Universidade de Aveiro Departamento de Ciências Médicas
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Doutor Ramiro de Almeida, Professor Auxiliar do Departamento de Ciências Médicas e coorientação do Doutor Rui O. Costa, Investigador de Pós-Doutoramento do Centro de Neurociências e Biologia Celular (CNC).

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Judges

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palavras-chave Tradução local de proteínas, axónios, neurónios, diferenciação pré-sináptica

Resumo O sistema nervoso humano é composto por uma rede neuronal intricada e complexa. As sinapses constituem a base da rede neuronal e, portanto, é necessária uma formação correta do terminal pré e pós-sináptico, que é impulsionado por uma organização dinâmica de proteínas *in locu*. Foi demonstrado que a tradução intra-axonal do mRNA fornece as proteínas necessárias para o crescimento axonal e o pathfinding. No entanto, ainda não está claro qual o papel da síntese local de proteínas na formação pré-sináptica. Com o intuito de responder a esta questão, foram plaqueados em câmaras microfluídicas neurónios embrionários do hipocampo de rato. Estes dispositivos permitem o isolamento físico e fluídico dos axónios distais e, assim, permitem o estudo dos mecanismos intrínsecos dos axónios. Descobrimos que a tradução intra-axonal do mRNA é necessária para a diferenciação pré-sináptica. Observamos ainda que a diferenciação pré-sináptica induzida pelo fator de crescimento de fibroblastos 22 e Beads revestidas com Poli-D-Lisina é acompanhada por um aumento de marcadores de tradução em locais pré-sinápticos recém-formados. Estes resultados demonstram a existência de tradução de mRNA intra-axonal local durante a formação de sinapses.

Keywords Local mRNA translation, axons, presynaptic differentiation, FGF22.

Abstract

The human nervous system is composed of an intricate and complex neuronal network. Synapses are the building blocks of this network and so a correct formation of the pre- and postsynaptic terminal is necessary, which is driven by a dynamic organization of proteins *in locu*. Intra-axonal mRNA translation has been shown to provide the proteins necessary for axonal growth and pathfinding. However, it is still unclear how local protein synthesis regulates presynaptic formation. To address this question, we cultured rat embryonic hippocampal neurons in microfluidic chambers. These devices allow the physical and fluidic isolation of distal axons and, thus, allow the study of axonal intrinsic mechanisms. We found that intra-axonal mRNA translation is required for presynaptic differentiation. We further observed that presynaptic differentiation induced by fibroblast growth factor 22 and Poly-D-Lysine-coated beads is accompanied by an increase of translational markers in newly formed presynaptic sites. Together, these results demonstrate the existence of local intra-axonal mRNA translation during synapse formation.

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List of Abbreviations, symbols and acronyms

5-FDU	5-fluoro-2’-deoxiuridina
BSA	Bovine Serum Albumin
CaMKII	Ca2+/calmodulin-dependent protein kinase II
DIV	Days in vitro
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF7	Fibroblast growth factor-7
FGF10	Fibroblast growth factor-10
FGF22	Fibroblast growth factor-22
FGFR	Fibroblast growth factor receptor
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF2	Insulin-like growth factor 2
LB	Luria-Bertani
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule-associated protein 2
OPP	O-propargyl-O-propargyl-puromycin
p-4E-BP1	Phosphorylated-4E-BP1
P/S	Penicillin/streptomycin
PBs	Processing bodies

PBS Phosphate buffered saline

PDL Poly-D-lysine

PDMS Poly-dimethlysiloxane

PFA Paraformaldehyde

PSD Postsynaptic density

RNP Ribonucleotide protein

SGs Stress granules

Sema3A Semaphoring-3A

VGlut1 Vesicular glutamate transporter 1

1 Introduction

1.1 Protein synthesis

1.1.1 Background

Marshall Nirenberg and Heinrich Matthaei studied and understood how the genetic code was composed in 1961 (Watson and Crick, 1953). When combined with the contents of broken E. coli cells, Nirenberg discovered that RNA, regardless of its source organism, could initiate protein synthesis. Nirenberg and Matthaei were able to establish that the codon UUU (the only one in poly-U) coded for the amino acid phenylalanine by applying poly-U to each of 20 test tubes (each tube containing a different "tagged" amino acid). The uracil chain acted as a signalling molecule that directed protein synthesis. The experiment proved that messenger RNA transcribes genetic information from DNA, directing the assembly of amino acids into complex proteins, despite this finding the question of how many units of U were required was still unanswered. A key step for understanding the genetic code had been discovered (Nirenberg and Matthaei, 1961) . Nirenberg and his colleagues identified nucleotide combinations for the incorporation of other amino acids using the poly-U experiment as a model. The researchers discovered that amino acid coding units contain three nucleotides (a triplet). Four nucleotides were combined into three-letter codes, yielding 64 possible combinations (4 x 4 x 4), enough to describe 20 amino acids. In 1964, Nirenberg and Philip Leder discovered a method for determining the letter sequence in each triplet word for amino acids (Nirenberg and Leder, 1964). By 1966, Nirenberg had deciphered the 64 RNA three-letter code words (codons) for all 20 amino acids. The language of DNA had been deciphered, and the code could now be represented graphically (Nirenberg *et al.*, 1966; Leder, 2010).

1.1.2 Overview

Protein synthesis consists in the decoding of a messenger RNA into a specific amino acid chain, denominated peptide. Translation requires several components such as

tRNAs, a mRNA molecule, amino acids, ribosomes and initiation factors. This process can be divided in three phases - ‘Initiation’, ‘Elongation’ and ‘Termination’, and it occurs in the cytoplasm of the cell. Protein synthesis culminates in the formation of a peptide, which can then suffer several post-translational modifications (Schweet and Heintz, 1966; Science and Lewis, 2013).

1.1.3 Protein Synthesis

Protein synthesis begins (Figure 1) with the specific codon AUG, which is a three-nucleotide sequence, and a specific charged tRNA is crucial to initiate translation. The initiator tRNA always transports the amino acid methionine, there is a distinct difference between this initiation tRNA and a tRNA that normally carries methionine. Newly synthesised proteins all start with this specific methionine amino acid at their N-terminal end, but it can be later removed by specific proteases. Regarding translation in eukaryotes, an initiator tRNA is loaded into the P site of the small ribosomal subunit, along with several proteins denominated translation initiation factors. In contrast with all the tRNAs present in the cell, the initiator tRNA as the unique capability of binding tightly to the P site in the absence of the larger ribosomal subunit. Afterwards, the small ribosomal subunit loaded with the initiator tRNA binds to the 5’ end of an mRNA sequence in the cap, which is presented in most eukaryotic mRNAs. The small ribosomal subunit then scans forward the mRNA searching for the AUG codon. When the AUG codon is encountered by the initiator tRNA, several initiation factors dissociate from the small ribosomal subunit so there is enough space for the large ribosomal subunit to bind and form the ribosomal complex. As the initiator tRNA is bound to the P site of the ribosome, protein synthesis is ready to start with the addition of the next tRNA to the A site. Ribosomes contain three binding sites for tRNA molecules, called the A site, the P site, and the E site. To add an amino acid to a growing peptide chain, the appropriate charged tRNA enters the A site by base-pairing with the complementary codon on the mRNA molecule. Its amino acid is then linked to the peptide chain held by the tRNA in the neighbouring P site. Next, the large ribosomal subunit shifts forward, moving the spent tRNA to the E site before ejecting it. This cycle of reactions is repeated each time an amino

acid is added to the polypeptide chain (Schweet and Heintz, 1966; Science and Lewis, 2013).

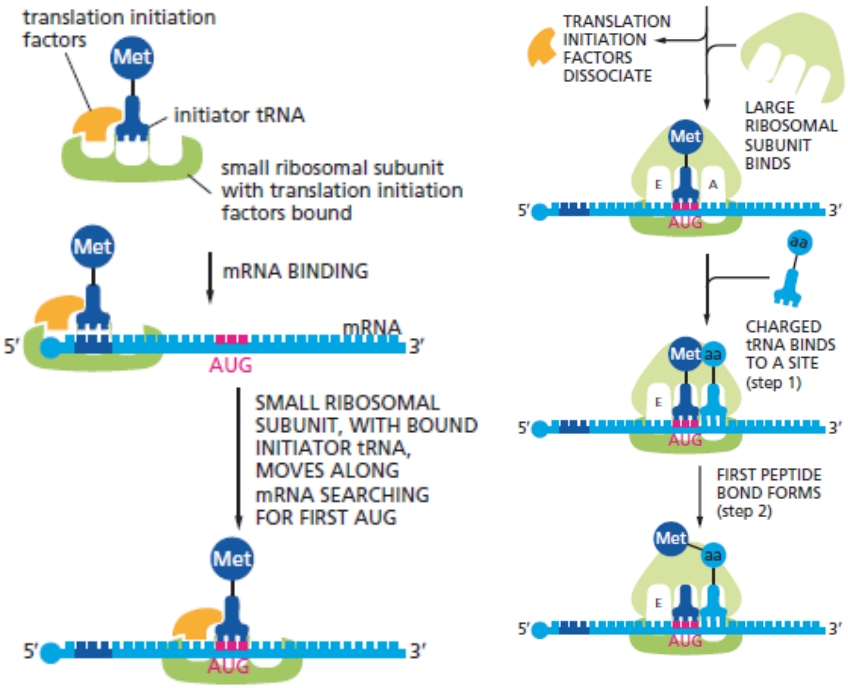


Figure 1 - Schematic representation of the initiation and elongation processes regarding protein synthesis (Adapted from Science and Lewis, 2013)

The end of translation (Figure 2) is signalled by the presence of one of the several stopping codons in the mRNA. The stopping codons -UAA, UAG and UGA- are not recognized by a tRNA and do not specify an amino acid, instead they signal the ribosome to terminate translation. Proteins known as release factors bind to any stopping codon that reaches the A site on the ribosome, this binding alters the activity of the peptidyl transferase in the ribosome, causing it to catalyse the addition of a water molecule instead of an amino acid to the peptidyl-tRNA. This reaction frees the carboxyl end of the polypeptide chain from its attachment to a tRNA molecule because this is the only attachment that holds the growing polypeptide to the ribosome, the completed protein chain is immediately released. At this point, the ribosome also releases the mRNA and dissociates into its two separate subunits, which can then assemble on another mRNA molecule to begin a new round of protein synthesis (Schweet and Heintz, 1966; Science and Lewis, 2013).

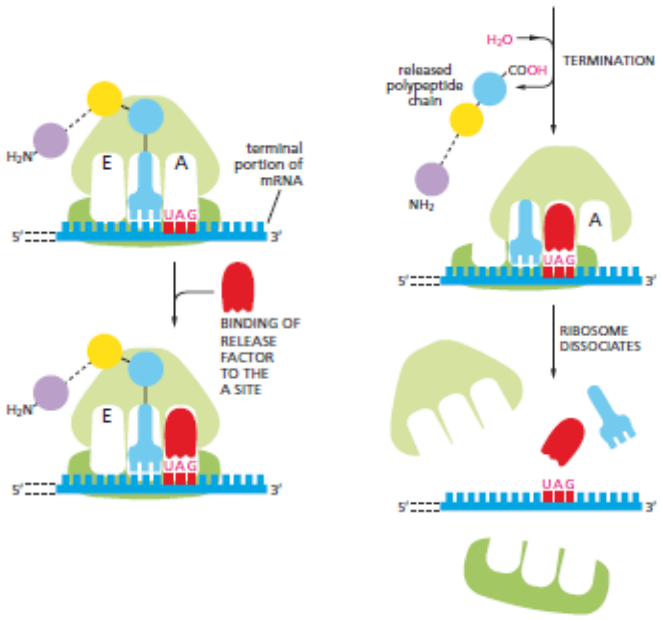


Figure 2 - Schematic representation of the termination processes regarding protein synthesis (Adapted from Science and Lewis, 2013)

1.2 Synaptic Differentiation

1.2.1 Synapse organization

Synapses are are responsible for the flow of information from one neuron to the next. They arise during nervous system development, and the construction of proper synapses is critical for the building of neural circuits that underlay behaviour and cognition (Tallafuss, Constable and Washbourne, 2010).

A presynaptic terminal is a specific part of the axon that clusters synaptic vesicles around a specialized location at the membrane, either at the ends of the axon or along the axon shaft (varicosities). The active zone, or presynaptic specialization, is physiologically described as the location of controlled synaptic vesicle fusion and neurotransmitter release (Couteaux, 1963). The release of neurotransmitters from presynaptic nerve terminals occurs in active zones, which are highly specialized locations. The active zone's architecture is constructed to allow synaptic vesicles to be tethered, docked, and fused to the plasma membrane in a controlled manner (Zhai and Bellen, 2004).

The postsynaptic density is a structure found in the postsynaptic plasma membrane that is made up of both membrane and cytoplasmic proteins. Several proteins have been found in the PSD through intense and meticulous research. For example, in excitatory neurons glutamate receptors, as well as membrane proteins involved in synaptic signal transduction and cell adhesion, are crucial components of the PSD. Scaffolding proteins interact with several membrane and cytoplasmatic proteins motifs to create the PSD (Shigeo, 2007). Figure 3 depicts a schematic representation of the different structures that compose the synapse, such as the presynaptic terminal, the active zone and the postsynaptic density.

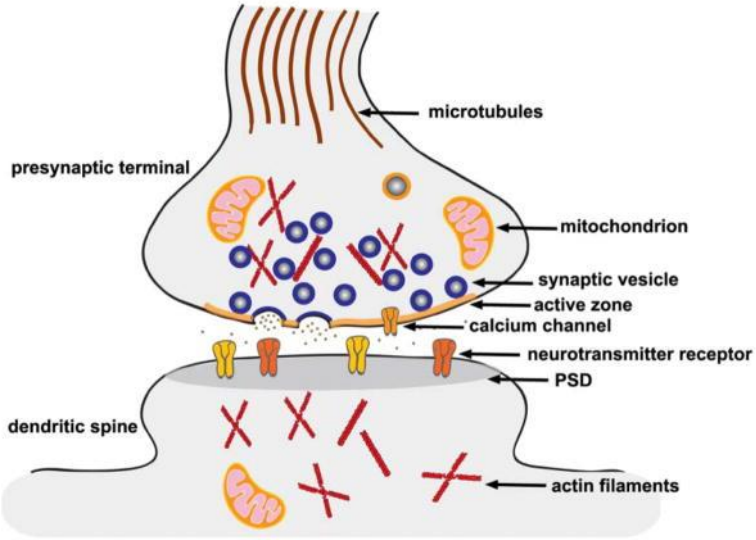


Figure 3 - Schematic representation of the synaptic organization (Adapted from Cai and Sheng, 2009)

The synapse has the crucial role to pass information from a presynaptic neuron to a postsynaptic cell, and it has the ability to convert an electrical signal into a chemical signal. The presynaptic terminal secretes neurotransmitters through exocytosis, whereas the postsynaptic specialization has specific receptors that can sense the neurotransmitters that were released onto the synaptic cleft. The binding between neurotransmitters and their specific receptors, leads to an influx of ions that's causes local depolarization and activation of voltage-gated ion channels. This process activates several signalling cascades and is commonly designated by synaptic transmission (Südhof, 2018).

1.2.2 Presynaptic differentiation

The establishment of chemical synapses requires an independent formation of presynaptic and postsynaptic structures, and also reciprocal induction between these two structures (Zhen *et al.*, 2004). Presynaptic differentiation of nerve terminals are characterized by machinery focused in the release of molecules that could vary in detail but are always formed by the same elements (Südhof, 2018).

During the initial stages of development neurons accommodate a vast array of transport packets, such as synaptic vesicles, tubulovesicular structures and precursors of the active zone (piccolo/bassoon transport vesicles), which are fundamental to the formation of nascent presynaptic structures (Figure 4a). It can occur the 'spontaneous' formation of primate vesicle releasing sites along the immature axon (Figure 4b). The contact between the axon and a postsynaptic target conducts to an accumulation of several transport vesicles in the precise location where they contacted. These 'primitive' presynaptic boutons have some ability to do synaptic vesicle recycling but they lack several structures in comparison to mature presynaptic boutons (Figure 4c). Axodendritic contact leads to a fast formation of an active zone with the fusion of active zone precursor vesicles and the recruitment of synaptic vesicles (Figure 4d). During maturation, there is an increase of the reserve pool of synaptic vesicles, and new synapses start to acquire functional and structural characteristics similar to mature synapses (Figure 4e). After the formation of presynaptic boutons, sometimes different vesicle clusters start to wander away to form new presynaptic sites (Figure 4f). This scheme is not temporally accurate because these are very fast and intricate processes with a lot of uncertainty (Noam and Craig, 2004).

