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Lysophosphatidic acid differentially regulates axonal mRNA translation through 5' UTR elements

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Abstract

Sensory neurons transport a complex population of mRNAs into their axons, including many encoding ER chaperone proteins. Transport of the mRNA encoding the ER chaperone protein calreticulin is regulated through 3'UTR elements. In other cellular systems, translation of chaperone protein mRNAs can be regulated by ER stress. Here, we have asked if the translation of axonal calreticulin mRNA is regulated in a different manner than its transport into axons. Treatment with lysophosphatidic acid, which is known to trigger axon retraction and stimulate ER Ca²⁺ release, caused a translation-dependent increase in axonal calreticulin protein levels. RNA sequences in the 5'UTR of calreticulin confer this translational control through a mechanism that requires an inactivating phosphorylation of eIF2 α . In contrast to calreticulin, these signaling events do not activate axonal translation through β -actin's 5'UTR. Together, these data indicate that stimulation of ER stress can regulate specificity of localized mRNA translation through 5'UTR elements.

Keywords

Chaperone protein; Calreticulin mRNA; Translational control; ER stress

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Introduction

The distances separating terminal axons from their cell body require that translation of mRNAs within these axons be regulated independently from cell body. This provides the distal axon with autonomy to rapidly respond to stimuli in its environment. Localized protein synthesis contributes both to developmental and regenerative axon growth (Donnelly et al., 2010). Protein synthesis can be regulated globally by modulating translation initiation factor activity, or at the level of individual mRNAs through RNA binding proteins modifying localization, sequestration from the translational machinery (e.g., through localization) or directly activating translation (Costa-Mattioli et al., 2009). Several groups have shown that axonal growth-regulating stimuli can also modulate localized protein synthesis. For example, an attractive gradient of netrin-1 or BDNF induces local translation of β -actin mRNA in axonal growth cones (Leung et al., 2006; Yao et al., 2006). In contrast, the growth cone repulsive cue Semaphorin3A (Sema3A) induces axonal synthesis of the small GTPase RhoA (Wu et al., 2005). The net effect of increasing these molecules could result in axon extension or retraction by modifying microfilament polymerization in the growth cone.

cis-Elements in the untranslated regions (UTR) of several axonally localized mRNAs, including calreticulin, appear to play central roles in post-transcriptional regulation through subcellular mRNA localization, translational efficiency, and/or mRNA stabilization (Andreassi and Riccio, 2009; Chatterjee and Pal, 2009). Such UTR *cis*-elements provide binding sites for *trans*-acting factors that are needed for the regulation of mRNA transport and translation. For example, the *cis*-elements in 5'UTR of axonally localized κ -opioid receptor confer netrin-dependent translation (Bi et al., 2007). In non-neuronal cells, translational regulation of the mRNAs encoding Hsp70 and Grp78/BiP chaperone proteins occurs through 5'UTR internal ribosome entry sites (IRES) (Macejak and Sarnow, 1991; Rubtsova et al., 2003).

In this study we used a physiological stress stimulus, lysophosphatidic acid (LPA), which is known to cause axonal retraction through proteolysis (Campbell and Holt, 2001), to examine the effects of stress on axonal mRNA translation. LPA is a bioactive phospholipid in serum that mediates multiple cellular responses including actin Ca^{2+} mobilization and cytoskeletal rearrangements (Moolenaar et al., 2004; Ye et al., 2002). Although it was thought to diffuse through the lipid bilayer to impart its intracellular effect, LPA seems to act predominantly by binding to one of four transmembrane receptors (LPA₁₋₄ receptors) (Moolenaar et al., 2004). LPA₁ receptor is a main subtype expressed in dorsal root ganglion (DRG) neurons (Elmes et al., 2004). LPA is released after tissue injury and is suggested to play a role in the development of neuropathic pain (Tokumura, 2002). Our data indicate that LPA stimulates translation of axonal but not cell body calreticulin mRNA through phosphorylation of eIF2 α and mechanisms that require the 5'UTR of calreticulin mRNA.

Results

Axonal calreticulin protein levels increase with LPA treatment

We have previously shown that inhibition of the smooth endoplasmic reticulum Ca-ATPase (SERCA) with thapsigargin increases synthesis of calreticulin protein (Wang et al., 2007). Here, we sought to determine the mechanism for this potential translational regulation of axonal calreticulin mRNA, by linking extracellular events with potential axonal mRNA translation. We used LPA as a more physiological stimulus since this has been shown to induce growth cone collapse in DRG neurons and cause release of ER Ca^{2+} through the activation of PLC (Elmes et al., 2004).

To determine if LPA can similarly increase axonal levels of calreticulin protein, we initially used immunostaining to detect axonal calreticulin in axons of cultured DRG neurons. Immunostaining for calreticulin showed an increase in signals in distal axons after 4 h treatment with LPA (Figs. 1A,C and Supplemental Figure S1). In our previous studies, the calreticulin antibody used here proved highly specific (Wang et al., 2007; Willis et al., 2005). Quantifying these signals showed a 2-fold increase in the axonal calreticulin immunoreactivity with the LPA treatment (Fig. 1C). Cell body immunoreactivity for calreticulin also increased over this period but did not reach statistical significance in our studies (Figs. 1B,C). Schwann cells also showed a clear increase in calreticulin protein immunoreactivity with LPA exposure (Fig. 1C). The LPA induced increase in axonal calreticulin immunoreactivity was significantly attenuated by anisomycin (Figs. 1A,C and Supplemental Figure S1), arguing that this is a protein synthesis-dependent event. However, we continued to see an increase in axonal calreticulin immunoreactivity upon LPA treatment in cultures where microtubule-dependent transport was blocked by colchicine (Figs. 1A,C). Together these data suggest that the LPA can trigger an increase in axonal calreticulin levels through protein synthesis-dependent mechanisms.

LPA stimulates translation of calreticulin mRNA in axons

To more directly test if LPA treatment can regulate translation of axonal mRNAs, we asked if axonal calreticulin mRNA shows altered association with polysomes after LPA treatment. As a more global assessment of axonal mRNA translation, we also analyzed distribution of axonal mRNAs encoding the ER chaperone protein Grp78/BiP, the cytoplasmic chaperone protein Hsp70, the mitochondrial chaperone protein Hsp60, and the growth-associated protein GAP-43. For this, 7 d injury-conditioned adult rat DRG neurons were cultured on a porous membrane to allow separation of axonal processes from cell body and non-neuronal cells (Willis et al., 2005). After LPA treatment for 4 h, the axonal compartment was lysed under conditions that maintain mRNA-ribosome association and then processed for fractionation of polysomal and sub-polysomal RNAs. The purity of axonal preparations was verified by the absence of cell body confined transcripts (γ -actin and MAP2) and glial cell-specific mRNAs (GFAP and ErbB3) (Fig. 2A).

RT-qPCR was then used to determine the absolute levels of the individual mRNAs in polysomal vs. sub-polysomal fractions. In untreated cultures, axonal calreticulin mRNA was seen in approximately equal distribution in the polysomal and subpolysomal fractions.

