrophoresis.

2.3.1. Electrophoresis and Western Blot

Total cell extracts obtained were electrophoresed in tris-glycine-SDS (TGS) buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) in a 7.5% (v/v) acrylamide gel 1.5 mm thick at 120-140 V. Each well was loaded with 20 µl of cell extract. Electrotransference onto a PVDF (polyvinylidene difluoride) membrane was performed overnight at 40 V at 4°C. Membranes were washed once with tris-buffered saline (TBS) (20 mM Tris, 137 mM NaCl) with 0.1% Tween 20 (TBS-T), and then blocked for 1 h at room temperature in TBS-T with 5% non-fat dry milk. Membranes were then washed three times (2×5 min and 1×15 min) with TBS-T and then incubated overnight at 4°C with the primary antibody diluted in TBS-T with 5% BSA. The following dilutions of primary antibodies were used: monoclonal anti-phospho-p44/42 MAPK (Erk1/2) 1:2000 and polyclonal anti-p44/42 MAPK (Erk1/2) 1:2000 (the first antibody applied was always for phospho-Erk1/2).

Membranes were then washed with TBS-T three times (2×5 min and 1×15 min) and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:20000) diluted in TBS-T with 3% non-fat dry milk. After

incubation with the secondary antibody, membranes were washed again with TBS-T (2×5 min and 1×15 min) and then resolved with ECF substrate for a maximum of 5 min or until the protein bands were visible. We scanned the membranes on Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK).

2.3.2. Stripping and reprobing

In order to reprobe the membranes for total Erk1/2, they were stripped with Restore western blot stripping buffer for 15 min and then washed several times with TBS-T. Membranes were blocked for 1 h in TBS-T with 5% non-fat dry milk and then washed twice briefly to remove the milk. The incubations with the primary and the secondary antibody (polyclonal anti-p44/42 MAPK (Erk1/2) 1:2000 and alkaline phosphatase-conjugated anti-rabbit 1:20000, respectively) and the membrane resolution were performed as described above.

2.4. Stimulation and protein synthesis inhibition

To look into the presynaptogenic effect of FGF22 and BNDF on hippocampal neurons, primary cultures at DIV6 (pseudo-explants on MW24) and DIV7 (microfluidic devices) were stimulated for 14h at 37^{o}C in conditioned medium. Recombinant human FGF22 was used at 10 nM (in MW24) and 20 nM or 100 nM (in microfluidic devices). Recombinant human BDNF was used at 100ng/ml. In microfluidic devices, these factors were added only to the axonal compartment. A minute volume difference between the somal compartment and the axonal compartment (≈25µl) was maintained during the incubation to prevent the diffusion of the applied factors from the axonal to

the somal side. The slightly higher volume on the somal side will cause a slow net flow of liquid from the somal to the axonal compartment and not the other direction.

When protein synthesis inhibitors were added, we performed 15 min incubation at 37^{o}C prior FGF22 stimulation, so that the drug could enter the cell before FGF22 exposure. FGF22 stimulation was carried in protein synthesis inhibitors containing medium. The inhibitors used were emetine and anysomicin at 2 μM (MW24) and 10 μM (microfluidic chambers). In microfluidic chambers, the inhibitors were added to the axonal compartment thus inhibiting protein synthesis only in axons.

2.4.1. Immunocytochemistry

After 14 h stimulation cells were fixed in 4% paraformaldehyde (in PBS with 4% sucrose) for 10 min at room temperature. In microfluidic chambers we did a pre-fixation of 5 min in 1% paraformaldehyde in order to reduce the aggressive effect of paraformalehyde in fragile axons of the axonal compartment. We washed cultures three times with tris-buffered saline (TBS) and then permeabilized them using TBS with 0.25% Triton X-100 for 5 min at room temperature. We washed once with TBS and we blocked non-specific binding with TBS with 5% goat serum and 5% FBS for 30 min at room temperature. We incubated overnight at 4^{o}C with primary antibodies in TBS with 5% goat serum and 5% fetal bovine serum. We used the following dilutions of primary antibodies: polyclonal anti-synapsin I 1:2000 in microfluidic devices and 1:4000 in MW24 coverslips, monoclonal anti-β-tubulin I 1:2000, monoclonal anti-neuronal class III β-tubulin (TUJ1) 1:1000 and monoclonal anti-MAP2 (2a + 2b) 1:200.

After incubation with the primary antibody, we washed preparations three times, 5 min each, with TBS to remove primary antibody, then incubated them with secondary

antibody in TBS with 5% goat serum and 5% FBS for 1 h at room temperature. We washed preparations twice, 5 min each, with TBS with 0.1% Triton X-100, then one 5-min wash in TBS, then we rinsed the glass slides with mQ H_2O and mounted them in prolong mounting media with DAPI. 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. For all experiments we used Texas Red anti-mouse 1:200 and Alexa Fluor 488 anti-rabbit 1:500.

2.4.2. Fluorescence microscopy and quantification

Fluorescent images were taken using an inverted microscope Zeiss Axiovert 200, an AxioCam HRm camera and AxioVision 4.8 software. Images were acquired with a Plan-Neofluar 63× oil objective (numerical aperture 1.3) and exposure times conserved in single experiments. In microfluidic chambers images were taken on the axonal side, and to be more accurate we selected regions equally distant from the microgrooves' entrance (approximately 300-400 μm). In pseudo-explants, images were taken on regions surrounding the central bulk of neurons, where growing axons could be found isolated.

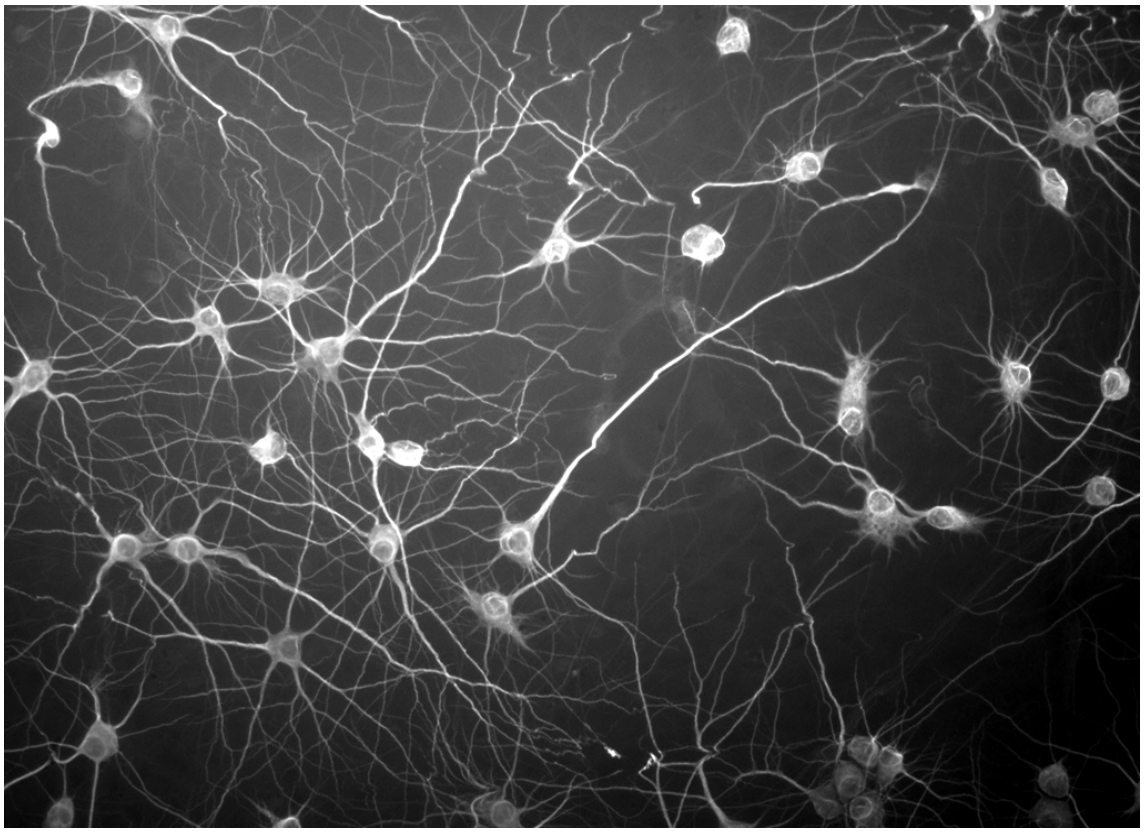
The images of the entire microfluidic chamber (both axonal and somal compartment) were taken using a confocal microscope Zeiss LSM 510 Meta and LSM 510 software. Images were acquired with an EC-PlanNeofluar 40× oil objective (numerical aperture 1.3) or with a Plan-ApoChromat 20× objective (numerical aperture 0.8).

Synapsin clustering was measured with Image J 1.42 software. Axons were randomly chosen from exported 16-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 β-tubulin images, without observation of synapsin puncta pattern. Images were thresholded (synapsin and background threshold values conserved in single experiments) and the number and area of synapsin puncta were quantified. The background intensity of each image was subtracted. Per condition, 25 or 30 axons were analyzed in each experiment. Per each selected axon we determined number of synapsin puncta/μm and total synapsin puncta area/μm. 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.

2.5. Statistical analysis

Results are presented as normalized means ± 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.

Chapter 3 Results and Discussion



3.1. Induction of presynaptic differentiation by FGF22

3.1.1. FGF22 stimulation of 293T cells and primary cultures of rat hippocampal neurons induces ERK1/2 phosphorylation

In humans, fibroblast growth factors (FGFs) family comprises twenty-two secreted proteins, commonly referred as FGF1-14 and FGF16-23, and arranged into seven subfamilies according to evolutionary distance (Itoh, 2007). The multifunctional FGF signaling has been demonstrated to be involved in a variety of biological processes, such as: wound healing (Komi-Kuramochi et al., 2005), bone formation (Lazarus et al., 2006), perimplantation and early placental function (Zhong *et al*., 2006), morphogenesis of embryonic tissues (for example Revest et al., 2001), proliferation of gland epithelial cells (Steinberg et al., 2004), among many others. FGF signaling has been suggested to be involved in CNS development and fcuntion, such as, in neural plate formation, patterning of specific CNS regions (neocortex and spinal cord), establishment of functional neuro-circuits, and repair of damaged tissues (reviewed in Dono, 2003). The recently discovered member of the FGF family, FGF22, is involved in neural differentiation of granule cells and acts as an organizer of presynaptic activity (Umemori et al., 2004).

FGF receptors (FGFR1-4) display extracellular immunoglobulin-like domains and cytoplasmic tyrosine kinase domain (Katoh, 2008). FGFR2 has two isoforms, FGFR2b and FGFR2c, with different affinities for FGFs (Katoh, 2008). FGFR2b is a high affinity receptor for FGF1, FGF7, FGF10 and FGF22 (Ornitz et al., 2001; Yeh et al., 2003; Umemori et al., 2004), while FGFR2c for FGF1, FGF2, FGF4, FGF6, FGF9, FGF16 and FGF20 (Ornitz et al., 1996; Zhang et al., 2006). The transduction of FGF

signals upon FGFR activation has been largely assessed with respect to three major pathways, PI3Kinase/ Akt pathway, PLCγ/ Ca^{2+} pathway and Ras/ MAPK pathway (reviewed in Böttcher and Niehrs, 2005). Upon FGF binding, receptors dimerize and autophosphorylation is triggered. Phosphorylated tyrosines function as binding sites for signaling proteins, thus triggering activation of signal transduction pathways. The most common pathway employed by FGFs is the MAPK pathway, in which Erk is promptly phosphorylated (reviewed in Böttcher and Niehrs, 2005, see also figure 5).

