Axonal and presynaptic protein synthesis: new insights into the biology of the neuron

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The presence of a local mRNA translation system in axons and terminals was proposed almost 40 years ago. Over the ensuing period, an impressive body of evidence has grown to support this proposal – yet the nerve cell body is still considered to be the only source of axonal and presynaptic proteins. To dispel this lingering neglect, we now present the wealth of recent observations bearing on this central idea, and consider their impact on our understanding of the biology of the neuron. We demonstrate that extrasomatic translation sites, which are now well recognized in dendrites, are also present in axonal and presynaptic compartments.

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In a recent study of axonal transport of neurofilament protein particles [1], the possibility was raised that such particles might represent '…complexes of newly (translated) protein…'. This proposition is noteworthy because it reflects a growing awareness that there is a protein-synthesizing machinery within axons [2–5] – a notion not generally shared, and one that is still excluded from textbooks [6,7]. We believe that this shifting frame of reference, from the cell body to the axon as an immediate source of axonal proteins, will ultimately have a strong impact on studies of the biology and pathobiology of the neuron. To stimulate a wider recognition of this perspective, we present a concise description of recent developments in the field, viewed in their historical context.

Objections to an exclusive somatic source of axonal and presynaptic proteins

The main objection to the soma being an exclusive source of axoplasmic proteins [8] arose from the fact that cytoskeletal and cytosolic proteins synthesized in the cell body reach their destinations via slow axoplasmic transport, at rates of a few millimeters per day [9,10]. As axons can extend distances of ≥1 m, years would be required to transport proteins to distal axonal segments and presynaptic terminals. Given that the typical half-life of brain cytoskeletal proteins is in the range of a few days to a few weeks [11–13], the requirements for metabolic stability on such a scale clearly would be unrealistic. Indeed, an earlier *ad hoc* hypothesis of sustained metabolic stability during transit [14] was invalidated by evidence for the metabolic decay of slowly transported proteins [15]. Moreover, the local re-utilization of released amino acid residues [13] supported the premise that there is local protein turnover. As considered elsewhere in detail [2–5,8,16], an exclusively somatic source of axonal proteins cannot explain the maintenance of, and local variations in, axoplasmic mass, nor the plasticity exhibited by axonal branches and terminal fields. Thus, as suggested by previous work [8], there must be an additional, local source of protein.

Local protein synthesis in axons and nerve endings

In early studies conducted mainly in large model axons, such as the goldfish Mauthner axon and the squid giant axon [2–5], axoplasm was shown to incorporate amino acids into proteins by a mechanism sensitive to inhibitors of protein synthesis. This mechanism was also present in nerve terminals, as indicated by studies of squid optic lobe synaptosomes [5]. Comparable observations have been made in several types of mature axons, including those from goldfish spinal cord, mammalian spinal nerve roots and peripheral nerves, as well as in cultured immature axons [5].

Axonal and presynaptic ribosomes

Despite the above evidence, axons are considered incapable of synthesizing proteins [6,7] – mainly because ribosomes have not generally been observed in axons by conventional electron microscopy (EM), except in the initial segment [17,18]. Nonetheless, the identification of axoplasmic rRNA in the Mauthner axon [19,20] and squid giant axon [21] provided indirect evidence for axonal ribosomes. This was confirmed by the isolation of active polysomes bearing nascent peptide chains from the axoplasm of squid giant axons [22], and by the demonstration of polysomes in proximal [23] and distal [22,24] axoplasmic regions using electron spectroscopic imaging (ESI), a method that removes the ambiguity inherent in conventional EM. ESI has provided direct evidence of phosphorus signals emitted by ribosomes in several additional studies [24–27]. The presence of ribosomes in the squid giant axon has also been confirmed using independent analytical methods [24,28]. In addition, there is now evidence that ribosomes are included in novel structural entities systematically distributed along vertebrate axons [25,27]. The presence of ribosomes in growing axons was reported in early EM studies [29,30] as well as in more recent investigations [31,32].

Restricted distributions of ribosomes were initially discovered in axoplasm isolated from goldfish myelinated fibers [25], and later shown in axoplasm isolated from spinal nerve roots of rabbit and rat [27].

