Local translation in neurons: visualization and function

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Abstract.
Neurons are amongst the most compartmentalized and interactive of all cell types. Like all cells, neurons use proteins as the main sensors and effectors. The modification of the proteome in axons and dendrites is used to guide the formation of synaptic connections and to store information. In this review, we discuss the data indicating that an important source of protein for dendrites, axons and their associated elements arises from the local synthesis of proteins. We review the data demonstrating the presence of the machinery required for protein synthesis, the direct visualization/demonstration of protein synthesis and the established functional roles for local translation for many different neuronal functions. Finally, we consider the open questions and future directions in this field.

The function of a neuron is to receive, process and potentially store information from other neurons, and then transmit an integrated signal to other cells in its network. A typical excitatory neuron receives information from 1-10,000 other neurons and transmits information to 50-100,000 neurons; information transfer occurs at specialized junctions called synapses. This complex morphology requires unique solutions to maintain and modify the proteins that are required for the correct wiring of the nervous system during development and for its function and plasticity during adulthood.

Neurons and numbers.

When compared to other cell types in the body, neurons possess a unique morphology. Each neuron has a cell body from which emanates a single axon and multiple dendrites. While the cell body contains the nucleus, the axons and dendrites of a neuron can comprise up to 99% of the cytoplasm. Axons can be extremely long, extending over hundreds of centimeters in length in vertebrates. Dendrites are highly branched and also quite long; for example, the average dendritic length of hippocampal pyramidal neuron is 13.5 mm[1] (Fig. 1).

The contacts between neurons occur at specialized junctions called synapses. This unique cellular junction usually forms between a specialized ending (axon bouton or axon terminal) of a neuron transmitting the information (the presynaptic neuron) and special compartments within dendrites (often protrusions called “spines”) of a receiving neuron (the postsynaptic neuron). During development, neurons send out long axons that navigate over hundreds or even thousands of cell body lengths to reach their postsynaptic targets. This navigational feat is accomplished by “growth cones”, the specialized tips of growing axons. These growth cones detect and react rapidly to local guidance cues that steers their movement along the route and when they reach their destination, axons branch and form. A single axon that has a length of ~ 200 cm makes about ~50,000 synapses (CA3 region of the hippocampus[2]). Axonal boutons contain synaptic vesicles filled with neurotransmitter as well as scaffolding and regulatory molecules and organelles that organize, promote and regulate the release of neurotransmitter. A typical pyramidal neuron hosts about 54,000 synapses its dendritic tree (~4.0 synapses per μm^2 in the CA1

region of the hippocampus[3]). The synaptic compartments within dendrites, for example the dendritic spines that form excitatory synapses, contain a special complement of proteins that include neurotransmitter receptors, as well as scaffolding, regulatory and specialized adhesion molecules. The union of proteomic studies of biochemical preparations that enrich for synapses (e.g. “synaptosomes”) has identified over 2500 proteins that are present at synapses[4]. This does not mean that all 2500 proteins are present at a single synapse- the proteome of a single synapse has not been measured. Within an individual pre- or postsynaptic compartment, however, the copy numbers of some proteins have been determined. For example, most excitatory pre-synaptic proteins (e.g. scaffolding molecules, synaptic vesicle proteins and membrane-associated proteins) have been reported to exhibit between 100-10,000 copies per bouton[5,6]. We have much less quantitative information about the postsynaptic compartment. Although the relative abundances of ~30 postsynaptic proteins have been determined[7], we have the rough copy number estimates per synapse for a relatively smaller number of proteins including CamKIIa (5000), the glutamate receptors (30-80 copies) and PSD-95 (300 copies); most other measured postsynaptic proteins exhibit copy numbers between 10- 1000[8] (Fig.1). In addition, like all proteins, neuronal proteins have a limited half-life. Neuronal proteins measured in vitro exhibit half-lives of ~ 5.5 days[9,10] and in vivo ~10 days[11]. Interestingly, as a group, proteins in synapses and axonal growth cones exhibit a half-life that is shorter than the average population of cellular proteins[9-12].

The number, size and strength of synapses in the brain changes with experience through a process known as synaptic plasticity. Experience-dependent changes in connectivity provide a means of forming and storing memories. Long lasting forms of synaptic plasticity, like long-term memory, require new RNA and protein synthesis[13,14]. Stimulation of individual synapses or synapse clusters has demonstrated that the stimulated synapses can undergo long-lasting changes in synaptic strength, while neighboring unstimulated synapses remain unchanged[15,16]. Since long-term plasticity can occur at some but not all synapses made by a given neuron, mechanisms must also exist to compartmentalize the changes in gene expression to those synapses undergoing plasticity.

Taken together, the morphological complexity of neurons, the volumetric dominance of the neuronal processes and their capacity for plasticity indicate that there are special challenges to set-up, maintain and modify the proteome in axonal and dendritic synaptic compartments. The fastest protein transport times (1µm/sec) measured (in axons) indicate that it would take 11.6 days to traffic a protein from the cell body to a distal (1 meter away) axon terminal[17]. The processing of local information and the ensuing response (e.g. turning in response to a navigational cue during development or synaptic plasticity in the adult) more often than not require responses on the time scale of minutes, rather than hours. As discussed below, the localization of mRNA and the on-site synthesis of proteins is a conserved mechanism that appears to have evolved to fulfill local demand for new proteins at short time scales and to provide subcellular functions for development, synaptogenesis, experience-dependent plasticity, and survival in dendrites and axons.

As a framework for the review, we consider which criteria must be met in order to establish that protein synthesis occurs in a particular subcellular compartment to supply proteins and to allow for proteome remodeling:

i) the localization of the protein template (mRNA), the machinery (ribosomes) and regulatory elements required for protein synthesis
ii) the detection of newly synthesized proteins
iii) the observation that blocking local synthesis reduces protein levels or affects plasticity.

We conclude the review with a discussion of some of the open questions and issues that are particularly interesting and timely.

The localization of protein synthesis machinery.

mRNAs.

The initial identification of mRNAs in distal dendrites was a fortuitous finding made in the course of in situ hybridization studies of a handful of transcripts, including MAP2[18] and

CamKIIa[19], and beta-actin mRNA in axons[20]. While the limited number of mRNAs detected in this manner at first suggested that only a select number of transcripts localized to synapses, the development of more sensitive fluorescent in situ hybridization (FISH) approaches and of modern genomic technologies subsequently allowed for more complete and unbiased detection of dendritically, axonally and synaptically localized transcripts (Fig. 2). Microarray analysis and/or qPCR of mRNAs present in isolated neuronal processes from Aplysia sensory neurons[21], rat DRG neurons[22], Xenopus or mouse retinal ganglion neurons[23,24] or hippocampal neurons[25-27] revealed the presence of up to hundreds of distally localized transcripts. In depth RNA sequencing combined with fluorescent in situ hybridization (FISH) and Nanostring of the stratum radiatum of rat hippocampus increased the resolution of detection to identify ~2500 mRNAs that localized to dendrites and axons of hippocampal pyramidal neurons[28] (Fig. 2a). More recently, purification of excitatory synaptosomes (from vGlut1-GFP mouse) identified over 450 transcripts that are enriched in excitatory presynaptic nerve terminals, including many components of the active zone[29] (Fig. 2e).

mRNAs are trafficked to distal compartments in a microtubule-dependent manner[20] and evidence indicates that the 3' and 5' untranslated (UTR) regions of mRNA play key roles in transport and localization selectivity[30-34]. However, despite the identification of multiple localization-required motifs[35], no single motif has emerged as a canonical sequence for targeting mRNAs specifically to axons, or parts of axon, or dendrites. Small changes in UTR sequences can change the secondary structure of mRNAs that, in turn, alter the binding of RNA-binding proteins [36]. Indeed, there is great diversity in the 3'UTRs of neuronal mRNAs: more than half of the mRNA population can express alternative 3'UTRs that give rise to differences in localization, half-life and presumably translational regulation, owing to the presence or absence of miRNA seed sequences[33]. Interestingly, plasticity changes 3'UTR choice[34] and there is also data suggesting that 3'UTRs of mRNAs can be locally remodeled following plasticity[33,37].