However, axonal calreticulin mRNA fractionated predominantly with polysomes after LPA treatment (Fig. 2C). The amount of RNA extracted from these axonal micro-ultracentrifugation fractions was extremely limited, thus we turned to cell body isolates to test the efficacy of polysomal RNA fractionation. We have previously shown that treating DRG lysate preparations with 50 mM EDTA prior to sucrose gradient fractionation effectively dissociates ribosome subunits (Zheng et al., 2001). Adding 50 mM EDTA to DRG cell body lysates prior to fractionation on sucrose gradients shifted nearly all of the polysomal calreticulin mRNA to the sub-polysomal fraction indicating that we had successfully fractionated polysomal mRNAs with these analyses (Fig. 2B). Interestingly, most of the cell body calreticulin mRNA associates with polysomes under basal conditions with minimal response to LPA (Fig. 2B). This suggests different translational regulations for the cell body and axonally localized calreticulin mRNA pools. Similar to calreticulin mRNA, axonal grp78/BiP mRNA shifted to predominantly polysomal with LPA treatment (Fig. 2C). However, axonal GAP-43, Hsp60, and Hsp70 mRNAs showed no significant redistribution between polysomal and subpolysomal fractions with LPA treatment (Fig. 2C). These data suggest that the response to LPA translationally activates axonal mRNAs encoding ER chaperone proteins.

LPA has been shown to increase cytoplasmic Ca^{2+} levels in DRG neurons through release of ER Ca^{2+} stores (Elmes et al., 2004). Release of ER Ca^{2+} is also seen during ER stress, a process known to induce transcription of mRNAs encoding ER chaperone proteins (Harding et al., 2002). A generalized increase in overall levels of calreticulin mRNA with LPA treatment could explain the increased translation seen above, particularly if the newly transcribed calreticulin mRNA selectively trafficked to the axonal compartment. This seemed unlikely since our cultures included the RNA polymerase II inhibitor DRB. Using quantitative PCR methods, we saw no increase in calreticulin or grp78/BiP mRNAs in the cell body following the same LPA treatment even when DRB was excluded from the medium (Supplementary Table 2). However, mRNA levels of calreticulin and grp78/BiP showed a significant increase after treatment with SERCA inhibitor thapsigargin (Supplementary Table 2), which triggers ER stress through depletion of ER Ca^{2+} (Llewellyn et al., 1996). This did not exclude the possibility of a shift in localization of these mRNAs and, we saw that transport of calreticulin mRNA into these axons is increased by neurotrophin 3 (NT3) and myelin-associated glycoprotein (MAG) in a prior work (Vuppalanchi et al., 2010). To determine if LPA can alter transport of calreticulin mRNA from the cell body, we analyzed axonal mRNA levels after LPA treatment. There was no increase in calreticulin or Grp78/BiP mRNA transport following LPA treatment; indeed, the axonal levels of these mRNAs decreased slightly in response to LPA, although this did not reach statistical significance (Supplementary Table 3). Taken together, these data argue that the shift of axonal mRNAs encoding ER chaperone proteins into the polysomal fraction upon LPA treatment reflects an increase in translational efficiency of these axonal mRNAs.

LPA alters the activity of translation initiation factor eIF2 α in axons

The above studies point to LPA as a stimulus for activating the translation of specific axonal mRNAs. As noted above, LPA is known to trigger release of ER Ca^{2+} stores. Decrease in ER Ca^{2+} perturbs protein folding and processing in the ER and results in a general

translational attenuation in part through phosphorylation of eIF2 α on serine 51 (Brostrom and Brostrom, 2003). This phosphorylation converts eIF2 α into an inhibitor of the guanine exchange factor activity of eIF2B causing a decrease in global translation (Hinnebusch and Natarajan, 2002). Thus, we asked if LPA treatment causes S51 phosphorylation of eIF2 α in cultured DRGs. Immunoblotting lysates from DRGs exposed to LPA for 30 min showed a clear increase in eIF2^{PS51} levels compared to vehicle treated cultures (Fig. 3A). To determine if the axonal eIF2 α is phosphorylated upon LPA treatment, we determined the eIF2 α ^{PS51} immunoreactivity along axons of injury-conditioned DRG cultures that were exposed to LPA immobilized onto polystyrene microparticles. The axonal eIF2 α ^{PS51} immunoreactivity was significantly increased adjacent to LPA microparticles compared to axons exposed to the control BSA-coated microparticles (Fig. 3B). These data indicate that LPA can induce an inactivating phosphorylation of axonal eIF2 α .

Although phosphorylation of eIF2 α impairs general translation, in non-neuronal cells it is associated with a paradoxical increase in translation of a subset of mRNAs including ER chaperone protein mRNAs (Brostrom and Brostrom, 1998; Brostrom et al., 1996; Kaufman et al., 1989). Since LPA increased the translation of axonal calreticulin mRNA and increased phosphorylation of eIF2 α in axons, we asked if expression of phosphomimetic and non-phosphorylatable forms of eIF2 α could alter calreticulin protein levels in axons. For this, we transduced DRG cultures with adenovirus expressing eIF2 α with serine 51 to aspartate (AV-eIF2 α ^{S51D}-GFP) vs. serine 51 to alanine mutations (AV-eIF2 α ^{S51A}-GFP) (Lee and Hollenbeck, 2003). Naïve DRG cultures were used in this case to give time for expression of the transduced eIF2 α . Over 2–3 DIV, axonal growth from naïve neurons shifts from arborizing to the elongating growth typical of injury-conditioned neurons (Smith and Skene, 1997).

DRGs expressing phosphomimetic eIF2 α ^{S51D}, which functions as a constitutively phosphorylated eIF2 α , showed significantly higher axonal calreticulin immunoreactivity than those expressing the non-phosphorylatable eIF2 α ^{S51A}, which functions as a constitutively active eIF2 α (Figs. 4A,C and Supplemental Figure S2). Neurons transduced with AV-GFP showed no changes in axonal or cell body calreticulin immunoreactivity (Fig. 4C) suggesting that the response to AV-eIF2 α ^{S51D}-GFP is due to expression of the eIF2 α ^{S51D} rather than a response to AV or GFP. Schwann cells and neuronal cell bodies also showed a robust increase in calreticulin immunoreactivity with eIF2 α ^{S51D} vs. eIF2 α ^{S51A} (Figs. 4B and D). These data are consistent with translation of calreticulin mRNA which have been increased when eIF2 α 's activity is inhibited by phosphorylation similar to what has been demonstrated in non-neuronal cells (Llewellyn et al., 1995).

5'UTR of calreticulin contains translational regulatory elements

The 5'UTRs of several mRNAs have been shown to confer translational regulation through secondary structure formation, alternate initiation sites, and direct protein interactions with RNA targets (Spriggs et al., 2008). Since we had previously shown that the 3' UTR of calreticulin drives transport of the mRNA into axons (Vuppalanchi et al., 2010), we asked if calreticulin's 5'UTR can confer LPA-dependent translational of calreticulin mRNA in axons. For this, we targeted a diffusion limited reporter, GFP^{myr}, into axons using the

axonally localizing 3'UTR of calreticulin mRNA (Vuppalanchi et al., 2010). The 5'UTR of the original reporter was replaced with the 5'UTRs of calreticulin or β -actin mRNAs (5'Cal-GFP^{myr}-3'Cal and 5' β -actin-GFP^{myr}-3'Cal, respectively). After 2 DIV, naïve cultures of DRG neurons expressing 5'Cal-GFP^{myr}-3'Cal or 5' β -actin-GFP^{myr}-3'Cal showed abundant fluorescent signals in the distal axons (Fig. 5) similar to what we have seen for other axonally localizing reporters (Vuppalanchi et al., 2010; Willis et al., 2007; Yudin et al., 2008).