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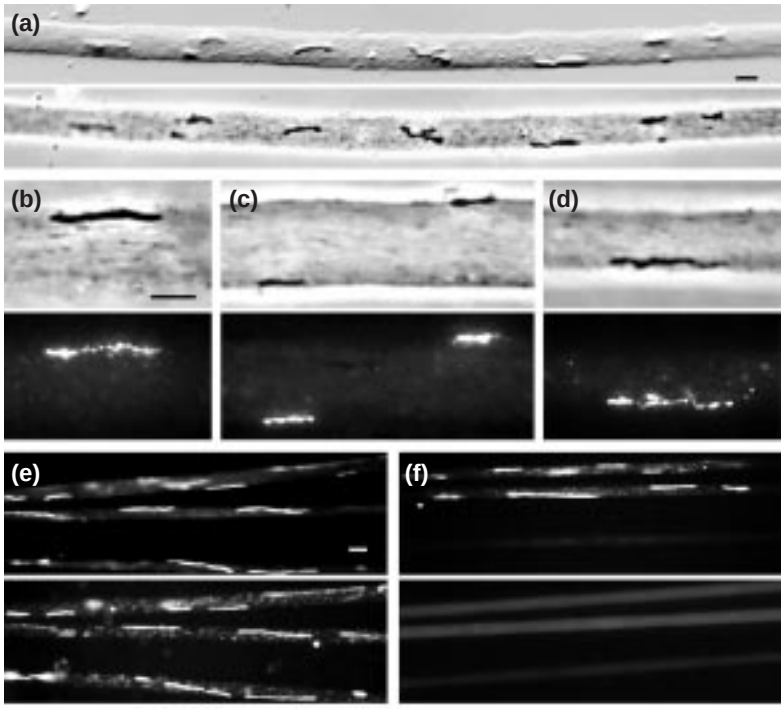

Fig. 1. Periaxoplasmic ribosomal plaques in the cortical zone of axoplasmic wholemounts isolated from rabbit ventral root myelinated fibers. (a) Low-power differential interference contrast (upper panel) and corresponding phase-contrast (lower panel) micrographs show a distribution of structural correlates, which appear as excrescences along the wholemount surface. The correspondence between structural plaques and ribosomal domains is shown by phase images of structural plaque correlates (b–d, upper panels) and by immunofluorescence of rRNAspecifically detected by monoclonal antibody (mAb) Y-10B (b–d, lower panels). At higher magnification, plaque ribosomal domains immunostained with mAb Y-10B reveal a punctate character, probably corresponding to polysomes [27]. (e,f) The specificity of mAb Y-10B immunoreactivity with rRNA. Two sets of wholemounts immunostained with mAb Y-10B [e,f (upper panels); emission maximum: 546 nm] reveal ribosomal plaque domains. RNA staining of the same two sets of wholemounts with YOYO-1, a high-affinity nucleic-binding dye [e,f (lower panels); emission maximum: 509 nm] show that ribosomal plaque domains are unaffected after incubation with buffer alone (e, lower panel), but are eliminated after incubation with RNase (f, lower panel). Scale bars, 10 μm. Modified, with permission, from Ref. [27].

Because isolated axoplasm is a visco-elastic solid that retains its shape, it is referred to as an 'axoplasmic wholemount'. Periaxoplasmic ribosomal domains appear plaque-like at the surface of the wholemount ('periaxoplasmic plaques'; Fig. 1), and their structural correlates appear in ESI as a dark matrix. The thinness and intermittent distribution of periaxoplasmic plaques make their detection by random search in conventional EM difficult, but not impossible [18]. Limited cortical ribosome distributions have been described after serial sectioning of sensory fibers [17]. Recently, confocal microscopy of myelinated fibers in rat nerve rootlets has revealed punctate cortical immunofluorescence of the translational co-factor eIF5, ribosomal L4 protein and rRNA [33].

