Universidade do Minho
Escola de Medicina

Andreia Filipa Rodrigues Batista

A role of local translation at presynaptic terminals

março de 2018

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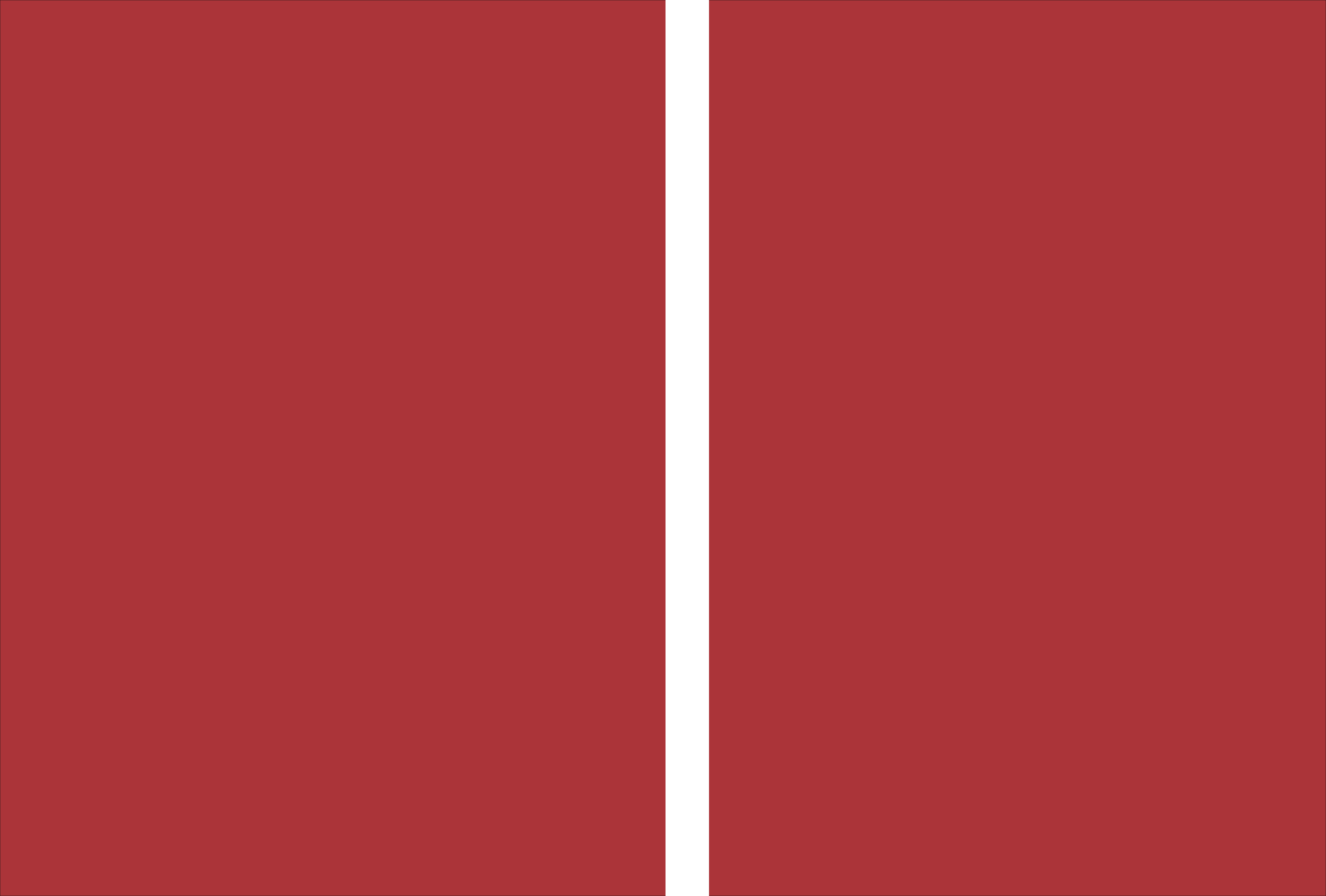

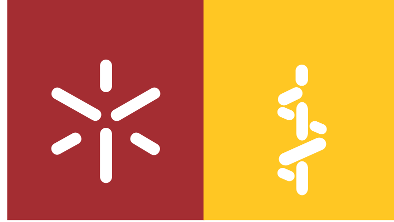

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A role of local translation at presynaptic terminals

Tese de Doutoramento em Medicina

Trabalho efetuado sob a orientação do
Professor Ulrich Hengst
e do
Professor Vitor Manuel da Silva Pinto

março de 2018

Declaração

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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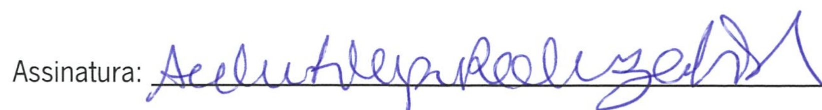

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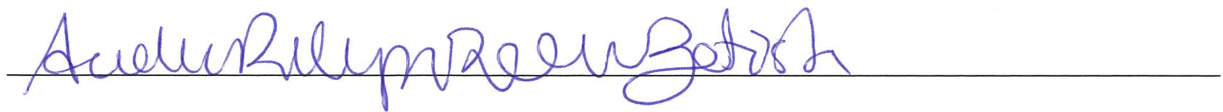

Em certa altura, chegou ao limite das terras até onde se aventurara sozinho.

(...) Dali para diante, para o nosso menino, será só uma pergunta:

«Vou ou não vou?» E foi.

A maior flor do mundo, José Saramago

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TITLE: A ROLE OF LOCAL TRANSLATION AT PRESYNAPTIC TERMINALS

ABSTRACT

Mechanisms of on-site protein production allow rapid subcellular responses to extracellular stimuli. This is especially relevant in polarized cells, such as neurons. During development, neurons extend axons to specific target areas and form an intricate network of connections, fundamental for nervous system function. Both axonal navigation and synapse formation are extremely fast processes that depend on target-derived cues. While local translation is important in regulating many aspects of axonal pathfinding, its role in presynaptic assembly is poorly understood.

We established that local translation is very rapidly induced in isolated axons upon contact with synaptogenic adhesive substrates. We were able to detect newly synthesized proteins within 15 minutes of contact and inhibiting protein synthesis interfered with the specific clustering of presynaptic proteins at contact sites. We found that the transcript for the t-SNARE protein SNAP25, required for vesicle exocytosis, localizes to axons and clusters around presynaptic specializations and we were able to directly visualize SNAP25 synthesis at these sites. We further demonstrated that inhibiting axonal SNAP25 synthesis interferes with presynaptic protein clustering and synaptic vesicles release dynamics.

This project uncovered a fundamental role for intra-axonal SNAP25 synthesis during synaptic terminal assembly. Furthermore, we have implicated local translation defects in delayed functional consequences, which opens up new possibilities relevant for the study of neurodevelopmental disorders.

TÍTULO: UM PAPEL DA TRADUÇÃO LOCAL NOS TERMINAIS PRÉ-SINÁPTICOS

RESUMO

Mecanismos de produção local de proteínas permitem respostas rápidas e compartimentalizadas a estímulos extracelulares. E são especialmente relevantes em células polarizadas, como neurónios. Durante o desenvolvimento, os neurónios projetam axónios para áreas específicas e formam uma rede de conexões, fundamental para o funcionamento do sistema nervoso. Tanto a navegação axonal como a formação sináptica são processos extremamente rápidos que dependem de sinais extracelulares derivados das células alvo. A tradução local é importante para muitos aspetos da navegação axonal, mas o seu papel na formação sináptica ainda não é bem conhecido.

Usámos um substrato adesivo para induzir diferenciação sináptica em axónios isolados e estabelecemos que a tradução local é rapidamente ativada após o contato. Conseguimos marcar e detetar proteínas sintetizadas *de novo* 15 minutos após o contato e a inibição da síntese proteica interferiu com a acumulação específica de proteínas pré-sinápticas nos locais de contacto. Mostrámos que o mRNA da proteína SNAP25, envolvida na exocitose de vesículas, é transportado para os axónios e acumula-se em torno das especializações pré-sináticas e conseguimos ainda visualizar diretamente a síntese de SNAP25 nestes locais. Mostrámos que a inibição da síntese axonal de SNAP25 afeta a acumulação de proteínas pré-sináticas e interfere com a exocitose de vesículas sinápticas.

Com este projeto demonstrámos que a síntese axonal de SNAP25 desempenha um papel fundamental na formação do terminal pré-sináptico. Adicionalmente, mostrámos que efeitos na tradução local podem ter consequências funcionais tardias, o que gera novas hipóteses de relevo no estudo de doenças do desenvolvimento.

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ABBREVIATION LIST

τ: time constant

μg: microgram

μL: microliter

μM: micromolar

μm: micrometer

°C: degrees celsius

4E-BP1: eukaryotic initiation factor 4E-binding protein 1

Aβ: β-amyloid

ADHD: adult attention deficit disorder

AUC: area under the curve

ATF4: activating transcription factor 4

AZ: active zone

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

Ca2+: calcium

CaMKIIα: Ca2+/calmodulin-dependent protein kinase alpha subunit

$CaCl_2$: calcium chloride

CB1: type 1 cannabinoid receptor

cDNA: complementary DNA

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CNS: central nervous system

CPEB1: cytoplasmic polyadenylation element-binding protein-1

CREB: cyclic AMP-responsive element-binding protein

DAPI: 4',6-diamidino-2-phenylindole

DIC: differential interference contrast

DIV: day *in vitro*

DL-AP5: DL-2-Amino-5-Phosphonovaleric acid

DNA: deoxyribonucleic acid

DRG: dorsal root ganglion

E17.5: embryonic day 17.5

eCB: endocannabinoid

EDTA: ethylenediaminetetraacetic acid

EGFP: enhanced green fluorescent protein

ER: endoplasmic reticulum

Erk: extracellular signal-regulates kinase

FISH: fluorescence *in situ* hybridization

FM4-64: (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide)

GABA: gamma-aminobutyric acid

GAP-43: growth associated protein 43

GFP: green fluorescent protein

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

HSPG: heparan sulfate proteoglycans

IF: immunofluorescence

IMPA-1: myo-inositol monophosphatase-1

IU: international unit

K^+: potassium

KCl: potassium chloride

LTD: long-term depression

LTF: long-term facilitation

LTP: long-term potentiation

M: molar

MAP2: microtubule-associated protein 2

MBP: myelin basic protein

MES: 2-(N-morpholino)ethanesulfonic acid

mg: milligram

$MgCl_2$: magnesium chloride

mL: milliliter

mM: millimolar

mRNA: messenger ribonucleic acid

mTOR: mammalian target of rapamycin

Munc13: mammalian uncoordinated 13

NaCl: sodium chloride

NGF: nerve growth factor

nM: nanomole

nm: nanometer

NMDA: N-methyl-D-aspartate receptor

n.s.: not significant

p-4EBP1: phospho-4EBP1

P0.5: postnatal day 0.5

P7.5: postnatal day 7.5

Par3: partitioning-defective 3

PBS: phosphate-buffered saline

PDL: poly-D-lysine

PFA: paraformaldehyde

PLA: proximity ligation assay

pmol: picomole

PNS: peripheral nervous system

PSD-95: postsynaptic density protein 95

PTV: piccolo-bassoon transport vesicle

Puro-PLA: puromycylation with proximity ligation assay

qPCR: quantitative polymerase chain reaction

RBP: RNA-binding protein

RGC: retinal ganglion cell

RhoA: ras homolog family member A

RIM: Rab3-interacting molecule

RIPA: radioimmunoprecipitation assay

RNA: ribonucleic acid

RNAi: RNA interference

rRNA: ribosomal ribonucleic acid

S6K: ribosomal protein S6 kinase

S. cerevisiae: Saccaromyces cerevisiae

SCG: superior cervical ganglion

SDS: sodium dodecyl sulfate

SEM: standard error of the mean

Sema3A: semaphorin 3A

siRNA: small interfering

RNA

SMA: spinal muscular atrophy

smiFISH: single-molecule inexpensive fluorescence *in situ* hybridization

SMN: survival motor neuron

SNAP25: synaptosomal-associated protein 25

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SNP: single nucleotide polymorphisms

SSC: saline sodium citrate

STAT 3: signal transducer and activator of transcription 3

STV: synaptic vesicle protein transport vesicle

SUnSET: surface sensing of translation

SV: synaptic vesicle

TBS-T: tris-buffered saline, tween 20

TH: tyrosine hydroxylase

TRAP: translating ribosome affinity purification

tRNA: transfer RNA

TRPV1: transient receptor potential channel V1

t-SNARE: target membrane SNARE

UTR: untranslated region

v-SNARE: vesicle SNARE

VAMP: vesicle-associated membrane protein

VGCC: voltage-gated calcium channels

VGluT1: vesicular glutamate transporter 1

X. laevis: Xenopus laevis

ZBP1: zipcode binding protein 1

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INTRODUCTION

LOCAL TRANSLATION

Proteins are not homogeneously distributed within a cell, and this asymmetrical expression is fundamental for cell function. The totality of a cell's information, including protein coding instructions, is stored in the nucleus in the form of DNA. In a very simplified manner, nuclear DNA is transcribed into RNA that is processed, exported from the nucleus, and translated in the cytoplasm into proteins by cellular machines called ribosomes. Intuitively, one would think that cells rely on a central production and export model, where all proteins are produced in the cell body and transported to where they are needed. Indeed, a lot of research has focused on peptide sorting signals, short amino acid sequences that, much like a stamp, guide protein transport to specific regions (Bauer et al., 2015; Blobel, 1980) Nevertheless, alternative mechanisms of RNA localization and on-site protein production coexist. mRNA, once thought of as merely a short-lived passive messenger, provides new important layers of local gene expression regulation (Figure1).

mRNA localization is an evolutionary conserved mechanism and it can be found in virtually all cells (Donnelly et al., 2010; Holt and Schuman, 2013; St Johnston, 2005). In the bacteria *Escherichia coli*, messenger RNAs (mRNAs) encoding membrane proteins localize to the periphery of the cell (Figure 2A), and migrate even when translation is inhibited (Nevo-Dinur et al., 2011). In the yeast *Saccaromyces cerevisiae*, several mRNAs encoding mitochondrial proteins localize to the mitochondria (Gadir et al., 2011; Luk et al., 2005; Yogev et al., 2007); and, similarly, several mRNAs encoding peroxisomal proteins colocalize with peroxisomes (Figure 2B) (Zipor et al., 2009). *S. cerevisiae* undergoes asymmetrical division and ASH1 mRNA localization to the budding tip is an important mechanism of sequestering protein activity, establishing mother-daughter polarity and influencing gene expression (Figure 2C) (Long et al., 1997).

When the mRNA localization is disrupted, ASH1 protein is distributed symmetrically between mother and daughter cells (Long et al., 1997; Takizawa et al., 1997). It should be noted that other asymmetrically expressed proteins in the budding yeast maintain their localization even in the absence

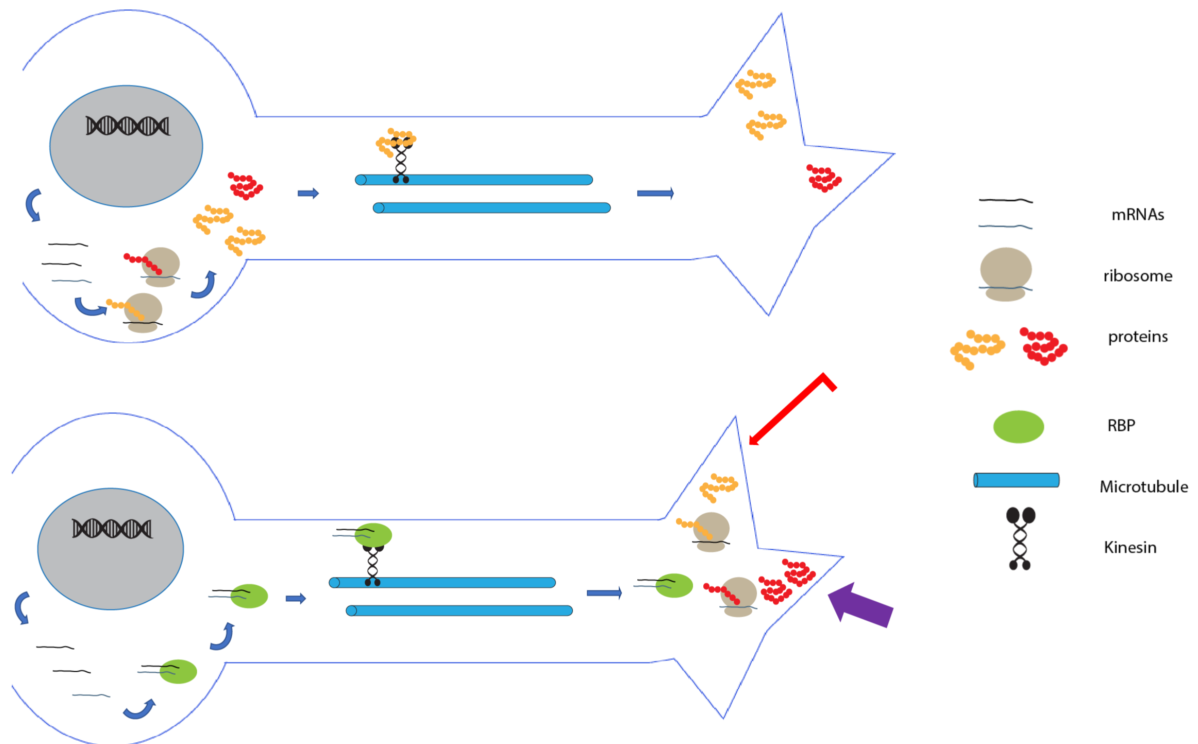

Figure 1: Two models of asymmetric protein expression. In the centralized model (upper neuron), mRNAs are translated in the cell body, and different proteins are transported into the periphery. In the local translation model (lower neuron), mRNAs are packaged into granules and transported, and proteins can be produced locally, where and when they are needed. We can already foresee some of the potential advantages of local translation that will be discussed later: One mRNA molecule can be used to produce many copies of each protein product; production can be minutely tailored to local demands/stimuli (red and purple arrows); the periphery can very rapidly react to changes in the environment, without the need for central input.

of RNA transport, indicating that both the mRNA and its encoded protein can have redundant targeting signals (Shepard et al., 2003).

RNA localization in oocytes and embryos is a means of establishing the embryonic body axes and determining cell fate (reviewed in (Medioni et al., 2012)). *Drosophila* oogenesis has been extensively studied and four maternal mRNAs, *oskar*, *nanos*, *bicoid* and *gurken*, that are differentially localized within the oocyte, are responsible for zygotic pattern specification (Figure 2D) (Becalska and Gavis, 2009; Kugler and Lasko, 2009; Weil, 2014). Strikingly, in *Drosophila* embryos, an *in situ* hybridization analysis showed that 71% of the more than three thousand analyzed transcripts were subcellularly localized and their distribution correlated closely with the expression pattern and function of their protein products (Lécuyer et al., 2007). In the vertebrate *Xenopus laevis* oogenesis, maternal mRNAs localized to the vegetal cortex are inherited by the vegetal blastomeres and are crucial for endoderm and mesoderm specification during embryogenesis (reviewed in (King et al., 2005). Other processes such as cell motility and migration are also influenced by and dependent upon asymmetrical RNA expression. The mRNA for the cytoskeletal β-actin protein isoform localizes to the peripheral protrusions of motile cells such as fibroblasts (Figure 2E) (Lawrence and Singer, 1986). In response to

a migratory stimulus, pseudopodial protrusions of fibroblasts contain more than 50 different enriched mRNAs (Mili et al., 2008), and interfering with the peripheral localization of a single one can impair directional migration (Liao et al., 2011). Differences in tumor cells RNA localization can similarly explain invasiveness and metastatic potential. Loss of RNA localization, with subsequent loss of intrinsic cell polarity, makes these cells more flexible in the direction of motility to chemotactic stimuli (Lapidus et al., 2007; Shestakova et al., 1999), while negatively influencing cell to cell adhesion (Gu et al., 2012).

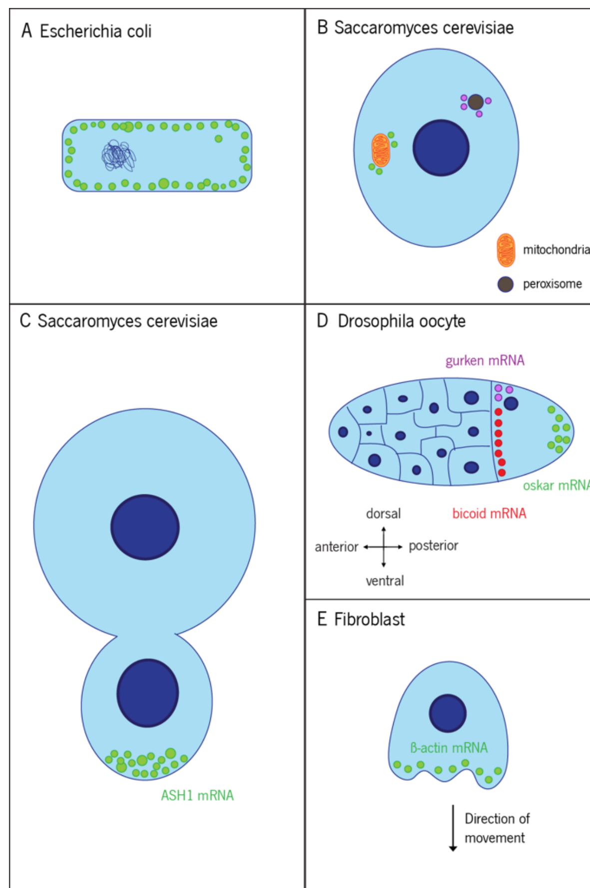

Figure 2: RNA localization. *Several examples of known localized transcripts. Several transcripts encoding membrane proteins localize to the Escherichia coli membrane (A), and, similarly, in the yeast several transcripts encoding mitochondrial and peroxisomal proteins localize to the mitochondria and peroxisome, respectively (B). During Saccaromyces cerevisiae division, ASH1 mRNA localized to the daughter cell (C). In the Drosophila oocyte, bicoid and oskar mRNAs define the antero-posterior axis, while gurken mRNA localizes to the dorsal pole (D). Migrating fibroblasts contain β-actin mRNA in their pseudopodial protrusions (E).*

While these are merely a few examples, they illustrate potential advantages of locally producing proteins (reviewed in (Jung et al., 2012, 2014; Medioni et al., 2012). First and foremost, local translation provides an extremely fast response to localized stimuli. And this response can be restricted to the stimulus-receiving area with a micrometric precision. It has been shown that transmembrane receptors can bind translational machinery, thus making a direct bridge between extracellular ligands

and intracellular translational events (Tcherkezian et al., 2010). Importantly, this could explain how external cues can lead to site-specific protein synthesis. Transporting and storing translationally repressed RNAs can also prevent the ectopic expression of proteins whose mislocalization could have dire consequences, such as embryonic pattern determinants. Furthermore, transport, localization, stability and translation of mRNAs can be regulated through multiple elements located in their 3' and 5' untranslated regions (UTR), without interfering with protein structure and function (Barrett et al., 2012). Additionally, local translation is an energy efficient process, since one mRNA molecule can potentially give rise to several protein molecules, through multiple rounds of translation. Also, locally produced proteins might have different post-translational modifications from centrally produced ones, which could condition their function. Or they might also find different binding partners and form distinct complexes. Functionally related mRNAs are thought to be transported within the same particle, which would allow the simultaneous translation of a functionally related cluster of proteins (Keene, 2007; Keene and Tenenbaum, 2002), thereby altering local stoichiometry, and favoring local interactions. This same principle might explain why it would be beneficial to locally produce ubiquitous and relatively abundant proteins such as β-actin. It is indeed puzzling why it would be necessary to locally synthesize proteins when there were already so much around in the first place. But in most cases preexisting proteins cannot compensate, however abundant they might be (Miller et al., 2002).

Local Translation in Neurons

Neurons are highly specialized cells in charge of receiving and conveying information through both chemical and electrical signals. They project several processes through which they form a multitude of connections, creating an intricate network (Figure 3). This network is not only able to integrate sensations and coordinate motor behavior, but also learn and store information, and even perform complex cognitive tasks. It is hard to grasp how all of this can be accomplished, and the study of the nervous system has fascinated biologists and doctors alike. The morphological complexity of this network and the innumerable intertwining neurites have led to the early belief that this network constituted a continuous reticular system (Cimino, 1999; Gerlach, J. von, 1872). And it is this complexity in both shape and task that makes neurons perfect subjects, in which to study RNA localization and local translation phenomena.

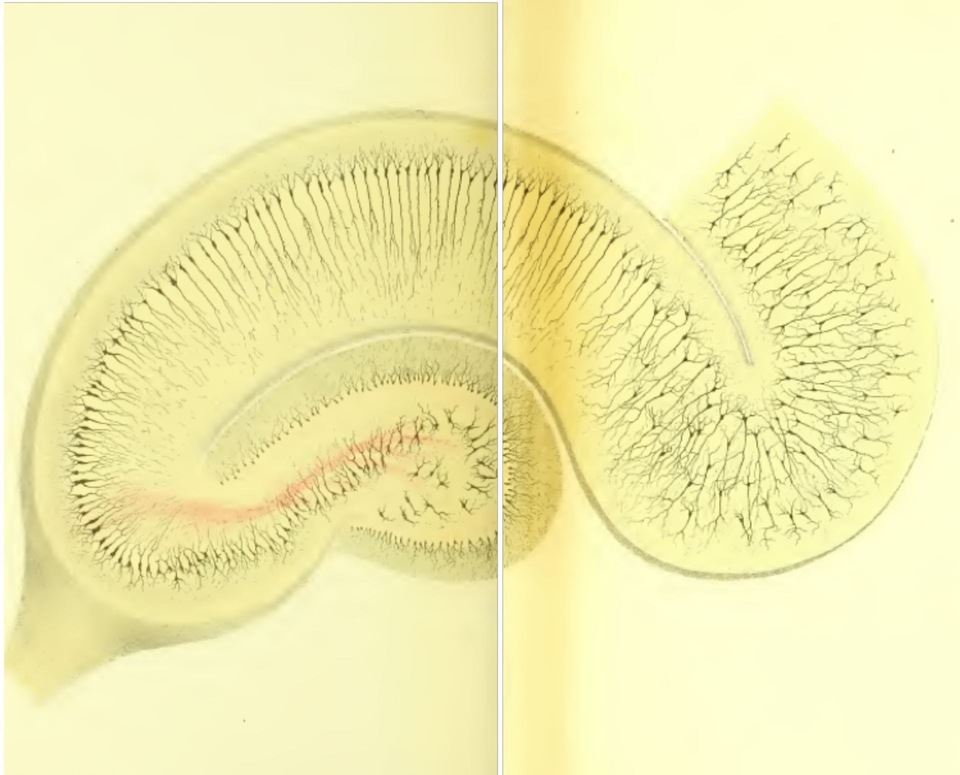

Figure 3: The structure of the hippocampus of a neonatal cat. *Drawing by Camillo Golgi. Based on Golgi, C., and Royal College of Physicians of Edinburgh (1886). Sulla fina anatomia degli organi centrali del sistema nervoso (Milano : U. Hoepli).*

Neurons have receiving and transmitter neurites. Dendrites are usually short and ramified and transmit information to the soma, while the axon is the longest process and conducts the nerve impulse from the cell body to the periphery (Figure 4). By the end of the 19th century it was first hypothesized that axons might be able to synthetize needed materials independently from the cell body (Barker, 1899). Indeed, we can use as an example the motor neurons that innervate our limbs, whose axons can easily span a meter. The fastest rate of anterograde transport reported is around 40 centimeters per day (Kaether et al., 2000), though most cytoskeletal and cytosolic proteins do not move more than a few millimeters per day (Campenot and Eng, 2000). So, in the best-case scenario, proteins would take a few days to arrive to these distal tips. Moreover, if we look at proteins life span, it seems unlikely that they would endure such a long trip (Yen et al., 2008). And although the first theories about axonal protein synthesis focused on this need to overcome slow transport rates (Alvarez, 1992), it soon became clear that local translation is not merely a mechanism to maintain proteome homeostasis far from the cell body. Neither is it exclusive to long neurites.

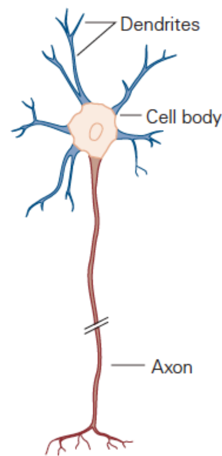

Figure 4: Neuronal structure. *Neurons usually possess several short, highly branched dendrites and a single long axon. Electrical signals generated in the receiving areas (dendrites or the cell body) are transmitted through the axon to other neurons or target cells. Adapted from: Kandel, E.R. (2013). Principles of neural science (New York: McGraw-Hill).*

Local translation in developing axons

During development axons extend towards their synaptic target, which might be located far away, in a complex process called pathfinding. A specialized 'fan-shaped' structure located at the distal tip of the axon, the growth cone, is responsible for sensing extracellular gradients and steering axonal growth (Figure 5). Growth cones are extremely dynamic, and they extend and retract filipodial projections at an astounding rate of seconds to minutes.

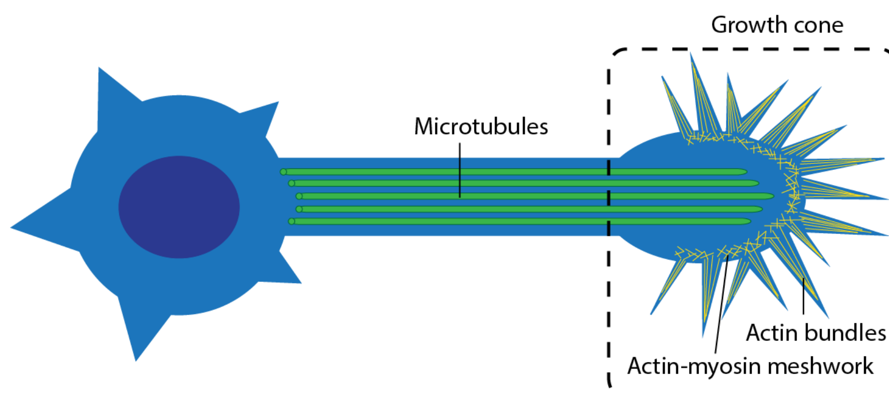

Figure 5: Axonal growth cone. *Growth cones are specialized structures at the distal tip of axons, in charge of sensing the extracellular environment and guiding axonal elongation. They possess a central domain, composed of microtubules, and a peripheral domain, filled with a dense actin meshwork (lamellipodia). Rapid actin polymerization into bundles leads to the formation of finger-like protrusions, the filopodia. Actin polymerization advances the growth cone leading edge, while microtubules invade the peripheral domain and membrane expands, resulting in axonal elongation.*

Cytoskeletal dynamics are intimately associated with pathfinding (Dent et al., 2011), and indeed cytoskeletal proteins were the first likely candidates for axonal synthesis. β-actin mRNA was shown to localize to neurites and growth cones of cultured cortical neurons (Bassell et al., 1998), and both actin and tubulin were found to be synthetized in rat sympathetic axons in cultures using a radioactive labelling technique (Eng et al., 1999). These last experiments were done using a microfluidic device able to isolate between cell bodies and axonal compartments. However, the estimated quantity of protein synthetized by distal axons was small (less than 1% of the anterogradely transported protein from the soma) and inhibiting protein synthesis did not have any effect on axonal elongation in these cultures. It was later shown that growth cones from *Xenopus laevis* retinal ganglion cells (RGCs) severed from their cell bodies continue to grow towards a netrin-1 gradient and away from a semaphoring 3A (sema3A) gradient for approximately 2 hours (Campbell and Holt, 2001). Blocking axonal protein synthesis could prevent these chemotropic responses. In fact, it had been previously shown *in vivo* that RGC axons from Xenopus embryos whose retinas had been removed still grew for up to 3 hours and were able to recognize and arborize in the tectum (Harris et al., 1987). Several subsequent studies clarified how local translation might mediate these cue-induced responses (Figure 6). A netrin-1 attractive stimulus leads to asymmetrical β-actin synthesis within the growth cone, preferential stabilization of actin filaments in the side of stimulus, and turning (Leung et al., 2006). On the contrary, repulsive signals such as sema3A and Slit2 lead to local synthesis of actin destabilizing proteins, such as RhoA (Wu et al., 2005) and cofilin (Piper et al., 2006).
Aside from this chemotropic turning, local translation is involved in other growth cone responses. Along their path, developing axons are exposed to changing concentrations of guidance cues, and they undergo continuous cycles of desensitization and resensitization to these extracellular stimuli, essential to maintain responsiveness in long-range chemotaxis. Resensitization to netrin-1 and brain-derived neurotrophic factor (BDNF) has been shown to be protein-synthesis dependent in *Xenopus* spinal nerves (Ming et al., 2002). Some axonal pathways involve intermediate targets, and the best characterized one is the central nervous system (CNS) midline, which presents special challenges for developing axons (Flanagan and Van Vactor, 1998). These growth cones are first attracted to midline signals, and, upon arriving to the midline, they need to lose their responsiveness to the previous attractant, in order to progress. At this point, they start following new guidance factors, and this has also been proposed to be dependent on axonal protein translation (Brittis et al., 2002). Intra-axonal protein synthesis can also influence different modalities of axonal growth, with impact on axonal

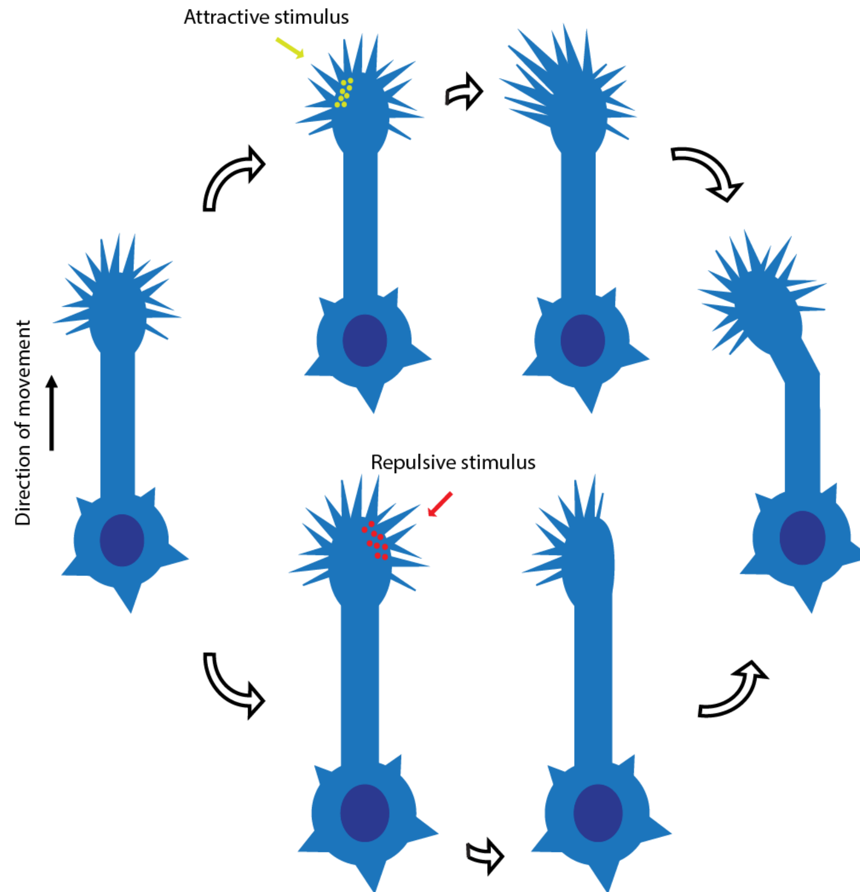

Figure 6: Local translation-dependent growth cone chemotaxis. *In response to attractive stimuli there is asymmetrical synthesis of β-actin within the growth cone, actin polymerization and stabilization on the side of the stimulus, and directional growth. On the contrary, in response to repulsive stimuli, there is synthesis of actin destabilizing molecules, growth cone collapse and asymmetrical growth.*

morphology. Increasing axonal synthesis of β-actin while decreasing axonal synthesis of growth associated protein 43 (GAP-43) results in short, highly branched axons (Donnelly et al., 2013). Conversely, increasing GAP-43 and decreasing β-actin local translation results in elongated axons with reduced branching. Axonal branching and arbor complexity have indeed been shown to correlate with mitochondria and RNA docking and to depend on local protein synthesis (Spillane et al., 2012, 2013; Wong et al., 2017). It has also been shown that nerve growth factor (NGF) can stimulate the synthesis of both partitioning-defective 3 (par3) and TC10 in dorsal root ganglion (DRG) growth cones (Gracias et al., 2014; Hengst et al., 2009). The first is a polarity complex protein localized to axonal growth cones and involved in cytoskeletal regulation, and the latter a member of the exocyst complex responsible for plasma membrane expansion. Cytoskeletal growth and membrane expansion are thus simultaneously activated in the same stimulus sensing area.

In all of the previous examples translated proteins were used locally, very close to their synthesis site, but that is not always the case. Sensory and sympathetic neurons are dependent upon NGF

neurotrophic signaling (Crowley et al., 1994). NGF-dependent survival of DRG neurons in culture is mediated through translation of the transcription factor cyclic AMP-responsive element-binding protein (CREB) (Cox et al., 2008). Locally translated CREB travels retrogradely to the nucleus and suppression of this process markedly reduces DRG survival in culture. Strikingly, cell body synthesized CREB cannot mediate this NGF response. Similarly, disturbing axonal synthesis of myo-inositol monophosphatase-1 (IMPA-1) in rat sympathetic neurons perturbs NGF survival signaling and leads to axonal degeneration (Andreassi et al., 2010). It is still unclear why an axon would locally synthesize a protein just to shuttle it back to the nucleus, but these are not isolated examples, and the same has been observed in axonal injury signaling (Rishal and Fainzilber, 2014) and neurodegenerative stimulation (Baleriola et al., 2014).

It is also worth pointing out that not all cue-induced growth cone responses are mediated through local translation. Inhibiting protein synthesis does not significantly affect ephrin-mediated growth cone collapse (Mann et al., 2003). Nevertheless, ephrin A stimulation in RGCs reduces the mammalian target of rapamycin mTOR activity and axonal protein synthesis (Nie et al., 2010). The mTOR kinase integrates several extracellular inputs, such as insulin, growth factors, cytokines and nutrients, as well as several intracellular indicators such as oxygen, energy and stress levels, and acts as a master regulator of cellular growth and proliferation through the control of general protein synthesis (Hay and Sonenberg, 2004). mTOR phosphorylates eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K), thereby activating translation initiation, the rate-limiting step in eukaryotes. And it has been shown that the mTOR pathway is also implicated in local translation regulation in axons (Campbell and Holt, 2001; Leung et al., 2006; Piper et al., 2006). It is possible that multiple localized signaling pathways in growth cones converge on mTOR, and other cues classified as nontranslational-dependent also influence axonal responses. Recent studies show that guidance cues concentrations can influence the growth cone dependence on protein synthesis (Manns et al., 2012; Nédelec et al., 2012). Above a certain level of Sema3A concentration, growth cone collapse responses are protein synthesis-independent, which explains discrepant results in other studies (Roche et al., 2009).

Local translation after development

After the developmental period, however, quantities of rRNA and mRNA in axons decrease (Bassell et al., 1994; Kleiman et al., 1994). Ribosomes are not easily identified by electron microscopy in mature

axons (Steward and Ribak, 1986; Zelená, 1970), and, in fact, the paucity of polyribosomes has been pointed out as one of the earliest features that allow the distinction between developing axons and other minor processes (Deitch and Banker, 1993). Subsequent immunohistological studies identified in mature mammalian peripheral nervous system (PNS) axons discrete, 'plaque-like' ribosome-containing structures in the peripheral axoplasm, close to the plasma membrane (Koenig et al., 2000; Kun et al., 2007). This submembranous distribution, together with the apparent absence of polyribosomes, could be the reason why they have been so hard to identify by ultrastructural analysis. Monosomes might also be more translationally active than previously thought (Heyer and Moore, 2016), and monosomal translation may predominate in axons. Intriguingly, ribosomes have been shown to be transferred from Schwann cells to sciatic nerves in mice and their numbers increase after sciatic nerve injury (Court et al., 2008), opening up the intriguing possibility that surrounding glia cells might be a source of translational machinery.