For FGF22 to exert its function in cultured cells, it must activate the signaling cascade downstream FGFR2b activation. On the other hand, the intracellular effects of a growth factor depend on the activation level of the signal transduction pathway, and so, in order to address the role of FGF22 in presynaptic differentiation, we should determine the optimal activation of the FGFR2b-signaling cascade. Sanes and colleagues already showed that 2 nM of FGF22 (purified endogenous protein) elicits near-maximal effects on cultured chick motoneurons (Umemori et al., 2004). Here, we aim to determine the optimal concentration range of recombinant human FGF22 to stimulate hippocampal neurons. Therefore, we stimulated HEK293T cells and hippocampal neurons with increasing doses of recombinant human FGF22 and we determined the levels of Erk1/2 phosphorylation using western blot analysis. In order to reduce the basal levels of phosphorylated Erk1/2, HEK 293T cells were starved for 3 h in DMEM supplemented with 0.5% BSA for 3 h (figure 6).

The results presented in panel A clearly show a strong phosphorylation of ERK1/2 upon stimulation with 10 nM of FGF22, much higher than the one observed with 2 nM of FGF22. There are no detectable changes in total ERK between all conditions, enabling us to ensure that differences in phosphorylated ERK are not due to different loading conditions. We then performed the same experiment with primary

cultures of rat embryonic hippocampal neurons at DIV 8 (panel B). This time, cells were not subjected to starvation before FGF22 stimulation, because previous experiments (data not show) with this treatment revealed that starvation induces high levels of ERK1/2 phosphorylation. This effect might be due to activation of cell survival signaling pathways that also involve phosphorylation of intermediates such as ERK. In fact, activation of MAPK pathway and ERK phosphorylation in hippocampus was shown to support neuronal survival in ischemia resistant neurons (Hu and Wieloch, 1994), and after lithium injection (Yan et al., 2007). Similarly, when neurons were subjected to the starvation period, MAPK survival pathway could have been activated, as a way to counteract the stress-induced conditions.

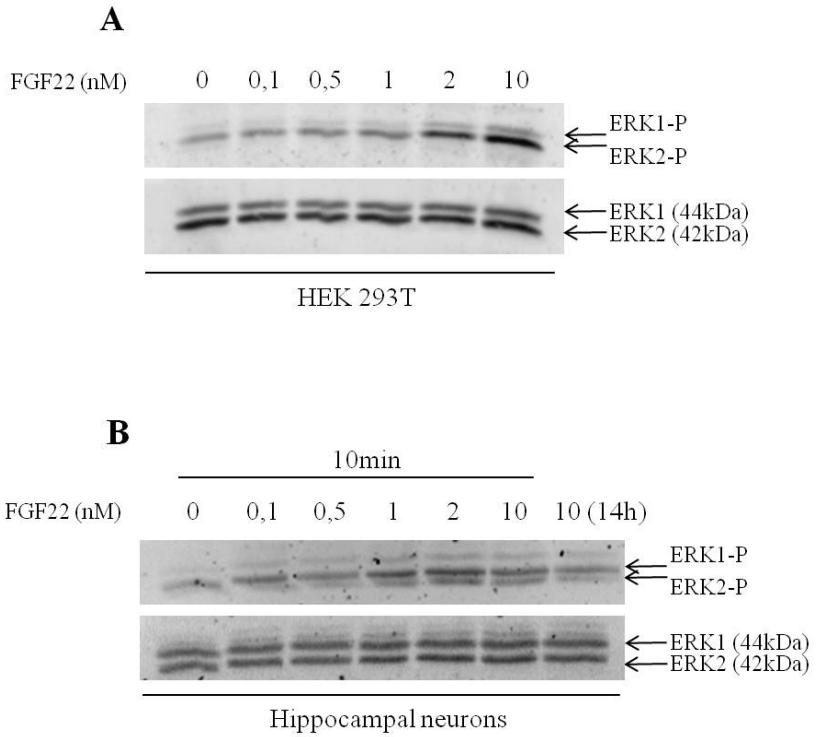


Figure 6 - FGF22-induced phosphorylation of ERK1/2. (A) 293T cells were starved for a period of 3 h in DMEM with 0.5% BSA, and then incubated for 5 min at 37°C with increasing doses of recombinant human FGF22 (0, 0.1, 0.5, 1,2 10 nM). Total cell extracts were prepared and Western Blot analysis for phosphorylated ERK1/2 was performed.

Membranes were reprobed for total ERK1/2. (B) Primary cultures of rat embryonic hippocampal neurons at DIV8 were incubated for 10 min at 37°C with increasing doses of recombinant human FGF22 (0, 0.1, 0.5, 1,2 10 nM). To evaluate the long-term effect of FGF22 on ERK1/2 phosphorylation we incubated neurons at DIV7 for 14 h at 37°C with the higher dose of FGF22 used (10nM). Total cell extracts were prepared and Western Blot analysis for phosphorylated ERK1/2 was performed. Membranes were reprobed for total ERK1/2.

The results obtained (figure 6B) show considerable levels of ERK phosphorylation when primary cultures of rat embryonic hippocampal neurons are stimulated with 1, 2 and 10 nM of FGF22. The signal obtained with 2 nM stimulation is slightly stronger than with 10 nM, difference that could probably be abolished if more experiments were done. Once more, there are no detectable changes in total ERK between all conditions, enabling us to ensure that differences in phosphorylated ERK are not due to different loading conditions. Concluding, figure 6B indicates that maximal activation of FGFR2b-signaling cascade in rat embryonic hippocampal neurons can be achieved by applying concentrations of recombinant human FGF22 equal or higher than 2 nM. So, taking both biological systems in consideration (HEK293T cells and hippocampal neurons), we decided 10 nM to be the ideal concentration to stimulate cultured cells.

3.1.2. Presynaptic differentiation assessed by synapsin clustering

Presynaptic differentiation comprises several events to occur in a coordinated manner: clustering of synaptic vesicles, establishment of the active zone, post- and pre-

synaptic membranes alignment, cytoskeletal restructuring and assembly of vesicle recycling machinery (Jin and Garner, 2008). In this work, we used synaptic vesicle clustering (synapsin I immunolabeling) as a marker of presynaptic differentiation.

Synapsins, the most abundant family of neuron-specific synaptic vesicle (SV)-associated phosphosproteins (Fornasiero et al., 2010), localize to the cytoplasmic surface of small SVs (figure 7C). Synapsin has long been used as a marker protein of synaptic vesicles (Thiel et al., 1993; Bixby et al., 1985), and nowadays it turned out to be a very useful tool to assess synaptic vesicles clustering in the study of presynaptic differentiation (Hall et al., 2000; Umemori et al., 2004; Fox et al., 2007; Umemori et al., 2008).

Cultured embryonic hippocampal neurons immunolabeled for synapsin exhibit a pronounced punctuated pattern (figure 7A). The magnified axonal segment in figure 2B shows that synapsin is arranged in distinct puncta along the axonal shaft, revealing clustering of synaptic vesicles. In this work, FGF22 effect on presynaptic differentiation was assessed by measuring synapsin puncta, a direct indicator of synaptic vesicle assembly and a hallmark of presynaptogenesis.

3.1.3. FGF22 and BNDF induce presynaptic differentiation of rat embryonic hippocampal neurons

To this date, several membrane and soluble proteins with functional roles in synaptogenesis have been identified (described in section 1.7.1). Fibroblast growth factors were recently associated to modulation of synaptogenesis. FGF22 was reported to induce presynaptic differentiation in cultured chick motoneurons and in cerebellum cultures (Umemori et al., 2004). Moreover, *in vivo* presynaptic differentiation of

cerebellar mossy fibers and chick embryonic neuromuscular junctions requires FGFR2b activity (Umemori et al., 2004; Fox et al., 2007). In this work, authors also concluded that FGF22 is expressed and secreted by postsynaptic neurons (such as granule cells), and interacts with its specific receptor located on the presynaptic membrane. BNDF is another key modulator of synaptic connectivity in the developing and adult vertebrate central nervous system. Several studies have suggested a role for BNDF/ TrkB signaling in the increase and refinement of hippocampal synaptic connections (Martinez et al., 1998; Jacobi et al., 2009), as well as a role in the modulation of presynaptic assembly in cultured hippocampal neurons (Bamji et al., 2006; Taniguchi et al., 2006).

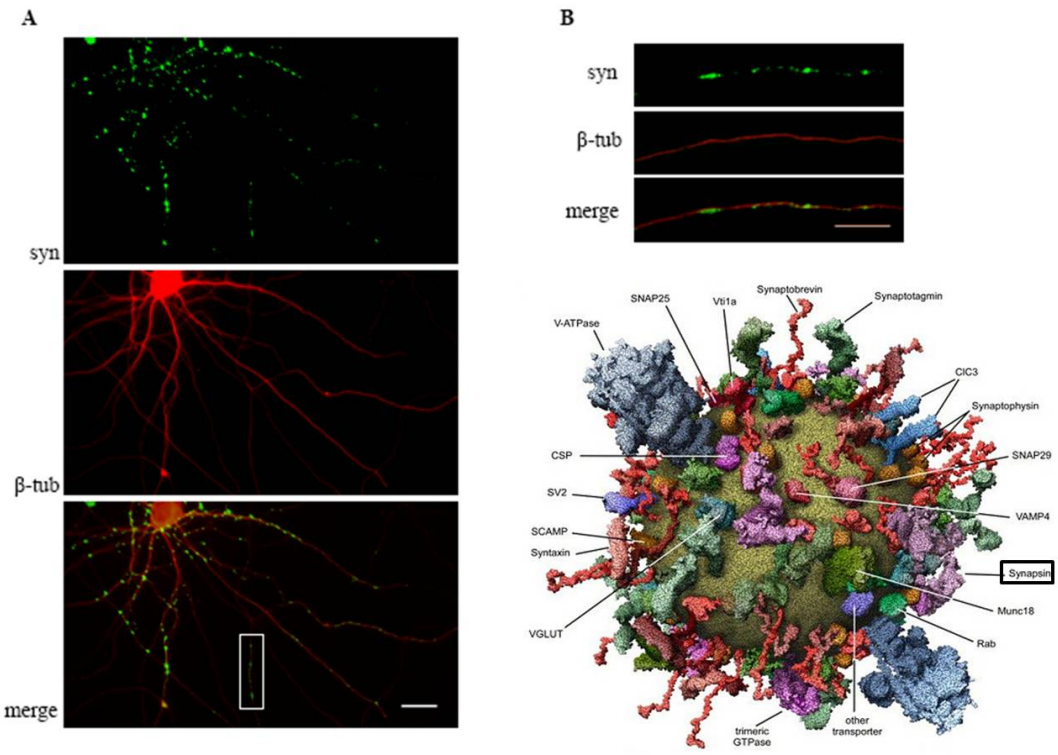


Figure 7 – Clustering of synaptic vesicles assessed by synapsin immunolabeling. (A) Primary cultures of rat embryonic hippocampal neurons at DIV7 were immunostained for synapsin and β-tubulin. Images were acquired using a Zeiss Axiovert 200 fluorescence microscope. A punctuated pattern of synapsin is observed in neurites of cultured neurons for

seven days. The scale bar is 25 μm. (B) Magnification of the area defined in A_{merge}. Bar is 10 μm. (C) Molecular model of an average synaptic vesicle (outside view) exhibiting synapsin to the cytosolic side (Takamori et al., 2006).

