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·Original Article·

Peripheral nerve axons contain machinery for co-translational secretion of axonally-generated proteins

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ABSTRACT

The axonal compartment of developing neurons and mature peripheral nervous system (PNS) neurons has the capacity to locally synthesize proteins. Axonally-synthesized proteins have been shown to facilitate axonal pathfinding and maintenance in developing central nervous system (CNS) and PNS neurons, and to facilitate the regeneration of mature PNS neurons. RNA-profiling studies of the axons of cultured neurons have shown a surprisingly complex population of mRNAs that encode proteins for a myriad of functions. Although classic-appearing rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (ER) and Golgi apparatus have not been documented in axons by ultrastructural studies, axonal RNA profiling studies show several membrane and secreted protein-encoding mRNAs whose translation products would need access to a localized secretory mechanism. We previously showed that the axons of cultured neurons contain functional equivalents of RER and Golgi apparatus. Here, we show that markers for the signal-recognition particle, RER, ER, and Golgi apparatus are present in PNS axons *in vivo*. Co-localization of these proteins mirrors that seen for cultured axons where locally-translated proteins are localized to the axoplasmic membrane. Moreover, nerve injury increases the levels and/or aggregation of these proteins, suggesting that the regenerating axon has an increased capacity for membrane targeting of locally synthesized proteins.

Keywords: rough endoplasmic reticulum; Golgi apparatus; membrane protein; axonal protein synthesis; mRNA transport

INTRODUCTION

Despite early studies of mature rodent hippocampus suggesting that only dendrites and not axons are capable of synthesizing proteins, work over the last decade has clearly shown that vertebrate axons, particularly growing and injured axons, contain ribosomes, translation factors, and mRNAs[1]. A wealth of earlier data existed for axonal protein synthesis in invertebrate neurons, but it was argued that incomplete polarity allowed mRNA templates and translational machinery to diffuse into these processes[2]. In the few instances where axonal mRNAs were identified in vertebrate neurons, no ribosomes were seen in ultrastructural studies, raising questions about the relevance of these axonal mRNAs. Elegant studies from the Singer and then Bassell, Hollenbeck, and Campenot groups in the mid-to-late 1990s showed that axonal mRNAs can be used in vertebrate neurons to locally generate proteins. Later studies in rodent sensory neurons, which can be cultured from adult animals, showed that this capacity for intra-axonal protein synthesis is maintained into adulthood. In developing neurons, most of the functional studies of axonal protein synthesis have pointed to a role in axon pathfinding. In adult neurons, axonally-generated proteins contribute to retrograde signaling and regeneration after injury[3]. However, these functions in adult sensory neurons are likely to be expanded as we learn

more of the proteins generated in axons. For example, there is evidence that capsaicin induces translation in peripheral axons for the propagation of neuropathic pain[4, 5]. Moreover, recent work has shown that retrograde transport of herpes simplex virus from axons but not viral entry into axons requires axonal protein synthesis[6].

Advances in detection and profiling methodologies for nucleic acids have precipitated an increase in the number of known axonal mRNAs by many-fold. Recent publications on sensory neurons, hippocampal neurons and retinal ganglion cell axons have shown that hundreds to a few thousand mRNAs are localized in the axons under different growth conditions[7-9]. A surprising revelation from these axonal RNA profiles is that several of the mRNAs encode membrane and secreted proteins that would presumably need access to the endoplasmic reticulum (ER) and Golgi apparatus for co-translational secretion. However, earlier electron microscopy (EM) studies failed to demonstrate the classical ultrastructural appearance of rough endoplasmic reticulum (RER) and Golgi apparatus in axons[10-13]. Considering this paradox, we previously asked if cultured neurons contain any protein markers classically associated with ER, RER, and Golgi apparatus. Although we are still uncertain of the ultrastructural appearance of these axoplasmic organelles, we clearly showed that growing sensory and retinal ganglion cell axons have functional equivalents of RER and Golgi apparatus[14]. Several publications have pointed to the role of axonally-generated proteins in axon regeneration in the mature rodent PNS[15-18]. Here, we asked if mature rat PNS axons contain ER, RER, and Golgi proteins, and if these organelles are regulated by regeneration.