Also central to this question is the stimulus-dependent nature of axonal local translation events. As discussed previously, even during development, where axonal translation is pronounced, inhibiting protein synthesis in axons does not seem to have an effect on unstimulated axonal elongation (Eng et al., 1999; Hengst et al., 2009). It is therefore not surprising that a strong stressor such as nerve injury has been shown to induce a translational response in post-developmental axons. The intra-axonal signaling cascade in injured nerves has been extensively studied and locally synthesized proteins have been identified (Gumy et al., 2010; Rishal and Fainzilber, 2014). An initial calcium wave triggers a first round of translation of several sensor mRNAs, among them importin β and the transcription factor signal transducer and activator of transcription 3 (STAT 3), that form an injury signaling complex (Ben-Yaakov et al., 2012; Hanz et al., 2003; Perry et al., 2012). This signaling complex travels retrogradely to the cell body and influences the regenerative response. Decreased protein synthetic capacity of CNS axons has been associated with decreased regenerative ability and, indeed, preconditioning an axon in vivo with a nerve lesion leads to enhanced local protein synthesis and regeneration in vitro (Gumy et al., 2010; Verma et al., 2005; Zheng et al., 2001).

Surprisingly, a recent study showed that axonal application of oligomeric β-amyloid (Aβ) could induce a translational response in axons of mature hippocampal cultures (Baleriola et al., 2014). Once more, local synthesis and retrograde transport of a transcription factor mediates axon to soma communication, in this case, ultimately leading to neuronal death. Several other neurodegenerative and neurodevelopmental disorders have been linked to axonal translation changes (Costa and Willis, 2017; Liu-Yesucevitz et al., 2011). Spinal muscular atrophy (SMA), for instance, is an autosomal disease

characterized by loss of motor neurons and progressive paralysis, eventually leading to premature death. It is caused by reduced levels of the survival motor neuron (SMN) protein. Although SMN is present in all cell types, and its total deletion is lethal, motor neurons are more sensitive to its reduction (Fallini et al., 2016; Pellizzoni et al., 1998). This selective motor neuron effect might be explained by SMN role as a RNA-binding protein (RBP) necessary for the transport and/or local translation of several axonal transcripts. Consistent with this, several studies have shown that its reduction can lead to decrease levels of several mRNAs in axons (Fallini et al., 2016; Rage et al., 2013; Saal et al., 2014).

More provocative studies report a function for local translation in axonal maintenance. Acute inhibition of local protein synthesis or mitochondrial protein import in axons of primary rat sympathetic cultures resulted in a decrease in axonal mitochondria membrane potential and axon retraction (Hillefors et al., 2007). Lamin B2, a component of the nuclear lamina, has an unexpected extranuclear role in the axon, and its local translation is required for mitochondrial function *in vitro* and RGC axonal maintenance *in vivo* (Yoon et al., 2012). Charcot-Marie-Tooth disease type 2 is a peripheral neuropathy characterized by progressive distal motor weakness and mild sensory loss, due to axonal degeneration and can be caused by mutations in both mitochondrial proteins or lamins (Capell and Collins, 2006; Pareyson and Marchesi, 2009). Indeed, several other nuclear-encoded mitochondrial proteins have been found in axonal transcriptomes and are, along with ribosomal proteins, among the most enriched classes of transcripts in axons (Gumy et al., 2011; Minis et al., 2014; Taylor et al., 2009; Zivraj et al., 2010).

Local translation at the synapse

Each neuron forms thousands of specialized connections, called synapses (Figure 7). Synaptic plasticity, or the ability to modulate synaptic strength in response to environmental stimuli, constitutes the molecular basis of memory consolidation and learning (Kandel, 2001). We can easily measure increases or decreases in synaptic strength that when persistent are called long-term potentiation (LTP) and long-term depression (LTD), respectively. These activity-induced long-lasting alterations in synaptic efficacy have been shown to rely on dendritic protein synthesis (Huber et al., 2000; Kang and Schuman, 1996; Miller et al., 2002). In fact, even though dendrites are shorter and very close to the cell body, local translation in the dendritic compartment has been far less controversial than axonal translation, mainly because it is easily detected. Polyribosomes localize to dendrites (Steward and Levy,

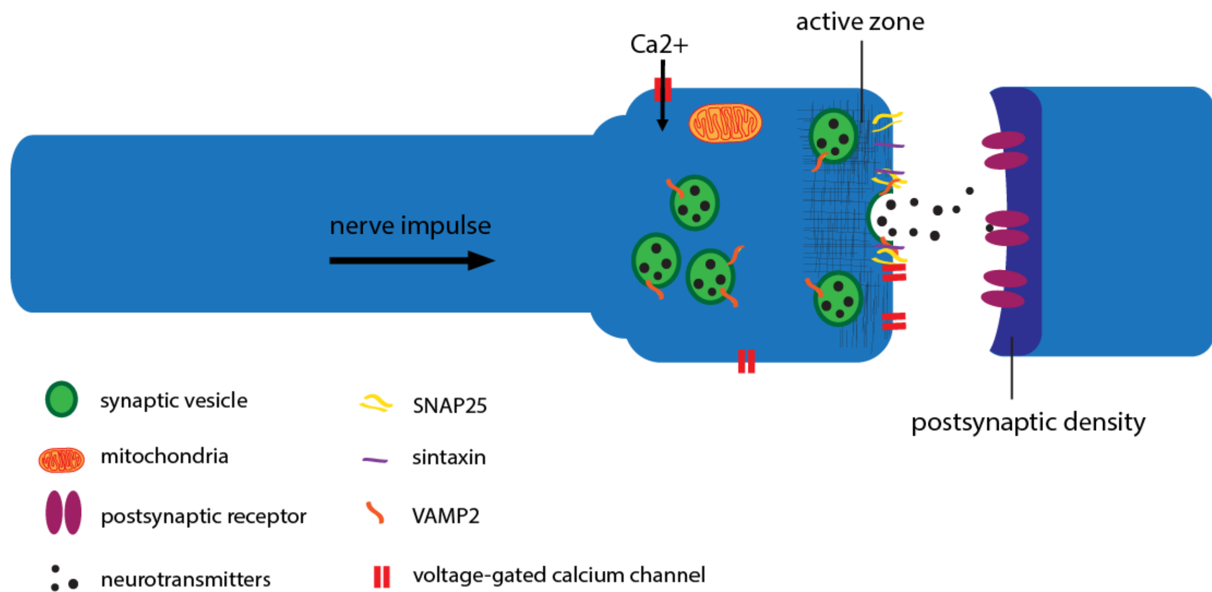

Figure 7: Chemical synapses. Neurons communicate with one another and with other cells at synapses. When an action potential, or nerve impulse, reaches the axon terminal it activates voltage-gated calcium channels and calcium flows into the terminal. This causes the fusion of synaptic vesicles with the plasma membrane and release of neurotransmitters in the synaptic cleft. Neurotransmitters then bind and activate receptors on the postsynaptic side. This fusion event happens at specialized areas, called active zones, and is mediated through the SNARE complex, composed of VAMP2, a vesicular SNARE, and sintaxin and SNAP25, two target membrane SNAREs.

1982), and they translocate from dendritic shafts to spines after the induction of LTP in rat hippocampal slices (Ostroff et al., 2002). *In situ* hybridization studies have also identified several different mRNAs in the dendritic compartment, such as the ones encoding Ca2+/calmodulin-dependent protein kinase alpha subunit (CaMKIIα) (Burgin et al., 1990), microtubule-associated protein 2 (MAP2) (Garner et al., 1988) and β-actin (Tiruchinapalli et al., 2003). The number of known dendritically localized mRNAs rapidly grew to hundreds with unbiased approaches (Miyashiro et al., 1994; Poon et al., 2006), and RNA sequencing analysis uncovered a surprising amount of more than 2500 transcripts localized to rat hippocampal neurites (Cajigas et al., 2012). In a recent study, parallel comparison of transcriptome and proteome in neurites and somas of neurons differentiated from mouse embryonic stem cells estimated that half of the neurite-enriched proteome is due to RNA localization and local translation mechanisms, and not protein transport (Zappulo et al., 2017). It is now established that dendritic translation plays a fundamental role in long-lasting synaptic plasticity, allowing the local control of gene expression (Costa-Mattioli et al., 2009; Tom Dieck et al., 2014). Very interesting questions remain about the size of the translation compartment (whether proteins are synthesized and used at a single spine, or diffuse and can be used by nearby spines), the mechanisms of regulation, the cell-type and circuit specificity of these local events, and how these molecular

changes can be translated to specific memory encoding (Poo et al., 2016; Rangaraju et al., 2017; Sutton and Schuman, 2006).

Whereas the postsynaptic arbor is considered to be the major effector of activity-induced changes in mammalian neuronal circuits, the axon and presynaptic terminals are mainly seen as passive transmitters of information. But a lot of what we know of LTP was first shown in invertebrate models that possess interesting particularities. In the giant marine snail *Aplysia californica*, a touch to the siphon elicits a defensive gill withdrawal, and this involuntary reflex can undergo different types of learning, such as habituation (where repeated stimulation leads to reduced withdrawal response), dishabituation, sensitization (where the presentation of a novel noxious results in increased response) and classical conditioning (where pairing of a neutral stimulus with a noxious one leads to an increased response to the neutral stimulus alone) (Carew et al., 1971, 1981; Pinsker et al., 1970). This reflex involves sensory neurons that make synapses with motor neurons and plasticity within these synapses accounts for these learned responses. Long-term facilitation (LTF) can be elicited in *Aplysia* sensory-motor synapses with local synapse-specific application of serotonin, and stimulated synapses show increased protein synthesis when compared with unstimulated ones (Casadio et al., 1999; Martin et al., 1997). LTF can still occur in nerve terminals separated from their cell bodies (Schacher and Wu, 2002), but transcriptional events are necessary for its sustained stabilization, suggesting the involvement of a retrograde signal (Guan and Clark, 2006; Liu et al., 2003). Even though transcription events are elicited and necessary, they do not seem to contribute to the spatial restriction of the response, since transcribed RNAs seem to be delivered throughout the neuron, but only translated at the previously stimulated synapses (Kim and Martin, 2015). The sensory neuron-specific neuropeptide sensorin is locally produced in *Aplysia* nerve terminals in response to LTF, and although interfering with its local synthesis does not reduce the protein levels at the presynapse, it prevents synapse formation, showing once again that local translation is much more than a means to maintain proteome homeostasis (Lyles et al., 2006). Sensorin levels decrease as the neurons mature, but it remains necessary for maintaining synapse efficacy (Hu et al., 2004). LTF in *Aplysia* is largely dependent on presynaptic protein synthesis, but there are fundamental differences between this invertebrate model and vertebrate neurons that complicate direct comparisons. These neurons lack the strict polarity of vertebrate neurons, and their axons can function as both transmitters and receivers of information (Martin et al., 2000). Indeed, LTF in Aplysia is elicited by serotonin binding to presynaptic receptors, thus acting functionally as postsynaptic (Mohr and Richter, 2000).

However, several presynaptic transcripts have been found in different axonal transcriptome studies. Laser capture microdissection of growth cones from pathfinding and target-arriving Xenopus RGCs found presynaptic protein mRNAs enriched in the target-arriving growth cones, as well as in mouse RGCs (Zivraj et al., 2010). A transcriptome obtained from axons of embryonic rat DRGs grown in compartmentalized cultures also found several synaptic vesicle and exocytosis related transcripts (Gumy et al., 2011). Axons from regenerating mature rat cortical neurons contained mRNAs involved in synaptic function (Taylor et al., 2009). And several presynaptic transcripts were also present in axons of mature hippocampal neurons grown in microfluidic devices and some showed significant increases after an Aβ challenge (Baleriola et al., 2014). In a recent study, isolation of ribosome-bound mRNAs in mouse RGC axons *in vivo*, with an axon-TRAP (translating ribosome affinity purification) approach, allowed the analysis of the axonal translatome at different time points, at embryonic day 17.5 (E17.5), postnatal day 0.5 (P0.5), postnatal day 7.5 (P7.5) and adult, corresponding to different stages of elongation, branching, pruning and maintenance, respectively (Shigeoka et al., 2016). Surprisingly, several mRNAs related to synaptic transmission were found enriched in adult axons, namely transcripts related to the presynaptic membrane, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and glutamate receptors. Transcripts related to synapse assembly, such as neurexins and presynaptic cell adhesion molecules, were translated at all ages.

A few studies do propose a more active function of the presynaptic terminal in circuit plasticity, and hint at a role for presynaptic translation. One common form of presynaptic plasticity is mediated through endocannabinoid (eCB) retrograde signaling (Castillo et al., 2012). These lipid messengers are produced upon postsynaptic stimulation and travel across the synaptic cleft, binding to presynaptic receptors. The hippocampal GABAergic inhibitory interneurons express type 1 cannabinoid receptor (CB1) on their axon terminals, and eCB release can result in both short-term and long-term reduction of GABA release (Kano et al., 2009). In rodent hippocampal slices, single-cell paired electrophysiological recordings in individual hippocampal interneurons and CA1 pyramidal cells showed that presynaptic protein synthesis is required for eCB mediated long-term depression (LTD) at these GABAergic interneurons (Younts et al., 2016). They further showed that this protein synthesis is most likely axonal, and they also provided anatomical evidence for the localization of ribosomes in CB1-expressing terminals of adult hippocampal rodent slices. Less robust studies suggest a role for presynaptic translation during LTP in the mossy fiber to CA3 synapses (Calixto et al., 2003; Hagena and Manahan-Vaughan, 2013) and in eCB-mediated LTD at rat corticostriatal synapses (Yin et al., 2006).

Another intriguing possibility is the local production of neuropeptides or even neurotransmitters at mature terminals. As mentioned above, the neuropeptide sensorin is produced in *Aplysia* nerve terminals during LTF (Hu et al., 2002; Lyles et al., 2006). In vertebrates, olfactory marker protein mRNA is found in mature axons of olfactory sensory neurons in rat, and an incredible amount of odorant receptors mRNAs associate with polysomes in nerve terminals of adult mouse bulb (Dubacq et al., 2009; Wensley et al., 1995). Nevertheless, no function has yet been attributed to the local synthesis of these transcripts (Dubacq et al., 2014). Mammalian distal axons of the hypothalamo-hypophyseal tract possess vasopressin and oxytocin mRNAs, but these also lack functional relevance (Jirikowski et al., 1990; Mohr and Richter, 1992; Mohr et al., 1991; Trembleau et al., 1996). Vasopressin, also called antidiuretic hormone, is an important hormone involved in the regulation of water reabsorption and the osmolality of body fluids. Curiously, vasopressin mRNA at rat neurohypophyseal presynaptic terminals are differentially regulated by dehydration and rehydration (Trembleau et al., 1995).

Similarly, it has been shown that tyrosine hydroxylase (TH), an enzyme involved in the rate-limiting step in the biosynthesis of catecholamine neurotransmitters, is locally translated in sympathetic axons (Gervasi et al., 2016). Consistent with this, TH mRNA was found in adult rat brain regions that receive catecholaminergic innervation but are devoid of catecholamine-synthetizing cells (Melia et al., 1994). In rat superior cervical ganglion (SCG) neurons, disrupting axonal TH mRNA trafficking diminished the axonal levels of dopamine and norepinephrine, whereas local translation of exogenous TH mRNA in distal axons enhanced norepinephrine levels (Gervasi et al., 2016).

Some studies also report the existence of mRNAs encoding surface receptors, such as the RGC axon-TRAP study mentioned previously (Shigeoka et al., 2016). DRGs send out peripheral branches that contain multiple noxious stimuli receptors. mRNA encoding transient receptor potential channel V1 (TRPV1), that senses thermal pain, is found in the axons of these neurons, and the axonal transport of this mRNA is enhanced after local tissue inflammation, indicating a possible role for local translation in mediating inflammation-induced pain hypersensitivity (Tohda et al., 2001). There is indeed ample evidence for the involvement of local translation in mediating nociceptive sensitization during the inflammatory response (Jiménez-Díaz et al., 2008; Melemedjian et al., 2014). This type of plasticity does not only involve PNS sensory neurons, but can also involve central neurons that receive their projections, resulting in chronic pain disorders that share a lot of similarities with the injury and neurodegenerative responses discussed before (Asante et al., 2009; Khoutorsky and Price, 2018; Price and Inyang, 2015).

All of the previously referred examples concerning membrane or secreted proteins are puzzling given that ultrastructural studies failed to identify conventional endoplasmic reticulum (ER) and Golgi apparatus in axons. These proteins enter or cross the ER membrane cotranslationally, during their synthesis in ER membrane-bound ribosomes. Nevertheless, both ER and Golgi-associated proteins have been detected in *Xenopus* RGCs, rat DRGs and rat sciatic nerve, and axonally synthesized proteins can be trafficked to the plasma membrane, suggesting that axons might possess an unconventional functional equivalent of the secretory pathway (Brittis et al., 2002; González et al., 2016; Merianda et al., 2009).

Even though the existence of constitutive or plasticity-induced translation in mature axons lacks generalized support, more convincing evidence links axonal translation and synaptogenesis. As growing axons reach their targets, they form complex structures, presynaptic terminals, specialized regions of the plasma membrane where synaptic vesicles dock and fuse, releasing neurotransmitters into the synaptic cleft. These presynaptic terminals are juxtaposed to complex postsynaptic receptive specializations, and both pre and postsynaptic assembly are coordinately regulated. Presynaptic terminals are composed of an electron-dense meshwork of proteins, called the active zone (AZ), and several neurotransmitter-filled synaptic vesicles (SVs) (Phillips et al., 2001; Takamori et al., 2006). A vast number of proteins localize to SVs, such as synapsin, synaptophysin, SV protein 2, synaptotagmin, vesicle-associated membrane protein (VAMP)/synaptobrevin, vesicular glutamate transporte 1 (VGluT1), just to name a few (Takamori et al., 2006); and to AZs, such as Piccolo, Bassoon, Rab3-interacting molecule (RIM), Munc13, SNAREs, as well as voltage-gated calcium channels, cell adhesion molecules and cytoskeleton elements (Ackermann et al., 2015). In spite of their extreme complexity, several studies have shown that synapses can form incredibly rapidly, within 30 minutes to a couple of hours after axodendritic contact, with presynaptic assembly preceding the postsynaptic assembly (Ahmari et al., 2000; Bresler et al., 2004; Friedman et al., 2000; Okabe et al., 2001). Presynaptic assembly is thought to result from clustering of pre-assembled mobile packets of vesicles generated in the soma and transported throughout the axon (Shapira et al., 2003; Vaughn, 1989; Ziv and Garner, 2004). Synaptic vesicle protein transport vesicles (STV), that corresponds to SVs and transports SV-associated proteins are one type of mobile packets found in axons and, when recruited to new contact sites, can become activity-competent in less than 1 hour (Ahmari et al., 2000; Kraszewski et al., 1995). Another major type of mobile precursor packet is the piccolo-bassoon transport vesicle (PTV), a larger 80 nm diameter dense core vesicle that contains most of the components of the active zone, namely piccolo, bassoon, synaptosomal-associated protein 25 (SNAP25) and N-cadherin (Shapira et

al., 2003; Zhai et al., 2001). On the contrary, postsynaptic assembly seems to result from the gradual recruitment and accumulation of individual proteins (Bresler et al., 2004; Waites et al., 2005). A plethora of trans-synaptic adhesion complexes and secreted molecules have been shown to induce recruitment and clustering of precursor vesicles and synaptic differentiation (Pinto and Almeida, 2016; Waites et al., 2005). Nevertheless, immature axons possess an inherent capacity for rudimentary vesicle recycling and form orphan presynaptic sites, without any postsynaptic contact (Ratnayaka et al., 2011). The precise sequence of events and the mechanisms regulating assembly, stabilization and maturation or elimination of putative synaptic sites are incompletely understood. There is also a surprisingly lack of knowledge of the intra-axonal mechanisms activated by these synaptic organizers, but both a series of hierarchical protein-protein interactions and actin cytoskeleton remodelling seem to be involved (Pinto and Almeida, 2016; Waites et al., 2011).

The first line of evidence connecting synaptogenesis and axonal translation comes from the close relationship between terminal branching and presynaptic formation. As RGCs reach the optic tectum, they arborize extensively and establish synaptic connections. *In vivo* studies in *Xenopus laevis* and zebrafish that followed the dynamics of both presynaptic puncta and axonal branches found that axonal branches continuously extend and retract at an incredibly fast rate, with most branches only persisting for mere minutes (Alsina et al., 2001; Meyer and Smith, 2006; Ruthazer et al., 2006). The same turnover was observed for presynaptic puncta, though synapses could stabilize axon branches, and branches would frequently form on newly added presynaptic puncta. A recent study used live imaging *in vivo* in the *Xenopus* visual system and showed that new axon branches formed at places where RNA granules and mitochondria dock, and that branch lifetime positively correlated with sustained RNA and mitochondria invasion (Wong et al., 2017). They further show that protein synthesis inhibitors can disrupt branching dynamics *in vivo*. *De novo* β-actin synthesis hotspots were visualized at branch points, within branches and at branch tips, in close proximity with docked RNA granules and knocking down β-actin reduced arbor complexity.

Indeed, many of the same clues that induce local translation during growth cone chemotropic responses, have been shown to influence *Xenopus* RGCs axonal arborization and synaptogenesis *in vivo*, such as BDNF and netrin-1 (Alsina et al., 2001; Manitt et al., 2009). In *Xenopus* nerve-muscle co-cultures, BDNF-coated beads were able to induce potentiation of neurotransmitter secretion at developing presynapses, and this potentiation was shown to be dependent on axonal protein synthesis (Zhang and Poo, 2002). Curiously, BDNF activity-induced secretion has also been shown to modulate synaptic transmission and plasticity in adult brains (Bramham and Messaoudi, 2005; Lu, 2003).

Netrin-1 has been shown to increase the number and strength of excitatory synaptic connections in rodent cortical neurons, through increased adhesion, actin cytoskeleton remodelling and mTOR-dependent protein translation (Goldman et al., 2013). Activity-driven presynapse assembly has also been experimentally demonstrated, both with KCl-induced depolarization and NMDA receptor activation (Diniz et al., 2012; Sceniak et al., 2012). In microfluidic cultures of rat cortical neurons, axonal treatment with glutamate, the main excitatory neurotransmitter in the mammalian CNS, was shown to induce mTOR activation and local protein synthesis through both ionotropic and metabotropic glutamate receptors, providing another link between neurotransmitter signalling and axonal translation (Hsu et al., 2015). Other studies inferred a role for local translation in synaptogenesis through RBP disruption. Loss of function of Vg1RBP, the *Xenopus* homolog of the zipcode binding protein 1, the main RBP involved in the axonal transport of β-actin, disrupts RGCs axon terminal arborization *in vivo* (Kalous et al., 2014). Similarly, disrupting the RBP Hermes in zebrafish *in vivo* results in RGC arborization defects and increased presynaptic puncta density (Hörnberg et al., 2013).

Direct evidence for the involvement of local translation in CNS synapse formation in mammalian neurons has been reported in a study that used poly-D-lysine (PDL)-coated beads to induce presynaptic clustering in axons of microfluidic hippocampal cultures (Taylor et al., 2013). Artificial presynaptic assembly can be induced with specific molecules, such as neuroligin expressed in non-neural cells (Scheiffele et al., 2000), but also without any specialized signalling, using adhesive substrates, such as PDL-coated latex beads (Lucido et al., 2009). These beads induce SV clustering very shortly after adhesion, and the bead-axon complexes are highly stable, contain presynaptic proteins in the correct disposition and are functionally capable of SV recycling as early as 1 hour after contact (Lucido et al., 2009). In axons of compartmentalized rat hippocampal cultures, addition of PDL-coated beads was shown to induce rRNA and β-catenin mRNA accumulation, and β-catenin protein production at contact sites (Taylor et al., 2013). Furthermore, interfering with axonal β-catenin synthesis using specific siRNA, added only to the axonal compartment, interfered with SV dynamics, resulting in increased SV release upon stimulation.

This was not the first study to report axonal synthesis of β-catenin. Hippocampal growth cones were shown to produce β-catenin in response to neurotrophin-3 and interfering with the RBP cytoplasmic polyadenylation element-binding protein-1 (CPEB1) abolishes the β-catenin protein increase in response to neurotrophin-3 and interferes with process branching (Kundel et al., 2009). β-catenin links cadherins to the actin cytoskeleton through the actin-binding protein α-catenin (Nishimura et al., 2002). Cadherins are trans-synaptic proteins involved in cell-cell adhesion and several studies implicate

them in synaptogenesis (Bozdagi et al., 2000; Togashi et al., 2002), and even in synapse plasticity (Manabe et al., 2000; Tang et al., 1998), though their role is not entirely clear. Besides this cadherin-actin anchor role, β-catenin also mediates Wnt-induced transcription and recruits PDZ proteins (Gujral et al., 2013; MacDonald et al., 2009). β-catenin has been shown to promote synapse formation in the cerebellum through the Wnt signalling pathway (Hall et al., 2000). A different study that used cell-wide conditional β-catenin ablation *in vivo* showed a reduction in the undocked vesicle pool, an increase in the number of synapses and an increase in basal transmission in mice hippocampus, but these effects were shown to be mediated through the PDZ binding domain (Bamji et al., 2003). Importantly, these effects are very similar to the ones reported with the axonal-specific knockdown of β-catenin (Taylor et al., 2013).

Given all of this, it is very tempting to speculate that intra-axonal protein synthesis might play other roles in synapse formation, and even in presynaptic plasticity. Local translation has been shown before to bridge localized extracellular stimuli and intracellular asymmetric events with extreme spatial precision. Many of the already described synapse-inducing factors have been previously shown to influence local translation in other instances. Additionally, the initial reliance of presynaptic assembly on actin cytoskeleton remodelling, as demonstrated by initial increased sensitivity to actin depolymerizing agents (Zhang and Benson, 2001), mimics growth cone local translation-dependent pathfinding and branching responses. Here, we focused on previously identified synapse-related axonal transcripts and used an *in vitro* compartmentalized approach to further elucidate the role of local translation on presynaptic assembly.

REFERENCES

Ackermann, F., Waites, C.L., and Garner, C.C. (2015). Presynaptic active zones in invertebrates and vertebrates. EMBO Rep. *16*, 923–938.

Ahmari, S.E., Buchanan, J., and Smith, S.J. (2000). Assembly of presynaptic active zones from cytoplasmic transport packets. Nat. Neurosci. *3*, 445–451.

Alsina, B., Vu, T., and Cohen-Cory, S. (2001). Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. Nat. Neurosci. *4*, 1093–1101.

Alvarez, J. (1992). Maintenance of the axoplasm: can neurones accord with the accepted notions? Neurosci. Lett. *144*, 1–3.

Andreassi, C., Zimmermann, C., Mitter, R., Fusco, S., De Vita, S., Devita, S., Saiardi, A., and Riccio, A. (2010). An NGF-responsive element targets myo-inositol monophosphatase-1 mRNA to sympathetic neuron axons. Nat. Neurosci. *13*, 291–301.

Asante, C.O., Wallace, V.C., and Dickenson, A.H. (2009). Formalin-induced behavioural hypersensitivity and neuronal hyperexcitability are mediated by rapid protein synthesis at the spinal level. Mol Pain *5*, 27.

Baleriola, J., Walker, C.A., Jean, Y.Y., Crary, J.F., Troy, C.M., Nagy, P.L., and Hengst, U. (2014). Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. Cell *158*, 1159–1172.

Bamji, S.X., Shimazu, K., Kimes, N., Huelsken, J., Birchmeier, W., Lu, B., and Reichardt, L.F. (2003). Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. Neuron *40*, 719–731.

Barker, L.F. (Lewellys F. (1899). The nervous system and its constituent neurones [microform] : designed for the use of practitioners of medicine and of students of medicine and psychology (New York : D. Appleton).

Barrett, L.W., Fletcher, S., and Wilton, S.D. (2012). Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. Cell. Mol. Life Sci. *69*, 3613–3634.

Bassell, G.J., Singer, R.H., and Kosik, K.S. (1994). Association of poly(A) mRNA with microtubules in cultured neurons. Neuron *12*, 571–582.

Bassell, G.J., Zhang, H., Byrd, A.L., Femino, A.M., Singer, R.H., Taneja, K.L., Lifshitz, L.M., Herman, I.M., and Kosik, K.S. (1998). Sorting of beta-actin mRNA and protein to neurites and growth cones in culture. J. Neurosci. *18*, 251–265.

Bauer, N.C., Doetsch, P.W., and Corbett, A.H. (2015). Mechanisms Regulating Protein Localization. Traffic *16*, 1039–1061.

Becalska, A.N., and Gavis, E.R. (2009). Lighting up mRNA localization in Drosophila oogenesis. Development *136*, 2493–2503.

Ben-Yaakov, K., Dagan, S.Y., Segal-Ruder, Y., Shalem, O., Vuppalanchi, D., Willis, D.E., Yudin, D., Rishal, I., Rother, F., Bader, M., et al. (2012). Axonal transcription factors signal retrogradely in lesioned peripheral nerve. EMBO J *31*, 1350–1363.

Blobel, G. (1980). Intracellular protein topogenesis. Proc Natl Acad Sci U S A *77*, 1496–1500.

Bozdagi, O., Shan, W., Tanaka, H., Benson, D.L., and Huntley, G.W. (2000). Increasing numbers of synaptic puncta during late-phase LTP: N-cadherin is synthesized, recruited to synaptic sites, and required for potentiation. Neuron *28*, 245–259.

Bramham, C.R., and Messaoudi, E. (2005). BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. Progress in Neurobiology *76*, 99–125.

Bresler, T., Shapira, M., Boeckers, T., Dresbach, T., Futter, M., Garner, C.C., Rosenblum, K., Gundelfinger, E.D., and Ziv, N.E. (2004). Postsynaptic Density Assembly Is Fundamentally Different from Presynaptic Active Zone Assembly. J. Neurosci. *24*, 1507–1520.

Brittis, P.A., Lu, Q., and Flanagan, J.G. (2002). Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. Cell *110*, 223–235.

Burgin, K.E., Waxham, M.N., Rickling, S., Westgate, S.A., Mobley, W.C., and Kelly, P.T. (1990). In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. J. Neurosci. *10*, 1788–1798.

Cajigas, I.J., Tushev, G., Will, T.J., tom Dieck, S., Fuerst, N., and Schuman, E.M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. Neuron *74*, 453–466.

Calixto, E., Thiels, E., Klann, E., and Barrionuevo, G. (2003). Early maintenance of hippocampal mossy fiber–long-term potentiation depends on protein and RNA synthesis and presynaptic granule cell integrity. J. Neurosci. *23*, 4842–4849.

Campbell, D.S., and Holt, C.E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. Neuron *32*, 1013–1026.

Campenot, R.B., and Eng, H. (2000). Protein synthesis in axons and its possible functions. J Neurocytol *29*, 793–798.

Capell, B.C., and Collins, F.S. (2006). Human laminopathies: nuclei gone genetically awry. Nature Reviews Genetics *7*, 940–952.

Carew, T.J., Castellucci, V.F., and Kandel, E.R. (1971). An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. Int. J. Neurosci. *2*, 79–98.

Carew, T.J., Walters, E.T., and Kandel, E.R. (1981). Classical conditioning in a simple withdrawal reflex in Aplysia californica. J. Neurosci. *1*, 1426–1437.

Casadio, A., Martin, K.C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C.H., and Kandel, E.R. (1999). A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell *99*, 221–237.

Castillo, P.E., Younts, T.J., Chávez, A.E., and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. Neuron *76*, 70–81.

Cimino, G. (1999). Reticular theory versus neuron theory in the work of Camillo Golgi. Physis Riv Int Stor Sci *36*, 431–472.

Costa, C.J., and Willis, D.E. (2017). To the end of the line: Axonal mRNA transport and local translation in health and neurodegenerative disease. Developmental Neurobiology.

Costa-Mattioli, M., Sossin, W.S., Klann, E., and Sonenberg, N. (2009). Translational control of long-lasting synaptic plasticity and memory. Neuron *61*, 10–26.

Court, F.A., Hendriks, W.T., MacGillavry, H.D., Alvarez, J., and van Minnen, J. (2008). Schwann cell to axon transfer of ribosomes: toward a novel understanding of the role of glia in the nervous system. J Neurosci *28*, 11024–11029.

Cox, L.J., Hengst, U., Gurskaya, N.G., Lukyanov, K.A., and Jaffrey, S.R. (2008). Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. Nat. Cell Biol. *10*, 149–159.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armaninl, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., et al. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell *76*, 1001–1011.

Deitch, J.S., and Banker, G.A. (1993). An electron microscopic analysis of hippocampal neurons developing in culture: early stages in the emergence of polarity. J. Neurosci. *13*, 4301–4315.

Dent, E.W., Gupton, S.L., and Gertler, F.B. (2011). The Growth Cone Cytoskeleton in Axon Outgrowth and Guidance. Cold Spring Harb Perspect Biol *3*.

Diniz, L.P., Almeida, J.C., Tortelli, V., Vargas Lopes, C., Setti-Perdigão, P., Stipursky, J., Kahn, S.A., Romão, L.F., de Miranda, J., Alves-Leon, S.V., et al. (2012). Astrocyte-induced synaptogenesis is mediated by transforming growth factor β signaling through modulation of D-serine levels in cerebral cortex neurons. J. Biol. Chem. *287*, 41432–41445.

Donnelly, C.J., Fainzilber, M., and Twiss, J.L. (2010). Subcellular communication through RNA transport and localized protein synthesis. Traffic *11*, 1498–1505.

Donnelly, C.J., Park, M., Spillane, M., Yoo, S., Pacheco, A., Gomes, C., Vuppalanchi, D., McDonald, M., Kim, H.H., Kim, H.K., et al. (2013). Axonally synthesized β-actin and GAP-43 proteins support distinct modes of axonal growth. J. Neurosci. *33*, 3311–3322.

Dubacq, C., Jamet, S., and Trembleau, A. (2009). Evidence for developmentally regulated local translation of odorant receptor mRNAs in the axons of olfactory sensory neurons. J. Neurosci. *29*, 10184–10190.

Dubacq, C., Fouquet, C., and Trembleau, A. (2014). Making scent of the presence and local translation of odorant receptor mRNAs in olfactory axons. Dev Neurobiol *74*, 259–268.

Eng, H., Lund, K., and Campenot, R.B. (1999). Synthesis of beta-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. J. Neurosci. *19*, 1–9.

Fallini, C., Donlin-Asp, P.G., Rouanet, J.P., Bassell, G.J., and Rossoll, W. (2016). Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons. J. Neurosci. *36*, 3811–3820.

Flanagan, J.G., and Van Vactor, D. (1998). Through the Looking Glass: Axon Guidance at the Midline Choice Point. Cell *92*, 429–432.

Friedman, H.V., Bresler, T., Garner, C.C., and Ziv, N.E. (2000). Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. Neuron *27*, 57–69.

Gadir, N., Haim-Vilmovsky, L., Kraut-Cohen, J., and Gerst, J.E. (2011). Localization of mRNAs coding for mitochondrial proteins in the yeast Saccharomyces cerevisiae. RNA *17*, 1551–1565.

Garner, C.C., Tucker, R.P., and Matus, A. (1988). Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. Nature *336*, 674–677.

Gerlach, J. von (1872). Über die struktur der grauen Substanz des menschlichen Grosshirns. Zentrallblatt Für Die Medicinischen Wissenschaften *10*, 273–275.

Gervasi, N.M., Scott, S.S., Aschrafi, A., Gale, J., Vohra, S.N., MacGibeny, M.A., Kar, A.N., Gioio, A.E., and Kaplan, B.B. (2016). The local expression and trafficking of tyrosine hydroxylase mRNA in the axons of sympathetic neurons. RNA *22*, 883–895.

Goldman, J.S., Ashour, M.A., Magdesian, M.H., Tritsch, N.X., Harris, S.N., Christofi, N., Chemali, R., Stern, Y.E., Thompson-Steckel, G., Gris, P., et al. (2013). Netrin-1 promotes excitatory synaptogenesis between cortical neurons by initiating synapse assembly. J. Neurosci. *33*, 17278–17289.

González, C., Cánovas, J., Fresno, J., Couve, E., Court, F.A., and Couve, A. (2016). Axons provide the secretory machinery for trafficking of voltage-gated sodium channels in peripheral nerve. Proc Natl Acad Sci U S A *113*, 1823–1828.

Gracias, N.G., Shirkey-Son, N.J., and Hengst, U. (2014). Local translation of TC10 is required for membrane expansion during axon outgrowth. Nat Commun *5*, 3506.

Gu, W., Katz, Z., Wu, B., Park, H.Y., Li, D., Lin, S., Wells, A.L., and Singer, R.H. (2012). Regulation of local expression of cell adhesion and motility-related mRNAs in breast cancer cells by IMP1/ZBP1. J Cell Sci *125*, 81–91.

Guan, X., and Clark, G.A. (2006). Essential role of somatic and synaptic protein synthesis and axonal transport in long-term synapse-specific facilitation at distal sensorimotor connections in Aplysia. Biol. Bull. *210*, 238–254.

Gujral, T.S., Karp, E.S., Chan, M., Chang, B.H., and MacBeath, G. (2013). Family-wide investigation of PDZ domain-mediated protein-protein interactions implicates β-catenin in maintaining the integrity of tight junctions. Chem. Biol. *20*, 816–827.

Gumy, L.F., Tan, C.L., and Fawcett, J.W. (2010). The role of local protein synthesis and degradation in axon regeneration. Exp. Neurol. *223*, 28–37.

Gumy, L.F., Yeo, G.S.H., Tung, Y.-C.L., Zivraj, K.H., Willis, D., Coppola, G., Lam, B.Y.H., Twiss, J.L., Holt, C.E., and Fawcett, J.W. (2011). Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. RNA *17*, 85–98.

Hagena, H., and Manahan-Vaughan, D. (2013). Differentiation in the protein synthesis-dependency of persistent synaptic plasticity in mossy fiber and associational/commissural CA3 synapses in vivo. Front Integr Neurosci *7*, 10.

Hall, A.C., Lucas, F.R., and Salinas, P.C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell *100*, 525–535.

Hanz, S., Perlson, E., Willis, D., Zheng, J.Q., Massarwa, R., Huerta, J.J., Koltzenburg, M., Kohler, M., van-Minnen, J., Twiss, J.L., et al. (2003). Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron *40*, 1095–1104.

Harris, W.A., Holt, C.E., and Bonhoeffer, F. (1987). Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibres in vivo. Development *101*, 123–133.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. *18*, 1926–1945.

Hengst, U., Deglincerti, A., Kim, H.J., Jeon, N.L., and Jaffrey, S.R. (2009). Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. Nat Cell Biol *11*, 1024–1030.

Heyer, E.E., and Moore, M.J. (2016). Redefining the Translational Status of 80S Monosomes. Cell *164*, 757–769.

Hillefors, M., Gioio, A.E., Mameza, M.G., and Kaplan, B.B. (2007). Axon viability and mitochondrial function are dependent on local protein synthesis in sympathetic neurons. Cell. Mol. Neurobiol. *27*, 701–716.

Holt, C.E., and Schuman, E.M. (2013). The central dogma decentralized: new perspectives on RNA function and local translation in neurons. Neuron *80*, 648–657.

Hörnberg, H., Wollerton-van Horck, F., Maurus, D., Zwart, M., Svoboda, H., Harris, W.A., and Holt, C.E. (2013). RNA-binding protein Hermes/RBPMS inversely affects synapse density and axon arbor formation in retinal ganglion cells in vivo. J. Neurosci. *33*, 10384–10395.