With bath application of LPA, the growth cones of 5'Cal-GFP^{myr}-3'Cal expressing DRG neurons showed a significant increase in GFP^{myr} signals compared to BSA treatment (Fig. 5A). This LPA-dependent increase in axonal 5'Cal-GFP^{myr}-3'Cal was protein synthesis dependent (Figs. 5A,B). Additionally, this increase in the GFP signals with LPA treatment was not attenuated by pre-treatment with colchicine, arguing that the elevated GFP levels were from axonal protein synthesis rather than transport of reporter mRNA or protein from the cell body via microtubule-dependent mechanisms (Fig. 5B). In contrast, the axons of DRG neurons expressing 5' β -actin-GFP^{myr}-3'Cal did not show any change in the GFP^{myr} signals in LPA treated compared to BSA treated neurons (Fig. 5C). These data suggest that calreticulin's 5'UTR imparts LPA-dependent translation of the axon-ally localized mRNA.

Phosphorylated eIF2 α regulates translation of calreticulin mRNA through its 5'UTR

Since LPA increased axonal eIF2 α ^{PS51} and introduction of the phosphomimetic eIF2 α ^{S51D} increased levels of endogenous calreticulin protein in the axons, we asked if phosphorylation of eIF2 α is needed for LPA-dependent translation of the 5'Cal-GFP^{myr}-3'Cal reporter mRNA in DRG axons. For this, we transduced DRG cultures with AV-eIF2 α ^{S51D} and used live cell imaging to monitor axonal reporter mRNA translation in 5'Cal-mCherry^{myr}-3'Cal transfected DRGs. Considering that the eIF2 α mutant could alter basal rates of translation, we used FRAP to test for LPA-dependent translation. The DRG neurons that were expressing eIF2 α ^{S51D}-GFP showed significant recovery of axonal mCherry signals after photobleaching in both LPA treated and control cultures (Figs. 6A,B). Pre-treatment with anisomycin attenuated the recovery of mCherry^{myr} fluorescence showing translation dependence. In contrast, 5' β -actin-mCherry^{myr}-3'Cal showed no significant recovery after photobleaching in neurons expressing eIF2 α ^{S51D} even in the absence of LPA (Figs. 6A,C). Since there were no statistical differences between basal and LPA induced fluorescence recoveries in the 5'Cal-mCherry^{myr}-3'Cal expressing neurons that were transduced with eIF2 α ^{S51D}, these data further support the conclusion that LPA-induced axonal translation through 5'UTR of calreticulin requires phosphorylation of eIF2 α . Moreover, recovery of fluorescence in axons for the 5' β -actin-mCherry^{myr}-3'Cal constructs was the same between LPA and BSA treated cultures when cultures were transduced with AV encoding the non-phosphorylatable eIF2 α ^{S51A} (Fig. 6E). Introducing eIF2 α ^{S51A} did not affect the LPA induced recovery of 5'Cal-mCherry^{myr}-3'Cal (Fig. 6D); however, this is likely due to LPA induced phosphorylation of endogenous eIF2 α . Taken together, these data show that axonal translation through 5'UTR of calreticulin occurs at a higher rate when eIF2 α is phosphorylated, an event that attenuates translation through the 5'UTR of β -actin.

Discussion

Neurons use cues in mRNAs, often in the 3'UTRs, to know which mRNAs should be targeted to their distal processes (Andreassi and Riccio, 2009). Once localized to a subcellular region, neuronal mRNAs can also be subjected to translational control. Several lines of evidence argue that translational regulation can be highly specific for individual mRNAs, but the mechanisms for this translational control of individual axonal mRNAs are largely unknown. Here, we show that axonal calreticulin mRNA is translationally regulated by LPA. This requires 5'UTR elements in calreticulin mRNA that impart specificity for this translational regulation after LPA-dependent phosphorylation of axonal eIF2 α , an event typically associated with attenuation of protein synthesis. This translational regulation occurs independent of any alterations in transport of calreticulin mRNA into the axons.

We have previously shown that the 3'UTRs of both calreticulin and Grp78/BiP mRNAs contain cis-elements that are required for their transport into axons (Vuppalanchi et al., 2010). However, these studies did not address their potential translational regulation in axons. The present study indicates that LPA can regulate translation of calreticulin mRNA in axons. Among other intracellular effects, LPA is known to cause release of ER Ca²⁺ in DRG neurons and other cellular systems (Elmes et al., 2004; Jalink et al., 1990, 1995). Decrease in ER Ca²⁺ levels has been shown activate PKR-like ER kinase (PERK) that subsequently phosphorylates and inactivates eIF2 α (Harding et al., 1999; Raven and Koromilas, 2008), but this has not been tested in neurons. Although this generally impedes translational initiation, mRNAs encoding the ER chaperone proteins show a paradoxical increase in their translation despite elevation of eIF2 α ^{PS51} (Brostrom and Brostrom, 2003). For the axonal mRNA translation studied here, LPA treatment increased the axonal levels of calreticulin through an inactivating phosphorylation of eIF2 α rather than through increasing RNA transport. Consistent with translational activation of resident axonal mRNA, inhibition of JNK has no effect on LPA-dependent translation of 5'Cal-GFP^{myr3}3'Cal reporter (data not shown), despite that both NT3- and MAG-dependent transport of calreticulin mRNA into axons required JNK activity (Vuppalanchi et al., 2010).

In contrast to the axons, LPA treatment did not alter calreticulin protein levels or increase translation of calreticulin mRNA in the cell body compartment of the DRG neurons. Concentration of LPA receptors in the axons could explain this, and LPA does result in a characteristic retraction of growth cones though this was not protein synthesis dependent (Campbell and Holt, 2001). Recent observations in developing brain have shown that neuroblasts have increased Ca²⁺ release in response to LPA compared to differentiated neurons (Dubin et al., 2010). This could point to distinct subcellular localization of LPA receptors in mature neurons. LPA has been shown to stimulate localization of multiple mRNAs into migratory protrusions of fibroblasts (Mili et al., 2008; Mingle et al., 2009), but specific translational regulation of localized mRNAs has not previously been documented in response to LPA. Different pools of calreticulin mRNA in cell body vs. axons with respect to basal translation could explain the cell body's lack of response to LPA based on the translation readouts studied here. At basal levels, cell body calreticulin mRNA is loaded onto ribosomes but the axons seem to maintain a pool of subpolysomal calreticulin mRNA that can be recruited to polysomes upon stimulation with LPA. The increased translation of

axonal importin $\beta 1$, RanBP1, and vimentin mRNAs upon axotomy (for review see Donnelly et al., 2010) would be consistent with storage of translationally inactive mRNAs in axons.

Several lines of evidence point to importance of Ca^{2+} -based signaling events in axons (Zheng and Poo, 2007). For example, a focal change in growth cone Ca^{2+} after BDNF exposure regulates local protein synthesis in axons (Yao et al., 2006). Other guidance and growth cues can exert their biological functions through modulating axonal Ca^{2+} and localized protein synthesis but there has not been a clear mechanistic link between these two (Yoon et al., 2009; Zheng and Poo, 2007). Axotomy-induced increase in intra-axonal Ca^{2+} levels is needed for translation of axonal Importin $\beta 1$, RanBP1, and vimentin mRNAs that encode proteins needed to help assemble retrograde injury signaling complexes (Perry and Fainzilber, 2009). Localized translation has been implicated for growth cone formation after in vitro axotomy (Verma et al., 2005) and studies from invertebrate neurons show Ca^{2+} dependence for growth cone formation (Gitler and Spira, 2002; Ziv and Spira, 1997). Although these studies point to Ca^{2+} as a mediator for axonal mRNA translation, a mechanistic link for conferring specificity to the axonal translational machinery has been lacking. Our studies show that LPA triggers phosphorylation of eIF2 α to activate translation through calreticulin's 5'UTR and inhibit translation through β -actin's 5'UTR. LPA increases axoplasmic Ca^{2+} in DRG neurons (Elmes et al., 2004), including the adult rat cultures utilized here (data not shown). Moreover, Dubin et al. (2010) recently showed that activation of LPA receptors in neuroblasts triggers release of thapsigargin-sensitive Ca^{2+} stores (Dubin et al., 2010), which is consistent with our observations that phosphorylation of eIF2 α is required for increased calreticulin mRNA translation through LPA.