The main aim of this work is to elucidate the role of axonal protein synthesis in presynaptic differentiation. Thus, to pursue our studies, we need to establish a functional, reliable and reproducible system of induction of presynaptic differentiation *in vitro*. Taking into consideration the highly pronounced presynaptogenic effect of FGF22 reported at both CNS and PNS level, and the fact that FGF22 is a target-derived soluble molecule, we decided to test the presynaptic organizing effect of this molecule in embryonic rat hippocampal neurons. Simultaneously, we also decided to stimulate hippocampal neurons with BDNF, neurotrophin already proven to affect hippocampal connectivity and synaptic formation, and whose receptor is highly expressed in hippocampus (Fryer et al., 1996) and present in the surface of hippocampal dendrites and axons (Yan et al., 1997).

In order to address the possible involvement of FGF22 and BNDF on the induction of presynaptic differentiation in hippocampus, we prepared primary cultures of embryonic rat hippocampal neurons in the form of pseudo-explants. Cultures at DIV6 were subjected to a 14 h-stimulation at 37^{o}C with 10 nM of FGF22 or 100 ng/ml of BNDF in conditioned medium. Cultures were then fixed and immunocytochemistry for synapsin and β-tubulin was performed. Fluorescence microscope images were taken at peripheral regions of the pseudo-explants, where extending axons are isolated. Quantification of number and area of synapsin puncta per axon length was carried out in Image J software.

The results obtained (figure 8) show that both FGF22 and BNDF significantly increase the number of synapsin puncta per axon length (figure 8B) (141.6%, $p<0.001$ and 132.7%, $p<0.05$, respectively), suggesting that these factors augment the number of synaptic vesicles-accumulating sites along the axon shaft. These sites with synaptic vesicles clusters represent potential spots for the formation of a functional and mature presynaptic terminal provided that one presynaptic differentiation event had already took place. Moreover, FGF22 also increased total synapsin puncta area per axon length (figure 8C) (123.27%, $p<0.05$), suggesting that FGF22 enlarges the area occupied by synapsin-positive puncta, or, in other words, increases the size of synaptic vesicles clusters. Conversely, BNDF does not increase synapsin puncta area per axon length (figure 8C) as statistical analysis using unpaired t-test showed no significant differences between control and BNDF-stimulated condition for total synapsin puncta area/μm.

In line with what has been reported, our results indicate that FGF22 and BNDF, when globally applied, induce presynaptogenesis in rat embryonic hippocampal neurons, with a specific role in the organization, distribution and clustering of synaptic vesicles in presynaptic terminals. The effect of FGF22 seems to be more robust than BNDF effect (considering that it alters two properties of SV clusters, number and area).

A very recent paper from Umemori and colleagues strongly supports and validates the results obtained and discussed for FGF22. Using hippocampal cultures of FGF22-knockout mice, authors observed that lack of FGF22 is characterized by a decrease in number and size of vesicular glutamate transporter 1 (VGLUT1)-positive puncta (Terauchi et al., 2010). This phenotype was reversed if FGF22 was subsequently added to cultures. Concluding, FGF22 induces differentiation of excitatory pre-synaptic terminals in CA3 pyramidal neurons (Terauchi et al., 2010). These results are in total agreement with our data (figure 8). However, FGF22 involvement in presynaptogenesis

was analyzed in different developmental stages. The authors, claiming that synaptogenesis peak is at P14, decided to use post-natal mice at P14. In this work, we used embryonic mice (E17-18), because dependence on axonal protein synthesis decreases with maturation of the neuronal network. It is known that mature presynaptic terminals show decreased sensitivity to protein synthesis inhibitors (Sebeo et al., 2009). Despite these differences, FGF22 induce similar responses, suggesting that hippocampal neurons conserve the ability to respond equally to extracellular FGF22 from embryonic state until, at least, 14 days after birth. Considering this, we can predict that *in vivo* FGF22 effect on presynaptic differentiation in CNS might occur before and/or after birth. In cerebellum (Umemori et al., 2004) and hippocampus (Terauchi et al., 2010) FGF22 and FGFR2 are highly expressed at P8, but decrease at P23 to barely detectable levels (Umemori et al., 2004). So, FGF22 presynaptogenic effect must be relevant only in the first weeks after birth. On the other hand, in mice neuromuscular junction FGF22 mRNA and FGFR2 levels are relatively high at E18 and decrease dramatically with birth (Fox et al., 2007). Summarizing, expression of FGF22 and its receptor seems to be transient and to correspond temporally to synaptogenesis period. Efforts should be made to analyze the expression levels of FGF22 and FGFR2 in hippocampus and cerebellum at embryonic stages.

To sum up, we were successful in establishing an *in vitro* system of presynaptogenesis induction by FGF22 in embryonic hippocampal neurons. Our data is validated by other studies, thus increasing its credibility and reliability.

Interestingly, the results obtained for BDNF are in total agreement with the working model proposed by Reichardt and colleagues. According to this model, activation of TrkB receptors localized on the presynaptic membrane culminates in disruption of the signals that localize SVs to the presynaptic compartment (Bamji et al.,

2006). As a result, synaptic vesicles splitting and dispersion will contribute to the formation of new synaptic vesicles-accumulating sites (Bamji et al., 2006). Summarizing, BNDF was proposed to enhance presynaptic differentiation by inducing formation of new synaptic zones from pre-existing and stable SV clusters. This effect of BNDF would lead to an increase in the number of sites with SV clusters, analogous to the observed increase in synapsin puncta number (figure 8B). However, these sites would be smaller than the initial ones, if new synaptic sites are created by splitting of pre-existing ones, the area occupied by synaptic vesicles in the axon would not vary, thus explaining the maintenance of total synapsin puncta area (figure 8C).

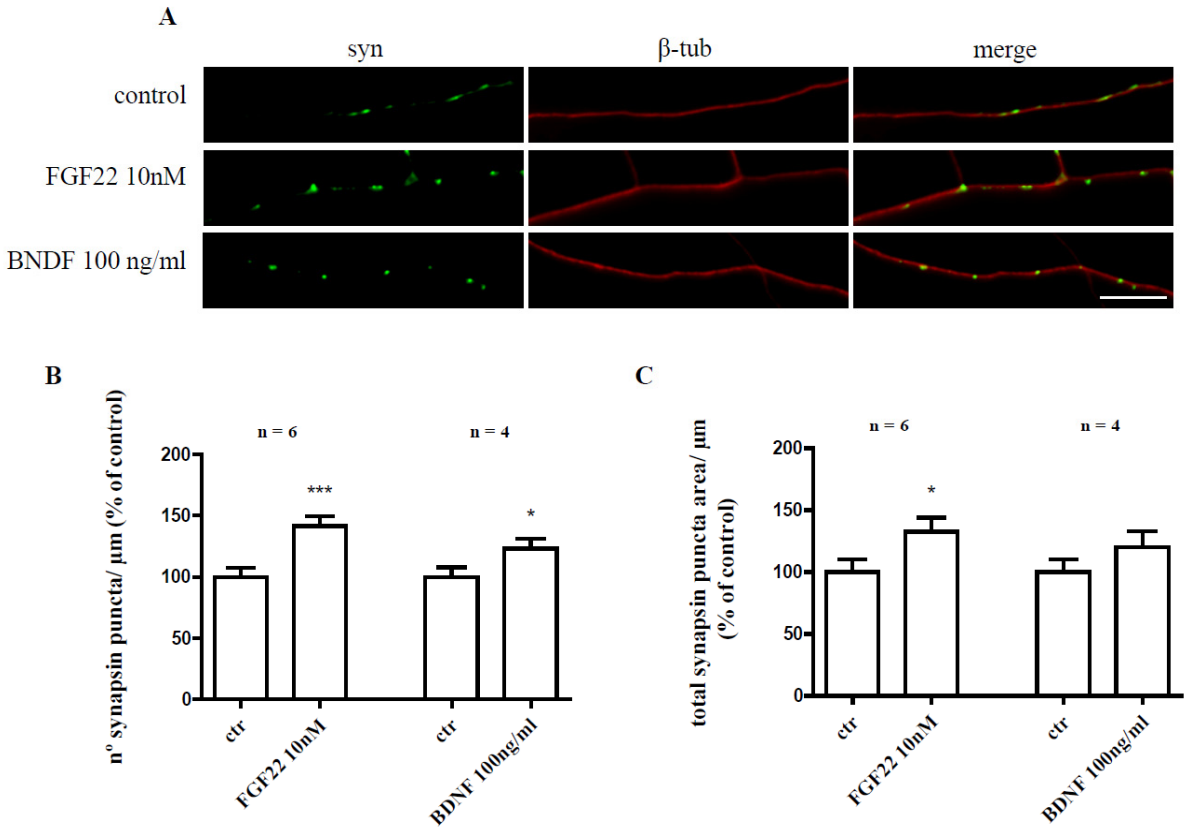


Figure 8 – Presynaptic organizing activity of FGF22 and BNDF. (A) Primary cultures of rat embryonic hippocampal neurons were stimulated at DIV6 for 14 h at 37°C in conditioned medium with either vehicle, FGF22 or

BNDF. Immunostaining for synapsin and β-tubulin was performed and images were taken using a Zeiss Axiovert 200 fluorescent microscope. Representative images of axons segments revealing a clear difference in synapsin clustering between analyzed conditions. Results show that global application of recombinant human FGF22 (10nM) or recombinant human BNDF (100 ng/ml) to hippocampal cultures induces a change in synapsin clustering/distribution pattern. The scale bar is 10 μm. (B, C) The number of synapsin puncta/ axon length and the total synapsin puncta area/ axon length were measured with Image J 1.42 software in randomly selected axons (25-30 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of 120-160 axons of at least four independent experiments (* represents $p<0.05$ by unpaired t-test and *** represents $p<0.001$ by unpaired t-test, when compared with control).

3.2. FGF22-induced presynaptic differentiation and axonal translation

3.2.1. Microfluidic devices for neuron cell culture

In the previous section, we observed that global application of FGF22 induces an increase in both number and total area of synapsin puncta per axon length. These results led us to conclude that FGF22 has a presynaptogenic effect when globally applied to cultured hippocampal neurons. Our next goal is to uncover the effect of FGF22 when it is locally applied, in other words, when only axons are exposed to the growth factor. In order to fulfill the need to only expose axons to different stimuli, we made use of microfluidic chambers, small multicompartment devices capable of creating physical and fluidical independent compartments.