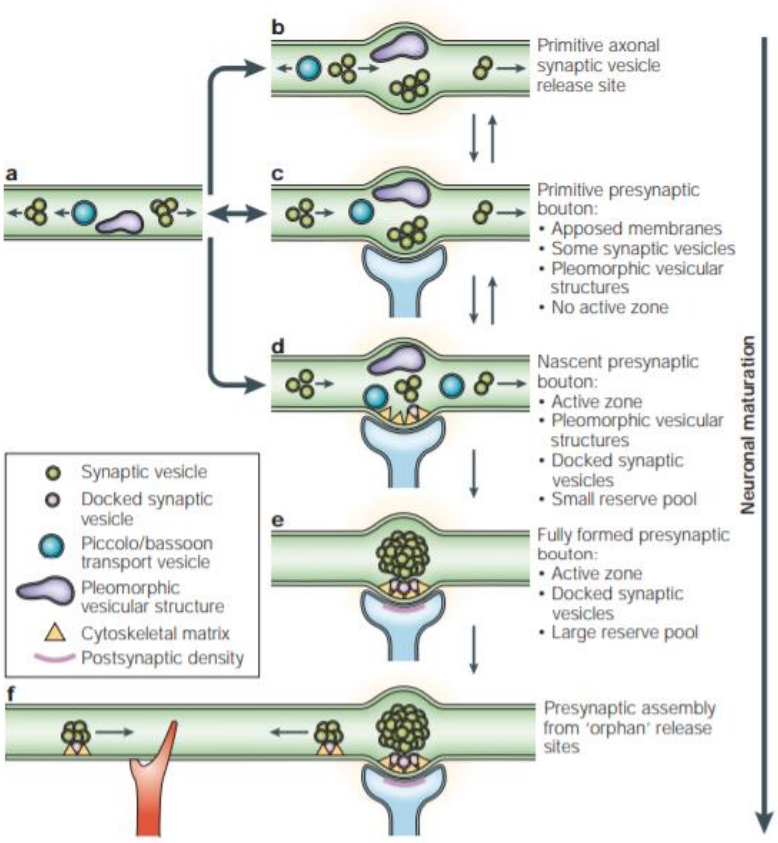


Figure 4 – Schematic representation of the multiple steps that occur during presynaptic differentiation (Adapted from Noam and Craig, 2004)

1.2.3 Synaptogenic cues

1.2.3..1 Fibroblast Growth Factor 22

Target-derived cues promote local differentiation of axons into nerve terminals at sites of synaptic contact (Fox *et al.*, 2007; Pinto and Almeida, 2016). Presynaptic differentiation can occur through specific target-derived organizers such as, WNT-7a, neuroligin, synaptic cell adhesion molecule (SynCAM) and FGF22. In addition, glial cells release factors which play a crucial role in regulating synapse assembly (Fox and Umemori, 2006). WNT proteins act as retrograde signals to control axon remodelling and presynaptic protein accumulation. Ahmad-Annuar reveals that these proteins could indirectly influence synapse development by speeding neuronal maturation via changes in synaptic component transcription and/or translation (Ahmad-Annuar *et al.*, 2006). Scheiffele demonstrated that nonneuronal cells engineered to express neuroligin, a cell adhesion molecule, are capable of inducing presynaptic differentiation in contacting axons (Scheiffele *et al.*, 2000). SynCAM belong to an immunoglobulin superfamily, which is expressed throughout the developing brain. It functions as a cell adhesion molecule and promotes functional synapses as it enhances excitatory neurotransmission (Fogel *et al.*, 2007). Glial cells influence synaptic characteristics by releasing neurologically active chemicals such as ATP and D-serine, this released factors are capable of presynaptic differentiation (Eroglu and Barres, 2010). Through the use of these specific cues, investigators are able to better understand the mechanism that underlies presynaptic differentiation at sites of synaptic contact.

Fibroblast Growth Factors (FGFs) have been involved in the regulation of several processes such as cell proliferation, differentiation, migration, tissue repair and injury response. During the development and the maturation of the nervous system, 14 FGFs and all four FGFs receptors are expressed and it was shown that they are key pieces in neural proliferation and survival, neural induction, neural plate patterning, and neuroprotection (Dono, 2003; Reuss and Von Bohlen Und Halbach, 2003).

FGF 22 is a specific fibroblast growth factor which incorporates a family of more than 20 intercellular signalling molecules (Umemori, Michael W Linhoff, *et al.*, 2004).

FGFs bind to and activate four tyrosine kinase FGF receptors that are alternatively spliced. The ability of certain ligand-receptor pairings to actively signal, as well as the spatial and temporal expression patterns of FGFs and FGFRs, are critical determinants governing FGF activity in several biological processes. The binding specificity of ligands and receptors regulates FGF signalling activity, which is modified by extrinsic cofactors like heparan sulphate proteoglycans (Zhang *et al.*, 2006).

Several studies also demonstrated that FGF22 is expressed in the skin and brain (Nakatake *et al.*, 2001; Beyer *et al.*, 2003). In the central and peripheral nervous system, it was shown that FGF22 messenger RNA is strongly expressed in the mouse hippocampus at 8 days after birth, which can be correlated with the time in which synapses start to form, there is also an expression of the messenger RNA of the major receptor FGFR2 in several neurons throughout the hippocampus. Regarding the FGF22 protein it is localized in synapse rich areas such as the stratum radiatum, stratum lucidum and in CA3 pyramidal neurons.

Terauchi and colleagues demonstrated that FGF22, as well as its close relatives FGF7 and FGF10, have been identified as molecules that stimulate the development of presynaptic nerve terminals. As shown in Figure 5, FGF22 induces vesicle clustering and neurite branching, indicating that it has the capability of inducing synapse formation (Umemori, Michael W. Linhoff, *et al.*, 2004).

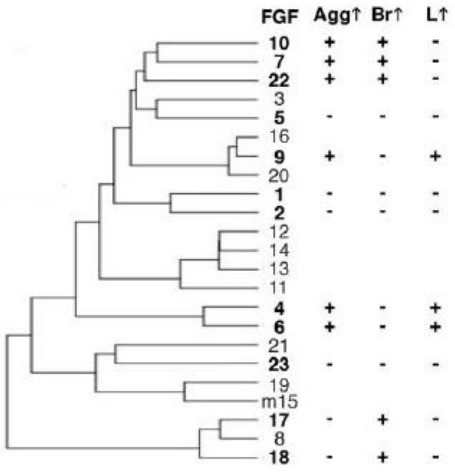


Figure 5 – Effects of different FGFs in cultured neurons (Adapted from Umemori, Linhoff, Ornitz, & Sanes, 2004)

Actually, FGF7, 10 and 22 signal through the same FGF receptor, FGFR2b, an alternatively spliced product of the FGFR2 gene (Ornitz *et al.*, 1996). In addition, FGF22 also binds to FGFR1b but with lower efficacy (Mason, 2007).

It was demonstrated that FGF22 is important in presynaptic differentiation in the cerebellum and at the neuromuscular junction (Terauchi *et al.*, 2010). Umemori and colleagues further showed that these newly formed synapses were functional using FM1-43 dye labelling, observing that FGF22-induced varicosities were capable of depolarization-dependent recycling (Umemori, Michael W. Linhoff, *et al.*, 2004).

Subsequent studies showed that FGF22 ia a specific inductor of excitatory synapses and FGF7 ia a inductor for inhibitory synapses (Terauchi *et al.*, 2010). In addition, FGF22-deficient mice are resistant to epileptic seizures, whereas, FGF7-deficient mice are prone to them, indicating that the FGF system regulate synapse formation and may underlie neurological disorders. FGF22 also regulates insulin-like growth factor 2 (IGF2) expression in presynaptic neurons. IGF2 helps to stabilize presynaptic synapses in an activity-dependent way. These findings indicate a novel feedback signal in mammalian presynaptic terminals that is critical for activity-dependent stability (Terauchi *et al.*, 2016).

To conclude, Fibroblast Growth Factor 22 is a target-derived soluble molecule, present in both the central and peripheral nervous system, which has the ability to induce the formation of presynapses, regulating neuronal circuit formation (Umemori, Michael W. Linhoff, *et al.*, 2004; Fox and Umemori, 2006; Fox *et al.*, 2007; Terauchi *et al.*, 2010; Singh *et al.*, 2012).

1.2.3..2 Poly-D-lysine coated Beads

The canonical model of synapse assembly occurs between an axon and a dendrite. To mimic this same contact and to better understand the dynamics behind synapses, investigators can use specific synaptogenic cues or target adhesion approaches. Burry and colleagues first demonstrated that neural processes contacting PDL-coated beads appear to create presynaptic elements, with the bead acting as a postsynaptic element (Burry, 1980). One of the early steps in the creation of a synaptic contact in culture is electrostatic interaction between poly-acidic groups on the presynaptic element and poly-basic groups on the postsynaptic element

(Burry, 1980). Burry also observed through the use of electron microscopy in younger rat cerebellums, that PDL-Beads when in contact with a neurites resulted in a swelling associated with vesicle accumulation and membrane thickening (Burry, 1982). In 1987, Peng and colleagues used light and electron microscopy to better understand the interaction between neurites and PDL-Beads. Using antibodies and light microscopy they showed that targeted synaptic vesicles had an increase of 60%, suggesting a clustering of synaptic vesicles at these specific sites. However, using negatively charged beads, this effect was not observed (Peng *et al.*, 1987). These findings suggest that in the absence of any specific cues, target adhesion alone can trigger the formation of presynaptic terminals. Early studies never demonstrated if these target adhesion varicosities were functional, whether their formation was intrinsic to axons or also valid for dendritic contact, or even if this model could be compared to *in vivo* models. Lucido and colleagues, addressed these questions using atomic force microscopy (AFM) (Figure 6). They demonstrated that PDL-coated beads can indeed induce the formation of presynaptic varicosities through a mechanism that is dependent on F-actin reorganization and the presence of heparan sulphate proteoglycans (HSPGs) (Lucido *et al.*, 2009).

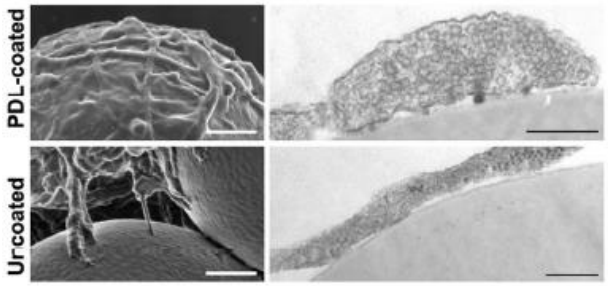


Figure 6 – Schematic representation of PDL coated and uncoated beads (Adapted from Lucido et al., 2009)

The authors also concluded that presynaptic differentiation could occur in the absence of postsynaptic partners, whereas postsynaptic assembly needs the presence of differentiated presynaptic structures. These presynaptic endings are functional and recycle synaptic vesicles in an activity-dependent manner. (Ryan *et al.*, 1993; Lucido *et al.*, 2009; Taylor *et al.*, 2013).

In sum, PDL-Beads can induce presynaptic differentiation through target adhesion, and this is one of the major differences to soluble synaptogenic cues such as FGF22. This approach has several benefits but it's not physiologically accurate. However, it can only be used in *in vitro* models and its action is extremely localized.

1.3 Local Protein synthesis in the axon

1.3.1 Background

The first studies that identified the existence of protein synthesis machinery in growth cones of developing axons were done in 1970s with the use of electron microscopy (Tennyson, 1970; Yamada, Spooner and Wessells, 1971). The discoveries by Steward and Levy (Steward and Levy, 1982) revealed the existence of protein machinery at synaptic sites on dendrites, more specifically the existence of polyribosomes at the base of dendritic spines. These revelations refuted the dominant model until date, which stated that all proteins are synthesized at the cell body and then shipped to specific sub compartments in the neuron, this model was denominated the 'cell body' model. However, this model had some critical flaws, because in some cases the transport from the nucleus to the axon was considerably longer than the protein half-life, which meant that some proteins wouldn't survive the entire journey (Varshavsky, 1996; Alvarez, Giuditta and Koenig, 1999).

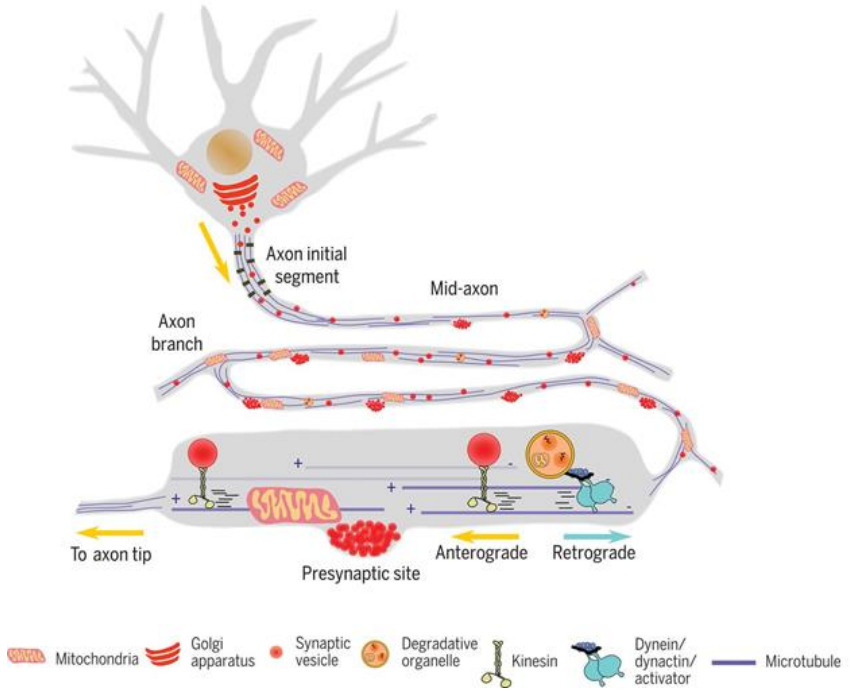


Figure 7– Schematic representation of the 'cell body' model for protein translation in axons (Guedes-Dias and Holzbaur, 2019)

Since then, several different mRNAs were shown to be transported out of the cell body such as MAP2, CamKIIα, Arc and RhoA. Local protein synthesis has also been shown to be involved in a variety of processes like cell survival, LTP, LTD, axonal guidance and growth cone collapse. Currently, the most consensual model accepts the existence of the 'cell body' theory but also the occurrence of local protein synthesis in dendrites and growth cones of immature neurons. Recently, there has been a detailed description of local protein synthesis in dendrites of cultured mammalian neurons, particularly in learning and synaptic plasticity. In 1996, Mayford and colleagues demonstrated through studies regarding CamKIIα protein, that neurons can manipulate the dendrite's local biochemistry by differential mRNA localization or variations in translational efficiency at different sites along the dendrite (Mayford *et al.*, 1996). An additional study, using electron microscopy, demonstrated that neuronal stimulation leading to long-term potentiation increased the number of dendritic spines containing polyribosomes. Strikingly, there was also an increase in the size of these spines, suggesting that local protein synthesis contributes to spine size (Ostroff *et al.*, 2002). A different study by Steward and

colleagues showed that both mRNA and translation machinery were present at dendritic spines (Schuman, Dynes and Steward, 2006). Schuman and colleagues found that the activation of D1/D5 Dopamine receptors increased local translation of AMPA receptor GluR1 subunit, and this protein was guided to the cell membrane where it led to an increase in the frequency of an excitatory postsynaptic potential (EPSP) (Smith *et al.*, 2005).

Besides the existence of a significative number of studies regarding dendritic local protein synthesis in the past years, few studies have their focus point on the synthesis of proteins in the axon. Axons have the capability of responding locally to guidance cues, but the mechanisms responsible for these phenomena just a few years ago started to be unveiled and understood in more detail. Studies involving axonal protein synthesis prioritize the role of local protein synthesis in growth cone collapse. One of these studies was performed by Campbell and Holt, using Xenopus retinal ganglion cells, it was shown that growth cone collapse induced by either Netrin-1 or Sema3A was dependent on local protein synthesis (Campbell and Holt, 2001). The same authors also showed that local protein synthesis was dependent on the activation of the Erk42/44 pathway (Campbell and Holt, 2003). Another study demonstrated the importance of RhoA mRNA translation, which is a small GTPase involved in several cellular processes such as cytoskeletal organization, cell development and cell cycle maintenance. The local translation of this mRNA regulates growth cone collapse induced by Sema3A in dorsal root ganglion neurons (Wu *et al.*, 2005).

The connection between synapse formation and local protein synthesis was first described in invertebrates. A study from Lyles and colleagues discovered that clustering of sensorin mRNA at nascent synapses in cultured *Aplysia* neurons is induced by synaptic partner recognition, but also necessary for synaptic development and maintenance (Lyles, Zhao and Martin, 2006; Shen, 2006). Previous studies from Martin and colleagues in 1997 showed that when a bifurcated sensory neuron from *Aplysia* Californica was co-cultured with two spatially separated motor neurons, branch specific long-term synaptic facilitation induced by serotonin was dependent on local protein synthesis (Lyles, Zhao and Martin, 2006).

These results show that when culturing sensory neurons with a non-physiological target neuron, there is no mRNA relocalization. Regarding mammals, there is no known homolog for sensorin. Younts using rodent hippocampal slices reported that long-term GABA released from axons requires presynaptic protein synthesis (Younts *et al.*, 2016). All these studies suggest that local protein synthesis has a crucial role in synapse formation.

1.3.2 Trafficking of mRNA in the axon

Neurons are highly polarized cells that have numerous dendrites and one axon that extends from the cell body. Until the discovery of polyribosomes at the base of dendritic spines (Steward and Levy, 1982), the widely held belief was that all proteins were translated in the cell body and subsequently carried to the neurites. Several studies in recent years have demonstrated the occurrence of localized protein production in axons and dendrites (Hirokawa, 2006). Synaptic plasticity, synapse development, axon guidance, and nerve regeneration can all be influenced by mRNA localization and translation (Kiebler and Bassell, 2006).

Nowadays, it is assumed the existence of mRNA trafficking from the cell body to the axon. Localized mRNAs are now largely believed to be carried in ribonucleoprotein particles (RNPs), sometimes known as RNA-containing granules (Kiebler and Bassell, 2006). Ribonucleoprotein particles (RNPs), stress granules (SG), and processing bodies (PB) are three forms of RNA-containing granules found in neurons. In terms of RNA binding proteins and mRNA, the contents of each of these granules varies (Sossin and Desgroseillers, 2006).

