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THE RNA BINDING AND TRANSPORT PROTEINS STAUFEN AND FRAGILE X MENTAL RETARDATION PROTEIN ARE EXPRESSED BY RAT PRIMARY AFFERENT NEURONS AND LOCALIZE TO PERIPHERAL AND CENTRAL AXONS

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Abstract

Neuronal proteins have been traditionally viewed as being derived solely from the soma; however, accumulating evidence indicates that dendritic and axonal sites are capable of a more autonomous role in terms of new protein synthesis. Such extra-somal translation allows for more rapid, on-demand regulation of neuronal structure and function than would otherwise be possible. While mechanisms of dendritic RNA transport have been elucidated, it remains unclear how RNA is trafficked into the axon for this purpose. Primary afferent neurons of the dorsal root (DRG) and trigeminal (TG) ganglia have among the longest axons in the neuraxis and such axonal protein synthesis would be advantageous, given the greater time involved for protein trafficking to occur via axonal transport. Therefore, we hypothesized that these primary sensory neurons might express proteins involved in RNA transport. Rat DRG and TG neurons expressed staufen (stau) 1 and 2 (detected at the mRNA level) and stau2 and fragile × mental retardation protein (FMRP; detected at the protein level). Stau2 mRNA was also detected in human TG neurons. Stau2 and FMRP protein were localized to the sciatic nerve and dorsal roots by immunohistochemistry and to dorsal roots by Western blot. Stau2 and FMRP immunoreactivities colocalized with transient receptor potential channel type 1 immunoreactivity in sensory axons of the sciatic nerve and dorsal root, suggesting that these proteins are being transported into the peripheral and central terminals of nociceptive sensory axons. Based on these findings, we propose that stau2 and FMRP proteins are attractive candidates to subserve RNA transport in sensory neurons, linking somal transcriptional events to axonal translation.

Keywords

primary afferent neuron; fragile X mental retardation protein; staufen; RNA binding protein; axonal translation; RNA cargo

The discovery that RNA is transported to synaptic sites by RNA transport proteins, where it can be translated on demand, has provided insight into how neurons handle the rapid synaptic

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architectural and signaling changes that are known to occur in brain areas associated with learning and memory (Job and Eberwine, 2001;Steward and Worley, 2002;Steward and Schuman, 2003;Martin, 2004;Tiedge, 2005). There is now compelling evidence that translation occurs in axons (Koenig, 1967;Nixon, 1980;Koenig, 1991;Eng et al., 1999;Brittis et al., 2002;Piper and Holt, 2004), as mRNA and translational machinery have been localized to CNS (Tiedge et al., 1993;Aronov et al., 2001;Brittis et al., 2002), sympathetic (Olink-Coux and Hollenbeck, 1996;Eng et al., 1999) and sensory (Zheng et al., 2001;Li et al., 2004a;Verma et al., 2005;Willis et al., 2005) axons. Moreover, an emerging physiological role for local translation in axons has been described for growth cone guidance and collapse (Zheng et al., 2001;Li et al., 2004a;Verma et al., 2005;Wu et al., 2005).

Primary sensory neurons of the dorsal root ganglion (DRG) and trigeminal ganglion (TG) have among the longest axons of the entire neuraxis (greater than one meter for some human DRG neurons). Given the relatively long time interval required for axonal transport of proteins to occur in these neurons following somal synthesis, it would be advantageous if regulable, on-demand protein synthesis would occur at distal sites (for review see: Alvarez et al., 2000;Alvarez, 2001). Extra-somal translation in neurons requires the presence of specific substrates, including mRNA and translational machinery, as well as mechanisms for their transport to these distal sites of protein synthesis. To this end, various components of the translational machinery have been identified in the axons of DRG neurons (Koenig, 1991;Pannese and Ledda, 1991;Verma et al., 2005), and translation itself has been shown to occur in these axons (Koenig, 1991;Zheng et al., 2001;Verma et al., 2005;Willis et al., 2005). However, with the exception of zipcode binding protein 1 (ZBP1), which transports β-actin mRNA to growth cones (Zhang et al., 2001), RNA binding and transport molecules have not been shown to be present in axons, and none, to our knowledge, have been localized to the axons of DRG afferents.

In this study, we tested the hypothesis that two of the best characterized RNA binding and transport proteins, staufen (stau) and fragile X mental retardation protein (FMRP), localize to extra-somal sites in sensory neurons, consistent with a role in RNA transport. Here, we provide evidence that DRG and TG neurons express a number of RNA binding and transport proteins. The specific localization of stau and FMRP to sensory neuronal axons, including nociceptors, suggests a role for these RNA transport proteins in linking somal transcription to axonal translation in primary afferents. Such a mechanism would have important implications for the ability of these neurons to rapidly alter their structure and function in subserving a variety of sensory modalities, including pain.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Adult, male, Sprague–Dawley rats (Harlan, Indianapolis, IN, USA), weighing 250–300 g were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and of McGill University and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health. Every effort was taken to reduce the number of animals used in this study and their suffering. Animals were anesthetized with halothane and then decapitated. The TGs were rapidly removed and placed in a tissue mold with OCT compound and placed on dry ice. The spinal cord was then pressure ejected and the lumbar dorsal horn was dissected and placed in liquid nitrogen. The sciatic nerve was exposed at the mid-thigh level and dissected free of connective tissue up to the spinal column. The sciatic nerve was then cut at the mid-thigh level and the nerve was used to gently pull the DRGs and dorsal roots out of the spinal column. The distal most 2 cm of the sciatic nerve (~3 cm from the DRGs), the DRGs and the distal most 1 cm of the dorsal roots (~1 cm from the DRGs)

were then removed and placed in liquid nitrogen for RNA and protein experiments or on dry ice in tissue molds with OCT for immunohistochemistry (IHC). All samples were stored at −80 °C until use.