Microfluidics can be defined as the study of flows (liquids or gases) that are circulating in tiny artificial microsystems (Tabeling, 2005). This area deals with the behavior, precise control and manipulation of small amounts of fluid (10^{-9} to 10^{-8} litres) that are constrained to sub-millimeter channels and valves (Tabeling, 2005). The accurate control of fluids dynamics and behavior allows microfluidic systems to monitor and regulate microenvironments, thereby allowing users to know exactly the content of individual compartments. In recent years, fluid management in microsystems has been very useful for cell biologists and neuroscientists, and several devices have been designed for *in vitro* cell cultures and biological studies.

The application of microfluidics in neuroscience is recent; nevertheless, microsystems have been employed in studies of: axon injury and regeneration (Taylor et al., 2005), localization and identification of mRNAs in axons (Taylor et al., 2005), synapse-to-nucleus signaling (Taylor et al., 2010), intracellular pH regulation in neuronal soma and neurites (Vitzhum et al., 2010), and axonal navigation and network formation (Millet et al., 2010). On the other hand, manipulation of this technology contributed to the appearance of microdevices specifically designed to visualize and manipulate synapses (Taylor et al., 2010), to orient growth of neuron processes through substrate patterning (Taylor et al., 2003), and to integrate networks of neurons with multielectrode arrays in an attempt to create neuron-based functional biosensors (Morin et al., 2005). Concluding, microfluidics is giving great contributions to neuroscience. In this work, we make use of these microfluidic devices to study presynaptic differentiation and axonal protein synthesis.

In the next sub-sections, I will describe succinctly the type of microfluidic chambers used, highlighting the special features that render them the ideal tool to address our goal.

3.2.1.1. Growth of embryonic rat hippocampal neurons in microfluidic chambers

In this work, we determined the effect of FGF22 on the presynaptic differentiation of rat embryonic hippocampal neurons (see 3.1.3). Since the next proposed aim is to look into the presynaptogenic effect of FGF22 in pure axonal populations, it was imperative to optimize long-term primary cultures of rat embryo hippocampal neurons in microfluidic devices. Up to now, few studies reported successful culturing of CNS neurons in microfluidic devices. Despite the difficulties, investigators were able to culture PNS and CNS neurons, such as chick dorsal root ganglion (DRG) (Park et al., 2009) and rat cortical neurons (Taylor et al., 2003, 2005; Vitzhum et al., 2010; Rhee et al., 2006; Park et al., 2009), respectively. While this study was being conducted, two independent studies also managed to culture viable primary hippocampal neurons in microfluidic chambers (Taylor et al., 2010; Millet et al., 2010).

Each microfluidic device is composed of a molded PDMS piece (figure 9A), bearing desired surface embossed designs, placed against a properly coated-glass coverslip where neurons adhere. Poly-dimethylsiloxane (PDMS) has been shown to be the most suitable material for the fabrication of microsystems in which to grow cells. PDMS's suitability for the job can be explained by a huge list of advantages: optical transparency, high permeability to oxygen and carbon dioxide, low cost and easy fabrication using standard soft-lithography (simple protocols described in Rhee et al., 2004 and Park et al., 2009), thermal stability[5], physiological and chemical inertness, and the most important biocompatibility (Wang et al., 2008 and Whitesides, 2006).

[5] Thermal stability is defined as the resistance to permanent change in properties caused by heat.

The microfluidic chambers used in this work have two compartments connected by arrays of microgrooves that physically confine cell bodies in the somal compartment, while allowing extending axons to penetrate into the opposite parallel division (figure 9B). Each compartment, measuring 1.5 mm wide, 7 mm long and 100 μm height, has two reservoirs at both edges to store culture medium. A permanent flow of medium between reservoirs should be maintained so that medium in the interior of the tiny compartments are continuously renewed, thus facilitating nutrient and gas exchange. A minimum volume of cell suspension is loaded into the upper reservoir of the somal side (orange) and the established flow drags cells to the interior of the compartment, where cell bodies settle, adhere and grow. Randomly growing neurites are able to pass through the microgrooves and reach the axonal compartment (blue side) (figure 9C). Microgrooves, which measure 450 μm long, 10 μm wide and 3 μm height, are extremely narrow tunnels filled with culture medium that bridge both compartments (figure 9C).

In order to observe a primary culture of rat embryonic hippocampal neurons inside microfluidic devices, cultures at DIV8 were immunostained for TUJ1, MAP2 and DNA and contiguous images were taken to a random area of the microfluidic device using a Zeiss LSM 510 Meta confocal microscope. A single image was obtained by assembling individual images using the LSM 510 software. The picture presented (figure 9E) depicts an example of hippocampal neurons grown on microfluidic chambers. Here, we observe the somal side (on the left), where cell bodies were plated, communicating through several axon-conducting channels (on the middle) with the axonal side (on the right). Axons are able to enter the microgrooves and cross them completely until they reach the opposing compartment, the axonal side.

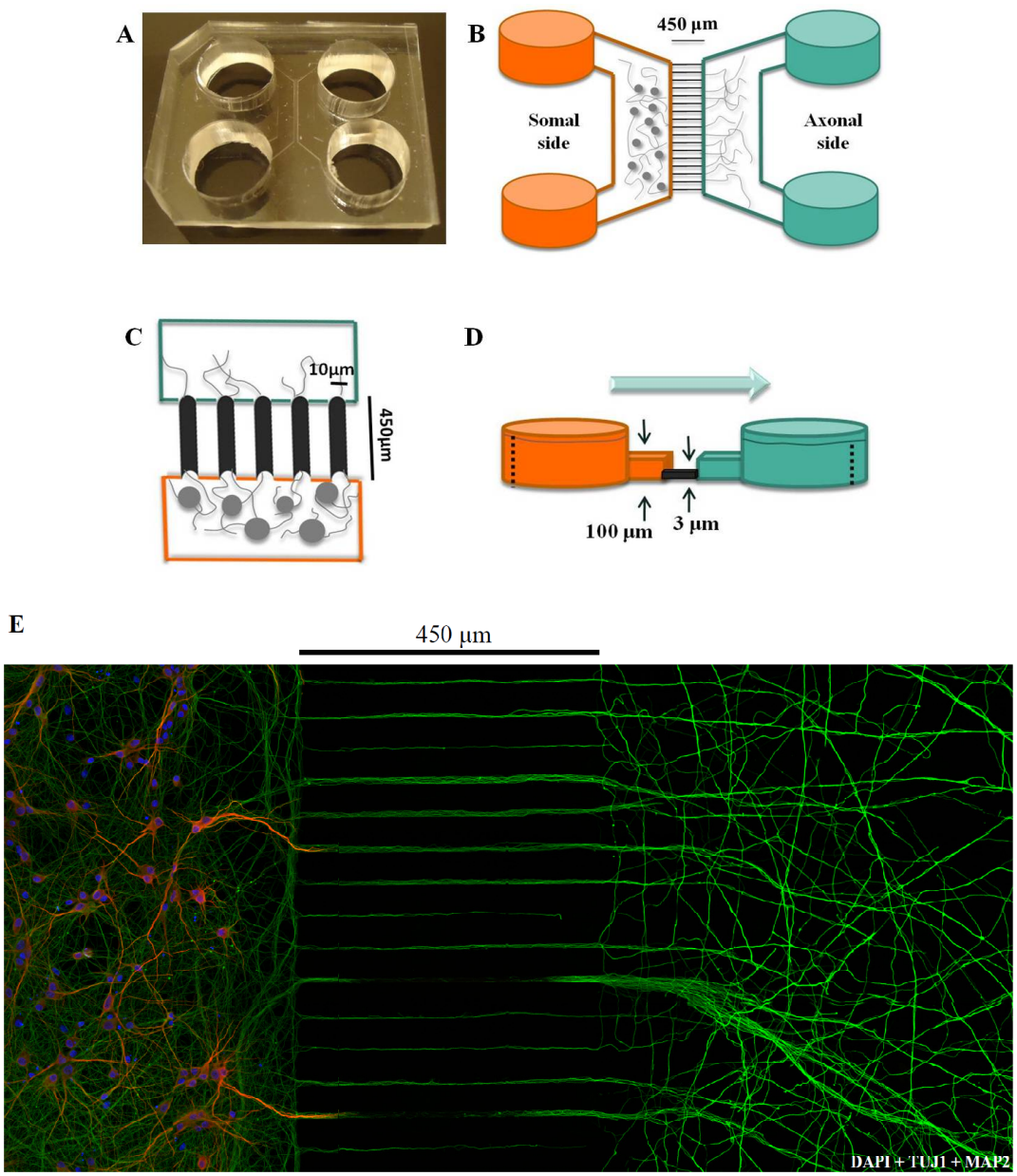


Figure 9 – Culture of hippocampal neurons in microfluidic devices. (A) These small systems (20 mm × 25 mm) consist of a molded PDMS chamber placed against a glass coverslip. (B, C) Front views of chamber. The chamber has two sides: the somal side (orange) and the axonal side (blue), each bearing a compartment (1.5 mm wide, 7 mm long) separated by a set of microgrooves (black) (450 µm long, 10 µm wide). Each compartment is connected by two reservoirs. Neurons are plated in the somal compartment, and randomly growing axons are guided to the axonal side through the

microgrooves. (D) Chamber side view: microgrooves' height (3 μm) is extremely small comparing to compartments' height (100 μm). If a volume difference is maintained (dotted lines), the high fluidic resistance of the microgrooves guarantees fluidic isolation for 20 h between microenvironments (Taylor et al., 2005). (E) Primary cultures of hippocampal neurons at DIV8 were immunostained for TUJ1, MAP2 and DNA. Contiguous images were taken from a random area of the microfluidic chamber using a Zeiss LSM 510 confocal microscope with an EC-PlanNeofluar 40× objective and assembled into a single image using the LSM 510 software. In this work, compartmentalization and isolation of axons was achieved by the use of microfluidic chambers.

It is important to emphasize the fact that conditions inside the chambers are not as appropriate to cell culture as conditions in multi-wells. Firstly, efficient gas exchange is compromised due to reduced contact between medium and surroundings. Secondly, the reduced size of the compartments (especially height – 100 μm) partially restricts fluidity, and so, accumulation of metabolism-derived toxic compounds in the interior of the compartment, as well as, dead cells, is likely to occur. Considering this, neurons confined to microfluidic chambers grow in a less pleasant environment, and so they are more sensitive to external stresses, such as: medium evaporation in the reservoirs, ineffective coating of the glass slides, temperature variations, substrate roughness, among others. Therefore, this extremely sensitive type of culture must be highly controlled and monitored.

Here, we report the well-succeeded growth of hippocampal neurons in microfluidic chambers. Our results show that neurons grow, develop and establish a complex and normal network of neurites when culture on microfluidic chambers (figure

10A). Moreover, cell viability inside the chambers, assessed by analysis of nuclear morphology, was shown unaffected when compared to control cultures (figure 10B and C). Hippocampal neurons were plated in microfluidic devices (40.000 cells in the somal compartment) and in coverslip-containing multi-wells (10.000 cells inside cloning cylinders). At DIV8 and DIV7, respectively, cultures were fixed and mounted in prolong mounting media with DAPI, and the number of apoptotic nuclei was counted. Our data shows that cells in the devices are morphologically equivalent to cells in the control culture (figure 10B) with only a slight non-statistically significant decrease in the percentage of viable cells cultured in the microfluidic chambers (figure 10C). The adverse conditions in microfluidic chambers do not affect neurons' viability and welfare. Moreover, our results show higher levels of cell viability than the pioneer studies performed with cortical neurons, where the authors only obtained survival rates around 70% (Taylor et al., 2003).