Ribosomes.

Ribosomes were first identified in the dendrites of primate spinal cord motoneurons[38] and then observed in the dendrites of hippocampal neurons[39]. Ribosomes were detected in axons as early as 1970[40] in embryonic rabbit spinal cord and *in vitro* [41] but were not detected in adult CNS axons until recently[29,42]. Polysomes (clusters of mRNA and 2 or more ribosomes) have been detected in dendritic spines and throughout the length of the dendrite[43]. Recently ribosomes were observed in axon terminals using EM detection of a tagged knock-in ribosomal protein in adult mouse retinal ganglion cell axons[42]. One of the reasons why ribosomes eluded discovery in adult CNS axons for so long is that they are likely sparsely scattered, and are only rarely seen grouped into polysomes, for example, in the nodes of Ranvier[44]. Even in growing axons and in growth cones where their presence is less controversial, polysomes are not numerous and instead ribosomes appear to be commonly scattered within the cytoplasm as monosomes (single ribosomes engaged with an mRNA), sometimes lined up along the plasma membrane (Figure 2f). In yeast, translation of short proteins or proteins subject to non-sense mediated decay can occur on monosomes[45]. In small cytoplasmic compartments, like dendritic spines, axonal growth cones or boutons, where the supply of only a few new molecules of a protein could make a large difference functionally (see protein copy numbers above), it is possible that monosomal translation could be sufficient. Indeed, translation on monosomes could allow for synthesis for a greater number of different protein species with relatively limited ribosomal resources.

Regulatory elements: co- and posttranslational processing machinery.

Ribosomes, and co-translational chaperones are sufficient to synthesize, fold and traffic cytoplasmic proteins but additional machinery is required for integral membrane and secreted proteins. Membrane and secreted proteins are processed through multiple membrane-bound organelles (including the endoplasmic reticulum, the ER-Golgi intermediate compartment (ERGIC), the Golgi apparatus, the *trans*-Golgi network), where they are folded, assembled, and glycosylated before their delivery to the plasma membrane. While ER and several of its intrinsic proteins have has been detected in dendrites and axons, e.g. [46-49], a conventional Golgi apparatus is largely lacking[50] and only sparse small Golgi outposts have been detected. Interestingly, many neuronal

membrane proteins appear to exhibit glycosylation patterns consistent with Golgi bypass[51]. Given the abundance of membrane proteins in the dendritic and axonal transcriptomes, this suggests that membrane proteins could indeed be synthesized locally and might have a glycosylation signature that would indicate their site of synthesis. In line with this hypothesis, a dendritic ER-ERGIC-Golgi satellite (GS)-retromer microsecretory system has been described recently.[52]

Detection of local protein synthesis.

Evidence for local protein synthesis in distal neuronal processes began with the demonstration of the metabolic incorporation of amino acids in axons in the 1960s[53,54]. Similar observations were made in a variety of biochemical fractions that were enriched for synaptic components (a.k.a. “synaptosomes” or “synaptoneurosomes”)[55-57]. These early experiments were often met with skepticism that pointed out a potential contribution of contaminating proteins synthesized in the cell body. Given these concerns, there are 4 basic approaches that have been taken to establish a local source of protein synthesis: i) physical isolation of the compartment of interest from the cell body, ii) use of photoconvertible or bleachable fluorescent reporters iii) high-resolution purification of the fraction or subcellular compartment, and iv) metabolic labelling with very short labeling periods, to eliminate the contributions of proteins made in the soma.

The physical isolation of the compartment is perhaps the most solid proof for local translation, coupled with a demonstration that the observed protein synthesis signal can be blocked by a protein synthesis inhibitor. In some brain slices (e.g. from the hippocampus), for example, where the synaptic neuropil can be physically isolated from the neuronal cell bodies, local protein-synthesis dependent plasticity was first observed[58,59]. In Aplysia neurons, physically isolated neurites were treated with ^{25}S-methionine and serotonin-induced protein synthesis was detected[60]. Physical isolation is easily accomplished in axons which can be surgically separated from their somas *in vitro* or *in vivo*[61-63]. For example, in isolated retinal ganglion growth cones, ^{3}H-leucine incorporation was observed that was stimulated by different guidance molecules[62]. More recently, 1000-2000 mRNAs undergoing active translation in isolated embryonic and adult

axons *in vivo* have been identified using an axon-specific Translating Ribosome Affinity Purification (TRAP) approach[42,64]. In addition, a highly sensitive proteomic approach combining Stable Isotope Labeling by Amino acids in Cell culture (pSILAC) with Single Pot Solid Phase-enhanced Sample Preparation (SP3) has revealed that hundreds of nascent proteins are rapidly synthesized in response to guidance molecules[65,66].

Fluorescent reporters which can be targeted to subcellular compartments and switched on or off or converted to different colors to define epochs of protein synthesis have been extremely useful. For example, expression of GFP-reporter in which the mRNA was targeted to the dendrites was followed by the cutting of a dendrite and stimulation of protein synthesis by a neurotrophin was observed[67] (Fig. 3a). Stimulus-induced protein synthesis has also been visualized in the growth cones of retinal axons and at synapses of Aplysia neurites, isolated from their cell bodies, using translational reporters that employ photoconvertible fluorophores[68,69]. At cultured Aplysia sensory-motoneuron synapses the guidance cue, netrin, stimulated protein synthesis in sensory neuron neurites isolated from their cell bodies[70] (Fig. 3). In addition, fluorescence recovery after photobleaching (FRAP) experiments have demonstrated the synthesis of beta-actin in branch tips in vivo[71] (Fig. 3b). Continuous FRAP/image capture with a fast-folding fluorescent reporter enabled single molecule imaging of newly synthesized proteins in dendrites of hippocampal neurons and retinal growth cones[72-74] and single molecule imaging of nascent peptides performed in conjunction with single molecule FISH to detect local mRNAs revealed 'bursting' translation in distal dendrites of hippocampal neurons[75].