As shown in Figure 8, the mRNA is transcribed in the nucleus and it is transported through the axon packaged into mRNP granules, when it reaches distal axons it is converted into polypeptides. Post-translational modification may occur (Figure 8A), or nascent polypeptides could have a role in specific activity-based needs of individual synapses (Figure 8B). Local extracellular signals may interact with specific synapses (Figure 8A), and these membrane receptors or interactions could have a crucial role in the local translation of some mRNAs. Regarding the synapse (Figure 8C), the mRNP granules can be stored for later use, or excluded (Figure

8D). The translation of local mitochondrial protein maintains a healthy supply of axonal mitochondria (Figure 8D).

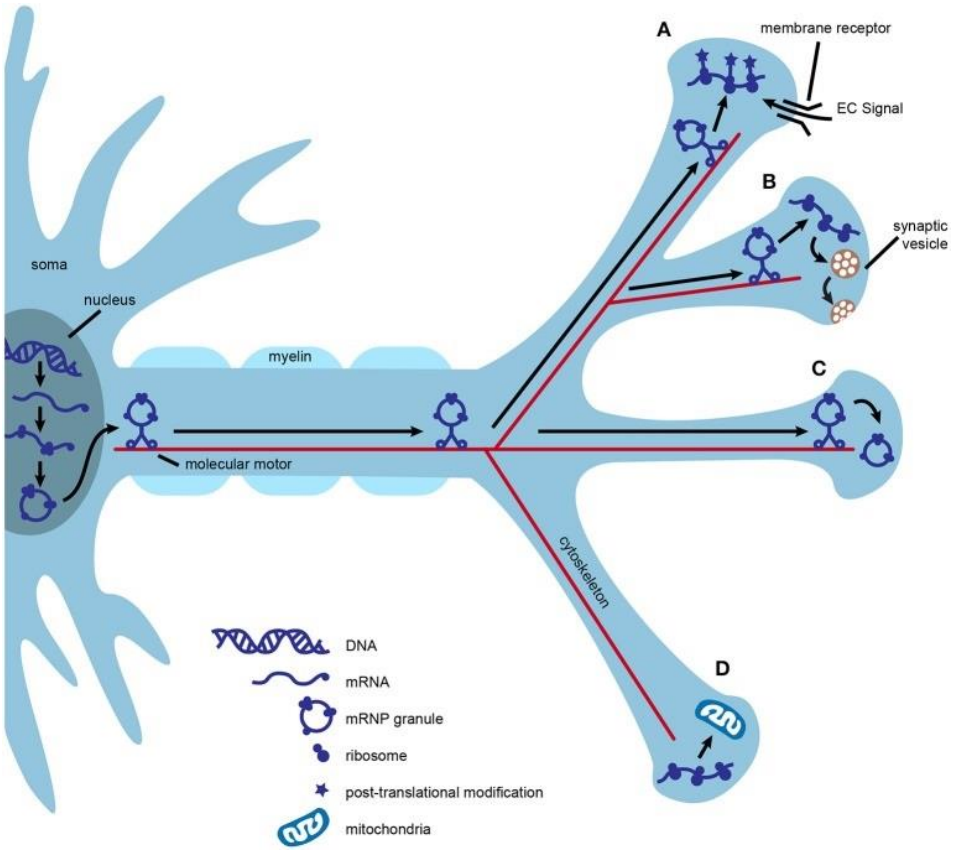


Figure 8 - Schematic representation of the alternative model for protein synthesis in axons (Adapted from Spaulding & Burgess, 2017)

1.4 Aims

The axon is a very complex and intricate structure that relies on local events to perform its functions. In the recent past it was demonstrated that intra-axonal protein synthesis regulates different aspects of axonal development and function. According to the literature several neuronal mechanisms require intra-axonal protein synthesis, such as axon guidance, synaptic plasticity, axonal regeneration and retrograde signalling. The aim of this master thesis is to investigate if nascent synapses form at sites of local translation in the axon. Our first objective is to demonstrate that FGF22 and the Poly-D-Lysine coated beads can induce presynaptic differentiation through intra-axonal mechanisms. We employed microfluidic chambers, to analyse axonal-specific events. FGF22 and PDL coated beads, were applied to isolated axons at different time points and we studied the dynamics of mRNA translation in axons. Secondly, we aim to analysed if nascent peptides colocalize with presynaptic sites, for that used a O-propargyl-O-propargyl-puromycin (OPP) method to measure on-going translation. In addition, we observed that phosphorylated-4E-BP1 colocalizes with presynaptic clusters, suggesting that intra-axonal mRNA translation coincides with newly formed presynaptic terminals. Finally, we aim to evaluate if intra-axonal mRNA translation precedes presynaptic sites formation. For this purpose, we transduced neurons with pRRL-eGFP-L10a, a ribosomal reporter and VGlut1-mCherry, a presynaptic reporter to evaluate in live neurons if colocalization between these two reporters occurs.

2 Methods

2.1 Reagents

Tabela 1- Reagents table

Reagent	Company/Source	Reagent	Company/Source
BSA	Sigma-Aldrich	Methanol	Fisher Scientific
$CaCl_2$	PanReac	Na_2HPO_4	Merck Millipore
FBS	Gibco® Life Technologies	NaCl	PanReac
Fluorescence Mounting Medium	Dako	$NaHCO_3$	Merck Millipore
Glucose	VWR Chemicals	Neurobasal Medium	Gibco® Life Technologies
Glutamate	Sigma-Aldrich	Paraformaldahyde	Sigma-Aldrich
Glutamine	Sigma-Aldrich	Phenol Red	Sigma-Aldrich
Glycerol	Amresco	Poly-D-Lysine	Sigma-Aldrich
Isopropanol	PanReac	SDS	Fisher Scientific
KCl	PanReac	Trypsin	Sigma-Aldrich
KH_2PO_4	PanReac		

2.1.1 Antibodies for immunocytochemistry

Tabela 2- Primary antibodies used in immunocytochemistry

Reagent	Reference	Company/ Source	Vendor	Stock Concentration	Final concentration	solvent
Chicken anti-neurofilament M (NF-M) polyclonal antibody	Cat# AB5735 RRID: AB_240806	Sigma-Aldrich	Milipore		1:1000	N/A
Rabbit Anti-synapsin polyclonal antibody	Cat# AB1543P RRID: AB_90757	Sigma-Aldrich	Milipore	1:10	1:4000	PBS 3% BSA
Mouse anti-β-Tubulin III monoclonal antibody	Cat# T8578 RRID: AB_1841228		Sigma-Aldrich		1:1000	N/A
Guinea Pig Anti-Vesicular Glutamate Transporter 1 polyclonal antibody	Cat# AB5905 RRID: AB_2301751	Sigma-Aldrich	Millipore	1:10	1:1500	PBS 3% BSA
Mouse anti-bassoon monoclonal antibody	Cat# ADI-VAM-PS003-D RRID: AB_2038857		Enzo Life Sciences		1:800	Glicerol
Rabbit anti-phospho-4E-BP1 (Thr37/46) monoclonal antibody	Cat# 2855 RRID: AB_560835		Cell Signaling		1:200	N/A

Tabela 3- Secondary antibodies used in immunocytochemistry

Reagent	Reference	Company/ Source	Vendor	Stock Concentration	Final concentration	solvent
AMCA secondary Ab goat anti-chicken 405	Cat# 103-155-155 RRID: AB_2337385		Jackson Immuno Research Labs		1:250	
Alexa fluor secondary Ab goat anti-mouse 488	Cat# A32723; RRID: AB_2633275	Invitrogen	Thermo Fisher	1:1	1:500	Glicerol
Alexa fluor secondary Ab goat anti-rabbit 568	Cat# AF568 RRID: Not available	Invitrogen	Thermo Fisher	1:1	1:500	Glicerol
Alexa fluor secondary Ab Goat anti-Guinea Pig 647	Cat# A-21450 RRID: AB_2735091	Invitrogen	Thermo Fisher	1:1	1:500	Glicerol

2.2 Hippocampal neurons

2.2.1 Culture of rat embryonic hippocampal neurons

Primary cultures of rat hippocampal neurons were prepared from the hippocampus of E17-18 Wistar rat embryos. The pregnant female was anesthetized and sacrificed by decapitation and the embryos were removed from the maternal womb. They were decapitated, their brains were removed, and the hippocampus was removal and kept on ice. After the hippocampus dissection, they were treated for 15 minutes at 37ºC with trypsin (0,25%) (to degrade the tissues) and with deoxyribonuclease (0,002%) (to cleave the DNA that was release by the lysed cells) in Hank's balanced salt solution (HBSS) (5.36 mM KCl, 0.44 mM KH2PO4, 137 mM NaCl, 4.16 mM NaHCO3, 0.34 mM Na2HPO4.2H_2O, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0,001% phenol red). This solution was then centrifugated for 1 minute at 1000 rpm, to deposit the hippocampus, then the trypsin-DNAse solution was removed and the hippocampus were washed with plating medium containing 10% FBS to block the trypsin action, centrifuged again for 1 minute at 1000 rpm, to deposit the hippocampus, and resuspended in plating media. In order to obtain an homogeneous cell suspension, the hippocampus was mechanically dissociated with the use of a P1000-pipette. The cells were then counted with the use of Trypan Blue Solution (0.4%), which helps to distinguish between dead and viable cells.

2.2.2 Microfluidic Chambers preparation

The microfluidic chambers, used for the hippocampal cell cultures, consisted of a molded PDMS chamber that was placed against a glass coverslip (Marienfeld No.1 24 mm × 24 mm). The chambers protocol consisted in the preparation of a PDMS silicon mixture which was then poured into a mould, the bubbles were removed with the use of vacuum and then the molds were placed in the incubator for 4h at 60ºC to cure. Coverslip's were placed into a glass container and 65% nitric acid was added. The containers were then placed on an agitator overnight, the next day the nitric acid was discarded, followed by a two times rinse with sterile mQ H_2O. Afterwards, the coverslips were washed five times during 30 minutes with sterile mQ

H_2O and finally washed with 70% ethanol and placed to dry at 50°C for 15 to 20 minutes. Before the plating step, the coverslips were coated with 0.1 mg/ml PDL overnight at 37°C and then washed several times with sterile mQ H_2O. The microfluidic chambers were rinsed with filtered 75% ethanol, then rinsed with sterile mQ H_2O, dried and assembled on to the glass coverslips. Finally, the coverslips were coated with laminin in neurobasal plain media for 2 hours at 37ºC and wash twice with plating media (minimum essential medium eagle (MEM) supplemented with 0,7% glucose and 10% FBS).

$7x10^5$ cells were added to the somal side of the microfluidic chambers for 15 minutes at 37ºC. After a 4 hours incubation at 37ºC, the plating media was removed and neurobasal media with glutamate (neurobasal medium supplemented with 2% B27, 25 µM glutamate, 0.5 mM glutamine and 1:400 penicillin/streptomycin) was added to the somal side and glutamate-free neurobasal media (neurobasal medium supplemented with 2% B27, 0.5 mM glutamine and 1:400 P/S) was added to the axonal side, because the high concentrations of glutamate could induce excitotoxicity in growing axons.

2.2.3 Pseudo explants preparation

The pseudo explants technique used for the hippocampal cell cultures, consisted of a single cloning cylinder placed on top of a glass coverslip (Marienfeld No.1 12 mm). The cloning cylinders were manufactured by SP Bel-Art. Coverslips were prepared as described above. Before the plating step, the coverslips were coated with 0.1 mg/ml PDL overnight at 37°C and then washed several times with sterile mQ H_2O. The cloning cylinders before being used, they were washed thoroughly with sterile mQ H_2O, then with 75% ethanol and were left to dry. After they were completely dry, the cloning cylinders were sterilized using a UV lamp for 30 minutes. The cylinders could then be used to plate cells.

Plating media is added to the microwells which had the coverslips that were previously coated with PDL (0.1 mg/ml). Then the cloning cylinders were placed on top of these coverslips very carefully with sterile tweezers and $2,5x10^5$ cells were then added inside the cylinders. After a 4 hours incubation at 37ºC, the cloning

cylinders and the plating media was removed and neurobasal media with glutamate (neurobasal medium supplemented with 2% B27, 25 µM glutamate, 0.5 mM glutamine and 1:400 penicillin/streptomycin) was added.

At DIV 3, 50 µl of 5-FDU in glutamate-free neurobasal media was added to the pseudo explants cultures at a final concentration of 10 µM.

2.3 Induction of synapse formation

The microfluidic chambers allow a fluidic isolation of the hippocampal neurons between the axonal and the somal compartment, and so this technique allows a better study of isolated axons. With the intent of studying protein synthesis, FGF22 (2 nM) and PDL coated Beads which are two different presynaptogenic approaches were used. These two synaptogenic cues were added only to the axonal compartment and a minimal volume difference between the somal and the axonal compartment was maintained during the stimulus time to avoid the diffusion of the applied cues to the other compartment. These cues were incubated during different time points depending on the experiment at 37ºC.

2.3.1 Immunocytochemistry

Neurons were fixed with 4% PFA for 10min at room temperature. The cells were then washed three times with PBS [1x] for 5 minutes and then PBS [1x] – triton 0,25% was added for 5 minutes to permeabilize the cells. The cells were washed once again with PBS [1x] for 5 minutes and then to block non-specific binding, BSA 3% was added for 40min. Primary antibodies solution was incubated overnight at 4°C in PBS with 3% BSA. After the incubation with primary antibodies, the coverslips were washed three times with PBS [1x] for 5 minutes, then incubated with secondary antibodies in PBS with 3% BSA for 1 hour at room temperature. Then the coverslips were washed two times with PBS [1x] – triton 0,1% for 5 minutes and once with PBS [1x] for 5 minutes. After all these steps, the coverslips were rinsed with mQ H_2O and they were mounted in the lamina using mounting media. The preparations were cured overnight at 4°C protected from light, sealed with nail polish and kept at 4°C until microscopy analysis.

2.3.2 Protein Synthesis Assay Kit

The hippocampal neurons were stimulated with FGF22 or PDL coated Beads under the described conditions and afterwards, were treated with the Click-iT Plus OPP Protein Synthesis Assay Kit from Thermo Fisher according to the manufacturer's instructions. Briefly, the Click-iT OPP (Component A) was diluted in cell culture medium to prepare a 20 µM working solution. The medium containing the stimulus was removed and the working solution was added to the neurons and incubated for 30 minutes at 37ºC. Afterwards, the medium was removed and the cells were washed once with PBS. Next, then the neurons were fixed with PFA 4% and incubated for 15 minutes at room temperature, washed and incubated with PBS [1x] – triton 0,25% for 15 minutes at room temperature.

The last step was the Click-It OPP detection, which involved the detection of the O-propargyl-puromycin with the use of a fluorescent antibody. A reaction cocktail needs to be prepared 15 minutes prior to its use (Table 3). The permeabilization buffer was removed and the neurons were washed once with PBS. Then the reaction cocktail was added to the neurons for 30 minutes at room temperature, protected from light. To finalize, the reaction cocktail was removed and the cells were washed with the Reaction Rinse Buffer (Component F). Afterwards, immunochemistry continued as described previously.

Tabela 4 - Click-iT Plus reaction cocktail (Adapted from Thermo Fisher - 'Click-iT Plus OPP Protein Synthesis Assay Kits' protocol)

Reaction components	Number of coverslips/Number of wells of a 96-well plate			
	1 coverslip/ 10 wells	5 coverslips/ 50 wells	10 coverslips/ 100 wells	20 coverslips/ 200 wells
Click-iT® OPP Reaction Buffer (1X concentrate, prepared in step 1.4)	880 µL	4.4 mL	8.8 mL	17.6 mL
Copper Protectant (Component D)	20 µL	100 µL	200 µL	400 µL
Alexa Fluor® picolyl azide (Component B)	2.5 µL	12.5 µL	25 µL	50 µL
Click-iT® Reaction Buffer Additive (1X solution, prepared in step 5.1)	100 µL	500 µL	1 mL	2 mL
Total reaction volume	**1 mL**	**5 mL**	**10 mL**	**20 mL**

2.3.3 Microscopy and quantification

The fluorescent images were taken using an inverted Zeiss Axiovert 200 microscope, an AxioCam HRm camera and ZEN Blue 2011 software. The objective used in all the experiments was a 63x oil objective (1.4 NA) and the exposure times were conserved between individual conditions in single experiments. The images regarding microfluidic chambers were taken from the axonal side. The proteins puncta were measured with the use of the Image J software. All the axons were randomly chosen from exported 8-bit images and the selected ones had similar morphology, the fragmented axons were rejected. To avoid a selection bias, this axonal selection was done in the neuronal marker channel, without the observation of any puncta channel. The threshold of every image was conserved in a single experiment and the number was then quantified. In each condition between 10 and 12 axons were analysed. The puncta number was determined in each selected axon by puncta per axonal length. Quantification process was performed in a condition-blind manner. For co-localization analysis, an in-house produced ImageJ macro was used. This allows to measure the overlap between two or more proteins of interest. Results can then be expressed as the number of particles that co-localized per axonal length. The values per axon were normalized against the control mean of

each experiment. Each graph has the indication of the number of experiments and the number of axons analysed.

2.3.4 Statistical analysis

Results are presented as normalized means ± SEM of at least 3 independent experiments performed in distinct preparations. Graphs and statistical analysis were performed in Graph Pad Prism 7 software. Statistical significance was assessed by unpaired t-test for the comparison of two groups, and one-way ANOVA analysis for comparisons between multiple groups followed by the Bonferroni´s post-test. A $p<0.05$ value was considered statistically significant.

