mRNA localization provides polarized cells with a locally renewable source of proteins. In neurons, mRNA translation can occur at millimeters to centimeters from the cell body, giving the dendritic and axonal processes a means to autonomously respond to their environment. Despite that hundreds of mRNAs have been detected in neuronal processes, there are no reliable means to predict mRNA localization elements. Here, we have asked what RNA elements are needed for localization of transcripts encoding endoplasmic reticulum chaperone proteins in neurons. The 3′-untranslated regions (UTRs) of calreticulin and Grp78/BiP mRNAs show no homology to one another, but each shows extensive regions of high sequence identity to their 3′UTRs in mammalian orthologs. These conserved regions are sufficient for subcellular localization of reporter mRNAs in neurons. The 3′UTR of calreticulin has two conserved regions, and either of these is sufficient for axonal and dendritic targeting. However, only nucleotides 1315–1412 show ligand responsiveness to neurotrophin 3 (NT3) and myelin-associated glycoprotein (MAG). This NT3- and MAG-dependent axonal mRNA transport requires activation of JNK, both for calreticulin mRNA and for other mRNAs whose axonal levels are commonly regulated by NT3 and MAG.

Targeting of mRNAs to subcellular regions is a highly selective process that provides spatial control to gene expression (1). Localized mRNA translation has been linked to polarized growth in fibroblasts and migration of cancer cells (2, 3). In neurons, protein products of dendritically localized mRNAs contribute to synaptic plasticity (4). Protein products of mRNAs transported into axons have been linked to growth and injury responses (5, 6). The mRNA population localizing into neuronal processes is quite complex, with several hundred mRNAs localizing into axonal and/or dendritic processes (7–11). However, it is clear that neurons do not send all mRNAs into their distal processes, and these cells must actively select which mRNAs to localize. For example, although β-actin and γ-actin mRNAs encode remarkably similar proteins, only β-actin mRNA is transported into neuronal processes (12–14). Moreover, some mRNAs are selectively transported into dendrites or axons (15, 16), whereas other mRNAs are targeted to both axons and dendrites (13, 17, 18).

Untranslated regions (UTR) of mRNAs, particularly the 3′UTRs (19, 20), often include elements that confer subcellular localization. These cis-elements are thought to provide binding sites for proteins needed for subcellular localization of mRNAs (21). There is little cumulative knowledge of what constitutes a cis-element responsible for targeting neuronal mRNAs to subcellular sites. RNA structures that drive localization along with the specific proteins that recognize these structures will undoubtedly play a key role in regulation of mRNA transport and localization. For example, the cis-element or “zipcode” of β-actin consists of a 54-nucleotide segment in its proximal 3′UTR that is bound by zipcode-binding protein 1 (ZBP1) (22, 23). Localization elements can also provide a means to regulate the translation of mRNAs by actively preventing translational initiation during their transport (24). The 3′UTR of rat β-actin is sufficient for axonal localization of reporter mRNAs in adult sensory neurons, including ligand-dependent mRNA transport (7). However, the β-actin zipcode shows no clear homology with the 3′UTRs of roughly 200 mRNAs that we have isolated from axons of cultured sensory neurons. Here, we have asked what cis-elements direct subcellular localization of mRNAs encoding the endoplasmic reticulum chaperone proteins calreticulin and Grp78/BiP. Despite the lack of homology between calreticulin and Grp78/BiP mRNA 3′UTRs, each shows high sequence identity with their mammalian orthologs. These conserved noncoding sequences are sufficient for subcellular local-

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The abbreviations used are: UTR, untranslated region; NT3, neurotrophin 3; MAG, myelin-associated glycoprotein; DRG, dorsal root ganglion; LV, lentivirus; FISH, fluorescence in situ hybridization; GFP, green fluorescent protein; GPPm5′, GFP with myristoylation element; NF, neurofilament; PBS, phosphate-buffered saline; ROI, region of interest; FRAP, fluorescent recovery after photobleaching; DIV, days in vitro; RT, transcriptase-coupled PCR; ANOVA, analysis of variance; NGF, nerve growth factor; JNK, c-Jun N-terminal kinase; BSA, bovine serum albumin; GFAP, glial fibrillary acidic protein; NF, neurofilament.
ization of both mRNAs. Calreticulin mRNA further contains two distinct elements that confer subcellular localization, but these elements are functionally distinguished by their responsiveness to ligand stimulation.

**MATERIALS AND METHODS**

**DNA Expression Constructs**—All reporter constructs for analyses of RNA localization were based on myristoylated, green fluorescent protein (GFP<sub>MYC</sub>) plasmid originally provided by Erin Schuman (7). GFP expression constructs containing the β-actin and γ-actin 3′UTRs have been described (7). 3′UTRs of rat calreticulin and Grp78/BiP mRNAs (GenBank<sup>TM</sup> accession numbers NM_022399 and M14050.1, respectively) were generated by RT-PCR and cloned into GFP<sub>MYC</sub> plasmid after sequence verification. The primer sequences and RT-PCR conditions are included in the supplemental material. The Kif5C<sub>560</sub>-dTomato, Kif1 CC-YFP, and pPrlSSmCherry constructs were kindly provided by Dr. Gary Banker.

**Cell Culture and Transfections**—Dissociated dorsal root ganglion (DRG) neurons were prepared as described previously (25). Neuron cultures were prepared from E18 embryonic rat cortexes using previously described dissociation and plating conditions (26). For expression of reporter constructs, both DRG and cortical neurons were transfected immediately after dissociation using the AMAXA Nucleofector<sup>TM</sup> apparatus in DRG and cortical neurons were transfected immediately after photobleaching using the Leica DMRXA2 microscope fitted with Orca-ER CCD camera (Hamamatsu) was used for epifluorescence microscopy.

**In Situ Hybridization**—The methods for fluorescence in situ hybridization (FISH) varied with the probe design and preparation; individual hybridization methods are described in detail in the supplemental material. Antisense oligonucleotide probes were designed to detect endogenous mRNAs in cultures and in tissue sections as described previously (7). These were synthesized with the 5′-amino modifier C6 at four thymidines per oligonucleotide, and then chemically labeled with digoxigenin succinimidyl ester (Roche Applied Science). Rat calreticulin probes spanned nucleotides 1360–1410 and 1637–1686 and rat Grp78/BiP probes spanned nucleotides 1422–1471 and 2164–2213; probes for rat β-actin and γ-actin mRNAs are as published previously (7). Scrambled oligonucleotide probes were used to test for specificity. cRNA probes were used to detect the GFP reporter mRNA in transfected neurons (27, 28). For this, linearized pcDNA3-EgFP (Addgene, Plasmid 13031) was in vitro transcribed with SP6 or T7 RNA polymerases using digoxigenin-labeled nucleotide mixture (Roche Applied Science) to generate antisense and sense riboprobes.