In situ hybridization (ISH)

All chemicals were from Sigma (St. Louis, MO, USA), unless otherwise stated. PCR products for each mRNA species were amplified from rat TG mRNA, ligated into pGEM®-T Easy (Promega, Madison, WI, USA) and subsequently sequenced. Stau1 (accession NM_053436) riboprobes corresponded to base-pairs 79–546 and stau2 (accession NM_134466) riboprobes corresponded to base-pairs 1073–1488. Digoxigenin-labeled sense and antisense riboprobes were generated and stored in hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate and 50 μg/ml yeast tRNA (Roche, Indianapolis, IN, USA). Fresh frozen, 20 μm tissue sections were prepared for hybridization by fixation in ice cold 3.7% formaldehyde for 1 h and permeabilization with 0.5% Triton X-100 in 0.1 M Tris/0.05 M EDTA for 30 min. Sections were then acylated with acetic anhydride for 10 min, dehydrated in ethanol, delipidated with chloroform and rehydrated in 2× SSC. For non-isotopic hybridization, sections were then hybridized with digoxigenin-labeled riboprobes (50 ng cRNA per slide) in hybridization buffer for 16 h at 55 °C. Following hybridization, sections were washed with 4× SSC four times and treated with 20 μg/ml RNase I (Roche) for 30 min at 37 °C. Washes were performed in decreasing concentrations of SSC, ending in 0.1× SSC at 55 °C for 30 min. Sections were then blocked in 4% sheep serum in B1 buffer (0.1 M NaCl, 25 mM Tris pH 7.4 and 20 mM KCl) and exposed to sheep anti-digoxigenin-AP fragments (Roche) for 1 h. Sections were then exposed to BCIP/NBT reaction overnight, according to the manufacturer's instructions (Roche). Sense probes were used as a negative control, and no signal was observed with any of these probes. For isotopic ISH on human TG, obtained from the National Disease Research Interchange (Philadelphia, PA, USA), the procedure was identical to that described previously (Price et al., 2003). For ISH on rat DRG and TG images are representative of samples from three animals.

IHC

Slide mounted sections were fixed in ice cold 3.7% paraformaldehyde in 1× PBS for 1 h and then washed 3×5 min in PBS. Slides were transferred to a solution containing 0.1 M sodium citrate and 0.05% Tween-20 and then microwaved on high power for 3 min in a 900 W microwave oven. After a 30-minute cooling period, slides were again transferred to 1× PBS, washed 3×5 min and then permeabilized in 1× PBS, containing 0.05% Triton X-100. Slides were then blocked for at least 1 h in 1× PBS, containing 10% normal goat serum, prior to the addition of a 1:1000 dilution of rabbit polyclonal anti-stau2 antiserum (kindly provided by Dr. Michael Kiebler, Vienna, Austria (Duchaine et al., 2002)) or a 1 μg/ml solution of mouse monoclonal anti-FMRP antibody (Iowa Developmental Studies Hybridoma Bank, University of Iowa (Brown et al., 2001;Lu et al., 2004)) overnight at 4 °C. Immunoreactivity was visualized following subsequent incubation with, respectively, goat anti-rabbit or goat anti-mouse Alexa-Fluor 488 antibody for one hour at room temperature. Supernatant from NS-1 cells (Iowa Developmental Studies Hybridoma Bank) was used at the same dilution factor as anti-FMRP as a negative control. In experiments where a second antigen was to be localized in the same tissue section, a 1:2000 dilution of guinea-pig polyclonal anti–transient receptor potential channel type 1 (TRPV1) antiserum (Neuromics, Northfield, MN, USA) (Guo et al., 1999) or a 1:1000 dilution of rabbit polyclonal anti-peripherin antiserum (Chemicon, Temecula, CA, USA) was then incubated for an additional overnight period and visualized following incubation with goat anti-guinea-pig or anti-rabbit Alexa-Fluor 568 secondary antibody, as described above. All IHC images are representative of samples taken from three animals.

Image acquisition and analysis

All brightfield ISH images were acquired using a Nikon E600 microscope (Melville, NY, USA) equipped with a Photometrics SenSys digital CCD camera (Roper Scientific, Tucson, AZ, USA). Confocal IHC images were taken using an Axiovert 100 M confocal microscope (Zeiss) at McGill University.

Western blotting

Frozen tissues were homogenized in standard RIPA buffer (Tris–HCl 50 mM, 1% Triton X-100, NaCl 150 mM and EDTA 1 mM at pH = 7.4) with a motorized dounce homogenizer, sonicated for 15 min at 4 °C and cleared of cellular debris and nuclei by centrifugation at 13,000 RCF for 5 min at 4 °C. Ten micrograms of protein per well was loaded and separated by standard SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Nepean, ON, Canada) and then blocked with 5% dry milk for 3 h at room temperature. Stau2 rabbit polyclonal antiserum (1:1000) or FMRP 7G1-1 mouse monoclonal antibody (1 μg/ml) was incubated overnight at 4 °C and detected the following day with donkey anti-rabbit or anti-mouse antibody conjugated to horse-radish peroxidase (Dako, Ottawa, Canada). Signal was detected by ECL-plus (Amersham/GE Health Care, Piscataway, NJ, USA) on chemiluminescent films (Kodak, Rochester, NY, USA). As a loading control, blots were then stripped using Restore reagent (Pierce, Rockford, IL, USA) and reblotted with a 1:1000 dilution of anti-β-III tubulin antibody (Tuj-1 clone, Promega). Western blots are representative of samples from three animals in which identical results were observed.

RT-PCR

Total RNA was isolated from frozen tissues using total-RNA kits (Ambion, Austin, TX, USA), and DNA was removed using DNA-free (Ambion). First strand cDNA synthesis was performed on 1 μg template RNA using iScript cDNA synthesis kits (Biorad, Hercules, CA, USA). PCR was then performed using 50 ng cDNA with iTAQ DNA polymerase kits (Biorad) for 35 cycles at an annealing temperature of 55 °C on an MJ mini thermal cycler (Biorad). Primers for stau1 were: fwd, 5′ gac tat ggc caa ggg atg aa 3′ and rev, 5′ gtc ctt gac taa ccc cga ca 3′; primers for stau2 were: fwd 5′ gac ccg agt atg gtc aag ga 3′ and rev 5′ gcc tag agt tgt gcc aga gg 3′; primers for FMRP were: fwd 5′ agt tgt gag ggt gag gat cg 3′ and rev 5′ cgt gcc ccc tst ttc tgt aa 3′. Rat GAPDH primers (Biorad) were used as a positive control. RT-PCR results are representative of samples taken from three animals in which identical results were observed.

RESULTS

Stau2 and FMRP expression in the DRG and TG

We addressed the hypothesis that sensory neurons might express certain RNA binding proteins thought to be involved in RNA transport or local translational control by performing ISH and IHC on TG and DRG sections from adult rats. Stau1 and 2 mRNAs were found in all rat DRG (Fig. 1A–B) and TG neurons (data not shown), and stau2 mRNA was found in all human TG neurons examined (Fig. 1C). No detectable signal was observed with matching sense riboprobes (data not shown). We also assessed FMRP mRNA expression in sensory ganglia, and despite the fact that sequence-confirmed FMRP PCR products could be reliably amplified by RT-PCR from DRG, TG, spinal cord and cortex, we did not observe a detectable ISH signal in any of these tissues with either of two distinct cRNA probes.