MATERIALS AND METHODS

Surgery

Male Sprague-Dawley rats (150–225 g) were used in this study. For nerve injury, rats (anesthetized with isoflurane) were subjected to a sciatic nerve crush at mid-thigh as previously described[19]. Seven days after injury, both ipsilateral and contralateral nerves were removed and a segment from the proximal thigh (i.e., proximal to the crush injury) was fixed in 4% paraformaldehyde overnight and processed for cryoprotection in 30% sucrose[20]. All surgery and experiments were conducted under IACUC-approved protocols at Alfred I. duPont Hospital for Children.

Immunofluorescence

For immunostaining, nerves were processed for cryosectioning at 10 μm. Sections were collected on Superfrost^{plus} glass slides (Fisher Scientific) and stored at –20°C until use. All subsequent steps were carried out at room temperature unless specified otherwise. Sections were thawed at room temperature and then treated to quench auto-fluorescence with 20 mmol/L glycine (BioRad) for 30 min (replenishing the solution every 10 min) and 0.25 mol/L sodium borohydride (Sigma) for another 30 min. Sections were then rinsed in phosphate-buffered saline (PBS), permeabilized with PBS + 0.2% Triton X-100 for 15 min, generously rinsed in fresh PBS, blocked with 10% serum to avoid non-specific binding, and then processed for immunofluorescent labeling as previously described[14]. The following antibodies were used: mouse anti-SRP54 (1:200; BD-Transduction Lab.), goat anti-ribophorin II (1:100; Santa Cruz Biotech.), rabbit anti-TRAPα (1:300; Christopher Nicchitta, Duke Univ., Durham, NC), mouse anti-calreticulin (1:200; BD-Transduction Lab.), rabbit anti-ERp29 (1:200; Affinity Bioreagents); mouse anti-PDI (1:200; Stressgen); mouse anti-KDEL-receptor (1:800; Stressgen), mouse anti-GM130 (1:200; Abcam or 1:50; BD Transduction), mouse anti-TGN38 (1:200; Affinity Bioreagents), rabbit anti-SERCA (1:100: Jonathan Lytton, University of Calgary, Canada), chicken anti-neurofilament (1:800; Chemicon), and rabbit anti-peripherin (1:500; Chemicon). Secondary antibodies consisted of FITC-, Alexa 555- or Cy5-conjugated donkey anti-mouse, anti-chick, or anti-rabbit IgGs (1:400; Jackson Immunoresearch). Samples were mounted with Vectashield (Vector Labs) and imaged using confocal microscopy with a Leica TCS/SP2 or Zeiss LSM700 confocal microscope. Image processing was performed using the Leica confocal or Volocity software packages as indicated below. ImageJ was used to quantitate protein signal intensity using images matched for exposure, gain, offset and post-processing. Student's *t*-test was used to calculate the significance for signal intensities shown in Fig. 3.

RESULTS

PNS Axons Contain ER, RER, and Golgi Apparatus Proteins *In Vivo*

Most proteins that need to be targeted for membrane localization or secretion are co-translationally targeted

through the action of the signal-recognition particle (SRP) that halts ribosome progression on the encoding mRNA. The SRP recognizes the nascent polypeptide for targeting through an N-terminal signal peptide sequence and the SRP-mRNA-ribosome complex is moved to the ER in areas that constitute the RER[21]. Newly-synthesized proteins then have access to the ER lumen for transit to the Golgi and cell surface. We had previously demonstrated that the axons of cultured dorsal root ganglion (DRG) and retinal ganglion cell neurons contain protein constituents of these organelles ranging from the SRP to RER to ER to *cis*- and *trans*-Golgi apparatus[14]. Each of the antibodies used in that study was validated by immunoblot showing high specificity for the anticipated molecular-weight proteins. Here, we have used immunofluorescence with these validated antibodies to components of the co-translational secretory apparatus proteins to ask if axons contain RER, ER and Golgi apparatus *in vivo*. By taking high-resolution optical sections, with confocal microscopy of longitudinal nerve sections, we were able to optically isolate the axoplasm from surrounding Schwann cells and other non-neuronal cells (Figs. 1 and 2).