Hsu, W.-L., Chung, H.-W., Wu, C.-Y., Wu, H.-I., Lee, Y.-T., Chen, E.-C., Fang, W., and Chang, Y.-C. (2015). Glutamate Stimulates Local Protein Synthesis in the Axons of Rat Cortical Neurons by Activating α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors and Metabotropic Glutamate Receptors. J. Biol. Chem. *290*, 20748–20760.

Hu, J.-Y., Meng, X., and Schacher, S. (2002). Target interaction regulates distribution and stability of specific mRNAs. J. Neurosci. *22*, 2669–2678.

Hu, J.-Y., Goldman, J., Wu, F., and Schacher, S. (2004). Target-dependent release of a presynaptic neuropeptide regulates the formation and maturation of specific synapses in Aplysia. J. Neurosci. *24*, 9933–9943.

Huber, K.M., Kayser, M.S., and Bear, M.F. (2000). Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. Science *288*, 1254–1257.

Jiménez-Díaz, L., Géranton, S.M., Passmore, G.M., Leith, J.L., Fisher, A.S., Berliocchi, L., Sivasubramaniam, A.K., Sheasby, A., Lumb, B.M., and Hunt, S.P. (2008). Local translation in primary afferent fibers regulates nociception. PLoS ONE *3*, e1961.

Jirikowski, G.F., Sanna, P.P., and Bloom, F.E. (1990). mRNA coding for oxytocin is present in axons of the hypothalamo-neurohypophysial tract. Proc. Natl. Acad. Sci. U.S.A. *87*, 7400–7404.

Jung, H., Yoon, B.C., and Holt, C.E. (2012). Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. Nat Rev Neurosci *13*, 308–324.

Jung, H., Gkogkas, C.G., Sonenberg, N., and Holt, C.E. (2014). Remote control of gene function by local translation. Cell *157*, 26–40.

Kaether, C., Skehel, P., and Dotti, C.G. (2000). Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons. Mol Biol Cell *11*, 1213–1224.

Kalous, A., Stake, J.I., Yisraeli, J.K., and Holt, C.E. (2014). RNA-binding protein Vg1RBP regulates terminal arbor formation but not long-range axon navigation in the developing visual system. Dev Neurobiol *74*, 303–318.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science *294*, 1030–1038.

Kang, H., and Schuman, E.M. (1996). A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science *273*, 1402–1406.

Kano, M., Ohno-Shosaku, T., Hashimotodani, Y., Uchigashima, M., and Watanabe, M. (2009). Endocannabinoid-mediated control of synaptic transmission. Physiol. Rev. *89*, 309–380.

Keene, J.D. (2007). RNA regulons: coordination of post-transcriptional events. Nat Rev Genet *8*, 533–543.

Keene, J.D., and Tenenbaum, S.A. (2002). Eukaryotic mRNPs may represent posttranscriptional operons. Mol. Cell *9*, 1161–1167.

Khoutorsky, A., and Price, T.J. (2018). Translational Control Mechanisms in Persistent Pain. Trends in Neurosciences *41*, 100–114.

Kim, S., and Martin, K.C. (2015). Neuron-wide RNA transport combines with netrin-mediated local translation to spatially regulate the synaptic proteome. Elife *4*.

King, M.L., Messitt, T.J., and Mowry, K.L. (2005). Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. Biol Cell *97*, 19–33.

Kleiman, R., Banker, G., and Steward, O. (1994). Development of subcellular mRNA compartmentation in hippocampal neurons in culture. J Neurosci *14*, 1130–1140.

Koenig, E., Martin, R., Titmus, M., and Sotelo-Silveira, J.R. (2000). Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons. J. Neurosci. *20*, 8390–8400.

Kraszewski, K., Mundigl, O., Daniell, L., Verderio, C., Matteoli, M., and De Camilli, P. (1995). Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. J. Neurosci. *15*, 4328–4342.

Kugler, J.M., and Lasko, P. (2009). Localization, anchoring and translational control of oskar, gurken, bicoid and nanos mRNA during Drosophila oogenesis. Fly (Austin) *3*, 15–28.

Kun, A., Otero, L., Sotelo-Silveira, J.R., and Sotelo, J.R. (2007). Ribosomal distributions in axons of mammalian myelinated fibers. J. Neurosci. Res. *85*, 2087–2098.

Kundel, M., Jones, K.J., Shin, C.Y., and Wells, D.G. (2009). Cytoplasmic polyadenylation element-binding protein regulates neurotrophin-3-dependent beta-catenin mRNA translation in developing hippocampal neurons. J. Neurosci. *29*, 13630–13639.

Lapidus, K., Wyckoff, J., Mouneimne, G., Lorenz, M., Soon, L., Condeelis, J.S., and Singer, R.H. (2007). ZBP1 enhances cell polarity and reduces chemotaxis. J Cell Sci *120*, 3173–3178.

Lawrence, J.B., and Singer, R.H. (1986). Intracellular localization of messenger RNAs for cytoskeletal proteins. Cell *45*, 407–415.

Lécuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T.R., Tomancak, P., and Krause, H.M. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell *131*, 174–187.

Leung, K.-M., van Horck, F.P.G., Lin, A.C., Allison, R., Standart, N., and Holt, C.E. (2006). Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. Nat. Neurosci. *9*, 1247–1256.

Liao, G., Simone, B., and Liu, G. (2011). Mis-localization of Arp2 mRNA impairs persistence of directional cell migration. Exp Cell Res *317*, 812–822.

Liu, K., Hu, J.-Y., Wang, D., and Schacher, S. (2003). Protein synthesis at synapse versus cell body: enhanced but transient expression of long-term facilitation at isolated synapses. J. Neurobiol. *56*, 275–286.

Liu-Yesucevitz, L., Bassell, G.J., Gitler, A.D., Hart, A.C., Klann, E., Richter, J.D., Warren, S.T., and Wolozin, B. (2011). Local RNA translation at the synapse and in disease. J Neurosci *31*, 16086–16093.

Long, R.M., Singer, R.H., Meng, X., Gonzalez, I., Nasmyth, K., and Jansen, R.P. (1997). Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. Science *277*, 383–387.

Lu, B. (2003). BDNF and Activity-Dependent Synaptic Modulation. Learn. Mem. *10*, 86–98.

Lucido, A.L., Suarez Sanchez, F., Thostrup, P., Kwiatkowski, A.V., Leal-Ortiz, S., Gopalakrishnan, G., Liazoghli, D., Belkaid, W., Lennox, R.B., Grutter, P., et al. (2009). Rapid assembly of functional presynaptic boutons triggered by adhesive contacts. J. Neurosci. *29*, 12449–12466.

Luk, E., Yang, M., Jensen, L.T., Bourbonnais, Y., and Culotta, V.C. (2005). Manganese activation of superoxide dismutase 2 in the mitochondria of Saccharomyces cerevisiae. J Biol Chem *280*, 22715–22720.

Lyles, V., Zhao, Y., and Martin, K.C. (2006). Synapse formation and mRNA localization in cultured Aplysia neurons. Neuron *49*, 349–356.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev Cell *17*, 9–26.

Manabe, T., Togashi, H., Uchida, N., Suzuki, S.C., Hayakawa, Y., Yamamoto, M., Yoda, H., Miyakawa, T., Takeichi, M., and Chisaka, O. (2000). Loss of cadherin-11 adhesion receptor enhances plastic changes in hippocampal synapses and modifies behavioral responses. Mol. Cell. Neurosci. *15*, 534–546.

Manitt, C., Nikolakopoulou, A.M., Almario, D.R., Nguyen, S.A., and Cohen-Cory, S. (2009). Netrin participates in the development of retinotectal synaptic connectivity by modulating axon arborization and synapse formation in the developing brain. J. Neurosci. *29*, 11065–11077.

Mann, F., Miranda, E., Weinl, C., Harmer, E., and Holt, C.E. (2003). B-type Eph receptors and ephrins induce growth cone collapse through distinct intracellular pathways. J. Neurobiol. *57*, 323–336.

Manns, R.P.C., Cook, G.M.W., Holt, C.E., and Keynes, R.J. (2012). Differing semaphorin 3A concentrations trigger distinct signaling mechanisms in growth cone collapse. J. Neurosci. *32*, 8554–8559.

Martin, K.C., Casadio, A., Zhu, H., Yaping, E., Rose, J.C., Chen, M., Bailey, C.H., and Kandel, E.R. (1997). Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. Cell *91*, 927–938.

Martin, K.C., Barad, M., and Kandel, E.R. (2000). Local protein synthesis and its role in synapse-specific plasticity. Curr. Opin. Neurobiol. *10*, 587–592.

Medioni, C., Mowry, K., and Besse, F. (2012). Principles and roles of mRNA localization in animal development. Development *139*, 3263–3276.

Melemedjian, O.K., Tillu, D.V., Moy, J.K., Asiedu, M.N., Mandell, E.K., Ghosh, S., Dussor, G., and Price, T.J. (2014). Local translation and retrograde axonal transport of CREB regulates IL-6-induced nociceptive plasticity. Mol Pain *10*, 45.

Melia, K.R., Trembleau, A., Oddi, R., Sanna, P.P., and Bloom, F.E. (1994). Detection and regulation of tyrosine hydroxylase mRNA in catecholaminergic terminal fields: possible axonal compartmentalization. Exp. Neurol. *130*, 394–406.

Merianda, T.T., Lin, A.C., Lam, J.S.Y., Vuppalanchi, D., Willis, D.E., Karin, N., Holt, C.E., and Twiss, J.L. (2009). A functional equivalent of endoplasmic reticulum and Golgi in axons for secretion of locally synthesized proteins. Mol. Cell. Neurosci. *40*, 128–142.

Meyer, M.P., and Smith, S.J. (2006). Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. J. Neurosci. *26*, 3604–3614.

Mili, S., Moissoglu, K., and Macara, I.G. (2008). Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. Nature *453*, 115–119.

Miller, S., Yasuda, M., Coats, J.K., Jones, Y., Martone, M.E., and Mayford, M. (2002). Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron *36*, 507–519.

Ming, G., Wong, S.T., Henley, J., Yuan, X., Song, H., Spitzer, N.C., and Poo, M. (2002). Adaptation in the chemotactic guidance of nerve growth cones. Nature *417*, 411–418.

Minis, A., Dahary, D., Manor, O., Leshkowitz, D., Pilpel, Y., and Yaron, A. (2014). Subcellular transcriptomics-dissection of the mRNA composition in the axonal compartment of sensory neurons. Dev Neurobiol *74*, 365–381.

Miyashiro, K., Dichter, M., and Eberwine, J. (1994). On the nature and differential distribution of mRNAs in hippocampal neurites: implications for neuronal functioning. Proc Natl Acad Sci U S A *91*, 10800–10804.

Mohr, E., and Richter, D. (1992). Diversity of mRNAs in the Axonal Compartment of Peptidergic Neurons in the Rat. Eur. J. Neurosci. *4*, 870–876.

Mohr, E., and Richter, D. (2000). Axonal mRNAs: functional significance in vertebrates and invertebrates. J. Neurocytol. *29*, 783–791.

Mohr, E., Fehr, S., and Richter, D. (1991). Axonal transport of neuropeptide encoding mRNAs within the hypothalamo-hypophyseal tract of rats. EMBO J. *10*, 2419–2424.

Nédelec, S., Peljto, M., Shi, P., Amoroso, M.W., Kam, L.C., and Wichterle, H. (2012). Concentration-dependent requirement for local protein synthesis in motor neuron subtype-specific response to axon guidance cues. J. Neurosci. *32*, 1496–1506.

Nevo-Dinur, K., Nussbaum-Shochat, A., Ben-Yehuda, S., and Amster-Choder, O. (2011). Translation-independent localization of mRNA in E. coli. Science *331*, 1081–1084.

Nie, D., Di Nardo, A., Han, J.M., Baharanyi, H., Kramvis, I., Huynh, T., Dabora, S., Codeluppi, S., Pandolfi, P.P., Pasquale, E.B., et al. (2010). Tsc2-Rheb Signaling Regulates EphA-Mediated Axon Guidance. Nat Neurosci *13*, 163–172.

Nishimura, W., Yao, I., Iida, J., Tanaka, N., and Hata, Y. (2002). Interaction of synaptic scaffolding molecule and Beta -catenin. J. Neurosci. *22*, 757–765.

Okabe, S., Miwa, A., and Okado, H. (2001). Spine formation and correlated assembly of presynaptic and postsynaptic molecules. J. Neurosci. *21*, 6105–6114.

Ostroff, L.E., Fiala, J.C., Allwardt, B., and Harris, K.M. (2002). Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. Neuron *35*, 535–545.

Pareyson, D., and Marchesi, C. (2009). Diagnosis, natural history, and management of Charcot–Marie–Tooth disease. The Lancet Neurology *8*, 654–667.

Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998). A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. Cell *95*, 615–624.

Perry, R.B., Doron-Mandel, E., Iavnilovitch, E., Rishal, I., Dagan, S.Y., Tsoory, M., Coppola, G., McDonald, M.K., Gomes, C., Geschwind, D.H., et al. (2012). Subcellular knockout of importin β1 perturbs axonal retrograde signaling. Neuron *75*, 294–305.

Phillips, G.R., Huang, J.K., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shan, W.S., Arndt, K., Frank, M., Gordon, R.E., et al. (2001). The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. Neuron *32*, 63–77.

Pinsker, H., Kupfermann, I., Castellucci, V., and Kandel, E. (1970). Habituation and dishabituation of the gill-withdrawal reflex in Aplysia. Science *167*, 1740–1742.

Pinto, M.J., and Almeida, R.D. (2016). Puzzling out presynaptic differentiation. J. Neurochem. *139*, 921–942.

Piper, M., Anderson, R., Dwivedy, A., Weinl, C., van Horck, F., Leung, K.M., Cogill, E., and Holt, C. (2006). Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones. Neuron *49*, 215–228.

Poo, M.-M., Pignatelli, M., Ryan, T.J., Tonegawa, S., Bonhoeffer, T., Martin, K.C., Rudenko, A., Tsai, L.-H., Tsien, R.W., Fishell, G., et al. (2016). What is memory? The present state of the engram. BMC Biol. *14*, 40.

Poon, M.M., Choi, S.-H., Jamieson, C.A.M., Geschwind, D.H., and Martin, K.C. (2006). Identification of process-localized mRNAs from cultured rodent hippocampal neurons. J. Neurosci. *26*, 13390–13399.

Price, T.J., and Inyang, K.E. (2015). Commonalities between pain and memory mechanisms and their meaning for understanding chronic pain. Prog Mol Biol Transl Sci *131*, 409–434.

Rage, F., Boulisfane, N., Rihan, K., Neel, H., Gostan, T., Bertrand, E., Bordonné, R., and Soret, J. (2013). Genome-wide identification of mRNAs associated with the protein SMN whose depletion decreases their axonal localization. RNA *19*, 1755–1766.

Rangaraju, V., Tom Dieck, S., and Schuman, E.M. (2017). Local translation in neuronal compartments: how local is local? EMBO Rep. *18*, 693–711.

Ratnayaka, A., Marra, V., Branco, T., and Staras, K. (2011). Extrasynaptic vesicle recycling in mature hippocampal neurons. Nat Commun *2*, 531.

Rishal, I., and Fainzilber, M. (2014). Axon-soma communication in neuronal injury. Nat Rev Neurosci *15*, 32–42.

Roche, F.K., Marsick, B.M., and Letourneau, P.C. (2009). Protein synthesis in distal axons is not required for growth cone responses to guidance cues. J. Neurosci. *29*, 638–652.

Ruthazer, E.S., Li, J., and Cline, H.T. (2006). Stabilization of axon branch dynamics by synaptic maturation. J. Neurosci. *26*, 3594–3603.

Saal, L., Briese, M., Kneitz, S., Glinka, M., and Sendtner, M. (2014). Subcellular transcriptome alterations in a cell culture model of spinal muscular atrophy point to widespread defects in axonal growth and presynaptic differentiation. RNA *20*, 1789–1802.

Sceniak, M.P., Berry, C.T., and Sabo, S.L. (2012). Facilitation of neocortical presynaptic terminal development by NMDA receptor activation. Neural Dev *7*, 8.

Schacher, S., and Wu, F. (2002). Synapse formation in the absence of cell bodies requires protein synthesis. J. Neurosci. *22*, 1831–1839.

Scheiffele, P., Fan, J., Choih, J., Fetter, R., and Serafini, T. (2000). Neuroligin Expressed in Nonneuronal Cells Triggers Presynaptic Development in Contacting Axons. Cell *101*, 657–669.

Shapira, M., Zhai, R.G., Dresbach, T., Bresler, T., Torres, V.I., Gundelfinger, E.D., Ziv, N.E., and Garner, C.C. (2003). Unitary Assembly of Presynaptic Active Zones from Piccolo-Bassoon Transport Vesicles. Neuron *38*, 237–252.

Shepard, K.A., Gerber, A.P., Jambhekar, A., Takizawa, P.A., Brown, P.O., Herschlag, D., DeRisi, J.L., and Vale, R.D. (2003). Widespread cytoplasmic mRNA transport in yeast: identification of 22 bud-localized transcripts using DNA microarray analysis. Proc Natl Acad Sci U S A *100*, 11429–11434.

Shestakova, E.A., Wyckoff, J., Jones, J., Singer, R.H., and Condeelis, J. (1999). Correlation of beta-actin messenger RNA localization with metastatic potential in rat adenocarcinoma cell lines. Cancer Res *59*, 1202–1205.

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, J.Q., Amieux, P.S., and Holt, C.E. (2016). Dynamic Axonal Translation in Developing and Mature Visual Circuits. Cell *166*, 181–192.

Spillane, M., Ketschek, A., Donnelly, C.J., Pacheco, A., Twiss, J.L., and Gallo, G. (2012). Nerve growth factor-induced formation of axonal filopodia and collateral branches involves the intra-axonal synthesis of regulators of the actin-nucleating Arp2/3 complex. J. Neurosci. *32*, 17671–17689.

Spillane, M., Ketschek, A., Merianda, T.T., Twiss, J.L., and Gallo, G. (2013). Mitochondria coordinate sites of axon branching through localized intra-axonal protein synthesis. Cell Rep *5*, 1564–1575.

St Johnston, D. (2005). Moving messages: the intracellular localization of mRNAs. Nat Rev Mol Cell Biol *6*, 363–375.

Steward, O., and Levy, W.B. (1982). Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J Neurosci *2*, 284–291.

Steward, O., and Ribak, C.E. (1986). Polyribosomes associated with synaptic specializations on axon initial segments: localization of protein-synthetic machinery at inhibitory synapses. J. Neurosci. *6*, 3079–3085.

Sutton, M.A., and Schuman, E.M. (2006). Dendritic protein synthesis, synaptic plasticity, and memory. Cell *127*, 49–58.

Takamori, S., Holt, M., Stenius, K., Lemke, E.A., Grønborg, M., Riedel, D., Urlaub, H., Schenck, S., Brügger, B., Ringler, P., et al. (2006). Molecular Anatomy of a Trafficking Organelle. Cell *127*, 831–846.

Takizawa, P.A., Sil, A., Swedlow, J.R., Herskowitz, I., and Vale, R.D. (1997). Actin-dependent localization of an RNA encoding a cell-fate determinant in yeast. Nature *389*, 90–93.

Tang, L., Hung, C.P., and Schuman, E.M. (1998). A role for the cadherin family of cell adhesion molecules in hippocampal long-term potentiation. Neuron *20*, 1165–1175.

Taylor, A.M., Berchtold, N.C., Perreau, V.M., Tu, C.H., Li Jeon, N., and Cotman, C.W. (2009). Axonal mRNA in uninjured and regenerating cortical mammalian axons. J Neurosci *29*, 4697–4707.

Taylor, A.M., Wu, J., Tai, H.-C., and Schuman, E.M. (2013). Axonal translation of β-catenin regulates synaptic vesicle dynamics. J. Neurosci. *33*, 5584–5589.

Tcherkezian, J., Brittis, P.A., Thomas, F., Roux, P.P., and Flanagan, J.G. (2010). Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation. Cell *141*, 632–644.

Tiruchinapalli, D.M., Oleynikov, Y., Kelic, S., Shenoy, S.M., Hartley, A., Stanton, P.K., Singer, R.H., and Bassell, G.J. (2003). Activity-dependent trafficking and dynamic localization of zipcode binding protein 1 and beta-actin mRNA in dendrites and spines of hippocampal neurons. J. Neurosci. *23*, 3251–3261.

Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O., and Takeichi, M. (2002). Cadherin regulates dendritic spine morphogenesis. Neuron *35*, 77–89.

Tohda, C., Sasaki, M., Konemura, T., Sasamura, T., Itoh, M., and Kuraishi, Y. (2001). Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. J. Neurochem. *76*, 1628–1635.

Tom Dieck, S., Hanus, C., and Schuman, E.M. (2014). SnapShot: local protein translation in dendrites. Neuron *81*, 958–958.e1.

Trembleau, A., Melia, K.R., and Bloom, F.E. (1995). BC1 RNA and vasopressin mRNA in rat neurohypophysis: axonal compartmentalization and differential regulation during dehydration and rehydration. Eur. J. Neurosci. *7*, 2249–2260.

Trembleau, A., Morales, M., and Bloom, F.E. (1996). Differential compartmentalization of vasopressin messenger RNA and neuropeptide within the rat hypothalamo-neurohypophysial axonal tracts: light and electron microscopic evidence. Neuroscience *70*, 113–125.

Vaughn, J.E. (1989). Fine structure of synaptogenesis in the vertebrate central nervous system. Synapse *3*, 255–285.

Verma, P., Chierzi, S., Codd, A.M., Campbell, D.S., Meyer, R.L., Holt, C.E., and Fawcett, J.W. (2005). Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. J Neurosci *25*, 331–342.

Waites, C.L., Craig, A.M., and Garner, C.C. (2005). Mechanisms of vertebrate synaptogenesis. Annu. Rev. Neurosci. *28*, 251–274.

Waites, C.L., Leal-Ortiz, S.A., Andlauer, T.F.M., Sigrist, S.J., and Garner, C.C. (2011). Piccolo regulates the dynamic assembly of presynaptic F-actin. J. Neurosci. *31*, 14250–14263.

Weil, T.T. (2014). mRNA localization in the Drosophila germline. RNA Biol *11*, 1010–1018.

Wensley, C.H., Stone, D.M., Baker, H., Kauer, J.S., Margolis, F.L., and Chikaraishi, D.M. (1995). Olfactory marker protein mRNA is found in axons of olfactory receptor neurons. J. Neurosci. *15*, 4827–4837.

Wong, H.H.-W., Lin, J.Q., Ströhl, F., Roque, C.G., Cioni, J.-M., Cagnetta, R., Turner-Bridger, B., Laine, R.F., Harris, W.A., Kaminski, C.F., et al. (2017). RNA Docking and Local Translation Regulate Site-Specific Axon Remodeling In Vivo. Neuron *95*, 852–868.e8.

Wu, K.Y., Hengst, U., Cox, L.J., Macosko, E.Z., Jeromin, A., Urquhart, E.R., and Jaffrey, S.R. (2005). Local translation of RhoA regulates growth cone collapse. Nature *436*, 1020–1024.

Yen, H.-C.S., Xu, Q., Chou, D.M., Zhao, Z., and Elledge, S.J. (2008). Global Protein Stability Profiling in Mammalian Cells. Science *322*, 918–923.

Yin, H.H., Davis, M.I., Ronesi, J.A., and Lovinger, D.M. (2006). The role of protein synthesis in striatal long-term depression. J. Neurosci. *26*, 11811–11820.

Yogev, O., Karniely, S., and Pines, O. (2007). Translation-coupled translocation of yeast fumarase into mitochondria in vivo. J Biol Chem *282*, 29222–29229.

Yoon, B.C., Jung, H., Dwivedy, A., O'Hare, C.M., Zivraj, K.H., and Holt, C.E. (2012). Local translation of extranuclear lamin B promotes axon maintenance. Cell *148*, 752–764.

Younts, T.J., Monday, H.R., Dudok, B., Klein, M.E., Jordan, B.A., Katona, I., and Castillo, P.E. (2016). Presynaptic Protein Synthesis Is Required for Long-Term Plasticity of GABA Release. Neuron *92*, 479–492.

Zappulo, A., van den Bruck, D., Ciolli Mattioli, C., Franke, V., Imami, K., McShane, E., Moreno-Estelles, M., Calviello, L., Filipchyk, A., Peguero-Sanchez, E., et al. (2017). RNA localization is a key determinant of neurite-enriched proteome. Nat Commun *8*, 583.

Zelená, J. (1970). Ribosome-like particles in myelinated axons of the rat. Brain Res. *24*, 359–363.

Zhai, R.G., Vardinon-Friedman, H., Cases-Langhoff, C., Becker, B., Gundelfinger, E.D., Ziv, N.E., and Garner, C.C. (2001). Assembling the presynaptic active zone: a characterization of an active zone precursor vesicle. Neuron *29*, 131–143.

Zhang, W., and Benson, D.L. (2001). Stages of synapse development defined by dependence on F-actin. J. Neurosci. *21*, 5169–5181.

Zhang, X., and Poo, M. (2002). Localized Synaptic Potentiation by BDNF Requires Local Protein Synthesis in the Developing Axon. Neuron *36*, 675–688.

Zheng, J.Q., Kelly, T.K., Chang, B., Ryazantsev, S., Rajasekaran, A.K., Martin, K.C., and Twiss, J.L. (2001). A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. J Neurosci *21*, 9291–9303.

Zipor, G., Haim-Vilmovsky, L., Gelin-Licht, R., Gadir, N., Brocard, C., and Gerst, J.E. (2009). Localization of mRNAs coding for peroxisomal proteins in the yeast, Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *106*, 19848–19853.

Ziv, N.E., and Garner, C.C. (2004). Cellular and molecular mechanisms of presynaptic assembly. Nat. Rev. Neurosci. *5*, 385–399.

Zivraj, K.H., Tung, Y.C.L., Piper, M., Gumy, L., Fawcett, J.W., Yeo, G.S.H., and Holt, C.E. (2010). Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. J. Neurosci. *30*, 15464–15478.

AIMS

The main goal of this proposal is to understand the role of local translation on presynaptic assembly.

SPECIFIC AIMS:

1. Determine whether axonal protein synthesis inhibition has an influence on synapse formation between axons and an artificial adhesive substrate.
2. Determine if any of the common synapse-related transcripts found in axonal transcriptomes cluster at axon contact sites and whether they are locally translated during synapse formation.
3. Determine if interfering with local protein synthesis of specific transcripts during synapse formation has an influence on presynaptic dynamics.

EXPERIMENTAL WORK

Intra-axonal synthesis of SNAP25 is required for the formation of presynaptic terminals

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Intra-axonal synthesis of SNAP25 is required for the formation of presynaptic terminals.

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Intra-axonal synthesis of SNAP25 is required for the formation of presynaptic terminals

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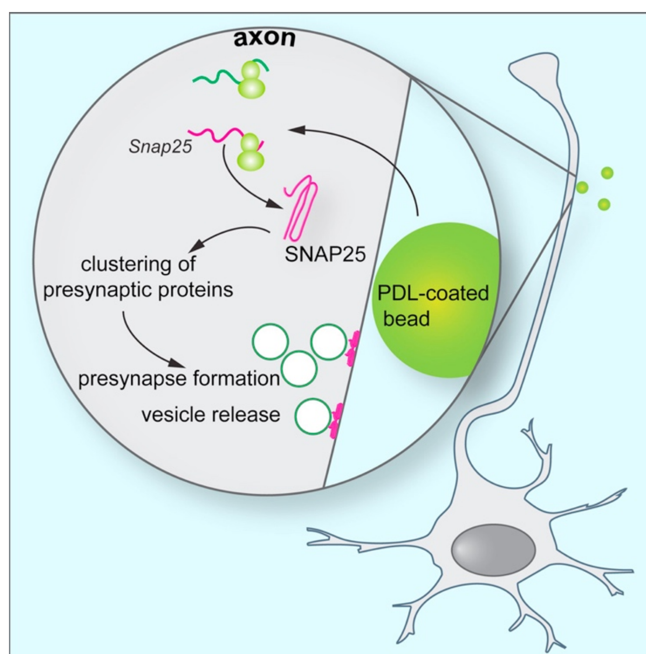
Graphical abstract



In brief

Batista et al. find that, during the assembly of presynaptic terminals, mRNA translation is upregulated at the nascent presynapses and required for the clustering of presynaptic proteins. Inhibition of local SNAP25 synthesis prevents proper formation of presynaptic terminal and interferes with synaptic vesicle release.

Highlights

- Protein synthesis is rapidly induced at contact sites with presynaptic organizers
- The presynaptic protein SNAP25 is locally synthesized at induced presynaptic sites
- Local SNAP25 synthesis is required for formation of presynapses
- Inhibition of local SNAP25 synthesis interferes with vesicle release

SUMMARY

Localized protein synthesis is a mechanism for developing axons to react acutely and in a spatially restricted manner to extracellular signals. As such it is important for many aspects of axonal development, but its role in the formation of presynapses remains poorly understood. We found that the induced assembly of presynaptic terminals required local protein synthesis. Newly synthesized proteins were detectable at nascent presynapses within 15 minutes of inducing synapse formation in isolated axons. The transcript for the t-SNARE protein SNAP25, which is required for the fusion of synaptic vesicles with the plasma membrane, was recruited to presynaptic sites and locally translated. Inhibition of intra-axonal SNAP25 synthesis affected the clustering of SNAP25 and other presynaptic proteins and interfered with the release of synaptic vesicles from presynaptic sites. This study reveals a critical role for the axonal synthesis of SNAP25 in the assembly of presynaptic terminals.

Keywords

presynapse, synapse formation, local protein synthesis, SNAP25, β-catenin, vesicle release, axon

INTRODUCTION

During the development of the nervous system axons project over long distances to their cognate targets, until upon contact with target-derived adhesive or soluble factors the assembly of a presynaptic terminal is initiated (Chia et al., 2013; Jin and Garner, 2008). Application of these presynaptic organizing molecules to isolated axons is sufficient to induce presynapse formation from components that have been transported from the neuronal soma. An alternative source for at least some of the presynaptic proteins might be the axon itself through the process of local translation. Protein synthesis in axons is required for proper axon development (Campbell and Holt, 2001; Gracias et al., 2014; Hengst et al., 2009; Wu et al., 2005) by providing a spatially and temporally tightly restricted source of protein in response to extracellular signals (Batista and Hengst, 2016). Transcripts coding for several presynaptic proteins have been found in developing cortical axons (Taylor et al., 2009), and in Aplysia, protein synthesis is required for the formation of presynapses (Schacher and Wu, 2002). Recently, the importance of presynaptic protein synthesis in the control of neurotransmitter release was reported for the mature mammalian brain (Younts et al., 2016), but the role of local translation in the formation of presynapses remains poorly understood. Specifically, it is unknown whether axonal protein synthesis is required for the assembly of presynaptic terminals. So far only one locally synthesized protein has been described that accumulates at nascent presynapses, β-catenin, where it regulates the release of synaptic vesicles (Taylor et al., 2013). Here, we report the induced intra-axonal synthesis of the t-SNARE protein synaptosomal-associated protein 25 (SNAP25) as a necessary, early step for the clustering of presynaptic proteins and the formation and function of presynapses.

RESULTS

Presynaptic proteins cluster within 1 hour at contact sites with PDL-coated beads

To investigate whether the formation of presynapses requires local protein synthesis we cultured embryonic hippocampal neurons in tripartite microfluidic chambers that allow the fluidic isolation of axons from cell bodies and dendrites (Figure 1A) (Baleriola et al., 2014; Taylor et al., 2005). Poly-D-lysine (PDL)-coated latex beads were applied selectively to the axonal compartments to induce the clustering of presynaptic proteins (Lucido et al., 2009; Taylor et al., 2013). Immunostaining revealed significantly increased levels of several presynaptic proteins (β-catenin, synaptophysin, GAP-43, SNAP25) and tau at contact sites between axons and PDL-coated beads after 24 hours of incubation, while β-III tubulin levels were unchanged (Figure 1B). As local protein synthesis is frequently an acute reaction to an external stimulus we next investigated how early after addition of the PDL-coated beads

we could detect clustering of presynaptic proteins. SNAP25 levels were significantly increased after 1 hour of contact with the PDL-coated beads (Figure 1C).

Inhibition of axonal protein synthesis prevents clustering of β-catenin and SNAP25 at 1 hour

Previously, it has been reported that the clustering of β-catenin at 3 hours after addition of PDL-coated beads requires local protein synthesis (Taylor et al., 2013). To determine whether the clustering of other presynaptic proteins is likewise dependent on axonal translation, we focused on SNAP25 and added the protein synthesis inhibitor emetine selectively to the axonal compartment during the treatment with uncoated or PDL-coated beads (Figure 2A). We quantified the fluorescent intensity within axons along a 30-μm-long line starting at the center of the beads (Figure 2B). Within 1 hour of treatment with PDL-coated beads, β-catenin and SNAP25 were significantly increased in the first 5 μm from the beads' centers, i.e. at contact sites (Figures 2C-E). The clustering of β-catenin and SNAP25 did not show any bias for either the proximal (i.e., toward the cell body) or distal side of the beads (Figure S1); thus, for our analyses we did not distinguish between the proximal and distal sides. Uncoated beads did not induce clustering, and addition of emetine completely prevented the clustering of β-catenin and SNAP25. Neither tau nor β-III tubulin clustered at this early time point (Figures 2F and 2G). To determine whether the observed protein synthesis dependency of β-catenin and SNAP25 clustering at 1 hour was limited to this early time point we analyzed their clustering at 3, 6 and 12 hours after addition of beads and emetine to the axons. While SNAP25 clustering required protein synthesis at all time points tested, β-catenin clustering was significantly affected by emetine only at 3 hours (as previously reported by Taylor et al. (2013) but not at the later time points [Figure 2H]).

PDL-coated beads induce protein synthesis at axonal contact sites within 15 minutes

The requirement for local translation for clustering after 1 hour of incubation with PDL-coated beads indicated that this treatment might trigger protein synthesis directly and acutely at contact sites with axons. We used puromycylation, also known as SUnSET (Figure 3A) (Schmidt et al., 2009), to detect protein synthesis events in axons. Puromycin is a tRNA analog that gets incorporated into the nascent protein chain (Yarmolinsky and Haba, 1959), allowing the detection of protein synthesis in situ with an anti-puromycin antibody. We detected a significant increase in the number of puromycin-positive puncta in axons at contact sites within 15 minutes of addition of PDL-coated beads (Figure 3B). The increase of the puromycin signal was accompanied by an increased presence of phosphor-EBP1, a marker for active translation, at contact sites with PDL-coated but not uncoated beads (Figure 3C). To investigate whether this immediate induction of protein synthesis was sustained over a longer time

period, we added the beads for 1 hour and shifted the puromycylation time window to the last 10 minutes of the assay. As before, the addition of PDL-coated beads was associated with a significantly higher number of puromycylation-positive puncta in their immediate vicinity compared to uncoated beads, and addition of the protein synthesis inhibitor anisomycin completely abolished this effect (Figure 3D).

Detection of SNAP25 mRNA in axons by fluorescent in situ hybridization

mRNA encoding SNAP25 has previously been found in axons of cortical neurons (Taylor et al., 2009), but it was below the detection threshold in other axonal transcriptome datasets (Baleriola et al., 2014, Zivraj et al., 2010). Here, we used single-molecule inexpensive fluorescent in situ hybridization (smiFISH; Tsanov et al., 2016) to directly visualize SNAP25 transcripts in axons of dissociated embryonic hippocampal neurons and to determine whether their intra-axonal localization changes in response to treatment with PDL-coated beads (Figure 4A). SNAP25 mRNA FISH-positive puncta were readily detectable at contact sites with PDL-coated beads, indicating that contact with PDL-coated beads recruits SNAP25 transcripts. The axonal smiFISH signal for SNAP25 mRNA was specific as transfection of axons with a SNAP25 small interfering RNA (siRNA) greatly reduced the number of positive puncta (Figure 4B).

SNAP25 is synthesized locally at contact sites of axons with PDL-coated beads

The protein synthesis-dependent clustering of SNAP25 and the presence of its mRNA in axons at contact sites with PDL-coated beads indicated that SNAP25 itself might be locally produced. To test this hypothesis, we adopted a different design of microfluidic chambers (Figure 4C) (Park et al., 2009). In these circular chambers, axons grow into the central open compartment, allowing the collection of axonal material in quantities required for biochemistry. SNAP25 protein was detectable by immunoblot in lysates of axons treated with uncoated beads. Application of PDL-coated beads for 24 hours greatly increased the presence of SNAP25 in axons and treatment of axons with emetine reduced SNAP25 below detection limit without affecting SNAP25 expression in the cell body compartment (Figure 4C). To directly visualize SNAP25 synthesis at contact sites with PDL-coated beads, we combined puromycylation with a proximity ligation assay (puro-PLA) (Figure 4D) (tom Dieck et al., 2015). Axons that were not treated with PDL-coated beads showed very few SNAP25 puro-PLA puncta, while treatment with PDL-coated beads induced the local synthesis of SNAP25 (Figure 4E). As before, we tested how persistent the induction of SNAP25 synthesis was by incubating the axons for 1 hour with PDL-coated beads and adding puromycin for the last 10 min. SNAP25 puro-PLA puncta were readily

detectable, and their presence was abolished by the presence of the protein synthesis inhibitor anisomycin (Figure 4F). Together, these results establish that SNAP25 is locally synthesized upon contact of axons with PDL-coated beads.

SNAP25 is locally synthesized at synapses

So far, our approach has been to induce formation of presynapses by applying PDL-coated beads. This approach allows us to study presynaptic events required for the clustering of presynaptic proteins in the absence of a postsynaptic cell, but it is necessarily artificial. To test whether SNAP25 is locally synthesized at synapses rather than at induced presynaptic specializations, we cultured embryonic hippocampal neurons in regular dissociated cultures and performed SNAP25 puro-PLA assays on DIV5, 10, and 15 (Figure 4G). SNAP25 puro-PLA puncta were visible in axons and dendrites at all developmental stages, and their frequency increased with the age of the cultures (Figure 4G). In DIV15 cultures, some SNAP25 puro-PLA puncta were found juxtapositioned to the postsynaptic protein PSD-95, indicating SNAP25 synthesis at established synapses. The appearance of SNAP25 puro-PLA puncta was prevented at all developmental stages if the cells were incubated with vehicle instead of puromycin or if the protein synthesis inhibitor anisomycin was added during the puromycylation (Figure 4H).

Axon-specific knockdown of SNAP25 mRNA reduces SNAP25 synthesis at contact sites with PDL-coated beads

To investigate the requirement of localized SNAP25 or β-catenin mRNAs, we selectively transfected the axons of hippocampal neurons grown in microfluidic chambers with siRNA (Figure 5A). Previously, we have found that RNAi is functional in axons (Hengst et al., 2006) and that localized mRNAs can be selectively knocked down using locally applied siRNAs without affecting protein expression dendrites or cell bodies (Baleriola et al., 2014; Gracias et al., 2014; Hengst et al., 2009; Villarin et al., 2016). When we transfected axons with a SNAP25 siRNA, the effect was restricted to axons (Figure 5B). The knockdown of SNAP25 mRNA in axons was only partial, likely because of the tight packaging of axonal mRNAs in granules (Buxbaum et al., 2014), which makes them inaccessible for the RNAi machinery while silenced but susceptible to siRNA under conditions that activate their translation, as we have observed previously (Baleriola et al., 2014; Villarin et al., 2016). Knockdown of axonal SNAP25 transcripts significantly reduced the appearance of SNAP25 puro-PLA puncta at contact sites with PDL-coated beads and increased that percentage of beads that had no puncta in their vicinity (Figure 5C).

Together, these results establish that axonal transfection of SNAP25 siRNA is an efficient method to prevent the local translation of SNAP25 at contact sites with PDL-coated beads.