2.4 Lentivirus

2.4.1 Cell transformation

The plasmid DNA of interest (pRR-EGFP-L10 or VGlut mcherry) was added to DH5α competent cells, gently mixed and incubate in ice for 30 minutes. Then the cells are placed in a 42ºC bath for 45 seconds and then in ice for 2 minutes, this thermic shock allows the entrance of the plasmid into the cells. LB broth was added to the cells and the solution was agitated at 225 rpm, 37ºC for 1 hour. This solution was then centrifuged for 30 seconds at 1000 rpm and the cells were resuspended in LB broth. The cells were then placed in LB agar plates with antibiotic (ampicillin) and were incubated overnight at 37ºC. An isolated colony was selected from the LB agar plate and it was added to LB broth supplemented with ampicillin 100 ug/mL and placed to grow in a shacking incubator at 37ºC overnight at 225 rpm/min. The following day the bacterial solution was centrifugated in a XYZ centrifuge with a JA 14 rotor at 4000 x g for 10 minutes, and then the cell pellet was stored at -20ºC.

2.4.2 Plasmid purification

The purification of the DNA plasmids consisted in the use of a Maxiprep kit from Invitrogen. The Maxiprep procedure consisted in the use of columns with filters to isolate de plasmid DNA of interest. The column was equilibrated with an

equilibration buffer and the cell pellet previously prepared was resuspended with a resuspension buffer supplemented with ribonuclease. Lysis buffer was added to the cell solution, this step promotes cell lyses and the release of the plasmid, and then it was added the precipitation buffer. This precipitated lysate was transferred to the column and allowed to flow by gravity through the column filter, which captures the DNA plasmid. The column was washed extensively with wash buffer. Finally, the elution buffer was added to the column to elute the DNA plasmid and the resulting solution mixed with isopropanol and centrifuged at 12000 x g for 30 minutes at 4ºC. The supernatant was discarded and the pellet containing pellet washed with 70%. The final step was to resuspend the plasmid pellet in DNase/RNase-Free Water.

2.4.3 HEK293T cell preparation

HEK293T cells were defrosted in a 37 °C bath, diluted in HEK culture media pH 7.2 (6.7g/500ml DMEM, 5ml/500ml sodium pyruvate, 1.85g/500ml $NAHCO_3$, 50ml/500ml FBS, 1250ml/500ml Pen/Strep), plated, and transferred to a cell culture incubator, after 2 hours the media was replaced with fresh one. When reaching 80-90 % confluency cells they were split, by removing the cell media and washing with PBS [1x]. The PBS was aspirated and a 0.05% trypsin solution was added and left to act for ~2 min, the cells were then released from the plate by dispensing culture media, dissociated and plated again at the desired concentration. The day before transfection the cells were split so that they would be 80% confluent the following day and HEK media without antibiotic was added 30 minutes to 3 hours before transfection.

2.4.4 Lentivirus production

i. Calcium phosphate transfection

For calcium phosphate transfection, two separate polystyrene tubes were prepared. In tube A was added ddH_2O, then the DNA plasmids (concentration of the plasmids of interest and helpers is indicated in Table 4) and finally 2M $CaCl_2$ drop-wise, In tube B contained 2x HBS pH 7.05 (50mM Hepes, 280mM NaCl, 1.5mM Na2HPO4). The solution from tube A containing the DNA plasmid was added drop-wise to the

tube B, then the solution was vortexed for a few seconds and incubated at room temperature for 30 minutes. Finally, the prepared mixture was added drop-wise onto the cells and after 5 hours it was removed and HEK media without antibiotic was added.

Tabela 5 - DNA plasmid concentration for transfection

PLASMID OF INTEREST	F(SYN)WRBN-VGLUT1MCHERRY	20 µg
	PRRL-EGFP-L10A	20 µg
HELPER PLASMIDS I	PMDLG/PRRE	10 µg
	PRSV-REV	10 µg
	PCMV-VSV-G	10 µg
HELPER PLASMIDS II	PLP1	10 µg
	PLP2	10 µg
	VSV	10 µg

ii. Virus harvesting

The cell media containing the virus was harvested after the transfection at 24/48/72 hours and fresh media without antibiotic was added on the cells after each harvest. The medium containing the virus was kept at 4ºC in between the harvests and after the final pool, it was filtered with a 45 µm cellulose syringe filter.

iii. Virus concentration

The virus was concentrated by ultracentrifugation of the solution from ii) in a refrigerated BeckMan Coulter L-100 XP ultracentrifuge with a swinging bucket rotor

(SW41) at 22,000 RPM, 22°c for 2 hours. Ultracentrifugation was performed in Ultra-clear sterile BeckMan tubes and the buckets for the rotors were washed thoroughly in Virkon before and after ultracentrifugation. After ultracentrifugation, the supernatant from the tubes was gently decanted into Virkon. In each tube it was added sterile PBS[1x]-1%BSA and the tubes were vortexed at room temperature for 40 minutes to resuspend the virus. Finally, virus aliquots of 10 µl and 20 µl were stored at -80ºC

2.4.5 Cell transduction

A sufficient volume of virus was stored on ice or at 4°C until it had almost entirely thawed before being used to transduce hippocampal neurons. The virus was diluted using the cell media, dropped into the cells dropwise and placed in the incubator at 37ºC for 72 hours.

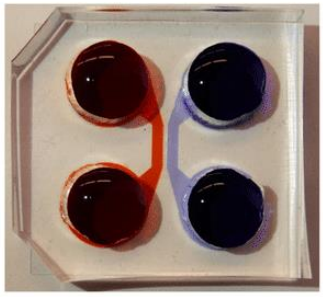
3 Results and Discussion

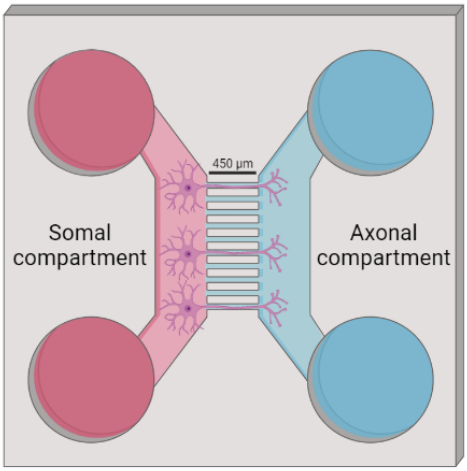
3.1 Microfluidic chambers

In order to study axons, microfluidic chambers are one of the most reliable techniques because it's a system that allows fluidic isolation and the physical separation between the cell body/dendrites and the axons (Taylor *et al.*, 2005; Pinto and Almeida, 2016).

The microfluidic chambers are made from molded PDMS and they are divided in two compartments, the somal compartment where the cell body is located and the axonal compartment where the axons grow into. These compartments are connected by microgrooves (450 µm long), which allow only the passing of fluids and axons because of their longer size. The compartments, each measuring 1.5 mm wide, 7 mm long and 100 µm height, have at the ends two reservoirs that store culture medium (Fig. 9A). Hippocampal neurons were grown for 5-6 days in the microfluidic chambers, at this point the neurons were fixed and immunostained for tubulin (red) and stained for DNA (blue). Several tiles of images were taken from a random area of the microfluidic device and then they were merged to obtain a representative image of the cultures (Fig. 9B) (Martins *et al.*, 2017). Analysing the image allows to have a better understanding of how microfluidic chambers work, on the left side we can observe the cell bodies and dendrites, whereas on the right side we can recognize isolated axons. This image demonstrates the physical barrier created by the microgrooves, which allows us to better study isolated axons.

A





B

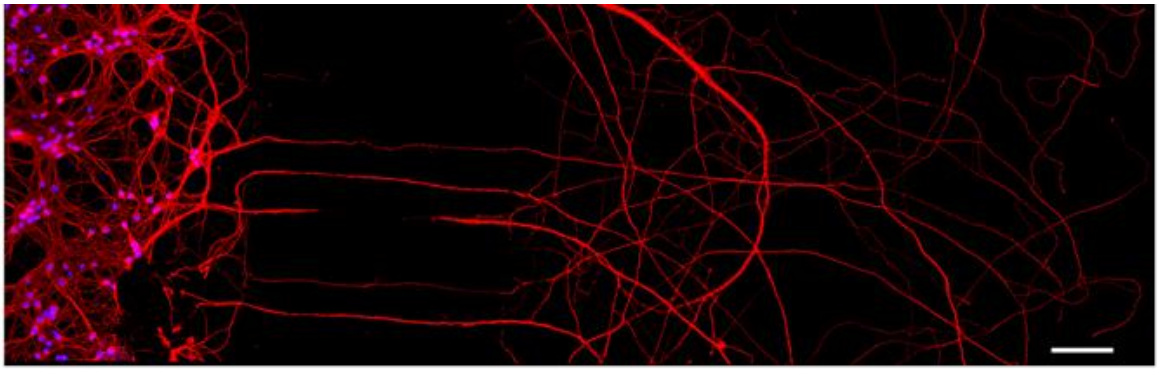


Figure 9 - Hippocampal neurons cultured in microfluidic chambers (A) Representative images of microfluidic chambers models. This model consists in a PDMS mold place against a glass coverslip. The chambers are composed of a somal compartment (red) and an axonal compartment (blue), each 1.5 mm wide, 7 mm long, that are divided by microgrooves (450 µm long, 10 µm wide). Hippocampal neurons are platted in the somal compartment and the axons start to grow through the microgrooves into the axonal compartment. The slight difference in heights between the compartments and the microgrooves, combined with the volume difference between both compartments, allow the fluidic isolation needed for the study of isolated axons. (B) Representative image of cortical neurons cultured in microfluidic chambers (Martins et al., 2017). Cortical neurons were immunostained for tubulin (red) and stained for DNA (blue) and images were taken using a Zeiss Axiovert 200 fluorescent microscope. The image shows that cell bodies are restricted to the somal compartment while in the opposite compartment only axons are observed. The scale bar is 100 µm.

3.2 Pseudo-explants

Pseudo-explants cultures are a reliable and easy technique, that allows the physical separation between the cell body and the axons (Fig. 10A). In comparation with the microfluidic chambers, this technique doesn't allow fluid separation between the somal and axonal compartment but is a more accessible technique to study axons. Hippocampal neurons were grown for 7-8 days, at this point the neurons were fixed and immunostained for MAP2 (red), β-Tubulin III (green), DAPI (blue) and several tiles of images were taken from a random area of the coverslip and then they were merged to obtain a representative image of the cultures (Fig. 10A). Analysing the image allows to have a better understanding of how the cells bodies are separated from the axons, to allow a better study of isolated axons.

A

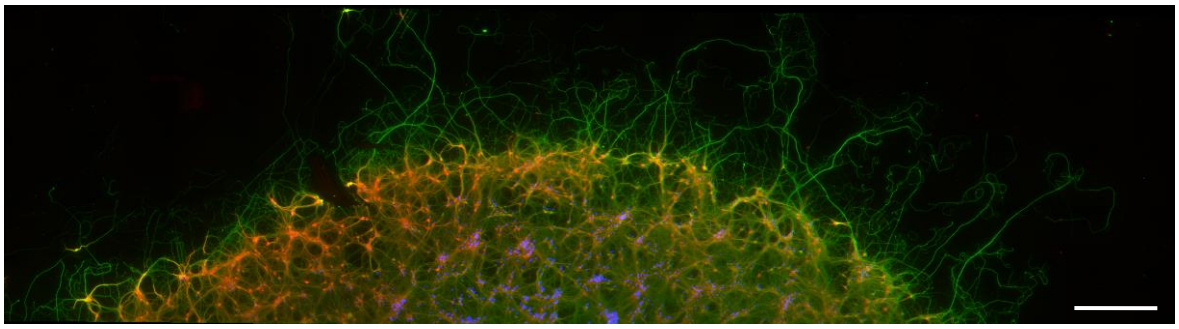


Figure 10 - Pseudo-explants cultures. (A) Representative image of hippocampal neurons grown as pseudo-explants. This representation allows to better understand how neurons are platted in pseudo-explants and we can observe in the middle of the petri dish the cell bodies and dendrites localized centrally, whereas the axons grow to the periphery. This model allows us study isolated axons. Primary cultures of rat embryonic hippocampal neurons were grown in pseudo-explants for 7/8 days. The cells were immunostained for Tubulin III (green), MAP2 (red) and DAPI (blue) and images were taken using a Zeiss Axiovert 200 fluorescent microscope. Scale bar is 500 µm.

3.3 Presynaptic Differentiation with FGF22 and PDL-Beads

3.3.1 FGF22 induced presynaptic differentiation

As previously mentioned in the introduction, FGF22 induces presynaptic differentiation (Fox *et al.*, 2007). Primary cultures of rat embryonic hippocampal neurons were grown in pseudo-explants until DIV7/8. The neuronal cultures were stimulated with 2 nM of FGF22 for 14 hours at 37ºC and they were immunostained for Synapsin a presynaptic marker, and β-Tubulin III, an neuronal marker (Fig. 11A). BSA 0,1% was used as a control. Synapsin are major peripheral proteins localized on the cytoplasmic face of synaptic vesicles and they have a crucial role in directing vesicles in the reserve pool and active zone, but also mediate at the latest stages of exocytosis the coupling release events associated with the action potentials (Thiel, 1993; Bykhovskaia, 2011). Stimulation of the neurons with 2 nM of FGF22 for 14h induced an increase in synapsin puncta number (150.1% ± 14.76) (Fig. 11B), puncta integrated density sum corrected (187.3% ± 22.09) (Fig. 11C), puncta area sum (166.9% ± 18.38) (Fig. 11D), puncta perimeter sum (156% ± 17.13) (Fig. 11E) and puncta mean area (144.7% ± 12.77) (Fig. 11F) per axonal length.

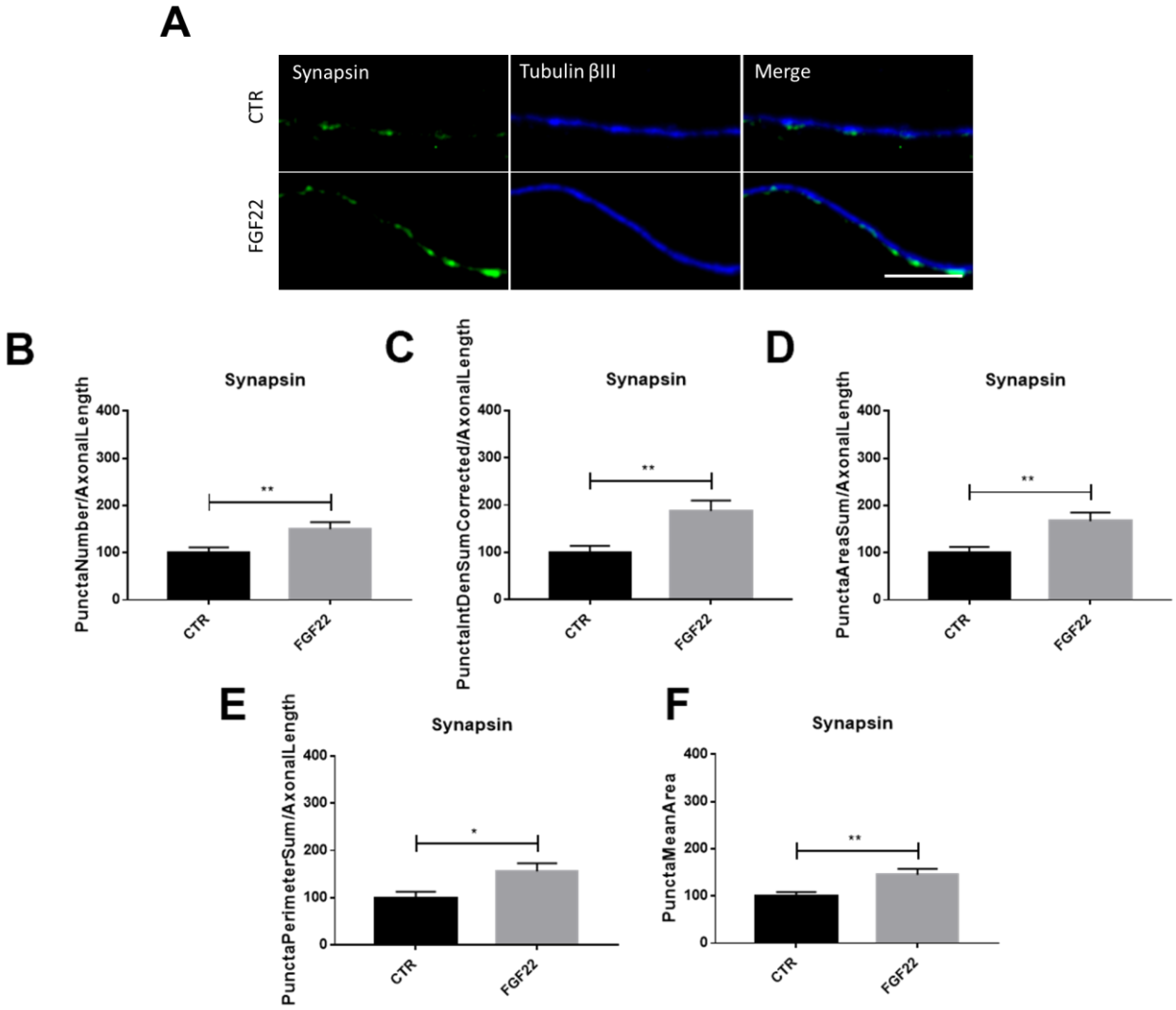


Figure 11- FGF22 induces synapsin clustering. (A) FGF22 induces the formation of presynaptic clusters. Primary cultures of rat embryonic hippocampal neurons were grown in pseudo-explants for 7/8 days. The neurons were then stimulated with 2 nM of FGF22 for 14 hours at 37ºC. The cells were immunostained for β-Tubulin III (blue) and Synapsin (green) and images were taken from axons using a Zeiss Axiovert 200 fluorescent microscope. (B-F) Presynaptic markers analysis. Several parameters were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from six independent experiments. The values indicate that FGF22 when applied in a neuronal culture increase the levels of synapsin. *Represents $p < 0.05$, **Represents $p<0.01$ by unpaired t-test when compared to

Ctr. Non-sense (ns.) represents p>0.05 by t test when compared to the control values. The scale bar is 5 µm.