It now appears that there is specific targeting of RNA to periaxoplasmic domains. For example, full-length or truncated 5′ sequences of neuronal BC1 RNA (an RNA polymerase III transcript) localize to cortical domains after microinjection into the Mauthner cell body, but a truncated 3′ sequence of

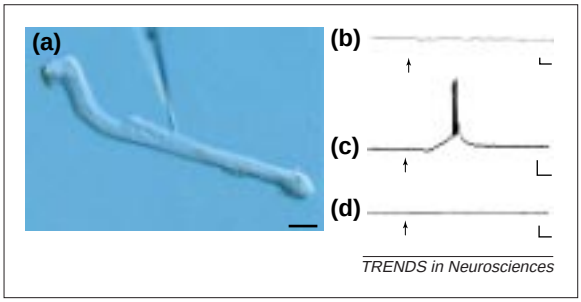

Fig. 2. Expression and insertion into the plasma membrane of a functionally active heterologous conopressin receptor in isolated snail axons. Isolated axons of *Lymnaea* neurons were injected *in vitro* with distilled water or mRNA for a G-protein-coupled conopressin receptor; a few hours later they were assessed for a functional response. (a) Injection into an isolated axon. Scale bar, 10 μm. (b–d) Electrical recordings of isolated axons before and after application of exogenous conopressin (arrows). (b) Conopressin elicits no response in a control axon injected with distilled water (the downward deflection at the start of the application represents a pressure artifact). Scale bars, 2 s (x-axis) and 10 mV (y-axis). By contrast, in an isolated axon injected with the mRNA (c), conopressin elicits a large and prolonged depolarizing response generating a burst of action potentials. (d) Intra-axonal injection of the G-protein inhibitor GDP-α-S suppresses the response to conopressin. Scale bars in (c,d), 5 s (x-axis) and 10 mV (y-axis). Modified, with permission, from Ref. [35].

BC1 RNA, and other non-cytoplasmic or nonsense RNAs of similar lengths, are not transported [34]. Thus, targeting depends on a specific 5′ sequence. RNA localization in plaque domains is mediated by a two-step transport process, initially requiring microtubules for rapid long-range axial transport, followed by local actin-dependent radial translocation.

Smaller ribosome-containing plaque-like formations are also present in the squid giant axon, where they are randomly distributed within axoplasm (at least in young squid), frequently in close proximity to mitochondria [22,24]. Vesicles and cisterns with smooth membranes are present near squid axoplasmic polysomes; membrane-bound ribosomes have not been observed [22–24], although there are examples of ribosomes being attached to axonal ER [18,27]. The lack of a morphologically detectable rough ER within axons does not mean that integral membrane proteins are not synthesized axonally. Indeed, when cultured motor axons (acutely separated from their cell bodies) of the snail *Lymnaea* were microinjected with a heterologous mRNA encoding a conopressin receptor [35], the newly synthesized receptor was present in the membrane and was functionally active (Fig. 2). These axonal capacities raise several fundamental questions worthy of future investigation.

Polysomes have also been isolated from synaptosomes derived from squid photoreceptor terminals [36] and found to be closely associated with mitochondria [26,37]. This spatial proximity is especially interesting in view of recent data demonstrating the local presynaptic synthesis of several mitochondrial proteins whose genes are in the nucleus (i.e. nuclear-encoded mitochondrial proteins) [38].