Clustering of SNAP25 and β-catenin requires the presence of their transcripts in axons

Next, we used this approach to investigated whether the local translation of SNAP25 or β-catenin was required for the clustering of these proteins. As before, we selectively knockdown SNAP25 or β-catenin transcripts in axons by locally applied siRNA and measured protein clustering 1 hour after the application of PDL-coated beads (Figure 5D). Knockdown of axonal SNAP25 mRNA significantly prevented the clustering of SNAP25 at contact sites with PDL-coated beads (Figure 5E). Knockdown of β-catenin mRNA did not prevent clustering of β-catenin directly at the contact sites (0-5 μm) but led to broadening of the peak with increased β-catenin levels at 5-10 μm (Figure 5F). This result indicates that the vast majority of β-catenin protein clustering at PDL-coated beads is not derived from acutely triggered local synthesis but rather is of somatic origin. However, a small amount of β-catenin whose local synthesis is prevented by the siRNA appears to be required to induce the clustering of β-catenin directly at contact sites. The β-catenin siRNA used here efficiently knocks down its target mRNA when applied to dissociated hippocampal neurons (Figure 5G). Together, these results suggest that most of the SNAP25 required during the early stages of presynapse formation is derived from local protein synthesis.

Clustering of SNAP25 and β-catenin require each other's local synthesis

Next, we used the same experimental approach to test the reciprocal requirement of SNAP25 and β-catenin synthesis for the clustering of these proteins. Clustering of β-catenin protein was significantly reduced in axons depleted of SNAP25 mRNA (Figure 6A). In axons with β-catenin mRNA knockdown, clustering of SNAP25 protein was reduced as well, and the peak for SNAP25 was broadened as we had seen before for β-catenin protein itself (Figure 6B).

Knockdown of axonal SNAP25 mRNA interferes with vesicle release from induced presynaptic sites

Lastly, we performed functional assays on induced presynaptic sites in axons transfected with siRNA similar to a previously described approach (Taylor et al., 2013). To study the release of synaptic vesicles at induced presynaptic sites we used FM 4-64. FM dyes are lipophilic dyes that can be endocytosed and incorporated into synaptic vesicles. We stimulated cells with a high potassium solution in the presence of FM 4-64 and let dye endocytosis occur. Cells were then imaged and stimulated a second time with high potassium. As vesicles are exocytosed, dye molecules are released

and rapidly diffuse, resulting in nerve terminal destaining. In axons transfected with the siRNA targeting SNAP25, release of synaptic vesicles as measured by the disappearance of fluorescence was significantly slower than in scrambled siRNA transfected axons while the loading was not different (Figure 7A and 7B). Again, this effect was limited to the siRNA treated axons, as the unloading dynamic was unchanged at synapses in the cell body compartment (Figure 7C). As an additional control, we transfected of axons with an siRNA targeting another presynaptic protein, piccolo (Fenster et al., 2000), whose transcript absent from axonal transcriptome or translatome data sets (Shigeoka et al., 2016; Taylor et al., 2009). Neuron-wide knockdown of piccolo causes enhanced synaptic vesicles exocytosis rates (Leal-Ortiz et al., 2008), but axon-specific delivery of piccolo siRNA had no effect on synaptic vesicle release, again demonstrating that the siRNAs act only locally in axons and do not interfere with protein expression and transport from the cell body (Figure 7D).

Discussion

Our results establish that SNAP25 synthesis is locally activated by a presynaptic organizing signal and required for presynaptic terminal assembly. Previously, it has been reported that β-catenin is locally synthesized at nascent presynaptic sites and that the locally produced protein regulates the release dynamics of synaptic vesicles (Taylor et al., 2013). The question of whether local protein synthesis was required for the assembly of presynaptic terminal was not directly addressed. Here, we demonstrate that localized protein synthesis is a required step in the formation of presynaptic sites.
In accordance with Taylor et al. (2013), we also detect that β-catenin is locally synthesized. We find, however, differences in the requirement for local SNAP25 and β-catenin synthesis. While inhibition of protein synthesis prevents the accumulation of SNAP25 protein at contact sites with PDL-coated beads at all time points tested (1–12 hours), the accumulation of β-catenin is only affected until the 3-hours time point, indicating that at this time anterograde transport sufficiently meets the demand for β-catenin at presynapses. This difference is not easily explained by differences in the stability of SNAP25 and β-catenin: the half-life of SNAP25 during synaptogenesis in cerebellar granule neurons was reported to be 16 hours (Sanders et al., 1998), slightly longer than the half-life of β-catenin in PC12 cells of 12 hours (Bareiss et al., 2010). Instead, the role of the locally synthesized proteins appears to differ. While axonal-specific siRNA treatment prevents the clustering of SNAP25 directly at contact sites with PDL-coated beads by around 50%, the analogous treatment leads to a broadening of the profile for β-catenin: the amount of protein found directly at beads is not significantly reduced but instead more β-catenin accumulates in the vicinity of the beads (5- to 15-μm distance from the bead center). These

findings indicate that 1 hour after contact with the beads nearly half of presynaptic SNAP25 protein is derived from local synthesis. In contrast, nearly all β-catenin accumulates at or near the beads independently of local protein synthesis, i.e., by anterograde transport from the cell body. The small amount of locally produced β-catenin is required to cluster the anterogradely transported protein directly at presynaptic sites. Thus, while local production of β-catenin is required only during the first steps of presynapse assembly and does not generate the bulk of presynaptic β-catenin protein, SNAP25 synthesis persists at least until 12 hours after initiation of presynapse formation, it generates a substantial amount of synaptic SNAP25 proteins, and it continues to be required even in established synapses, as demonstrated by the effect of axonal SNAP25 mRNA knockdown on synaptic vesicle release.

A recent translatome analysis of retinal ganglion cells axons identified SNAP25 as highly expressed not only in developing, but also in mature axons (Shigeoka et al., 2016), and inhibition of protein synthesis at established presynaptic terminals deregulates GABA release (Younts et al., 2016). The stability of SNAP25 at presynapses is controlled by activity induced ubiquitination and proteasome-dependent degradation (Sheehan et al., 2016). SNAP25 synthesis at established synapses might therefore be an important mechanism for the control of synaptic SNAP25 levels and synapse function.

mRNA localization to axons is generally understood to be controlled by RNA-binding proteins that associated with mRNAs through sequence elements nearly always found in the 3′ UTRs of the transcripts. Interestingly, several SNPs located in the 3′ UTR of SNAP25 are linked to adult attention deficit disorder (ADHD) (Barr et al., 2000, Brophy et al., 2002, Kustanovich et al., 2003, Mill et al., 2004). In the context of our findings, it is tempting to speculate that these mutations might interfere with the local synthesis of SNAP25 at presynaptic sites. Future investigation of the potentially changes in subcellular localization and synaptic translation of SNAP25 mRNA might provide a mechanistic understanding of how these mutations are linked to hyperactive disorders.

In conclusion, we describe an alternative source for presynaptic proteins, intra-axonal synthesis, that is required the formation presynaptic terminals.

Experimental Procedures

Compartmentalized culture of embryonic hippocampal neurons

All work involving animals was performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and was approved by The Animal Care and Use Committee of Columbia University. All reagents were purchased from ThermoFisher Scientific unless otherwise noted. Hippocampal neurons were harvested from Sprague-Dawley embryonic day 17/18 rat embryos (Kaech and Banker, 2006). Embryonic rat neurons were grown in tripartite microfluidic chambers with 500-μm-long microgrooves connecting the three fluidically isolated compartments. Microfluidic chambers were produced according to published protocols (Gracias et al., 2014; Park et al., 2006). Primary hippocampal neurons (50,000 to 60,000 cells per chamber) were cultured in one of the side compartments and axons were allowed to grow into the other two compartments. Chambers were coated with 0.1 mg ml^{-1} poly-D-lysine (Sigma-Aldrich) and 2 μg ml^{-1} laminin (Trevigen). After 24 hours plating medium (neurobasal, 10% fetal bovine serum, 100 mM glutamine, 1mM sodium pyruvate, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) was completely exchanged for growth medium (neurobasal, 1x B27, 100 mM glutamine). Half of this growth medium was replaced with fresh growth medium on DIV 4 and DIV 8. All experiments were performed at DIV 9-11. Whenever stated, axonal compartments were treated with emetine (100 nM, EMD Millipore) or anisomycin (10 μM, Sigma-Aldrich).

Presynaptic clustering with PDL-coated beads

Bead preparation and treatments were performed as described (Lucido et al., 2009; Taylor et al., 2013). Surfactant-free aliphatic amine latex microspheres 4-5 μm in diameter (Invitrogen) were coated in 50 μg ml^{-1} poly-D-lysine (Sigma-Aldrich) at 37°C for at least 2 hours, rinsed twice with sterile water and diluted in growth medium. Uncoated beads were incubated in sterile water. In order to increase the number of beads that reached the chamber compartments, around 150,000 PDL-coated beads were added to each axonal compartment through the side access ports. As adhesion of uncoated beads to axons is much lower for uncoated than for PDL-coated beads they were added at 5-10 times excess.

Immunofluorescence and line scans

Neurons grown in microfluidic chambers were treated on DIV9-11 and fixed for 20 minutes at room temperature in 4% paraformaldehyde (PFA) in cytoskeleton buffer (10 mM MES, 3 mM $MgCl_2$, 138 mM

KCl, 2 mM EGTA, 0.32 M sucrose, pH 6.1). Neurons were washed with PBS, permeabilized and blocked for 30 minutes with 3 mg ml^{-1} BSA, 100 mM glycine and 0.25% Triton X-100. Coverslips were incubated overnight at 4°C with primary antibodies: rabbit anti-β-catenin (1:500, Invitrogen, 71-2700), mouse anti-synaptophysin (1:500, BioLegend, SY38), mouse anti-GAP-43 (1:500, Invitrogen, 7B10), mouse anti-SNAP25 (1:1,000, BioLegend, SMI 81), rabbit anti-tau (1:500, GenScript, phospho-Ser235), rabbit anti-βIII-tubulin (1:1,000, BioLegend, Poly18020), mouse anti-βIII-tubulin (1:500, Abcam, TU-20). Neurons were washed with PBS and incubated with fluorophore-conjugated Alexa secondary antibodies (1:200) for 1 hour at room temperature. Samples were mounted with ProLong Diamond Antifade Mountant (Invitrogen) and images were acquired in Z-stacks using a 63x/1.3 oil objective on an Axio-Observer.Z1 microscope equipped with an AxioCam MRm Rev. 3 camera (Zeiss). Acquisition settings were kept the same for all samples in any given experiment. 5 random axonal fields containing beads were imaged per coverslip. To quantify average fluorescence intensity at axon-beads contact sites, a 5-µm-diameter circle around the bead center was draw in AxioVision and average pixel intensity was determined inside that circle. For off-bead values, average pixel intensity was determined in a 5 µm circle that encompassed axons not in proximity with beads. For each image, background fluorescence intensity was determined in an area with no axons and subtracted from all bead and off-bead values. To quantify fluorescence along the axons, starting at the center of the bead a 3-pixels-wide line was draw along the axon using ImageJ. Average pixel intensity in that line was determined for 30 µm. The intensity along the last 15 µm of each segment was averaged and the resulting off-bead mean axonal fluorescence intensity was subtracted from all values.

Immunoblot analysis

Hippocampal neurons were culture in circular microfluidic chambers modified after Park et al. (2009), in which the axon grow across a 500-µm-long microgroove barrier into the inner open compartment (6 mm diameter). For protein isolation, medium was carefully removed from axonal compartment and axons were collected in 50 µl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1%SDS, 1mM EDTA, supplemented with protease and phosphatase inhibitors (Pierce)), the chamber was then removed and somatic material was collected in 100 µl of RIPA buffer. The material from 6 different chambers was collected this way using the same buffer to increase the amount of protein. 20 µl of lysate from axons and 2 µl of lysate from cell bodies was used for Western blotting. Nitrocellulose membrane was incubated with anti-SNAP-25 (1:1,000, BioLegend, 836304) or anti-β-III tubulin (1:10,000, BioLegend, 802001) at 4°C overnight in TBS-T

with 4% milk. For detection, blots were incubated with respective secondary antibodies (1:10,000, anti-Ms-HRP or anti-Rb-HRP, Invitrogen) and developed with 1-Shot Digital-ECL (KindleBio, R1003) and images were taken with the KwikQuant Imager (KindleBio, D1001) according to the manufacturer's instructions.

Puromycylation and puromycylation-proximity ligation assays

To detect newly synthesized proteins, puromycin (1.8 μM, Sigma-Aldrich) or growth medium was added to axons in compartmentalized cultures or to dissociated neurons in regular cultures for 10-15 minutes, depending on the experiment, in the absence or presence of the protein synthesis inhibitor anisomycin (10 μM for axons, 40 μM for dissociated cultures). After incubation, cells were washed twice with warm PBS and fixed for 20 minutes at room temperature in 4% PFA in cytoskeleton buffer. Cells were washed with PBS, blocked and permeabilized in 3 mg ml^{-1} BSA, 100 mM glycine and 0.25% Triton X-100, and incubated overnight at 4°C with mouse anti-puromycin antibody (1:250, Millipore, MABE343) and rabbit anti-SNAP25 antibody (1:250, Sigma-Aldrich, S9684) for the puro-PLA assay. Detection of newly synthesized SNAP25 through PLA was performed according to the manufacturer's recommendations, using rabbit PLA^{plus} and mouse PLA^{minus} probes, and red Duolink detection reagents (Sigma-Aldrich). Before mounting with Duolink In Situ Mounting Medium with DAPI (Sigma-Aldrich), coverslips were incubated with anti-βIII-tubulin (1:100, Abcam, 2G10; conjugated to Alexa Fluor 488) or anti- βIII-tubulin (1:100, Abcam, EP1569Y; Alexa Fluor 647) and anti-PSD-95 (1:50, Abcam, EP2652Y; Alexa Fluor 488) for 1 hour at room temperature. Images were acquired as described previously and quantified by counting the number of puromycin-positive or PLA puncta. SNAP25 puro-PLA experiments in dissociated cultures were imaged in a Zeiss LSM 800 confocal microscope using a 40x oil objective and Zen Blue 2.1 software.

Single-molecule inexpensive Fluorescence *In Situ* Hybridization

Oligonucleotide probes were designed using Oligostan software (Tsanov et al., 2016). For Snap25, we obtained 30 probes, while for Egfp we designed 15 probes due to the smaller coding sequence (Table S1). The probes were hybridized to a digoxigenin-labeled FLAP oligonucleotide to create FLAP-structured duplex probes (Tsanov et al., 2016). smiFISH was performed as described (Tsanov et al., 2016), with minor changes. On DIV10, beads were added to the axonal compartments of hippocampal neurons grown in microfluidic devices. Cells were fixed after 24 hours for 20 minutes in 4% PFA in cytoskeleton buffer. Coverslips were washed in PBS, permeabilized with 0.3% Triton X-100 in PBS for 5 minutes, and washed again with PBS. Coverslips were equilibrated at 37°C in 15% formamide, 1 ×

saline sodium citrate (SSC). Samples were incubated with 10 pmol of FLAP-structured duplex probes in 50 µL of hybridization buffer (15% formamide, 1 × SSC, 10% dextran, 350 ng µL−1 yeast tRNA, 0.2 mg mL−1 BSA, 2 mM vanadyl ribonucleoside complex) overnight at 37°C. Coverslips were washed twice with 15% formamide in 1 × SSC for 30 minutes at 37°C. Afterward samples were washed three times with PBS-T (0.1% Tween 20) for 5 min, blocked with 3% BSA in PBS-T for 30 minutes, and incubated with goat anti-digoxigenin (1:500, Vector Laboratories) overnight at 4°C. Samples were washed three times with PBS-T for 5 minutes and incubated with Alexa 488 anti-goat secondary (1:1,000), and anti-βIII-tubulin was conjugated to Alexa-594 (Abcam, ab201740) for 1 hour at room temperature. They were then washed with PBS and mounted with ProLong Diamond Antifade Mountant (Invitrogen). Egfp fluorescence was used as a control for nonspecific hybridization and subtracted from all Snap25 values. The specificity of the Egfp probes was verified by performing smiFISH on cells transfected by a Gfp plasmid or control (data not shown). The specificity of the Snap25 probe was verified by RNAi in axons (Figure 4B).

siRNA transfections

Axon-specific silencing of *Snap25*, *ctnnb1* and *pclo* mRNAs was achieved using the following siRNAs: *Snap25* (NM_001270575.1) 5′-CGUGUCGAAGAAGGGAUGAACCAUA-3′ and 5′-UAUGGUUCAUGCCUUCUUCGACACG-3′; *ctnnb1* (NM_053357.2) 5′-UCUGCAUGCCCUCAUCUAGUGUCUC-3′ and 5′-GAGGUCGAAGAAGGCAUGAACCAUA-3′; *pclo* (NM_020098.1) 5′-CACCUUGCUGGUCUCUCACAUUAUU-3′ and 5′-AAUAAUGUGAGAGACCAGCAAGGUG-3′. Negative control siRNA was purchased from ThermoFisher Scientific (Stealth RNAi siRNA Negative Control Med GC Duplex #3). siRNAs were transfected into axons of DIV 10 neurons grown in microfluidic chambers using NeuroPORTER transfection reagent (Genlantis). Final siRNA concentration was 50 nM. Beads were added 24 hours after transfection.

Real Time RT-PCR

Total RNA from cell bodies (4 chambers or 150,000 cells) and axonal compartments (from a minimum of 6 chambers) was extracted with TRIzol, and RNA was purified using the Direct-zol RNA MiniPrep kit (Zymo Research). Axonal RNA was eluted in 10 µl of nuclease-free water and reverse transcribed to cDNA using the Superscript VILO cDNA synthesis kit. For cell body RNA, a total of 100 ng was reverse transcribed to cDNA. Axonal cDNA was preamplified using the TaqMan PreAmp kit, following manufacturer's instructions for the 14 cycles preamplification. Real Time RT-PCR was performed with TapMan Fast Advanced master mix in a StepOnePlus Real-Time PCR instrument, using pre-designed

TapMan probes. Amplification conditions were as follow: initial denaturing step at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute. *Snap25*, *ctnnb1* and *pclo* levels were normalized to *gapdh*.

FM 4-64 release assay

After DIV 11 the chamber medium was exchanged with tyrode's solution (125 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4) and cells were let to equilibrate for at least 30 minutes. Then FM 4-64 (15 µM, N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide, Invitrogen), was loaded for 90 seconds in a high K^+ solution (37 mM NaCl, 90 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4) with 10 µM CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione disodium, Santa Cruz Biotechnology) and 50 µM DL-AP5 (DL-2-Amino-5-phosphonovaleric acid, Santa Cruz Biotechnology). Cells were loaded for 10 more minutes in tyrode's solution with FM 4-64, CNQX and DL-AP5. Chambers were then briefly washed twice in low Ca^{2+} solution (112 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 30 mM glucose, 25 mM HEPES, pH 7.4) with 0.5 mM advasep-7 (Sigma-Aldrich), CNQX and DL-AP5, and three times in tyrode's solution with CNQX and DL-AP5. Unloading was then evoked with high K^+ solution. During imaging neurons were kept in a CO_2 and humidity controlled incubation chamber at 37°C. Images were acquired every 15 seconds, for a total of 4 minutes. The high potassium solution was added after 30 seconds. The intensity of FM 4-64 puncta around beads was quantified over time. Relative fluorescence values were plotted and entered in SPSS (IBM). A monoexponential fit was calculated for each individual bead data with the equation,

$F = F_{max} \times e^{-time/\tau} + F_{final}$, where F_{max} represents the fluorescence value right before K^+ stimulation, τ the exponential decay constant and F_{final} the final plateau. The exponential decay constant values were plotted and compared between conditions.

Statistical methods

Statistical analysis was performed using Prism 7 (GraphPad). The means of two groups were compared with unpaired t tests, and the means of three groups were compared using ANOVA with Tukey's multiple comparison test. The means of multiple groups with two independent variables were compared using two-way ANOVA with Bonferroni's multiple comparisons tests.

Author Contributions

A.F.R.B. and U.H. designed the experiments. A.F.R.B. and J.C.M. performed the experiments and data analysis. A.F.R.B. and U.H. wrote the paper.

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REFERENCES

Baleriola, J., Walker, C.A., Jean, Y.Y., Crary, J.F., Troy, C.M., Nagy, P.L., and Hengst, U. (2014). Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. Cell *158*, 1159-1172.

Bareiss, S., Kim, K., and Lu, Q. (2010). Delta-catenin/NPRAP: A new member of the glycogen synthase kinase-3beta signaling complex that promotes beta-catenin turnover in neurons. J Neurosci Res *88*, 2350-2363.

Barr, C.L., Feng, Y., Wigg, K., Bloom, S., Roberts, W., Malone, M., Schachar, R., Tannock, R., and Kennedy, J.L. (2000). Identification of DNA variants in the SNAP-25 gene and linkage study of these polymorphisms and attention-deficit hyperactivity disorder. Mol Psychiatry *5*, 405-409.

Batista, A.F., and Hengst, U. (2016). Intra-axonal protein synthesis in development and beyond. Int J Dev Neurosci *55*, 140-149.

Brophy, K., Hawi, Z., Kirley, A., Fitzgerald, M., and Gill, M. (2002). Synaptosomal-associated protein 25 (SNAP-25) and attention deficit hyperactivity disorder (ADHD): evidence of linkage and association in the Irish population. Mol Psychiatry *7*, 913-917.

Buxbaum, A.R., Wu, B., and Singer, R.H. (2014). Single beta-actin mRNA detection in neurons reveals a mechanism for regulating its translatability. Science *343*, 419-422.

Campbell, D.S., and Holt, C.E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. Neuron *32*, 1013-1026.

Chia, P.H., Li, P., and Shen, K. (2013). Cell biology in neuroscience: cellular and molecular mechanisms underlying presynapse formation. J Cell Biol *203*, 11-22.

Fenster, S.D., Chung, W.J., Zhai, R., Cases-Langhoff, C., Voss, B., Garner, A.M., Kaempf, U., Kindler, S., Gundelfinger, E.D., and Garner, C.C. (2000). Piccolo, a presynaptic zinc finger protein structurally related to bassoon. Neuron *25*, 203-214.

Gracias, N.G., Shirkey-Son, N.J., and Hengst, U. (2014). Local translation of TC10 is required for membrane expansion during axon outgrowth. Nat Commun *5*, 3506.

Hengst, U., Cox, L.J., Macosko, E.Z., and Jaffrey, S.R. (2006). Functional and selective RNA interference in developing axons and growth cones. J Neurosci *26*, 5727-5732.

Hengst, U., Deglincerti, A., Kim, H.J., Jeon, N.L., and Jaffrey, S.R. (2009). Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. Nat Cell Biol *11*, 1024-1030.

Jin, Y., and Garner, C.C. (2008). Molecular mechanisms of presynaptic differentiation. Annu Rev Cell Dev Biol *24*, 237-262.

Kaech, S., and Banker, G. (2006). Culturing hippocampal neurons. Nat Protoc *1*, 2406-2415.

Kustanovich, V., Merriman, B., McGough, J., McCracken, J.T., Smalley, S.L., and Nelson, S.F. (2003). Biased paternal transmission of SNAP-25 risk alleles in attention-deficit hyperactivity disorder. Mol Psychiatry *8*, 309-315.

Leal-Ortiz, S., Waites, C.L., Terry-Lorenzo, R., Zamorano, P., Gundelfinger, E.D., and Garner, C.C. (2008). Piccolo modulation of Synapsin1a dynamics regulates synaptic vesicle exocytosis. J Cell Biol *181*, 831-846.

Lucido, A.L., Suarez Sanchez, F., Thostrup, P., Kwiatkowski, A.V., Leal-Ortiz, S., Gopalakrishnan, G., Liazoghli, D., Belkaid, W., Lennox, R.B., Grutter, P.*, et al.* (2009). Rapid assembly of functional presynaptic boutons triggered by adhesive contacts. J Neurosci *29*, 12449-12466.

Mill, J., Richards, S., Knight, J., Curran, S., Taylor, E., and Asherson, P. (2004). Haplotype analysis of SNAP-25 suggests a role in the aetiology of ADHD. Mol Psychiatry *9*, 801-810.

Park, J., Koito, H., Li, J., and Han, A. (2009). Microfluidic compartmentalized co-culture platform for CNS axon myelination research. Biomed Microdevices *11*, 1145-1153.

Park, J.W., Vahidi, B., Taylor, A.M., Rhee, S.W., and Jeon, N.L. (2006). Microfluidic culture platform for neuroscience research. Nat Protoc *1*, 2128-2136.

Sanders, J.D., Yang, Y., and Liu, Y. (1998). Differential turnover of syntaxin and SNAP-25 during synaptogenesis in cultured cerebellar granule neurons. J Neurosci Res *53*, 670-676.

Schacher, S., and Wu, F. (2002). Synapse formation in the absence of cell bodies requires protein synthesis. J Neurosci *22*, 1831-1839.

Schmidt, E.K., Clavarino, G., Ceppi, M., and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods *6*, 275-277.

Sheehan, P., Zhu, M., Beskow, A., Vollmer, C., and Waites, C.L. (2016). Activity-Dependent Degradation of Synaptic Vesicle Proteins Requires Rab35 and the ESCRT Pathway. J Neurosci *36*, 8668-8686.

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, J.Q., Amieux, P.S., and Holt, C.E. (2016). Dynamic Axonal Translation in Developing and Mature Visual Circuits. Cell *166*, 181-192.

Taylor, A.M., Berchtold, N.C., Perreau, V.M., Tu, C.H., Li Jeon, N., and Cotman, C.W. (2009). Axonal mRNA in uninjured and regenerating cortical mammalian axons. J Neurosci *29*, 4697-4707.

Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2005). A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat Methods *2*, 599-605.

Taylor, A.M., Wu, J., Tai, H.C., and Schuman, E.M. (2013). Axonal translation of beta-catenin regulates synaptic vesicle dynamics. J Neurosci *33*, 5584-5589.

tom Dieck, S., Kochen, L., Hanus, C., Heumuller, M., Bartnik, I., Nassim-Assir, B., Merk, K., Mosler, T., Garg, S., Bunse, S.*, et al.* (2015). Direct visualization of newly synthesized target proteins in situ. Nat Methods *12*, 411-414.

Tsanov, N., Samacoits, A., Chouaib, R., Traboulsi, A.M., Gostan, T., Weber, C., Zimmer, C., Zibara, K., Walter, T., Peter, M., et al. (2016). smiFISH and FISH- quant - a flexible single RNA detection approach with super-resolution capability. Nucleic Acids Res. *44*, e165.

Villarin, J.M., McCurdy, E.P., Martinez, J.C., and Hengst, U. (2016). Local synthesis of dynein cofactors matches retrograde transport to acutely changing demands. Nat Commun *7*, 13865.

Wu, K.Y., Hengst, U., Cox, L.J., Macosko, E.Z., Jeromin, A., Urquhart, E.R., and Jaffrey, S.R. (2005). Local translation of RhoA regulates growth cone collapse. Nature *436*, 1020-1024.

Yarmolinsky, M.B., and Haba, G.L. (1959). Inhibition by Puromycin of Amino Acid Incorporation into Protein. Proc Natl Acad Sci U S A *45*, 1721-1729.

Younts, T.J., Monday, H.R., Dudok, B., Klein, M.E., Jordan, B.A., Katona, I., and Castillo, P.E. (2016). Presynaptic Protein Synthesis Is Required for Long-Term Plasticity of GABA Release. Neuron *92*, 479-492.

Zivraj, K.H., Tung, Y.C., Piper, M., Gumy, L., Fawcett, J.W., Yeo, G.S., and Holt, C.E. (2010). Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. J Neurosci *30*, 15464-15478.

Figure Legends

Figure 1. Clustering of presynaptic markers at PDL-coated beads in axons

(A) Scheme of a tripartite microfluidic chamber used to selectively treat axons. Embryonic hippocampal neurons were seeded in the upper compartment of the chamber (teal) and axons cross through two microgroove barriers (200-µm-long) into the middle and lower axonal compartments (yellow). After DIV 10, PDL-coated or uncoated beads were added to the axonal compartments for 15 minutes to 1 hour, and clustering of presynaptic proteins (i.e. β-catenin, SNAP25) adjacent to the bead-axon contact sites was determined by IF.

(B) Axons were incubated with PDL-coated beads for 24 hours and immunostained for presynaptic and axonal proteins. Levels of β-catenin, synaptophysin, SNAP25, GAP43, and tau are significantly increased at axon-bead contact sites. IF and IF merged with DIC images are shown; yellow dashed circles outline beads. Quantifications are relative to off-bead fluorescence values. Mean ± SEM of 30-120 axonal fields (n = 3 biological replicates per condition). Unpaired t tests. n.s., not significant; $^{**}p < 0.01$; $^{****}p < 0.0001$.

(C) Axons were incubated with PDL-coated and uncoated beads for the indicated times and immunostained for SNAP25. SNAP25 immunoreactivity is significantly increased at contact sites with PDL-coated beads at all time points. Fluorescence is normalized to off-bead values. Mean ± SEM of 30-50 axonal fields (n = 3-4 different biological replicates per condition). Two-way ANOVA with Bonferroni's multiple comparison test.

$^{*}p < 0.05$; $^{**}p < 0.01$.

Scale bars, 5 µm.

Figure 2. Clustering of β-catenin and SNAP25 at PDL-coated beads requires local protein synthesis

(A) Experimental design: on DIV 11 axons were treated with PDL-coated or uncoated beads in the presence of a protein synthesis inhibitor (emetine, 100 nM) or vehicle. Cultures were fixed at different time points after treatment.

(B) A 3-pixels-wide line was drawn along the axon, starting at the center of the bead. Fluorescence along this line was quantified for 30 µm and normalized against the average fluorescence in last 15 µm. Beads have a diameter of 5 µm.

(C-G) Axon were immunostained forβ-catenin (D), SNAP25 (E), tau (F), and β-III tubulin (G) after 1 hour of treatment. β-catenin and SNAP25 increased in the direct vicinity of the beads in a protein synthesis inhibitor sensitive manner, while tau and β-III tubulin levels remained unchanged. IF and IF merged

with DIC images are shown; yellow dashed circles outline beads. Fluorescence intensities obtained in the lines scans were averaged and normalized. The area under the curve (AUC) was then calculated for the first 5 μm and 5-15 μm from the bead center. Mean ± SEM of 30-120 beads (n = 3 biological replicates per condition).

(H) Axons were immunostained for β-catenin or SNAP25 after 3, 6 or 24 hours of treatment. SNAP25 levels were significantly increased at bead contact sites at all times tested. The effect for β-catenin was significant only at 3 hours and emetine did not affect β-catenin levels at 24 hours.

One-way ANOVA with Tukey's multiple comparison tests and unpaired t tests. n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001. Scale bars, 5 μm.

Figure 3. PDL-coated beads induce axonal protein synthesis

(A) Puromycylation assay: ribosomes incorporate puromycin into nascent polypeptide chains, leading to elongation termination and premature chain release. An antibody against puromycin is used to label nascent proteins in situ.

(B) Axons were treated with PDL-coated beads and puromycin or puromycin alone for 15 min and immunostained for puromycin and β-III tubulin. The level of puromycylation, indicating de novo protein synthesis is significantly increased at contact sites with PDL-coated beads. Mean ± SEM of 30 axonal fields per condition (n = 3 biological replicates). Unpaired t test. ∗p < 0.05.

(C) Axons were treated with uncoated or PDL-coated beads and puromycin for 15 min and immunostained for phospho-4EBP1, β-III tubulin, and puromycin. The signal for p-4EBP1, indicating activation of translation, co-localizes with the puromycin signal at contact sites with PDL-coated beads.

(D) Uncoated beads, PDL-coated beads, or PDL-coated beads and the protein synthesis inhibitor anisomycin (10 μM) were added to the axonal compartments. Puromycin was added to axons during the last 10 min of the assay. The number of puromycin-positive puncta in a circle of 5 μm around each bead center and the percentage of all beads imaged in each condition with no puncta or at least one puncta in the 5-μm circle were plotted. Means ± SEM of 40–142 beads in 30 axonal fields (n = 3 different biological replicates). One-way ANOVA with Tukey's multiple comparison test. ∗p < 0.05; ∗∗∗p < 0.001.

Yellow dashed line represents bead location. Scale bars, 5 μm.

Figure 4. SNAP25 is locally synthesized in axons

(A) After DIV11, PDL-coated beads were added to the axonal compartments, and 24 hr later cells were fixed and processed for Snap25 or Gfp smiFISH, and Snap25 and Gfp positive puncta at bead contact

sites were counted. SNAP25 transcripts accumulated at bead contact sites. Mean ± SEM of ten axonal fields per condition (n = 3 biological replicates). Unpaired t test. $^{****}p < 0.0001$. Scale bar, 5 μm.

(B) Hippocampal neurons were cultured in microfluidic chambers for 10 days. siRNA was applied only to the axonal compartments for 24 hr, and the neurons were processed for Snap25 smiFISH. Axonal transfection with SNAP25 siRNA greatly reduced the number of SNAP25 puncta, establishing the specificity of the axonal Snap25 smiFISH signal. Mean ± SEM of ten axonal fields per condition. Unpaired t tests.

$^{*}p < 0.05$. Scale bar, 5 μm.

(C) Scheme of a circular microfluidic chamber used for selective axonal treatments and protein isolation. Embryonic hippocampal neurons are seeded in the inner compartment and axons cross through a microgroove barrier (500-μm-long) into an open circular (6-mm-diameter) compartment. After DIV11, uncoated beads or PDL-coated beads with or without emetine (100 nM) were added to the axonal compartment only, and 24 hr later proteins were obtained from both somatic and axonal compartments and analyzed for immunoblot blot. Selective treatment of axons with PDL-coated beads resulted in an increase of SNAP25 protein only in axons but not in cell bodies. This increase was blocked with local application of the protein synthesis inhibitor emetine.

(D) Principle of puro-PLA: puromycin is incorporated by active ribosomes into nascent polypeptide chains. A proximity ligation assay with antibodies against puromycin and the protein of interest—here: SNAP25—is used to detect synthesis of this protein.

(E) Axons of hippocampal neurons cultured in microfluidic chambers for 11 days were treated with PDL-coated beads and puromycin or puromycin alone for 15 min and processed for puro-PLA against SNAP25. The number of SNAP25 puro-PLA puncta is significantly increased in axons incubated with PDL-coated beads. Mean ± SEM of ten axonal fields per condition (n = 3 biological replicates). Unpaired t test.

$^{**}p < 0.01$.

(F) PDL-coated beads or PDL-coated beads and the protein synthesis inhibitor anisomycin (10 μM) were added to the axonal compartments for 1 hr. Puromycin was added to axons during the last 10 min of the assay. The number of SNAP25 puro-PLA-positive puncta in a circle of 5 μm around each bead center and the percentage of all beads imaged in each condition with no puncta or at least one puncta in the 5-μm circle were plotted. Means ± SEM of 76–84 beads in five to ten axonal fields per condition (n = 4 different biological replicates). Scale bars, 5 μm.

(G) Dissociated hippocampal neurons were cultured for 5, 10, or 15 days and incubated with puromycin for 10 min before fixation. Closed arrowheads indicate puro-PLA puncta and opened arrowheads PSD-95 puncta. Scale bars, 10 μm.

(H) Dissociated hippocampal neurons were cultured for 5, 10, or 15 days and processed for SNAP25 puro-PLA without puromycin incubation or after incubation with puromycin and anisomycin for 10 min before fixation. Closed arrowheads indicate puro-PLA puncta and opened arrowheads PSD-95 puncta. Scale bars, 10 μm.

Figure 5. Effect of local siRNA on β-catenin and SNAP25 clustering

(A) Experimental design for C: hippocampal neurons were cultured in microfluidic chambers for 10 days. siRNA was applied only to the axonal compartments for 24 hours before PDL-coated beads were added for 1 hour. Puromycin was added to the axons for the last 10 minutes before fixation and processing for SNAP25 puro-PLA.

(B) SNAP25 mRNA levels were quantified by RT-PCR in lysates obtained from the axonal and the cell body compartment 48 hours after transfection of the axons with siRNA. SNAP25 levels were reduced significantly reduced in axons but not in the cell bodies, demonstrating that the axonally applied siRNAs act exclusively locally. Mean ± SEM (n = 3 biological replicates).

(C) Transfection of axons with SNAP25 siRNA greatly reduced the synthesis of SNAP15 at contact sites with PDL-coated beads as measured with SNAP25 puro-PLA. Mean ± SEM of 80-103 beads in 10 axonal fields per condition (n = 3 biological replicates).

(D) Experimental design for E and F: Hippocampal neurons were cultured and their axons selectively transfected with siRNAs and incubated with PDL-coated beads as in A. The neurons were fixed after 1 hour and immunostained for SNAP25 (E) or β-catenin (F).

(E) SNAP25 immunostaining in the direct vicinity of PDL-coated beads is significantly reduced in axons transfected with SNAP25 siRNA. Average fluorescence values for 30 μm from the bead center, and the mean AUC for the 0-5 μm and 5-15 μm regions are plotted.

(F) β-catenin immunostaining in the 0-5 μm region is not significantly decreased in axons transfected with β-catenin siRNA. The signal intensity is increased in the 5-15 μm region.

Mean ± SEM of 80-100 beads (n = 3 biological replicates).

(G) Dissociated hippocampal neurons were cultured for 11 days, transfected with siRNA and β-catenin mRNA levels were determined by RT-PCR after 48 hours.

Unpaired t tests. n.s., not significant; $^{*}p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$. Yellow dashed lines represent bead outline. Scale bars, 5 μm.

Figure 6. Reciprocal effects of local β-catenin and SNAP25 siRNA on clustering

Hippocampal neurons were cultured and treated as in Figure 5D.

(A) Axons were transfected with scrambled or SNAP25 siRNA. 24 hours later, PDL-coated beads were added for 1 hour and β-catenin clustering was determined by IF. Fluorescence values were quantified for 30 μm starting at the bead center and mean AUC for the 0-5 μm and 5-15 μm regions are plotted.

(B) Axons were transfected with scrambled or β-catenin siRNA. 24 hours later, PDL-coated beads were added for 1 hour and SNAP25 clustering was determined by IF. Fluorescence values were quantified for 30 μm starting at the bead center and mean AUC for the 0-5 μm and 5-15 μm regions are plotted. Mean ± SEM of 80-100 beads (n = of 3 different biological replicates). Unpaired t test. $^{*}p < 0.05$ $^{****}p < 0.0001$. Yellow dashed lines represent bead outline. Scale bars, 5 μm.

Figure 7. Knockdown of axonal SNAP25 mRNA affects vesicle release of newly formed synaptic terminals

(A) Experimental design: hippocampal neurons were cultured in microfluidic chambers for 10 days. Axons were transfected with siRNAs 24 hours before adding PDL-coated beads. 24 hours after bead incubation, cells were loaded with FM dyes and imaged.

(B) Sequential imaging and stimulation of FM4-64 puncta in scrambled or SNAP25 siRNA treated-axons at contact sites with PDL-coated beads. SNAP25 siRNA slows vesicle release after stimulation with potassium but does not impede FM4-64 loading. Pictures were taken every 15 seconds, high potassium was added after 30 seconds (black arrow), and axons were imaged for three and a half minutes more. Fluorescence at PDL-coated beads (yellow dashed circle) was quantified over time, and mean fluorescence, normalized to initial FM4-64 intensity, is shown with fitted exponential decay curves. The exponential destaining time constant (τ) for each bead and initial fluorescence values are plotted.

(C) FM 4-64 puncta (white arrows) of neurites in the cell body compartment of chambers where siRNA was applied only to axons were also stimulated and imaged. Knockdown of SNAP25 mRNA in axons does not affect vesicle dynamics in the cell body compartment. Exponential decay time constant (τ) and initial fluorescence are shown.