We next used IHC to assess the localization of stau2 and FMRP in the DRG and TG utilizing previously characterized antibodies against these proteins (Brown et al., 2001;Duchaine et al., 2002;Lu et al., 2004). Stau2 immunoreactivity was detected in all DRG (Fig. 2A and J) and TG neurons (data not shown). Likewise, FMRP immunoreactivity was present in all DRG (Fig.

2D and K) and TG neurons (data not shown), while the negative control (NS-1 cell supernatant), used at the same dilution factor, yielded no signal above background (Fig. 2G–I). Because small diameter DRG and TG neurons can be difficult to detect without a specific marker, we utilized an anti-TRPV1 antibody to visualize a subset of small diameter nociceptive neurons. There was a 100% overlap between both TRPV1 and stau2 immunoreactivities (Fig. 2A–C) as well as TRPV1 and FMRP immunoreactivities (Fig. 2D–F) in all DRG and TG (data not shown) sections analyzed. Higher magnification images for stau2 (Fig. 2J) and FMRP (Fig. 2K) immunoreactivities illustrate that expression was restricted to neurons.

Localization of stau2 and FMRP protein in DRG axons *in vivo*

We tested the hypothesis that stau2 and FMRP are present in the peripheral and central processes of DRG neurons *in vivo*. We chose stau2 rather than stau1 for these studies, because stau2 is neuron-specific, whereas stau1 is ubiquitously expressed, and its mRNA was present at low levels in the dorsal root (Fig. 3A). Stau2 exists as at least three isoforms, with distinct molecular weights generated through alternative splicing (Duchaine et al., 2002). Using Western blot, we detected the 62, 59 and 52 kDa isoforms of stau2 in the spinal dorsal horn and DRG, but only the 59 and 52 kDa isoforms in the dorsal root (Fig. 3A) or sciatic nerve (data not shown). Stau2 mRNA was detected only in the spinal dorsal horn and DRG by RT-PCR (Fig. 3B). The positive control GAPDH was present in all samples. FMRP protein was also present as an ~80 kDa band in the dorsal horn, dorsal root and DRG (Fig. 3C). Cerebral cortex protein was used as a positive control for the known protein size of FMRP, as detected with the same antibody (Lu et al., 2004), and this molecular species matched samples from the other tissues tested (Fig. 3C). FMRP mRNA was detected by RT-PCR in the dorsal horn, dorsal root and DRG (Fig. 3D).

We then used IHC to demonstrate the presence of stau2 and FMRP protein in the sciatic nerve and dorsal root. We examined colocalization of stau2 and FMRP with TRPV1 because, in the peripheral nervous system (PNS), TRPV1 expression is restricted to sensory fibers and because TRPV1 immunoreactivity defines the specific function of those neurons as nociceptors (Tominaga et al., 1998;Caterina et al., 2000). Stau2 immunoreactivity was localized to axons throughout the sciatic nerve. Many stau2-immunoreactive axons colocalized TRPV1, indicating that they are nociceptive in character (Fig. 4A–C). A representative photomicrograph of stau2 and TRPV1-immunoreactivities colocalized in a single sensory axon is shown in Fig. 4D–F. In sciatic nerve sections analyzed from three animals all TRPV1-positive axons also contained stau2-immunoreactivity. Additionally, many TRPV1-negative axons contained stau2-immunoreactivity. FMRP immunoreactivity was also present throughout the sciatic nerve. FMRP immunoreactivity colocalized with many peripherin-immunoreactive (Fig. 4G–I) and TRPV1-immunoreactive (Fig. 4J–L) sensory axons. As shown in Fig. 4J, FMRP immunoreactivity was observed as distinct puncta throughout axons, including TRPV1-immunoreactive axons (Fig. 4K and L), consistent with its localization in RNA granules. In sciatic nerve sections analyzed from three animals all peripherin- or TRPV1-positive axons also contained FMRP-immunoreactivity. Conversely, there were many FMRP-positive axons that were not immunoreactive for either TRPV1 or peripherin. No immunoreactivity was observed in the sciatic nerve or dorsal root with NS-1 cell supernatant (data not shown).

Stau2- (Fig. 5A) and FMRP- (Fig. 5G) immunoreactive axons were also found in the dorsal root, and many of these axons colocalized TRPV1 immunoreactivity (stau2: Fig. 5B and 5C; FMRP: Fig. 5H and 5I). However, as opposed to the sciatic nerve, there were clear examples of TRPV1-positive axons in the dorsal root that did not contain either stau2 or FMRP. We also examined the localization of the stau2 (Fig. 5D) and FMRP (Fig. 5J) in the dorsal horn. While stau2 and FMRP were expressed by many dorsal horn neurons, including neurons of lamina I

and IIi, where TRPV1-positive afferents terminate (Fig. 5E and 5K), we did not observe colocalization of stau2 (Fig. 5F) or FMRP (FIg 5L) in the central terminals of TRPV1-positive afferents.

DISCUSSION

DRG and TG neurons express stau and FMRP

Two of the best characterized RNA binding and transport proteins are stau and FMRP, which coexist in RNA/protein granules (Ohashi et al., 2002;Kanai et al., 2004;Villace et al., 2004). Stau is involved in RNA transport to synaptic sites in neurons (Kiebler et al., 1999;Tang et al., 2001) and appears to be involved in both learning and memory (Dubnau et al., 2003) and spine morphogenesis (Goetze et al., 2006). FMRP binds and is involved in transport of RNA to synapses, where it also regulates translation (for review, see Antar and Bassell, 2003). FMRP is critically involved in synaptic plasticity, acting downstream of group I metabo-tropic glutamate receptors to regulate synapse-specific RNA translocation and translation (Antar et al., 2004,2005;Bear et al., 2004;Weiler et al., 2004;Aschrafi et al., 2005). We have demonstrated here that stau is expressed by neurons in the DRG and TG of the rat and in the human TG. We have also shown that stau2 protein is expressed by all DRG and TG neurons. FMRP immunoreactivity was localized to all DRG and TG neurons by IHC, and FMRP protein and mRNA were identified in the DRG by Western blot and RT-PCR, respectively. Taken together, these results indicate that DRG and TG neurons express stau and FMRP, proteins thought to be involved in RNA transport and local regulation of translation. These proteins are found in RNA granules that localize to distal sites in CNS neurons (Ohashi et al., 2002;Kanai et al., 2004;Villace et al., 2004), suggesting that they may serve a similar function in axons of the PNS.