After hybridization, samples were processed for immunodetection. The following primary antibodies were used: chicken anti-neurofilament (NF) H (1:1,000; Chemicon), chicken anti-NF M (1:1,000; Aves Laboratories), chicken anti-MAP2 (1:10,000; Abcam), rabbit anti-Tau (1:5,000; Sigma), and rabbit anti-S100 (1:500; Dako). Probes were detected using Cy3-conjugated mouse anti-digoxigenin antibody, and proteins were detected using Cy5- and fluorescein isothiocyanate-conjugated anti-rabbit and anti-chicken antibodies (1:400 each; Jackson ImmunoResearch). To increase sensitivity for the tissue sections, an additional Cy3-conjugated anti-mouse IgG F(ab′) fragment antibody (1:400; Jackson ImmunoResearch) was included with secondary antibody incubation.

Epifluorescent microscopy was used to capture the pixel intensity of the FISH signals for cultured neurons. ImageJ was used to quantitate pixel intensities for the GFP mRNA in growth cone images (matched for exposure, gain, and offset). For this, the growth cone was visualized by differential interference contrast, and NF signal was used to determine the end of the axon shaft.

Leica DMRXA2 microscope fitted with Orca-ER CCD camera (Hamamatsu) was used for epifluorescence microscopy. Leica SP2 confocal microscope was used to image tissue sections and for live cell imaging (see below).

**Immunofluorescence**—For immunostaining only, sciatic nerve sections were equilibrated in phosphate-buffered saline (PBS) and then incubated in 20% mouse glycine three times for 10 min followed by 0.25 M NaBH<sub>4</sub> for 30 min to quench autofluorescence. Samples were rinsed in PBS, permeabilized in 0.2% Triton X-100 for 15 min, and then incubated in 5% donkey serum for 1 h to block nonspecific binding. Sections were then incubated overnight at 4 °C in the following primary antibodies diluted in blocking buffer: rabbit anti-calreticulin (1:100; StressGen), rabbit anti-Grp78/BiP (1:100; StressGen), and chicken anti-NF H (1:1,000; Chemicon). Tissues were rinsed several times in PBS and incubated for 1 h in the following secondary antibodies diluted in blocking buffer as follows: fluorescein isothiocyanate-conjugated anti-chicken (1:400; Jackson ImmunoResearch) and Alexa555-conjugated anti-rabbit (1:750; Invitrogen). Samples were rinsed in PBS and then mounted with Prolong Gold Antifade (Invitrogen).

**Fluorescence Recovery after Photobleaching**—GFP expression in transfected neurons was confirmed by epifluorescence, and then distal processes were subjected to fluorescence recovery after photobleaching (FRAP) sequence at 37 °C using a Leica TCS/SP2 confocal microscope fitted with an environmental chamber (29). 40× oil immersion objective (numerical aperture = 0.7) was used for imaging, with the confocal pinhole set to 4 airy units to ensure full thickness of the axon/growth cone was photobleached. Prior to bleaching, neurons were imaged every 30 s for 2 min with a 488-nm laser and 15% power to establish baseline intensity. For photobleaching, the region of interest (ROI) was exposed to a 488-nm laser and 75% power every 1.6 s over 40 frames. GFP emission was then monitored every 60 s using a 488-nm laser, 15% power, 498–530-nm collection filter, with photomultiplier tube energy, offset, and gain matched for all collection sets. Photobleaching for Kif5C<sub>560</sub>-dTomato was performed with a 543 nm laser, 75% power, and recovery was monitored every 60 s at 15% power (595–640-nm band filter). To test for translation-dependent recovery, cultures were pretreated with 50 µM anisomycin for 30 min prior to photobleaching (29).

“Double-bleach” FRAP was performed as described with minor modifications (29). Briefly, an ROI in the distal axon was photobleached with a 488-nm laser, 100% power over...
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60 s. The proximal half of the original ROI was then repeatedly bleached by 15 successive scans at 70% power, 2-s intervals (continuous bleaching), whereas the distal half of the original ROI was monitored for the recovery by collecting GFP emissions (488-nm laser, 7% power, 498–530-nm collection filter) at the end of the 15th scan (monitoring scan). Continuous bleaching and monitoring scans were repeated for 61 cycles (30 s between each monitoring scan) to give an overall 30-min time-lapse sequence.

To determine the rate of vesicular transport, a 20-μm length of mid-axon shaft of pPrlSSmCherry-transfected DRG neurons was subjected to photobleaching and then monitored for recovery through anterograde transport of fluorescent vesicles. The laser and filter parameters for photobleaching and recovery were the same as those for dTomato outlined above. Image sequences were equally thresholded to minimize background using ImageJ. Each bleached ROI was then divided into 1-μm bins, and the duration for each bin to reach 50% recovery threshold was calculated by measuring the pixel intensity of the bleached ROI at 0–30 min.

Imagel was used to calculate average pixels/μm² in the ROIs of the raw confocal images. The residual fluorescence signal at t = 0 min (post-bleach) was subtracted from intensity values. The corrected values were then normalized to the pre-bleach (set to 100%). For converting time-lapse sequences to video, the original WMV file was converted into AVI format using the Power Video Converter, version 1.5.43 (Apus).

Analyses of Ligand-dependent mRNA Transport—Both local and bath applied neurotrophin 3 (NT3) and myelin-associated glycoprotein (MAG) were used to evaluate regulation of axonal mRNA transport. For local application, NT3 (100 ng; Alomone Labs, Ltd.) and MAG-Fc (2.5 μg; R & D Systems) were covalently coupled to 2.5 × 10⁷ Fluoresbrite YO Carboxylate Microspheres (Polysciences, Inc.). Equimolar amounts of BSA (Sigma) and human IgG Fc (Sigma) were immobilized to microparticles as controls for NT3 and MAG, respectively. Efficiency of coupling was determined by the Bradford assay as described previously (7). For bath application, 100 ng/ml NT3 or 2.5 μg/ml MAG was added directly to the culture medium.

Accumulation of mRNA directly at sites of ligand stimulation was tested by FISH/immunofluorescence of dissociated DRG cultures grown overnight in the presence of immobilized microparticles (4.5 μm diameter) as described previously (7). Cultures were fixed and processed for FISH/immunofluorescence as outlined above. For quantifying signals, the pixel intensity was measured in bins (5 μm each 7 times) of the axon proximal to and distal 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-μm binned region. Mean normalized intensity of each bin was calculated for 30 axons over at least three separate culture experiments.