An alternative approach makes use of new metabolic labeling approaches that enable brief labeling and/or the ability to mark or tag nascent proteins. For example, non-canonical amino acids (e.g. methionine analogs like azidohomoalanine; AHA) can be taken up by the cell, charged by the cell's own tRNA synthetase, and incorporated in protein[76]. Using click-chemistry and fluorescent label, newly synthesized proteins have been detected in situ (fluorescence noncanonical amino acid tagging, FUNCAT; [77]). Many demonstrations of local translation have made use of the antibiotic puromycin, which gets covalently associated with the nascent chain during protein synthesis[78]. Following very

brief (~5-10 min) treatment with low concentrations of puromycin, protein synthesis can be visualized in situ by recognition with antibodies[70,79,80]. In brain tissue subjected to expansion microscopy to increase the spatial resolution, positive protein synthesis signal was detected in ~60% of dendritic spines and ~40% of excitatory and inhibitory nerve terminals following just 5 min of metabolic labeling with puromycin[29]. The combination of puromycin labeling with the proximity ligation assay (puro-PLA) has allowed the direct visualization of newly synthesized proteins of interest[81]. To date, several locally newly synthesized have been detected at synapses and in growing axons using puro-PLA, such as CamKIIa[81,82], Bassoon[29,81], SNAP25[83], RapGEF[29], Tau protein[84], LB2[49] and VDAC2[49], Limk1[85], and several others[65] (Table 1).

How specific is localized translation? There is evidence for tight control over mRNA translation specificity in neuronal compartments, especially in signal-regulated translation. In growing axons, there appears to be a high degree of specificity to translation, with specific extrinsic cues triggering the synthesis of distinct proteins[65]. Hints of this specificity were first seen using a candidate approach where different guidance cues that exerted translation-dependent responses in turning growth cones, were found to induce the synthesis of different proteins. Netrin-1, an attractant, triggered the local synthesis of proteins that build-up the cytoskeleton (e.g. beta-actin; [69]) whereas repellents Sema3A and Slit-2 induced synthesis of cytoskeletal disassembly factors[86,87]. Unbiased proteomic analyses has extended this differential translation model revealing that a single cue typically triggers the rapid and specific regulation of ~100 proteins, and that different cues induce distinct proteomic signatures[65]. Another likely important factor in the specificity of cue-induced translation is receptor activation. Different guidance cues or different neurotransmitters bind to and activate specific receptors, some of which are directly coupled to ribosomes[88]. In Aplysia neurons, local translation of the neuropeptide sensorin was induced by stimuli that produce translation-dependent long-term facilitation but not by stimuli that induce translation-dependent long-term depression of sensory-motor synapses[55]. In rodent neurons, a large-scale proteomic analysis of two opposite forms of homeostatic scaling revealed both commonly and differentially regulated newly synthesized proteins[89,90]. Another level of specificity may be the individual synaptic

compartment in which protein synthesis is regulated by plasticity. For example, a recent study demonstrated that 3 different forms of plasticity (e.g. induced by brain-derived neurotrophic factor (BDNF), mGluR activation, or endocannabinoids) result in different signatures of protein synthesis in post-synaptic, excitatory or inhibitory presynaptic compartments[29]. Several mechanisms might underlie this specificity, such as microRNA regulation[91], mRNA modification[92], modulation of the phosphorylation of eukaryotic initiation factors[93], and/or RNA-binding protein (RBP) phosphorylation[94,95]. This important topic here has been recently reviewed in more detail: [96,97].

Established roles for local translation in protein supply and plasticity.

The detection of mRNAs and translational machinery in axonal, dendritic and synaptic compartments and the visualization of local protein synthesis at synapses indicate that translation can occur at synapses, which in turn leads to questions about the physiological function of local translation in neural circuit development, survival and plasticity. An experimental challenge in demonstrating functions for local translation is the need to differentiate local translation from somatic translation with protein transport to synapses. For example, synaptic potentiation elicited by the neurotrophin BDNF requires local translation and this was demonstrated by recording from hippocampal slices in which the synaptic neuropil was physically isolated from the cell body layer[58] (Fig. 3a). A similar approach demonstrated a requirement for local translation in mGluR-dependent long-term depression of synaptic transmission[59] and late-phase long-term potentiation (LTP)[98]. In electron microscopy analyses, induction of long-term potentiation is associated with an increased number of polyribosomes detected in dendritic spines[43]. Restricted extracellular application of protein synthesis inhibitors to different synaptic layers has also been used to demonstrate that local protein synthesis is required for late-phase long-term potentiation[99]. Another form of plasticity, local homeostatic scaling, is mediated via regulation local protein synthesis. Acute blockade of synaptic activity at the synapse results in a stimulation of local translation[100,101]. At the Calyx of Held synapse, located ~3 mm from the cell body in mouse, a block of protein synthesis leads to an upregulation of spontaneous release events[102]. In addition, long-term depression of inhibitory transmission induced by endocannabinoids requires protein synthesis in the inhibitory

presynaptic nerve terminal[103]. In the above experiments, protein synthesis inhibitors were injected, bath applied or perfused in a restricted manner. Clearly, a new generation of protein synthesis inhibitors which can be expressed in individual cells or enabled with light (e.g. [104,105]) would enable better spatial and temporal control of protein synthesis inhibition and hence allow us to ascertain more readily roles for local translation.

As noted above, CamKIIa is one of the most prominent dendritically localized mRNAs. Within 5 minutes of LTP induction, newly synthesized CamKIIa was detected in the dendrites[106]. To probe the function of local translation of dendritically localized CamKIIa mRNA in vivo, Miller et al.[107] generated a mouse in which the CamKIIa 3'UTR that targeted the mRNA to dendrites was deleted, such that the protein was expressed but the mRNA was not dendritically localized. The phenotype included impairments in hippocampal-dependent forms of memory, reduced late-phase LTP, and a striking reduction in the amount of CamKIIa protein present in post-synaptic densities. Using a similar approach, Kuklin et al observed that loss of the CamKIIa 3'UTR in Drosophila showed reduced spontaneous neurotransmission and capacity for plasticity[108]. Together, these findings indicate that local translation of one dendritically-localized mRNA is required for functional and structural synaptic plasticity in vivo.

Cultured Aplysia sensory-motor synapses provide a simple reduced preparation for investigating the function of local translation in synapse formation and plasticity. Reduction of a localized mRNA—without altering the protein levels—was found to block synapse formation[109], indicating that synaptic translation of that single localized mRNA was required for synapse formation. Local perfusion of translational inhibitors to distal sensory neurites was found to inhibit serotonin-induced plasticity at the translationally-inhibited synapse but not at other stimulated synapses formed by that neuron, and sensory neurons severed from their cell bodies were capable of undergoing serotonin-induced, translation-dependent long-term facilitation that lasted 24 hours[60]. Together, these findings demonstrate a role for translation of localized mRNAs in both synapse formation and plasticity in this reduced, culture preparation.

Local translation plays an important role during axonal development. When retinal ganglion cell axons grow towards their targets, local translation is required for them to respond appropriately (e.g. turn) to secreted guidance molecules[62]. Indeed, guidance molecule-driven translation of beta actin is asymmetrically required on the near-side of the growth cones (the side closest to the attractive guidance cue) to help growth cones turn in that direction[69], as is the interaction with a specific RNA-binding protein that interacts with elements in the 3'UTR of the beta-actin mRNA in its transport to the near side[110]. In developing retinal axons *in vivo*, acute inhibition of protein synthesis or electroporation of beta-actin translation-blocking morpholinos in isolated axons (i.e. no somal contribution) impairs axonal branching in the optic tectum[71]. In adult sensory axons, Perry et al. (2012)[111] took advantage of the finding that the mRNA encoding the nuclear transport factor importin beta 1 had two distinct 3'UTRs, including a long 3'UTR that localized the mRNA to axons of sensory neurons and a short 3'UTR that localized the mRNA to cell bodies of sensory neurons. They targeted the long 3'UTR to generate a mouse that lacked importin beta 1 mRNA in axons, without altering the expression or function of importin beta 1 in the sensory cell body. The phenotype of this mouse included delayed axonal loss in response to axonal injury, indicative of a function for local, axonal translation of importin beta 1 in initiating the response to injury. Indeed, there are multiple examples where local axonal translation is required for the axon to mount an injury response[112,113]. Another important role for local translation in axons is the promotion of axon maintenance and survival. The axonal translation of LaminB2 and Bclw, for example, has been shown to be required for axon viability[63,114,115].