Stau2 and FMRP proteins localize to DRG axons: implications for RNA transport in the PNS

Stau and FMRP were found exclusively in the cell bodies and dendrites, but not axons, of CNS neurons, leading to the conclusion that stau- and/or FMRP-mediated, extra-somal RNA transport in the CNS is primarily directed to dendrites (Feng et al., 1997;Kiebler et al., 1999;Tang et al., 2001). We have demonstrated here that stau2 and FMRP are present in DRG axons *in vivo*. While three stau2 isoforms were found to be present in both the DRG and spinal dorsal horn, only the 52 and 59 kDa isoforms were present in the dorsal roots, wherein stau2 mRNA was completely absent. This finding suggests that stau2 isoforms are differentially trafficked into DRG axons. Interestingly, the 62 kDa stau2 isoform is found mostly in ribosome-free fractions, whereas the 59 and 52 kDa isoforms fractionate into higher density complexes that contain ribosomes (Duchaine et al., 2002). The presence of the 52 and 59 kDa isoforms of stau2 in the dorsal roots, wherein the only neural component is sensory axons, coupled with the complete absence of stau2 mRNA and the presence of stau2 immunoreactivity in TRPV1-immunoreactive axons, strongly supports the conclusion that stau2 protein is present in sensory axons. FMRP immunoreactivity was found in peripherally and centrally projecting axons that colocalized peripherin (a marker for unmyelinated sensory axons) and TRPV1 (a marker for nociceptive sensory axons), suggesting that FMRP is also present in sensory axons. In addition, FMRP protein was found in the dorsal roots by Western blot, although FMRP mRNA was also present in this tissue. To the extent that FMRP mRNA and protein were found in the dorsal roots, we cannot exclude the possibility that its source is intrinsic (i.e. extra-neuronal); however, the finding that FMRP immunoreactivity localizes to peripherally and centrally projecting sensory axons by IHC suggests that at least a portion of this protein band represents axonal FMRP.

To localize stau2 and FMRP to DRG axons by IHC we focused on nociceptive, TRPV1-positive axons because they are readily labeled with an anti-TRPV1 antibody and their functional role

(nociception) is well defined. In the sciatic nerve, all TRPV1-positive axons also contained stau2- or FMRP-immunoreactivity. On the other hand, in the dorsal root, only a subset of TRPV1-positive axons also contained stau2 or FMRP. Moreover, we did not observe any colocalization between TRPV1 and stau2 or FMRP in the central terminals of nociceptive afferents. These findings suggest that both stau2 and FMRP are differentially trafficked into the peripheral or central branches of nociceptors such that the peripheral direction is preferred in the normal animal. In aplysia sensory neurons stau and cytoplasmic polyadenylation element binding protein (CPEB) cooperate to modify the distribution of syntaxin mRNA to different regions of sensory neurons upon 5-HT stimulation (Liu et al., 2006). Rat DRG neurons also express CPEB (Price and Cervero, unpublished observations), hence, it is tempting to speculate that trafficking of stau or FMRP might be altered in pathophysiologies that lead to nociceptive plasticity, especially in the direction of the central projection.

Stau2 and FMRP are generally considered to be restricted to the somato-dendritic domains of neurons. In this regard, it is worth noting that there are substantial differences in the morphology of CNS and PNS sensory neurons. While CNS neurons have distinct axonal and dendritic domains, the primary afferent axon bifurcates to form two projections, one peripheral and one central, both of which are considered axons. However, these peripheral and central axons serve both afferent and efferent functions (Willis, 1999); hence, on a neurophysiologic level, they are both axon- and dendrite-like. Therefore, it is possible that the axonal localization of stau2 and FMRP in DRG neurons represents a unique property of these neurons based on their distinct morphological and functional properties. On the other hand, a recent study indicates that FMRP is found in the axons of hippocampal neurons (Antar et al., 2006). Thus, it remains to be seen if the axonal localization of stau2 and FMRP occurs on a more general basis or if this is largely limited to sensory axons and exceptional cases in the CNS.

To date, a number of RNA binding and transport proteins have been localized to dendrites, but only one, ZBP1, has been conclusively localized to axons (Zhang et al., 2001). This scarcity of data on candidate proteins for RNA transport to axons presents an obstacle for understanding the role of intra-axonal protein synthesis, because, aside from ZBP1, many of the proteins thought to be involved in RNA localization have not been demonstrated there. Our findings indicate that stau2 and FMRP are axonally trafficked in DRG neurons and implicate these two proteins as candidates to subserve a RNA transport function in these axons. Interestingly, both FMRP- and stau2-containing granules have been shown to contain BC1 RNA (Mallardo et al., 2003;Zalfa et al., 2003), a non-coding RNA polymerase III product that has been localized to axons (Tiedge et al., 1993;Trembleau et al., 1995;Muslimov et al., 2002) and implicated in facilitating the transport of a diverse number of RNAs through complementary base pairing (Zalfa et al., 2003).

Stau2 and FMRP localization in nociceptors: implications for nociception

Stau2 and FMRP colocalized with TRPV1 in the peripheral and central axons of DRG neurons. These findings indicate that stau2 and FMRP are present in the arborations of a major subclass of nociceptors and raise the possibility that stau2 and FMRP might be associated with an RNA transport function in these specialized sensory neurons. The role of local translation and RNA transport in synaptic plasticity (Steward and Worley, 2002;Wang and Tiedge, 2004;Tiedge, 2005) and the results presented here are consistent with the suggestion that similar mechanisms may be involved in the process of peripheral sensitization leading to persistent pain (Woolf and Salter, 2000). Several lines of evidence support this reasoning. First, local, axonal protein synthesis has been shown to be critical for sensitization of aplysia nociceptive tail neurons by nerve crush or depolarization (Weragoda et al., 2004). Second, in rodents, TRPV1 mRNA is axonally transported in DRG neurons in response to injury and its local translation has been implicated in inflammatory pain responses (Tohda et al., 2001;Schicho et al., 2004). Finally,

calcitonin gene-related peptide mRNA has also been detected in axons (Denis-Donini et al., 1998), and this peptide neurotransmitter, released by sensory neurons, including nociceptors, has been shown to be critical for neurogenic inflammation (Brain et al., 1985;Kilo et al., 1997), displaying considerable plasticity after nerve crush (Li et al., 2004b) and inflammation (Galeazza et al., 1995).

CONCLUSIONS

An increasing body of evidence indicates that DRG axons contain mRNA and that these axons are translationally competent (Zheng et al., 2001;Verma et al., 2005;Willis et al., 2005). In this study, we have shown that DRG and TG neurons express the RNA binding and transport proteins stau and FMRP and that these proteins localize to DRG axons. We suggest that stau2 and FMRP proteins are attractive candidates to subserve the requisite function of trafficking RNA to sensory axons and, therefore, as targets for therapeutic intervention in sensory dysfunction, including pain.