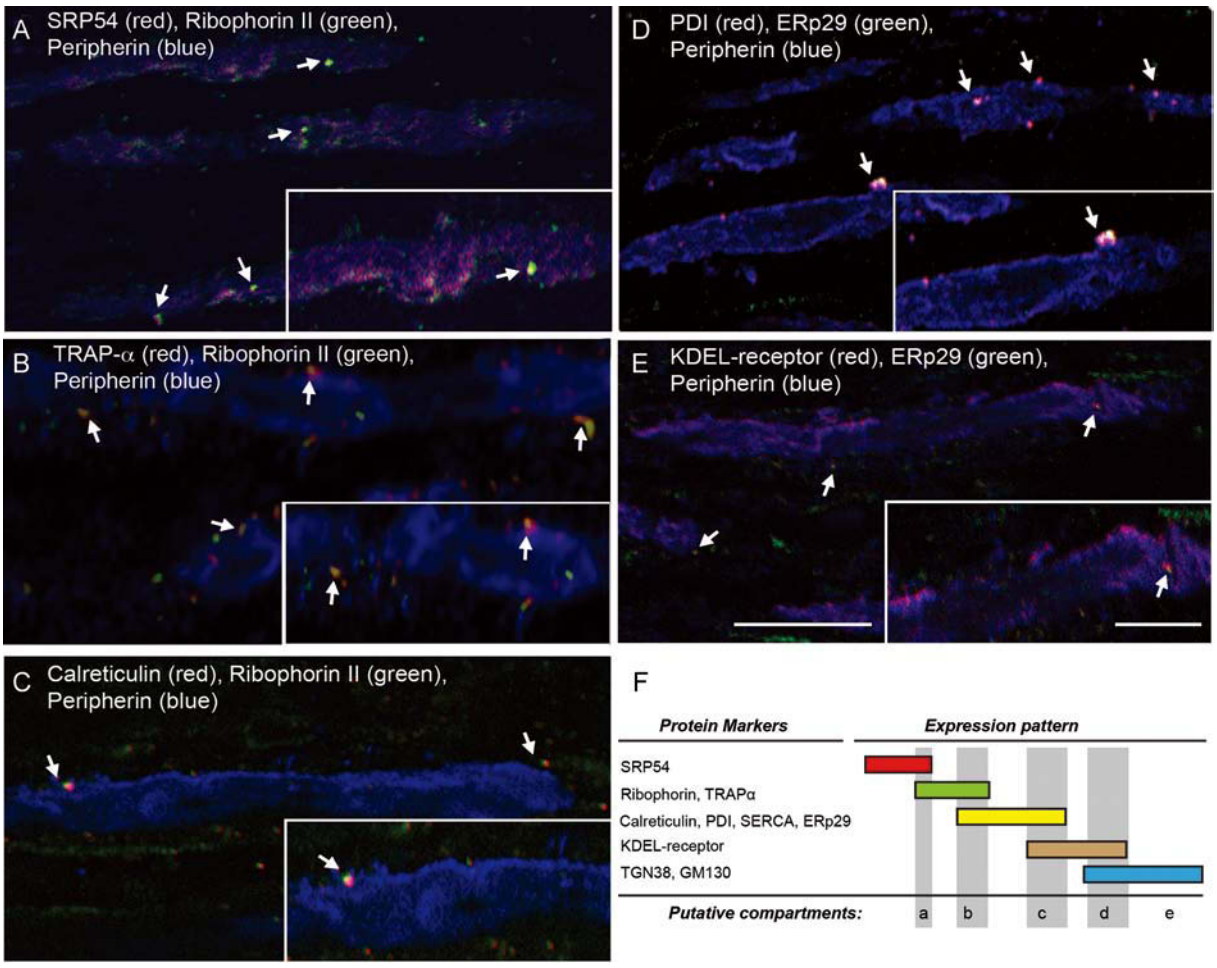

Fig. 1. SRP and ER components co-localize in axons *in vivo*. A: SRP54 shows focal co-localization (arrows) with ribophorin II. B: Co-labeling for TRAPα and ribophorin II, and overlap at the periphery of the axon (arrows) in some regions. C: Signals for calreticulin and ribophorin II show overlap along the axon (arrows). D: Three-dimensional average projection of axons from a nerve stained for PDI and ERp29. E: Sciatic nerve showing co-localization for KDEL-receptor and ERp29 in the regenerating axon (arrows). Signals for peripherin are shown in blue. F: Summary of SRP, RER, ER, and Golgi apparatus protein co-localization in PNS axons. 'Putative compartments a–e' refer to the regions of overlapping colocalizations that would be seen for RER, ER, ER to *cis*-Golgi trafficking, *cis*-Golgi, and *trans*-Golgi. Scale bars, 10 μm for main panels and 5 μm for inset panels. Images were processed using the Leica confocal software package.