Live cell imaging of GFPmmyr-transfected cultures was used to visualize ligand-dependent changes in mRNA localization with accumulation of GFP as a readout. For this, ligand-coupled microparticles were contacted to axons using a pulled glass micropipette mounted onto a micromanipulator (Sutter Instruments). Microparticles prepared as above were held at the tip of the mounted micropipette using gentle suction pressure. For analyses, GFP signal intensity (pixels/μm²) was quantified in a 10-μm axon segment spanning 5 μm proximal to and distal from the center of the microparticle in each frame of the time-lapse sequence.

To quantify overall changes in axonal mRNA levels in response to these ligands, dissociated DRGs were cultured on porous membranes (8 μm diameter pores) as described previously (30). After 20 h in culture, the axonal compartment was selectively exposed to microparticles (20 μm diameter; 4.7 × 10⁵) for 4 h. Based on efficiency of protein coupling to microparticles and microparticles/ml used, an equivalent of 84 ng/ml NT3 and 4.16 μg/ml MAG-Fc was used. The axons were then sheared from cell bodies, and this “axonal preparation” was used for preparation of RNA as outlined below.

In some experiments, cultures were treated with pharmacological inhibitors to evaluate downstream signaling events for ligand-dependent axonal RNA transport. The following agents were used: K252A (200 nM; Calbiochem), PD98059 (50 μM; Biomol), and SP600125 (10 μM; Biomol). Additionally, some cultures were treated with colchicine (1 μg/ml; Sigma) to evaluate microtubule-dependent events.

RT-PCR Analyses of Axonal mRNA Levels—Axonal RNA was extracted using the RNAqueous Micro kit (Ambion). All axonal RNA samples were normalized to protein content before RT-PCR analyses as described previously (30). Normalized axonal RNA samples were reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). The purity of axonal preparations was assessed by RT-PCR for γ-actin, MAP2, ErbB3, and GFAP mRNAs; amplification of β-actin mRNA was used as a positive control (see supplemental material for primer sequences).

After verification of purity, reverse-transcribed axonal RNA samples were processed for quantitative PCR using Prism 7900HT (Applied Biosystems) with 2× SYBR Green Master Mix (Qiagen). Axonal mRNA signals were analyzed as described previously with 12 S mitochondrial RNA amplification product providing normalization between isolates (7).

Statistical Analyses—Multiple statistical measures were used to analyze live cell imaging data. FRAP data were analyzed by one-way and repeated measures ANOVA. One-way ANOVA with Bonferroni post-hoc comparisons was performed using GraphPad Prism 5 software package. Significance of each time point post-bleach was compared with t = 0 min post-bleach. For repeated measures ANOVA, the differences of means between groups and differences within group mean over time were tested. The significance of difference of overall treatment group means as well as the difference of treatment group means for each assessment point, including within group difference from base line, were examined. The sphericity assumption of the model was checked using Mauchly test and Greenhouse-Geisser corrected method in case of violation of this model assumption. All analyses were two-tailed at 5% level of significance. The analyses were performed using statistical package SPSS version 17.0 (SPSS Inc., Chicago). The more conservative of the two statistical measures for FRAP data is indicated in the figures, with less conservative measure noted in the figure legends. For axonal GFP response to stimuli, two-way ANOVA was used to compare GFP signal intensities at different time.
RESULTS

**Chaperone Protein mRNAs Are Transported into Axons of Peripheral Nervous System Neurons**—mRNAs encoding the endoplasmic reticulum chaperone proteins calreticulin and Grp78/BiP were previously detected in sensory axons by RT-PCR and array hybridization (7, 30). Because these analyses used lysed axons that could contain proximal axonal segments, we used FISH to directly visualize the mRNAs in dissociated cultures of DRG neurons. DRG neurons showed punctate FISH signals for β-actin, calreticulin, and Grp78/BiP mRNAs that extended into growth cones (Fig. 1A). This granular distribution is similar to other mRNAs localizing to dendrites and axons (21).

Sotelo-Silveira et al. (31) recently provided some in vivo evidence for intra-axonal localization of β-actin mRNA in myelinated peripheral nervous system axons. By FISH analyses of adult rat sciatic nerve, both calreticulin and Grp78/BiP mRNA signals overlap with NF signals in regions distinct from the S100 immunoreactivity that marks the Schwann cell cytoplasm (Fig. 1B). XZ and YZ views generated from multiple optical XY planes through the nerve sections confirmed that the FISH signals for calreticulin and Grp78/BiP mRNAs are indeed within the axoplasm (supplemental Fig. S1, A and B). We had previously shown that axons of cultured DRG neurons contain calreticulin and Grp78/BiP proteins (30). Sciatic nerve axons also showed immunoreactivity for calreticulin and Grp78/BiP proteins that again overlapped with NF immunoreactivity (Fig. 1C). Taken together, these data indicate that endogenous calreticulin and Grp78/BiP mRNAs are transported into peripheral nervous system axons both in culture and in vivo, where they are likely used to generate proteins.

**3′UTRs of Calreticulin and Grp78/BiP mRNAs Are Sufficient for Axonal Localization**—mRNA localization is frequently driven by sequences within the 3′UTR. Analyses of rat calreticulin and Grp78/BiP mRNA 3′UTRs showed no clear homology to the zipcode element or any other region of rat β-actin mRNA. Calreticulin and Grp78/BiP 3′UTRs also showed no homology to one another (data not shown). However, by BLAST analyses, calreticulin and Grp78/BiP 3′UTRs show considerable homology to 3′UTRs from their mammalian orthologs. 3′UTRs of available mammalian calreticulin mRNAs contain two spans of >150 nucleotides in length showing ≥85% sequence identity, with shorter spans showing ≥95% sequence identity (supplemental Fig. S2A). 3′UTRs

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**FIGURE 1.** Chaperone protein mRNAs and protein products localize into peripheral nervous system axons. A, representative images are shown for cultured DRG neurons that were processed for FISH/imunofluorescence to visualize calreticulin, Grp78/BiP, β-actin, or γ-actin mRNAs (red) and neurofilament (NF) protein (green). The larger panels show high power images of distal axons, and inset panels are a lower magnification to show the neuronal cell body. β-Actin and γ-actin mRNAs show characteristic localization, with β-actin mRNA extending into the distal axons and γ-actin mRNA being restricted to the proximal segment of the axon. Calreticulin and Grp78/BiP mRNAs show granular signals along the axon shaft (arrowheads) extending into the growth cone (arrows). Images from neurons hybridized with scrambled probe show no FISH signal in images matched for exposure, gain, and offset. B, single optical planes of sciatic nerves that were hybridized for NF/green, S100/green, calreticulin (red), and Grp78/BiP (red). NF (green) and S100 (green) are as indicated. Granular signals for calreticulin and Grp78/BiP mRNAs are seen both in the axons (arrows) and Schwann cells (arrowheads). The scrambled probe shows no specific signal in identically processed samples. C, immunolabeling of sciatic nerve sections for calreticulin (red), Grp78/BiP (red), and NF (green) proteins are shown as indicated. The images are single confocal planes taken through the center of the axoplasm of the axons traversing the center of the panel. Calreticulin and Grp78/BiP proteins are seen both in the axons (arrows) and Schwann cells (arrowheads) (scale bars, main panels = 5 μm; insets = 20 μm).