(D) Neurons were treated as in (A) but their axons were transfected with a piccolo siRNA. The piccolo siRNA does not change axonal vesicle dynamics. Mean fluorescence at beads over time with fitted exponential decay curves, exponential decay constant (τ) and initial fluorescence are represented. Mean ± SEM of 45 beads per condition (n = 3 different biological replicates). Decay plots: Two-way ANOVA with multiple comparison test. Bar graphs: Unpaired t tests. n.s., not significant; **$p < 0.01$; ***$p < 0.001$****; $p < 0.001$. Scale bars, 5 μm.

(E) Dissociated hippocampal neurons were cultured for 11 days, transfected with siRNA and piccolo mRNA levels were determined by RT-PCR after 48 hours.

Mean ± SEM (n= 3 different biological replicates). Unpaired t test. *$p<0.05$

Figure 1

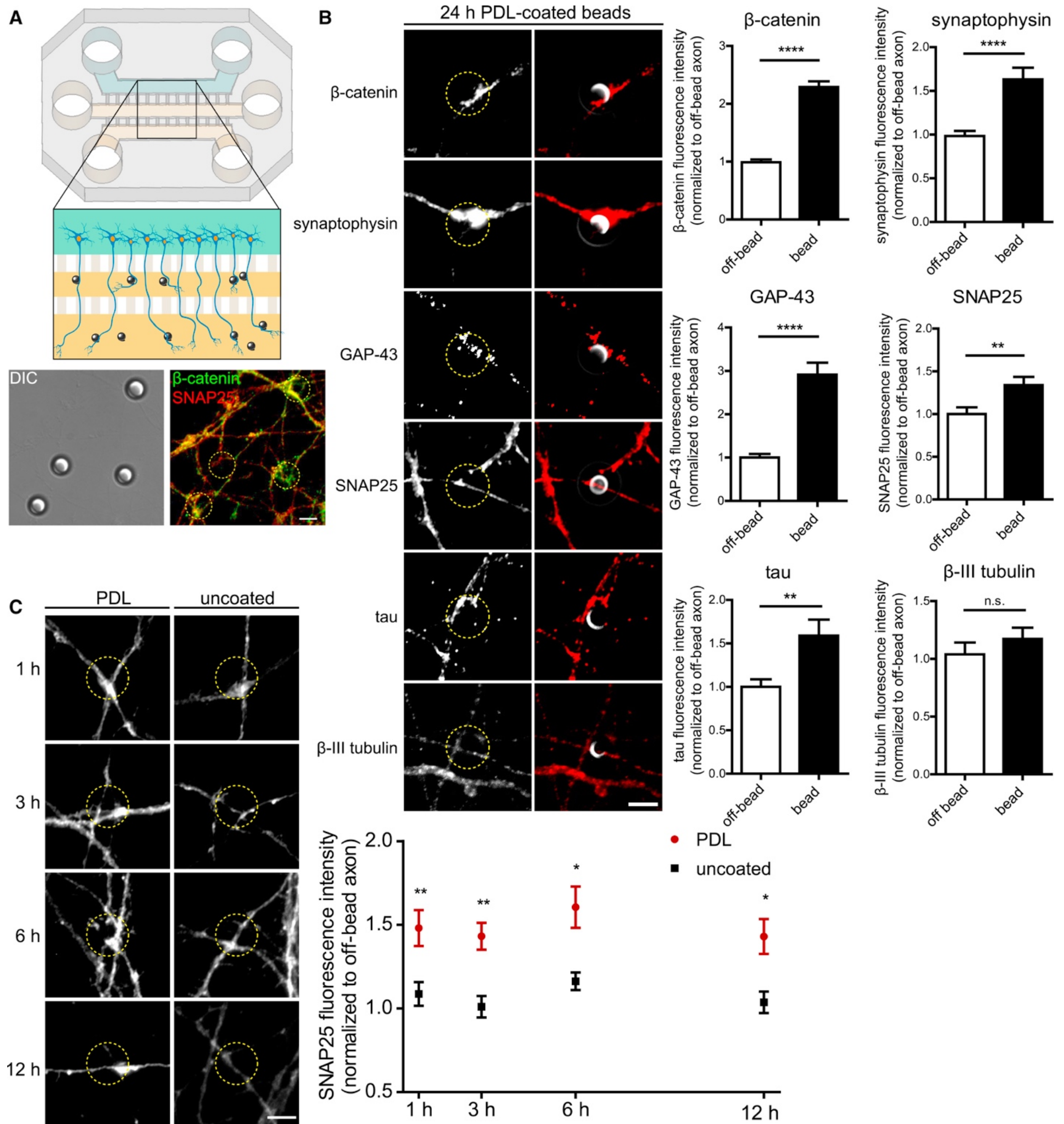

Figure 2

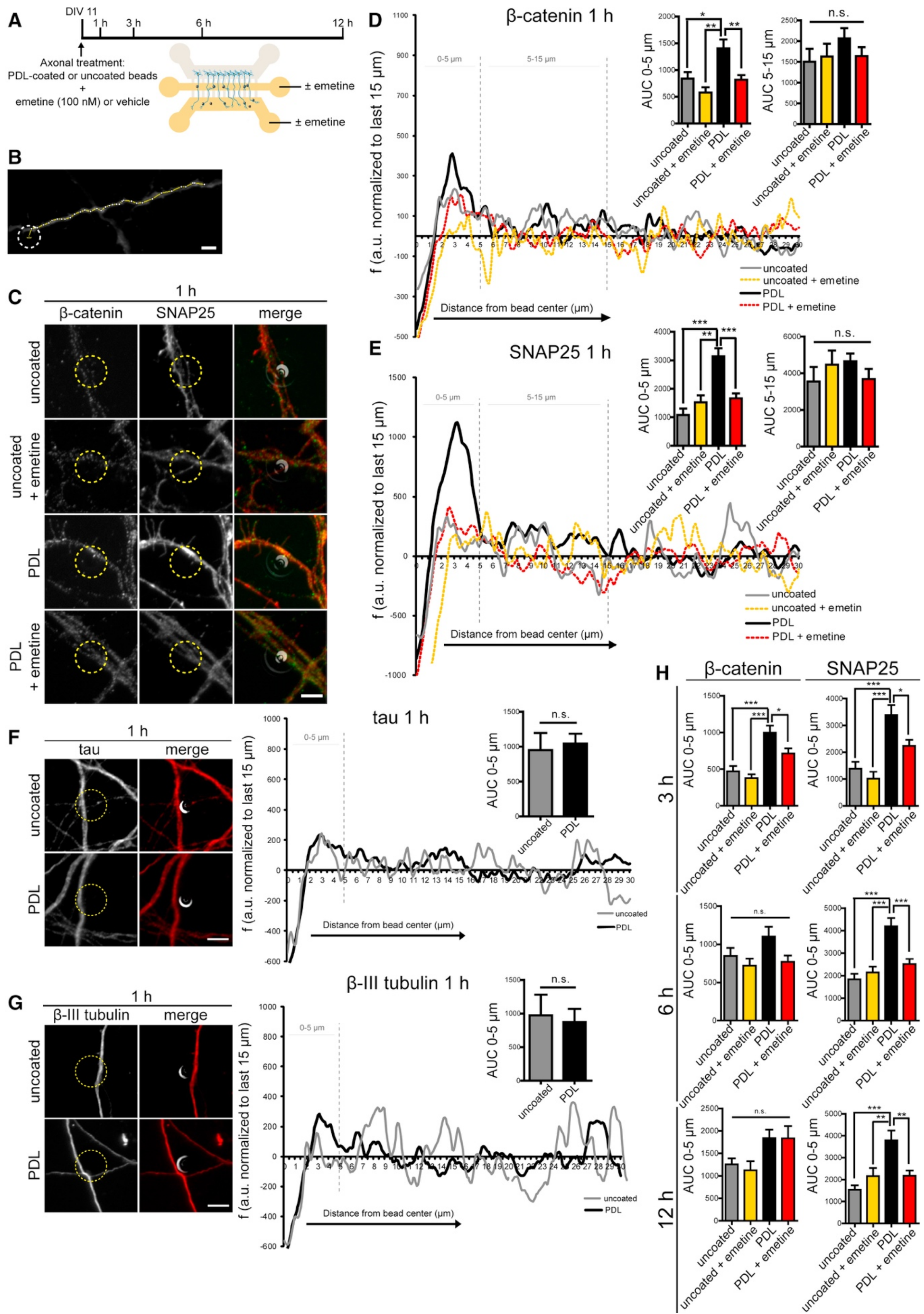

Figure 3

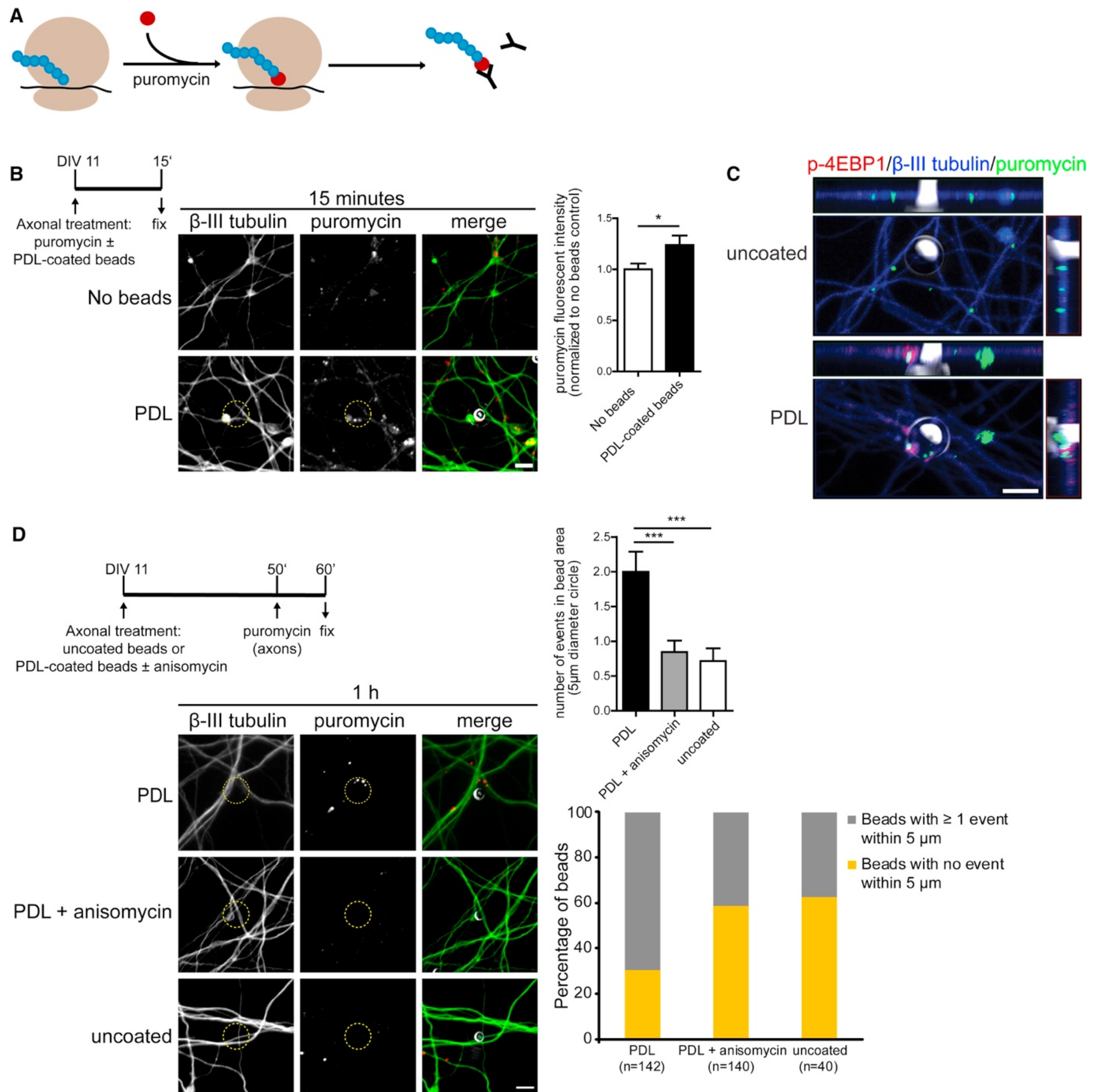

Figure 4

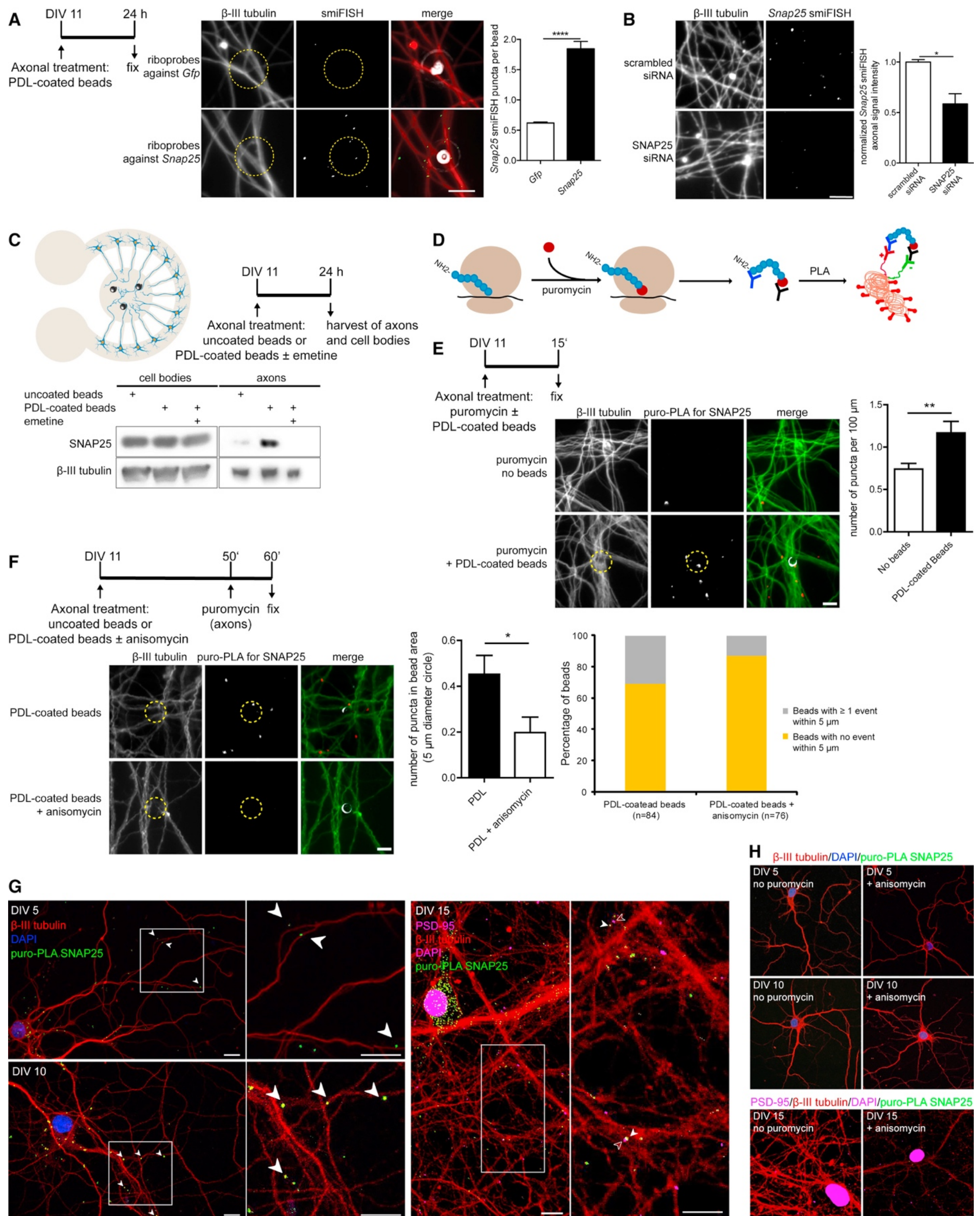

Figure 5

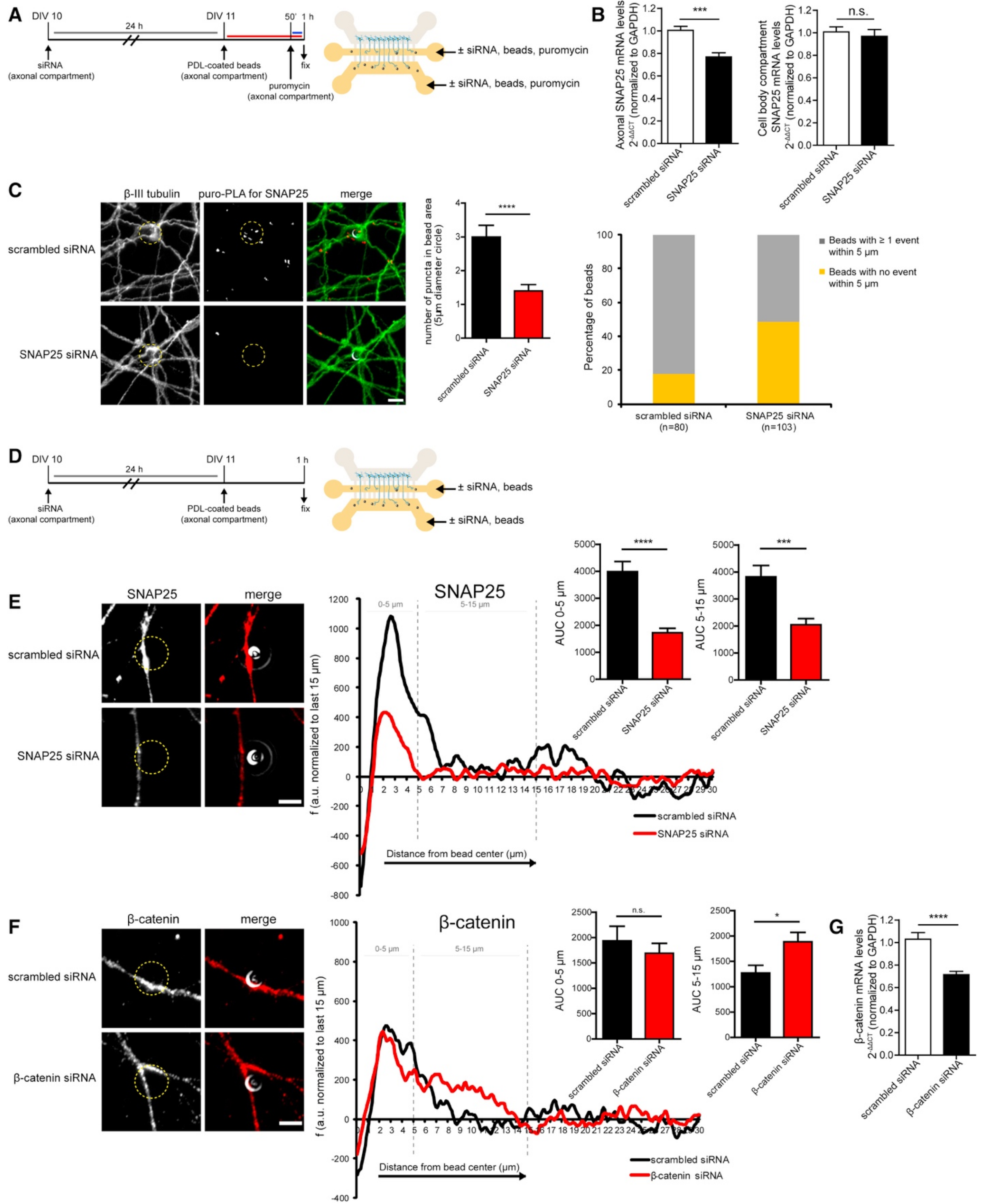

Figure 6

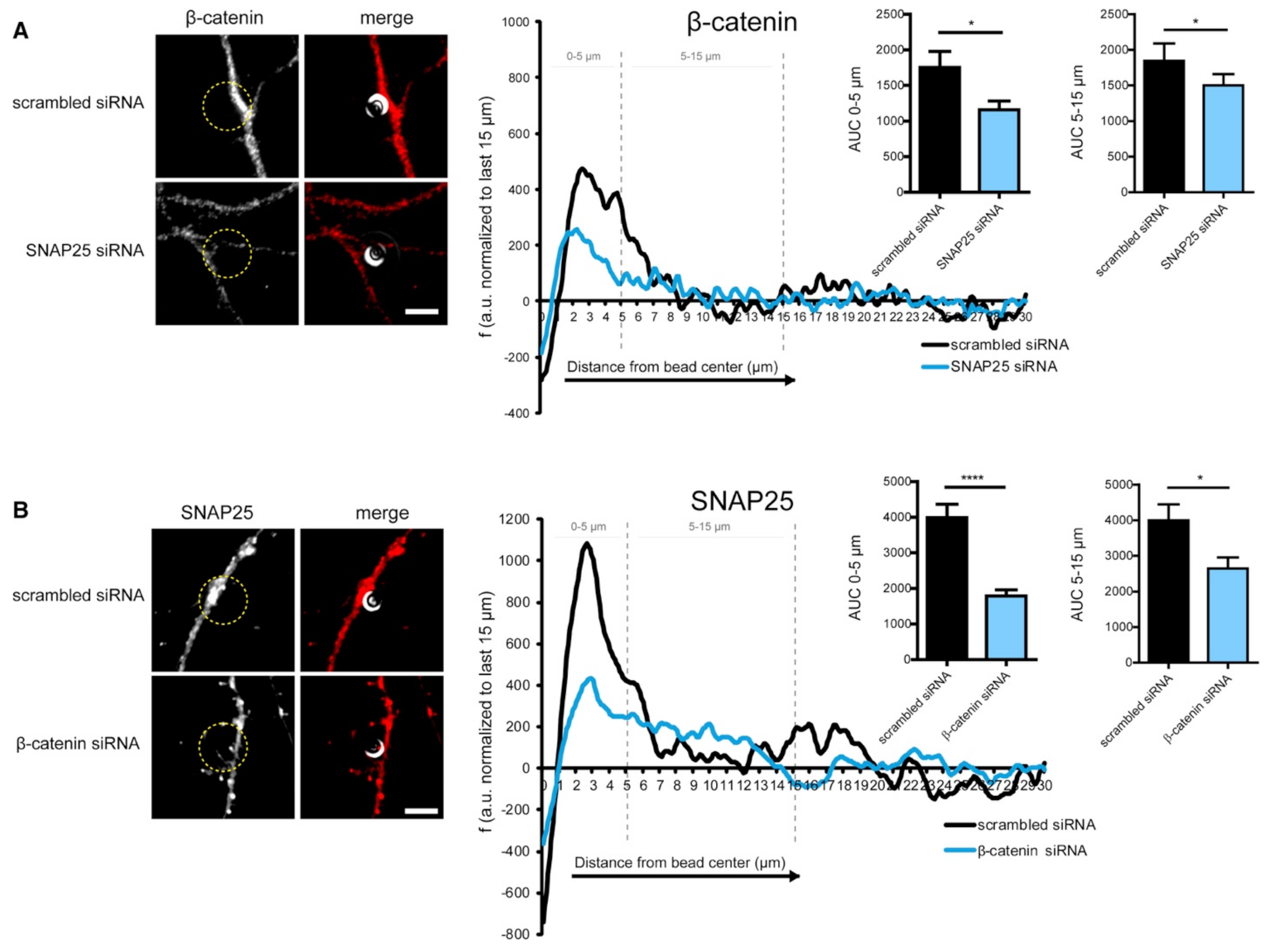

Figure 7

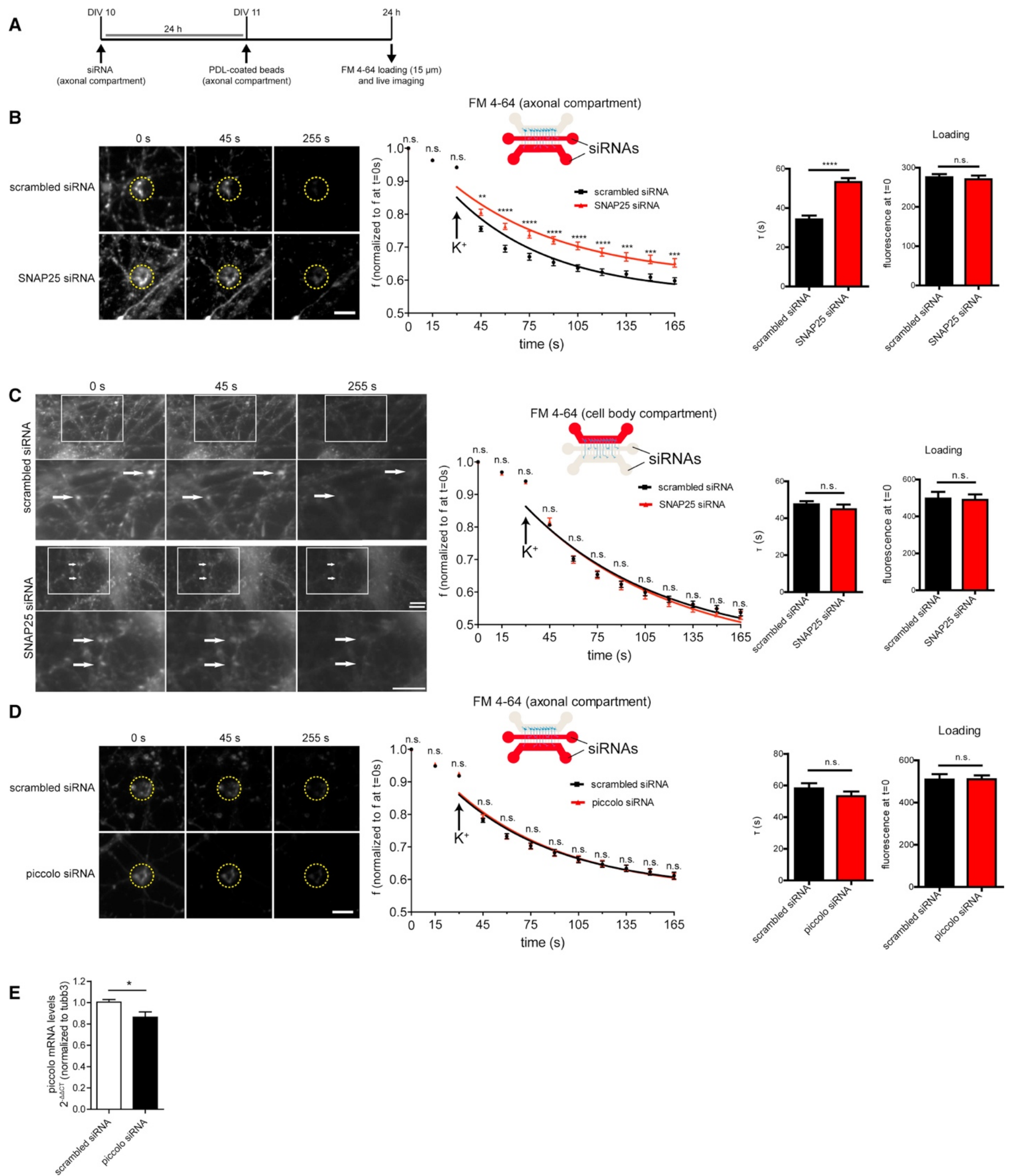

Figure S1. Clustering of β-catenin and SNAP25 at PDL-coated beads is equal in proximal and distal direction. Related to Figure 2

On DIV 11 axons were treated with PDL-coated or uncoated beads in the presence of a protein synthesis inhibitor (emetine, 100 nM) or vehicle. Cultures were fixed 1 hour after treatment, and axon were immunostained for β-catenin (A) or SNAP25 (B). 3-pixels-wide lines were drawn along the axons in proximal (i.e. cell body) and distal direction, starting at the center of the bead. Fluorescence along this line was quantified for 30 μm and normalized against the average fluorescence in last 15 μm. Beads have a diameter of 5 μm. The area under the curve (AUC) was then calculated for the first 5 μm and 5-15 μm from the bead center. β-catenin and SNAP25 increased in the direct vicinity of the beads in a protein synthesis inhibitor sensitive manner, and the increase was indistinguishable on the proximal and distal side of the beads. Mean ± SEM of 30-120 beads (n = 3 biological replicates per condition). One-way ANOVA with Tukey's multiple comparison tests. n.s., not significant; **$p < 0.01$; ***$p < 0.001$.

Table S1. Sequences of oligonucleotides used in smiFISH. Related to Figure 4

Figure S1

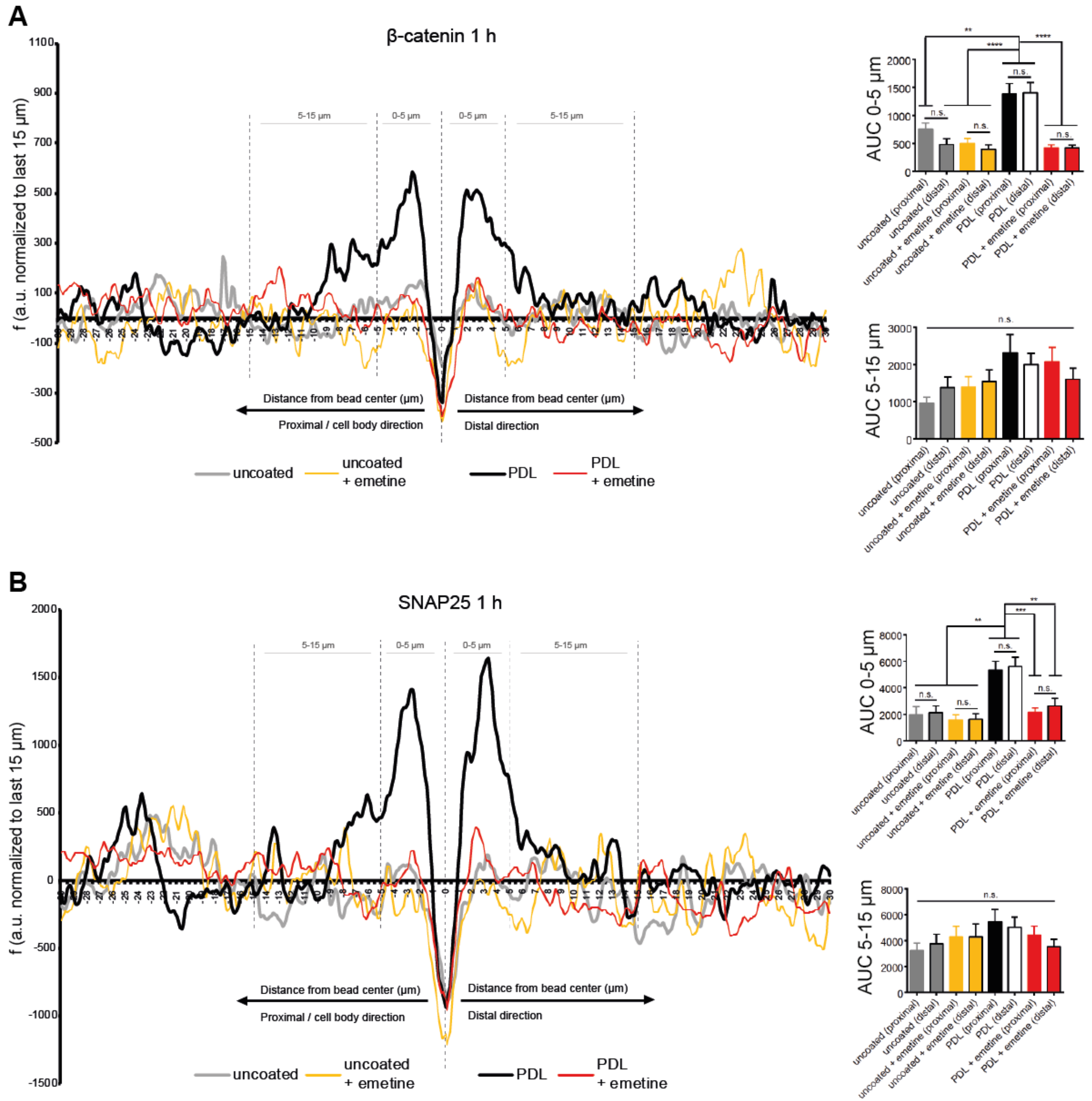

Table S1

Name	Sequence
EGFP_1	CGGTCACGAACTCCAGCAGGACCATGCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_2	CTGAAGCACTGCACGCCGTAGGTCAGCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_3	TTACTTGTACAGCTCGTCCATGCCGAGACCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_4	ATCGCGCTTCTCGTTGGGGTCTTTGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_5	TGAAGTTCACCTTGATGCCGTTCTTCTGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_6	GTCGGCCATGATATAGACGTTGTGGCTGTCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_7	TTCAGCTCGATGCGGTTCACCAGGGTCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_8	GTTGCCGTCGTCCTTGAAGAAGATGGTGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_9	GAAGTCGTGCTGCTTCATGTGGTCGGGGCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_10	GGCAGCTTGCCGGTGGTGCAGATGAACCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_11	TCAGGGTCAGCTTGCCGTAGGTGGCACCTCCTAAGTTTCGAGCTGGACTCAGTGB12
EGFP_12	CCGGACACGCTGAACTTGTGGCCGTTCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_13	CTGCCGTCCTCGATGTTGTGGCGGATCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_14	TAGTTGTACTCCAGCTTGTGCCCCAGCCTCCTAAGTTTCGAGCTGGACTCAGTG
EGFP_15	TGTTGCCGTCCTCCTTGAAGTCGATGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_01	ATTTGGAGAGATTCAGAGCCTGACACCCTCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_02	GAAAGGACTGTGGTAGTAGCTCTGTGGAATGTCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_03	CTTCTCTCTTGTCCAACAACATTGGAGGAGACCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_04	AATCTCATTGCCCATGTCTAGGGCCACCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_05	AGTTGTTCGCCTTGCTCATCCAACATAACCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_06	AAGTCCTGATGCCAGCATCTTTACTCTCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_07	ATCCATTTGTGTGTGTGTGTGTGTGTGTGTGTCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_08	TTTGCTGGTATGACTTAATCTTGACAGTGTCCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_09	TTGCTGTTGTGTGTGTTTTCTTGTCAGTATGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_10	GACTCTGTGTGTGTGTGTGTGTGTGTGTGTGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_11	GCATCTTTGTTGCACGTTGGTTGGCTTCACCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_12	AATTCTGGTTTTGTTGGAGTCAGCCTTCTCCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_13	GATCCTGTCAATCTGGCGATTCTGGGTGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_14	TGACGGAGGTTTCCGATGATGCCGCTCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_15	GGCATCGTTTGTTACCCTGCGGATGAAGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_16	CTGGCCACTACTCCATCCTGATTATTGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_17	AAATTTTTCTCGGCCTCCTTCATGTCTTGGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_18	GATATGGTTCATGCCTTCTTCGACACGATCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_19	TCCTCTGCATCTCCTCCAGTTCATTACCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_20	ATGTCAAATCACTTCACAAACCAACAGAGGAGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_21	TGGTGTGCATAGCTCTAGATTAAGCAACAAAGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_22	TTGTACTGATGACAAACCACAGGGAAATGGTGCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_23	CAACAAGAGCCAGACTTAGAAGATCTTCACGTCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_24	GTCTGTATGTTGACAGAAGAAGGTGACACAGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_25	ACAAAGAATAGCCATGAGGAACCAAAGACAGCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_26	TAAGTCAGGAAATAGAGTCACCAGATGACCACCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_27	AGGTAATACCATGAGAGAAGCATGAAGGAGCTCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_28	ACAGCAGAAGGGCAGATTTAACCACTTCCCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_29	AGCATCACTGGATTTAAGCTTGTTACAAGGACCCTCCTAAGTTTCGAGCTGGACTCAGTG
SNAP25_30	AACCACACAGGACATTTACAATGGGGGTGACCTCCTAAGTTTCGAGCTGGACTCAGTG

DISCUSSION, STANDING QUESTIONS AND FUTURE DIRECTIONS

DISCUSSION

THE RATIONALE BEHIND COMPARTMENTALIZED APPROACHES

Correct network wiring is an essential step in neurodevelopment. Understanding the sequence of events involved in synaptogenesis is therefore critical and might provide insight into many poorly understood neurodevelopmental disorders.

The directional nature of synaptic transmission, from axons of transmitting neurons to dendrites of receiving neurons, results in marked neuronal polarity and compartmentalization. Since dendritic protein synthesis has been linked to memory formation, a lot of research has focused on the study of local events. And even though dendrites and axons are very dissimilar, both are capable of autonomous information processing, and of protein synthesis. Recent research has expanded the repertoire of known axonally synthesized proteins and their specific functions, and it is now clear that it is important to study axonal events in isolation. Especially since signaling cascades can be unique to axons and some locally produced proteins might have distinct, axon-specific functions. An example of the first situation is the axonal injury response (Hanz et al., 2003; Perlson et al., 2005). After axotomy, both vimentin and importin β1 are locally translated and form a unique signaling complex with importin α and phosphorylated Erk proteins. Vimentin is an intermediate filament protein, expressed mainly in glial cells and during early neuronal development phases (Boyne et al., 1996; Menet et al., 2003), and has not been described in other instances in mature neurons. However, we currently know very little about the dendritic regenerative response to injury, and it will be interesting to compare dendritic and axonal responses in the future (Tao and Rolls, 2011; Thompson-Peer et al., 2016). An example of the latter situation is the recently described intra-axonal response to Aβ oligomers exposure (Baleriola et al., 2014). After an acute Aβ treatment, axons produce several proteins, among them the transcription factor ATF4, that travels to the nucleus and influences gene transcription, ultimately resulting in neuronal death. Treating axons with an ATF4 siRNA can block the neuronal death induced by this Aβ treatment, whereas silencing cell body-produced ATF4 has no effect. AFT4 has also been described as prosurvival in other instances (Ameri and Harris, 2008), so axonal production, in this context, greatly influences ATF4 function. Moreover, one of the major difficulties in studying these axonal events and

cascades is the inherently low levels of mediators. In axonal subcompartments, synthesis of very few molecules has been shown to induce significant changes, and whole-neuron studies might dilute these changes. This problem is particularly relevant when studying the synapse, given the abundance of postsynaptic translation.

Time frame of adhesion, clustering and translation events

Presynaptic formation occurs very rapidly and, since axons can travel large distances until they reach their targets, it is highly dependent on intra-axonal events. In this study we cultured hippocampal neurons in tripartite microfluidic devices and used PDL-coated latex beads to induce presynaptic assembly in isolated axons. Adding beads to axons physically separated from dendrites and neuronal cell bodies allowed us to induce synchronous synapse assembly, without the need of a postsynaptic cell, and thus manipulate axons without also inducing confounding postsynaptic changes. PDL is an artificial polymer used to promote *in vitro* adhesion of cells to a substrate. Beads coated with PDL have been shown to adhere to the axon 5 minutes after contact, and become highly resistant to detachment (Lucido et al., 2009; Suarez et al., 2013). This adhesion is dependent on specific interactions between PDL and cell surface heparan sulfate proteoglycans (HSPG), and uncoated beads neither adhere nor induce presynaptic differentiation (Lucido et al., 2009). SVs and AZ proteins accumulate at these contact points, and the domains at which these proteins cluster both mimic the ultrastructural organization of native presynaptic boutons and are able of SV depolarization-induced recycling (Lucido et al., 2009). Analysis of PTV and STV recruitment following bead-axon contact showed that PTVs cluster earlier than STVs (23 vs 43 minutes) (Suarez et al., 2013). As soon as 1 hour after first contact these presynaptic sites are already capable of FM dye uptake and release. Using puromycin to tag newly synthesized proteins, we detected an increase in protein synthesis after just 15 minutes of PDL-coated bead treatment. Puromycin puncta were frequently located near bead-axon contacts and co-localized with p-4EBP1, a marker of active translation downstream of mTOR. This time point is shorter than average reported PTVs and STVs recruitment times, consistent with the idea that local translation does indeed precede presynaptic transport packets clustering.