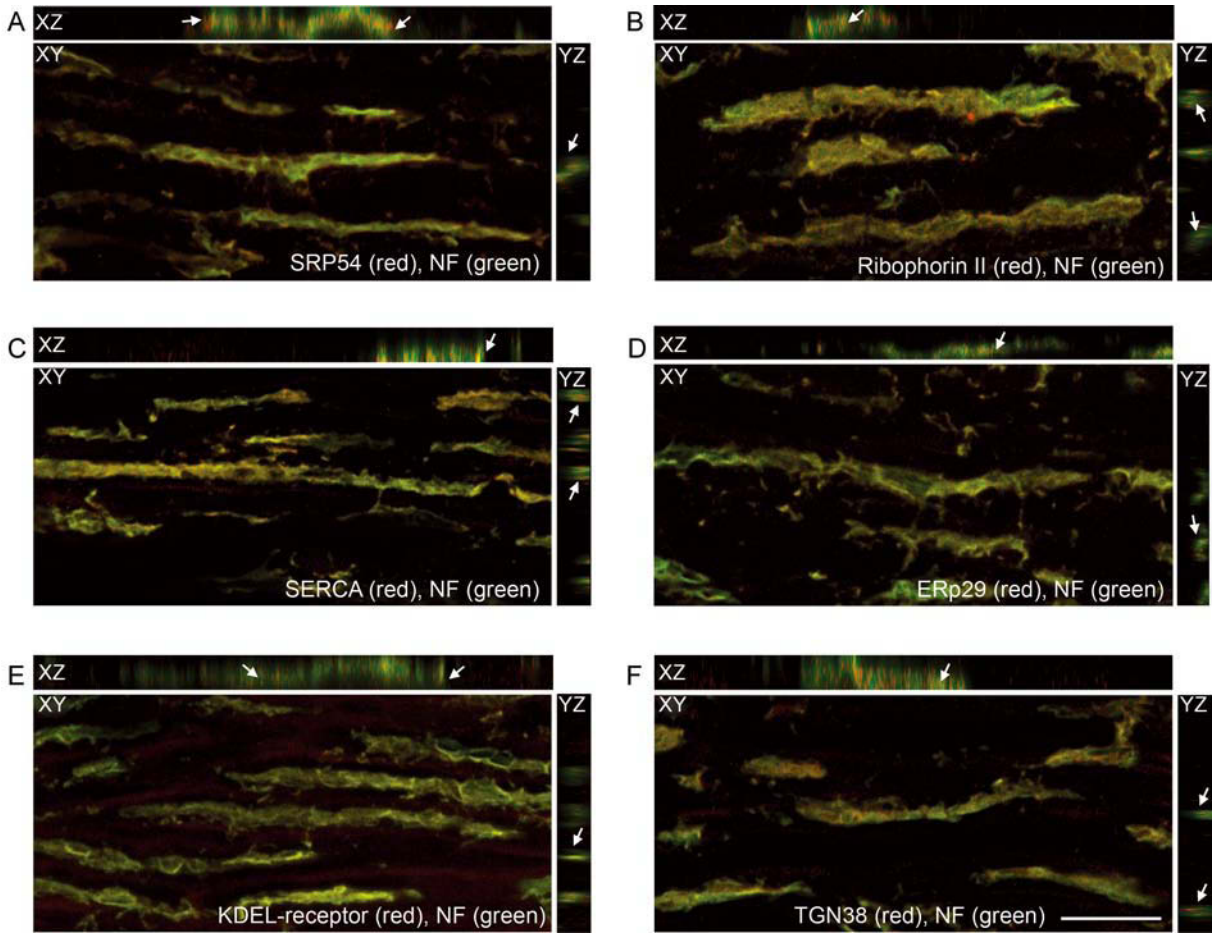

Fig. 2. SRP, ER and Golgi proteins are intra-axonal *in vivo*. Sections of sciatic nerve from mid-thigh, immunostained with the indicated antibodies. Confocal sections are shown as orthogonal XZ (top) and YZ (side) projections for the corresponding XYZ image stack. Arrows indicate clear intra-axonal signals for SRP54 (A), ribophorin II (B), SERCA (C), ERp29 (D), KDEL receptor (E), and TGN38 (F) overlapping neurofilament (NF) signals in the orthogonal projections. Scale bar, 10 μm. Images processed using the Perkin-Elmer Volocity software package.