points. All live cell imaging sequences were performed on at least 10 axons over at least three separate transfection experiments. For quantifying axonal mRNA levels by FISH or RT-qPCR, significance was determined by Student-Newman-Keul test over at least three replicate experiments.
of mammalian Grp78/BiP mRNA show even higher homology, with ≥95% identity over nearly their entire 3′UTR (supplemental Fig. S2B). In contrast, the 3′UTR of γ-actin mRNA, which does not localize into axons (Fig. 1A) (12, 14), shows <50% sequence homology between mammalian orthologs using the same BLAST parameters (data not shown).

To determine whether subcellular mRNA localization is driven by these conserved regions of rat calreticulin and Grp78/BiP mRNAs, the 3′UTRs were cloned downstream of the coding sequence of the diffusion-limited GFP<sup>myr</sup> (7, 29, 33). We have previously used axonal fluorescence of GFP<sup>myr</sup> as an estimate of localized protein synthesis (7, 29, 33). As positive and negative controls for axonal localization, we used GFP<sup>myr</sup> constructs containing the 3′UTRs of rat β-actin and γ-actin mRNAs, respectively (GFP<sup>myr</sup>-β-actin and GFP<sup>myr</sup>-γ-actin) (7). DRG cultures transfected with GFP<sup>myr</sup>-β-actin and GFP<sup>myr</sup>-γ-actin plasmids showed the anticipated axonal localization of GFP, with the 3′UTR of β-actin mRNA giving cell body and axonal signals and the 3′UTR of γ-actin mRNA giving cell body restricted signals (Fig. 2, A and B). The GFP mRNA distribution mirrored the localization of GFP fluorescence, with cell body and axonal FISH signals in GFP<sup>myr</sup>-β-actin expressing neurons and only cell body signals in GFP<sup>myr</sup>-γ-actin expressing neurons (Fig. 2, E and F). Neurons transfected with GFP<sup>myr</sup> carrying the 3′UTRs of calreticulin and Grp78/BiP mRNAs (GFP<sup>myr</sup>-Cal and GFP<sup>myr</sup>-BiP) showed axonal GFP fluorescence and mRNA localization that was comparable with the β-actin 3′UTR construct (Fig. 2, C, D, G, and H). These data suggest that the 3′UTRs of rat calreticulin and Grp78/BiP mRNAs have axonal localization elements.

To test whether the axonal GFP<sup>myr</sup>-Cal and GFP<sup>myr</sup>-BiP mRNAs are locally translated, we performed FRAP analyses of transfected DRG cultures. For this, the terminal axons of neurons were photobleached, and the bleached ROI was then monitored for recovery of GFP fluorescence by time-lapse imaging (29). GFP<sup>myr</sup>-Cal expressing neurons showed significant recovery by 6 min after photobleaching (Fig. 3, A and C; supplemental Video 1), and GFP<sup>myr</sup>-BiP expressing neurons showed significant recovery by 8 min after photobleaching (Fig. 3, B and D). Fluorescence for both UTR reporter constructs showed a steady increase in intensity over the 20-min observation period, and the recovery for both was protein synthesis-dependent (Fig. 3, A–D; supplemental Video 2).

Despite the above protein synthesis-dependent recovery from photobleaching in these axons, closer inspection of the GFP signals in the more proximal segments of the anisomycin-treated cultures showed a decline in intensity over time (supplemental Fig. S3A). This could reflect movement of existing reporter protein from proximal axonal segments into the bleached ROI. As outlined below, several independent experimental approaches were used to rule out the possibility of protein diffusion versus localized translation of reporter.

First, we co-transfected DRG neurons with GFP<sup>myr</sup>-Cal and a constitutively active kinesin isoform (Kif5C<sup>560</sup>) that was shown to accumulate selectively in axonal growth cones (34). In contrast to cortical neurons expressing Kif5C<sup>560</sup>-dTomato, DRG neurons showed robust accumulation of the Kif5C<sup>560</sup>-dTomato in every growth cone (supplemental Fig. S4); this is consistent with the previously published axonal nature of DRG processes in culture (14, 35). Photobleaching growth cones with both GFP<sup>myr</sup>-Cal and Kif5C<sup>560</sup>-dTomato signals showed rapid recovery of GFP fluorescence but no recovery of dTomato fluorescence over the 30-min observation (data not shown; also see Fig. 6 below). Although Kif5C<sup>560</sup>-dTomato would move by fast axonal transport, the post-bleach observation period might not adequately reflect transport if detection thresholds for the dTomato and GFP<sup>myr</sup> were vastly different. To more directly measure fast axonal transport rates in these DRG cultures, we transfected DRG neurons with an expression construct consisting of the signal peptide of prolactin fused to mCherry (pPrLSsmCherry). The protein generated from pPrLSsmCherry is translated in the cell body with the signal peptide directing the nascent polypeptide into the default secretory pathway with some vesicular mCherry transported into axons. Monitoring rates of mCherry movement in the axon shaft gave a transport rate...
of 2.6 ± 0.3 μm/s, consistent with known rates of fast axonal transport (36). With an average axon length of 1,200 μm by 3 DIV for the cultured rat DRGs, GFP could reach from cell body to the growth cone in 7–9 min through microtubule-based transport. Thus, we could not exclude the possibility that transport of cell body-derived GFPmyr protein contributed to the recovery from photobleaching.

To distinguish locally synthesized GFPmyr from transported and diffusing GFPmyr protein, we used a double-bleach FRAP sequence to continuously photobleach signals in the proximal axon of the GFPmyr-Cal transfected DRGs, whereas recovery was monitored in distal axons. Although the GFP recovery is overall lower and more variable with the near continual laser exposure required for this double-bleach sequence (29), significant recovery from photobleaching (supplemental Fig. S3).