Although we show that the 5'UTR of calreticulin clearly provides translational specificity in response to LPA compared to β -actin's 5' UTR, the molecular mechanism for this translational regulation is not clear. The attenuation of translation through β -actin's 5'UTR may relate to LPA's effects on other translation factors beyond eIF2 α . Previous work has shown that LPA can decrease phosphorylation of eIF4E and the eIF4E binding protein in axons (Campbell and Holt, 2001). Phosphorylated eIF4E is associated with increased cap-dependent protein synthesis and dephosphorylated 4EBP1 binds eIF4E and represses translation (Gingras et al., 1999). Thus, together with the inactivating phosphorylation of eIF2 α , LPA treatment would result in a generalized attenuation of cap-dependent protein synthesis. This raises the question of whether calreticulin 5'UTR-dependent translation could be regulated through cap-independent mechanisms. 5'UTRS of Grp78/BiP and HSP70 mRNAs were reported to have IRES activity (Rubtsova et al., 2003; Sarnow, 1989). However, the calreticulin 5'UTR used here is comparatively short (63 nucleotides) and has no initiation codons upstream of its open reading frame as is often seen in cellular mRNAs showing IRES activity (Stoneley and Willis, 2004). Future studies will be needed to determine the molecular events triggering translation of calreticulin with this LPA-dependent inhibition of eIF2 α and the potential contributions of any 5'UTR secondary structures.

Since PERK has been detected in DRG axons and LPA triggers ER Ca^{2+} release (Wang et al., 2007), a Ca^{2+} -dependent activation of axonal PERK likely leads to phosphorylation of eIF2 α and translation of axonal calreticulin mRNA. Calreticulin functions as a molecular

chaperone involved in the normal folding process for nascent proteins and it has a very high Ca^{2+} binding affinity (Michalak et al., 1998, 2002; Ostwald and MacLennan, 1974). The ER's Ca^{2+} storage capacity is reduced in calreticulin-null fibroblasts and is increased with overexpression of calreticulin (Mery et al., 1996). Interestingly, Mantuano et al. (2011) very recently showed that transcriptional upregulation of Grp78/BiP in Schwann cells following sciatic nerve crush provides a mechanism to attenuate the unfolded protein response and prevent apoptosis (Mantuano et al., 2011). Considering the distances separating the cell body and distal axons and that the axon has no genes to induce, translational regulation may represent a key adaptive response in the axon. For injured neurons, an axonal source for new calreticulin could either increase chaperone activity or to buffer axoplasmic Ca^{2+} to provide a means for the severed axons to recover.

Experimental methods

Cell culture

Neurons were cultured from L4–6 dorsal root ganglia (DRGs) of 150–225 g male Sprague Dawley rats. Dissociated DRG cultures were prepared as previously described (Willis et al., 2005). DRG neurons were plated on substrates coated with poly-L-lysine (Sigma) and laminin (Millipore) and maintained in growth medium. For imaging, neurons were cultured on coated-glass coverslips. For isolation of DRG axons from cell bodies and non-neuronal cells, injury-conditioned dissociated DRGs were plated onto coated polyethylene terephthalate (PET) membrane with 8 μm diameter pores (BD Falcon) (Willis et al., 2005). Naïve DRG cultures were used for expression of transfected or transduced mRNAs and proteins (see below); these cultures were analyzed after 2–3 DIV to allow for expression of the exogenously encoded mRNAs and proteins. Cultures of 7 d injury-conditioned neurons were used to assess endogenous mRNAs and proteins when no transfections or transductions were performed; these cultures were evaluated after 1 day in vitro (DIV). For injury-conditioning, sciatic nerve was crushed at mid thigh level 7 d prior to culture using a Dumont 0 forceps (Smith and Skene, 1997).

Axons were isolated from neurons cultured on PET membrane after 16 – 20h in culture by carefully scraping the top membrane surface using a cotton-tipped applicator to remove all cell bodies and non-neuronal cells. The purity of the axonal preparations was tested by reverse transcription coupled polymerase chain reaction (RT-PCR) for microtubule-associated protein 2 (MAP2), γ -actin, β -actin, glial fibrillary acidic protein (GFAP), and erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ErbB3) mRNAs.

For transfection of sensory neurons, dissociated DRGs were centrifuged at $100\times g$ for 5 min and gently resuspended in 'nucleofector solution' (*Rat Neuron Nucleofector kit*; Lonza, Inc.). 5 μg of each plasmid DNA was electroporated using *AMAXA Nucleofector* apparatus. After electroporation, the cells were resuspended in culture medium and plated; medium was then replaced 4h later to remove any non-adherent cells and debris.

For transduction with adenovirus (AV), dissociated DRGs were allowed to attach to the culture plate for 4h and then 100 MOI AV-eIF2 α ^{S51D}-GFP or AV-eIF2 α ^{S51A}-GFP was added to the media. The AV used in these studies encoded an in frame GFP fused to the C-

terminus of eIF2 α ^{S51D} or eIF2 α ^{S51A} (Lee and Hollenbeck, 2003). After 16 h, the culture media were replaced, and the cells were allowed to grow for 48 h.

Expression vectors

The 5'UTR of rat calreticulin was cloned using overlapping oligonucleotides that spanned the entire 5'UTR with 5' NheI and 3' BamHI sites added for subcloning. Oligonucleotides (2.5 μ M each) were annealed and extended using *HotTaq Master Mix* (Qiagen); PCR conditions were 94 °C for 5 min, 94°C×30s, 56°C×30s, 68 °C×30 s for 3 cycles with a final extension of 68 °C×5 min. The 5'UTR of rat β -actin was amplified by standard reverse transcriptase coupled PCR (RT-PCR) with primers to add 5' AflIII and 3' BamHI restriction sites. The 5'UTR calreticulin and 5'UTR β -actin PCR products were restriction digested and cloned up-stream of GFP^{myr}-Cal (5'Cal-GFP^{myr}-3'Cal or 5' β -actin-GFP^{myr}-3'Cal). Similar to the GFP^{myr}-Cal constructs, the 5'UTRs of rat calreticulin or β -actin were cloned up-stream of mCherry^{myr} that included the 3'UTR of calreticulin down-stream of the mCherry^{myr} coding sequence (5'Cal-mCherry^{myr}-3'Cal or 5' β -actin-mCherry^{myr}-3'Cal). All primer sequences for cloning and RNA level analyses (see below) are included in Supplementary Table 1.