We initially asked if the components needed for ribosome docking with the ER are present in axons of the mature, uninjured sciatic nerve. SRP54, a protein constituent of the SRP[22], co-localized with ribophorin II, an ER membrane protein involved in the glycosylation of nascent proteins[23], in the sciatic nerve sections and these signals focally overlapped with the peripherin signal used to mark axons (Fig. 1A). Newly-synthesized proteins enter the ER lumen by interaction with the translocon-associated protein (TRAPα)[24]. TRAPα and ribophorin II showed focal co-localization overlapping with peripherin (Fig. 1B). The ribophorin II immunoreactivity overlapped with calreticulin protein, an ER chaperone protein that plays a role in protein folding[25] (Fig. 1C). The lumenal ER chaperone protein ERp29 also showed focal overlap with protein disulfide isomerase (PDI), which plays a role in the formation of disulfide bridges in the ER[26] (Fig. 1D). Finally, we asked if the axons contain the KDEL receptor, a lumenal ER protein that shuttles resident ER proteins from *cis*-Golgi back to the ER[27]. Focal immunoreactivity for the KDEL receptor overlapped with peripherin signals and these co-localized with ERp29 immunoreactivity in the axons (Fig. 1E). Markers for *cis*- and *trans*-Golgi proteins similarly showed axonal localization, but did not co-localize with any of the ER proteins except for KDEL receptor that shuttles between the ER and *cis*-Golgi (data not shown).