Diffusion of abundant mRNAs was previously suggested to account for axonal localization in hypophyseal and olfactory nerve axons (37–40). To determine whether GFPmyr-Cal and GFPmyr-BiP transcripts nonspecifically diffuse into the axons, we used LV-mediated transduction to express GFPmyr-Cal. With serial dilution of the LV, we obtained near homogeneous expression in the DRG neurons at much lower overall GFP fluorescence levels than by the electroporation method used above. Despite low GFP fluorescence in the cell body, axonal GFP signals were seen as long as GFP fluorescence was visible in the cell body (supplemental Fig. S3C). This observation plus the restriction of the GFPmyr γ-actin mRNA to the neuronal cell...
body (see Fig. 2B) argues that the axonally localized GFP mRNA and protein are the result of specific mRNA transport driven by the conserved 3’UTR of calreticulin mRNA.

Finally, we performed FRAP analyses on the GFP<sup>myr</sup>-Cal- or GFP<sup>myr</sup>-BiP-transfected DRGs after microtubule depolymerization to determine the contribution of microtubule-based transport to the recovery of GFP fluorescence above. Even in the presence of colchicine, both GFP<sup>myr</sup>-Cal- and GFP<sup>myr</sup>-BiP-expressing neurons showed significant fluorescence recovery after photobleaching (Fig. 3, C and D). Although diffusion rather than transport of GFP protein could contribute to this recovery, the double photobleach experiments shown in supplemental Fig. S3B effectively rule out this possibility. Moreover, the myristoylation tag greatly reduces diffusion of the GFP (33); FRAP performed on axons of DRGs expressing soluble GFP shows recovery in seconds compared with the minutes required for recovery of the GFP<sup>myr</sup> with the axonally localizing 3’UTRs shown here (data not shown). Finally, GFP<sup>myr</sup> constructs with γ-actin 3’UTR and deletions of calreticulin 3’UTR (see below) show no axonal fluorescence despite expressing exactly the same protein. Taken together, these data point to a microtubule-dependent transport of GFP<sup>myr</sup>-Cal reporter to axons of cultured DRG neurons. Because axonal localization of a heterologous mRNA could be driven by the 3’UTR of calreticulin or Grp78/BiP mRNAs and these show high sequence identity between mammalian orthologs, we hypothesized that the conserved regions of these UTRs contain RNA structures needed for axonal localization. We generated 3’UTR deletion mutants of the GFP<sup>myr</sup>-Cal reporter to test this possibility (Fig. 4A).

Conserved RNA Elements Drive Axonal Localization of Calreticulin mRNA—Because axonal localization of a heterologous mRNA could be driven by the 3’UTR of calreticulin or Grp78/BiP mRNAs and these show high sequence identity between mammalian orthologs, we hypothesized that the conserved regions of these UTRs contain RNA structures needed for axonal localization. We generated 3’UTR deletion mutants of the GFP<sup>myr</sup>-Cal reporter to test this possibility (Fig. 4A). Because the 3’UTR of calreticulin showed less conservation than Grp78/BiP, we reasoned that this 3’UTR should provide a more stringent test for functionality of conserved regions. GFP<sup>myr</sup> constructs that included nucleotides 1315–1412 or 1682–1780 of rat calreticulin mRNA showed axonal fluorescence with protein synthesis-dependent recovery from...
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FIGURE 5. Chaperone protein mRNAs localize into axons and dendrites of cortical neurons. A, representative co-labeling of cultured cortical neurons (9 DIV) is shown for MAP2 (red) and Tau (green) to distinguish dendritic and axonal processes, respectively. B and C, high magnification images of axons (B) and dendrites (C) displaying FISH signals for calreticulin and Grp78/BiP mRNAs (red) are shown as indicated. Both calreticulin and Grp78/BiP mRNAs show granular signals (arrows) in the axonal and dendritic processes. Scrambled probe (bottom right) shows no signal in these processes. All images were matched for exposure, gain, and offset. Scale bars; A = 20 μm; B and C = 5 μm.

photobleaching (Fig. 4, B, D, and E). GFP<sup>myr</sup> with nucleotides 1413–1681 of calreticulin mRNA showed only weak axonal fluorescence with no recovery from photobleaching (Fig. 4C). To determine whether the reporter mRNA with nucleotides 1413–1681 localized into the axons but was not efficiently translated, we performed FISH for GFP mRNA on the transfected DRGs. No axonal GFP mRNA was seen in the axons of cultures expressing GFP<sup>myr</sup> plus nucleotides 1413–1681, but the neuronal cell bodies showed strong FISH signals for the reporter mRNA (Fig. 4C). In contrast, each construct that showed axonal GFP fluorescence recovery also showed axonally localized reporter mRNA (Fig. 4A, B, D, and E). These data suggest that nucleotides 1315–1412 or 1682–1780 of rat calreticulin mRNA (Fig. 4A) are necessary for subcellular localization.

To further define the cis-elements needed for calreticulin mRNA localization, we generated GFP reporter mutants that included just nucleotides 1315–1412 and 1675–1780 in the 3′UTR. Both reporters showed axonal localization of GFP mRNA and protein synthesis-dependent recovery from photobleaching in distal axons (Fig. 4, F and G). Thus, either of the conserved 3′UTR segments of nucleotides 1315–1412 or 1682–1780 from rat calreticulin mRNA are sufficient for axonal mRNA localization.

Calreticulin mRNA 3′UTR Is Sufficient for Localization in Central Nervous System Neurons—To determine whether calreticulin and Grp78/BiP mRNAs also localize into processes of more polarized neurons, we performed FISH on cortical neuron cultures prepared from rat embryos. Co-labeling for Tau and MAP2 was used to distinguish axons and dendrites, respectively. After 9 DIV, both the axonal and dendritic processes showed granular FISH signals for calreticulin and Grp78/BiP mRNAs (Fig. 5, A–C). Thus, these endogenous chaperone protein mRNAs can also localize into axons and dendrites of cultured central nervous system neurons.

We next asked if the 3′UTR of rat calreticulin mRNA is sufficient for axonal or dendritic localization in central nervous system neurons. For this, we co-transfected dissociated cortical neurons with GFP<sup>myr</sup>-Cal and Kif5C<sup>560</sup>dTomato plasmids. The Kif5C<sup>560</sup> mutant selectively concentrates in axonal growth cones of hippocampal neurons at stage 3 and above (~4 DIV) (34). After 5 DIV, the cortical neurons showed a single process with prominent Kif5C<sup>560</sup>-dTomato signal in its growth cone. These Kif5C<sup>560</sup>-positive processes were considered as axons, and the other neurites were presumed to be dendritic. Both the axonal and dendritic processes of the cortical neurons showed prominent GFP<sup>myr</sup>-Cal fluorescence. As in the DRG axons, the Kif5C<sup>560</sup>-dTomato-positive axonal processes showed significant recovery of GFP fluorescence within 6 min after photobleaching. This recovery was also protein synthesis-dependent and preceded recovery of the dTomato fluorescence (Fig. 6, A and C).