To further confirm our results, we next stimulated primary cultures of rat embryonic hippocampal neurons with 2 nM of FGF22 for 14 hours at 37ºC, and cells were immunostained against VGlut1 (red), a presynaptic marker, and β-Tubulin III (blue), an neuronal marker (Fig. 12A). Vesicular glutamate transporter 1 (VGlut1) is an active transporter that is responsible for glutamate vesicular storage in synaptic vesicles (Satomi *et al.*, 2017). The immunostaining of this protein, which is associated to synaptic vesicles, allows the study of vesicle clustering in presynaptic differentiation studies. We observed an increase in puncta number (210.3% ± 26.91) (Fig. 12B), puncta integrated density sum corrected (290.2% ± 69.52) (Fig. 12C) puncta area sum (227.5% ± 52.78) (Fig. 12D), puncta perimeter sum (297.4% ± 66.68) (Fig. 12E) per axonal length. Puncta mean area per axonal length is not significatively different (82.5% ± 10.29) (Fig. 12F). The fact that the puncta mean area of the VGlut1 is not significative, maybe be explained by the increase of the other parameters, which show that there is an increase in the number of puncta but their area is not significatively changed.

The increase in the clustering of synapsin and VGlut1, both synaptic vesicles markers, show a sign of mature and functional presynaptic terminals. In conclusion, our results demonstrate that stimulation with FGF22 induces an increase in presynaptic differentiation.

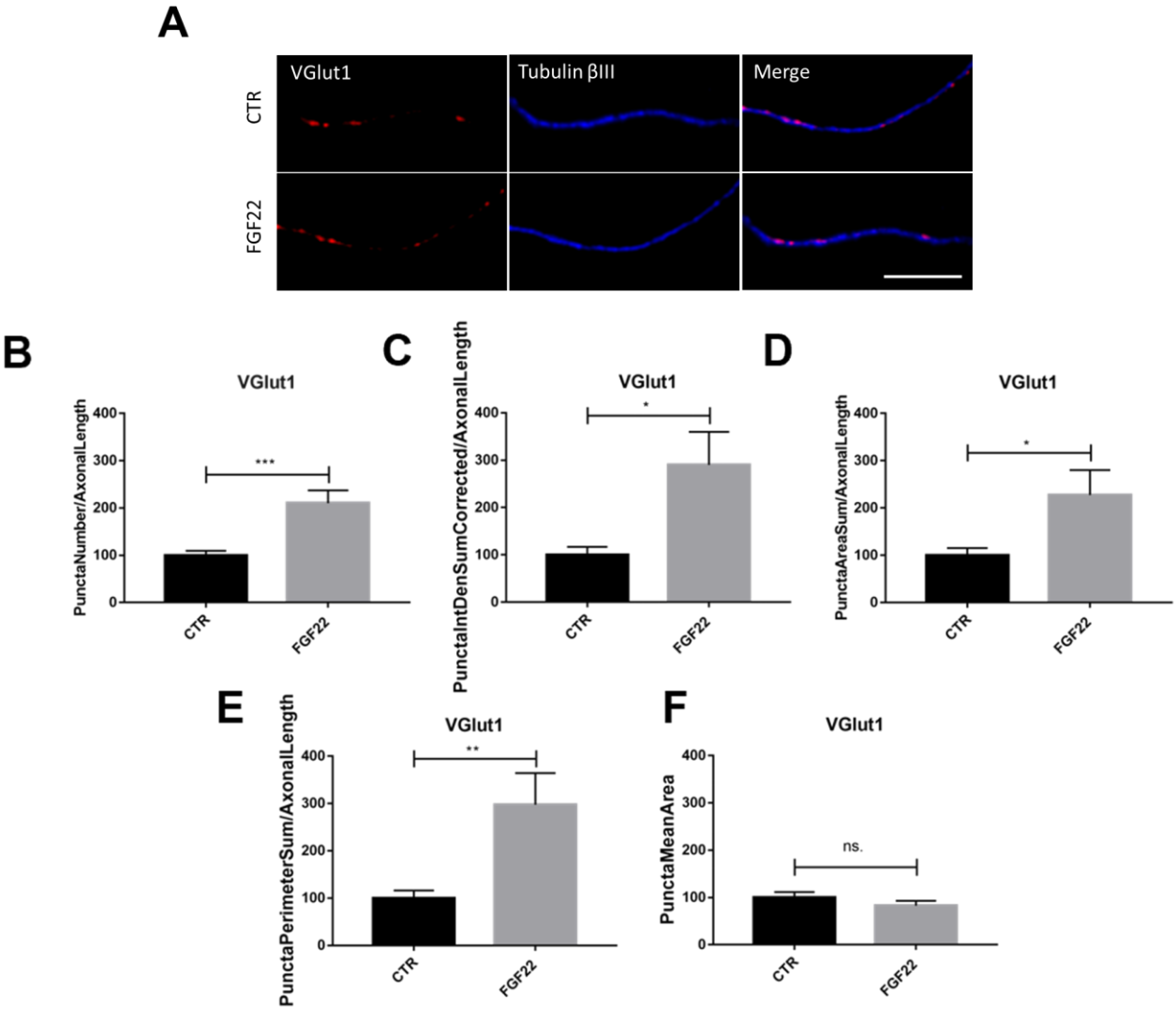


Figure 12- FGF22 induces Vesicular glutamate transporter 1 clustering. (A) FGF22 induces the formation of presynaptic boutons. Primary cultures of rat embryonic hippocampal neurons were grown in pseudo-explants for 7/8 days. The cultures were then stimulated with 2 nM of FGF22 for 14 hours at 37ºC. The cells were immunostained for β-Tubulin III (blue) and VGlut1 (red) and images were taken from axons using a Zeiss Axiovert 200 fluorescent microscope. (B-F) Presynaptic marker analysis. Several parameters were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from six independent experiments. The values indicate that FGF22 when applied in a neuronal culture have a synaptogenic effect on axons. *Represents $p < 0.05$, **Represents $p<0.01$, ***Represents

p<0.001 by unpaired t-test when compared to Ctr. Non-sense (ns.) represents p>0.05 by t test when compared to the control values. The scale bar is 5 µm.

3.3.2 PDL Beads induced presynaptic differentiation

Previous studies show that PDL coated Beads are capable of inducing presynaptic differentiation. The beads have a more localized action and this presynaptic capability is induced through contact (Lucido *et al.*, 2009).

Primary cultures of rat embryonic hippocampal neurons were grown in pseudo-explants until DIV7/8. The cell cultures were stimulated with 30 µl of PDL coated Beads for 14 hours at 37ºC and they were immunostained for Synapsin, VGlut1 and β-Tubulin III (Fig. 13A). A ROI similar to the dimension of the beads was delineated around the bead and considered “On-bead”. As a control we used a ROI with the same dimensions in an adjacent axonal region, which we designated as “Off Bead”. The mean fluorescence in the On-bead/Off-bead was analysed. We observed that PDL coated Beads increases synapsin mean fluorescence per bead (228.1% ± 16.72) (Fig. 13B). Regarding VGlut1 values we can observe an increase in the mean fluorescence per bead (354.6% ± 28.26) (Fig. 13C). The clustering of both these two proteins is significantly increased in the regions where a bead is contacting the axon, which indicates that presynaptic differentiation is occurring in those specific sites.

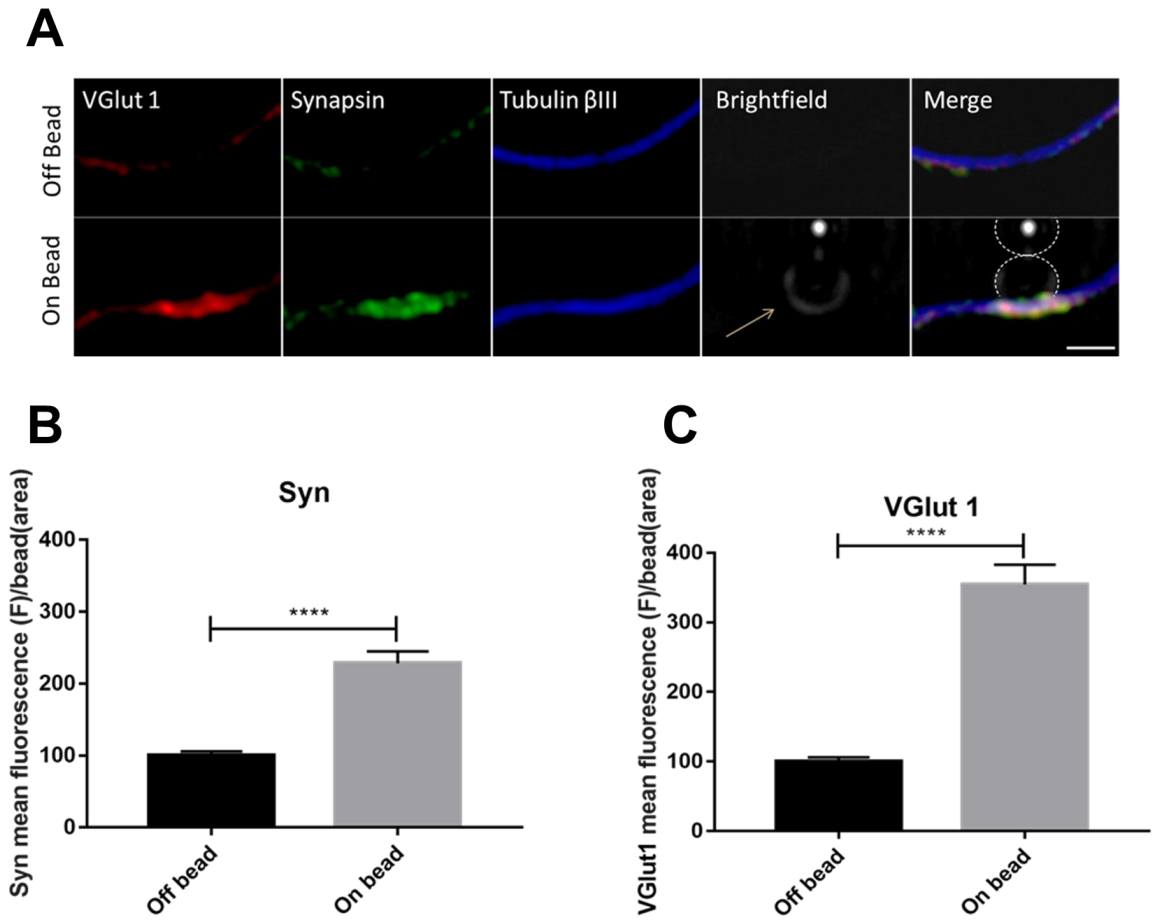


Figure 13 – PDL-coated beads induced presynaptic differentiation. (A) PDL-coated beads induce the formation of presynaptic clusters. Primary cultures of rat embryonic hippocampal neurons were grown in pseudo-explants for 7/8 days. The axons were then stimulated with 30 µl of PDL-coated Beads for 14 hours at 37ºC. The cells were immunostained for Tubulin III (blue), VGlut1 (red) and Synapsin (green) and images were taken from axons using a Zeiss Axiovert 200 fluorescent microscope. The arrow of the brightfield channel and the circle in the merge channel indicate the localization of a Bead. (B-C) Measurement of presynaptic markers. The mean fluorescence per bead were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 30 axons from three independent experiments. The values indicate that PDL Beads when applied in a neuronal culture have a synaptogenic effect on axons. ****Represents $p<0.0001$ by unpaired t-test when compared to Ctr. The scale bar is 2 µm.

3.4 Analyze if synaptogenic cues induce local mRNA translation

3.4.1 FGF22 induces newly synthetised proteins

Lyles et al. using invertebrates neurons demonstrated that clustering of sensorin mRNA at nascent synapses is necessary for synaptic development and maintenance (Lyles, Zhao and Martin, 2006). We wanted to access at what time point there was an increase in protein synthesis, to achieve this goal we used OPP as a protein synthesis marker. Primary rat embryonic hippocampal neurons were cultured until DIV 7/8 in microfluidic chambers. The axons were stimulated with 2 nM of FGF22 in the axonal compartment for 5, 10, 20, 30 and 60 minutes at 37ºC and then immunostained for OPP (yellow), Neurofilament (blue) (Fig. 14B). BSA 0,1% was used as a control. OPP method consists in the incorporation of a O-propargyl-puromycin (OPP) in the nascent peptides, causing premature chain termination during translation taking place in the ribosome. O-propargyl-puromycin resembles tRNA, it enters the ribosome and transfers to the growing chain, causing premature chain release. Then the antibodies recognize O-propargyl-puromycin and this technique allows to mark the sites where protein synthesis is occurring. Afterwards, an antibody recognizes OPP and we can detect the formation of newly synthetised proteins (Fig. 14A).

The results show an increase in OPP puncta integrated density sum corrected (323.1 ± 48.57) (Fig.14D) and in the puncta area sum (214.3 ± 33.77) (Fig.14E) at 30 minutes. Whereas in the levels of puncta number there is no significative difference (Fig.14C).

Analysing these results, allow us to conclude that with a 30 minutes stimulus with FGF22 there is the formation of newly synthetised proteins.

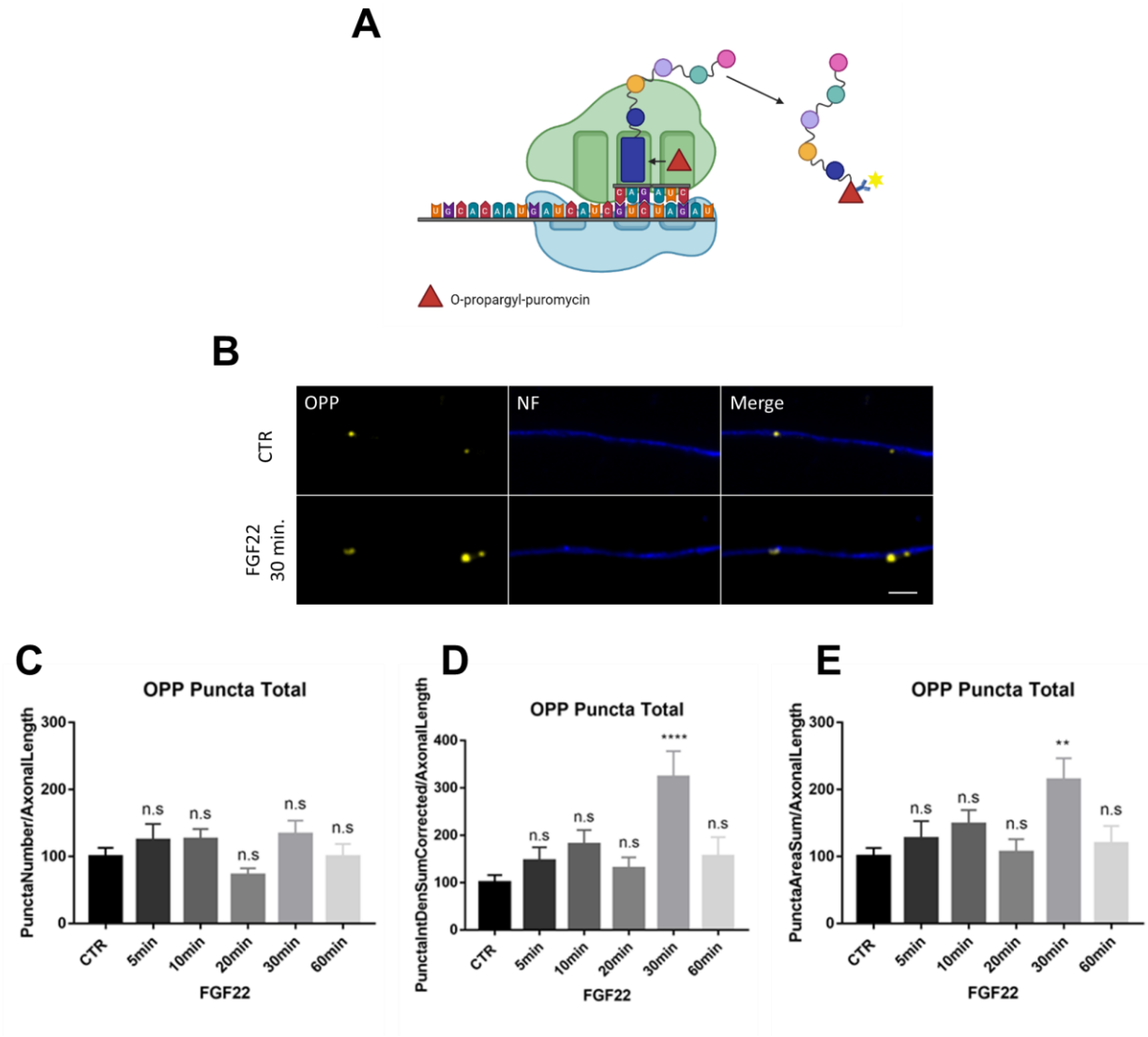


Figure 14 - FGF22 induces new proteins synthesis. (A) OPP method. Incorporation of O-propargyl-puromycin (red) in the nascent peptides, causing premature chain termination during translation taking place in the ribosome. (B) FGF22 induces newly protein synthesis. Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers for 7/8 days. The axons were then stimulated with 2 nm for 5, 10, 20, 30 and 60 minutes at 37ºC. The cells were immunostained for Neurofilament (blue), OPP (yellow), and images were taken of the axons using a Zeiss Axiovert 200 fluorescent microscope. (C-E) Measurement of protein synthesis. Several parameters were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate that

with a stimulus with FGF22 for 30 minutes there is an increase of protein synthesis. **Represents p<0.01, ****Represents p<0.0001 by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents p>0.05 by ANOVA using Bonferroni´s post-test when compared to control values. The scale bar is 2 µm.