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References

Alvarez J. The autonomous axon: a model based on local synthesis of proteins. Biol Res 2001;34:103–109. [PubMed: 11715201]

Alvarez J, Giuditta A, Koenig E. Protein synthesis in axons and terminals: significance for maintenance, plasticity and regulation of phenotype. With a critique of slow transport theory. Prog Neurobiol 2000;62:1–62. [PubMed: 10821981]

Antar LN, Afroz R, Dictenberg JB, Carroll RC, Bassell GJ. Metabo-tropic glutamate receptor activation regulates fragile × mental retardation protein and FMR1 mRNA localization differentially in dendrites and at synapses. J Neurosci 2004;24:2648–2655. [PubMed: 15028757]

Antar LN, Bassell GJ. Sunrise at the synapse: the FMRP mRNP shaping the synaptic interface. Neuron 2003;37:555–558. [PubMed: 12597853]

Antar LN, Dictenberg JB, Plociniak M, Afroz R, Bassell GJ. Localization of FMRP-associated mRNA granules and requirement of microtubules for activity-dependent trafficking in hippocampal neurons. Genes Brain Behav 2005;4:350–359. [PubMed: 16098134]

Antar LN, Li C, Zhang H, Carroll RC, Bassell GJ. Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. Mol Cell Neurosci. 200610.1016/j.mcn.2006.02.001Epub ahead of print

Aronov S, Aranda G, Behar L, Ginzburg I. Axonal tau mRNA localization coincides with tau protein in living neuronal cells and depends on axonal targeting signal. J Neurosci 2001;21:6577–6587. [PubMed: 11517247]

Aschrafi A, Cunningham BA, Edelman GM, Vanderklish PW. The fragile X mental retardation protein and group I metabotropic glutamate receptors regulate levels of mRNA granules in brain. Proc Natl Acad Sci U S A 2005;102:2180–2185. [PubMed: 15684045]

Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. Trends Neurosci 2004;27:370–377. [PubMed: 15219735]

Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I. Calcitonin gene-related peptide is a potent vasodilator. Nature 1985;313:54–56. [PubMed: 3917554]

Brittis PA, Lu Q, Flanagan JG. Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. Cell 2002;110:223–235. [PubMed: 12150930]

Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT, Tenenbaum SA, Jin X, Feng Y, Wilkinson KD, Keene JD, Darnell RB, Warren ST. Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell 2001;107:477–487. [PubMed: 11719188]

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000;288:306 –313. [PubMed: 10764638]

Denis-Donini S, Branduardi P, Campiglio S, Carnevali MD. Localization of calcitonin gene-related peptide mRNA in developing olfactory axons. Cell Tissue Res 1998;294:81–91. [PubMed: 9724458]

Dubnau J, Chiang AS, Grady L, Barditch J, Gossweiler S, McNeil J, Smith P, Buldoc F, Scott R, Certa U, Broger C, Tully T. The staufen/pumilio pathway is involved in Drosophila long-term memory. Curr Biol 2003;13:286–296. [PubMed: 12593794]

Duchaine TF, Hemraj I, Furic L, Deitinghoff A, Kiebler MA, DesGro-seillers L. Staufen2 isoforms localize to the somatodendritic domain of neurons and interact with different organelles. J Cell Sci 2002;115:3285–3295. [PubMed: 12140260]

Eng H, Lund K, Campenot RB. Synthesis of beta-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. J Neurosci 1999;19:1–9. [PubMed: 9870932]

Feng Y, Gutekunst CA, Eberhart DE, Yi H, Warren ST, Hersch SM. Fragile X mental retardation protein: nucleocytoplasmic shuttling and association with somatodendritic ribosomes. J Neurosci 1997;17:1539–1547. [PubMed: 9030614]

Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS. Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. Neuroscience 1995;66:443–458. [PubMed: 7477885]

Goetze B, Tuebing F, Xie Y, Dorostkar MM, Thomas S, Pehl U, Boehm S, Macchi P, Kiebler MA. The brain-specific double-stranded RNA-binding protein Staufen2 is required for dendritic spine morphogenesis. J Cell Biol 2006;172:221–231. [PubMed: 16418534]

Guo A, Vulchanova L, Wang J, Li X, Elde R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur J Neurosci 1999;11:946–958. [PubMed: 10103088]

Job C, Eberwine J. Localization and translation of mRNA in dendrites and axons. Nat Rev Neurosci 2001;2:889–898. [PubMed: 11733796]

Kanai Y, Dohmae N, Hirokawa N. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. Neuron 2004;43:513–525. [PubMed: 15312650]

Kiebler MA, Hemraj I, Verkade P, Kohrmann M, Fortes P, Marion RM, Ortin J, Dotti CG. The mammalian staufen protein localizes to the somatodendritic domain of cultured hippocampal neurons: implications for its involvement in mRNA transport. J Neurosci 1999;19:288–297. [PubMed: 9870958]

Kilo S, Harding-Rose C, Hargreaves KM, Flores CM. Peripheral CGRP release as a marker for neurogenic inflammation: a model system for the study of neuropeptide secretion in rat paw skin. Pain 1997;73:201–207. [PubMed: 9415506]

Koenig E. Synthetic mechanisms in the axon. IV. In vitro incorporation of [3H]precursors into axonal protein and RNA. J Neurochem 1967;14:437–446. [PubMed: 5336968]

Koenig E. Evaluation of local synthesis of axonal proteins in the goldfish Mauthner cell axon and axons of dorsal and ventral roots of the rat in vitro. Mol Cell Neurosci 1991;2:384.

Li C, Sasaki Y, Takei K, Yamamoto H, Shouji M, Sugiyama Y, Kawakami T, Nakamura F, Yagi T, Ohshima T, Goshima Y. Correlation between semaphorin3A-induced facilitation of axonal transport and local activation of a translation initiation factor eukaryotic translation initiation factor 4E. J Neurosci 2004a;24:6161–6170. [PubMed: 15240808]

Li XQ, Verge VM, Johnston JM, Zochodne DW. CGRP peptide and regenerating sensory axons. J Neuropathol Exp Neurol 2004b;63:1092–1103. [PubMed: 15535136]

Liu J, Hu JY, Wu F, Schwartz JH, Schacher S. Two mRNA-binding proteins regulate the distribution of syntaxin mRNA in aplysia sensory neurons. J Neurosci 2006;26:5204–5214. [PubMed: 16687512]

Lu R, Wang H, Liang Z, Ku L, O'Donnell WT, Li W, Warren ST, Feng Y. The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. Proc Natl Acad Sci U S A 2004;101:15201–15206. [PubMed: 15475576]