SNAP25 and other presynaptic transcripts

We ultimately focused on SNAP25, a member of the SNARE complex, responsible for membrane fusion and vesicle exocytosis. SNAP25 is a plasma membrane or target membrane SNARE (t-SNARE), and,

together with syntaxin 1, another t-SNARE, and VAMP2, a vesicle SNARE (v-SNARE), form a complex responsible for mediating SV exocytosis and neurotransmitter release (Söllner et al., 1993; Weber et al., 1998). SNAP25 transcript had been previously detected in axons of rat cortical neurons, although not in other transcriptomes (Baleriola et al., 2014; Taylor et al., 2009; Zivraj et al., 2010). Surprisingly, in mice RGC axons, SNAP25 transcript was recently found to be associated with ribosomes not only during development, but also in adults (Shigeoka et al., 2016). Here we show that SNAP25 transcript can be specifically detected in naive hippocampal axons with *in situ* hybridization, and that it accumulates at bead-axon contact points, similarly to β-catenin (Taylor et al., 2013). It is likely that more transcripts might also localize to induced synaptic specializations. The first such candidate to come to mind is β-actin. β-actin local synthesis has been associated with several growth cone responses such as stimulus-induce elongation, chemotropic responses, and, importantly, branching (Donnelly et al., 2013; Leung et al., 2006; Piper et al., 2006; Wong et al., 2017; Wu et al., 2005). Terminal arbor branching and presynaptic puncta appearance are closely related, with new branches frequently forming on newly added presynaptic sites, and synapses stabilizing branches (Alsina et al., 2001; Meyer and Smith, 2006; Ruthazer et al., 2006). RNA docking and β-actin synthesis correlate with branch emergence and stabilization *in vivo* (Wong et al., 2017). Actin dynamics do appear to underlie this connection between both processes (Chia et al., 2014), and early synapses are sensitive to actin depolymerizing agents (Zhang and Benson, 2001). The synaptic specializations induced by PDL-coated beads show this same sensitivity to actin depolymerization (Lucido et al., 2009).

Other candidates are comprised by several proteins whose transcripts have been previously detected in CNS axons, and that we found to accumulate early at axon-bead contacts. GAP-43, for instance, localizes to early axons and growth cones, and is involved in axonal growth, regeneration and mechanisms of synaptic plasticity (Benowitz and Routtenberg, 1997). Its local translation has been shown to support axonal elongation (Donnelly et al., 2013). Its levels after development decrease, but it continues to participate in LTP and is required for normal learning (Rekart et al., 2005; Routtenberg et al., 2000). Its transcript significantly increases in axons of cortical neurons following axotomy, and in hippocampal axons after an Aβ challenge (Baleriola et al., 2014; Taylor et al., 2009). It is possible that GAP-43 local synthesis might also be involved in synapse formation and that at least part of its role in plasticity is mediated by on site translation. Several lines of evidence correlate GAP-43 dysregulation with schizophrenia and epileptogenesis (Eastwood, 2004; Nemes et al., 2017), so it would be interesting to clarify whether local translation might be involved. Curiously, several schizophrenia

studies consistently report a discrepancy between transcript and protein levels in patients, which would argue for with preponderant alterations in posttranscription regulation (Eastwood, 2004).
Synaptophysin, the most abundant SV membrane protein, also accumulates around beads. There is still no clear role for this protein, but it seems to participate in the regulation of endocytosis and in synaptogenesis (Kwon and Chapman, 2011; Tarsa and Goda, 2002). Though its local translation has never been described, its transcript is present in hippocampal axons, and increases significantly upon axonal Aβ treatment (Baleriola et al., 2014). However, synaptophysin is an integral membrane protein, and, as already referred, it remains unclear how that can be accomplished without a conventional secretory pathway which requires both ER and the Golgi apparatus.

SNAP25 and β-catenin translation as distinct events

We examined SNAP25 accumulation at bead-axon contacts at several time points, and its dependence on axonal protein synthesis. We were able to detect significant SNAP25 increases around coated-beads as early as 1 hour after contact. This SNAP25 accumulation required local protein synthesis, and axonal emetine treatment significantly reduced SNAP25 accumulation around beads to control levels at 1, 3, 6 and 12 hours. Using microfluidic devices with an open axonal compartment (circular microfluidic chambers) we demonstrate that PDL-coated beads induce a very pronounced increase in SNAP25 protein in axons after 24 hours and that blocking axonal protein synthesis almost completely blocks this increase, without affecting SNAP25 levels in the cell bodies. Given all of this, we show that blocking protein synthesis in axons does not merely delay SNAP25 clustering in these newly assembled presynaptic sites but may interfere with functional synapse assembly.
Taylor *et al.* reported a continuous increase in bassoon fluorescence around beads until 48 hours after treatment and showed that bassoon still clustered, at 3 hours, in the presence of protein synthesis inhibitors (Taylor et al., 2013). In our study we did not evaluate bassoon clustering, but both SNAP25 and bassoon are transported in PTVs. One possible explanation for this discrepancy is that at this early time point SNAP25 clustering is more dependent on local synthesis than protein transport, and that PTV transport is not totally blocked. PTV bassoon content greatly exceeds SNAP25 content and it has been estimated that a mere 2 to 3 PTVs might be needed to assemble a presynaptic bouton (Shapira et al., 2003). Therefore, some clustering of bassoon might happen without significant SNAP25 clustering. Some studies also support the idea that synaptogenic factors might induce the formation of a stable platform where transport packets get trapped (Bury and Sabo, 2014). We did not dissect the mechanism through which PDL interaction leads to protein synthesis and blocking protein synthesis

might not completely block the synaptogenic cascade. It is possible that several parallel pathways are activated, or that there are redundant mechanisms, and that both active recruitment and local entrapment coexist (Pinto and Almeida, 2016). In order to fully understand this difference, bassoon clustering should be studied at longer time points, with concomitant protein synthesis inhibition.

In contrast to these SNAP25 results, in our time course, β-catenin clustering was dependent on local protein synthesis at 1 hour and 3 hours, but it was not affected by protein synthesis inhibitors at later time points. Taylor et al., who first reported β-catenin synthesis at these presynaptic specializations, only looked at 3 hours after bead-axon contact (Taylor et al., 2013). This delayed clustering probably translates β-catenin recruitment from the soma. But even though transported β-catenin can compensate at these later times, β-catenin siRNA was shown to significantly impair SV release dynamics 1 day after presynaptic specialization induction (Taylor et al., 2013). Surprisingly, β-catenin siRNA was not able to block β-catenin protein clustering at 1 hour, although significantly decreasing SNAP25 clustering at the same time point. Once again, we show that β-catenin protein clustering does not depend on its local synthesis, and previously made protein can be anterogradely transported. But β-catenin local synthesis does seem to possess other roles, perhaps as a signal mediator.

β-catenin has several functions, as cadherin-actin anchor, PDZ protein recruitment and participates in the Wnt signaling pathway. Pre-existing protein might be able to cluster, but only partially fulfil its functions. β-catenin peaks and AUC were smaller than SNAP25 ones, and although unlikely, we cannot exclude that only a very low amount of translation might be needed to cluster β-catenin protein and that siRNA inhibition was not sufficient. It is also possible that both SNAP25 and β-catenin transcripts are packaged and transported together, in the same granule and that β-catenin siRNA could interfere with SNAP25 translation.

SNAP25 siRNA, on the other hand, is able to interfere with both SNAP25 and β-catenin proteins clustering at 1 hour after bead treatment. SNAP25 local synthesis seems to be necessary for the clustering of at least these two proteins. We did not analyze the clustering of other proteins, or looked at later time points, both of which would be interesting to further answer remaining questions about the necessity of SNAP25 local translation for synapse assembly.

Overall, it seems that a small amount of β-catenin local synthesis is necessary for initiating the clustering of other presynaptic proteins, though this small amount does not seem to be responsible for the fluorescence increase observed in the bead vicinity. Pre-existing β-catenin accumulates through a parallel mechanism, that possibly involves local translation of other proteins, and positive feedback mechanisms, that justify the delayed clustering in the presence of synthesis inhibitors in the axon.

SNAP25 local synthesis seems to be involved in these positive feedback mechanisms and greatly influences clustering of both analyzed proteins. The circular chamber data also supports the existence of translation-dependent feedback mechanisms. Since SNAP25 is transported in PTVs and, in the emetine condition no SNAP25 is locally synthesized, protein amounts reflect PTVs recruitment. So, in the emetine condition, the very low amounts of SNAP25 probably reflect diminished PTVs recruitment, comparable to uncoated beads level. Therefore, we can infer that inhibiting axonal protein synthesis greatly perturbs PTVs recruitment to axons in response to the beads synaptogenic stimulus.
One other curious observation is that β-catenin siRNA appears to elongate the clustering area (from 5 μm to 15 μm). Although this could just be artificial, β-catenin could also play a role in defining the synapse area, and this is consistent with the reports of increased number of synapses and increased basal transmission in β-catenin conditional knockdown mice, and could also justify the increased SV release upon stimulation after axonal siRNA treatment (Bamji et al., 2003; Taylor et al., 2013).

Direct visualization of SNAP25 synthesis

Coupling puromycylation with a proximity ligation assay (puro-PLA), we were able to directly visualize newly synthesized SNAP25 protein within bead-axon contact sites as early as 15 minutes after initial contact. This SNAP25 synthesis is sustained, at least for the first hour of bead contact, since we could still detect SNAP25 puro-PLA signal using a 10 minutes puromycin pulse at the end of the 1 hour time point. However, the average number of puro-PLA puncta per bead area is about a fourth of the number of puncta we obtained with puromycin alone. This might be because there is concomitant synthesis of other proteins, like β-catenin, or because PLA puncta, that result from circular amplification of a DNA fragment, and are usually bigger, might mask multiple translation hotspots. Also, some information can be lost since SNAP25 antibodies might not be able to recognize smaller fragments with premature puromycin incorporation. It might also just reflect fluctuations between biological replicates.
Using the same technique, we observed SNAP25 local synthesis in neurites of dissociated hippocampal cultures at DIV5, 10 and 15. Some of these puncta appear to be located on dendrites rather than axons. Though SNAP25 had been previously shown to be exclusively localized to axons, some recent reports have shown that SNAP25 has postsynaptic functions, although clear demonstration for its dendritic localization is lacking, possibly because it is expressed at much lower levels (Antonucci et al., 2016; Fossati et al., 2015; Ovsepian and Dolly, 2011; Tao-Cheng et al., 2000). It has been previously shown that SNAP25 is required for the dendritic growth of hippocampal pyramidal and granule neurons (Grosse et al., 1999). A function for SNAP25 local synthesis in dendritic morphogenesis would be a

very interesting possibility, although it lacks any supporting evidence. This somewhat puzzling incidental finding of SNAP25 local synthesis in growing dendrites, in light of the increasing reports of new postsynaptic functions (Antonucci et al., 2016), justifies further research.

At DIV 15, several SNAP25 puro-PLA puncta were juxtaposed to PSD-95 signal, a postsynaptic density marker. Cultured axons start to form immature connections at DIV 5, though postsynaptic specializations only appear later (Basarsky et al., 1994; Fletcher et al., 1994; Mozhayeva et al., 2002). At DIV 15 there are already mature synapses in dissociated cultures, and so these puro-PLA puncta might indicate SNAP25 synthesis at established synapses.

A FUNCTIONAL ROLE FOR SNAP25 TRANSLATION

Finally, after selective ablation of axonal SNAP25 with SNAP25 siRNA, we added the beads for 24 hours and analyzed SV dynamics with FM4-64 dye. We loaded the terminals and subsequently induced synaptic release with a high K+ solution (Iwabuchi et al., 2014). Presynaptic specializations induced by PDL-coated beads have been shown before to be able of SV recycling with the use of FM dyes (Lucido et al., 2009). Specific axonal knockdown of β-catenin has been reported to change unloading kinetics of FM dyes in these axon-bead specializations (Taylor et al., 2013). Since we determined that SNAP25 was locally translated at these contacts, and that inhibition of SNAP25 axonal synthesis markedly reduced both SNAP25 and β-catenin early clustering, we wanted to determine whether there were other long-lasting effects. FM dye fluorescence after loading was similar between scrambled and SNAP25 siRNA conditions, but in the presence of SNAP25 siRNA vesicle release was slower, with a significantly increased destaining time constant (τ). The average τ values obtained in axons were comparable to the ones we obtained in the cell body compartment, where dendrites and axons freely contact, and to previous ones reported in mature synapses of other culture systems (Kavalali et al., 1999; Mozhayeva et al., 2002; Ryan and Smith, 1995). High K+ solutions result in sustained elevations of the presynaptic Ca2+ and the entire recycling vesicle pool is mobilized (Cohen and Segal, 2011; Nimmervoll et al., 2013). Cell-wide SNAP25 knockdown completely abolishes Ca2+-dependent synaptic transmission, but not spontaneous vesicle cycling, and homozygous mutation is lethal *in utero* (Bronk et al., 2007; Washbourne et al., 2002). Our axonal knockdown has a completely different phenotype, with reduced vesicle release, and a higher destaining constant. Higher τ are characteristic of more immature synapses, which at first lack a readily releasable pool (docked vesicles), and subsequently increase both the docked and undocked vesicles of the recycling pool size as they mature (Mozhayeva et al., 2002). The total variation in FM dye destaining appears to be smaller, which would

reflect a smaller recycling pool. Nevertheless, a single potassium pulse might not have been sufficient to completely mobilize the entire recycling pool. Also, FM dye loading was apparently unaffected by SNAP25 siRNA treatment, which would mean there is no difference in recycling pools between conditions. SNAP25 has been shown to play a role in the exocytosis-endocytosis coupling, being involved in both rapid and slow clathrin-dependent endocytosis (Xu et al., 2013; Zhang et al., 2013). When loading the dye, we used a 90 seconds potassium-stimulation followed by a 10 minutes endocytosis period in the presence of the dye, and this post-stimulation period is considerably more than most protocols. This might have masked any loading defects. Alternatively, SNAP25 local translation could differently affect endocytosis and exocytosis.

From these SV release experiments, we can only conclude that SNAP25 local synthesis has a role in the functional assembly of these presynaptic specializations, and, after 24 hours, more than enough time for anterogradely transported SNAP25 to compensate, the terminals show considerably different responses. Whether these changes are due to an immature terminal, an exocytosis defect or any other presynaptic change is unclear. We did not analyze clustering or terminal composition at this later time point (24 hours) after siRNA treatment, though 1 hour is in principle sufficient for presynapse assembly (Ahmari et al., 2000; Friedman et al., 2000). From the FM dye staining and destaining profile, it seems that SVs were able to cluster around beads, and to recycle at axon-bead contacts. At 1 hour after bead contact, no SNAP25 or β-catenin clustered in the SNAP25 siRNA condition. Also, inhibiting total axonal protein synthesis inhibited SNAP25 clustering for up to 24 hours. In addition, the much reduced amount of SNAP25 protein in the circular chambers in the synthesis inhibitors condition is unlikely to correspond to this FM dye profile. Even balancing the fact that inhibiting axonal synthesis for a whole day might interfere with axonal health, though it should be noted that no axonal disintegration was observed at the end of the period. Thus, inhibiting the local synthesis of other proteins, such as β-catenin, seems to have an additive effect over SNAP25 axonal silencing alone. Or the SNAP25 synthesis inhibition elicited by emetine is greater than the one elicited by the SNAP25 siRNA. PDL-coated beads adhesion is likely to elicit changes in transmembrane proteins and act as a constant synaptogenic inducer that might overcome low levels of inhibition. Synaptogenic organizers also appear to be associated with a high level of redundancy, and *in vivo* deletion studies of several synaptogenic proteins did not have a large effect on synapse formation (Pinto and Almeida, 2016). In the same way, downstream parallel pathways are likely activated, and some might not be greatly affected by local translation, thereby ameliorating the defects.

STANDING QUESTIONS AND FUTURE DIRECTIONS

ELUCIDATING THE INTRA-AXONAL CASCADE

Our experimental work establishes an extremely rapid induction of protein synthesis, faster than average synaptic packets transport times. Several questions remain about the exact intra-axonal cascade of events and their causality. First, how does PDL-adhesion elicit translation activation? PDL seems to interact with transmembrane HSPGs in the axonal membrane, specifically syndecan 2 (Lucido et al., 2009). HSPG are a known class of synaptic organizer molecules and have been shown to regulate several signaling pathways, such as the Wnt pathway (Condomitti and de Wit, 2018). The translation machinery could directly bind to these transmembrane molecules, as was shown for the receptor DCC (Tcherkezian et al., 2010). Other examples in the literature involve calcium-signaling, as in the axonal injury model (Rishal and Fainzilber, 2014), but in the majority of described cases, the exact mechanism through which extracellular signals induce translation activation is unknown. Evoked action potentials along the axon have been shown to lead to PTVs and STVs stalling and calcium-induced exocytosis and to the formation of orphan presynaptic sites (Ratnayaka et al., 2011), and in this case calcium signaling could simultaneously activate translation. Some studies do suggest that synapses form selectively at these sites, and, similarly to what has been described for RGC terminal branching, RNA docking might predetermine future synapse sites, and the availability of protein synthesis machinery might have a permissive role. Nevertheless, this does not seem to be the case in our synaptogenesis assay. Further analysis of the precise molecular interactions of syndecan 2 are needed, specifically whether it interacts with RNA granules or translation machinery.

Another unresolved issue is the mechanism through which protein synthesis in general, and SNAP25 synthesis in particular, can influence presynaptic protein clustering, and perhaps PTVs clustering (if we assume that an absence of SNAP25 clustering can be used to infer about PTVs mobilization). And whether STVs clustering is also affected. The latter could be easily answered with specific analysis of PTV and STV proteins clustering in the presence of translation inhibitors in the axon. And mechanistical insight could be obtained by analyzing actin and cytoskeleton dynamics, since some studies do propose that local entrapment is responsible for transport vesicles clustering.

A recent study has showed that proteasome inhibition, through the accumulation of polyubiquitinated chains, has a synaptogenic effect on axons, and, using microfluidic devices and PDL-coated beads, they showed a rapid increase of polyubiquitinated chains 10 minutes after bead-axon contact (Pinto et

al., 2016, 2016). This time point is very close to our 15 minutes one, and it seems that both local translation induction and proteasome inhibition are simultaneously stimulated. Coupling of local synthesis and degradation has been described to regulate growth cone collapse in response to Sema3A (Deglincerti et al., 2015). In this case, inhibiting the degradation of the locally synthesized protein RhoA is sufficient to remove the RhoA synthesis dependence for Sema3A collapse response. SNAP25 shows a wide range of ubiquitination events, and its stability at the synapse is controlled by activity-induced ubiquitination and proteasome degradation (Na et al., 2012; Sheehan et al., 2016). It would be interesting to assess whether SNAP25 undergoes ubiquitination at nascent synaptic specializations and whether local translation and proteasome inhibition are linked events during presynaptic assembly.

The function of locally translated SNAP25

The most important remaining question concerns the specific SNAP25 function in synapse assembly. SNAP25 is fundamental for vesicle exocytosis, and has been implicated in endocytosis, but also possesses other non-SNARE roles, in regulating voltage-gated calcium channels (VGCCs) and at the postsynapse (Antonucci et al., 2016). SNAP25 undergoes palmitoylation, a reversible lipid modification that functions as a lipid anchor and targets SNAP25 to the plasma membrane (Gonzalo et al., 1999). The reversible nature of palmitoylation allows proteins to shuttle between different compartments and palmitoylation-depalmitoylation cycles are involved in several signaling pathways (Iwanaga et al., 2009). Palmitoyl transferase enzimes are usually localized to the ER, Golgi and plasma membrane (Ohno et al., 2006), and depalmitoylation enzymes can localize to axons and nerve terminals (Kim et al., 2008). Since the axons does not possess classical ER or Golgi apparatus, it is unclear whether locally synthesized SNAP25 can get palmitoylated, and whether it targets to the membrane. It is, nevertheless, possible that locally translated SNAP25 does not need palmitoylation to fulfil its specific function. Palmitoylation has important roles in pre- and postsynaptic regulation, and in axonal growth, degeneration and regeneration, and though initial studies reported that axonal proteins were very stably palmitoylated, in contrast with the highly dynamic palmitoylation state of somatodendritic proteins, recent studies contradict this idea (Holland and Thomas, 2017; Holland et al., 2016; Kang et al., 2008). As an example, the extremely fast growth cone collapse induced by nitric oxide, resulting from inhibition of protein palmitoylation, strongly suggests a local effect (Hess et al., 1993). It would be easy to assess the existence of a local axonal palmitoylation mechanism using microfluidic devices and *in vivo* metabolic labeling with radiolabeled palmitate or ω-azido-fatty acids and subsequent click-chemistry (Fukata et al., 2004; Martin and Cravatt, 2009). Specific SNAP25 palmitoylation assays,

such as immunoprecipitation coupled with acyl-biotin exchange, would be technically challenging, but could be attempted in circular chambers after PDL-coated bead stimulation (Brigidi and Bamji, 2013). In order to better understand the function of locally translated SNAP25, it would be important to better characterize the induced presynapses in the axonal siRNA conditions, analyzing protein clustering at longer time points, and SV markers. One outstanding question is whether the defects we observed in FM dye destaining would be transient or persist. But inhibiting protein synthesis or using SNAP25 siRNA for longer time points is not a viable option and our artificial synaptogenesis induction would not be a good control for more mature terminals. Ultimately, SNAP25 axonal silencing should be performed *in vivo*. A simple system, amenable to manipulation, is the *Xenopus* visual system. SNAP25 has been shown to be expressed in mice RGC axons during synaptogenesis (Shigeoka et al., 2016), though not in the frog.

Study of SNAP25 mRNA transport, and identification of localizing sequences and interacting RBPs, could open the possibility of specifically mutating the SNAP25 transcript region responsible for its axonal localization, thus specifically inhibiting SNAP25 local translation *in vitro* and *in vivo*.

Finally, there is still the exciting possibility that SNAP25 might continue to get translated in adult axons. SNAP25 transcript showed high reads in adult axons in the recent mice RGC translatome (Shigeoka et al., 2016). And we detected SNAP25 puro-PLA signal in hippocampal cultures at DIV 15, when most presynapses have already matured. As discussed in the introduction, several anecdotal reports point collectively to a function for intra-axonal synthesis in presynaptic plasticity. Ubiquitination is an essential mechanism for maintaining presynaptic integrity, and, as referred previously, SNAP25 stability is regulated by activity-induced ubiquitination (Sheehan et al., 2016; Waites et al., 2013). Coupled SNAP25 local synthesis and activity-induced ubiquitination would be a very elegant mechanism to regulate synapse function. However, experimentally testing SNAP25 synthesis at mature nerve terminals might be technically challenging and supporting evidence is still scarce.

LOCAL SYNTHESIS OF OTHER PROTEINS AT THE NERVE TERMINAL

Other presynaptic protein-encoding mRNAs have been detected in axonal transcriptomes and translatomes (Baleriola et al., 2014; Shigeoka et al., 2016; Taylor et al., 2009; Zivraj et al., 2010). It is possible that, much like SNAP25 and β-catenin, other proteins are also locally translated during synaptogenesis. The likely candidates and rationale have already been discussed above. Among them, β-actin is a likely candidate, and it might elucidate the role of cytoskeleton dynamics, and the similarity to other translation-dependent axonal responses. Furthermore, one could address these possibilities

with simple and fast experiments and help generate a clearer picture of this extremely complex assembly event.

SNAP25 is a ubiquitous neuronal protein and does not show neuron type or circuit specificity. But axonal transcriptomes, and described local translation events, do appear to change between different types of neurons. Neurons, and synapses, are very heterogeneous. Some circuits have been shown to rely more on presynaptic plasticity, such as the eCB-dependent signaling in hippocampal GABAergic inhibitory interneurons, which might have specific assembly cascades (Younts et al., 2016). There are also synapses with particular characteristics, such as the neuro-muscular junction. A vast amount of different synaptogenic mediators have been identified and maybe assembly pathways show the same variability. It would be interesting to understand whether local synthesis of SNAP25 also occurs at distinct synapses and whether other proteins might be locally translated at specific terminals.

A possible role for SNAP25 at dendrites

SNAP25 has been shown to possess several other functions in the dendritic compartment, in spine morphogenesis, in receptor trafficking, and in LTP (Antonucci et al., 2016). SNAP25 puro-PLA puncta were readily observed in hippocampal neurites in culture at DIV 5 and DIV 10. Most of the times, several processes from the same neuron showed puncta, so, forcibly, some of these represented dendrites. It is highly unlikely that these events are culture artefacts, and this warrants further research.

Disease relevance

Current knowledge points to a general mechanism for axonal mRNA localization, involving the specific recognition of target sequences located in the 3'UTR region by RBPs. SNAP25 mutations and SNPs have been associated with different psychiatric disorders, namely attention-deficit hyperactivity disorder (ADHD), schizophrenia and early-onset bipolar disorders (Antonucci et al., 2016). Interestingly, two different mutations in the SNAP25 3' UTR have been described that confer disease risk (Barr et al., 2000; Brophy et al., 2002; Carroll et al., 2009; Dai et al., 2014; Pazvantoğlu et al., 2013; Wang et al., 2015). In light of our finding that SNAP25 is locally synthesized in axons, and has a role in synapse assembly, dysregulation of local translation might be the underlying disease physiopathology.

REFERENCES

Ahmari, S.E., Buchanan, J., and Smith, S.J. (2000). Assembly of presynaptic active zones from cytoplasmic transport packets. Nat. Neurosci. *3*, 445–451.

Alsina, B., Vu, T., and Cohen-Cory, S. (2001). Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. Nat. Neurosci. *4*, 1093–1101.

Ameri, K., and Harris, A.L. (2008). Activating transcription factor 4. Int J Biochem Cell Biol *40*, 14–21.

Antonucci, F., Corradini, I., Fossati, G., Tomasoni, R., Menna, E., and Matteoli, M. (2016). SNAP-25, a Known Presynaptic Protein with Emerging Postsynaptic Functions. Front Synaptic Neurosci *8*.

Baleriola, J., Walker, C.A., Jean, Y.Y., Crary, J.F., Troy, C.M., Nagy, P.L., and Hengst, U. (2014). Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. Cell *158*, 1159–1172.

Bamji, S.X., Shimazu, K., Kimes, N., Huelsken, J., Birchmeier, W., Lu, B., and Reichardt, L.F. (2003). Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. Neuron *40*, 719–731.

Barr, C.L., Feng, Y., Wigg, K., Bloom, S., Roberts, W., Malone, M., Schachar, R., Tannock, R., and Kennedy, J.L. (2000). Identification of DNA variants in the SNAP-25 gene and linkage study of these polymorphisms and attention-deficit hyperactivity disorder. Mol. Psychiatry *5*, 405–409.

Basarsky, T.A., Parpura, V., and Haydon, P.G. (1994). Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. J. Neurosci. *14*, 6402–6411.

Benowitz, L.I., and Routtenberg, A. (1997). GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci. *20*, 84–91.

Boyne, L.J., Fischer, I., and Shea, T.B. (1996). Role of vimentin in early stages of neuritogenesis in cultured hippocampal neurons. Int. J. Dev. Neurosci. *14*, 739–748.

Brigidi, G.S., and Bamji, S.X. (2013). Detection of Protein Palmitoylation in Cultured Hippocampal Neurons by Immunoprecipitation and Acyl-Biotin Exchange (ABE). JoVE (Journal of Visualized Experiments) e50031–e50031.

Bronk, P., Deák, F., Wilson, M.C., Liu, X., Südhof, T.C., and Kavalali, E.T. (2007). Differential effects of SNAP-25 deletion on Ca2+ -dependent and Ca2+ -independent neurotransmission. J. Neurophysiol. *98*, 794–806.

Brophy, K., Hawi, Z., Kirley, A., Fitzgerald, M., and Gill, M. (2002). Synaptosomal-associated protein 25 (SNAP-25) and attention deficit hyperactivity disorder (ADHD): evidence of linkage and association in the Irish population. Mol. Psychiatry *7*, 913–917.

Bury, L.A., and Sabo, S.L. (2014). Dynamic mechanisms of neuroligin-dependent presynaptic terminal assembly in living cortical neurons. Neural Dev *9*, 13.

Carroll, L.S., Kendall, K., O'Donovan, M.C., Owen, M.J., and Williams, N.M. (2009). Evidence that putative ADHD low risk alleles at SNAP25 may increase the risk of schizophrenia. Am. J. Med. Genet. B Neuropsychiatr. Genet. *150B*, 893–899.

Chia, P.H., Chen, B., Li, P., Rosen, M.K., and Shen, K. (2014). Local F-actin network links synapse formation and axon branching. Cell *156*, 208–220.

Cohen, D., and Segal, M. (2011). Network bursts in hippocampal microcultures are terminated by exhaustion of vesicle pools. J. Neurophysiol. *106*, 2314–2321.

Condomitti, G., and de Wit, J. (2018). Heparan Sulfate Proteoglycans as Emerging Players in Synaptic Specificity. Front Mol Neurosci *11*.

Dai, D., Wang, Y., Yuan, J., Zhou, X., Jiang, D., Li, J., Zhang, Y., Yin, H., and Duan, S. (2014). Meta-analyses of 10 polymorphisms associated with the risk of schizophrenia. Biomed Rep *2*, 729–736.

Deglincerti, A., Liu, Y., Colak, D., Hengst, U., Xu, G., and Jaffrey, S.R. (2015). Coupled local translation and degradation regulate growth cone collapse. Nat Commun *6*, 6888.

Donnelly, C.J., Park, M., Spillane, M., Yoo, S., Pacheco, A., Gomes, C., Vuppalanchi, D., McDonald, M., Kim, H.H., Kim, H.K., et al. (2013). Axonally synthesized β-actin and GAP-43 proteins support distinct modes of axonal growth. J. Neurosci. *33*, 3311–3322.

Eastwood, S.L. (2004). The Synaptic Pathology of Schizophrenia: Is Aberrant Neurodevelopment and Plasticity to Blame? In International Review of Neurobiology, (Academic Press), pp. 47–72.

Fletcher, T.L., De Camilli, P., and Banker, G. (1994). Synaptogenesis in hippocampal cultures: evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. J. Neurosci. *14*, 6695–6706.

Fossati, G., Morini, R., Corradini, I., Antonucci, F., Trepte, P., Edry, E., Sharma, V., Papale, A., Pozzi, D., Defilippi, P., et al. (2015). Reduced SNAP-25 increases PSD-95 mobility and impairs spine morphogenesis. Cell Death Differ. *22*, 1425–1436.

Friedman, H.V., Bresler, T., Garner, C.C., and Ziv, N.E. (2000). Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. Neuron *27*, 57–69.

Fukata, M., Fukata, Y., Adesnik, H., Nicoll, R.A., and Bredt, D.S. (2004). Identification of PSD-95 palmitoylating enzymes. Neuron *44*, 987–996.

Gonzalo, S., Greentree, W.K., and Linder, M.E. (1999). SNAP-25 Is Targeted to the Plasma Membrane through a Novel Membrane-binding Domain. J. Biol. Chem. *274*, 21313–21318.

Grosse, G., Grosse, J., Tapp, R., Kuchinke, J., Gorsleben, M., Fetter, I., Höhne-Zell, B., Gratzl, M., and Bergmann, M. (1999). SNAP-25 requirement for dendritic growth of hippocampal neurons. J. Neurosci. Res. *56*, 539–546.

Hanz, S., Perlson, E., Willis, D., Zheng, J.Q., Massarwa, R., Huerta, J.J., Koltzenburg, M., Kohler, M., van-Minnen, J., Twiss, J.L., et al. (2003). Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron *40*, 1095–1104.

Hess, D.T., Patterson, S.I., Smith, D.S., and Skene, J.H. (1993). Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. Nature *366*, 562–565.

Holland, S.M., and Thomas, G.M. (2017). Roles of Palmitoylation in Axon Growth, Degeneration and Regeneration. J Neurosci Res *95*, 1528–1539.

Holland, S.M., Collura, K.M., Ketschek, A., Noma, K., Ferguson, T.A., Jin, Y., Gallo, G., and Thomas, G.M. (2016). Palmitoylation controls DLK localization, interactions and activity to ensure effective axonal injury signaling. PNAS *113*, 763–768.

Iwabuchi, S., Kakazu, Y., Koh, J.-Y., Goodman, K.M., and Harata, N.C. (2014). Examination of Synaptic Vesicle Recycling Using FM Dyes During Evoked, Spontaneous, and Miniature Synaptic Activities. J Vis Exp.

Iwanaga, T., Tsutsumi, R., Noritake, J., Fukata, Y., and Fukata, M. (2009). Dynamic protein palmitoylation in cellular signaling. Prog. Lipid Res. *48*, 117–127.

Kang, R., Wan, J., Arstikaitis, P., Takahashi, H., Huang, K., Bailey, A.O., Thompson, J.X., Roth, A.F., Drisdel, R.C., Mastro, R., et al. (2008). Neural Palmitoyl-Proteomics Reveals Dynamic Synaptic Palmitoylation. Nature *456*, 904–909.

Kavalali, E.T., Klingauf, J., and Tsien, R.W. (1999). Activity-dependent regulation of synaptic clustering in a hippocampal culture system. PNAS *96*, 12893–12900.

Kim, S.-J., Zhang, Z., Sarkar, C., Tsai, P.-C., Lee, Y.-C., Dye, L., and Mukherjee, A.B. (2008). Palmitoyl protein thioesterase-1 deficiency impairs synaptic vesicle recycling at nerve terminals, contributing to neuropathology in humans and mice. J Clin Invest *118*, 3075–3086.

Kwon, S.E., and Chapman, E.R. (2011). Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron *70*, 847–854.

Leung, K.-M., van Horck, F.P.G., Lin, A.C., Allison, R., Standart, N., and Holt, C.E. (2006). Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. Nat. Neurosci. *9*, 1247–1256.

Lucido, A.L., Suarez Sanchez, F., Thostrup, P., Kwiatkowski, A.V., Leal-Ortiz, S., Gopalakrishnan, G., Liazoghli, D., Belkaid, W., Lennox, R.B., Grutter, P., et al. (2009). Rapid assembly of functional presynaptic boutons triggered by adhesive contacts. J. Neurosci. *29*, 12449–12466.

Martin, B.R., and Cravatt, B.F. (2009). Large-scale profiling of protein palmitoylation in mammalian cells. Nat. Methods *6*, 135–138.

Menet, V., Prieto, M., Privat, A., and Giménez y Ribotta, M. (2003). Axonal plasticity and functional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. Proc. Natl. Acad. Sci. U.S.A. *100*, 8999–9004.

Meyer, M.P., and Smith, S.J. (2006). Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. J. Neurosci. *26*, 3604–3614.

Mozhayeva, M.G., Sara, Y., Liu, X., and Kavalali, E.T. (2002). Development of Vesicle Pools during Maturation of Hippocampal Synapses. J. Neurosci. *22*, 654–665.

Na, C.H., Jones, D.R., Yang, Y., Wang, X., Xu, Y., and Peng, J. (2012). Synaptic protein ubiquitination in rat brain revealed by antibody-based ubiquitome analysis. J. Proteome Res. *11*, 4722–4732.

Nemes, A.D., Ayasoufi, K., Ying, Z., Zhou, Q.-G., Suh, H., and Najm, I.M. (2017). Growth Associated Protein 43 (GAP-43) as a Novel Target for the Diagnosis, Treatment and Prevention of Epileptogenesis. Sci Rep *7*.

Nimmervoll, B., Flucher, B.E., and Obermair, G.J. (2013). Dominance of P/Q-type calcium channels in depolarization-induced presynaptic fm dye release in cultured hippocampal neurons. Neuroscience *253*, 330–340.

Ohno, Y., Kihara, A., Sano, T., and Igarashi, Y. (2006). Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids *1761*, 474–483.

Ovsepian, S.V., and Dolly, J.O. (2011). Dendritic SNAREs add a new twist to the old neuron theory. PNAS *108*, 19113–19120.

Pazvantoğlu, O., Güneş, S., Karabekiroğlu, K., Yeğin, Z., Erenkuş, Z., Akbaş, S., Sarısoy, G., Korkmaz, I.Z., Böke, O., Bağcı, H., et al. (2013). The relationship between the presence of ADHD and certain candidate gene polymorphisms in a Turkish sample. Gene *528*, 320–327.

Perlson, E., Hanz, S., Ben-Yaakov, K., Segal-Ruder, Y., Seger, R., and Fainzilber, M. (2005). Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron *45*, 715–726.

Pinto, M.J., and Almeida, R.D. (2016). Puzzling out presynaptic differentiation. J. Neurochem. *139*, 921–942.

Pinto, M.J., Pedro, J.R., Costa, R.O., and Almeida, R.D. (2016). Visualizing K48 Ubiquitination during Presynaptic Formation By Ubiquitination-Induced Fluorescence Complementation (UiFC). Front Mol Neurosci *9*, 43.

Piper, M., Anderson, R., Dwivedy, A., Weinl, C., van Horck, F., Leung, K.M., Cogill, E., and Holt, C. (2006). Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones. Neuron *49*, 215–228.

Ratnayaka, A., Marra, V., Branco, T., and Staras, K. (2011). Extrasynaptic vesicle recycling in mature hippocampal neurons. Nat Commun *2*, 531.

Rekart, J.L., Meiri, K., and Routtenberg, A. (2005). Hippocampal-dependent memory is impaired in heterozygous GAP-43 knockout mice. Hippocampus *15*, 1–7.

Rishal, I., and Fainzilber, M. (2014). Axon-soma communication in neuronal injury. Nat Rev Neurosci *15*, 32–42.

Routtenberg, A., Cantallops, I., Zaffuto, S., Serrano, P., and Namgung, U. (2000). Enhanced learning after genetic overexpression of a brain growth protein. Proc. Natl. Acad. Sci. U.S.A. *97*, 7657–7662.

Ruthazer, E.S., Li, J., and Cline, H.T. (2006). Stabilization of axon branch dynamics by synaptic maturation. J. Neurosci. *26*, 3594–3603.

Ryan, T.A., and Smith, S.J. (1995). Vesicle pool mobilization during action potential firing at hippocampal synapses. Neuron *14*, 983–989.

Shapira, M., Zhai, R.G., Dresbach, T., Bresler, T., Torres, V.I., Gundelfinger, E.D., Ziv, N.E., and Garner, C.C. (2003). Unitary Assembly of Presynaptic Active Zones from Piccolo-Bassoon Transport Vesicles. Neuron *38*, 237–252.

Sheehan, P., Zhu, M., Beskow, A., Vollmer, C., and Waites, C.L. (2016). Activity-Dependent Degradation of Synaptic Vesicle Proteins Requires Rab35 and the ESCRT Pathway. J Neurosci *36*, 8668–8686.

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, J.Q., Amieux, P.S., and Holt, C.E. (2016). Dynamic Axonal Translation in Developing and Mature Visual Circuits. Cell *166*, 181–192.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993). SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318–324.

Suarez, F., Thostrup, P., Colman, D., and Grutter, P. (2013). Dynamics of presynaptic protein recruitment induced by local presentation of artificial adhesive contacts. Dev Neurobiol *73*, 98–106.

Tao, J., and Rolls, M.M. (2011). Dendrites Have a Rapid Program of Injury-Induced Degeneration That Is Molecularly Distinct from Developmental Pruning. J. Neurosci. *31*, 5398–5405.

Tao-Cheng, J.-H., Du, J., and McBain, C.J. (2000). Snap-25 is polarized to axons and abundant along the axolemma: an immunogold study of intact neurons. J Neurocytol *29*, 67–77.

Tarsa, L., and Goda, Y. (2002). Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A. *99*, 1012–1016.

Taylor, A.M., Berchtold, N.C., Perreau, V.M., Tu, C.H., Li Jeon, N., and Cotman, C.W. (2009). Axonal mRNA in uninjured and regenerating cortical mammalian axons. J Neurosci *29*, 4697–4707.

Taylor, A.M., Wu, J., Tai, H.-C., and Schuman, E.M. (2013). Axonal translation of β-catenin regulates synaptic vesicle dynamics. J. Neurosci. *33*, 5584–5589.