Polysome RNA fractionation

Polysomal and subpolysomal RNAs were fractionated on 20% discontinuous sucrose gradients as previously described with minor modifications (Rajasekaran et al., 2004). Briefly, cultured cells were rinsed with cold PBS containing 0.1 mM cycloheximide and then lysed under conditions that maintain mRNA-ribosome association using 'polysome lysis buffer' (300 mM KCl, 2 mM MgCl₂, 20 mM Tris HCl [pH 7.4], 2 mM DTT, 0.05% deoxycholate, 0.1 mM cycloheximide, 50 U/ml RNase-In). Insoluble components were cleared from the lysate by centrifugation at 13,000 $\times$ g for 15 min at 4 °C. An aliquot of the lysate was used to test for purity of the axonal isolate (see below). The cleared lysate was layered onto 1 ml 20% sucrose in polysome lysis buffer. Gradients were then fractionated by ultracentrifugation at 105,000 $\times$ g, 4 °C, in a RP55S swinging bucket rotor (Sorvall) for 2h using slow acceleration and deceleration to prevent disruption of the gradient. To test for isolation of polysomal mRNAs, 50 mM EDTA was added to sister lysates to dissociate ribosome sub-units before centrifugation. The upper 80% the gradient was collected as the subpolysomal fraction and the lower 20% was collected as the polysomal fraction. RNA was extracted from each fraction using *TRI-ZOL* (Ambion) reagent and then precipitated with isopropanol with *glycogen-blue* (Ambion) as a carrier. The RNA was processed for quantitative RT-PCR (RTqPCR, see below).

RT-PCR was used to test for purity of the axonal isolate. For this, RNA was isolated using the *RNAqueous Micro* kit (Ambion) and quantified by fluorimetry using *Ribogreen* reagent (Invitrogen). After normalization, RNA was processed for standard RT-PCR using primers for β -actin, γ -actin, MAP2, GFAP, and ErbB2 (Supplemental Table 1). Reactions were analyzed by standard ethidium bromide stained agarose gels.

Axonal mRNA transport analyses

DRGs were cultured on a porous membrane for analyses of axonal mRNA transport as previously described (Willis et al., 2007). To restrict analyses to existing mRNAs, the RNA synthesis inhibitor 5,6-dichlorobenzimidazole riboside (DRB) (80 μ M, Sigma) was included in the culture medium. After 16–20h in culture, the neurons were treated with 30 μ M LPA (BioMol, reconstituted in 0.1% BSA) for 4h, after which the membranes were rinsed in PBS and the axonal compartment was isolated as described above. RNA was extracted from the fractionated cultures using the *RNAqueous Micro* kit and quantified by fluorimetry with the *Ribogreen reagent* (Molecular Probes). To normalize the axon mass between samples, RNA samples were normalized to axonal protein content before RT-PCR analyses. Protein content was measured in the flow-through from the affinity-based RNA isolation using fluorimetry with the *NanoOrange* reagent (Molecular Probes). For RT-PCR, $\sim$ 100 ng of RNA from axonal compartment was used as a template and the RT reaction was performed using the *iScript cDNA synthesis kit* (Biorad). After purity of axonal isolates was verified by standard RT-PCR (see above), samples were processed for RTqPCR to evaluate axonal mRNA levels (see below).

RNA quantification by RTqPCR

qPCR was performed with the *Prism 7900HT* sequence detection system (ABI) with 2x *SybrGreen Master Mix* (Qiagen) with standard cycling parameters. All samples were assayed in quadruplicate over at least three independent experiments. The following primers were used to amplify from reverse transcribed total RNA: Calreticulin, Grp78/BiP, Hsp70, Hsp60, β -actin, GAP-43, and 12S mitochondrial rRNA (see Supplementary Table 1).

Absolute quantification of PCR products was used to analyze mRNA levels from the polysome fractions. For this, RNA concentration was calculated from standard curves for each specific PCR product generated using serial dilutions of cDNA subcloned into pCR2.1TOPO vector for each mRNA. RNA isolated from non-fractionated DRGs was used for standard curves. All standard curves demonstrated a strong linear relationship with a correlation coefficient 0.98 between the fractional cycle number and the log of the starting copy number.

Relative quantification of PCR products was used for analyzing potential changes in axonal RNA levels with stimulation (Willis et al., 2005). For this, the levels of each test mRNA were normalized to the 12S mitochondrial rRNA using the comparative threshold method (C_t) method to provide an internal control for RT efficiency. From this, the calculation of

C_t was determined by subtracting the C_t calibrator value (BSA for LPA and DMSO for thapsigargin). The fold difference was then expressed as 2^{-C_t} .

Immunofluorescence

DRG cultures were rinsed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min, and then permeabilized in PBS plus 0.3% Triton X-100 for 5 min. After permeabilization, samples were incubated in blocking buffer (PBS plus 5% donkey serum) for 1 h. The samples were then incubated overnight at 4 °C in the following primary antibodies in blocking buffer: rabbit anti-calreticulin (1:200; Stressgen), chicken

anti-neurofilament (NF) H (1:1000; Millipore), rabbit eIF2 α ^{PS51} (1:100; Cell Signaling). Cultures were rinsed several times in PBS and incubated for 1h in the following secondary antibodies in blocking buffer: FITC-conjugated anti-chicken (1:400; Jackson ImmunoRes.) and Texas-red conjugated anti-rabbit or anti-goat (1:400; Jackson ImmunoRes.). Samples were rinsed in PBS and then mounted with *Prolong Gold Antifade* reagent (Invitrogen).

Epifluorescent microscopy with Leica DMRXA2 was used to image cultured neurons. The exposure times, gain and offset were kept constant between samples such that matched images were obtained. Quantitative analysis for protein levels within growth cones was analyzed using fluorescence (pixel) intensities of at least 15 growth cones for each condition over at least three independent experiments. The outlines of the growth cones were traced from DIC images and fluorescence intensity within the growth cone was calculated using *ImageJ*. Accumulation of eIF2 α ^{PS51} directly at sites of ligand stimulation was tested by immunofluorescence of dissociated DRG cultures exposed overnight to LPA- or control-microparticles (4.5 μ m diameter; Polysciences) (Willis et al., 2007). For quantifying signals, the pixel intensity was measured in seven 5 μ m length bins along the axon shaft proximally and distally to the center of the microparticle. Background was subtracted from the intensity values, and intensity in each bin was normalized to the mean intensity over the entire 35 μ m binned region. Mean normalized intensity of each bin was calculated for 25 axons over at least three separate culture experiments.

Immunoblotting

For detection of eIF2 α in cell lysates, cultures were lysed in RIPA buffer at 4 °C for 20 min. Lysates were cleared by centrifugation at 13,000 $\times$ g, 4 °C; cleared lysates were normalized for protein concentration by Bradford assay and then processed for SDS/PAGE. Fractionated proteins were transferred to PVDF membranes, blocked for non-specific binding using 5% non-fat dry milk in Tris-buffered saline (TBS) and then probed with anti-eIF2 α (1:1000) or anti-eIF2 α ^{PS51} (1:1000) overnight at 4 °C in blocking buffer. After washing in TBS, blots were incubated in HRP-conjugated anti-rabbit secondary antibodies (1:3000; Cell Signaling) and developed with *ECL^{Plus}* (Amersham).

Time-lapse imaging

Naïve DRG cultures transfected with the GFP^{myr} constructs were used for time-lapse imaging. To test for translation vs. RNA transport dependent recovery, cultures were pre-treated for 30 min prior to time lapse imaging sequences with 50 μ M anisomycin or 200 nM col-chicine, respectively. Imaging was performed at 37 °C using a TCS/SP2 confocal microscope with a 40 $\times$ oil immersion objective. The 488 nm laser line (10% power) was used to excite GFP^{myr} and the emission was collected at 498–530 nm. Images were collected every 120 s using the LCS confocal software package (Leica). The axon shaft including the growth cones in the distal 50 μ m of the axon was traced and the average pixels/ μ m² within the area was calculated using *ImageJ*.

Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) was used to test for localized translation of mCherry^{myr} mRNAs. Neurons were imaged every 30s for 2 min with 488 nm

and 543 nm laser set at 10% power to establish a baseline fluorescence intensity. For photobleaching, a region of interest (ROI) comprising the distal 30–45 μm of the axon including growth cone was exposed to 75% power of 488 and 543 laser for 20 frames at 1.6s intervals. GFP and mCherry levels were monitored every 60 s over 30 min by excitation with 10% power of 488 and 543 nm with emissions collected at 498–530 nm and 595–640 nm, respectively. To test for translation dependent recovery, cultures were pre-treated with 50 μM anisomycin for 30 min prior to photobleaching.

ImageJ was used to calculate average pixels/ μm^2 in the ROIs of the raw confocal images. The residual fluorescence signal at $t=0$ min (post-bleach) was subtracted from all time point intensity values. The corrected values were then normalized to the pre-bleach (set to 100%) and graphed as mean $\pm$ standard error of the mean.

Statistical analyses

For immunofluorescence and polysomal fractionation studies Student's Newman–Keul test was used to compare protein and RNA levels in the distal axons and cell bodies; $p < 0.05$ was considered significant. One way ANOVA was used to determine significance of fluorescence in live cell imaging sequences with Bonferroni post-hoc test; $p < 0.01$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AV	adenovirus
Ct	comparative threshold
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DRG	dorsal root ganglion
ER	endoplasmic reticulum
FRAP	fluorescence recovery after photobleaching
GAP-43	43 kDa growth associated protein
GFAP	glial fibrillary acidic protein
IRES	internal ribosome entry site
JNK	c-Jun N-terminal kinase
LPA	lysophosphatidic acid

MAG	myelin associated glycoprotein
MAP2	microtubule associated protein 2
NF	neurofilament
PERK	PKR-like ER kinase
NT3	neurotrophin 3
PET	polyethylene terephthalate
qPCR	quantitative PCR
RT-PCR	reverse transcriptase coupled PCR
Sema3A	semaphorin 3A
SERCA	sarcoplasmic endoplasmic reticulum calcium ATPase
UTR	untranslated region

References

- Andreassi C, Riccio A. To localize or not to localize: mRNA fate is in 3'UTR ends. *Trends Cell Biol.* 2009; 19:465–474. [PubMed: 19716303]
- Bi J, Tsai NP, Lu HY, Loh HH, Wei LN. Copb1-facilitated axonal transport and translation of kappa opioid-receptor mRNA. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:13810–13815. [PubMed: 17698811]
- Brostrom CO, Brostrom MA. Regulation of translational initiation during cellular responses to stress. *Prog. Nucleic Acid Res. Mol. Biol.* 1998; 58:79–125. [PubMed: 9308364]
- Brostrom MA, Brostrom CO. Calcium dynamics and endoplasmic reticular function in the regulation of protein synthesis: implications for cell growth and adaptability. *Cell Calcium.* 2003; 34:345–363. [PubMed: 12909081]
- Brostrom CO, Prostko CR, Kaufman R, Brostrom MA. Inhibition of translational initiation by activators of the glucose-regulated stress response and heat shock protein stress response systems. *J. Biol. Chem.* 1996; 271:24995–25002. [PubMed: 8798781]
- Campbell D, Holt C. Chemotropic responses of reginal growth cones mediated by rapid local protein synthesis and degradation. *Neuron.* 2001; 32:1013–1016. [PubMed: 11754834]
- Chatterjee S, Pal JK. Role of 5'- and 3'-untranslated regions of mRNAs in human diseases. *Biol. Cell.* 2009; 101:251–262. [PubMed: 19275763]
- Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N. Translational control of long-lasting synaptic plasticity and memory. *Neuron.* 2009; 61:10–26. [PubMed: 19146809]
- Donnelly CJ, Fainzilber M, Twiss JL. Subcellular communication through RNA transport and localized protein synthesis. *Traffic.* 2010; 11:1498–1505. [PubMed: 21040295]
- Dubin AE, Herr DR, Chun J. Diversity of lysophosphatidic acid receptor-mediated intracellular calcium signaling in early cortical neurogenesis. *J. Neurosci.* 2010; 30:7300–7309. [PubMed: 20505096]
- Elmes SJ, Millns PJ, Smart D, Kendall DA, Chapman V. Evidence for biological effects of exogenous LPA on rat primary afferent and spinal cord neurons. *Brain Res.* 2004; 1022:205–213. [PubMed: 15353230]
- Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* 1999; 68:913–963. [PubMed: 10872469]
- Gitler D, Spira M. Short window of opportunity for Calpain induced growth cone formation after axotomy in *Aplysia* neurons. *J. Neurobiol.* 2002; 52:267–279. [PubMed: 12210094]

- Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature*. 1999; 397:271–274. [PubMed: 9930704]
- Harding HP, Calton M, Urano F, Novoa I, Ron D. Transcriptional and translational control in the mammalian unfolded protein response. *Annu. Rev. Cell Dev. Biol.* 2002; 18:575–599. [PubMed: 12142265]
- Hinnebusch AG, Natarajan K. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot. Cell.* 2002; 1:22–32. [PubMed: 12455968]
- Jalink K, van Corven EJ, Moolenaar WH. Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+} -mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action. *J. Biol. Chem.* 1990; 265:12232–12239. [PubMed: 2373690]
- Jalink K, Hengeveld T, Mulder S, Postma FR, Simon MF, Chap H, van der Marel GA, van Boom JH, van Blitterswijk WJ, Moolenaar WH. Lysophosphatidic acid-induced Ca^{2+} mobilization in human A431 cells: structure–activity analysis. *Biochem. J.* 1995; 307(Pt 2):609–616. [PubMed: 7733903]
- Kaufman RJ, Davies MV, Pathak VK, Hershey JW. The phosphorylation state of eucaryotic initiation factor 2 alters translational efficiency of specific mRNAs. *Mol. Cell. Biol.* 1989; 9:946–958. [PubMed: 2657393]
- Lee S, Hollenbeck P. Organization and translation of mRNA in sympathetic axons. *J. Cell Sci.* 2003; 116:4467–4478. [PubMed: 13130093]
- Leung KM, van Horck FP, Lin AC, Allison R, Standart N, Holt CE. Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat. Neurosci.* 2006; 9:1247–1256. [PubMed: 16980963]
- Llewellyn DH, Sheikh FN, Kendall JM, Campbell AK. Upregulation of calreticulin expression in HeLa cells by calcium-stress. *Biochem. Soc. Trans.* 1995; 23:330S. [PubMed: 7672361]
- Llewellyn DH, Kendall JM, Sheikh FN, Campbell AK. Induction of calreticulin expression in HeLa cells by depletion of the endoplasmic reticulum Ca^{2+} store and inhibition of N-linked glycosylation. *Biochem. J.* 1996; 318(Pt 2):555–560. [PubMed: 8809046]
- Macejak D, Sarnow P. Internal initiation of translation mediated by the 5' leader of a cellular mRNA. *Nature*. 1991; 353:90–94. [PubMed: 1652694]
- Mantuano E, Henry K, Yamauchi T, Hiramatsu N, Yamauchi K, Orita S, Takahashi K, Lin JH, Gonias SL, Campana WM. The unfolded protein response is a major mechanism by which LRP1 regulates schwann cell survival after injury. *J. Neurosci.* 2011; 31:13376–13385. [PubMed: 21940431]
- Mery L, Mesaeli N, Michalak M, Opas M, Lew DP, Krause KH. Overexpression of calreticulin increases intracellular Ca^{2+} storage and decreases store-operated Ca^{2+} influx. *J. Biol. Chem.* 1996; 271:9332–9339. [PubMed: 8621597]
- Michalak M, Mariani P, Opas M. Calreticulin, a multifunctional Ca^{2+} binding chaperone of the endoplasmic reticulum. *Biochem. Cell Biol.* 1998; 76:779–785. [PubMed: 10353711]
- Michalak M, Parker J, Opas M. Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium*. 2002; 32:269–278. [PubMed: 12543089]
- Mili S, Moissoglu K, Macara IG. Genome-wide screen reveals APC-associated RNAs enriched in cell protrusions. *Nature*. 2008; 453:115–119. [PubMed: 18451862]
- Mingle LA, Bonamy G, Barroso M, Liao G, Liu G. LPA-induced mutually exclusive subcellular localization of active RhoA and Arp2 mRNA revealed by sequential FRET and FISH. *Histochem. Cell Biol.* 2009; 132:47–58.
- Moolenaar WH, van Meeteren LA, Giepmans BN. The ins and outs of lysophosphatidic acid signaling. *Bioessays*. 2004; 26:870–881. [PubMed: 15273989]
- Ostwald TJ, MacLennan DH. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *J. Biol. Chem.* 1974; 249:974–979. [PubMed: 4272851]
- Perry RB, Fainzilber M. Nuclear transport factors in neuronal function. *Semin. Cell Dev. Biol.* 2009; 20:600–606. [PubMed: 19409503]
- Rajasekaran SA, Gopal J, Willis D, Espineda C, Twiss JL, Rajasekaran AK. Na, K-ATPase beta1-subunit increases the translation efficiency of the alpha1-subunit in MSV-MDCK cells. *Mol. Biol. Cell.* 2004; 15:3224–3232. [PubMed: 15133131]