On the other hand, as it is shown in figure 9E, after the long crossing confined to the microgrooves' limits, when axons reach the axonal side they spread in every direction and cover the whole surface of the axonal compartment. We observed no axon bundles or bead-bearing axons in the axonal side which reveals that axons are healthy, stable and in good conditions.

3.2.1.2. Microfluidic chambers: fluidic isolation of pure axonal populations

Microfluidic chambers usefulness to the field of neuroscience is due to two distinct features: axonal isolation and fluidic isolation. In this section we will explain succinctly these properties and their relevance to the proposed work.

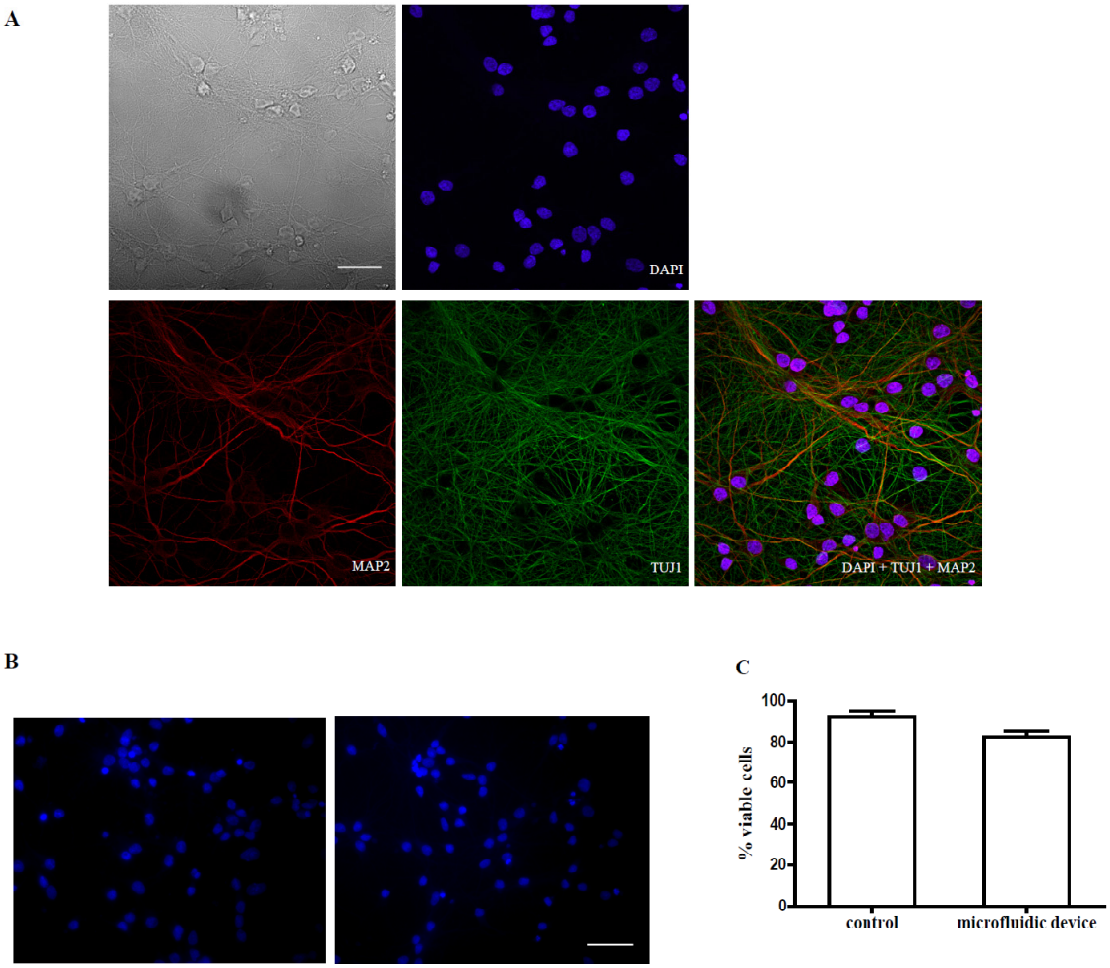


Figure 10 – The microfluidic environment does not alter cell viability. Primary cultures of rat hippocampal neurons at DIV8 were immunostained for TUJ1 and MAP2, and then fixed and mounted with DAPI. (A) Images were taken from the somal compartment using a Zeiss LSM 510 Meta confocal microscope (EC-PlanNeofluar 40× objective). No nuclear condensation or neurite fragmentation was observed. The scale bar is 25 µm. (B) To examine viability of hippocampal neurons, primary cultures of rat embryonic hippocampal neurons grown in multi-wells (DIV7) (control) and in microfluidic devices (DIV8) were fixed and stained as described above. Preparations were examined under a Zeiss Axiovert 200 fluorescence microscope and images were acquired with a Plan-Neofluar 40× objective. The scale bar is 50 µm. (C) Quantification of results shown in B. 400-500

cells were counted per experiment and the percentage of viable cells determined. Bars represent the mean ± SEM of three independent experiments. Statistical analysis was preformed with unpaired t-test.

As discussed in the previous section (3.2.1.1), dissociated neurons are plated in the somal compartment and randomly growing axons arise in the axonal compartment by traversing narrow microchannels. Neuron cell bodies are known to vary from 4 to 100 μm in diameter and microgrooves height is 3 μm, thus preventing passage of cell bodies through these microtunnels. As a result, cell bodies get trapped on the somal side (figure 11A). Importantly, when neurons are plated, axonal reservoirs are totally filled with medium, in contrast to somal reservoirs, inducing the formation of an unidirectional flow (from axonal to somal side). This flow counteracts completely diffusion of suspended components from the somal to the axonal side.

Oppositely, dendrites are as narrower as axons, and they can easily enter the microgrooves. However, dendrites length is much smaller. Garruto and colleagues reported that the mean length of longest radiolabeled dendrite at DIV12 of hippocampal neurons was 168.3 μm (Wakayama et al., 1997). According to this, the presence of dendrites in a 450μm-distant axonal compartment is surely impossible. Indeed, we can detect dendrites inside the microgrooves, but they were never detected beyond the middle of the microgrooves, or more importantly, in the axonal compartment (figure 11B). These results are consistent with observations reported by Jeon and colleagues for cultures of embryonic rat cortical neurons in microfluidic devices with 450 μm micropores (Taylor et al., 2005).

On the other hand, fluidic isolation of the axonal microenvironment can be achieved if a minute volume difference between the two compartments is established (Taylor et al., 2003 and 2005). The reduced height of the microgrooves (3 μm) creates a

high fluidic resistance between compartments (whose height is 100 μm), thus reducing fluidic flow to minimal levels between microenvironments. Theoretically (see figure 9D), if a compartment has higher volume (dotted line, orange side) than the other (dotted line, blue side), a small but sustained unidirectional flow of liquids arises, thus ensuring retention of soluble components that might be present only in the blue side. Aiming at testing this hypothesis, Texas Red and [^{35}S] methionine were added to the axonal compartment of a microfluidic device under a volume difference of 25 μl between compartments. After 20 h there was no trace of these components in the somal side (Taylor et al., 2005). Considering these experiments, we can stimulate or apply a particular drug only to the axonal side, thus producing a localized effect.

Concluding, a pure population of functional and living axons without contamination of somata or dendrites can be obtained inside the axonal compartment of a microfluidic device. Furthermore, microfluidic chambers allow us to fluidically isolate axons for up to 20 h, thus enabling localized exposure to insults. In the next sections, we make great use of this amazing property to induce local stimulation with FGF22 and local inhibition of protein synthesis.

3.2.2. Local FGF22-presynaptogenic effect is specific

Before, we have shown that FGF22 and BNDF, when globally applied, induce presynaptic differentiation in hippocampal neurons. Both factors were capable of inducing changes in synaptic vesicles clustering pattern (figure 8). These results lead us to ask if FGF22 and BNDF presynaptogenic effect occurs locally. So, are these soluble molecules able to induce presynaptic differentiation when applied only to axons? In fact, FGF22 was proven to induce presynaptic differentiation in fluidically isolated

axons of chick ciliary ganglia (unpublished data, Almeida RD et al.). Conversely, to our knowledge, no study addressed the role of local BNDF stimulation in presynaptogenesis.

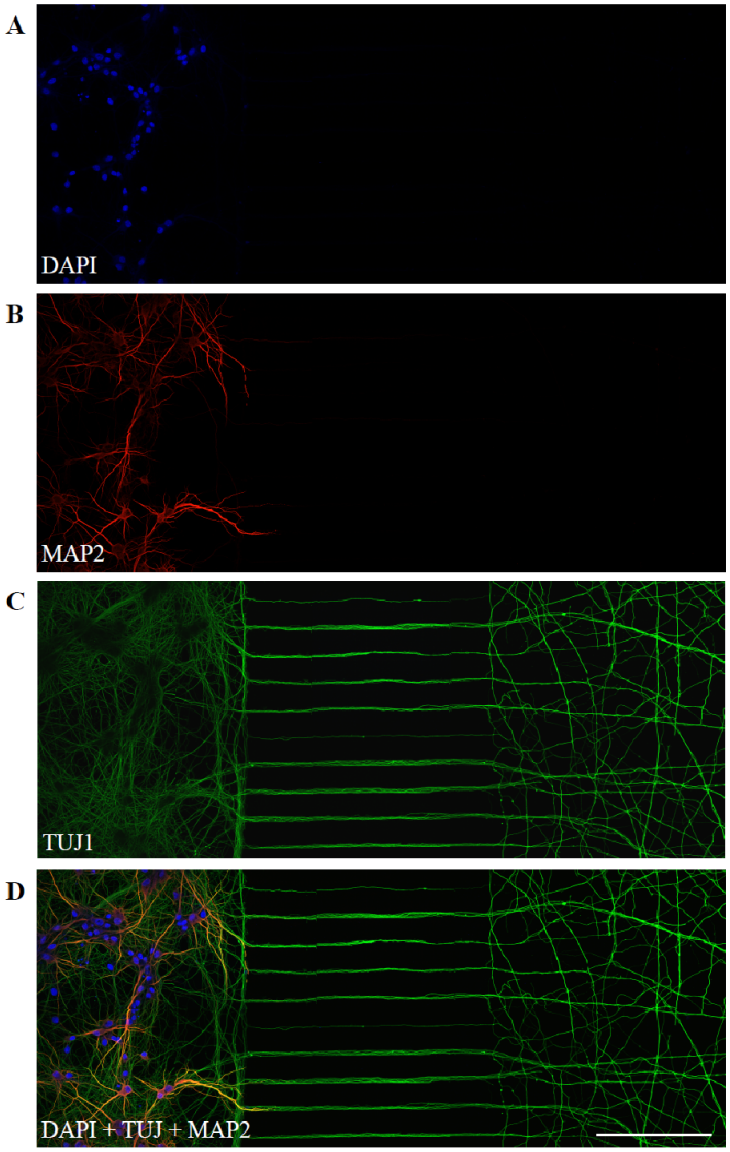


Figure 11 – Isolation of pure axonal populations without contamination of cell bodies and dendrites. Primary rat hippocampal neurons were plated in the somal compartment of microfluidic chambers and allowed to develop for 8 days and then cells were immunostained for TUJ1, MAP2 and DNA. Contiguous images were taken from a random area of the microfluidic chamber using a Zeiss LSM 510 Meta confocal microscope with an EC-PlanNeofluar 40× objective, and assembled into a single image using the

LSM 510 software. Cell bodies are unable to pass through the narrow microgrooves, and become trapped on the somal side. Dendrites are able to enter the microgrooves, but they are not long enough to reach the axonal side. As a result, only axons are able to cross to the opposite compartment (the axonal compartment) making these microfluidic devices an essential tool to compartmentalize and isolate axons *in vitro*. The scale bar is 200 μm.