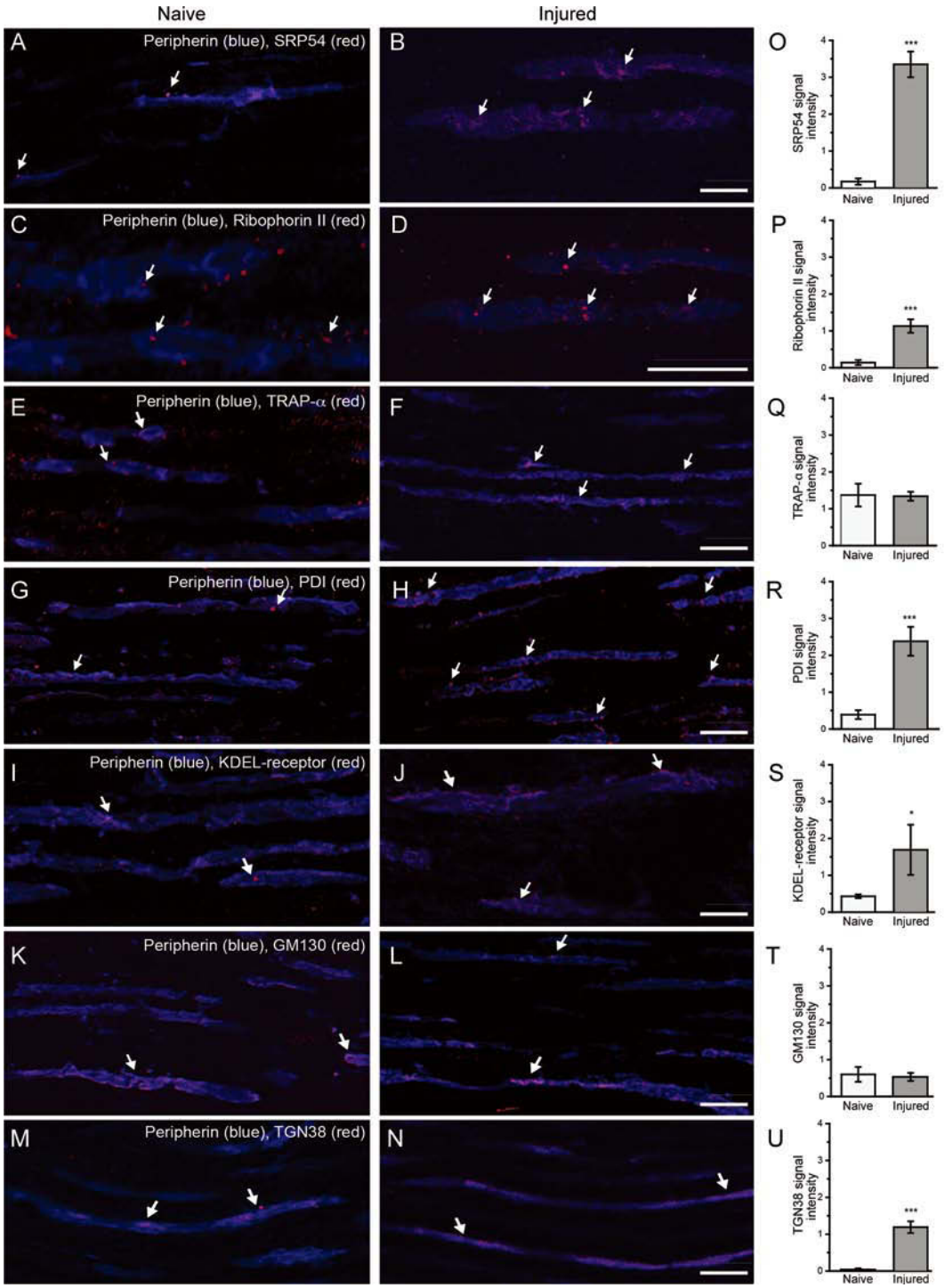

Fig. 3. Distribution of co-translational targeting machinery in the axons of sciatic nerve after injury. Seven days after injury, protein levels were assessed in naïve *versus* 7-day injured sciatic nerve as indicated by immunostaining with antibodies to peripherin and SRP54 (A, B), ribophorin II (C, D), TRAPα (E, F), PDI (G, H), KDEL receptor (I, J), GM130 (K, L), and TGN38 (M, N). Each row shows representative exposure-matched merged image-pairs showing peripherin signals in blue and SRP, RER, ER and Golgi proteins in red. The immunoreactivity for SRP54, ribophorin II, PDI, KDEL receptor, and TGN38 were relatively increased in the axons of injured nerve compared with those of naïve nerve. Quantitation of the indicated immunoreactivity overlapping with peripherin is shown in panels O–U. *$P \leq 0.05$; ***$P \leq 0.001$ for injured *vs* naïve by Student's *t*-test; Scale bars, 10 µm. Images were processed using the Leica confocal software package.

A summary of these SRP-RER-ER-Golgi marker co-localization studies from sciatic nerve axons is schematically illustrated in Fig. 1F. These data showed the trend of protein co-localization that we visualized in the axons of cultured DRG neurons[14], and strongly argue that PNS axons have the capacity to appropriately target locally-synthesized membrane and secreted proteins *in vivo*. Although the localization studies shown in Fig. 1 are based on single optical planes, there remained a possibility that these signals were derived from closely-apposed non-neuronal cells in these nerves. Thus, we examined orthogonal sections from Z stacks to determine if these RER, ER, and Golgi apparatus proteins co-localized in the XZ and YZ planes and found clear co-localization of these proteins with neurofilament in both planes (Fig. 2).

Protein-trafficking Machinery in the Axons of Rat Sciatic Nerve Is Altered by Injury

The above data suggested that PNS axons contain the necessary components for co-translational secretion of axonally-synthesized proteins. Several lines of evidence indicate that PNS axons synthesize proteins *in vivo*[17, 18, 28]. Using a viral transduction system coupled with an axonally-targeted fusion protein, we recently showed that the mRNA for neuronal membrane protein 35 (NMP35) is localized to PNS axons *in vivo* and its protein product appears to be inserted into axoplasmic membrane *in vivo*[20]. In our hands, locally-generated NMP35 increases axon growth from cultured DRG neurons. Thus, we asked if nerve injury with the subsequent spontaneous axonal regeneration in the PNS might trigger changes in the RER, ER or Golgi protein profiles in axons.