3.4.2 FGF22 induces the intra-axonal mRNA translation

A parallel strategy was used to study mRNA translation, the levels of p-4E-BP1, a mRNA translation marker. All nuclear transcribed RNAs have a 5' methylated GTP cap that binds to the translation factor eIF4E. In the unphosphorylated state 4E-BP1 is bound to eIF4E, which sequesters this initiating factor and prevents translation. Specific stimuli lead to the phosphorylation of the mTOR which, in turn, phosphorylates and inactivates 4E-BP1, releasing eIF4E and enhancing translation. Therefore, p-4E-BP1 staining is commonly used as a model for local translation (Fig.15A).

Primary rat embryonic hippocampal neurons were cultured until DIV 7/8 in microfluidic chambers. The neurons were stimulated with 2 nM FGF22 in the axonal compartment for 20 and 30 minutes at 37ºC and they were immunostained for p-4E-BP1 (yellow), Neurofilament (blue) (Fig. 15B). Regarding the values of the p-4E-BP1, we highlight the increase in puncta integrated density sum corrected of p-4E-BP1 (150.5 ± 19.9) (Fig. 15D). However, there is no significative difference regarding the puncta number (Fig.15C) and puncta area sum (Fig.15E). We can conclude that there is an increase in mRNA translation at 20 minutes.

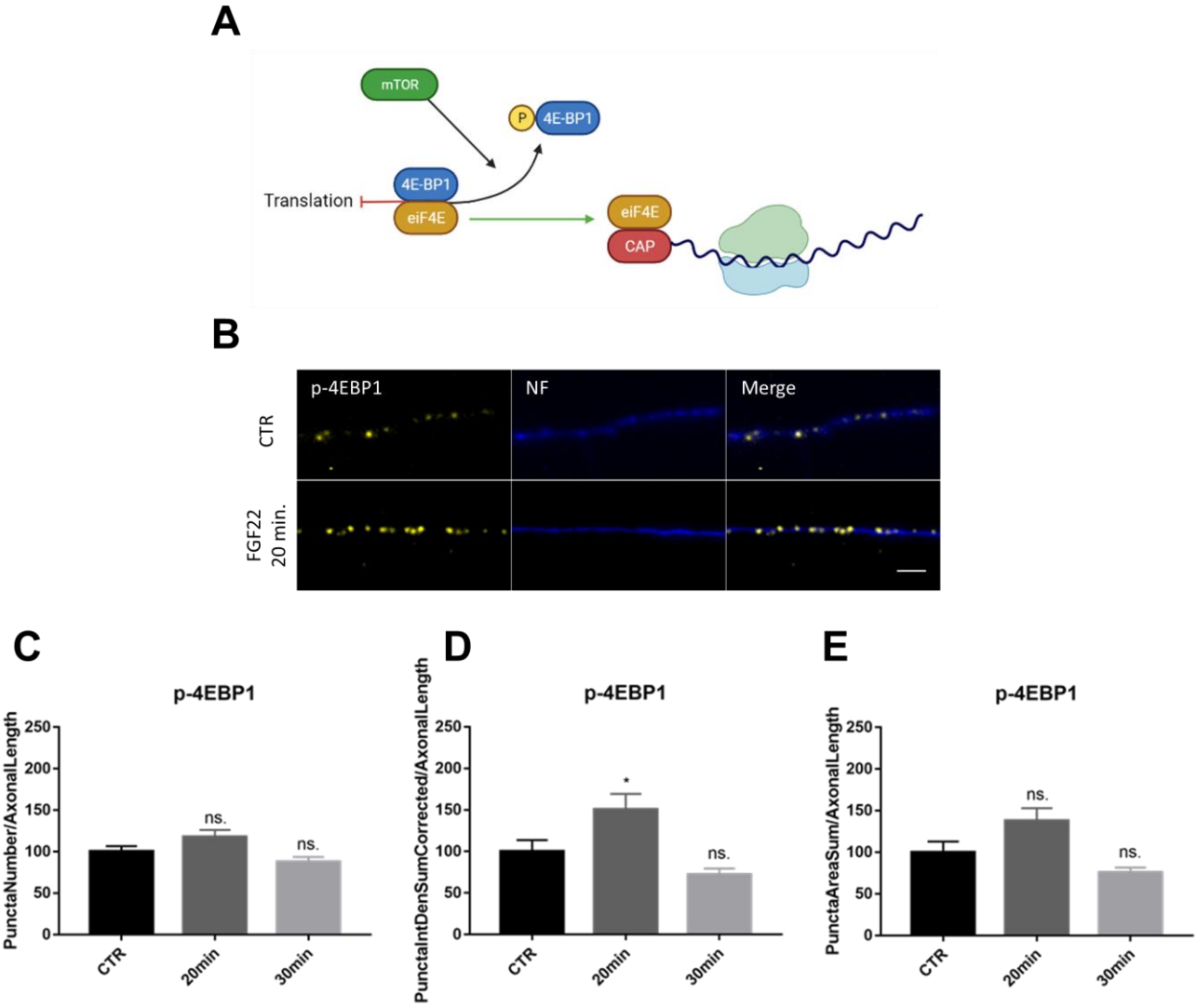


Figure 15 - FGF22 induces the intra-axonal mRNA translation. (A) Phosphorylated-4E-BP1 pathway. The immunostaining against p-4E-BP1 allows the detection of mRNA translation. A specific stimulus allows the phosphorylation of 4E-BP1, which in terms permits the releasing of the initiation factor eiF4E and it allows mRNA translation to occur. (B) FGF22 induces mRNA translation. Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers for 7/8 days. The axons were then stimulated with 2 nm for 20 and 30 minutes at 37ºC. The cells were immunostained for Neurofilament (blue), p-4E-BP1 (yellow), and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. (C-E) Measurement of mRNA translation during presynaptic differentiation. Several parameters were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were

normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate that with a stimulus with FGF22 for 20 minutes there is an increase of mRNA translation. *Represents $p< 0.05$ by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents $p>0.05$ by ANOVA using Bonferroni´s post-test when compared to control values. The scale bar is 2 µm.

3.4.3 Contact-induced synapse formation induces intra-axonal mRNA translation

A parallel strategy we used PDL coated Beads, which also promotes the formation of presynaptic differentiation but their action mechanisms are distinct. PDL coated Beads induce synaptogenesis through contact with axons. Also, their action is very localized allowing to pinpoint the site of contact.

Primary rat embryonic hippocampal neurons were cultured until DIV 7/8 in microfluidic chambers. The neurons were stimulated with 30 µl of PDL coated Beads in the axonal compartment for 5, 15, 35 and 60 minutes at 37ºC and they were immunostained for OPP (yellow), Neurofilament (blue) (Fig. 16B),. During the 35 and 60 minutes stimulus, we took in account the 30 minutes required for OPP binding(Fig. 16A). The immunostaining of OPP allows the detection of newly synthetised proteins.

The results allow us to conclude that a PDL coated beads stimulus promotes a significative increase in OPP. We observe t that with a 60 min. stimulus, the OPP mean fluorescence per bead (117.6 ± 4.08) (Fig. 16C) had a significative increase. Regarding the experiment in Figure 16D, we used a ROI (region of interest) with a radius of 6 µm to quantify the mean fluorescence, which is the approximated radius of the bead (5µm). Whereas, in the experiment in Figure 16C, we used a ROI with double the radius with the objective to analyse if we were losing any signal to the vicinity. In the experiment of Figure 16D, we used the same images as the previously described and we also observed a significant increase in all the markers, and at 60 minutes stimulus there was a peak in the increase in OPP mean fluorescence per

bead (150.3 ± 8.54).. We can conclude that the PDL-coated beads are able to induce through contact the formation of newly synthetised proteins.

In the experiment of Figure 14E, we wanted to check if there was a consistency with the previous results and so, we stimulated the axons with the PDL coated Beads in the last 5/15 minutes of the 30 minutes incubation with the OPP in an independent experiment. The results demonstrate that there is a tendency to an increase in the OPP mean fluorescence per bead.

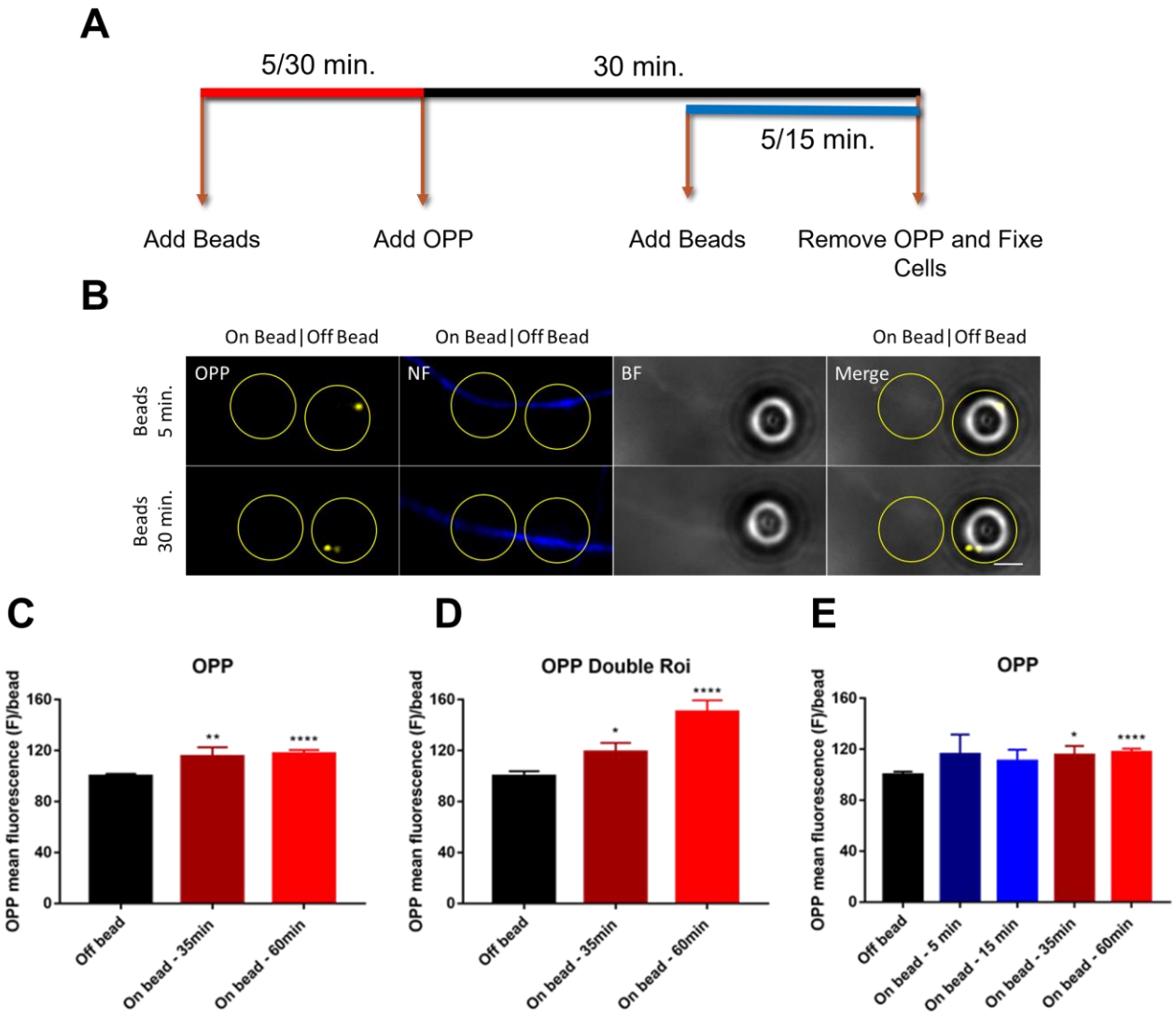


Figure 16 - PDL Coated Beads induces the formation of newly synthetised proteins in presynaptic boutons. (A) Schematic representation of the experiments. The black bar represents the 30 minutes in which the OPP is added to the axonal compartment, the red

bar indicates when the beads were added to the axons for 5 *and* 30 minutes plus the 30 minutes required for the OPP takes his action and the blue bar represents the addition of the beads in the last 5 *and* 15 minutes of the 30 minutes incubation with the OPP. (B) Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers for 7/8 days. The axons were then stimulated with 30 µl of PDL coated Beads for 5, 15, 35 and 60 hours at 37ºC. The neurons were immunostained for Neurofilament (blue), OPP (yellow), and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. The yellow circles represent the on bead and off bead. In the brightfield channel we can identify the bead. (C-E) Measurement of the protein synthesis marker. The mean fluorescence per bead were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Black and Red bars represent the mean ± SEM of a minimum of 30 axons from three independent experiments, whereas blue bars represent the mean ± SEM of a minimum of 10 axons from a single experiment. The values indicate that stimulus with PDL Beads promote an increase in protein synthesis. *Represents $p< 0.05$, ****Represents $p<0.0001$ by ANOVA using Bonferroni´s post-test when compared to control. The scale bar is 2 µm.

3.5 Determine if intra-axonal mRNA translation colocalizes with presynaptic clusters

3.5.1 FGF22 induces the formation of newly synthetised proteins in presynaptic sites

We next sought to investigate if local protein synthesis and presynaptic differentiation co-localize in developing axons. For this, we used OPP as a protein synthesis marker and as presynaptic markers VGlut1 and Synapsin. Primary rat embryonic hippocampal neurons were cultured until DIV 7/8 in microfluidic chambers. The axons were stimulated with 2 nM of FGF22 in the axonal compartment for 5, 10, 20, 30 and 60 minutes at 37ºC and they were immunostained for OPP (cyan), Synapsin (green), VGlut1(red) and Neurofilament (blue) (Fig. 17A). BSA 0,1% was used as a control.

We observed colocalization between OPP, Synapsin and VGlut1 at 30 minutes regarding puncta number (210.4 ± 39.32) (Fig. 17B), puncta integrated density sum corrected (422 ± 81.12) (Fig. 17C) and puncta area sum (268.6 ± 58.74) (Fig. 17D). There was also colocalization between OPP and VGlut1 at 30 minutes in the puncta number (215.6 ± 38.83) (Fig. 17F), puncta integrated density sum corrected (457.3 ± 87.43) (Fig. 17G) and puncta area sum (295 ± 60.52) (Fig. 17H). These results demonstrate an increase of co-localization of local protein synthesis and newly formed presynaptic clusters.