- Raven JF, Koromilas AE. PERK and PKR: old kinases learn new tricks. *Cell Cycle*. 2008; 7:1146–1150. [PubMed: 18418049]
- Rubtsova P, Sizova D, Dmitriev S, Ivanov D, Prassolov V, Shatsky I. Distinctive properties of the 5'-untranslated region of human HSP70 mRNA. *J. Biol. Chem.* 2003; 278:22350–22356. [PubMed: 12682055]
- Sarnow P. Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. *Proc. Natl. Acad. Sci. U. S. A.* 1989; 86:5795–5799. [PubMed: 2548189]
- Smith DS, Skene P. A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *J. Neurosci.* 1997; 17:646–658. [PubMed: 8987787]
- Spriggs KA, Stoneley M, Bushell M, Willis AE. Re-programming of translation following cell stress allows IRES-mediated translation to predominate. *Biol. Cell*. 2008; 100:27–38. [PubMed: 18072942]
- Stoneley M, Willis AE. Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene*. 2004; 23:3200–3207. [PubMed: 15094769]
- Tokumura A. Physiological and pathophysiological roles of lysophosphatidic acids produced by secretory lysophospholipase D in body fluids. *Biochim. Biophys. Acta*. 2002; 1582:18–25. [PubMed: 12069806]
- Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, Holt CE, Fawcett JW. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J. Neurosci.* 2005; 25:331–342. [PubMed: 15647476]
- Vuppalanchi D, Coleman J, Yoo S, Merianda TT, Yadhati AG, Hossain J, Blesch A, Willis DE, Twiss JL. Conserved 3'-untranslated region sequences direct subcellular localization of chaperone protein mRNAs in neurons. *J. Biol. Chem.* 2010; 285:18025–18038. [PubMed: 20308067]
- Wang W, van Niekerk E, Willis DE, Twiss JL. RNA transport and localized protein synthesis in neurological disorders and neural repair. *Dev. Neurobiol.* 2007; 67:1166–1182. [PubMed: 17514714]
- Willis D, Li KW, Zheng JQ, Chang JH, Smit A, Kelly T, Merianda TT, Sylvester J, van Minnen J, Twiss JL. Differential transport and local translation of cyto-skeletal, injury-response, and neurodegeneration protein mRNAs in axons. *J. Neurosci.* 2005; 25:778–791. [PubMed: 15673657]
- Willis DE, van Niekerk EA, Sasaki Y, Mesngon M, Merianda TT, Williams GG, Kendall M, Smith DS, Bassell GJ, Twiss JL. Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J. Cell Biol.* 2007; 178:965–980. [PubMed: 17785519]
- Wu KY, Hengst U, Cox LJ, Macosko EZ, Jeromin A, Urquhart ER, Jaffrey SR. Local translation of RhoA regulates growth cone collapse. *Nature*. 2005; 436:1020–1024. [PubMed: 16107849]
- Yao J, Sasaki Y, Wen Z, Bassell GJ, Zheng JQ. An essential role for beta-actin mRNA localization and translation in Ca(2+)-dependent growth cone guidance. *Nat. Neurosci.* 2006; 9:1265–1273. [PubMed: 16980965]
- Ye X, Fukushima N, Kingsbury MA, Chun J. Lysophosphatidic acid in neural signaling. *Neuroreport*. 2002; 13:2169–2175. [PubMed: 12488791]
- Yoon BC, Zivraj KH, Holt CE. Local translation and mRNA trafficking in axon pathfinding. *Results Probl. Cell Differ.* 2009; 48:269–288. [PubMed: 19343311]
- Yudin D, Hanz S, Yoo S, Iavnilovitch E, Willis D, Segal-Ruder Y, Vuppalanchi D, Ben-Yaakov K, Hieda M, Yoneda Y, Twiss J, Fainzilber M. Localized regulation of axonal RanGTPase controls retrograde injury signaling in peripheral nerve. *Neuron*. 2008; 59:241–252. [PubMed: 18667152]
- Zheng JQ, Poo MM. Calcium signaling in neuronal motility. *Annu. Rev. Cell Dev. Biol.* 2007; 23:375–404. [PubMed: 17944572]
- Zheng J-Q, Kelly T, Chang B, Ryazantsev S, Rajasekaran A, Martin K, Twiss J. A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. *J. Neurosci.* 2001; 21:9291–9303. [PubMed: 11717363]
- Ziv N, Spira M. Localized and transient elevations of intracellular Ca²⁺ induce dedifferentiation of axonal segments into growth cones. *J. Neurosci.* 1997; 17:3568–3579. [PubMed: 9133380]

Fig. 1. LPA increases axonal calreticulin levels. Cultures of 7 d injury-conditioned DRG neurons at 1 DIV were treated with LPA for 4h and then processed for immunostaining with antibodies to calreticulin (red) and neurofilament ([NF] green). Exposure matched images of growth cones are shown. *A*, Immunoreactivity for calreticulin is increased in growth cones LPA treated compared to BSA treated cultures. Pretreatment with 50 μ M anisomycin attenuates the LPA-induced increase in axonal calreticulin. However, the LPA-induced increase in axonal calreticulin is not affected by pretreatment with 200 nM colchicine (also see Supplemental Figure S1). *B*, Representative images of DRG cell body and Schwann cells for LPA vs. BSA treated cultured. For DRG cell bodies, main panel shows calreticulin in red and inset shows NF in green; for Schwann cells, calreticulin is shown in red and DAPI signals are shown in blue. LPA increases calreticulin protein immunoreactivity in the Schwann cells in these cultures but only a modest elevation is seen in the cell body. *C*,