Open questions and future directions

We highlight the following areas as particularly interesting owing to recent progress, new ideas, or persistent questions that still remain unanswered.

Ribosome repair/remodeling. Little is known about the origin and life of ribosomes in distant neuronal compartments. It is presumed that ribosomal proteins (RPs) synthesized in the cell body are assembled into ribosomes in the nucleolus and then transported to the cytoplasm and ultimately to distal neuronal processes but recent findings point to the

possibility of on-site ribosome remodeling. Transcriptome studies have repeatedly shown the abundant presence of RP-encoding mRNAs in axons and dendrites[21,23,25,28] raising the possibility that RP mRNAs are locally translated. A translatome analysis has confirmed this by showing that RPs constitute the most enriched functional category of mRNAs translated in mouse retinal axons *in vivo*[42]. A proteomic study has further shown that RPs are the most enriched functional group of basally translated proteins in cultured retinal axons and that extrinsic cues, such as Netrin, trigger the rapid regulation of newly synthesised RPs[65]. In line with this, a recent subcellular transcriptome/proteome analysis of mouse callosal axons identified the ribosome as one of the six most enriched categories[116]. Several RPs are known to serve extra-ribosomal functions but the sheer number of RP mRNAs detected (>50) in dendrites and axons suggests that at least some may serve a ribosomal function. The gold standard experimental approach to answering this question directly is to perform proteomic analysis on biochemically isolated ribosomes. However, this approach presents a major challenge in neuronal compartments because of the very small amounts of purified axon/dendrite material from ribosomes can be isolated. Recent analysis of this sort has been performed on isolated axons by growing hundreds of embryonic Xenopus eyes on filters in which the cell bodies reside on one side and the axons extend to the other[64]. SILAC labeling combined with axonal ribosome purification and mass spec analysis suggests that locally synthesized RPs may join pre-existing ribosomes in axons, in response to cue (Netrin-1) stimulation[117].

If new RPs are added to ribosomes on-site in dendrites/axons, what could be their role and how would it fit with classical views on ribosome biogenesis and function? In cultured neurons, ribosomal proteins exhibit remarkably variable half-lives that range from 3-9 days[10], inconsistent with the entire ribosome being synthesized and degraded together. Little is known about neuronal ribosome biogenesis and the trafficking of ribosomes to dendrites and axons. It is conceivable that a mechanism exists for maintaining/prolonging ribosomal function in distant compartments. One possibility is that basally translated RPs may be needed for the constant, on-site homeostasis of ribosomes. A second possibility follows from compelling evidence showing that mutations in individual RPs give rise to

distinct, sometimes tissue-specific, phenotypes rather than a general non-thriving phenotype expected from a global loss of protein synthesis has given rise to the 'specialized ribosome' hypothesis[118]. In line with this idea is the observation that distinct guidance cues induce a specific signature of RP translational changes in axons[65], raising the possibility that localized RP synthesis could 'tune' ribosomes in a highly localized way in axons/dendrites, perhaps determining which mRNAs are translated.

RNA methylation and partitioning of transcripts at synapses.

The discovery and development of tools to study reversible chemical modifications of RNAs[119,120] raised interest in the possibility that epitranscriptomic modifications might play a distinct role in regulating local translation at synapses. The most abundant mRNA modification in mammalian cells involves the addition of a methyl group to the N^6 position of adenine to form N^6-methyladenine (m^6A). Methylation of RNA has been shown to affect many aspects of RNA metabolism, including stability, transport and translation[121], and the level of m^6A expression is higher in the brain than in any other organ in the body[120]. Using m^6A immunoprecipitation to sequence the m^6A epitranscriptome from purified mouse forebrain synaptosomes, Merkurjev et. al (2018)[122] identified 4,469 m^6A peaks in 2,921 genes that were selectively enriched in synaptosomes as compared to whole forebrain extracts. Over 40% of these mRNAs were not themselves enriched at synapses, suggesting either that the methyl modification was deposited at synapses or that methylation directed the synaptic localization of the transcript. These authors also showed that several of the enzymes involved in m^6A modification ("m^6A writers, erasers and readers") are present at synapses, and that shRNA-mediated reduction of m^6A readers resulted in immature spine morphology and in synaptic transmission deficits. Together, these findings indicate that chemical modification of mRNAs serves to partition specific mRNAs at synapses in a manner that could alter multiple steps of mRNA metabolism (e.g. stability, translation) to control the synaptic proteome.

Intercellular RNA transfer via extracellular vesicles.

The capacity of synaptic compartments to autonomously regulate their proteome via local translation decentralizes the regulation of gene expression from the nucleus to the

synapse[123]. A series of intriguing and compelling studies over the past decade have revealed a role for extracellular vesicles in transporting mRNAs and miRNAs from one cell to another. Studies conducted in *Drosophila melanogaster*[124] and in mouse[125] revealed that the neuronal Arc protein assembles into viral-like capsids that contain the *Arc* mRNA, and that these viral-like capsids are transferred from one neuron to a target muscle (at the *Drosophila* neuromuscular junction) or to a target neuron (in cultured mouse cortical and hippocampal neurons). Moreover, the transferred *Arc* mRNA was shown to be translated in the recipient cell. The remarkable implication of these findings is that one cell can directly regulate the proteome of a neighboring cell via the local delivery of RNA. In the context of local translation at synapses, this is a particularly provocative idea because it suggests that the regulation of gene expression during the development and plasticity of neural circuits is not just regulated in a compartmentalized fashion within neurons, but that it is regulated at the level of local spatial neighborhoods comprising multiple neurons.

Liquid-liquid phase separation and RNA localization.

The regulated assembly of proteins and RNAs into membraneless organelles or compartments via liquid-liquid phase separation (LLPS, [126]) has emerged as an important mechanism underlying RNA trafficking and metabolism in neurons. Many RNA binding proteins associated with neurodegenerative disease, including FUS, TDP-43, tau, TIA1 and hnRNPA1, have been found to undergo LLPS in a manner that contributes to pathology[127]. One consequence of increased gel formation in hyperphosporylated FUS mutants is the impairment of new protein synthesis in axon terminals[128], and reducing gelation of mutant FUS by overexpression of the nuclear transport protein Transportin rescues protein synthesis in these axons[129]. Further elucidation of the biochemistry of this phase separation process promises to provide therapeutic insights into these diseases (e.g. [130]). By dynamically compartmentalizing RNAs and RNA binding proteins, LLPS is also likely to play regulatory roles in synaptic RNA localization and translation.