Mallardo M, Deitinghoff A, Muller J, Goetze B, Macchi P, Peters C, Kiebler MA. Isolation and characterization of Staufen-containing ribonucleoprotein particles from rat brain. Proc Natl Acad Sci U S A 2003;100:2100–2105. [PubMed: 12592035]

Martin KC. Local protein synthesis during axon guidance and synaptic plasticity. Curr Opin Neurobiol 2004;14:305–310. [PubMed: 15194110]

Muslimov IA, Titmus M, Koenig E, Tiedge H. Transport of neuronal BC1 RNA in Mauthner axons. J Neurosci 2002;22:4293–4301. [PubMed: 12040034]

Nixon RA. Protein degradation in the mouse visual system. I. Degradation of axonally transported and retinal proteins. Brain Res 1980;200:69–83. [PubMed: 6158362]

Ohashi S, Koike K, Omori A, Ichinose S, Ohara S, Kobayashi S, Sato TA, Anzai K. Identification of mRNA/protein (mRNP) complexes containing Puralpha, mStaufen, fragile X protein, and myosin Va and their association with rough endoplasmic reticulum equipped with a kinesin motor. J Biol Chem 2002;277:37804–37810. [PubMed: 12147688]

Olink-Coux M, Hollenbeck PJ. Localization and active transport of mRNA in axons of sympathetic neurons in culture. J Neurosci 1996;16:1346–1358. [PubMed: 8778286]

Pannese E, Ledda M. Ribosomes in myelinated axons of the rabbit spinal ganglion neurons. J Submicrosc Cytol Pathol 1991;23:33–38. [PubMed: 2036627]

Piper M, Holt C. RNA translation in axons. Annu Rev Cell Dev Biol 2004;20:505–523. [PubMed: 15473850]

Price TJ, Helesic G, Parghi D, Hargreaves KM, Flores CM. The neuronal distribution of cannabinoid receptor type 1 in the trigeminal ganglion of the rat. Neuroscience 2003;120:155–162. [PubMed: 12849749]

Schicho R, Florian W, Liebmann I, Holzer P, Lippe IT. Increased expression of TRPV1 receptor in dorsal root ganglia by acid insult of the rat gastric mucosa. Eur J Neurosci 2004;19:1811–1818. [PubMed: 15078554]

Steward O, Schuman EM. Compartmentalized synthesis and degradation of proteins in neurons. Neuron 2003;40:347–359. [PubMed: 14556713]

Steward O, Worley P. Local synthesis of proteins at synaptic sites on dendrites: role in synaptic plasticity and memory consolidation? Neurobiol Learn Mem 2002;78:508–527. [PubMed: 12559831]

Tang SJ, Meulemans D, Vazquez L, Colaco N, Schuman E. A role for a rat homolog of staufen in the transport of RNA to neuronal dendrites. Neuron 2001;32:463–475. [PubMed: 11709157]

Tiedge H. RNA reigns in neurons. Neuron 2005;48:13–16. [PubMed: 16273717]

Tiedge H, Zhou A, Thorn NA, Brosius J. Transport of BC1 RNA in hypothalamo-neurohypophyseal axons. J Neurosci 1993;13:4214 – 4219. [PubMed: 7692010]

Tohda C, Sasaki M, Konemura T, Sasamura T, Itoh M, Kuraishi Y. Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. J Neurochem 2001;76:1628–1635. [PubMed: 11259480]

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 1998;21:531–543. [PubMed: 9768840]

Trembleau A, Melia KR, Bloom FE. BC1 RNA and vasopressin mRNA in rat neurohypophysis: axonal compartmentalization and differential regulation during dehydration and rehydration. Eur J Neurosci 1995;7:2249–2260. [PubMed: 8563974]

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]

Villace P, Marion RM, Ortin J. The composition of Staufen-containing RNA granules from human cells indicates their role in the regulated transport and translation of messenger RNAs. Nucleic Acids Res 2004;32:2411–2420. [PubMed: 15121898]

Wang H, Tiedge H. Translational control at the synapse. Neuroscientist 2004;10:456–466. [PubMed: 15487031]

Weiler IJ, Spangler CC, Klintsova AY, Grossman AW, Kim SH, Bertaina-Anglade V, Khaliq H, de Vries FE, Lambers FA, Hatia F, Base CK, Greenough WT. Fragile X mental retardation protein is necessary for neurotransmitter-activated protein translation at synapses. Proc Natl Acad Sci U S A 2004;101:17504–17509. [PubMed: 15548614]

Weragoda RM, Ferrer E, Walters ET. Memory-like alterations in Aplysia axons after nerve injury or localized depolarization. J Neurosci 2004;24:10393–10401. [PubMed: 15548654]

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 cytoskeletal, injury-response, and neurodegeneration protein mRNAs in axons. J Neurosci 2005;25:778–791. [PubMed: 15673657]

Willis WD Jr. Dorsal root potentials and dorsal root reflexes: a double-edged sword. Exp Brain Res 1999;124:395–421. [PubMed: 10090653]

Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. Science 2000;288:1765–1769. [PubMed: 10846153]

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]

Zalfa F, Giorgi M, Primerano B, Moro A, Di Penta A, Reis S, Oostra B, Bagni C. The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. Cell 2003;112:317–327. [PubMed: 12581522]

Zhang HL, Eom T, Oleynikov Y, Shenoy SM, Liebelt DA, Dictenberg JB, Singer RH, Bassell GJ. Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility. Neuron 2001;31:261–275. [PubMed: 11502257]

Zheng JQ, Kelly TK, Chang B, Ryazantsev S, Rajasekaran AK, Martin KC, Twiss JL. A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. J Neurosci 2001;21:9291–9303. [PubMed: 11717363]