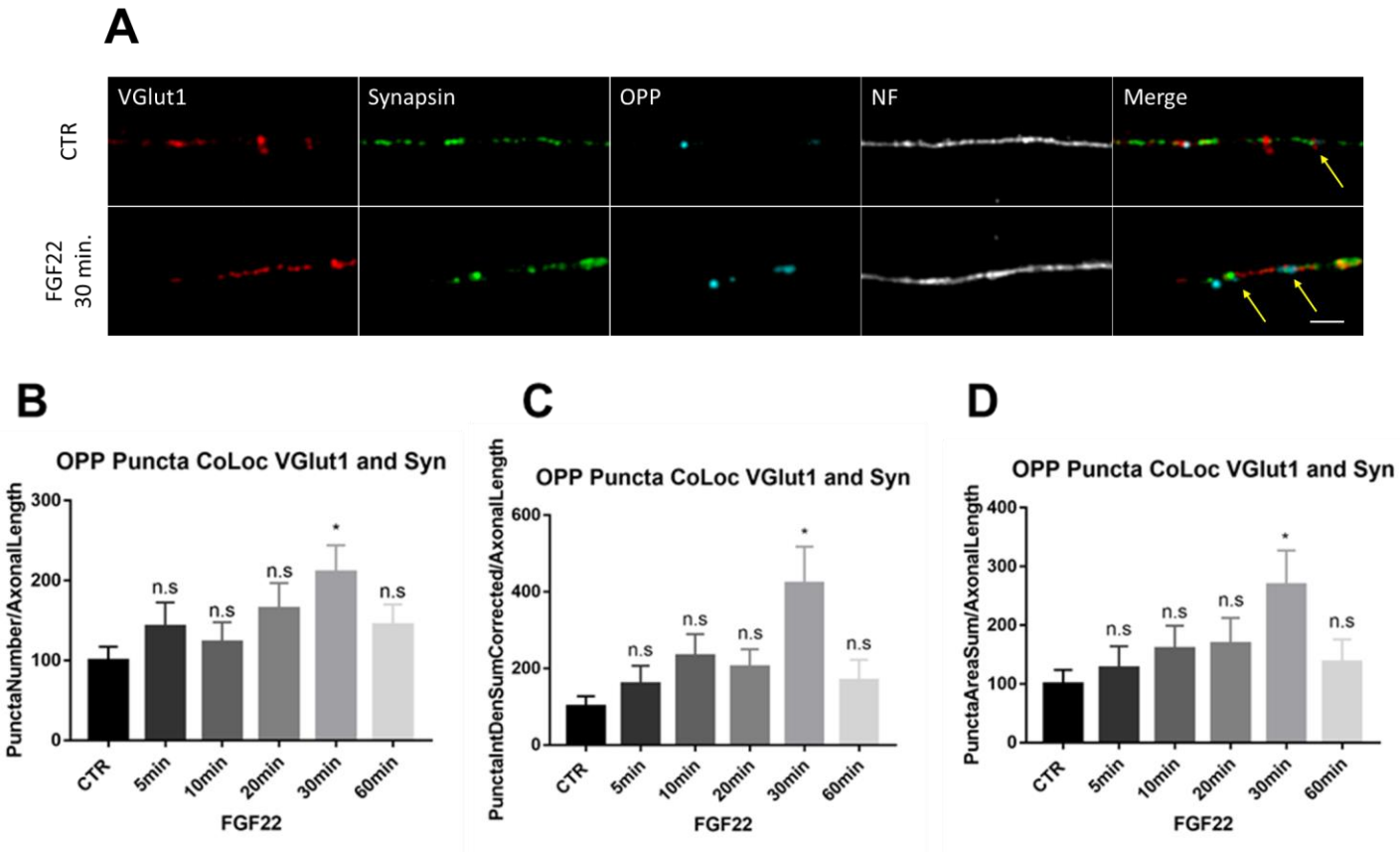


Figure 17 - FGF22 induces the formation of newly synthetised proteins in presynaptic sites. (A) FGF22 induces protein synthesis in presynaptic clusters. Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers for 7/8 days. The axons were then stimulated with 2 nm for 5, 10, 20, 30 and 60 minutes at 37ºC. The cells were immunostained for Neurofilament (blue), OPP (cyan), VGlut1 (red) and Synapsin (green) and images were taken of the axons using a Zeiss Axiovert 200 fluorescent microscope. The yellow arrows indicate triple colocalization. (B-D) Measurement of co-localization of protein translation in presynaptic sites. Several parameters were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate that with a stimulus with FGF22 for 30 minutes there is an increase of protein synthesis in presynaptic clusters. **Represents p<0.01, ****Represents p<0.0001 by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents p>0.05 by ANOVA using Bonferroni´s post-test when compared to control value. The scale bar is 2 µm.

3.5.2 FGF22 induces mRNA translation in presynaptic clusters

It is known that mRNA is transported to the axon, in addition, it was shown that protein synthesis machinery is also present in axons. We next aimed to evaluate if there was a colocalization between a mRNA translation marker (p-4E-BP1), a presynaptic marker (VGlut1) and an active zone marker (Bassoon). Bassoon is a scaffolding protein of the cytomatrix assembled at the active zone (CAZ) where neurotransmitters are released.. Primary rat embryonic hippocampal neurons were cultured until DIV 7/8 in microfluidic chambers. The neurons were stimulated with 2 nM FGF22 in the axonal compartment for 20 and 30 minutes at 37ºC and then immunostained for p-4E-BP1 (cyan), Bassoon, VGlut1 and Neurofilament (blue) (Fig. 18A). By analysing the results, we can conclude that FGF22 stimulation for 20 min induces a significant increase in the colocalization between p-4E-BP1, Bassoon and VGlut1 puncta number (149 ± 20.54) (Fig. 18B), puncta integrated density sum corrected (184.2 ± 36.55) (Fig. 18C) and puncta area sum (170.7 ± 31.65) (Fig. 18D). These results indicate that there is an increase in mRNA translation in presynaptic sites at 20 minutes.

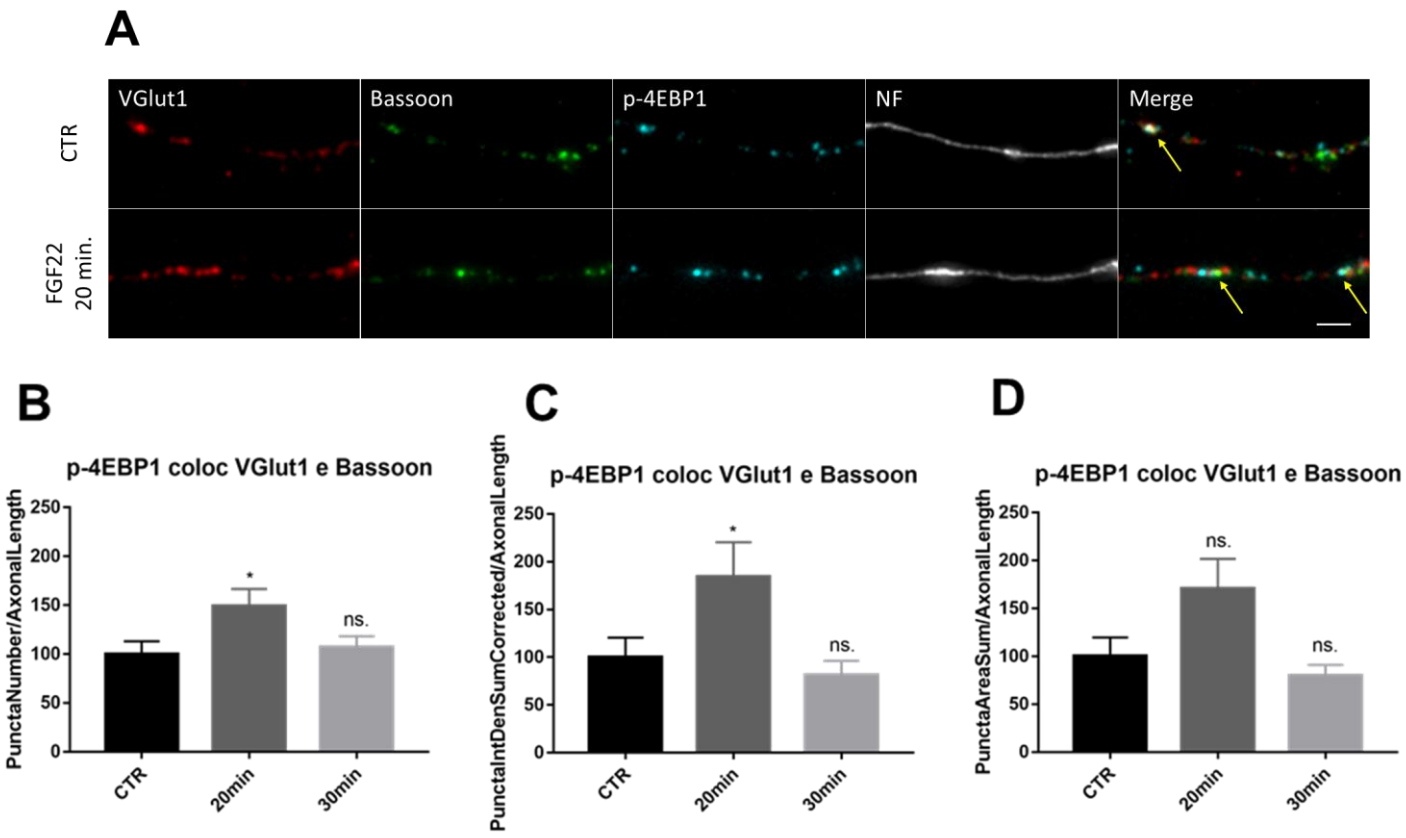


Figure 18 - Intra-axonal mRNA translation colocalizes with presynaptic clusters. (A) FGF22 induces mRNA translation in presynaptic clusters. Primary cultures of rat embryonic hippocampal neurons were grown in microfluidic chambers for 7/8 days. The axons were then stimulated with 2 nm for 20 or 30 minutes at 37ºC. The cells were immunostained for Neurofilament (blue), p-4E-BP1 (cyan), VGlut1 (red) and Bassoon (green) and images were taken from the axonal compartment using a Zeiss Axiovert 200 fluorescent microscope. The yellow arrows indicate triple colocalization. (B-D) Measurement of mRNA translation in presynaptic sites. Several parameters were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate that with a stimulus with FGF22 for 20 minutes there is an increase of mRNA translation in presynaptic clusters. *Represents $p < 0.05$ by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents $p > 0.05$ by ANOVA using Bonferroni´s post-test when compared to control values. The scale bar is 2 µm.

4 Conclusion and Future Perspectives

4.1 Conclusions

In this study, we demonstrated the existence of protein synthesis in presynaptic clusters. We firstly wanted to demonstrate that FGF22 and PDL coated Beads are capable of presynaptic differentiation as previously described in the literature. Secondly, we wanted to analyse of the dynamics of mRNA translation using a translational marker (OPP) and two presynaptic markers (Synapsin and VGlut1) in neurons stimulated with FGF22 or PDL-coated beads. We observed an increase in the colocalization levels of all three markers at 30 minutes in neurons stimulated with the FGF22 and indirectly we observed a similar increase when PDL coated Beads are added. However, PDL-coated Beads induce a stronger co-localization then FGF22. Strikingly we saw an increase firstly in the OPP levels at 30 minutes and then in the Synapsin and VGlut1 levels at 60 minutes with FGF22, suggesting that local mRNA translation may precede the formation of a presynaptic terminal.

Next, we used as a parallel strategy, we labelled neurons with an antibody against p-4E-BP1, which is part of the pathway that regulates/activates mRNA translation but is not connected to the protein synthesis machinery. We observed that with the use of FGF22 at 20 minutes, there was a significative increase in the levels of p-4E-BP1. This is in accordance with our hypothesis because the phosphorylation of 4E-BP1 precedes the OPP labelling in the mRNA translation pathway.

In conclusion, our results demonstrate that two distinct synaptogenic stimuli induce co-localization between translation reporters and presynaptic clusters. The levels of p-4E-BP1 increase at 20 minutes, suggesting that translation machinery is being activated, then at 30 minutes there was an increase in the OPP identifying newly synthetised proteins and after at 60 minutes there was an increase in the levels of presynaptic proteins (VGlut1 and Synapsin).

4.2 Future perspectives

According to our findings, it would be interesting to use of a live imaging approach to further explore our results. A potential experiment would consist in the production of a ribosomal reporter pRRL-eGFP-L10a and a presynaptic reporter VGlut1-mCherry in neurons stimulated with PDL-coated Beads. We expect that the ribosome reporter will appear in the axonal region contacting the Bead and immediately before VGlut1-mCherry accumulates at this spot. This would allow us to conclude that protein synthesis occurs in sites of presynaptic assembly (Fig. 19A). The next step would be to investigate if nascent β-actin peptides are produced (accumulated) at emerging synapses. To monitor the spatial distribution of protein synthesis we would use a sindbis translation reporter assay. The sindbis virus reporter gene-based assay consists of a myr-dEGFP fused to the 3'UTR of Beta-Actin, a destabilized EGFP containing a myristoylation domain sequence that limits the diffusion of the fluorescent protein from the site of translation (Fig.19B). The myr-dEGFP fusion protein has a half-life of one hour, thus fluorescent signals represent newly synthesized protein. We will infect organotypic slice cultures with the sindbis virus expressing myr-dEGFP-β-actin-3'UTR. If this reporter is translated just prior to synapse formation we expect to see an increase in GFP fluorescence when axons synapse with the target dendrites (Fig.19B).

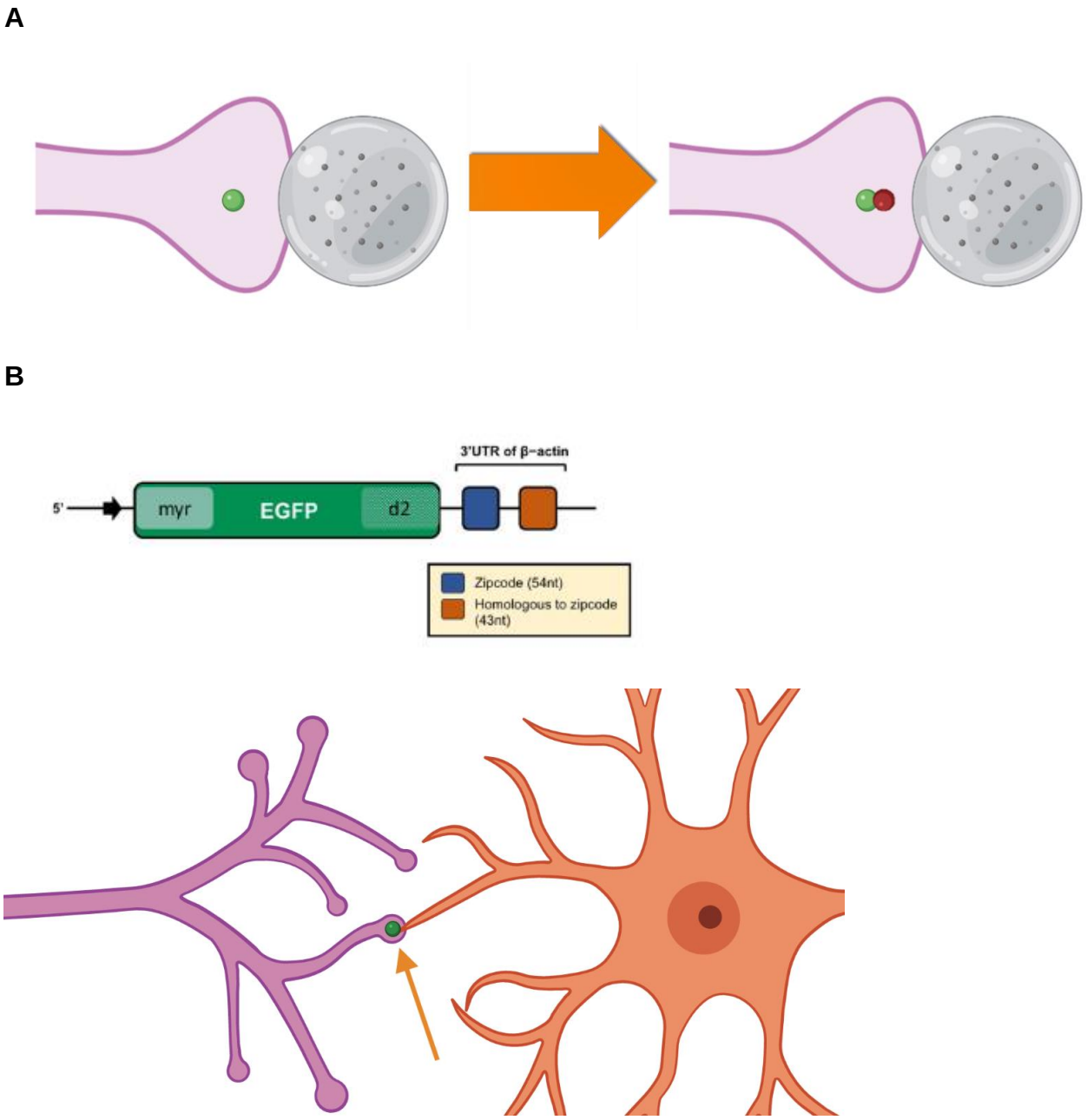


Figure 19 – Live imaging approach (A) Live imaging scheme to access if intra-axonal mRNA translation precedes the formation of a presynaptic bouton. With the use of a ribosomal reporter pRRL-eGFP-L10a (green) and a presynaptic reporter VGlut1-mCherry (red), this method will allow us to correlate local translation to sites of presynaptic assembly. (B) Nascent β-actin peptides dynamics at emerging synapses. The reporter expresses a destabilized (d2) form of EGFP fused to the 3'UTR of β-actin that gets anchored to the plasma membrane at the site of translation due to a C-terminal myristoylation sequence (myr). The 3'UTR of β-actin

contains a zipcode sequence described to be responsible for axonal localization of the mRNA. The yellow arrow indicates the expected results with the synthesis of Beta-actin mRNA during synapse formation with an increase in GFP fluorescence (green bead) when axons synapse with the target dendrites.