Tcherkezian, J., Brittis, P.A., Thomas, F., Roux, P.P., and Flanagan, J.G. (2010). Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation. Cell *141*, 632–644.

Thompson-Peer, K.L., DeVault, L., Li, T., Jan, L.Y., and Jan, Y.N. (2016). In vivo dendrite regeneration after injury is different from dendrite development. Genes Dev. *30*, 1776–1789.

Waites, C.L., Leal-Ortiz, S.A., Okerlund, N., Dalke, H., Fejtova, A., Altrock, W.D., Gundelfinger, E.D., and Garner, C.C. (2013). Bassoon and Piccolo maintain synapse integrity by regulating protein ubiquitination and degradation. EMBO J. *32*, 954–969.

Wang, Q., Wang, Y., Ji, W., Zhou, G., He, K., Li, Z., Chen, J., Li, W., Wen, Z., Shen, J., et al. (2015). SNAP25 is associated with schizophrenia and major depressive disorder in the Han Chinese population. J Clin Psychiatry *76*, e76-82.

Washbourne, P., Thompson, P.M., Carta, M., Costa, E.T., Mathews, J.R., Lopez-Benditó, G., Molnár, Z., Becher, M.W., Valenzuela, C.F., Partridge, L.D., et al. (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat. Neurosci. *5*, 19–26.

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T.H., and Rothman, J.E. (1998). SNAREpins: minimal machinery for membrane fusion. Cell *92*, 759–772.

Wong, H.H.-W., Lin, J.Q., Ströhl, F., Roque, C.G., Cioni, J.-M., Cagnetta, R., Turner-Bridger, B., Laine, R.F., Harris, W.A., Kaminski, C.F., et al. (2017). RNA Docking and Local Translation Regulate Site-Specific Axon Remodeling In Vivo. Neuron *95*, 852–868.e8.

Wu, K.Y., Hengst, U., Cox, L.J., Macosko, E.Z., Jeromin, A., Urquhart, E.R., and Jaffrey, S.R. (2005). Local translation of RhoA regulates growth cone collapse. Nature *436*, 1020–1024.

Xu, J., Luo, F., Zhang, Z., Xue, L., Wu, X.-S., Chiang, H.-C., Shin, W., and Wu, L.-G. (2013). SNARE Proteins Synaptobrevin, SNAP-25, and Syntaxin Are Involved in Rapid and Slow Endocytosis at Synapses. Cell Reports *3*, 1414–1421.

Younts, T.J., Monday, H.R., Dudok, B., Klein, M.E., Jordan, B.A., Katona, I., and Castillo, P.E. (2016). Presynaptic Protein Synthesis Is Required for Long-Term Plasticity of GABA Release. Neuron *92*, 479–492.

Zhang, W., and Benson, D.L. (2001). Stages of synapse development defined by dependence on F-actin. J. Neurosci. *21*, 5169–5181.

Zhang, Z., Wang, D., Sun, T., Xu, J., Chiang, H.-C., Shin, W., and Wu, L.-G. (2013). The SNARE proteins SNAP25 and synaptobrevin are involved in endocytosis at hippocampal synapses. J. Neurosci. *33*, 9169–9175.

Zivraj, K.H., Tung, Y.C.L., Piper, M., Gumy, L., Fawcett, J.W., Yeo, G.S.H., and Holt, C.E. (2010). Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. J. Neurosci. *30*, 15464–15478.

CONCLUSIONS

We proposed to analyze the role of local translation for presynaptic terminals assembly. Our main conclusions are as follows:

1- **Local translation is very rapidly induced upon axon contact with synaptogenic adhesive substrates.**

2- **Inhibiting axonal synthesis interferes with the clustering of specific synaptic proteins.**

3- **SNAP25 is locally synthesized at induced presynaptic specializations and in neurites of hippocampal neurons.**

4- **SNAP25 local translation at presynaptic specializations regulates vesicle release dynamics.**

Overall, we identify mRNA localization and axonal synthesis as an important regulatory mechanism for synapse formation. Several poorly understood neurodevelopmental disorders might be caused by dysregulation of axonal RNA transport or local translation defects and dissecting the axonal translatome is fundamental.

ANNEXES

Annex 1: Intra-axonal protein synthesis in development and beyond (2016).

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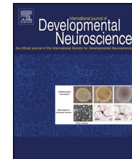

Review

Intra-axonal protein synthesis in development and beyond

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ABSTRACT

Proteins can be locally produced in the periphery of a cell, allowing a rapid and spatially precise response to the changes in its environment. This process is especially relevant in highly polarized and morphologically complex cells such as neurons. The study of local translation in axons has evolved from being primarily focused on developing axons, to the notion that also mature axons can produce proteins. Axonal translation has been implied in several physiological and pathological conditions, and in all cases it shares common molecular actors and pathways as well as regulatory mechanisms. Here, we review the main findings in these fields, and attempt to highlight shared principles.

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1. Introduction

Asymmetric expression of proteins through the subcellular localization and translation of their mRNAs is an evolutionary conserved mechanism found in essentially all cells (Donnelly et al., 2010; Holt and Schuman, 2013; St Johnston, 2005). In the bacterium *Escherichia coli*, some transcripts are differentially localized between the cytoplasm and the inner membrane, according to the destination of their protein products (Nevo-Dinur et al., 2011). In Drosophila embryos, *in situ* hybridization analysis showed that 71% of the more than three thousand analysed transcripts were specifically localized, and their distribution was tightly correlated with

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the expression pattern of their protein products (Lécuyer et al., 2007). In rat hippocampal neurons an astonishing greater than 50% of the total mRNA content is estimated to be localized to neurites (Cajigas et al., 2012).

Neurons are highly specialized cells, able of conveying information over large distances. They typically possess several highly branched dendrites and a single long effector process, the axon. Due to this extremely polarized morphology, neurons are an ideal cell type to study the regulation and functional consequences of localized protein synthesis. In fact, their unique shape and the extreme distances covered by their neurites prompted already at the end of the 19th century the suggestion that axons might be able to synthesize at least parts of their materials in a manner independent of their cell bodies (Barker, 1899). Axons can span over distances thousands times the neuronal soma's diameter, and the fastest anterograde velocity reported in living neurons is a little less than 40 cm in a day (Kaether et al., 2000), with most cytoskeletal and cytosolic proteins moving only a few millimetres per day (Campenot and Eng, 2000). As a consequence, it can take days for somatically derived proteins to reach their targets in distal axons, raising the issue of protein stability during these long transport times. Slowly transported proteins are indeed degraded over time within mouse retinal ganglion axons (Nixon, 1980). Based on these considerations, the hypothesis was formulated that axonal protein synthesis was needed to overcome the slow transport rates of proteins from the neuronal cell bodies (Alvarez, 1992). Intensive research efforts aimed at understanding the functional requirements of individual axonally localized mRNAs, and more recently transcriptome analyses have changed this view. Instead of being merely an adaptation to slow axonal transport (Alvarez et al., 2000), local protein synthesis is now understood as a mechanism for the spatial-temporal regulation of gene expression, allowing neurons to react acutely to changes in their environment, to compartmentalize signalling events, and to communicate over long distances from the periphery to the cell body. Here we review the functional importance of local translation in developing axons and discuss the rapidly emerging evidence for the requirement of axonal translation beyond the developmental period.

2. Local translation in dendrites

Even though intuitively the longer length of axons means that they may benefit most from a local source of proteins, the existence of local translation in dendrites has been far less controversial, likely due to the ease which with it is detected. It has been known for more than 50 years that disrupting mRNA transcription or translation impedes the long-term storage of memory (Agranoff et al., 1967; Flexner et al., 1963). In the same decade, Bodian (1965) identified ribosome particles in proximal dendrites in monkey spinal cord motorneurons and speculated about a possible functional significance for local protein synthesis in synaptic function. Roughly twenty years later, using electron microscopy, polyribosomes were identified under the base of dendritic spines (Steward and Levy, 1982), and their number and localization showed changes following lesion and during synaptogenesis (Steward, 1983; Steward and Falk, 1986). Metabolic labelling studies in both synaptosomal fractions and compartmentalized cultures soon established that proteins could actually be produced outside of the neuronal soma (Rao and Steward, 1991; Torre and Steward, 1992; Weiler and Greenough, 1991). Initially, the number of proposed dendritically localized mRNAs was no more than a few dozens. New interest in the matter soon revealed an unexpectedly greater number of localized RNAs (Miyashiro et al., 1994), and large-scale microarray studies identified hundreds of transcripts (Poon et al., 2006; Zhong et al., 2006). More recently, RNA-sequencing analysis uncovered 2550 mRNAs localized to neurites of rat hippocampal neurons (Cajigas et al., 2012).

A first functional role for dendritic protein synthesis was revealed when it was found to be involved in a neurotrophin-dependent form of long-lasting synaptic enhancement in the hippocampus: neuropil isolated from its cell bodies could undergo brain-derived neurotrophic factor (BDNF)-induced long term plasticity, and, more importantly, this process was sensitive to inhibitors of protein synthesis (Kang and Schuman, 1996). Dendritic protein synthesis is now considered a requisite process in synaptic plasticity (Sutton and Schuman, 2005), especially for the late phase of long-term potentiation (LTP). LTP reflects a persistent strengthening of synaptic connections and is regarded as the main molecular mechanism underlying memory consolidation, or the conversion of short-term and working memory into long-term memory (Kandel, 2001; McGaugh, 2000). Accordingly, the dysregulation of dendritic protein synthesis has been implied in several neurological disorders (Swanger and Bassell, 2013).

3. Local translation in developing axons

3.1. Early evidence and controversy

Unlike dendritic translation, the existence of a localized system of protein synthesis in axons and nerve terminals was not easily accepted. The first experiments that argued against the exclusive somatic origin of axonal proteins were conducted on vertebrate neurons. If the soma was the only source of an axonal protein such as acetylcholinesterase (AChE), after an irreversible inactivation of AChE, its reappearance in axons should follow a proximal to distal gradient. However, in cat cholinergic neurons both proximal and distal axonal segments displayed an homogeneous recovery rate (Koenig and Koelle, 1960). Also, metabolic labelling studies showed incorporation of [^{3}H]leucine into proteins of desomatized rabbit axons (Koenig, 1967). But after initial enthusiasm and several studies conducted on synaptosomal fractions, the idea lost widespread support mainly because, differently from dendrites, only in very few studies were ribosomes identified in axons by electron microscopy (Peters et al., 1970; Zelená, 1972). Thus based mainly on the perceived absence of clearly identified ribosomes in electron micrographs of mature axons the source of newly synthesized proteins found in synaptosomal fractions was assumed to be exclusively glial, post-synaptic, free mitochondria and membranous contaminants (reviewed in Alvarez et al., 2000). On the contrary, ribosomes were easily identified in growth cones of developing dorsal root ganglion neurons (Tennyson, 1970; Yamada et al., 1971) and in the neurite endings of sympathetic neurons (Bunge, 1973). Brain synaptosomes prepared from young rats had a higher proportion of polyadenylated RNAs and seemed to show higher protein synthetic ability (DeLarco et al., 1975). Further evidence showed isolated axonal growth cones could incorporate radiolabelled amino acids (Davis et al., 1992). Polyribosomes were later identified in the growth cones of developing hippocampal axons by ultrastructural analysis (Deitch and Banker, 1993), even though at a much lower density. Indeed, this paucity of polyribosomes was pointed as one of the earliest and most reliable feature that distinguishing a developing axon from other minor processes (Deitch and Banker, 1993).

Evidence for axonal protein synthesis also started to accumulate from invertebrate models. The squid giant axon was found to possess ribosomal RNAs (Giuditta et al., 1980), mRNAs (Giuditta et al., 1986), tRNAs (Ingoglia et al., 1983), and actively translating polysomes (Giuditta et al., 1991). More compelling still, squid axons separated from their soma could incorporate radioactive amino acids into newly synthesized proteins (Giuditta et al., 1968). Similar

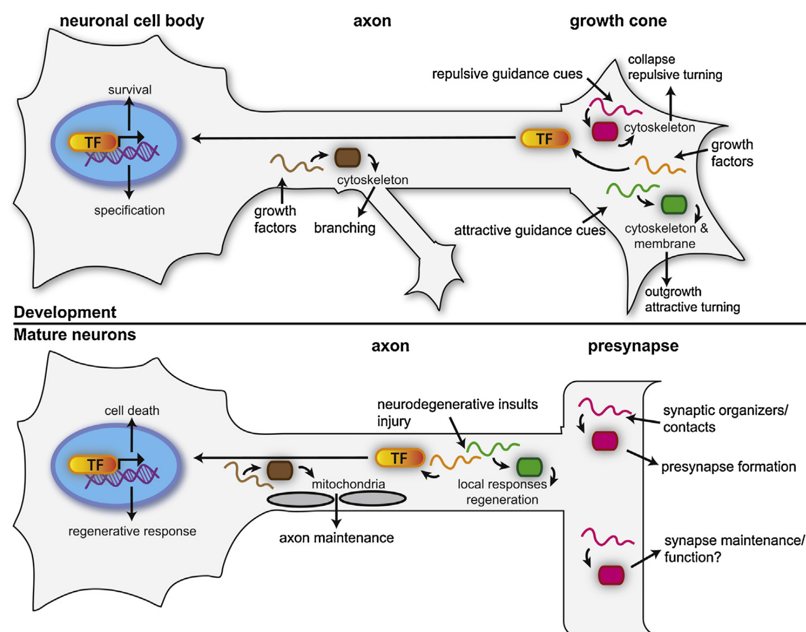

Fig. 1. Roles of intra-axonal protein synthesis in developing and mature axons. In developing axons, local protein synthesis of cytoskeletal regulators supports: axons branching in response to growth factors (brown), axonal elongation and attractive turning triggered by attractive guidance cues (green), and growth cone collapse and axonal retraction induced by repulsive guidance cues (magenta). Local growth factor signalling triggers intra-axonal synthesis of transcription factors that are required for cell survival or specification (yellow). In mature neurons, local protein synthesis supports axon maintenance through the local synthesis of mitochondria-associated proteins (brown). Neurodegenerative insults and nerve injury activate local protein synthesis of transcription factors that can cause cell death or initiate a regenerative response, respectively (green, yellow). Local synthesis of in presynaptic terminals is involved in synapse formation but its role in synapse function or maintenance remains unknown (magenta). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

experiments detected protein synthesis in isolated large myelinated Mauthner goldfish axons (Edström and Sjöstrand, 1969), and in cultured mollusc axons separated from their soma (Van Minnen et al., 1997). Electron spectroscopy imaging together with immunohistological studies of axoplasm whole-mounts showed discrete, intermittent 'plaque-like' ribosome-containing structures closely associated with the plasma membrane in the Mauthner axons (Koenig and Martin, 1996). These same periaxoplasmic ribosomal plaques (PARPs) were also seen on mammalian peripheral nervous system (PNS) axons (Koenig et al., 2000; Kun et al., 2007). In fact, a very early report of few 'ribosome-like' particles close to the periphery of myelinated mammalian axons is in agreement with these later descriptions (Zelená, 1970). Individual RNAs were studied and *in situ* hybridization confirmed the presence of β-*actin* and β-*tubulin* mRNAs in squid giant axons (Kaplan et al., 1992), and of β-*actin* mRNA in the Mauthner axon, associated with the newly identified PARPs (Sotelo-Silveira et al., 2008). Several later studies additionally demonstrated the presence of ribosomal proteins and translation factors in vertebrate axonal growth cones (Bassell et al., 1998; Campbell and Holt, 2001). Taken together, these studies challenged the widely held view at the time that only dendrites but not axons were able to synthesize proteins locally, but it remained unclear to which extent local translation was required for axons.

3.2. Local translation in axonal growth and guidance

Elongating neurites terminate in specialized structures, the growth cones, responsible for directional outgrowth into the synaptic target. Growth cones are highly motile and react to extracellular cues in a strictly regulated temporally and spatially-specific way. Both microtubule dynamics and actin filament remodelling are involved in correct growth cone navigation (Dent et al., 2011), and cytoskeletal proteins were the first likely candidates for local synthesis. *In situ* hybridization analysis showed that β-*actin* mRNA localized to neurites and growth cones of cultured cortical neurons (Bassell et al., 1998), and both actin and tubulin were found to be synthesized in vertebrate growth cones (Eng et al., 1999). Nevertheless, inhibiting local synthesis did not inhibit axonal elongation (Eng et al., 1999), and its functional significance remained elusive. A major breakthrough was the discovery of the role of local translation in a growth cone's response to guidance cues. Cultured *Xenopus laevis* retinal growth cones severed from their cell body continue to grow and turn towards a netrin-1 gradient and away from semaphorin 3A gradient. Blocking local protein synthesis prevented these chemotropic responses (Campbell and Holt, 2001). In a series of studies motivated by this finding, individual mRNAs have been identified whose local translation is required for growth cone responses to extracellular guidance cues (Fig. 1). The attractive guidance cues netrin-1 and nerve growth factor (NGF) trigger local synthesis of a variety of proteins including β-actin (Leung et al., 2006; Yao et al., 2006), Par3 (Hengst et al., 2009), and TC10 (Gracias et al., 2014). On the other hand, the repulsive guidance cues Slit2 and Sema3A induce the translation of actin destabilizing proteins, such as RhoA (Piper et al., 2006; Wu et al., 2005) and cofilin (Piper et al., 2006). A growing body of evidence connects local axonal translation with directional and stimulated growth during development, and disruption of some pathways can lead to axonal morphological changes. β-catenin is synthesized in developing hippocampal growth cones following neurotrophin-3 (NT-3) stimulation and its translation is regulated by the cytoplasmic polyadenylation element-binding protein, whose inhibition affects axonal growth and branching (Kundel

et al., 2009). Axonal branching in response to NGF in chicken embryonic sensory neurons is also dependent on intra-axonal synthesis of the actin nucleating Arp2/3 complex activator WAVE1 and the complex stabilizer cortactin (Spillane et al., 2012). In cultured rat DRG neurons, increasing axonal synthesis of β-actin protein while decreasing growth associated protein 43 (GAP-43) results in short, highly branched axons, while increasing GAP-43 axonal expression over β-actin increases the length and reduces branching of axons (Donnelly et al., 2013a). NGF-induced axon elongation is mediated by local synthesis of microtubule associated protein 1B and calmodulin (Wang et al., 2015). Axonal pathfinding often involves more complex environmental interactions and more demanding responses. While migrating axonal growth cones can be attracted to intermediate targets, the classical example being the central nervous system (CNS) midline, but upon reaching their intermediate targets, they need to change their responsiveness to the guidance cues, in order to progress. Besides losing responsiveness to the intermediate attractant, they need to gain responsiveness to a new set of cues, and axonal protein synthesis was suggested as a mechanism for the latter (Brittis et al., 2002). Axon growth is guided by extracellular gradients of guidance cues, and growth cones need to adapt successively to different concentrations, in order to respond to further increases or decreases in the signal. Indeed, cultured *Xenopus* spinal neurons show desensitization and resensitization in response to netrin-1 and brain-derived neurotrophic factor, and resensitization required local protein synthesis (Ming et al., 2002). It is worth noting that not all growth cone or axonal responses to extracellular factors are the result of local translation; for example L-α-lysophosphatidic acid-induced growth cone collapse is not abolished by protein synthesis inhibitors (Campbell and Holt, 2001). In another study, chick retinal, sympathetic and DRG neurons, and mouse DRG neurons, attractive responses to NGF and NT-3, as well as collapsing ephrin-A2, slit3 and sema3A effects were not dependent on local translation (Roche et al., 2009). Axonal protein synthesis was elicited, but its inhibition failed to affect growth cone responses for 24–48 h. Factors such as a higher metabolic activity or differences in developmental stages might explain these discrepancies (Nédelec et al., 2012).

The stimulus-dependent nature of these local translation events does appear to be typical for intra-axonal protein synthesis. Severed axons grow normally for hours in the presence of protein synthesis inhibitors (Campbell and Holt, 2001), and basal axonal elongation does not require axonal protein synthesis (Hengst et al., 2009). Further, the vast majority of β-actin protein is transported anterogradely from the cell body (Eng et al., 1999), and yet disrupting axonal β-*actin* mRNA interaction with zipcode binding protein 1 (ZBP1) (Yao et al., 2006) or genetically deleting ZBP1 (Welshhans and Bassell, 2011) interferes with directional growth. ZBP1 is a RBP that is required for the trafficking and localized translation of β-*actin* mRNA in axons (Hüttelmaier et al., 2005). Thus, β-*actin* mRNA translation at the growth cone is clearly not merely a means to overcome long distances from the cell body and slow axonal transport, especially as a similar asymmetrical β-actin synthesis is also seen in lamellipodia of fibroblast (Shestakova et al., 2001), and in these cells there is no obvious temporal advantage for *de novo* synthesis of β-actin.

Instead, local translation affords axons advantages that are specific to the protein products. For example, in the case of attractive guidance cues, both Par3 and TC10 are locally synthesized. Par3 is the major component of the PAR polarity complex regulating cytoskeletal dynamics while TC10 is a member of the exocyst complex, that is essential for the addition of new membrane to the plasma membrane in growing axons (Dupraz et al., 2009). The temporally and spatially coincident local synthesis of Par3 and TC10 downstream of attractive guidance cues is thus a mechanism to ensure the simultaneous and spatially restricted activation of these two pathways during stimulated axon outgrowth (Gracias et al., 2014). In this context it is especially interesting that the netrin-1 receptor DCC and the plus-end microtubule tracking protein APC can act as translational hubs (Preitner et al., 2014; Tcherkezian et al., 2010) that at least in the case of APC interact with a defined subset of localized mRNAs. Similarily, stalled axonal mitochondria can also generate hot spots of localized mRNA translation, with proven functional significance in axonal branching (Spillane et al., 2013), and, likely, in other axonal responses, given the high energy demand of translation. These local translation hubs might provide an answer to the question how the local translation of a specific cohort of localized mRNAs is triggered in response to the extracellular stimuli.

3.3. *Local translation in neuron specification and survival*

Besides influencing local morphological responses, neurotrophins acting at distal axonal tips can also signal retrogradely to influence neuronal survival or specification through changes of nuclear gene expression (Zweifel et al., 2005). An increasing body of evidence supports the notion that local translation within axons and growth cones is a crucial mechanism for retrograde communication from the cellular periphery to the nucleus. Local synthesis of retrogradely trafficked signals, especially transcription factors, is triggered by several neurotrophins. In response to axonal treatment with NGF, cAMP-responsive element (CRE)-binding protein (CREB) is locally synthesized and retrogradely transported to the nucleus in dorsal root ganglion (DRG) neurons, and suppression of this process markedly reduced NGF-dependent survival of DRGs in culture (Cox et al., 2008). It remains unclear why a transcription factor would be locally synthesized just to be transported back to the nucleus. A possible answer might be that local synthesis and retrograde transport of transcription factor allow to very tightly control a signalling event. For example, BDNF induces local synthesis of SMAD1/5/8 followed by their retrograde transport in trigeminal neurons. But importantly, these transcription factors were activated only in the cell bodies by retrogradely transported BMP4-induced signalling endosomes leading subtype specification (Ji and Jaffrey, 2012). This mechanism provides a means to ensure subtype specification of trigeminal neurons occurs only if two signals, BDNF and BMP-4 are detected by an axon at the same time.

4. Axonal translation after development

4.1. *Local translation in nerve terminals and synapse formation*

While the requirement of local translation in developing axons has been demonstrated in several instances during the last 15 years and is now widely accepted, its presence and significance during synaptogenesis and in mature axons has remained in comparison much more controversial. As the axon matures, and upon encountering proper connectivity targets, it forms specialized structures, the presynaptic terminals, that contains neurotransmitters inside synaptic vesicles, and upon a nerve impulse, release them near the postsynaptic cell. Each neuron makes thousands of synapses, and the capacity of the neurons to modulate the strength of their synapses in response to their environment is fundamental for several processes already highlighted, such as memory formation and learning (Bliss and Collingridge, 1993).

After the discovery of the enrichment of presynaptic nerve terminals in synaptosome preparations from vertebrate brains, the heterogeneity of these preparations raised doubts about the actual origin of the newly synthesized proteins, and efforts in identifying presynaptic translated proteins in mature vertebrate terminals using synaptosomal preparations were largely abandoned (Crispino et al., 2001). Again, studies in invertebrate systems

provided early evidence for protein synthesis in nerve terminals and pre-synapses. Preparations from squid optic lobes generate large presynaptic nerve terminals with low level of contaminating structures (Crispino et al., 1997). Squid photoreceptor nerve terminals contain active polysomes (Crispino et al., 1997) and polysome-associated mRNAs encoding translation factors, cytoskeletal and ribosomal proteins, and nuclear-encoded mitochondrial proteins (Gioio et al., 2001, 2004). The presynaptic terminals are capable of synthesizing approximately 80 different proteins (Jiménez et al., 2002), and this synthesis appears to be under the control of cell signalling pathways, as it is strongly modulated by cytosolic calcium (Crispino et al., 1993). In the giant marine snail *Aplysia*, a touch to the siphon elicits a reflexive gill withdrawal. This reflex shows different forms of learning, such as habituation, dishabituation, sensitization, and classical conditioning (Carew et al., 1971, 1981; Pinsker et al., 1970). It involves sensory neurons that make synapses with motor neurons and plasticity in these synapses accounts for the learning responses (Purves et al., 2012). In a culture system where a bifurcated sensory axon makes synapses with two different motor neurons, administration of serotonin specifically only to one synapse induces long term facilitation (LTF) at that synapse, but not on the other. The nerve terminal of the stimulated synapse shows enhanced protein synthesis when compared with the unstimulated terminal and inhibiting local protein synthesis abolished LTF (Casadio et al., 1999; Martin et al., 1997). LTF occurs in terminals separated from their cell bodies (Schacher and Wu, 2002), but transcriptional events are necessary for its sustained stabilization (Liu et al., 2003), suggesting that a retrograde signal might travel to the nucleus (Guan and Clark, 2006). The sensory cell-specific neurotransmitter sensorin is locally produced and both the mRNA and the peptide accumulate in nerve terminals after LTF. Interference with its local production does not decrease the protein levels at the presynapse, but it prevents synapse formation (Lyles et al., 2006). It is produced in response to interaction with specific post synaptic targets and as the synapse matures, its levels decline, but it remains necessary for maintenance of synapse efficacy (Hu et al., 2004). These experiments in *Aplysia* established a role for local translation in synaptic plasticity, and its ability to modify synapses with great spatial specificity. They also hinted at a complex interaction between nerve terminals and cell body, requiring general transcriptional changes while maintaining branch-specific regulation, and they once again showed that local protein synthesis is far more than a way to maintain auto sufficiency, as presynthesized sensorin protein could not fulfil the same role as the locally translated one.

There are however fundamental differences between invertebrate and vertebrate neurons that complicate the comparison of these findings with the situation in vertebrate neurons. In invertebrate models, LTF is largely dependent on presynaptic protein synthesis. The regulation of the most intensively studied protein synthesis-dependent form of synaptic plasticity in mammalian neurons, late phase long-term potentiation (L-LTP), on the other hand, is largely postsynaptic (Colbran, 2015; Sutton and Schuman, 2006). Invertebrate neurons lack the strict polarity of vertebrate neurons, and their axons are both transmissive and receptive surfaces (Martin et al., 2000). LTF induction in *Aplysia* neurons is mediated by serotonin binding to receptors on the presynaptic neuron membrane, thus acting functionally as postsynaptic (Mohr and Richter, 2000).

As *Xenopus* retinal ganglion cell (RGC) growth cones mature in culture, their mRNA population changes, and specific presynaptic protein mRNAs, undetectable in young growth cones, become enriched (Zivraj et al., 2010). Local translation could provide a means for the developing nerve terminal to sense and respond to extrinsic signals, playing an essential role in synaptogenesis, synapse strengthening and elimination, and even relay signals to the cell soma and influence neuronal survival. Indeed, in *X. laevis* neuro-muscular junction neurons, presynaptic protein synthesis is needed for neurotrophin-induced synaptic potentiation of vesicle release (Je et al., 2011; Zhang and Poo, 2002) and studies on rodent neurons appear to confirm these findings. In compartmentalized cultures of mature rat hippocampal neurons, where poly-d-lysine-coated beads were added to induce the formation of functional presynaptic terminals (Lucido et al., 2009), 18S rRNA and β-*catenin* mRNA were detected within these terminals (Taylor et al., 2013). The β-*catenin* mRNA localized preferentially to newly formed terminals, was locally translated during terminal formation, and knockdown of it interfered with in synaptic vesicle release. A recent study proposed a role for axonal protein synthesis in regulating vesicle recycling pool size in developing CNS synapses (Hsiao et al., 2014). Another study suggested that presynaptic local translation, and not postsynaptic, is required for a type of long-term depression in rat striatum (Yin et al., 2006). In the fly visual system, taking advantage of the synchronous conversion of R cells' growth cones into synaptic terminals (Chen et al., 2014), global changes in gene expression during presynaptic differentiation were analyzed by tandem tagged ribosome affinity purification of RNA (Zhang et al., 2016). Presynaptic mRNAs showed lenghtning of their 3′ untranslated regions (UTRs) and enrichment in RBP binding motifs. Several RBPs known to regulate mRNA localization and translation were also dynamically regulated. Post-transcriptional regulation mechanisms could be especially important during this time period, and local translation might play a role.

4.2. Axonal synthesis of secreted and membrane proteins

Connected to the issue of a potential role of local protein synthesis in synapse formation and function is the question which protein are expressed in mature axons. A local pathway for the production of neuropeptides and hormones, or even neurotransmitters, could arguably have special benefits. Indeed, invertebrate examples for the production of secreted proteins or peptides in nerve terminals include the hormone sensorin in *Aplysia* sensory neurons (Hu et al., 2002; Lyles et al., 2006; Schacher et al., 1999), the egg-laying hormone in *Aplysia* peptidergic neurons (Lee et al., 2002), and the caudodorsal hormone in the central nervous system of the mollusc *Lymnae stagnalis* (Dirks et al., 1993). In vertebrates, olfactory marker protein mRNA is found in mature axons of the olfactory receptor neurons in rat (Wensley et al., 1995), and a significant amount of odorant receptors' mRNAs associate with polysomes in nerve terminals of axons of adult mouse bulb (Dubacq et al., 2009). Similarly, posterior hypophysis mammalian nerve terminals possess transcripts encoding vasopressin and oxytocin (Jirikowski et al., 1990; Mohr et al., 1991; Mohr and Richter, 1992; Trembleau et al., 1996), and *vasopressin* mRNA at the presynapse is differentially regulated by dehydration and rehydration (Trembleau et al., 1995). Mature DRG terminals express *κ-opioid receptor* mRNA, are able to translate it, and KCl depolarization can regulate transcript quantities (Bi et al., 2006).

These examples of locally produced secreted or membrane proteins are somewhat puzzling because of the lack of a conventional Golgi apparatus in axons that is normally required for the secretory pathway. Yet, also in developing axons several transcripts encoding membrane and secreted proteins localize to axons including for example Ephrin type-A receptor 2 (EphA2) (Brittis et al., 2002). Despite the absence of a Golgi apparatus, many Golgi and ER markers are present in axons and a yet poorly understood secretory mechanism appears to be available for locally produced proteins (González et al., 2016; Merianda et al., 2009).

4.3. Local protein synthesis and axonal maintenance

Local translation also mediates axon maintenance in both developing and mature axons. In cultured sympathetic neuron axons, myo-inositol monophosphatase 1 (Impa-1) is locally produced in response to NGF stimulation and inhibiting its synthesis resulted in axonal degeneration (Andreassi et al., 2010). This study, along with other axonal transcriptome analysis, identified several mitochondrial proteins (Andreassi et al., 2010; Gumy et al., 2011; Taylor et al., 2009; Zivraj et al., 2010). Nuclear-encoded mitochondrial proteins mRNAs are present in the nerve terminals of squid photoreceptor cells (Gioio et al., 2001), as well as in the axons of sympathetic vertebrate neurons (Aschrafi et al., 2010). Inhibition of axonal translation or axonal mitochondrial import of proteins decreases mitochondrial membrane potential in cultured sympathetic neurons (Hillefors et al., 2007). It also impairs the mitochondria ability to restore ATP levels in these neurons after stimulated depolarization. All this implies a function for local translation in maintaining mitochondrial function and axon viability. This idea has been validated *in vivo* by the finding that lamin B2, a protein commonly thought to be exclusively found at the nuclear membrane, is synthesized in *Xenopus* RGC axons in response to engrailed 1 (Yoon et al., 2012). Axonally derived lamin B2 associates with mitochondria and is necessary for mitochondrial maintenance and axon survival.

4.4. Mature axons and injury response

After the developmental period the quantity of rRNA and mRNA in axons decreases (Bassell et al., 1994; Hengst and Jaffrey, 2007; Kleiman et al., 1994). This decreased protein synthetic capacity has been associated with decreased ability to regrow after axotomy (Gumy et al., 2010). Central nervous system neurons show decreased regenerative potential as compared with PNS axons and this is related to their intrinsic protein synthetic ability (Verma et al., 2005). Indeed, preconditioning an axon *in vivo* with nerve injury, leads to enhanced protein synthesis and regeneration *in vitro* (Verma et al., 2005; Zheng et al., 2001). The signalling cascade induced by nerve injury has been extensively studied in peripheral axons and some of the locally synthesized proteins and signalling cascades have been uncovered (Gumy et al., 2010; Rishal and Fainzilber, 2010). An initial calcium wave triggers a first round of translation of several sensor mRNAs, forming a signalling complex that is retrogradely trafficked and influences transcription (Rishal and Fainzilber, 2014). Importin β, whose classical function is to facilitate nuclear import of nuclear localization signal (NLS)-bearing proteins, is locally translated in the injury site and a core component of the injury signalling complex. Its upregulation leads to the formation of a NLS binding complex that associates with the motor protein dynein and travels retrogradely to the cell body (Hanz et al., 2003). Deleting axonal localization sequence in the importin β1 transcript in mice causes a subcellular loss of both importin β1 mRNA and protein in axons, affects the transcriptional response, and delays functional recovery to nerve injury (Perry et al., 2012), confirming its significance *in vivo*. Once again the local synthesis of transcription factors, such as signal transducer and activator of transcription 3 (STAT 3), is a necessary requirement for the retrograde communication with from the periphery to the cell body, in this case leading to the mounting of an injury response (Ben-Yaakov et al., 2012). Boosting protein synthesis might restore the regenerative potential of CNS axons (Park et al., 2008), and importantly, mRNAs and protein synthetic machinery can localize to regenerating mature CNS axons when they are provided with a growth supporting substrate (Kalinski et al., 2015).

Tissue injury induces the release of pro-inflammatory mediators that are critical for the initiation of the repair and regeneration mechanisms, but also induce nociceptive sensitization, a mechanism which might seem maladaptive, but is crucial for organism survival (Price and Dussor, 2014). Inflammatory mediators such as interleukin-6 and NGF are released in response to tissue or nerve injury and sensitize nociceptors (Dina et al., 2008; Pezet and McMahon, 2006), by regulating local protein synthesis (Melemedjian et al., 2010). Increases in the sensory neurons' translational capacity are associated with persistent pain, and several mechanisms involved are similar to those seen in other axonal stimulated conditions, such as mTOR activation (Jiménez-Díaz et al., 2008), and local CREB synthesis and retrograde trafficking (Melemedjian et al., 2014). This nociceptive plasticity affects not only the peripheral pain sensing neurons, but also the central neurons that receive their projections, and this spinal cord central sensitization is also dependent upon local translation (Asante et al., 2009). Molecular mechanisms underlying nociceptive sensitization show remarkable resemblance with those involved in learning and memory processes, and chronic pain disorders are nervous system disorders, differing from acute pain syndromes, with treatment implications (Price and Inyang, 2015). Interfering with local axonal translation is a promising treatment option (Mao-Ying et al., 2014; Price et al., 2007; Russe et al., 2013), but we are far from understanding the complexity of the pain pathway regulation and the identity of the locally relevant transcripts (Melemedjian and Khoutorsky, 2015).

4.5. Role in neurodevelopmental disorders and neurodegeneration

Several neurodevelopmental disorders such as fragile X mental retardation and autism spectrum disorders seem to have underlying local translation deficits (Bear et al., 2008; Kelleher and Bear, 2008). Fragile X mental retardation protein (FMRP) is a well documented plasticity regulator in dendritic spines (Bassell and Warren, 2008). It is found in growth cones and axons (Antar et al., 2006; Christie et al., 2009; Li et al., 2009), where it regulates the presynaptic proteome (Akins et al., 2012), and its loss affects the formation of presynaptic nerve terminals in organotypic mouse slices (Hanson and Madison, 2007).

It is also not at all surprising that changes in local translation have been proposed to play a role in several neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Liu-Yesucevitz et al., 2011). Two related RBPs have been associated with ALS, FUS and TDP-43, which impair axonal trafficking of mRNA granules (Alami et al., 2014). SMA is caused by reduced level of survival motor neuron (SMN) protein. SMN is present in all cell types, and its total deletion is lethal, but somehow motor neurons are more sensitive to its reduction (Fallini et al., 2012). This might be because SMN reduction causes the reduction in the axonal localization of several mRNAs (Rage et al., 2013), and inhibits mTOR activity in axons (Kye et al., 2014).

Perhaps more unexpected was the recent discovery for a role in axonal protein synthesis in response to β-amyloid (Aβ) stimulation, indicating a functional role for local translation in the pathogenesis Alzheimer's disease (AD) (Baleriola et al., 2014). In compartmentalized hippocampal cultures, application of oligomeric $A\beta_{1\text{-}42}$ specifically to axons elicited the recruitment and axonal translation of many mRNAs, including the transcript for the transcription factor activating transcription factor 4 (ATF4). Locally synthesized ATF4 is retrogradely transported to the cell soma, where it changes nuclear transcription, ultimately leading to cell death. Once more, a locally translated transcription factor mediates axon-soma communication. AD follows a stereotypical pattern of neuronal dysfunction (Braak and Braak, 1991), and several studies hypothesize that there might be transsynaptic spread of the pathology in neural circuits (Harris et al., 2010; Palop and Mucke, 2010). For example, synaptic changes precede neuronal

dysfunction (Shankar and Walsh, 2009), and Aβ oligomers interfere with presynaptic vesicle release machinery (Abramov et al., 2009; Nimmrich and Ebert, 2009). The discovery that Aβ oligomers change intra-axonal protein synthesis could indicate that part of the presynaptic alterations might be caused by changes in intra-axonal translation.

The translation of *Atf4* mRNA is controlled by the phosphorylation of the elongation initiation factor 2α (eIF2α), which constitutes the pivotal component in the integrated stress response (Donnelly et al., 2013b; Harding et al., 2003). Brain ER stress (De Felice and Lourenco, 2015) and eIF2α kinases have been implicated in AD (Ohno, 2014). Importantly, activation of the integrated stress response is not limited to AD, and it might be a possible therapeutic target not only in AD, but in other neurodegenerative disorders (Baleriola and Hengst, 2015). In fact, the first oral treatment to have an effect in preventing neurodegeneration in a prion mouse model targeted one eIF2α kinase (Moreno et al., 2013).