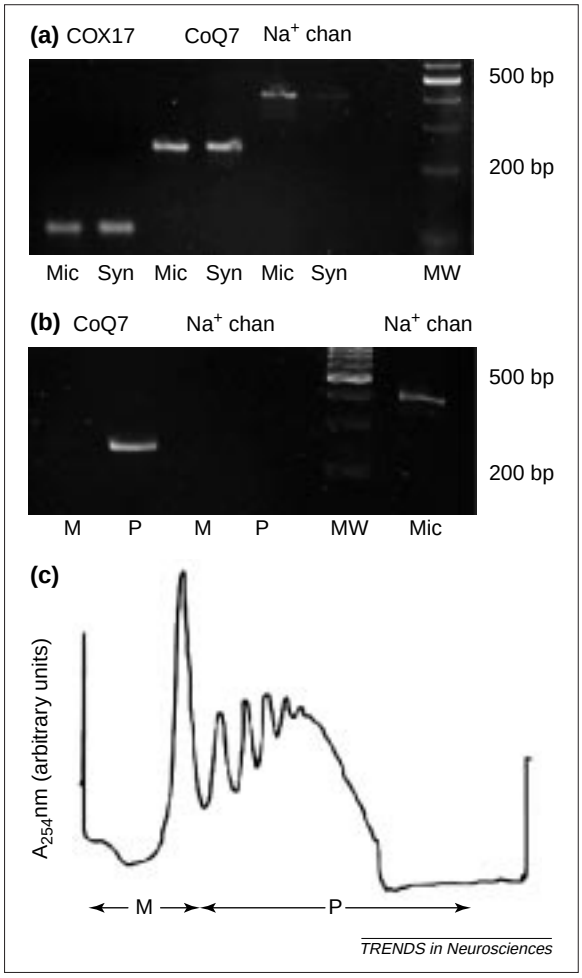

Fig. 3. Nuclear-encoded mitochondrial proteins are translated in squid presynaptic synaptosomes. (a) RT–PCR amplicons of nuclear-encoded mitochondrial proteins (COX 17 and CoQ 7) are present in squid optic lobe microsomes (Mic) and synaptosomes (Syn), but amplicons of the voltage-sensitive Na^+ channel (Na^+ chan) are only present in microsomes. The latter observation indicates that synaptosomes are not contaminated by microsomes. (b) Amplicons of CoQ 7 are associated with synaptosomal polysomes (P) but are absent from the monomer fraction (M). This indicates that CoQ 7 mRNA is being translated. Purified synaptosomal polysomes were fractionated by sedimentation on a sucrose density gradient, and their optical absorption profile (A_{254} nm) was recorded in arbitrary units using a gradient fractionator (c). The absence of amplicons generated from gene-specific primers for the voltage-sensitive Na^+ channels from either P or M, but their presence in microsomes, confirms the purity of synaptosomal polysomes. Abbreviation: MW, molecular weight markers. Modified, with permission, from Ref. [37].

Axonal and presynaptic mRNAs and translation products

Earlier demonstrations of the presence of a heterogeneous family of mRNAs in the squid giant axon [39,40], and the complexity of these mRNA sequences [41], led to the identification of several mRNAs [42]. These included those encoding β-actin and β-tubulin [43], neurofilament proteins [22], enolase [44], kinesin [45] and a novel protein [46]. The relative abundance of axoplasmic mRNAs was markedly different from that in the corresponding cell bodies, thus indicating the occurrence of selective sorting [42]. Squid axoplasm was also shown to contain all species of tRNA [47] and the complete set of protein factors required for protein synthesis [48].

Squid synaptosomal mRNAs derived from photoreceptor terminals code for several nuclear-encoded mitochondrial-associated proteins [38], including cytochrome oxidase subunit 17, propionyl-CoA carboxylase, dihydrolipoamide dehydrogenase, coenzyme Q subunit 7 and heat shock protein 70 (HSP 70; a molecular chaperone). Some of these proteins are synthesized in nerve terminals, as shown by the presence of the coding mRNAs on purified presynaptic polysomes [38] (Fig. 3). Mitochondria-associated proteins account for a large fraction of the proteins synthesized by squid synaptosomes [38]. More recently, a proteomic investigation of metabolically labeled presynaptic synaptosomes [49] confirmed the *de novo* synthesis of ~80 protein species, including nuclear-encoded mitochondrial proteins, cytoskeletal proteins, various enzymes and HSP 70.

The data from metabolically labeled presynaptic synaptosomes have important implications. They indicate that mitochondrial maintenance could be supported by local mechanisms, rather than being exclusively dependent on somatic sources. In addition, they focus attention on the mutual relationship that exists between presynaptic translation processes and local energy supply. In this context, the inhibition of squid synaptosomal protein synthesis by high cytosolic Ca^{2+} concentrations [50] suggests that, in addition to other mechanisms, a sustained increase in presynaptic Ca^{2+} could trigger a deleterious cascade, by impairing mitochondrial energy output and eventually leading to degeneration of the nerve terminal.