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Supplementary Figures

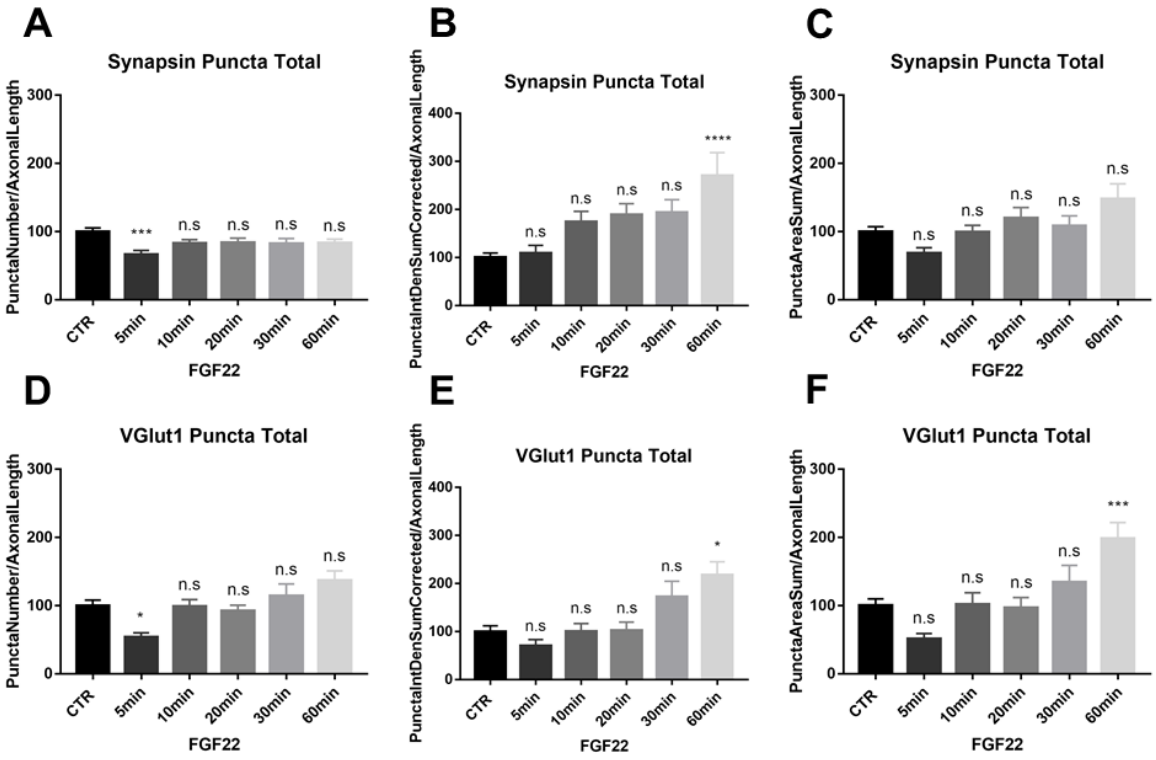

Figure 20 - Supplementary figure S1. FGF22 induces the formation of presynaptic sites. The levels of presynaptic markers were analysed by immunocytochemistry in axons of rat hippocampal primary cultures. Neurons (DIV7/8) were treated in the axonal compartment of microfluidic chambers with 2nM FGF22 for 5/10/20/30/60 minutes. (A-F) Quantitative data of the integrated density, number and area of VGlut1 and Synapsin per axonal length were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate that with a stimulus with FGF22 for 60 minutes there is an increase in the formation of presynaptic sites. *Represents $p < 0.05$, ***Represents $p<0.001$, ****Represents $p<0.0001$ by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents $p>0.05$ by ANOVA using Bonferroni´s post-test when compared to control values

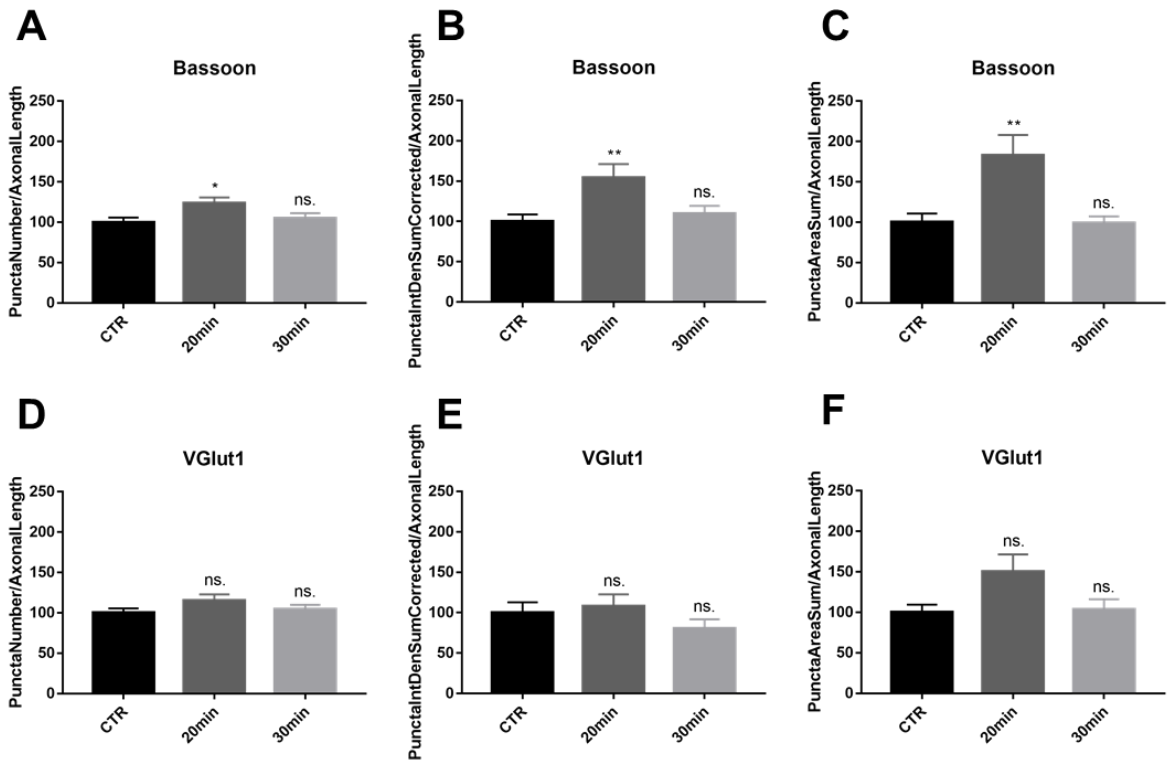

Figure 21 - Supplementary figure S2. FGF22 induces the formation of presynaptic clusters. The levels of presynaptic markers were analysed by immunocytochemistry in axons of rat hippocampal primary cultures. Neurons (DIV7/8) were treated in the axonal compartment of microfluidic chambers with 2nM FGF22 for 20 and 30 minutes. (A-F) Quantitative data of the integrated density, number and area of VGlut1 and Bassoon per axonal length were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate that with a stimulus with FGF22 for 20 minutes there is an increase of mRNA translation. *Represents $p< 0.05$, **Represents $p<0.01$ by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents $p>0.05$ by ANOVA using Bonferroni´s post-test when compared to control values.

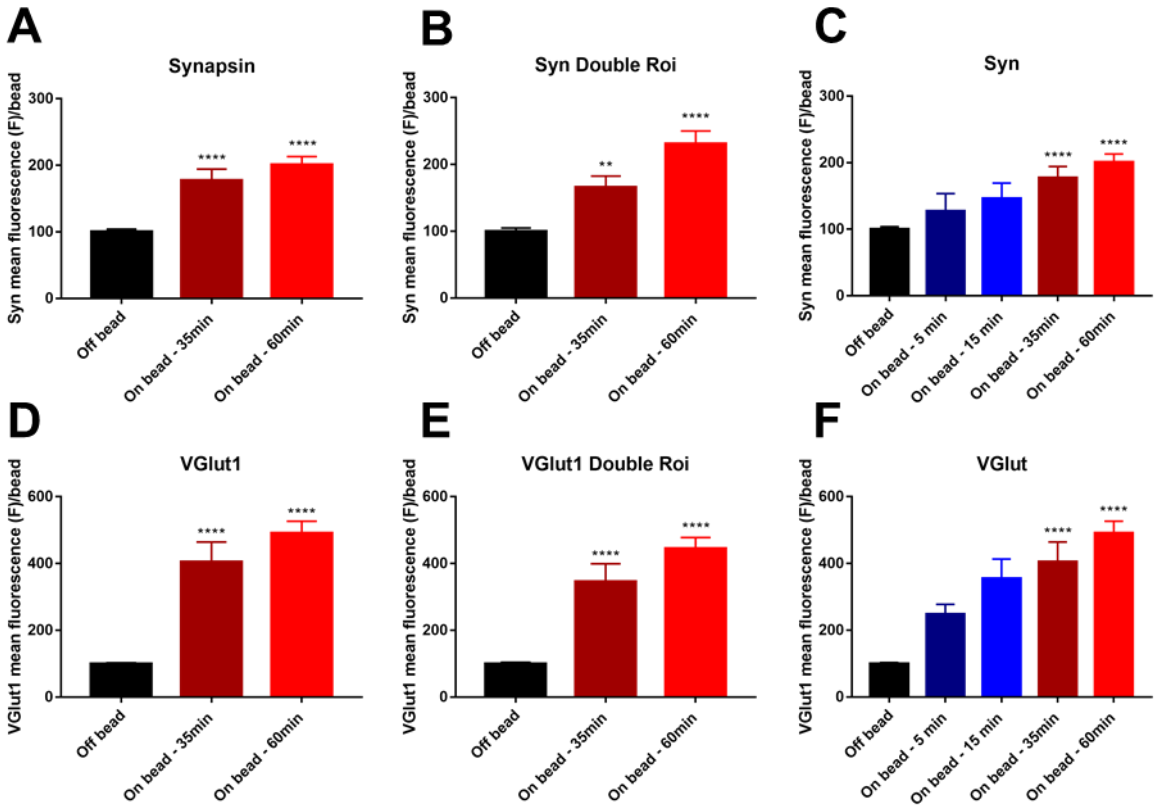

Figure 22 - Supplementary figure S3. PDL Coated Beads induces of presynaptic boutons. The levels of presynaptic markers were analysed by immunocytochemistry in axons of rat hippocampal primary cultures. Neurons (DIV7/8) were treated in the axonal compartment of microfluidic chambers with 30µl of PDL coated Beads for 5/15/35/60 minutes. (A-F) Measurement of the presynaptic boutons. The mean fluorescence per bead were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Black and Red bars represent the mean ± SEM of a minimum of 30 axons from three independent experiments, whereas blue bars represent the mean ± SEM of a minimum of 10 axons from a single experiment. The values indicate that stimulus with PDL Beads promote an increase in presynaptic differentiation. **Represents $p < 0.01$, ****Represents $p<0.0001$ by ANOVA using Bonferroni´s post-test when compared to control.

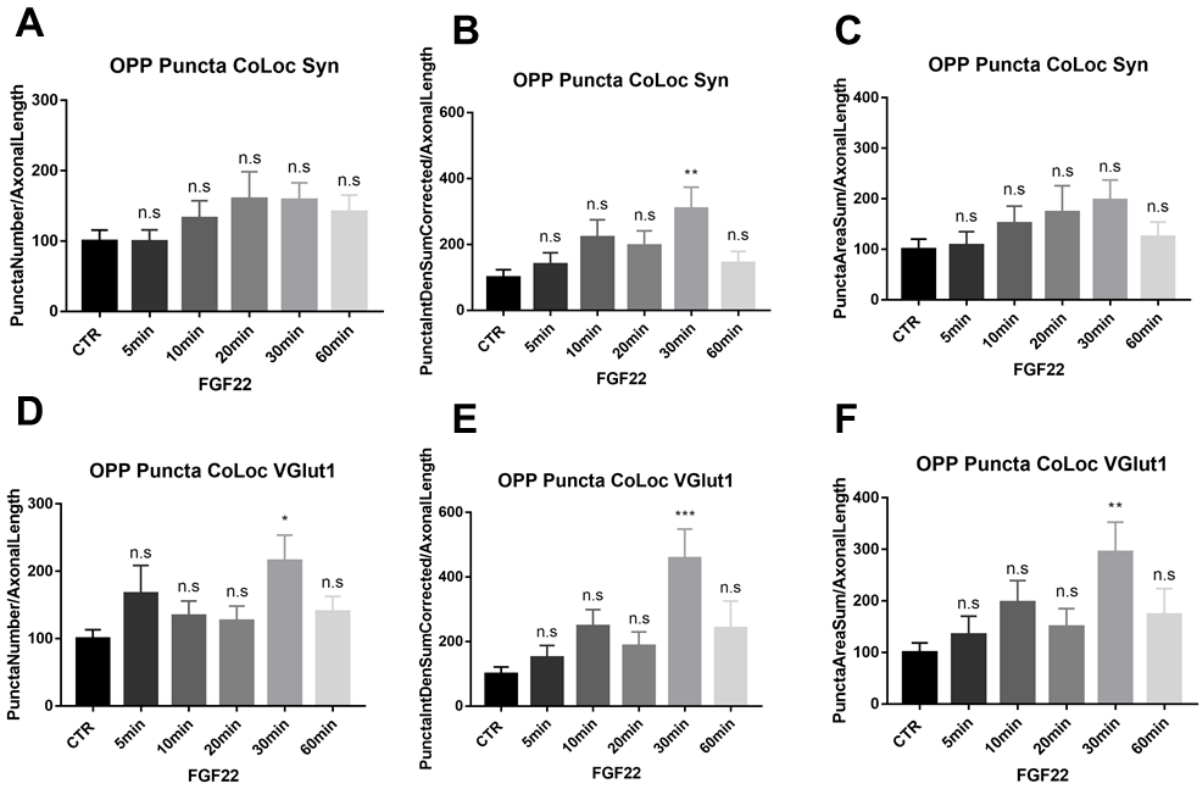

Figure 23 – Supplementary figure S4. FGF22 induces the formation of new proteins in presynaptic clusters. The levels of colocalization between a translational marker (OPP) and presynaptic markers (Synapsin and Vglut1) were analysed in axons of hippocampal primary cultures. Neurons were treated in the axonal compartment of microfluidic chambers with 2Nm FGF22 for 5/10/20/30/60 minutes. (A-F) Quantitative data of the colocalization regarding integrated density, number and area between OPP/Vglut1 and OPP/Synapsin per axonal length were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate the existence of colocalization between OPP and Synapsin at 30 minutes, and also between OPP and Vglut1 at the same time point. *Represents $p< 0.05$, **Represents $p<0.01$, ***Represents $p<0.001$ by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents $p>0.05$ by ANOVA using Bonferroni´s post-test when compared to control values.

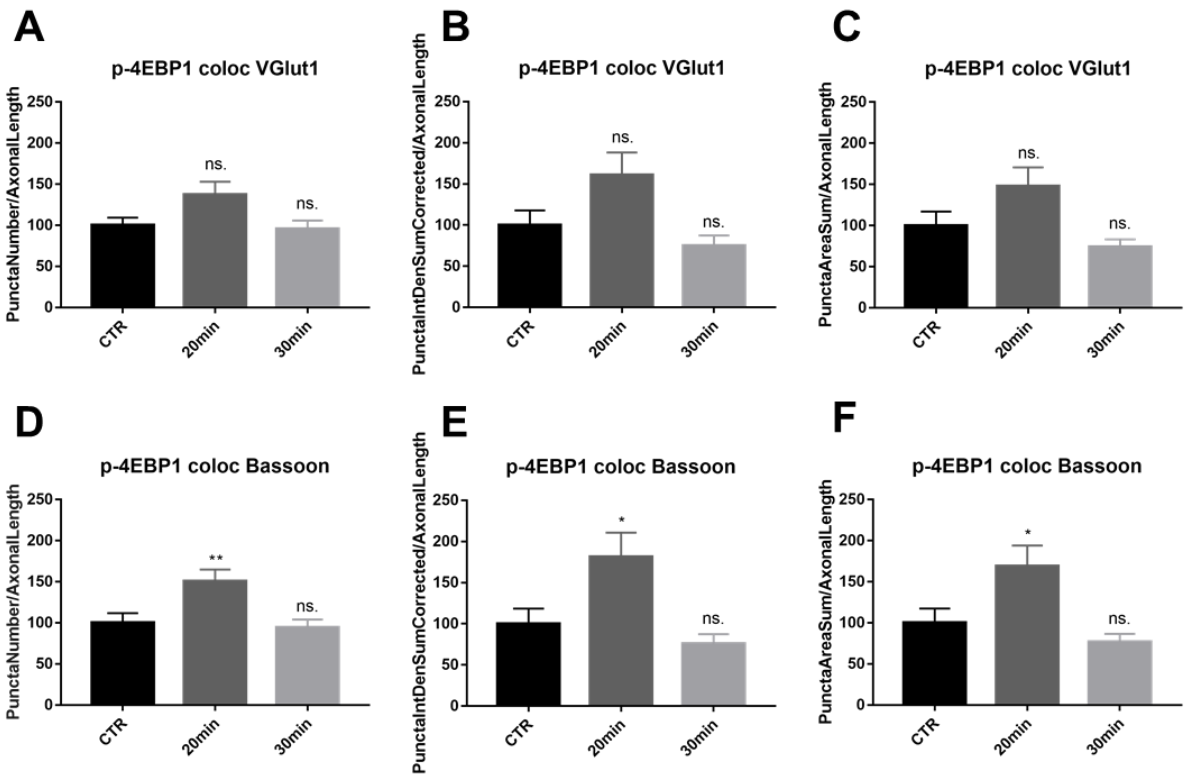

Figure 24 - Supplementary figure S5. FGF22 induces mRNA translation in presynaptic clusters. The levels of colocalization between a translational marker (p-4E-BP1) and presynaptic markers (Bassoon and VGlut1) were analysed in axons of hippocampal primary cultures. Neurons were treated in the axonal compartment of microfluidic chambers with 2nM FGF22 for 20 and 30 minutes. (A-F) Quantitative data of the colocalization regarding integrated density, number and area between p-4E-BP1/VGlut1 and p-4E-BP1/Bassoon per axonal length were measured using the software Image J 1.53 in randomly selected neurons (minimum 10 axons per experiment). Values were normalized to the control mean of each experiment. Bars represent the mean ± SEM of a minimum of 60 axons from three independent experiments. The results indicate the existence of colocalization between OPP and Bassoon at 20 minutes. *Represents $p < 0.05$, **Represents $p<0.01$ by ANOVA using Bonferroni´s post-test when compared to control. Non-sense (ns.) represents $p>0.05$ by ANOVA using Bonferroni´s post-test when compared to control values.