Concluding remarks

The unique anatomy of neurons enables the vast information processing they conduct. However, it also poses huge challenges for how to manage protein supply and proteome remodeling at the temporal and spatial scales needed for axon guidance, branching and viability[131], the basic supply of an integral synaptic protein[107], the ongoing maintenance of synaptic function and the modification of individual proteins or proteomes required for synaptic plasticity. In each of these cases, the supporting data arise from studies both in vitro and in vivo, though in vivo visualizations of protein synthesis are relatively rare owing to the extreme difficulty of the experiments (e.g. [71,132]). Therefore, it is clear that the ability to assess further the necessity of local translation will require new tools with enhanced spatial and temporal resolution.

Figure Legends.

Figure 1. Neurons and numbers. A. Anatomy of a neuron. Neuron-specific cell parts. The sites of local protein translation and neuronal functions dependent on local translation are indicated. B. Localization, functions and per synapse copy numbers of various synaptic proteins. Protein copy numbers taken from Wilhelm et al. 2014 and Sheng and Hoogenraad, 2007.

Figure 2. The localization of the protein template (mRNA), the machinery (ribosomes) and regulatory elements required for protein synthesis.
a, Scheme of a postsynaptic compartment highlighting some of the transcript families for synaptically-relevant proteins identified by RNA-seq that are present in the synaptic neuropil in area CA1 of the hippocampus. Reproduced with permission from Cajigas et al., 2012. **b,** Fluorescence in situ hybridization (FISH) signal in cultured rat hippocampal neurons (upper panels) or adult rat hippocampal slices (lower panels) using probes designed to detect the indicated mRNAs. Dendritic processes were immunostained with an anti-MAP2 antibody (purple). Scale bars represent 15 and 25 μm, for upper and lower panels, respectively. Reproduced with permission from Tushev et al., 2018. **c,** FISH for sensorin mRNA (red; left panel) in Aplysia sensory-motoneuron cultures showing the localization of the mRNA at presynaptic sites, labeled by VAMP-GFP (green; right panel). An Alexa dye (blue) represents the membrane. Scale bar = 50 um. **d,** the stem-loop structure present in the sensorin mRNA 5-UTR that is responsible for localization (Meers et al., 2012). **e,** Schematic representation of a vGLUT1^{+} presynaptic terminal with the localization of a subset of proteins coded by mRNA detected using Next Generation RNA sequencing of fluorescently-sorted synaptosomes. Note that many presynaptic active zone-related mRNAs are enriched in vGluT1+ presynaptic terminals relative to unsorted (generic) synaptosomes whereas synaptic vesicle-related mRNAs are either significantly depleted (magenta) or not enriched by sorting. Reproduced with permission from Hafner, Donlin-Asp, et al., 2019. **f,** HA immuno-gold electron microscopy (EM) to label ribosomes containing the Rpl22-HA tagged protein. HA-tagged ribosomes localize to and RGC axon terminals in the superior colliculus SC from embryonic day 18.5 mice (left) or adults (right). Scale bars = 500 nm. Reproduced with permission from Shigeoka et al., 2016.

Figure 3. Visualization of local protein synthesis.
a, Fluorescence reporter combined with physical isolation. A cultured hippocampal neuron was transfected with a GFP reporter containing the 3'UTR from CamKIIa for dendritic targeting of the mRNA. Images of a transfected neuron before (left) and 120 min after (right) BDNF treatment; arrow points to the region of transfection. The fluorescent signal in the transected dendrite increases following BDNF treatment indicating a stimulation of local protein synthesis. Scale bar = 15 um. Reproduced with

permission from Aakalu et al., 2001. **b,** Fluorescence reporter combined with photobleaching (FRAP). Venus-beta-actin was electroporated into Xenopus retinal ganglion cells and fluorescence was monitored in axonal branches. Shown are Venus-beta-actin hotspots forming at different sub-compartments of an axonal branch. The Kymograph displays the FRAP after 300s, along the magenta arrow. At least 4 distinct translational hotspots can be identified in this single branch.Reproduced with permission from Wong et al., 2017. **c and d,** Fluorescence reporter combined with physical isolation. A reporter consisting of sensorin mRNA fused to the photoconvertible fluorescent protein dendra2 was expressed in cultured Aplysia sensory neurons (SN). c, The SN soma was removed (dotted circle), and 12–18 hr later photoconverted the dendra2 signal from green to red (right panel). **d,** Higher magnification panels show the green (top) and the the red channel (volume control, bottom). Increased green signal (yellow arrowheads) represents newly translated reporter after spaced application of 5-hydroxytryptamine. Reproduced with permission from Wang et al., 2009. **e,** A newly synthesized protein-of-interest demonstrating the utility of Puro-PLA, a metabolic labeling strategy that couples puromycin as a metabolic tag (recognized by an anti-puromycin antibody) together with an antibody against the protein-of-interest, followed by the proximity-ligation-assay (see tom Dieck et al., 2015). Shown is a cultured hippocampal neuron labelled for 15 min with puromycin; newly synthesized CamKIIa is detected. Scale bar = 25 um.

Table 1. Locally synthesized individual proteins detected with puro-PLA.

Table 2. Established roles for local translation in neural circuit development, survival and plasticity.

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Table 1 Newly synthesized proteins of interest detected with Puro-PLA