Consistent with the situation described for the giant axon [42], notably with regard to the different sets of neurofilament proteins synthesized in squid axoplasm (M. Crispino *et al.*, unpublished) and synaptosomes [51], analyses of RNA transcripts or translation products in vertebrate axons have highlighted the selectivity of the sorting process. Although mRNAs encoding the neurofilament proteins NF-M and NF-L have been identified in mature axons, such as the Mauthner axon [52] and neurohypophyseal axons [53], most studies on vertebrate preparations have focused on immature axons of cultured explants or dissociated neurons [31,32,54–58]. It is now clear that growing neurites and growth cones contain β-actin mRNA, but α-tubulin and γ-actin mRNAs are excluded [31,32,56]. Polysomes isolated from sensory axons regenerating *in vitro* are also associated with β-actin and NF-L mRNAs [32]. Indications that β-actin and β-tubulin, but not α-tubulin, are synthesized locally [55] have been recently confirmed by immunoprecipitation of newly synthesized translation products [58]. Nonetheless, the synthesis of axoplasmic α-tubulin and β-tubulin by goldfish Mauthner axons and rat spinal root fibers [59] suggests that findings

concerning immature axons *in vitro* do not necessarily apply to mature intact axons. In immature axons, mRNAs appear as punctate structures closely associated with [31,32], and actively transported along [56], microtubules. Indeed, neurotrophin-3 induces a short-latency cAMP-dependent localization of β-actin mRNA in growth cones that is mediated by microtubules [57].

Less conclusive data are available for mammalian presynaptic mRNAs [5], but recently mRNA encoding the presynaptic GAT-1 protein was found to be significantly enriched in rat synaptosomes [60]. Interestingly, as demonstrated in cultured *Aplysia* sensory neurons [61], target interaction and synapse formation result in accumulation of mRNAs destined for translocation, and positively affect the stability of translocated mRNAs.

Local translation processes in axonal growth, axonal regeneration and synaptic plasticity

Translational machinery is present in growing axons [54,55], where it plays crucial roles in axon guidance, regeneration and synaptic plasticity. For example, isolated retinal growth cones immediately lose their ability to turn in a chemotropic gradient of netrin-1 or Sema3A when translation is inhibited. Inhibition of protein synthesis does not affect axon elongation [62], but it promotes growth-cone retraction if transport between cell bodies and axons is interrupted [33]. Moreover, netrin-1 and Sema3A activate initiation factors and stimulate a marked rise in protein synthesis within minutes [62]. Thus, the directional growth potential of immature axons appears to depend on local synthesis.

A more marked response occurs in the adult sciatic nerve, in which local inhibition of protein synthesis reduces the regeneration rate by as much as 60% [63]. The site of inhibition is clearly local because the growth rate is unaffected when the region proximal to the lesion is treated with cycloheximide. In addition, nerve transection induces a delayed, but marked, elevation in protein synthesis that is restricted to the extremity of regenerating axons [64] and is under local regulation [5,65].

Importantly, in primary cultures of *Aplysia* neurons, axons deprived of their cell bodies can grow and develop synaptic connections for up to three days, an effect reversibly blocked by anisomycin [66]. In addition, the formation of long-term facilitation is crucially dependent on presynaptic protein synthesis [67,68]. A comparable dependence has been reported for the induction of long-term facilitation in the crayfish [69]. Clearly, these observations indicate that synaptic plasticity is supported by local translation processes.

Conclusions

The presence of endogenous protein-synthesizing machinery in axons and nerve terminals creates an entirely new perspective in studies of the biology and pathobiology of neurons. As these and other data [5] suggest, the growth, differentiation, maintenance, plasticity and pathobiology of axons and nerve endings must now also be viewed in the context of local mechanisms that endow axons and nerve terminals with the capacity to respond in a semi-autonomous manner to local challenges [70]. This new frame of reference opens the door to a wide range of novel experimental approaches in the quest for a better understanding of neuronal cell biology.

Perhaps one of the more intriguing questions concerning axonal and presynaptic protein synthesis centers on mechanisms of local regulation. Such mechanisms might not be limited to the cognate cell body as an exclusive source of RNAs, but could also include the glial cell as a potential source, as indicated by evidence of local transcription of axonal RNAs [5,19]. The possibility that periaxonal glial cells could transfer RNAs, including mRNA, to the subjacent axon is clearly worthy of future investigation, as it might shed light on local modulatory mechanisms.

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