In order to clarify this question, we prepared primary cultures of rat embryonic hippocampal neurons in microfluidic chambers. At DIV7 neurons were stimulated with either FGF22 or BNDF in conditioned medium. A volume difference of 25 μl was kept between compartments to guarantee total fluidic isolation. Cultures were then fixed and immunocytochemistry was performed.

The results obtained clearly show an increase in number (288%, $p<0,001$) (figure 12B) and total area of synapsin puncta (328%, $p<0,001$) (figure 12C) per axon length when 100 nM of FGF22 is applied to the axonal compartment of microfluidic devices. However, stimulation of hippocampal neuron axons with BNDF 100 ng/ml or FGF22 20 nM doesn't alter synapsin clustering, neither number of synpasin puncta/μm or total puncta area/μm showed significant differences when compared to control conditions, indicating that FGF22, but not BNDF, is able to induce presyaptogenesis when locally applied.

It should be noted that FGF22-induced presynaptic differentiation was obtained at a concentration of 100 nM, higher than what we previously reported to induce presynaptic differentiation when globally applied (figure 8).

An adequate explanation for this discrepancy lies on PDMS properties. Succinctly, PDMS, which is the main component of microfluidic chambers, is a polymer consisting of repeating -OSi(CH3)2- units. The huge number of CH_3 groups

makes solid PDMS samples surface extremely hydrophobic (Sia and Whitesides, 2003; McDonald et al., 2000). This hydrophobicity repels polar solvents and makes the surface prone to nonspecific adsorption[6] of hydrophobic contaminants (Sia and Whitesides, 2003). FGF22 added to the medium might be adsorbed at high levels by the chamber, thus reducing drastically FGF22 free concentration in contact with axons. Further experiments would be useful to test this idea, such as, determination, by ELISA or Western Blot, of the relative quantities of FGF22 present in the medium along 14 h in microfluidic devices.

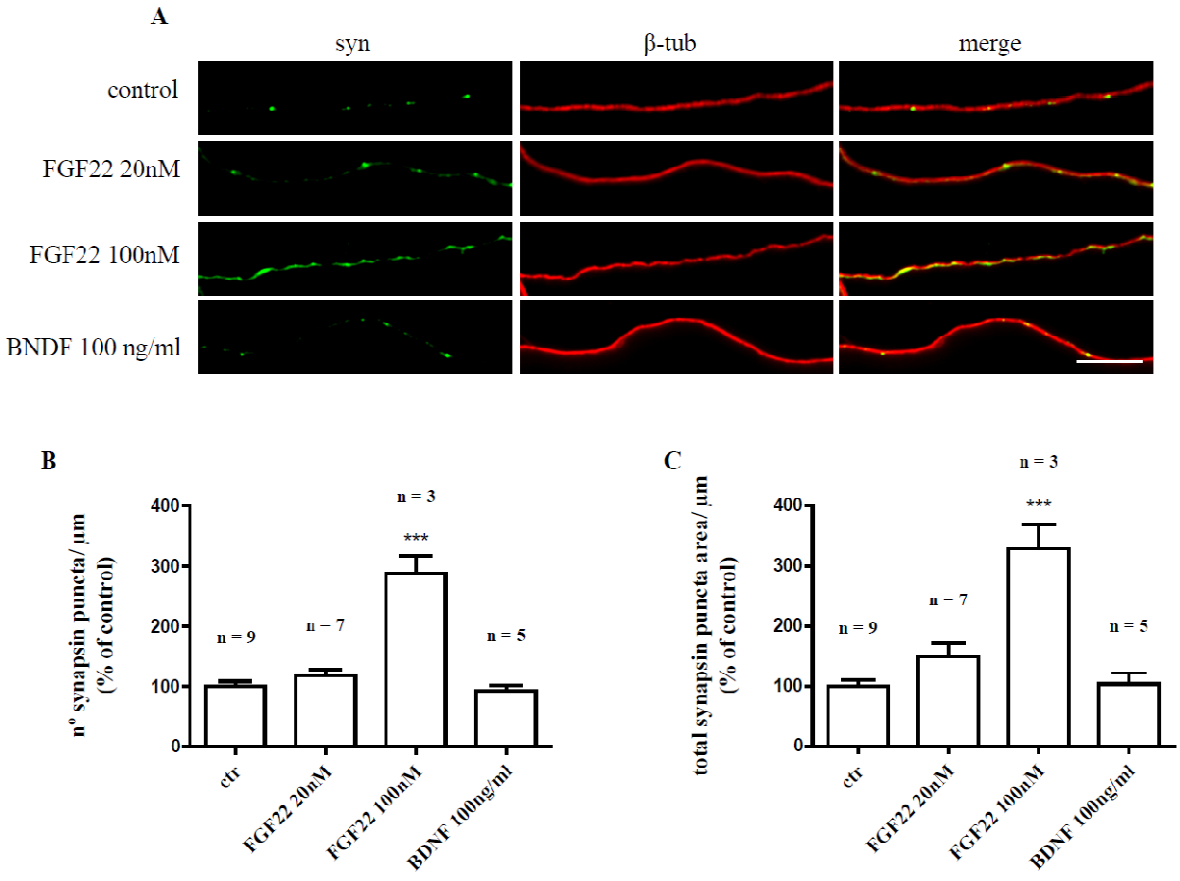


Figure 12 – Local application of FGF22 induces presynaptic assembly.

Primary cultures of rat embryonic hippocampal neurons in microfluidic

[6] Adsorption is the process of attraction of molecules present in an adjacent gas or liquid to an exposed solid surface, thus diminishing the amount of the adsorbed molecule available on the gas or liquid.

devices were stimulated at DIV7 for 14 h at 37^{o}C in conditioned medium with either vehicle, FGF22 (20 nM and 100 nM) or BNDF (100 ng/ml). Stimuli were added to the axonal compartment only. Immunostaining for synapsin and β-tubulin was performed and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (A) Representative images of axons segments revealing a clear difference in synapsin clustering when conditioned medium of the axonal compartment is supplemented with 100 nM of FGF22 for 14 h. Results show that local application of recombinant human FGF22 to hippocampal cultures, as opposed to local application of recombinant human BNDF, induces a pronounced increase in synapsin clustering. The scale bar is 10 μm. (B,C) The number of synapsin puncta/axon length and total synpasin puncta area/axon length were measured with Image J 1.42 software in randomly selected axons (30 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of 90-200 axons of at least three independent experiments (*** represents $p<0.001$ by ANOVA using Bonferroni's post test when compared to control).

On the other hand, based on the working model proposed by Umemori and colleagues, FGF22 is a post-synaptic derived soluble molecule and so secreted locally upon synaptic contact (Terauchi et al., 2010). As a result of this local secretion, concentration of FGF22 must be elevated on the areas surrounding the presynaptic terminal and gradually smaller in remote places. According to this, local stimulation might require extremely high concentrations of FGF22, thus partially justifying the need of 100 nM of FGF22 to induce presynaptogenesis in fluidically isolated axons. It would be interesting to look into FGFR2b activation by FGF22 in pure axonal populations and determine the concentration of FGF22 that would lead to optimal activation of FGFR2b,

although this would be a very challenging experiment due to the small amounts of protein that can be obtained from the axonal compartment.

Unlike FGF22 our results show that local stimulation with BNDF does not induce presynaptic differentiation, enabling us to reason that BNDF contact with axons is not sufficient to trigger remodeling of synaptic vesicles clusters (figure 12).

To sum up, when applied specifically to axons FGF22 induces differentiation of the presynaptic terminal as determined by clustering of synaptic vesicles, a hallmark of presynaptogenesis. Moreover, this localized presynaptogenic effect of FGF22 is specific because when axons are stimulated with BNDF no increase in synapsin puncta number is observed. We predict that this different local responsiveness to FGF22 and BNDF results from differential localization and distribution of the corresponding receptor. To date, no efforts have been made to determine the localization of FGFR2b receptors in neurons, a question that could be addressed by ultrastructural studies of the synapse.

3.2.3. FGF22-induced presynaptic differentiation is dependent on axonal protein synthesis

We next decided to study the role of intra axonal translation in the formation of synapses, particularly the differentiation of the presynaptic terminal. Preliminary evidences suggest that local translation in axons might be a fundamental mechanism in synaptogenesis (Schacher and Wu, 2002; Zhang and Poo, 2002; Lyles et al., 2006; Sebeo et al., 2009). So, considering that FGF22 is capable of inducing presynaptic assembly when locally applied to axons of hippocampal neurons (figure 12), we next asked if this local FGF22-induced presynaptic differentiation depends on axonal protein

synthesis. In fact, local protein synthesis was shown to be required for FGF22-induced presynaptic assembly in chick cilliary ganglia (unpublished data, Almeida RD et al.).

To this purpose, we prepared primary cultures of rat embryonic hippocampal neurons in microfluidic devices. The axonal compartment of cultures at DIV7 was subjected to a 14 h stimulation with 100 nM of FGF22 in the absence or presence of protein synthesis inhibitors (emetine or anisomycin, 10 μM). A pre-incubation of 15 min with the inhibitors was performed, so that the drug could enter the cells prior to FGF22 sitmulation. Total fluidic isolation was guaranteed by a volume difference of 25 μl between compartments. Cultures were then fixed and immunocytochemistry for synapsin and β-tubulin was performed. Images were taken from the axonal compartment and quantification of number and area of synapsin puncta per axon length was carried out in Image J software.

The results obtained show that when protein synthesis inhibitors are present in the axonal compartment at the time of FGF22 stimulation, presynaptic differentiation is abolished (figure 13). Emetine decreased synapsin puncta number and area to 84.95% ($p<0.001$) and 73.92% ($p<0.001$) respectively, and anisomycin decreased synapsin puncta number and area to 107% ($0<0.01$) and 89.98% ($p<0.001$) respectively. These inhibitors exert their action through different mechanisms, while emetine blocks protein synthesis by binding to the 40S ribosomal subunit, anisomycin binds to the 60S subunit, indicating that the loss FGF22-induced puncta is protein synthesis-dependent and not due to a non-specific effect of the pharmacological inhibitor. It is also important to notice that 14h-incubation with the inhibitors does not alter presynaptic assembly pattern (neither number nor area of synapsin puncta). Thus, guaranteeing that the observed effect is entirely due to abrogation of FGF22-induced synapsin clustering. Therefore, we can conclude that FGF22-induced presynaptic assembly requires axonal

translation, indicating that local protein translation can regulate the formation of new synapses.