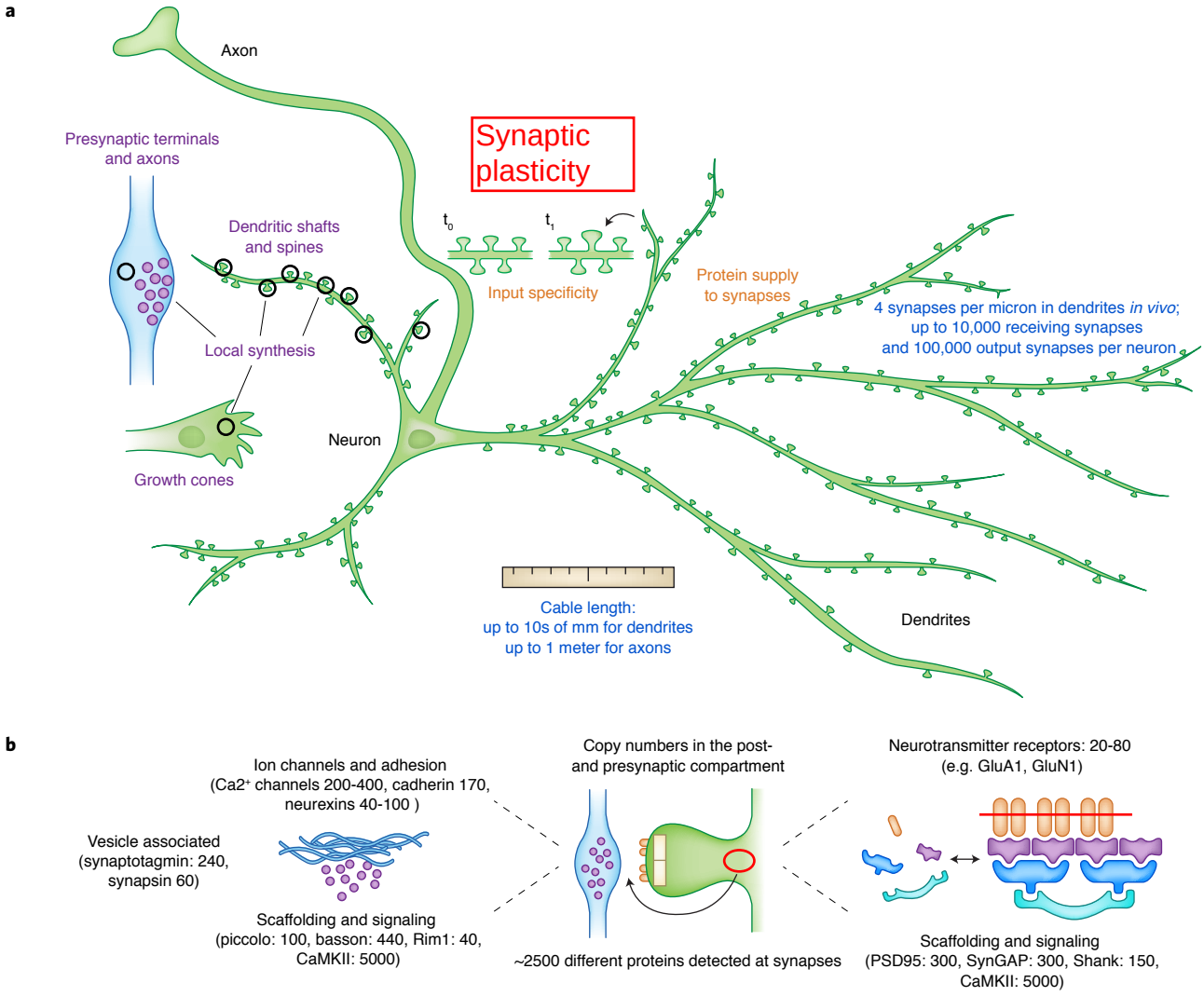
Protein detected	**Species/ model system**	**compartment**	**method**	**references**
CamK2a	rat hippocampal neuron culture	dendrite	endogenous	tom Dieck et al 2015 Sambandan et al 2017 (Ref 81, 82)
Bassoon	rat hippocampal neuron culture	along dendrites VGluT1 positive presynaptic terminals	endogenous	tom Dieck et al 2015 Hafner et al 2019 (Ref 81, 29)
RapGEF	rat hippocampal neuron culture	VGluT1 positive presynaptic terminals	endogenous	Hafner et al 2019 (Ref 29)
Tau	mouse hippocampal neuron culture	dendrite	endogenous	Li & Götz 2017 (Ref 84)
LaminB2	Xenopus retinal ganglion cell culture	axon	endogenous	Cioni et al 2019 (Ref 49)

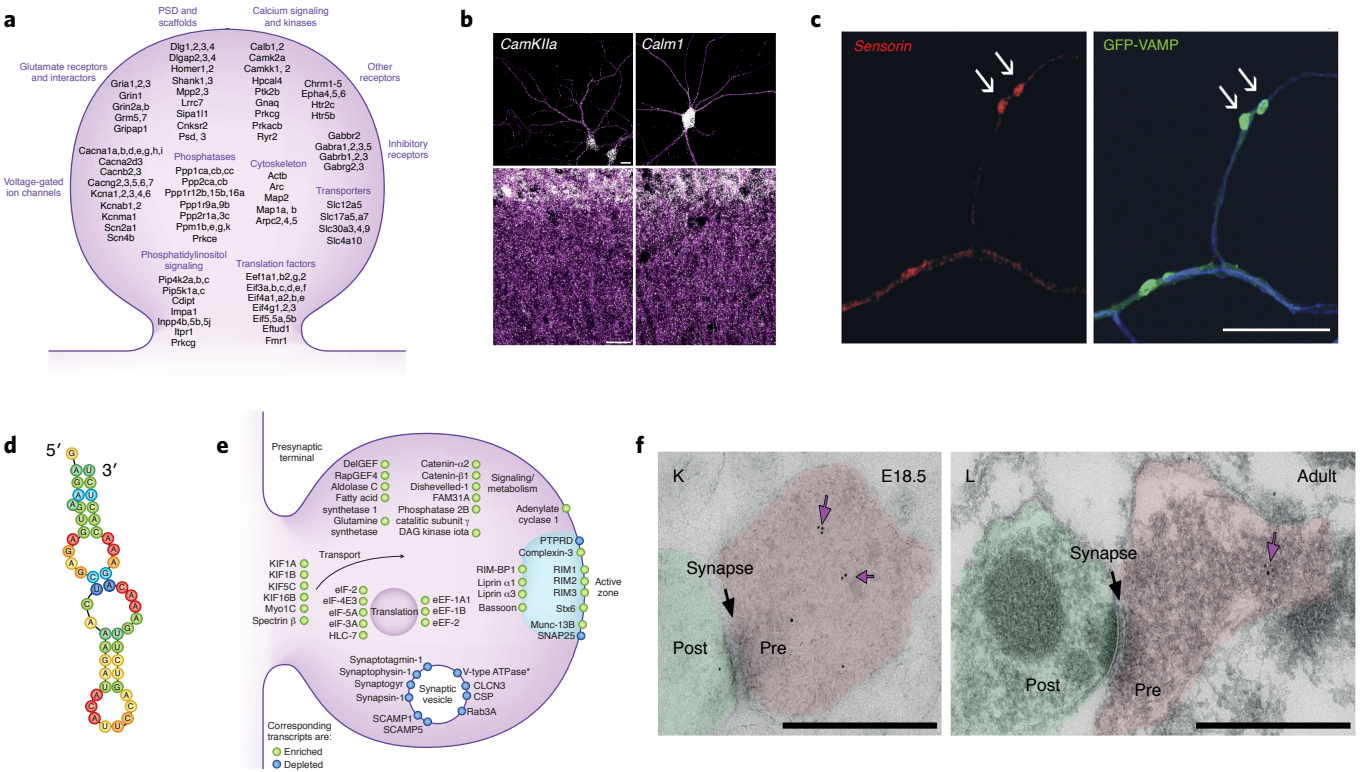
VDAC2	Xenopus retinal ganglion cell culture	axon	endogenous	Cioni et al 2019 (Ref 49)
LimK1	rat cortical neuron culture	dendrite	endogenous	Rajgor et al 2018 (Ref 85)
DSCR1.4	mouse hippocampal neuron culture	axon	5‘3‘-UTR-containing expression reporter	Seo et al., 2019 (Ref 133)
DAP5	mouse hippocampal neuron culture	axon	endogenous	Seo et al., 2019 (Ref 133)
CDC42 isoform	mESC-derived neuron culture	neurites	CDS-E7-3’UTR expression reporter	Mattioli et al., 2019 (Ref 134)
Neurogranin	mouse cortical neuron culture	dendrite	endogenous	Jones et al. 2018 (Ref 135)
Calmodulin	mouse cortical neuron culture	dendrite	endogenous	Jones et al. 2018 (Ref 135)
SNAP25	rat hippocampal neuron culture	axon nascent presynapses	endogenous	Batista et al 2017 (Ref 83)