The dendritic processes also showed significant recovery of fluorescence within 6 min that was similarly attenuated by protein synthesis inhibition (Fig. 6, B and C). Although not reaching significance, there was an initial increase in GFP fluorescence in the bleached ROI of the dendrites despite the anisomycin pretreatment (Fig. 6C). This raised the possibility that diffusion of GFP from cell body or more proximal regions of the dendrites could explain the recovery in dendrites. However, GFP mRNA was present in both axonal and dendritic processes of GFP<sup>myr</sup>-Cal-transfected cortical cultures by FISH analysis (Fig. 6D). Furthermore, transfection of these cortical cultures with the GFP<sup>myr</sup> reporter 3′UTR deletion mutants containing nucleotides 1315–1587 and 1586–1863 showed similar axonal and dendritic GFP mRNA localization, but the UTR mutant containing nucleotides 1413–1681 only showed cell body GFP mRNA similar to DRG neurons (Fig. 6D). Thus, like the DRG neurons, either of the conserved regions of the 3′UTR of calreticulin is sufficient for localization in the cortical neurons, and at least one of these elements is needed for localization. These data suggest that localization elements for calreticulin mRNA are
shared between the peripheral and central nervous system neurons as well as between axons and dendrites.

Calreticulin mRNA Levels Are Altered at the Sites of Tropic Stimulation—We showed previously that axonal levels of calreticulin mRNA are specifically increased by exposure to NT3 and MAG by RT-quantitative PCR assays (7). By the quantitative in situ hybridization approach, calreticulin mRNA also accumulates focally in axons at sites of NT3 and MAG stimulation, with 4–5 times more abundant FISH signal intensity in the axon directly adjacent to the stimuli (supplemental Fig. S1, C and D). In DRG neurons transfected with the GFPmvr-Cal construct, axonal GFP fluorescence accumulated adjacent to NT3 microparticles (Fig. 7A). The GFP signals showed a significant increase within 15 min immediately adjacent to the ligand source (Fig. 7D; supplemental Video 3). This NT3-dependent increase in GFP fluorescence was attenuated by inhibition of protein synthesis (Fig. 7A), and there was no response in axons exposed to microparticles with equimolar quantity of BSA (Fig. 7B; supplemental Video 4). Using a micromanipulator to apply the microparticles allowed us to directly correlate responses with neuronal subtypes in these DRG cultures. Interestingly, only the large diameter neurons responded to NT3. The small diameter DRG neurons, which are typically NGF-responsive, showed no NT3-dependent increase in GFP fluorescence (Fig. 7D). Thus, this NT3 response is restricted to a neuronal subpopulation in the DRG neurons, consistent with the restricted expression of TrkC, the high affinity receptor for NT3, in the large diameter sensory neurons.

The response to MAG was similarly translation-dependent but was overall smaller than that seen with NT3, not reaching...
significance until 20 min after exposure (Fig. 7, C and D). However, in contrast to NT3, there was no difference in the responses of the large and small diameter DRG neurons to MAG stimulation (data not shown). GFPmyr-Cal transfected neurons exposed to human IgG Fc microparticles showed no change in axonal GFP fluorescence adjacent to microparticles over the 40-min analysis period (Fig. 7D). Taken together, these data indicate that the 3′UTR of calreticulin mRNA is sufficient for ligand-dependent localization of calreticulin mRNA in DRG axons.

Because calreticulin mRNA has two distinct localization elements, we asked if either of these UTR subregions confers ligand-dependent localization in sensory neurons. Only the GFPmyr containing the proximal element of calreticulin 3′UTR (nucleotides 1315–1587) showed accumulation of axonal GFP fluorescence in response to NT3 and MAG (Fig. 8). Neurons expressing GFPmyr with the distal element from calreticulin (nucleotides 1586–1863) showed no change in signal intensity with NT3 and MAG treatment (Fig. 8, C and D). However, GFPmyr containing just nucleotides 1315–1412 of the 3′UTR of calreticulin was responsive to both NT3 and MAG (Fig. 8, C and D).

Because RNA localization elements can also confer translational control (41, 42), we quantified axonal levels of the GFP reporter mRNAs in cultures treated with NT3 and MAG. FISH signal intensity in the growth cones of neurons expressing GFPmyr with the full 3′UTR of calreticulin (nucleotides 1315–1863) or just nucleotides 1315–1412 increased with both NT3 and MAG treatment (Fig. 8E and supplemental Fig. S5). This increase in mRNA levels is comparable

FIGURE 7. 3′UTR of calreticulin confers ligand-dependent localization with NT3 and MAG stimulation. A, representative time-lapse sequences are shown for DRG cultures expressing GFPmyr-Cal that were exposed to microparticles with immobilized NT3. The microparticles are shown in gray, and GFP signal intensity is shown as indicated. With NT3 treatment, GFP accumulates directly adjacent to the ligand source (upper row; also see supplemental Video 3). The NT3-dependent increase in axonal GFP signal intensity is prevented by pretreatment with 50 μM anisomycin (lower row). B, no change in GFP intensity was seen adjacent to BSA (see supplemental Video 4). C, GFP similarly accumulates directly adjacent to MAG sources (upper row), and this is prevented by anisomycin pretreatment (lower panel). D, average GFP signal intensity ± S.D. in a 10 μm ROI adjacent to the ligand sources is illustrated graphically for NT3, MAG, BSA, and IgG Fc (n = 6 per condition over ≥3 transfection experiments each). For the NT3 experiments, only large diameter neurons (black line) show an increase in GFP signals; GFP signals in axons of small diameter DRG neurons exposed to NT3 (red line) are indistinguishable from the BSA and IgG Fc controls (* = p = 0.05; ** = p ≤ 0.01 by one-way ANOVA versus t = 0 min). The GFP signals at t = 40 min for NT3 and MAG are also significantly greater than t = 40 min signals for the BSA and Fc controls, respectively (1.56 ± 0.04-fold for NT3 versus BSA; 1.43 ± 0.07-fold for MAG versus Fc; p ≤ 0.001 by Student-Neuman-Keul test). Scale bars, 5 μm.
with the fold increase in GFP fluorescence for GFPmyr-Cal observed in response to NT3 and MAG observed in Fig. 7. GFP accumulates in the axons directly adjacent to the NT3 and MAG microparticles only in neurons expressing GFPmyr-Cal1315–1587 and GFPmyr-Cal1586–1863 showed no increase in axonal GFP signals in response NT3 or MAG (center rows in A and B). Normalized GFP signal intensities adjacent to the NT3 (C) and MAG (D) are illustrated as described in Fig. 7D (n ≥ 10 per condition; ***, p < 0.001, comparison between GFPmyr-Cal1315–1587 and GFPmyr-Cal1586–1863; † † † p < 0.001 by two-way ANOVA analysis). E, fluorescence intensity quantification for GFP mRNA signal in growth cones is shown. A statistically significant increase in GFP mRNA localization is seen with NT3 or MAG treatment for neurons expressing GFPmyr-Cal1315–1587 and Cal1315–1412 compared with the BSA and IgG Fc controls (2.11 ± 0.5-fold for NT3 versus BSA and 1.96 ± 0.3-fold for MAG versus Fc for GFPmyr-Cal1315–1863; 1.97 ± 0.3-fold for NT3 versus BSA and 2.05 ± 0.3-fold for MAG versus Fc for Cal1315–1412, **, p < 0.01, and † † † p < 0.001 by Student-Neuman-Keul test). The neurons expressing GFPmyr-Cal1413–1780 showed no significant change in GFP mRNA signals in response NT3 or MAG (see supplemental Fig. S5 for representative images of these FISH analyses). Scale bars, 5 μm.