Quantification of fluorescent intensity for calreticulin protein in the distal axons normalized to NF signal is shown. Anisomycin pretreated samples show no significant difference compared to control. Pretreatment with colchicine followed by LPA shows a significant increase in calreticulin in distal axons compared to control. The cell bodies treated with LPA show no significant change in the calreticulin compared to control. (N = 15 over 3 separate experiments; * $p < 0.05$, *** $p < 0.001$ by Student's Newman–Keul test) [scale bars=5 μm]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Calreticulin is actively translated upon LPA treatment. Cultures of 7 d injury-conditioned DRG neurons were grown on porous membrane for isolation of axons as previously described (Willis et al, 2005). After 16 h, the axonal compartment was stimulated with 30 μ M LPA for 4h and then axonal lysates were used for fractionation of polysomal RNA by discontinuous 20% sucrose gradients. **A**, Aliquot of axonal lysates was used to test for purity of axonal isolates by RT-PCR. Rat brain was used as a positive control template. β -actin mRNA was amplified from the axons, but cell body confined MAP2 and γ -actin were not detected. The Schwann cell restricted mRNAs, ErbB3 and GFAP, were not detected in the axons. **B**, Lysates from the cell body compartment were used to confirm successful polysome fractionation. In standard polysome fractionation, the majority of calreticulin mRNA from these cell body lysates fractionated at the bottom of the gradient consistent

with a predominantly polysomal distribution. Addition of 50 mM EDTA prior to fractionation shifted calreticulin mRNA to a predominantly subpolysomal distribution. C, Absolute levels of mRNAs from equivalent fractions of polysomal and subpolysomal RNA were quantified using RTqPCR. Average distribution of the indicated mRNAs in polysomal vs. subpolysomal fractions is illustrated. Axonal calreticulin and Grp78/BiP mRNAs showed a significant shift to polysomes after LPA treatment Hsp70, Hsp60 and GAP43 mRNAs showed no significant change in their distributions with LPA treatment. Error bars represent the standard deviation of three replicate experiments with each sample measured in quadruplicate (* = $p < 0.05$, ** = $p < 0.01$, Student's Newman-Keul test).

Fig. 3.

LPA stimulates phosphorylation of axonal initiation factor eIF2 α . **A**, Lysates from naïve DRG cultures (1 DIV) that had been exposed to 30 μ M LPA for 30 min were processed for immunoblotting with anti-eIF2 α ^{PS51} and anti-eIF2 α antibodies. LPA triggers increase in eIF2 α ^{PS51} with approximate equal levels of total eIF2 α seen upon probing with non-selective anti-eIF2 α antibody. **B**, 7 d injury-conditioned DRG cultures were grown in the presence of microparticles coated with BSA+LPA or BSA alone for 16h and then processed for immunostaining as described previously (Willis et al., 2007). The average signal intensities for eIF2 α ^{PS51} in 5 μ m bins over a 35 μ m segment of axon shaft centered on the microparticles ('Bead') are shown; the signals for both LPA and BSA-treated axons are normalized to the overall average axonal eIF2 α ^{PS51} immunoreactivity over the 35 μ m of the BSA-treated cultures.

Fig. 4.

Expression of phosphomimetic eIF2 α increases calreticulin levels. **A**, Representative images of distal axons of DRG neurons transduced with 100 MOI recombinant AV-eIF2 α ^{S51A}-GFP or AV-eIF2 α ^{S51D}-GFP are shown at 2 DIV. Note that the axonal signals for calreticulin protein (red) are increased in the AV-eIF2 α ^{S51D}-GFP transduced neurons. GFP signal is shown in green. These images are matched for exposure, gain, offset and post-processing (also see Supplemental Figure S2). **B**, Schwann cells also show increased calreticulin protein (red; GFP signal from AV shown in green) levels in the cultures infected with AV-eIF2 α ^{S51D}-GFP (asterisk represents infected cell). Note that the Schwann cells without GFP expression show much lower calreticulin immunoreactivity. **C**, Quantification of calreticulin immunoreactivity from exposure matched images of distal axons vs. cell bodies is shown. Both cellular regions show significantly higher fluorescent intensity for calreticulin protein in AV-eIF2 α ^{S51D}-GFP vs. AD-eIF2 α ^{S51A}-GFP transduced neurons; transduction with AV-eGFP caused no change in calreticulin immunoreactivity (*= p 0.05, **= p 0.01, Student's Newman-Keul test) [scale bars: B=5 μ m, C=10 μ m]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

5'UTR of calreticulin contains translational regulatory elements. Cultures of naïve DRG neurons were transfected with 5'Cal-GFP^{myr}3'Cal or 5'β-actin-GFP^{myr}3'Cal and used for live cell imaging at 2–3 DIV. **A**, Image sequences for the axonal GFP signal displayed as a spectrum after control, LPA, and LPA + 50 μM anisomycin are shown for 30 min segments with images collected every 2 min. Regions of interest corresponding to ~50 μm of distal axon that was used for quantification of GFP signal intensities over time for **B** and **C** are shown as boxes in each image panel. **B**, Relative change in axonal GFP fluorescence for 5'Cal-GFP^{myr}3'Cal transfected neurons is shown as mean ± SEM. Note that LPA treatment results in a significant increase in reporter fluorescence in distal axons within 10 min that is only minimally reduced by colchicine and completely blocked by the protein synthesis

inhibitor anisomycin. *C*, Relative change in axonal GFP fluorescent for 5'β-actin-GFP^{myr}-3'Cal is shown. There is no significant change in GFP signal intensity with LPA treatment (N>6 for each condition over 3 transfection experiments each, * = p 0.05, ** = p 0.01, *** = p 0.001 by ANOVA vs. t = 0 min for LPA; †† = p<0.01 for LPA plus colchicine by one-way ANOVA vs. t = 0 min) [scale = 10 μm].

Fig. 6.

Phosphomimetic eIF2 α differentially regulates axonal translation through 5'UTRs. FRAP sequences were performed on 2–3 DIV cultures of naïve DRG neurons that had transfected with 5'Cal-GFP^{myr}-3'Cal or 5'β-actin-GFP^{myr}-3'Cal and transduced with the indicated AV-eIF2 α variants. **A**, Representative time-lapse sequences from FRAP experiments for mCherry fluorescence in axons of 5'Cal-mCherry^{myr}-3'Cal (top and middle rows) or 5'β-actin-mCherry^{myr}-3'Cal (bottom row) expressing DRG neurons that were transduced with AV-eIF2 α ^{S51D}-GFP are shown. The boxed regions represent the ROIs that were photobleached. The mCherry signal is displayed as a pixel intensity spectrum as indicated in the bottom row of images. **B–C**, Quantification of the mCherry signals from multiple FRAP sequences for the AV-eIF2 α ^{S51D}-GFP transduced DRG cultures is graphically illustrated as the mean relative signal $\pm$ SEM. The 5'Cal-mCherry^{myr}-3'Cal expressing cultures (**B**) show a significant protein synthesis-dependent recovery within 10 min after photobleaching

regardless of the presence of LPA. The 5'β-actin-mCherry^{myr}-3'Cal expressing cultures (C) show no significant recovery even in the absence of LPA. *D–E*, mCherry signals from multiple FRAP sequences for the AV-eIF2α^{S51A}-GFP transduced DRG cultures are shown as in B. Note that 5'β-actin-mCherry^{myr}3'Cal shows significant recovery despite the presence of LPA when eIF2α^{S51A} is expressed (N = 6 for each condition over at least 3 independent culture preparation; ** = p<0.01, *** = p<0.001 for LPA and †† = p<0.01, ††† = p<0.005 for BSA by one-way ANOVA vs. t = 0 min) [scale bars = 10 μm].