5. Challenges and future perspectives

Axonal transcriptome studies (Andreassi et al., 2010; Baleriola et al., 2014; Gumy et al., 2011; Minis et al., 2014; Taylor et al., 2009; Zivraj et al., 2010) identified thousands of transcripts, encoding a huge variety of proteins of very different functional classes, in both embryonic and mature, CNS and PNS, uninjured and regenerating axons (Deglincerti and Jaffrey, 2012). Local translation in the axonal compartment is far more complex than previously though and changes dynamically in response to extracellular and developmental factors. It has been implied in several processes from development to degeneration. Contrary to what was previously thought, mature axons contain mRNAs and are able to rapidly activate their translational capacity in response to stimuli or stress. The elaborate neuronal environment, where nerve terminals, postsynaptic targets and glial cells all interact is one that is not accurately reproducible in culture. There is some evidence that glial cells could contribute with the transfer of translational machinery and even RNA to axons and nerve terminals (Giuditta et al., 2008; Twiss and Fainzilber, 2009). Also, many compartmentalized cultures lack postsynaptic targets, so they are inadequate for the study of the nerve terminal. The study of axonal protein synthesis is further hindered by the low levels of mRNAs in axons, when compared with the somatodendritic compartment. New sensitive tools with single-molecule resolution are needed to detect endogenous levels of expression of biomolecules in axons. These will also enable investigation into translation localized in even smaller sites such as organelle surfaces. An interesting question that remains is the role of local translation at the mature nerve terminal in vertebrates, specifically in presynaptic plasticity and the response to pathological and adaptive stimuli. The synapse being such a dynamic structure, and in light of recent evidence pointing for a possible role of local translation at the nerve terminal, it is likely that, similarly to what happens in dendrites, there are translationally regulated mechanisms at the presynapse. One cannot view the nerve terminal as a translational stagnant compartment, especially in stimulated/pathological conditions. Additionally, the vast diversity of neuronal populations in biological systems warrants more research into how different neuron types react to these stimuli. Our knowledge about local translation in mature axons is still limited and even general principles are not fully comprehended. To understand how the composition of the axonal transcriptome is regulated and how specificity both in mRNA recruitment and local translation is established, remain major research challenges.

Conflict of interest

The authors state that the present manuscript presents no conflict of interest.

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References

Abramov, E., Dolev, I., Fogel, H., Ciccotosto, G.D., Ruff, E., Slutsky, I., 2009. Amyloid-beta as a positive endogenous regulator of release probability at hippocampal synapses. Nat. Neurosci. 12, 1567–1576.
Agranoff, B.W., Davis, R.E., Casola, L., Lim, R., 1967. Actinomycin D blocks formation of memory of shock-avoidance in goldfish. Science 158, 1600–1601.
Akins, M.R., Leblanc, H.F., Stackpole, E.E., Chyung, E., Fallon, J.R., 2012. Systematic mapping of fragile X granules in the mouse brain reveals a potential role for presynaptic FMRP in sensorimotor functions. J. Comp. Neurol. 520, 3687–3706.
Alami, N.H., Smith, R.B., Carrasco, M.A., Williams, L.A., Winborn, C.S., Han, S.S., Kiskinis, E., Winborn, B., Freibaum, B.D., Kanagaraj, A., Clare, A.J., Badders, N.M., Bilican, B., Chaum, E., Chandran, S., Shaw, C.E., Eggan, K.C., Maniatis, T., Taylor, J.P., 2014. Axonal transport of TDP-43 mRNA granules is impaired by ALS-causing mutations. Neuron 81, 536–543.
Alvarez, J., Giuditta, A., Koenig, E., 2000. Protein synthesis in axons and terminals: significance for maintenance, plasticity and regulation of phenotype. With a critique of slow transport theory. Prog. Neurobiol. 62, 1–62.
Alvarez, J., 1992. Maintenance of the axoplasm: can neurones accord with the accepted notions? Neurosci. Lett. 144, 1–3.
Andreassi, C., Zimmermann, C., Mitter, R., Fusco, S., De Vita, S., Saiardi, A., Riccio, A., 2010. An NGF-responsive element targets myo-inositol monophosphatase-1 mRNA to sympathetic neuron axons. Nat. Neurosci. 13, 291–301.
Antar, L.N., Li, C., Zhang, H., Carroll, R.C., Bassell, G.J., 2006. Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. Mol. Cell. Neurosci. 32, 37–48.
Asante, C.O., Wallace, V.C., Dickenson, A.H., 2009. Formalin-induced behavioural hypersensitivity and neuronal hyperexcitability are mediated by rapid protein synthesis at the spinal level. Mol. Pain 5, 27.
Aschrafi, A., Natera-Naranjo, O., Gioio, A.E., Kaplan, B.B., 2010. Regulation of axonal trafficking of cytochrome c oxidase IV mRNA. Mol. Cell. Neurosci. 43, 422–430.
Baleriola, J., Hengst, U., 2015. Targeting axonal protein synthesis in neuroregeneration and degeneration. Neurotherapeutics 12, 57–65.
Baleriola, J., Walker, C.A., Jean, Y.Y., Crary, J.F., Troy, C.M., Nagy, P.L., Hengst, U., 2014. Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. Cell 158, 1159–1172.
Barker, L.F., 1899. The Nervous System and its Constituent Neurones—Designed for the Use of Practitioners of Medicine and of Students of Medicine and Psychology. D. Appleton and Company, New York.
Bassell, G.J., Warren, S.T., 2008. Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron 60, 201–214.
Bassell, G.J., Singer, R.H., Kosik, K.S., 1994. Association of poly(A) mRNA with microtubules in cultured neurons. Neuron 12, 571–582.
Bassell, G.J., Zhang, H., Byrd, A.L., Femino, A.M., Singer, R.H., Taneja, K.L., Lifshitz, L.M., Herman, I.M., Kosik, K.S., 1998. Sorting of β-actin mRNA and protein to neurites and growth cones in culture. J. Neurosci. 18, 251–265.
Bear, M.F., Dolen, G., Osterweil, E., Nagarajan, N., 2008. Fragile X: translation in action. Neuropsychopharmacology 33, 84–87.
Ben-Yaakov, K., Dagan, S.Y., Segal-Ruder, Y., Shalem, O., Vuppalanchi, D., Willis, D.E., Yudin, D., Rishal, I., Rother, F., Bader, M., Blesch, A., Pilpel, Y., Twiss, J.L., Fainzilber, M., 2012. Axonal transcription factors signal retrogradely in lesioned peripheral nerve. EMBO J. 31, 1350–1363.
Bi, J., Tsai, N.P., Lin, Y.P., Loh, H.H., Wei, L.N., 2006. Axonal mRNA transport and localized translational regulation of kappa-opioid receptor in primary neurons of dorsal root ganglia. Proc. Natl. Acad. Sci. U. S. A. 103, 19919–19924.
Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39.
Bodian, D., 1965. A suggestive relationship of nerve cell rna with specific synaptic sites. Proc. Natl. Acad. Sci. U. S. A. 53, 418–425.
Braak, H., Braak, E., 1991. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 82, 239–259.
Brittis, P.A., Lu, Q., Flanagan, J.G., 2002. Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. Cell 110, 223–235.
Bunge, M.B., 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. J. Cell Biol. 56, 713–735.

Cajigas, I.J., Tushev, G., Will, T.J., tom Dieck, S., Fuerst, N., Schuman, E.M., 2012. The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. Neuron 74, 453–466.
Campbell, D.S., Holt, C.E., 2001. Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. Neuron 32, 1013–1026.
Campenot, R.B., Eng, H., 2000. Protein synthesis in axons and its possible functions. J. Neurocytol. 29, 793–798.
Carew, T.J., Castellucci, V.F., Kandel, E.R., 1971. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. Int. J. Neurosci. 2, 79–98.
Carew, T.J., Walters, E.T., Kandel, E.R., 1981. Classical conditioning in a simple withdrawal reflex in Aplysia californica. J. Neurosci. 1, 1426–1437.
Casadio, A., Martin, K.C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C.H., Kandel, E.R., 1999. A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell 99, 221–237.
Chen, Y., Akin, O., Nern, A., Tsui, C.Y., Pecot, M.Y., Zipursky, S.L., 2014. Cell-type-specific labeling of synapses in vivo through synaptic tagging with recombination. Neuron 81, 280–293.
Christie, S.B., Akins, M.R., Schwob, J.E., Fallon, J.R., 2009. The FXG: a presynaptic fragile X granule expressed in a subset of developing brain circuits. J. Neurosci. 29, 1514–1524.
Colbran, R.J., 2015. Thematic minireview series: molecular mechanisms of synaptic plasticity. J. Biol. Chem. 290, 28594–28595.
Cox, L.J., Hengst, U., Gurskaya, N.G., Lukyanov, K.A., Jaffrey, S.R., 2008. Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. Nat. Cell Biol. 10, 149–159.
Crispino, M., Castigli, E., Perrone Capano, C., Martin, R., Menichini, E., Kaplan, B.B., Giuditta, A., 1993. Protein synthesis in a synaptosomal fraction from squid brain. Mol. Cell. Neurosci. 4, 366–374.
Crispino, M., Kaplan, B.B., Martin, R., Alvarez, J., Chun, J.T., Benech, J.C., Giuditta, A., 1997. Active polysomes are present in the large presynaptic endings of the synaptosomal fraction from squid brain. J. Neurosci. 17, 7694–7702.
Crispino, M., Capano, C.P., Aiello, A., Iannetti, E., Cupello, A., Giuditta, A., 2001. Messenger RNAs in synaptosomal fractions from rat brain. Brain Res. Mol. Brain Res. 97, 171–176.
Davis, L., Dou, P., DeWit, M., Kater, S.B., 1992. Protein synthesis within neuronal growth cones. J. Neurosci. 12, 4867–4877.
De Felice, F.G., Lourenco, M.V., 2015. Brain metabolic stress and neuroinflammation at the basis of cognitive impairment in Alzheimer's disease. Front. Aging Neurosci. 7, 94.
DeLarco, J., Nakagawa, S., Abramowitz, A., Bromwell, K., Gurogg, G., 1975. Polyadenylic acid-containing RNA from rat brain synaptosomes. J. Neurochem. 25, 131–137.
Deglincerti, A., Jaffrey, S.R., 2012. Insights into the roles of local translation from the axonal transcriptome. Open Biol. 2, 120079.
Deitch, J.S., Banker, G.A., 1993. An electron microscopic analysis of hippocampal neurons developing in culture: early stages in the emergence of polarity. J. Neurosci. 13, 4301–4315.
Dent, E.W., Gupton, S.L., Gertler, F.B., 2011. The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harb. Perspect. Biol. 3.
Dina, O.A., Green, P.G., Levine, J.D., 2008. Role of interleukin-6 in chronic muscle hyperalgesic priming. Neuroscience 152, 521–525.
Dirks, R.W., van Dorp, A.G., van Minnen, J., Fransen, J.A., van der Ploeg, M., Raap, A.K., 1993. Ultrastructural evidence for the axonal localization of caudodorsal cell hormone mRNA in the central nervous system of the mollusc Lymnaea stagnalis. Microsc. Res. Tech. 25, 12–18.
Donnelly, C.J., Fainzilber, M., Twiss, J.L., 2010. Subcellular communication through RNA transport and localized protein synthesis. Traffic 11, 1498–1505.
Donnelly, C.J., Park, M., Spillane, M., Yoo, S., Pacheco, A., Gomes, C., Vuppalanchi, D., McDonald, M., Kim, H.H., Merianda, T.T., Gallo, G., Twiss, J.L., 2013a. Axonally synthesized beta-actin and GAP-43 proteins support distinct modes of axonal growth. J. Neurosci. 33, 3311–3322.
Donnelly, N., Gorman, A.M., Gupta, S., Samali, A., 2013b. The eIF2α kinases: their structures and functions. Cell. Mol. Life Sci. 70, 3493–3511.
Dubacq, C., Jamet, S., Trembleau, A., 2009. Evidence for developmentally regulated local translation of odorant receptor mRNAs in the axons of olfactory sensory neurons. J. Neurosci. 29, 10184–10190.
Dupraz, S., Grassi, D., Bernis, M.E., Sosa, L., Bisbal, M., Gastaldi, L., Jausoro, I., Cáceres, A., Pfenninger, K.H., Quiroga, S., 2009. The TC10-Exo70 complex is essential for membrane expansion and axonal specification in developing neurons. J. Neurosci. 29, 13292–13301.
Edström, A., Sjöstrand, J., 1969. Protein synthesis in the isolated Mauthner nerve fibre of goldfish. J. Neurochem. 16, 67–81.
Eng, H., Lund, K., Campenot, R.B., 1999. Synthesis of β-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. J. Neurosci. 19, 1–9.
Fallini, C., Bassell, G.J., Rossoll, W., 2012. Spinal muscular atrophy: the role of SMN in axonal mRNA regulation. Brain Res. 1462, 81–92.
Flexner, J.B., Flexner, L.B., Stellar, E., 1963. Memory in mice as affected by intracerebral puromycin. Science 141, 57–59.
Gioio, A.E., Eyman, M., Zhang, H., Lavina, Z.S., Giuditta, A., Kaplan, B.B., 2001. Local synthesis of nuclear-encoded mitochondrial proteins in the presynaptic nerve terminal. J. Neurosci. Res. 64, 447–453.
Gioio, A.E., Lavina, Z.S., Jurkovicova, D., Zhang, H., Eyman, M., Giuditta, A., Kaplan, B.B., 2004. Nerve terminals of squid photoreceptor neurons contain a heterogeneous population of mRNAs and translate a transfected reporter mRNA. Eur. J. Neurosci. 20, 865–872.
Giuditta, A., Dettbarn, W.D., Brzin, M., 1968. Protein synthesis in the isolated giant axon of the squid. Proc. Natl. Acad. Sci. U. S. A. 59, 1284–1287.
Giuditta, A., Cupello, A., Lazzarini, G., 1980. Ribosomal RNA in the axoplasm of the squid giant axon. J. Neurochem. 34, 1757–1760.
Giuditta, A., Hunt, T., Santella, L., 1986. Messenger RNA in squid axoplasm. Neurochem. Int. 8, 435–442.
Giuditta, A., Menichini, E., Perrone Capano, C., Langella, M., Martin, R., Castigli, E., Kaplan, B.B., 1991. Active polysomes in the axoplasm of the squid giant axon. J. Neurosci. Res. 28, 18–28.
Giuditta, A., Chun, J.T., Eyman, M., Cefaliello, C., Bruno, A.P., Crispino, M., 2008. Local gene expression in axons and nerve endings: the glia-neuron unit. Physiol. Rev. 88, 515–555.
González, C., Canovas, J., Fresno, J., Couve, E., Court, F.A., Couve, A., 2016. Axons provide the secretory machinery for trafficking of voltage-gated sodium channels in peripheral nerve. Proc. Natl. Acad. Sci. U. S. A. 113, 1823–1828.
Gracias, N.G., Shirkey-Son, N.J., Hengst, U., 2014. Local translation of TC10 is required for membrane expansion during axon outgrowth. Nat. Commun. 5, 3506.
Guan, X., Clark, G.A., 2006. Essential role of somatic and synaptic protein synthesis and axonal transport in long-term synapse-specific facilitation at distal sensorimotor connections in Aplysia. Biol. Bull. 210, 238–254.
Gumy, L.F., Tan, C.L., Fawcett, J.W., 2010. The role of local protein synthesis and degradation in axon regeneration. Exp. Neurol. 223, 28–37.
Gumy, L.F., Yeo, G.S., Tung, Y.C., Zivraj, K.H., Willis, D., Coppola, G., Lam, B.Y., Twiss, J.L., Holt, C.E., Fawcett, J.W., 2011. Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. RNA 17, 85–98.
Hüttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X., Bassell, G.J., Condeelis, J., Singer, R.H., 2005. Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. Nature 438, 512–515.
Hanson, J.E., Madison, D.V., 2007. Presynaptic FMR1 genotype influences the degree of synaptic connectivity in a mosaic mouse model of fragile X syndrome. J. Neurosci. 27, 4014–4018.
Hanz, S., Perlson, E., Willis, D., Zheng, J.Q., Massarwa, R., Huerta, J.J., Koltzenburg, M., Kohler, M., van-Minnen, J., Twiss, J.L., Fainzilber, M., 2003. Axoplasmic importins enable retrograde injury signaling in lesioned nerve. Neuron 40, 1095–1104.
Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D.F., Bell, J.C., Hettmann, T., Leiden, J.M., Ron, D., 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell 11, 619–633.
Harris, J.A., Devidze, N., Verret, L., Ho, K., Halabisky, B., Thwin, M.T., Kim, D., Hamto, P., Lo, I., Yu, G.Q., Palop, J.J., Masliah, E., Mucke, L., 2010. Transsynaptic progression of amyloid-beta-induced neuronal dysfunction within the entorhinal-hippocampal network. Neuron 68, 428–441.
Hengst, U., Jaffrey, S.R., 2007. Function and translational regulation of mRNA in developing axons. Semin. Cell Dev. Biol. 18, 209–215.
Hengst, U., Deglincerti, A., Kim, H.J., Jeon, N.L., Jaffrey, S.R., 2009. Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. Nat. Cell Biol. 11, 1024–1030.
Hillefors, M., Gioio, A.E., Mameza, M.G., Kaplan, B.B., 2007. Axon viability and mitochondrial function are dependent on local protein synthesis in sympathetic neurons. Cell. Mol. Neurobiol. 27, 701–716.
Holt, C.E., Schuman, E.M., 2013. The central dogma decentralized: new perspectives on RNA function and local translation in neurons. Neuron 80, 648–657.
Hsiao, K., Bozdagi, O., Benson, D.L., 2014. Axonal cap-dependent translation regulates presynaptic p35. Dev. Neurobiol. 74, 351–364.
Hu, J.Y., Meng, X., Schacher, S., 2002. Target interaction regulates distribution and stability of specific mRNAs. J. Neurosci. 22, 2669–2678.
Hu, J.Y., Goldman, J., Wu, F., Schacher, S., 2004. Target-dependent release of a presynaptic neuropeptide regulates the formation and maturation of specific synapses in Aplysia. J. Neurosci. 24, 9933–9943.
Ingoglia, N.A., Giuditta, A., Zanakis, M.F., Babigian, A., Tasaki, I., Chakraborty, G., Sturman, J.A., 1983. Incorporation of 3H-amino acids into proteins in a partially purified fraction of axoplasm: evidence for transfer RNA-mediated, post-translational protein modification in squid giant axons. J. Neurosci. 3, 2463–2473.
Je, H.S., Ji, Y., Wang, Y., Yang, F., Wu, W., Lu, B., 2011. Presynaptic protein synthesis required for NT-3-induced long-term synaptic modulation. Mol. Brain 4, 1.
Ji, S.J., Jaffrey, S.R., 2012. Intra-axonal translation of SMAD1/5/8 mediates retrograde regulation of trigeminal ganglia subtype specification. Neuron 74, 95–107.
Jiménez, C.R., Eyman, M., Lavina, Z.S., Gioio, A., Li, K.W., van der Schors, R.C., Geraerts, W.P., Giuditta, A., Kaplan, B.B., van Minnen, J., 2002. Protein synthesis in synaptosomes: a proteomics analysis. J. Neurochem. 81, 735–744.
Jiménez-Díaz, L., Geranton, S.M., Passmore, G.M., Leith, J.L., Fisher, A.S., Berliocchi, L., Sivasubramaniam, A.K., Sheasby, A., Lumb, B.M., Hunt, S.P., 2008. Local translation in primary afferent fibers regulates nociception. PLoS One 3, e1961.
Jirikowski, G.F., Sanna, P.P., Bloom, F.E., 1990. mRNA coding for oxytocin is present in axons of the hypothalamo-neurohypophysial tract. Proc. Natl. Acad. Sci. U. S. A. 87, 7400–7404.
Kaether, C., Skehel, P., Dotti, C.G., 2000. Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons. Mol. Biol. Cell 11, 1213–1224.

Kalinski, A.L., Sachdeva, R., Gomes, C., Lee, S.J., Shah, Z., Houle, J.D., Twiss, J.L., 2015. mRNAs and protein synthetic machinery localize into regenerating spinal cord axons when they are provided a substrate that supports growth. J. Neurosci. 35, 10357–10370.
Kandel, E.R., 2001. The molecular biology of memory storage: a dialogue between genes and synapses. Science 294, 1030–1038.
Kang, H., Schuman, E.M., 1996. A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 273, 1402–1406.
Kaplan, B.B., Gioio, A.E., Capano, C.P., Crispino, M., Giuditta, A., 1992. beta-Actin and beta-Tubulin are components of a heterogeneous mRNA population present in the squid giant axon. Mol. Cell. Neurosci. 3, 133–144.
Kelleher 3rd, R.J., Bear, M.F., 2008. The autistic neuron: troubled translation? Cell 135, 401–406.
Kleiman, R., Banker, G., Steward, O., 1994. Development of subcellular mRNA compartmentation in hippocampal neurons in culture. J. Neurosci. 14, 1130–1140.
Koenig, E., Koelle, G.B., 1960. Acetylcholinesterase regeneration in peripheral nerve after irreversible inactivation. Science 132, 1249–1250.
Koenig, E., Martin, R., 1996. Cortical plaque-like structures identify ribosome-containing domains in the Mauthner cell axon. J. Neurosci. 16, 1400–1411.
Koenig, E., Martin, R., Titmus, M., Sotelo-Silveira, J.R., 2000. Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons. J. Neurosci. 20, 8390–8400.
Koenig, E., 1967. Synthetic mechanisms in the axon. IV. In vitro incorporation of [3H]precursors into axonal protein and RNA. J. Neurochem. 14, 437–446.
Kun, A., Otero, L., Sotelo-Silveira, J.R., Sotelo, J.R., 2007. Ribosomal distributions in axons of mammalian myelinated fibers. J. Neurosci. Res. 85, 2087–2098.
Kundel, M., Jones, K.J., Shin, C.Y., Wells, D.G., 2009. Cytoplasmic polyadenylation element-binding protein regulates neurotrophin-3-dependent beta-catenin mRNA translation in developing hippocampal neurons. J. Neurosci. 29, 13630–13639.
Kye, M.J., Niederst, E.D., Wertz, M.H., Goncalves Ido, C., Akten, B., Dover, K.Z., Peters, M., Riessland, M., Neveu, P., Wirth, B., Kosik, K.S., Sardi, S.P., Monani, U.R., Passini, M.A., Sahin, M., 2014. SMN regulates axonal local translation via miR-183/mTOR pathway. Hum. Mol. Genet. 23, 6318–6331.
Lécuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T.R., Tomancak, P., Krause, H.M., 2007. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell 131, 174–187.
Lee, W., Jones, A.M., Ono, J.K., Wayne, N.L., 2002. Regional differences in processing of locally translated prohormone in peptidergic neurons of Aplysia californica. J. Neurochem. 83, 1423–1430.
Leung, K.M., van Horck, F.P., Lin, A.C., Allison, R., Standart, N., Holt, C.E., 2006. Asymmetrical β-actin mRNA translation in growth cones mediates attractive turning to netrin-1. Nat. Neurosci. 9, 1247–1256.
Li, C., Bassell, G.J., Sasaki, Y., 2009. Fragile x mental retardation protein is involved in protein synthesis-Dependent collapse of growth cones induced by semaphorin-3A. Front Neural Circuit. 3, 11.
Liu, K., Hu, J.Y., Wang, D., Schacher, S., 2003. Protein synthesis at synapse versus cell body: enhanced but transient expression of long-term facilitation at isolated synapses. J. Neurobiol. 56, 275–286.
Liu-Yesucevitz, L., Bassell, G.J., Gitler, A.D., Hart, A.C., Klann, E., Richter, J.D., Warren, S.T., Wolozin, B., 2011. Local RNA translation at the synapse and in disease. J. Neurosci. 31, 16086–16093.
Lucido, A.L., Suarez Sanchez, F., Thostrup, P., Kwiatkowski, A.V., Leal-Ortiz, S., Gopalakrishnan, G., Liazoghli, D., Belkaid, W., Lennox, R.B., Grutter, P., Garner, C.C., Colman, D.R., 2009. Rapid assembly of functional presynaptic boutons triggered by adhesive contacts. J. Neurosci. 29, 12449–12466.
Lyles, V., Zhao, Y., Martin, K.C., 2006. Synapse formation and mRNA localization in cultured Aplysia neurons. Neuron 49, 349–356.
Mao-Ying, Q.L., Kavelaars, A., Krukowski, K., Huo, X.J., Zhou, W., Price, T.J., Cleeland, C., Heijnen, C.J., 2014. The anti-diabetic drug metformin protects against chemotherapy-induced peripheral neuropathy in a mouse model. PLoS One 9, e100701.
Martin, K.C., Casadio, A., Zhu, H., Yaping, E., Rose, J.C., Chen, M., Bailey, C.H., Kandel, E.R., 1997. Synapse-specific, long-term facilitation of *aplysia* sensory to motor synapses: a function for local protein synthesis in memory storage. Cell 91, 927–938.
Martin, K.C., Barad, M., Kandel, E.R., 2000. Local protein synthesis and its role in synapse-specific plasticity. Curr. Opin. Neurobiol. 10, 587–592.
McGaugh, J.L., 2000. Memory–a century of consolidation. Science 287, 248–251.
Melemedjian, O.K., Khoutorsky, A., 2015. Translational control of chronic pain. Prog. Mol. Biol. Transl. Sci. 131, 185–213.
Melemedjian, O.K., Asiedu, M.N., Tillu, D.V., Peebles, K.A., Yan, J., Ertz, N., Dussor, G.O., Price, T.J., 2010. IL-6- and NGF-induced rapid control of protein synthesis and nociceptive plasticity via convergent signaling to the eIF4F complex. J. Neurosci. 30, 15113–15123.
Melemedjian, O.K., Tillu, D.V., Moy, J.K., Asiedu, M.N., Mandell, E.K., Ghosh, S., Dussor, G., Price, T.J., 2014. Local translation and retrograde axonal transport of CREB regulates IL-6-induced nociceptive plasticity. Mol Pai. 10, 45.
Merianda, T.T., Lin, A.C., Lam, J.S., Vuppalanchi, D., Willis, D.E., Karin, N., Holt, C.E., Twiss, J.L., 2009. A functional equivalent of endoplasmic reticulum and Golgi in axons for secretion of locally synthesized proteins. Mol. Cell. Neurosci. 40, 128–142.
Ming, G.L., Wong, S.T., Henley, J., Yuan, X.B., Song, H.J., Spitzer, N.C., Poo, M.M., 2002. Adaptation in the chemotactic guidance of nerve growth cones. Nature 417, 411–418.
Minis, A., Dahary, D., Manor, O., Leshkowitz, D., Pilpel, Y., Yaron, A., 2014. Subcellular transcriptomics-dissection of the mRNA composition in the axonal compartment of sensory neurons. Dev. Neurobiol. 74, 365–381.
Miyashiro, K., Dichter, M., Eberwine, J., 1994. On the nature and differential distribution of mRNAs in hippocampal neurites: implications for neuronal functioning. Proc. Natl. Acad. Sci. U. S. A. 91, 10800–10804.
Mohr, E., Richter, D., 1992. Diversity of mRNAs in the Axonal Compartment of Peptidergic Neurons in the Rat. Eur. J. Neurosci. 4, 870–876.
Mohr, E., Richter, D., 2000. Axonal mRNAs: functional significance in vertebrates and invertebrates. J. Neurocytol. 29, 783–791.
Mohr, E., Fehr, S., Richter, D., 1991. Axonal transport of neuropeptide encoding mRNAs within the hypothalamo-hypophyseal tract of rats. EMBO J. 10, 2419–2424.
Moreno, J.A., Halliday, M., Molloy, C., Radford, H., Verity, N., Axten, J.M., Ortori, C.A., Willis, A.E., Fischer, P.M., Barrett, D.A., Mallucci, G.R., 2013. Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. Sci. Transl. Med. 5, 206ra138.
Nédelec, S., Peljto, M., Shi, P., Amoroso, M.W., Kam, L.C., Wichterle, H., 2012. Concentration-dependent requirement for local protein synthesis in motor neuron subtype-specific response to axon guidance cues. J. Neurosci. 32, 1496–1506.
Nevo-Dinur, K., Nussbaum-Shochat, A., Ben-Yehuda, S., Amster-Choder, O., 2011. Translation-independent localization of mRNA in E coli. Science 331, 1081–1084.
Nimmrich, V., Ebert, U., 2009. Is Alzheimer's disease a result of presynaptic failure? Synaptic dysfunctions induced by oligomeric beta-amyloid. Rev. Neurosci. 20, 1–12.
Nixon, R.A., 1980. Protein degradation in the mouse visual system. I. Degradation of axonally transported and retinal proteins. Brain Res. 200, 69–83.
Ohno, M., 2014. Roles of eIF2α kinases in the pathogenesis of Alzheimer's disease. Front. Mol. Neurosci. 7, 22.
Palop, J.J., Mucke, L., 2010. Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. Nat. Neurosci. 13, 812–818.
Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., He, Z., 2008. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322, 963–966.
Perry, R.B., Doron-Mandel, E., Iavnilovitch, E., Rishal, I., Dagan, S.Y., Tsoory, M., Coppola, G., McDonald, M.K., Gomes, C., Geschwind, D.H., Twiss, J.L., Yaron, A., Fainzilber, M., 2012. Subcellular knockout of importin beta1 perturbs axonal retrograde signaling. Neuron 75, 294–305.
Peters, A., Palay, S.L., Webster, H., 1970. The Fine Structure of the Nervous System, 1st ed. Harper & Row, New York.
Pezet, S., McMahon, S.B., 2006. Neurotrophins: mediators and modulators of pain. Annu. Rev. Neurosci. 29, 507–538.
Pinsker, H., Kupfermann, I., Castellucci, V., Kandel, E., 1970. Habituation and dishabituation of the gill-withdrawal reflex in Aplysia. Science 167, 1740–1742.
Piper, M., Anderson, R., Dwivedy, A., Weinl, C., van Horck, F., Leung, K.M., Cogill, E., Holt, C., 2006. Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones. Neuron 49, 215–228.
Poon, M.M., Choi, S.H., Jamieson, C.A., Geschwind, D.H., Martin, K.C., 2006. Identification of process-localized mRNAs from cultured rodent hippocampal neurons. J. Neurosci. 26, 13390–13399.
Preitner, N., Quan, J., Nowakowski, D.W., Hancock, M.L., Shi, J., Tcherkezian, J., Young-Pearse, T.L., Flanagan, J.G., 2014. APC is an RNA-binding protein, and its interactome provides a link to neural development and microtubule assembly. Cell 158, 368–382.
Price, T.J., Dussor, G., 2014. Evolution: the advantage of 'maladaptive' pain plasticity. Curr. Biol. 24, R384–386.
Price, T.J., Inyang, K.E., 2015. Commonalities between pain and memory mechanisms and their meaning for understanding chronic pain. Prog. Mol. Biol. Transl. Sci. 131, 409–434.
Price, T.J., Rashid, M.H., Millecamps, M., Sanoja, R., Entrena, J.M., Cervero, F., 2007. Decreased nociceptive sensitization in mice lacking the fragile X mental retardation protein: role of mGluR1/5 and mTOR. J. Neurosci. 27, 13958–13967.
Purves, D., Augustine, G.J., Fitzpatrick, D., Hall, W.C., LaMantia, A.S., White, L.E., 2012. Neuroscience, 5th ed. Sinauer Associates, Sunderland, MA.
Rage, F., Boulisfane, N., Rihan, K., Neel, H., Gostan, T., Bertrand, E., Bordonne, R., Soret, J., 2013. Genome-wide identification of mRNAs associated with the protein SMN whose depletion decreases their axonal localization. RNA 19, 1755–1766.
Rao, A., Steward, O., 1991. Evidence that protein constituents of postsynaptic membrane specializations are locally synthesized: analysis of proteins synthesized within synaptosomes. J. Neurosci. 11, 2881–2895.
Rishal, I., Fainzilber, M., 2010. Retrograde signaling in axonal regeneration. Exp. Neurol. 223, 5–10.
Rishal, I., Fainzilber, M., 2014. Axon-soma communication in neuronal injury. Nat. Rev. Neurosci. 15, 32–42.
Roche, F.K., Marsick, B.M., Letourneau, P.C., 2009. Protein synthesis in distal axons is not required for growth cone responses to guidance cues. J. Neurosci. 29, 638–652.

Russe, O.Q., Moser, C.V., Kynast, K.L., King, T.S., Stephan, H., Geisslinger, G., Niederberger, E., 2013. Activation of the AMP-activated protein kinase reduces inflammatory nociception. J. Pain 14, 1330–1340.
Schacher, S., Wu, F., 2002. Synapse formation in the absence of cell bodies requires protein synthesis. J. Neurosci. 22, 1831–1839.
Schacher, S., Wu, F., Panyko, J.D., Sun, Z.Y., Wang, D., 1999. Expression and branch-specific export of mRNA are regulated by synapse formation and interaction with specific postsynaptic targets. J. Neurosci. 19, 6338–6347.
Shankar, G.M., Walsh, D.M., 2009. Alzheimer's disease: synaptic dysfunction and Abeta. Mol. Neurodegener. 4, 48.
Shestakova, E.A., Singer, R.H., Condeelis, J., 2001. The physiological significance of beta –actin mRNA localization in determining cell polarity and directional motility. Proc. Natl. Acad. Sci. U. S. A. 98, 7045–7050.
Sotelo-Silveira, J., Crispino, M., Puppo, A., Sotelo, J.R., Koenig, E., 2008. Myelinated axons contain beta-actin mRNA and ZBP-1 in periaxoplasmic ribosomal plaques and depend on cyclic AMP and F-actin integrity for in vitro translation. J. Neurochem. 104, 545–557.
Spillane, M., Ketschek, A., Donnelly, C.J., Pacheco, A., Twiss, J.L., Gallo, G., 2012. Nerve growth factor-induced formation of axonal filopodia and collateral branches involves the intra-axonal synthesis of regulators of the actin-nucleating Arp2/3 complex. J. Neurosci. 32, 17671–17689.
Spillane, M., Ketschek, A., Merianda, T.T., Twiss, J.L., Gallo, G., 2013. Mitochondria coordinate sites of axon branching through localized intra-axonal protein synthesis. Cell Rep. 5, 1564–1575.
St Johnston, D., 2005. Moving messages: the intracellular localization of mRNAs. Nat. Rev. Mol. Cell Biol. 6, 363–375.
Steward, O., Falk, P.M., 1986. Protein-synthetic machinery at postsynaptic sites during synaptogenesis: a quantitative study of the association between polyribosomes and developing synapses. J. Neurosci. 6, 412–423.
Steward, O., Levy, W.B., 1982. Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J. Neurosci. 2, 284–291.
Steward, O., 1983. Alterations in polyribosomes associated with dendritic spines during the reinnervation of the dentate gyrus of the adult rat. J. Neurosci. 3, 177–188.
Sutton, M.A., Schuman, E.M., 2005. Local translational control in dendrites and its role in long-term synaptic plasticity. J. Neurobiol. 64, 116–131.
Sutton, M.A., Schuman, E.M., 2006. Dendritic protein synthesis, synaptic plasticity, and memory. Cell 127, 49–58.
Swanger, S.A., Bassell, G.J., 2013. Dendritic protein synthesis in the normal and diseased brain. Neuroscience 232, 106–127.
Taylor, A.M., Berchtold, N.C., Perreau, V.M., Tu, C.H., Li Jeon, N., Cotman, C.W., 2009. Axonal mRNA in uninjured and regenerating cortical mammalian axons. J. Neurosci. 29, 4697–4707.
Taylor, A.M., Wu, J., Tai, H.C., Schuman, E.M., 2013. Axonal translation of beta-catenin regulates synaptic vesicle dynamics. J. Neurosci. 33, 5584–5589.
Tcherkezian, J., Brittis, P.A., Thomas, F., Roux, P.P., Flanagan, J.G., 2010. Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation. Cell 141, 632–644.
Tennyson, V.M., 1970. The fine structure of the axon and growth cone of the dorsal root neuroblast of the rabbit embryo. J. Cell Biol. 44, 62–79.
Torre, E.R., Steward, O., 1992. Demonstration of local protein synthesis within dendrites using a new cell culture system that permits the isolation of living axons and dendrites from their cell bodies. J. Neurosci. 12, 762–772.
Trembleau, A., Melia, K.R., Bloom, F.E., 1995. BC1 RNA and vasopressin mRNA in rat neurohypophysis: axonal compartmentalization and differential regulation during dehydration and rehydration. Eur. J. Neurosci. 7, 2249–2260.
Trembleau, A., Morales, M., Bloom, F.E., 1996. Differential compartmentalization of vasopressin messenger RNA and neuropeptide within the rat hypothalamo-neurohypophysial axonal tracts: light and electron microscopic evidence. Neuroscience 70, 113–125.
Twiss, J.L., Fainzilber, M., 2009. Ribosomes in axons–scrounging from the neighbors? Trends Cell Biol. 19, 236–243.
Van Minnen, J., Bergman, J.J., Van Kesteren, E.R., Smit, A.B., Geraerts, W.P., Lukowiak, K., Hasan, S.U., Syed, N.I., 1997. De novo protein synthesis in isolated axons of identified neurons. Neuroscience 80, 1–7.
Verma, P., Chierzi, S., Codd, A.M., Campbell, D.S., Meyer, R.L., Holt, C.E., Fawcett, J.W., 2005. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. J. Neurosci. 25, 331–342.
Wang, B., Pan, L., Wei, M., Wang, Q., Liu, W.W., Wang, N., Jiang, X.Y., Zhang, X., Bao, L., 2015. FMRP-Mediated axonal delivery of miR-181d regulates axon elongation by locally targeting map1b and calm1. Cell Rep. 13, 2794–2807.
Weiler, I.J., Greenough, W.T., 1991. Potassium ion stimulation triggers protein translation in synaptoneurosomal polyribosomes. Mol. Cell. Neurosci. 2, 305–314.
Welshhans, K., Bassell, G.J., 2011. Netrin-1-induced local beta-actin synthesis and growth cone guidance requires zipcode binding protein 1. J. Neurosci. 31, 9800–9813.
Wensley, C.H., Stone, D.M., Baker, H., Kauer, J.S., Margolis, F.L., Chikaraishi, D.M., 1995. Olfactory marker protein mRNA is found in axons of olfactory receptor neurons. J. Neurosci. 15, 4827–4837.
Wu, K.Y., Hengst, U., Cox, L.J., Macosko, E.Z., Jeromin, A., Urquhart, E.R., Jaffrey, S.R., 2005. Local translation of RhoA regulates growth cone collapse. Nature 436, 1020–1024.
Yamada, K.M., Spooner, B.S., Wessells, N.K., 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. J. Cell Biol. 49, 614–635.
Yao, J., Sasaki, Y., Wen, Z., Bassell, G.J., Zheng, J.Q., 2006. An essential role for β-actin mRNA localization and translation in Ca^{2+}-dependent growth cone guidance. Nat. Neurosci. 9, 1265–1273.
Yin, H.H., Davis, M.I., Ronesi, J.A., Lovinger, D.M., 2006. The role of protein synthesis in striatal long-term depression. J. Neurosci. 26, 11811–11820.
Yoon, B.C., Jung, H., Dwivedy, A., O'Hare, C.M., Zivraj, K.H., Holt, C.E., 2012. Local translation of extranuclear lamin B promotes axon maintenance. Cell 148, 752–764.
Zelená, J., 1970. Ribosome-like particles in myelinated axons of the rat. Brain Res. 24, 359–363.
Zelená, J., 1972. Ribosomes in myelinated axons of dorsal root ganglia. Z. Zellforsch. Mikrosk. Anat. 124, 217–229.
Zhang, X., Poo, M.M., 2002. Localized synaptic potentiation by BDNF requires local protein synthesis in the developing axon. Neuron 36, 675–688.
Zhang, K.X., Tan, L., Pellegrini, M., Zipursky, S.L., McEwen, J.M., 2016. Rapid changes in the translatome during the conversion of growth cones to synaptic terminals. Cell Rep. 14, 1258–1271.
Zheng, J.Q., Kelly, T.K., Chang, B., Ryazantsev, S., Rajasekaran, A.K., Martin, K.C., Twiss, J.L., 2001. A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. J. Neurosci. 21, 9291–9303.
Zhong, J., Zhang, T., Bloch, L.M., 2006. Dendritic mRNAs encode diversified functionalities in hippocampal pyramidal neurons. BMC Neurosci. 7, 17.
Zivraj, K.H., Tung, Y.C., Piper, M., Gumy, L., Fawcett, J.W., Yeo, G.S., Holt, C.E., 2010. Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. J. Neurosci. 30, 15464–15478.
Zweifel, L.S., Kuruvilla, R., Ginty, D.D., 2005. Functions and mechanisms of retrograde neurotrophin signalling. Nat. Rev. Neurosci. 6, 615–625.