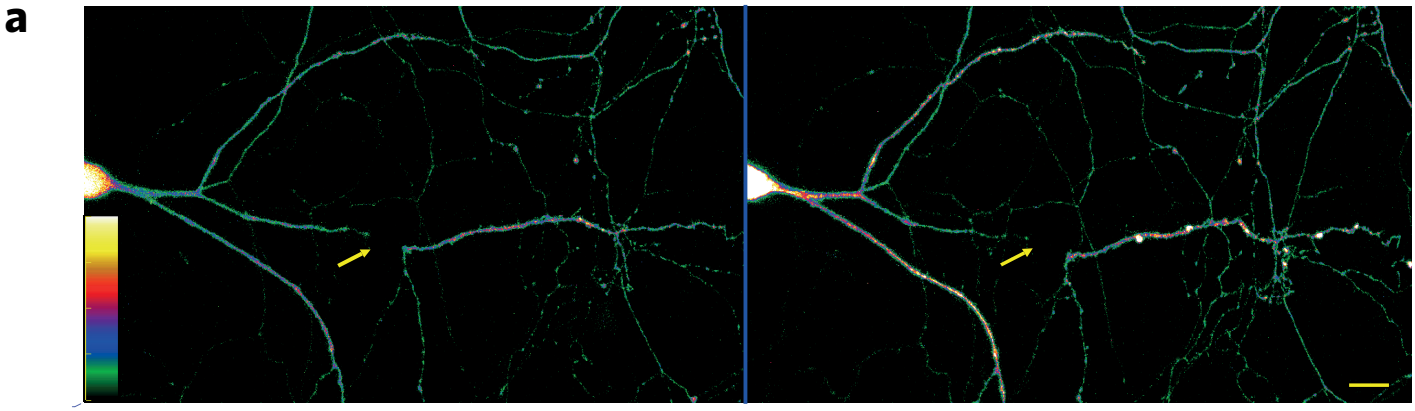
Table 2: Established Roles for Local Translation in Protein Supply and Plasticity

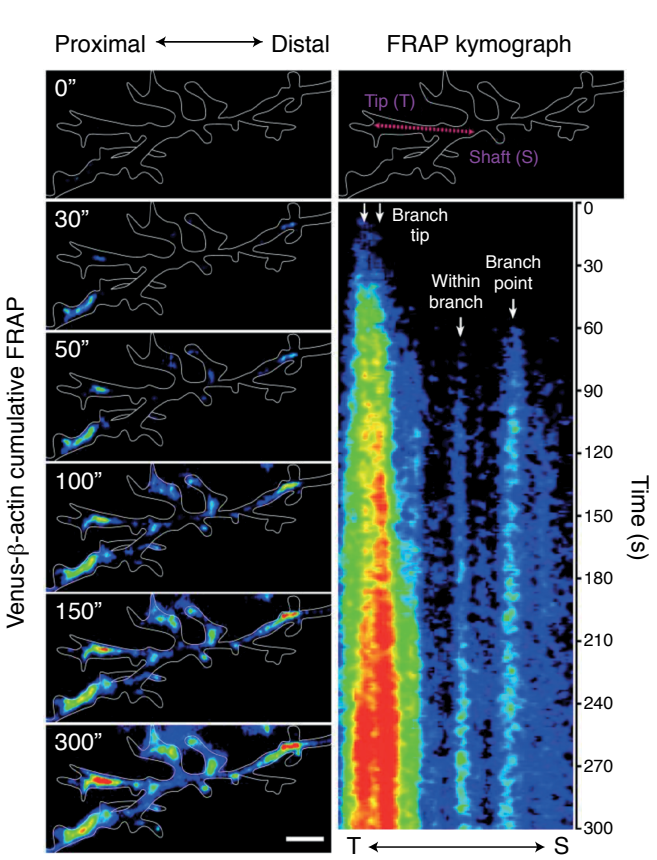
Function	Species/ model system	Compartment	Method	Reference
BDNF-induced synaptic potentiation	rat hippocampal slices	Synaptic neuropil of area CA1	Physical isolation	Kang and Schuman, 1996 (ref 58)
Serotonin-induced synaptic facilitation	cultured Aplysia sensory and motoneurons	Sensory to motoneuron synapses	Physical isolation	Martin et al., 1997 (ref 60)
mGluR1/5-induced long-term depression	rat hippocampal slices	Synaptic neuropil of area CA1	Physical isolation	Huber et al., 2000 (ref 59)
Guidance decisions	*Xenopus* retinal ganglion cells in culture	Axonal growth cones	Physical isolation and protein synthesis inhibitors and translation inhibition of □-actin	Campbell & Holt, 2001 (ref 62); Leung et al, 2006.(ref 69)
Maintenance of synaptic CamKIIa	Mouse synapses	Postsynaptic density of forebrain synapses	Deletion of CamKIIa 3‘UTR	Miller et al., 2002 (Ref 107)
Late-phase long-term potentiation	rat hippocampal slices	Synaptic neuropil of area CA1	Restricted perfusion of a protein synthesis inhibitor	Bradshaw et al., 2003 (Ref 99)
Late-phase long-term potentiation	rat hippocampal slices	Synaptic neuropil of area CA1	Physical isolation	Vickers et al., 2005 (Ref 98)
Local homeostatic scaling	Rat hippocampal neuron cultures	dendrites	Local perfusion of a protein synthesis inhibitor	Sutton et al., 2006 (Ref 100)
Synapse formation	cultured Aplysia sensory and motoneurons	Sensory to motoneuron synapses	mRNA knockdown	Lyles et al., 2006 (Ref 109)
Injury response	Mouse sensory neuron axons	axons	Deletion of long 3’UTR of importin beta 1	Perry et al., 2012 (Ref 111)
Maintenance of presynaptic beta-catenin	Cultured hippocampal neurons in microfluidic chambers	Axon terminals	Fluidically isolated application of a protein synthesis inhibitor	Taylor et al., 2013 (Ref 27)
Endocannabinoid-induced plasticity of inhibitory transmission	Rat hippocampal slices	Inhibitory axon terminals	Injection of a protein synthesis inhibitor	Younts et al. 2016 (ref 103)
Presynaptic protein clustering and neurotransmistter release	Cultured hippocampal neurons in microfluidic chambers	Axon terminals	Fluidically isolated application of a protein synthesis inhibitor	Batista et al., 2017 (Ref 83)
Spontaneous neurotrasmitter release	Mouse Calyx of Held synapse	Axon terminals	Bath application of protein synthesis inhibitor	Scarnati et al., 2018 (Ref 102)

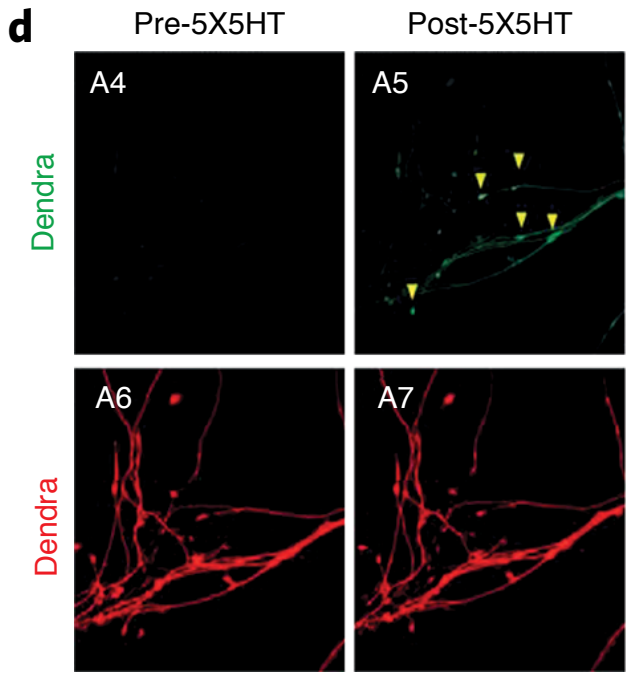
Axon branching	*Xenopus* retinal neurons *in vivo*	Axon terminals	Translation inhibition of □-actin in somaless axons	Wong et al., 2017 (Ref 71)
Axon maintenance	*Xenopus* retinal neurons *in vivo*	Axons	Translation inhibition of LB2 in somaless axons	Yoon et al., 2012 (Ref 63)
Axon maintenance	Rat dorsal root ganglion sensory neurons *in vitro*	Axons	Compartmentalized axon cultures and shRNA to Sfpq, LaminB2 or Bclw	Cosker et al., 2016 (Ref 114)
Axon maintenance	Rat sympathetic superior cervical ganglion neurons *in vitro*	Axons	Compartmentalized axon cultures and protein synthesis inhibitors	Hillefors et al., 2007 (Ref 115)