Abbreviations

CPEB
: cytoplasmic polyadenylation element binding protein

DRG
: dorsal root ganglion

FMRP
: fragile X mental retardation protein

IHC
: immunohistochemistry

ISH
: *in situ* hybridization

PNS
: peripheral nervous system

stau
: staufen

TG
: trigeminal ganglion

TRPV1
: transient receptor potential channel type 1

ZBP1
: zipcode binding protein 1

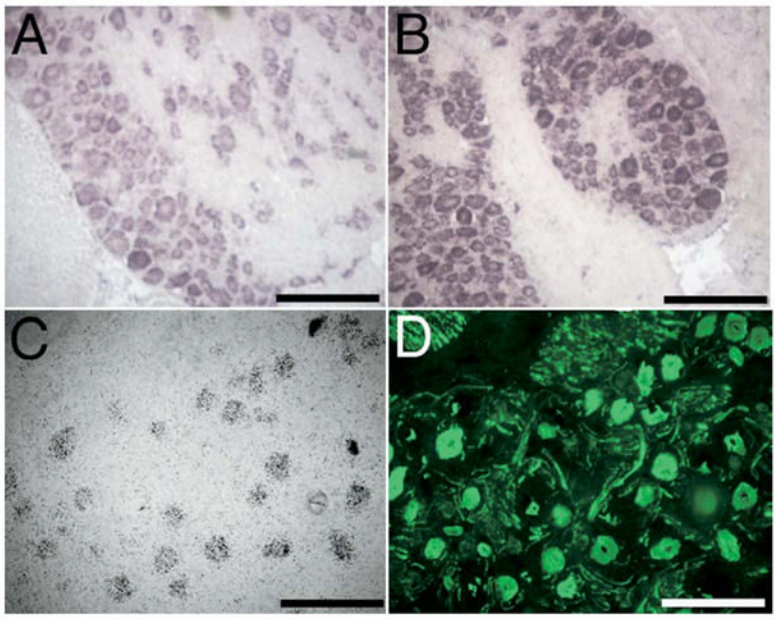

Fig 1.

Stau expression in rat DRG and human TG. Both Stau1 (A) and stau2 (B) mRNAs were expressed by all DRG neurons. Human TG neurons also express stau2 (C) as shown by silver grains from isotopic ISH. Matching image of neurofilament 200 protein (D) is shown to demonstrate the location of neurons in the ganglia. Scale bars=200 μm in all panels.

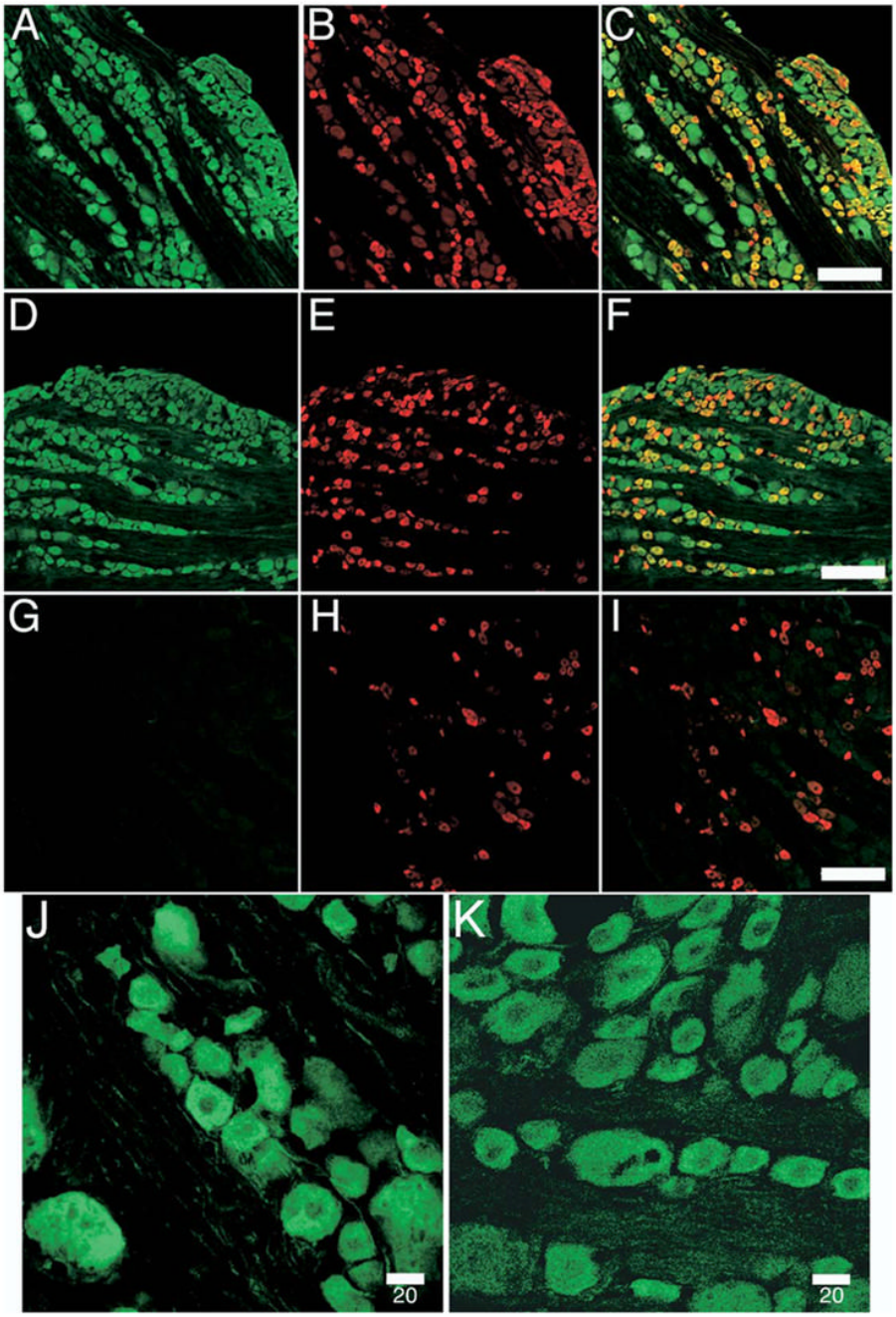

Fig 2.

Stau2 and FMRP protein expression in the DRG and dorsal horn. Stau2 protein was expressed by all DRG neurons (A), while expression of TRPV1 (B) demonstrates colocalization in small nociceptive neurons (C). FMRP protein was also expressed by all DRG neurons (D), while expression of TRPV1 (E) demonstrates colocalization in small nociceptive neurons (F). Negative control for FMRP (G) with TRPV1 immunoreactivity (H and merged in I). Scale bars=200 μm (A–I) or 20 μm (J–K). High magnification images for stau2 (J) and FMRP (K) show the neuron-specific expression of these proteins in the DRG.