Stimulus-dependent Axonal Transport of Calreticulin mRNA Requires Activation of JNK—Previous work showed that NGF modulates transport of mRNAs into axons through activation of MEK1, at least for most mRNAs tested (7). To determine whether NT3 and MAG use similar signaling pathways to modulate calreticulin transport, we quantified endogenous axonal calreticulin mRNA levels after exposing axons to immobilized sources of ligand as described previously (7). After 4 h of exposure to ligand ± kinase inhibitors, axons were isolated from the cell body and used for RNA extraction. Purity of the axonal RNA isolates was verified by the absence of MAP2, γ-actin, ErbB3, and GFAP mRNAs (Fig. 9A). In DRG neurons, MAP2 and γ-actin mRNAs are restricted to the neuronal cell body, and ErbB3 and GFAP mRNAs provide a test for glial contents in these axonal preparations. As anticipated, the NT3-dependent increase in axonal calreticulin mRNA was attenuated by inhibition of the Trk receptor with K252A, but the response to MAG was not affected by K252A (Fig. 9B). This is consistent with the selective effect of NT3 on large diameter DRG neurons shown in Fig. 7. In contrast to the effects of NGF on axonal mRNA transport (7), inhibition of MEK1 with PD98059 had no effect on the NT3- and MAG-dependent increases in axonal calreticulin mRNAs (Fig. 9B).

In our previous work, several mRNAs showed a similar transport profile to calreticulin, with regulation by MAG and NT3 in parallel but no response to brain-derived neurotrophic factor or NGF (7). Thus, we asked whether axonal localization of these other mRNAs might also require activation of JNK. Shunts in axonal levels of HSP60 mRNA, whose transport is increased by NT3 and MAG similar to calreticulin mRNA, required activation of JNK. Inhibition of the JNK pathway also prevented NT3- and MAG-dependent shifts in axonal levels of HSP90 mRNA. Interestingly, axonal HSP90 mRNA are increased by MAG but decreased by NT3. Axonal levels of...
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**FIGURE 9.** JNK signaling pathway provides specificity for the transport of calreticulin mRNA into axons. The axonal compartments of DRG neuron cultures were selectively treated with BSA, NT3, Fc, or MAG-Fc-absorbed microparticles for 4 h in the presence of the pharmacological inhibitors of MEK1 (PD98059) or JNK (SP600125)-signaling pathways. A, RNA isolated from the axonal compartment was used for detection of an axonal mRNA β-actin, γ-actin, and MAP2 as cell body-restricted mRNAs and GFAP and ErbB3 for Schwann cell mRNAs to assess purity of the axonal preparations. β-Actin was amplified from axonal RNA templates. MAP2, γ-actin, GFAP, and ErbB3 were not detected in the axonal RNA isolates but were clearly amplified from control RNA preparations. B, real time RT-PCR was used to quantitate the level of calreticulin mRNA in each condition. The calreticulin mRNA values for NT3 are expressed relative to axons treated with BSA. Values for MAG are expressed relative to human IgG Fc. The increase of axonal calreticulin mRNA with NT3 or MAG was not affected by the inhibition of MEK1 pathway but was blocked completely by JNK inhibition. Error bars represent the S.D. of three independent experiments with each sample in quadruplicate. Significance was calculated by a Student-Newman-Keul test compared with the control (*, p < 0.01 for NT3 versus NT3 + SP600125 and MAG versus MAG + SP600125; † p < 0.01 for NT3 versus NT3 + K252A; there was no significant difference for MAG and MAG + K252A or for PD98059 treatments versus ligand only).

### TABLE 1

JNK signaling provides a common pathway for NT3- and MAG-dependent RNA transport regulation

RT-quantitative-PCR analyses of axonal mRNA levels in response to NT3 or MAG were in the presence of the JNK inhibitor SP600125. Axonal levels of the mRNAs are compared with axons treated with controls (BSA for NT3; Fc for MAG); mean values are displayed ± S.D. for three replicate experiments. Values in boldface are statistically different between NT3 versus NT3 + SP600125 and MAG versus MAG + SP600125 (p < 0.01 by the Student-Newman-Keul test). Those values shown in lightface are not statistically different.

| mRNA          | Treatment               | NT3   | NT3 + SP600125 | MAG   | MAG + SP600125 |
|---------------|-------------------------|-------|----------------|-------|----------------|
| αB-crystallin | NT3                     | 0.69 ± 0.15   | 0.51 ± 0.09   | 0.58 ± 0.12 | 0.56 ± 0.12   |
| HSP60        | NT3                     | 2.61 ± 0.098  | 1.22 ± 0.12   | 2.12 ± 0.13 | 1.29 ± 0.16   |
| HSP90        | NT3                     | −2.19 ± 0.077 | −1.28 ± 0.092 | 2.28 ± 0.082 | 1.24 ± 0.115  |
| Kv3.1a       | NT3                     | 1.98 ± 0.094  | 2.16 ± 0.109  | 1.06 ± 0.078 | 1.17 ± 0.083  |

αB-crystallin and Kv3.1a mRNAs, which are selectively regulated by MAG or NT3, respectively, were not affected by the JNK inhibitor.

### DISCUSSION

Several hundred different neuronal mRNAs have been shown to be transported into axons and dendrites (7–11). Despite increasing interest in RNA localization, there have been only a few RNA elements identified that distinguish localizing from nonlocalizing mRNAs. Moreover, only a handful of elements have been identified for neurons and even fewer for axonally localized mRNAs (43). Here, we have used complementary methods to show that neuronal calreticulin and Grp78/BiP mRNAs are localized into neuronal processes, and this is driven through conserved 3′ UTR elements.