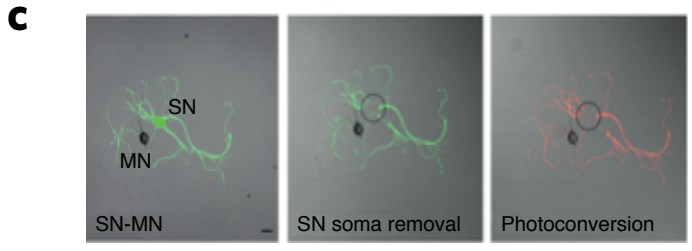

a
Axon
Presynaptic terminals and axons
Dendritic shafts and spines
Local synthesis
Growth cones
Neuron
Synaptic plasticity
t_0
t_1
Input specificity
Protein supply to synapses
4 synapses per micron in dendrites *in vivo*; up to 10,000 receiving synapses and 100,000 output synapses per neuron
Cable length: up to 10s of mm for dendrites up to 1 meter for axons
Dendrites
b
Ion channels and adhesion (Ca^{2+} channels 200-400, cadherin 170, neurexins 40-100)
Vesicle associated (synaptotagmin: 240, synapsin 60)
Scaffolding and signaling (piccolo: 100, basson: 440, Rim1: 40, CaMKII: 5000)
Copy numbers in the post- and presynaptic compartment
~2500 different proteins detected at synapses
Neurotransmitter receptors: 20-80 (e.g. GluA1, GluN1)
Scaffolding and signaling (PSD95: 300, SynGAP: 300, Shank: 150, CaMKII: 5000)

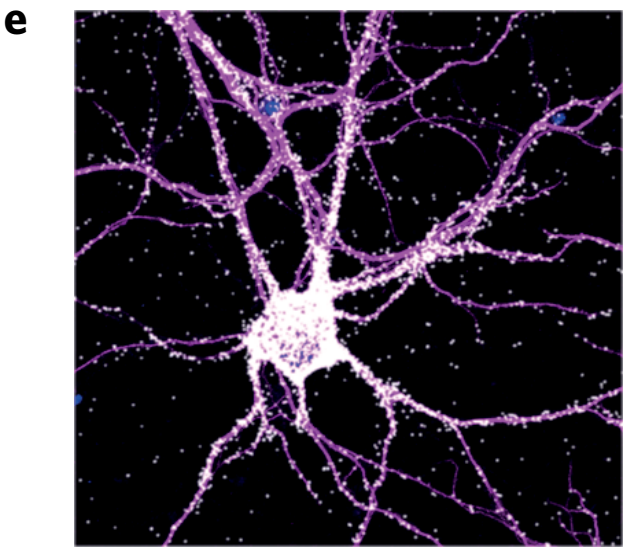

a
PSD and scaffolds
Calcium signaling and kinases
Glutamate receptors and interactors
Other receptors
Inhibitory receptors
Voltage-gated ion channels
Dlg1,2,3,4
Dlgap2,3,4
Homer1,2
Shank1,3
Mpp2,3
Lrrc7
Sipa1l1
Cnksr2
Psd, 3
Calb1,2
Camk2a
Camkk1, 2
Hpca4
Ptk2b
Gnaq
Prkcg
Prkacb
Ryr2
Gria1,2,3
Grin1
Grin2a,b
Grm5,7
Gripap1
Chrm1-5
Epha4,5,6
Htr2c
Htr5b
Gabbr2
Gabra1,2,3,5
Gabrb1,2,3
Gabrg2,3
Cacna1a,b,d,e,g,h,i
Cacna2d3
Cacnb2,3
Cacng2,3,5,6,7
Kcna1,2,3,4,6
Kcnab1,2
Kcnma1
Scn2a1
Scn4b
Phosphatases
Ppp1ca,cb,cc
Ppp2ca,cb
Ppp1r12b, 15b,16a
Ppp1r9a,9b
Ppp2r1a,3c
Ppm1b,e,g,k
Prkce
Cytoskeleton
Actb
Arc
Map2
Map1a, b
Arpc2,4,5
Transporters
Slc12a5
Slc17a5,a7
Slc30a3,4,9
Slc4a10
Phosphatidylinositol signaling
Pip4k2a,b,c
Pip5k1a,c
Cdipt
Impa1
Inpp4b,5b,5j
Itpr1
Prkcg
Translation factors
Eef1a1,b2,g,2
Eif3a,b,c,d,e,f
Eif4a1,a2,b,e
Eif4g1,2,3
Eif5,5a,5b
Eftud1
Fmr1
b
CamKIIa
Calm1
c
Sensorin
GFP-VAMP
d
5′
3′
e
Presynaptic terminal
DelGEF
RapGEF4
Aldolase C
Fatty acid synthetase 1
Glutamine synthetase
Catenin-α2
Catenin-β1
Dishevelled-1
FAM31A
Phosphatase 2B catalitic subunit γ
DAG kinase iota
Signaling/ metabolism
Adenylate cyclase 1
PTPRD
Complexin-3
RIM-BP1
Liprin α1
Liprin α3
Bassoon
RIM1
RIM2
RIM3
Active zone
Stx6
Munc-13B
SNAP25
Transport
KIF1A
KIF1B
KIF5C
KIF16B
Myo1C
Spectrin β
eIF-2
eIF-4E3
eIF-5A
eIF-3A
HLC-7
Translation
eEF-1A1
eEF-1B
eEF-2
Synaptotagmin-1
Synaptophysin-1
Synaptogyr
Synapsin-1
Synaptic vesicle
V-type ATPase*
CLCN3
CSP
Rab3A
SCAMP1
SCAMP5
Corresponding transcripts are:
Enriched
Depleted
f
K
E18.5
Synapse
Post
Pre
L
Adult
Synapse
Post
Pre


a


b
Proximal
Distal
FRAP kymograph
0”
30”
50”
100”
150”
300”
Tip (T)
Shaft (S)
Branch tip
Within branch
Branch point
Venus-β-actin cumulative FRAP
Time (s)
0
30
60
90
120
150
180
210
240
270
300
T
S


d
Pre-5X5HT
Post-5X5HT
A4
A5
A6
A7
Dendra
Dendra


c
SN
MN
SN-MN
SN soma removal
Photoconversion


e