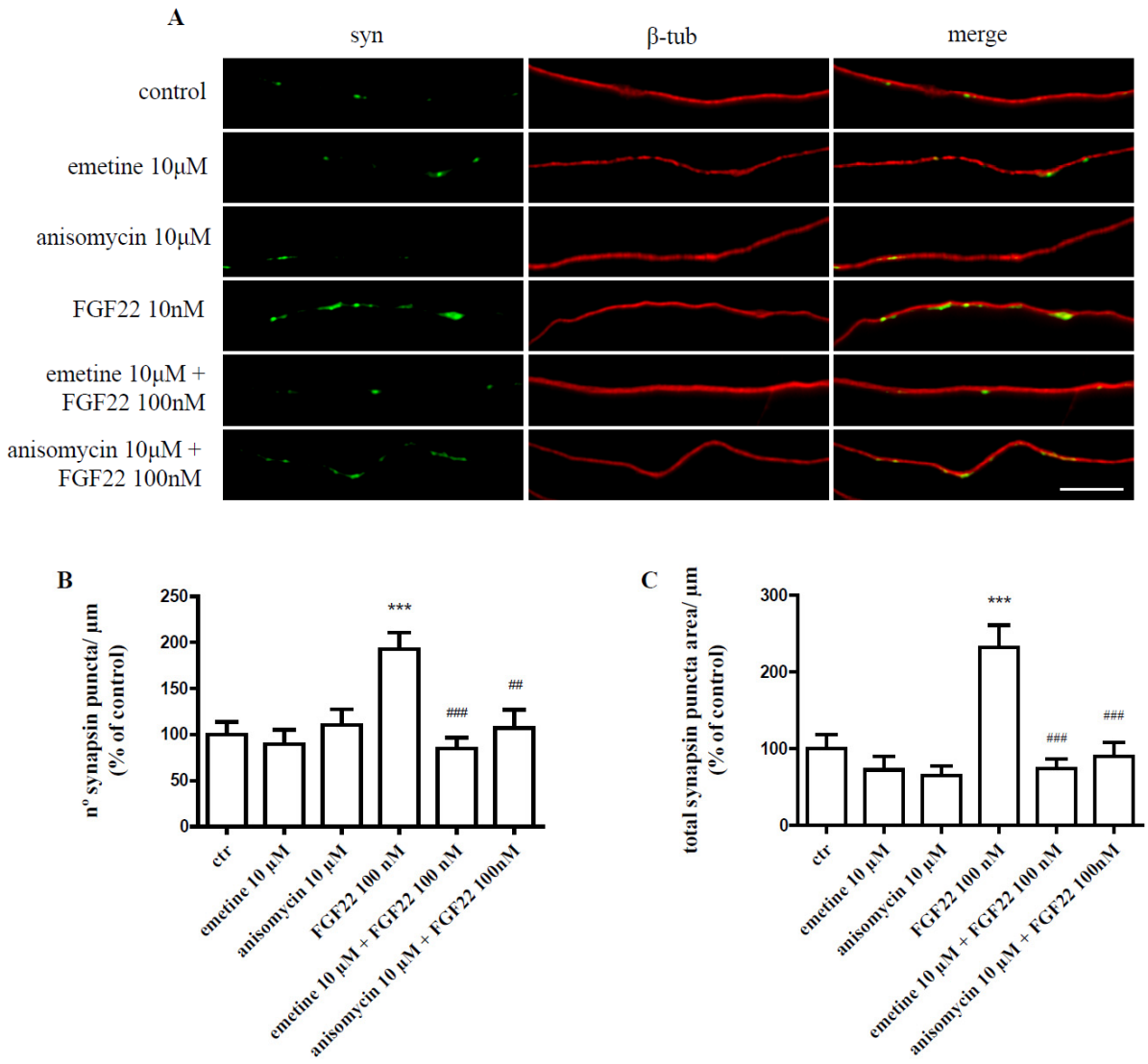


Figure 13 – Intra-axonal protein synthesis is required for FGF22-induced presynaptic differentiation. (A) Primary cultures of rat embryonic hippocampal neurons in microfluidic devices were allowed to develop for 7 days, then emetine or anisomycin were added to the axonal compartment for 15 min. Axons located in the axonal compartment were then stimulated with 100 nM of FGF22 in protein synthesis inhibitor-containing medium for 14 h at 37ºC. Immunostaining for synapsin and β-tubulin was performed and images were taken to the axonal compartment using a Zeiss Axiovert 200

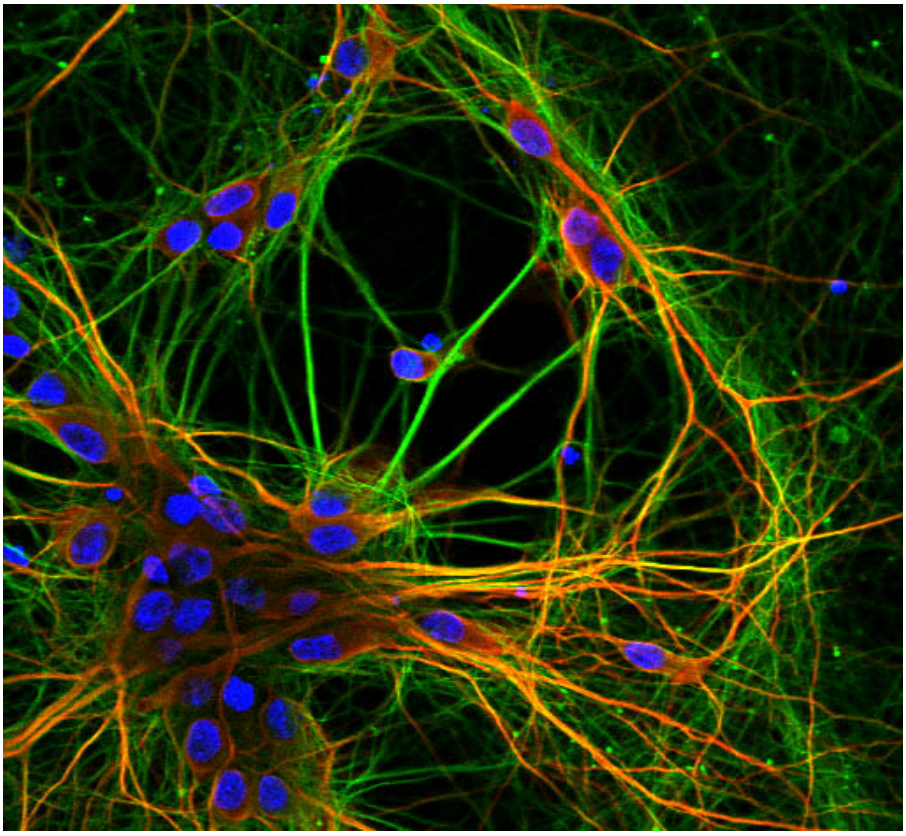
fluorescent microscope. Representative images of axons segments revealing a clear reduction in synapsin clustering when protein synthesis inhibitors are added. Results show that local inhibition of protein synthesis with emetine or anisomycin abolishes FGF22-induced synapsin clustering. The scale bar is 10 μm. (B, C) The number of synapsin puncta/axon length and total synpasin puncta area/axon length were measured with Image J 1.42 software in randomly selected axons (30 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of 120 axons of four independent experiments (*** represents $p<0.001$ by ANOVA using Bonferroni´s post test when compared to control; ## represents $p<0.01$ and ### represents $p<0.001$ by ANOVA using Bonferroni´s post test when compared to FGF22 100nM).

If FGF22 induces synapsin clustering in a local protein synthesis dependent manner, then it depends on the presence of local mRNAs to fulfill its presynaptogenic effect. Thus, we may wonder if FGF22 is capable of redistributing mRNAs, promoting their recruitment to synaptic sites, and their translation in a spatial and temporal regulated manner. Indeed, using *Aplysia* sensory neurons it was reported that a specific mRNA (sensorin) immediately localizes to sites of contact between pre- and postsynaptic neurons, in other words, to sites of newly forming synapses (Lyles et al., 2006). Moreover, knockdown of sensorin mRNA abolishes synapse formation (Lyles et al., 2006). According to this study and the results shown here, it is reasonable to assume that, upon synaptic contact, specific targeting of mRNAs to presynaptic sites could be promoted by target derived presynaptogenic molecules (such as FGF22, WNT-7a, neuroligin and SynCAM).

Interestingly, Benson and colleagues showed that rat hippocampal neurons require local protein synthesis to maintain the pool of synaptic vesicles available for fusion, thus regulating presynaptic function (Sebeo et al., 2009). However, it is not clear if the protocol used by the authors to block axonal protein synthesis is only specific to axons and does not inhibit somal protein synthesis, compromising the entire study. To our knowledge, this study is the first work to report an indispensable role for intra-axonal translation in the differentiation of the presynaptic terminal in a mammalian system. Furthermore, this study represents the first attempt to establish a link between the activity of presynaptogenic molecules and protein synthesis occurring at the axoplasm.

In order to further extend our findings it would be interesting to show, using a puromycin derivative assay, that FGF22 induces local protein synthesis in live neurons. Puromycin, a common translation reporter, is covalently bound to a fluorescence group that shows no signal in this form. When protein translation is initiated, puromycin inserts itself into the nascent peptide and releases its fluorescence group. Consequently, an increase in fluorescence intensity correlates to an increase in protein translation. We would expect to observe an increase in fluorescence intensity when FGF22 is added to fluidically isolated axons.

Chapter 4 Closing Remarks



4.1. Conclusion

In this study, using fluidically isolated axons, we have examined the requirement of axonal protein synthesis in FGF22-induced presynaptic differentiation of hippocampal neurons in culture. We found that (1) FGF22 induces activation of FGFR2b signaling cascade in embryonic rat hippocampal neurons and, consequently, phosphorylation of ERK1/2 (figure 1); (2) FGF22 and BNDF induce differentiation of the presynaptic terminal (figure 3); (3) FGF22 has a localized presynaptogenic effect (figure 7); and (4) FGF22-induced presynaptic assembly is dependent on axonal protein synthesis (figure 8).

Taken together, our data suggest that the spatially directed action of FGF22 on presynaptic assembly is dependent on axonal translation. This dependence was also previously observed in chick ciliary ganglia (unpublished data, Almeida RD et al.) thus revealing a conserved mechanism of action of FGF22 between different species (rat and chicken) and between different types of neurons (hippocampal neurons (CNS) and ciliary ganglia (PNS)).