The 3′UTRs of localizing mRNAs frequently contain sequence elements responsible for their transport to subcellular domains. Farina et al. (23) defined the minimal consensus mRNA-binding site for ZBP1 by SELEX analyses as RCACCC. Rat and mouse calreticulin 3′UTRs include the ACACCC motif (see supplemental Fig. S2), but this is not seen in the 3′UTR of Grp78/BiP or in the other mammalian orthologs of calreticulin mRNA. Moreover, neither calreticulin nor Grp78/BiP mRNAs were detected in a recent screen of mRNAs co-purifying with IMP1, the human ortholog of ZBP1 (44). These observations plus the studies here on ligand-dependent transport suggest that other neuronal RNA-binding proteins beyond ZBP1 contribute to subcellular localization of calreticulin and Grp78/BiP mRNAs.

Secondary structure rather than primary sequence more typically defines RNA localization elements. Detecting commonalities in RNA secondary structure has proven difficult for more than just a few transcripts such that biologically testing for localization elements has been the most successful approach (19, 45). Sequence comparisons of calreticulin and Grp78/BiP mRNA 3′UTRs to their mammalian orthologs show more than 95% primary sequence identity. For Grp78/BiP, this spans nearly its entire 3′ UTR; for calreticulin mRNA, this consisted of two ~100-nucleotide segments with >80% identity in adjacent regions (supplemental Fig. S2). Analogous queries among
mammalian orthologs for the 3’ UTR of the nonlocalizing γ-actin mRNA showed much lower conservation (≤50%). The conserved UTR segments exceed homology of mRNA sequence in the coding regions of these chaperone protein mRNAs, and both of the 3’UTRs are sufficient for subcellular localization in neurons. However, we did not see any homology comparing calreticulin and Grp78/BiP mRNA sequences available for amphibian, flies, and nematodes.

Dendrite-specific localization elements have been described for the 3’UTRs of the mRNAs encoding microtubule-associated protein 2 (MAP2), calcium-calmodulin kinase IIα (CAMKIIα), and protein kinase Mg (PKMg) (46–48). Considering that the DRGs do not develop dendritic processes in culture, we anticipated that the two distinct localization elements in calreticulin mRNA would provide differential transport into axons versus dendrites. However, either localization element was sufficient for localization into axons and dendrites of polarized central nervous system neurons. To date, there has been no report of neuronal mRNA transcript that contains separate dendritic and axonal localization elements. The zipcode element of β-actin confers both axonal and dendritic localization (13). Map1b mRNA similarly shows both axonal and dendritic localization, but the RNA elements responsible for these have not been fully determined (17, 18, 49). Interestingly, early PCR-based studies of dendritic growth cones showed a number of mRNAs that are now known to localize to axons, including the mRNA encoding GAP-43, a classic axonal growth cone protein (50). Thus, axons and dendrites clearly share some RNA transport mechanisms, with the same RNA elements and likely the same RNA-binding proteins used for both.

The 3’UTR elements in calreticulin mRNA are distinguished by their ligand responsiveness. Other localizing mRNAs have been shown to contain more than one element used for subcellular localization. For example, nanos mRNA contains four elements in its 3’UTR that function cooperatively to confer localization in Drosophila oocytes (51, 52). Localization of protein kinase Mg mRNA into dendrites is specified by two cis-elements. One element in the 5’UTR, and the coding sequence is needed for export from the cell body, and a second element in the 3’UTR element is required for delivery of the mRNA into distal dendrites (48). The 3’UTR of myelin basic protein mRNA contains two cis-elements for localization in oligodendrocytes. One element is needed for transport of the mRNA along the processes, and a second element is required for localization of the mRNA to sites of myelin formation within the processes (53). For calreticulin mRNA, either cis-element is sufficient for transport into distal neuronal processes, and without ligand stimulation, both show axonal GFP localization that appears comparable with the full-length 3’UTR. We cannot exclude the possibility that autocrine or paracrine production of NT3 in the DRG cultures or spontaneous activity in the cortical cultures provides a stimulus for localization of GFPmyr through the proximal ligand-dependent segment of calreticulin mRNA.

The distinct responses of calreticulin mRNA elements to NT3 versus MAG are intriguing. The large diameter neurons that classically express TrkC were the only ones to show a neurotrophin-dependent localization of calreticulin mRNA, even though calreticulin mRNA is also expressed in the NGF- and brain-derived neurotrophic factor-responsive neurons. Other mRNAs that show ubiquitous expression in DRG neuronal subpopulations are selectively regulated by neurotrophins. For example, axonal levels of GAP-43 mRNA are increased by NGF, but not by brain-derived neurotrophic factor or NT3, despite that GAP-43 is also expressed by TrkB- and TrkC-expressing DRG neurons (7). Although it is possible that expression of the RNA-binding proteins needed to mobilize these transcripts is restricted to the TrkC- and TrkA-expressing neurons, our data indicate that nucleotides 1315–1412 are both necessary and sufficient for the NT3- and MAG-dependent effects on axonal localization of calreticulin mRNA. Because the MAG effect was not restricted to neuronal subpopulations, common activation of signaling pathways by NT3 and MAG that converge on a shared transport apparatus for the proximal 3’UTR calreticulin localization element is a more likely explanation for the data shown here. Consistent with this notion, activation of JNK is required for both the NT3- and MAG-dependent modulation of axonal calreticulin mRNA levels.

JNK has been shown to play a role in axonal development and regeneration (54–56). However, for the neurotrophins, JNK activation is typically considered to occur through activation of the nonselective p75 receptor rather than through TrkA, TrkB, and TrkC that selectively bind to NGF, brain-derived neurotrophic factor, and NT3, respectively (57). On the other hand, MAG is known to signal through p75 (58, 59), and MAG-dependent increase in transport of the GFPmyr-Cal was not restricted to neuronal subtypes consistent with expression of p75 in all neuronal subtypes in the DRG. The inhibition of NT3-dependent transport of calreticulin mRNA by the K252A Trk inhibitor and the selective response of axon diameter TrkC-expressing neurons argues against a p75-dependent effect for calreticulin transport. TrkC-dependent activation of JNK has been demonstrated in developing Schwann cells (32), and our data indicate that this can occur in neurons as well. Although it is not clear why NT3 and MAG share this signaling pathway for modulating calreticulin mRNA transport, other axonal mRNAs showing parallel transport regulation by MAG and NT3 also required JNK activation. Thus, our data suggest the JNK signaling pathway converges on an RNA transport machinery that is shared with other mRNAs to specifically modulate the localization of groups or families of mRNAs in tandem.

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