Nerve-crush injury was used to activate regeneration programs in peripheral nerve. We examined the nerves at 7 days post-crush, a time when axons have recovered from the initial injury and started regenerating. In exposure-matched optical planes of naïve, uninjured (contralateral), and injured/regenerating sciatic nerve, there were clear changes in the relative intensity of immunoreactivity for several of the SRP, RER, ER, and Golgi apparatus proteins tested (Fig. 3). SRP54, ribophorin II, PDI, KDEL receptor, and the *trans*-Golgi network protein 38 (TGN38) showed increased immunoreactivity in the injured/regenerating compared to the uninjured sciatic nerve axons (Fig. 3A–D, G–J, M–N). This change in immunoreactivity was not as apparent for the other proteins tested. However, the axonal signals for the 130-kDa *cis*-Golgi marker protein (GM130) showed increased aggregation in the injured/regenerating compared to the uninjured sciatic nerve axons (Fig. 3K, L). Quantitation of the intra-axonal signals for these proteins confirmed a significant increase in SRP54, ribophorin II, PDI, KDEL receptor, and TGN38 immunoreactivity in the injured compared to naïve nerve sections (Fig. 3O, P, R, S, and U). There were no significant differences between injured and naïve for intra-axonal TRAPα and GM130 immunoreactivity (Fig. 3Q and T). Taken together, these data suggest that injury triggers a shift in the ability of PNS axons to co-translationally direct new proteins for membrane targeting and secretion.

DISCUSSION

Although previous EM studies have not detected the classical ultrastructure of the ER and Golgi apparatus in distal axons, previous work has clearly shown that the axons of cultured neurons have functional equivalents of RER, ER, and Golgi[14]. Here, we showed that PNS axons contain co-localizing SRP, RER, ER, and Golgi apparatus protein immunoreactivity *in vivo* that is comparable to what we previously saw in the axons of cultured neurons[14]. This strongly suggests that axonally-synthesized proteins can gain access to a classical co-translational secretory mechanism in the intact animal. In addition to ruling out the potential confounds that culture conditions present for localization studies like these, the data further emphasize that these structures can be localized centimeters away from the cell body. Indeed, we estimate that the mid-thigh region analyzed here was 5–6 cm from the L4–5 DRGs and anterior horns where the cell bodies of these sciatic nerve axons reside.

The localization studies performed here do not address the functions of the intra-axonal RER, ER and Golgi complex, but there are reports that link locally-translated proteins to transmembrane signaling events. Work from the Flanagan lab showed that locally-synthesized EphA2 protein alters the trajectory of commissural axons in the developing spinal cord[29]. The classic function of this receptor in axonal pathfinding would require its membrane localization. There is increasing evidence for localized translation in adult neurons *in vivo*. The Fainzilber lab

recently showed that the translation of axonally-targeted importin β1 mRNA is needed for PNS injury-induced gene regulation in the DRG *in vivo*[17]. Donnelly *et al*. showed that localized translation of axonal β-actin and GAP-43 mRNAs is needed for axonal outgrowth after nerve injury[15]. The Jaffrey lab used a Sindbis viral transduction approach to show that adult spinal axons can synthesize proteins[30]. Though none of these studies in adult systems addressed membrane or secreted proteins, there is circumstantial evidence for the function of these co-translational secretory mechanisms in PNS axons *in vivo*. Toth *et al*. (2009) showed that CGRP mRNA is enriched in axons after injury, and localized translation of CGRP triggers the migration of Schwann cells *via* a mechanism requiring the expression of CGRP-receptor components in these glial cells[31]. This effect would require that the locally-synthesized CGRP protein has access to a secretory mechanism. Moreover, the protein product of an axonally-targeted NMP35-AcGFP fusion protein mRNA is clearly inserted into the axoplasmic membrane of sciatic nerve axons, based on confocal imaging of nerve sections and teased nerve preparations[20]. This would again require access to a secretory mechanism, suggesting that the RER, ER, and Golgi apparatus protein constituents that we showed here to be localized in PNS axons *in vivo* are undoubtedly functional. In addition, mRNAs encoding ER chaperone proteins localize to PNS axons *in vitro* and *in vivo*[32], and translational upregulation of these could provide a means for increasing the axon's capacity for co-translational secretion of locally-generated proteins after injury[33].

As noted above, a few studies indicate that spinal cord axons can translate proteins, both in development[29, 34] and in the adult[30, 35]. Importantly for the theme of this edition of *Neuroscience Bulletin*, Willis *et al*. (2011) reported that sensory axons in adult mouse spinal cord show injury-induced translation of a GFP reporter transgene targeted to axons through the 3'UTR of β-actin[36]. There is a clear need to determine if membrane localization and secretion occurs in axons after spinal cord injury, both for the ascending axons studied by Willis *et al*. (2011)[36] and descending axons that show overall poorer regeneration after injury.

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