Firstly, this work aimed at characterizing FGF22 effect on the differentiation of presynaptic terminals in cultured hippocampal neurons. For some time, there is evidence that FGFs play a role in the formation of the presynaptic terminal, particularly in synaptic vesicles clustering (Dai and Peng, 1995). Application of exogenous FGFs, FGF7, FGF10 and FGF22 promote vesicle clustering and neurite branching (Umemori et al., 2004). Moreover, FGF22-knockout mice have impaired differentiation of excitatory nerve terminals (Terauchi et al., 2010). The present study demonstrates that FGF22 acts locally to induce presynaptic differentiation. Upon contact to nascent presynaptic sites, FGF22 acts as a localized neuromodulator capable of inducing

synaptic vesicles assembly. This is consistent with findings from Sanes and Umemori, who showed that FGF22 is secreted by the postsynaptic terminal, thus promoting a spatially restricted effect on closely located nascent presynaptic sites (Umemori et al., 2004; Terauchi et al., 2010). In order to reinforce the idea of a localized presynaptic action, it would be interest to determine the exact localization of FGFR2b receptors in neurons. In a similar way, potentiation of neurotransmitter secretion at developing synapses by BNDF was shown to occur in a spatially restricted manner (Zhang and Poo, 2002).

Secondly, we aimed to determine the dependence of FGF22-induced presynaptogenesis on proteins synthesized locally. Neurons are highly complex cells, capable of extending axons to remote places, frequently far away from the cell body. Developing axons undergo long-range pathfinding to seek for a synaptic target, and, upon initial contact, morphological and functional remodeling of terminals guarantees the perfect establishment of synaptic connections. Formation of the neuronal network is a highly demanding period in terms of requirement of newly synthesized components. Indeed, during development, protein synthesis levels peak during synaptogenesis (Phillips et al., 1990), suggesting that immature and newly-forming synapses have a particular high demand for new proteins. The required set of newly synthesized proteins could be generated at the cell body and then translocated to nascent synaptic sites, or, alternatively, synthesized locally along the axon shaft. In these last years, accumulating evidences support a role for axonal protein synthesis in synaptogenesis (Schacher and Wu, 2002; Lyles et al., 2006; Zhang and Poo, 2002; Boylan et al., 2008; Sebeo et al., 2009 – for a detailed description see introduction, section 1.5.1.2.). Our work strengthens this emerging hypothesis. Here, we demonstrate that FGF22-induced presynaptogenesis relies on axonally synthesized proteins. We can speculate that

extracellular presynaptogenic factors, by interacting with membrane receptors, activate specific intracellular signaling pathways that culminate in modulation of local translation initiation. In fact, it was already reported that activation of guidance cues receptors leads to eIF-4E and eIF4EBP phosphorylation (Campbell and Holt, 2003; Piper et al., 2006), known to de-repress local translation, and so, contribute with newly-synthesized proteins needed for the structural alterations inherent to the induced growth cone response.

Based on the current knowledge and on the new findings presented here, an interesting working model could be formulated where the postsynaptic cell secrets FGF22, which in turn interacts with FGFR2b, located at the presynaptic membrane, activating downstream signaling pathways that will probably induce translation of localized mRNAs. We may speculate that activation of FGFRs induces de-repression of local translation and/or translocation of specific mRNAs, present at RNA granules, at the nascent presynaptic site. Newly-synthesized proteins will then promote the clustering of freely-moving synaptic vesicles at these specific sites along the axon shaft leading to presynaptic assembly (figure 14). Future work will be necessary to elucidate how FGF22 signaling leads to the translation of specific mRNAs necessary for the assembly of the presynaptic terminal.

4.2. Future perspectives

In this work, we investigated the role of local protein synthesis in synapse formation using fluidically isolated axons. We assessed synaptic vesicles clustering by immunolabeling primary cultures of rat embryonic hippocampal neurons for synapsin,

and we showed that FGF22-induced presynaptic assembly is dependent on axonal protein synthesis.

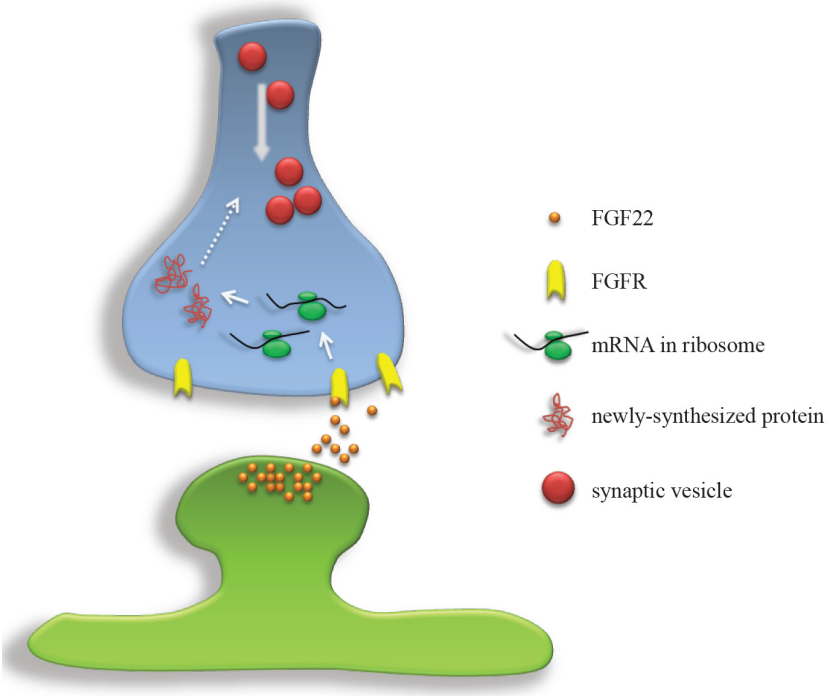


Figure 14 – A model for FGF22-induced presynaptic differentiation. Activation of FGFRs by target-derived FGF22 induces local protein synthesis required for synaptic vesicles clustering at nascent presynaptic sites.

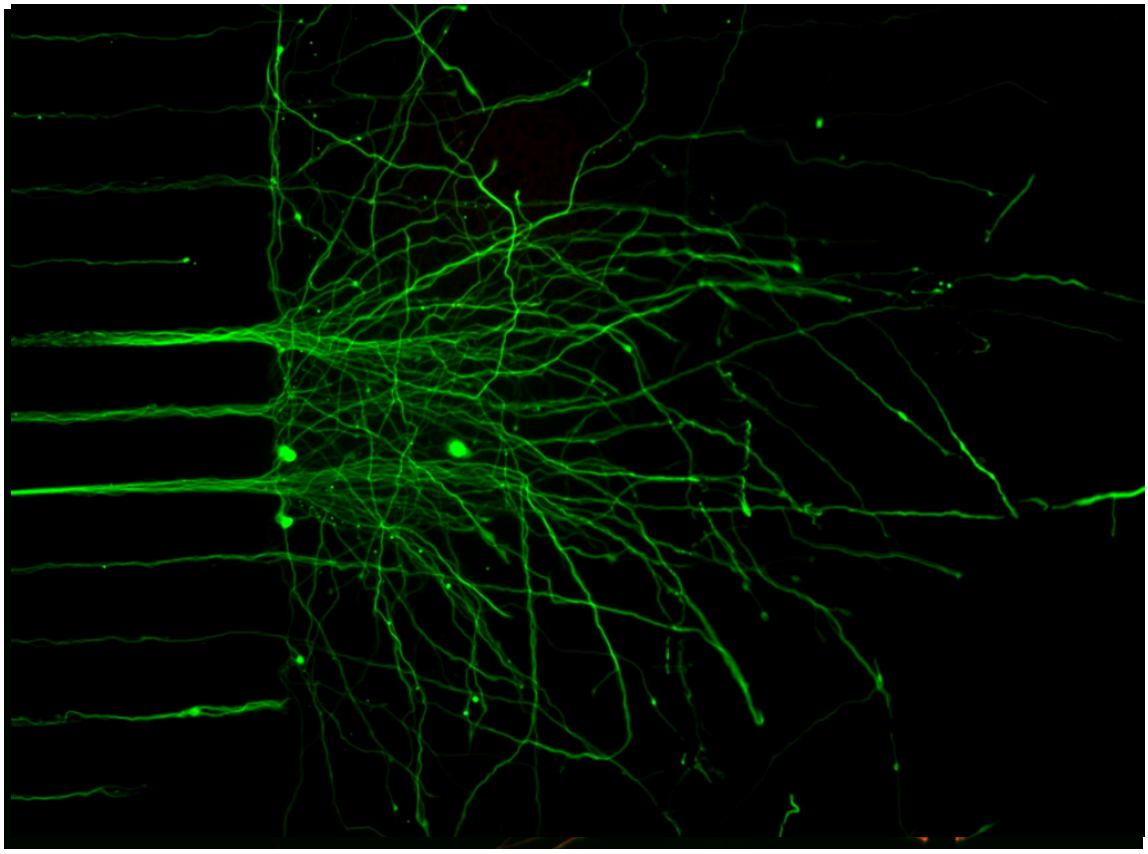
Little is known about FGFR2b localization in neurons. To better understand and characterize the mechanism of presynaptic differentiation induced by FGF22 it is crucial to determine the spacial localization of FGFR2b. I propose to immunolabel cultured hippocampal neurons for FGFR2b and a presynaptic marker. Since FGF22 induces clustering of synaptic vesicles, FGFR2b-enriched areas at the presynaptic membrane are more likely to be nascent presynaptic sites, and so, the localization of these receptors is determinant for differentiation of presynaptic terminals.

Protein synthesis, both in dendrites and axons, is known to be tightly regulated. It has been shown that external stimuli activate intracellular cascades that de-repress translation of dormant mRNAs (Campbell and Holt, 2003; Piper et al., 2006). Similarly, FGF22/FGFR2b signaling must modulate local protein synthesis. It would be interesting to unravel the intracellular signaling pathways activated by FGF22 that, in turn, activate translation pathways and/or machinery. We propose to use specific inhibitors for intermediates of the intracellular cascades known to be activated by FGFR2b (figure 5), and evaluate their requirement for FGF22-induced presynaptic differentiation and for FGF22-induced intra axonal protein synthesis.

Having characterized the requirement of local protein synthesis on FGF22-induced presynaptic differentiation, it would be interesting to find out which mRNAs are translated upon this stimulus. This information would give us important clues to understand the mechanism of action of FGF22 and the mechanisms leading to presynaptogenesis. To achieve this, I propose to purify distal axons and growth cones of rat embryonic hippocampal neurons, subjected or not to FGF22 stimulation, and screen for mRNAs using microarray technology. Differences in mRNAs expression between conditions will indicate which mRNAs are locally regulated, and possibly translated, upon FGFR2b activation by FGF22.

If we succeed on identifying mRNAs whose axonal expression is upregulated upon FGF22 stimulation, several questions may then be raised: how are these mRNAs related to presynaptogenesis? Is their local translation required for FGF22-induced presynaptic terminal formation? These questions can then be answered by knocking down specific mRNAs located in axons by adding siRNAs to the axonal compartment of microfluidic devices, determining how and when presynaptogenesis is affected.

Further understanding the signaling pathways and mechanism of FGF22-induced presynaptogenesis, and the exact role of axonal protein synthesis in synapse formation may prove relevant to better understand the formation and establishment of neuronal connections in the central nervous system, both in neurodevelopmental and pathophysiological conditions.



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