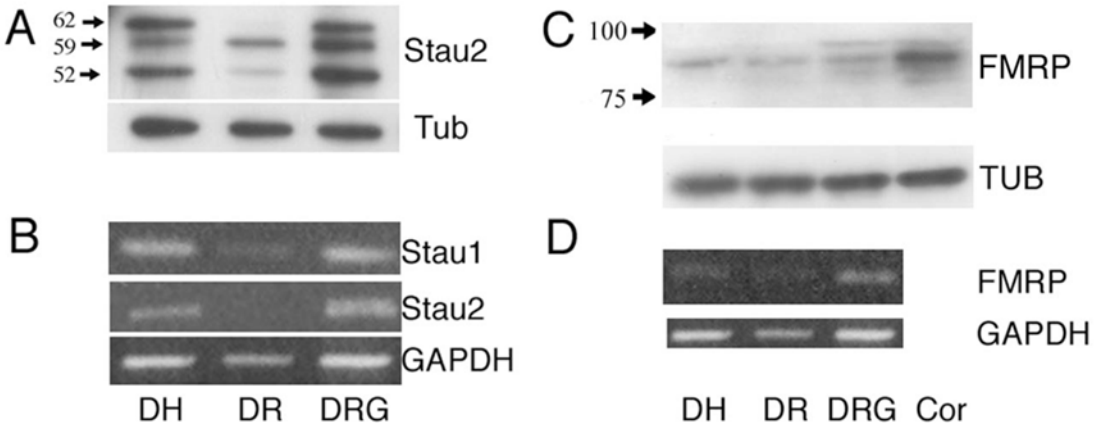

Fig 3.

Assessment of stau2 and FMRP protein and mRNA expression in the dorsal horn (DH), dorsal root (DR) and DRG by Western blot and RT-PCR. (A) Stau2 protein Western blot illustrating that the 62, 59 and 52 kDa isoforms of the protein are present in the DH and DRG, whereas only the 59 and 52 kDa isoforms are present in the DR. *β*III-Tubulin (tub) was used as a loading control. (B) Stau1 mRNA was present in the DH, DR and DRG by RT-PCR, whereas stau2 mRNA was only present in the DH and DRG. GAPDH was used as a loading and positive control. (C) FMRP protein, detected as an ~80 kDa protein band, was present in DH, DR and DRG by Western blot. Cortex (Cor) protein was used as a positive control for the known size of FMRP. Tub was used as a loading control. (D) FMRP mRNA was detected by RT-PCR in the DH, DR and DRG. GAPDH was used as a loading and positive control. All Western blot and RT-PCR experiments are representative of samples run from three different animals per tissue.

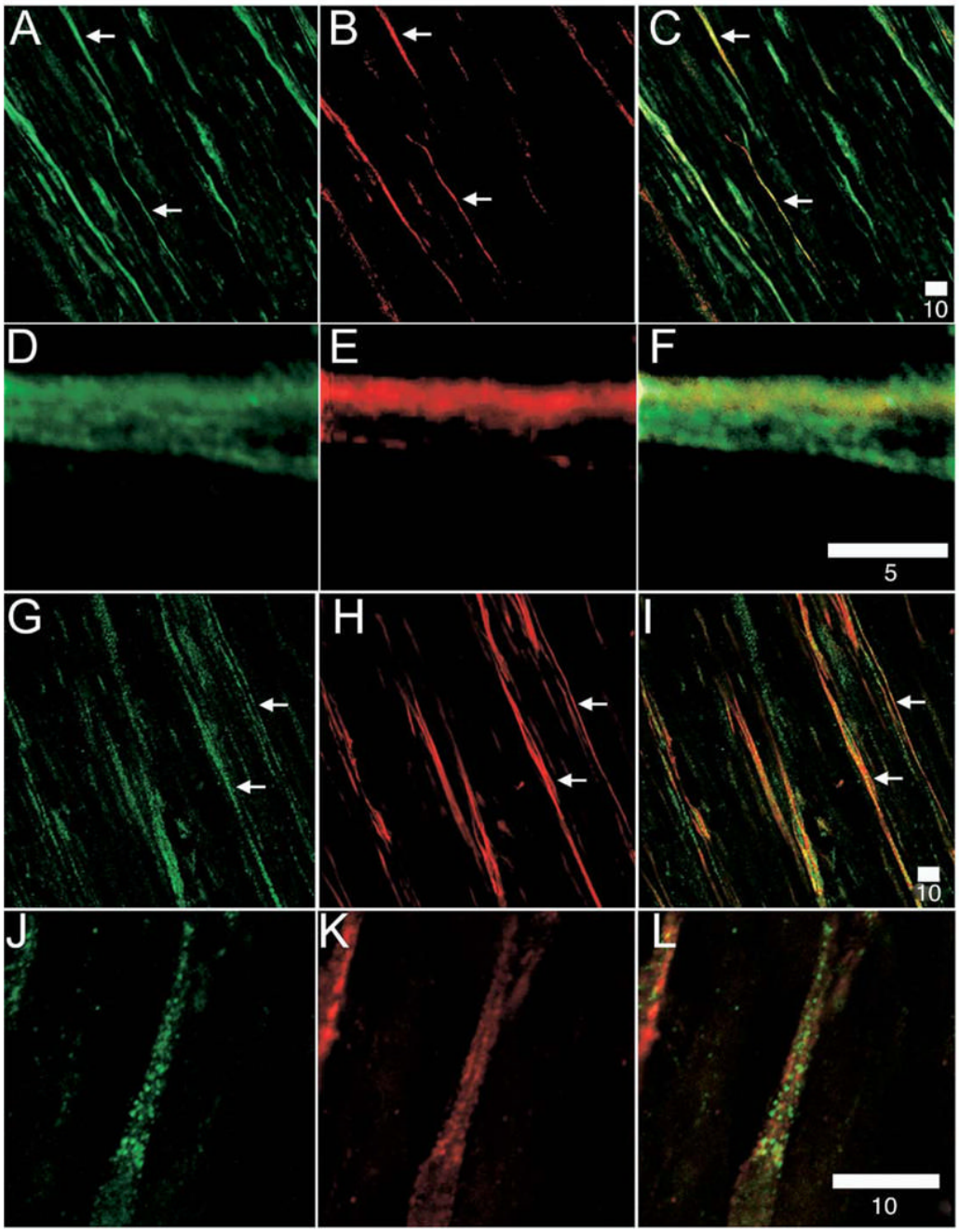

Fig 4.

Stau2 and FMRP immunoreactivities in the sciatic nerve. Stau2 (A) and TRPV1 (B and merge in C) immunoreactivities observed in the sciatic nerve. Examples of fibers that colocalized stau2 and TRPV1 are illustrated with arrows. High magnification image of a single TRPV1-immunoreactive fiber (E) that colocalized stau2 (D and merge in F). FMRP (G) and peripherin (H and merge in I) immunoreactivities observed in the sciatic nerve. Examples of fibers that colocalized FMRP and peripherin are illustrated with arrows. High magnification image of a single TRPV1-immunoreactive fiber (K) that colocalized FMRP (J and merge in L). Scale bars=10 μm (A–C, G–I and J–L) or 5 μm (D–F).

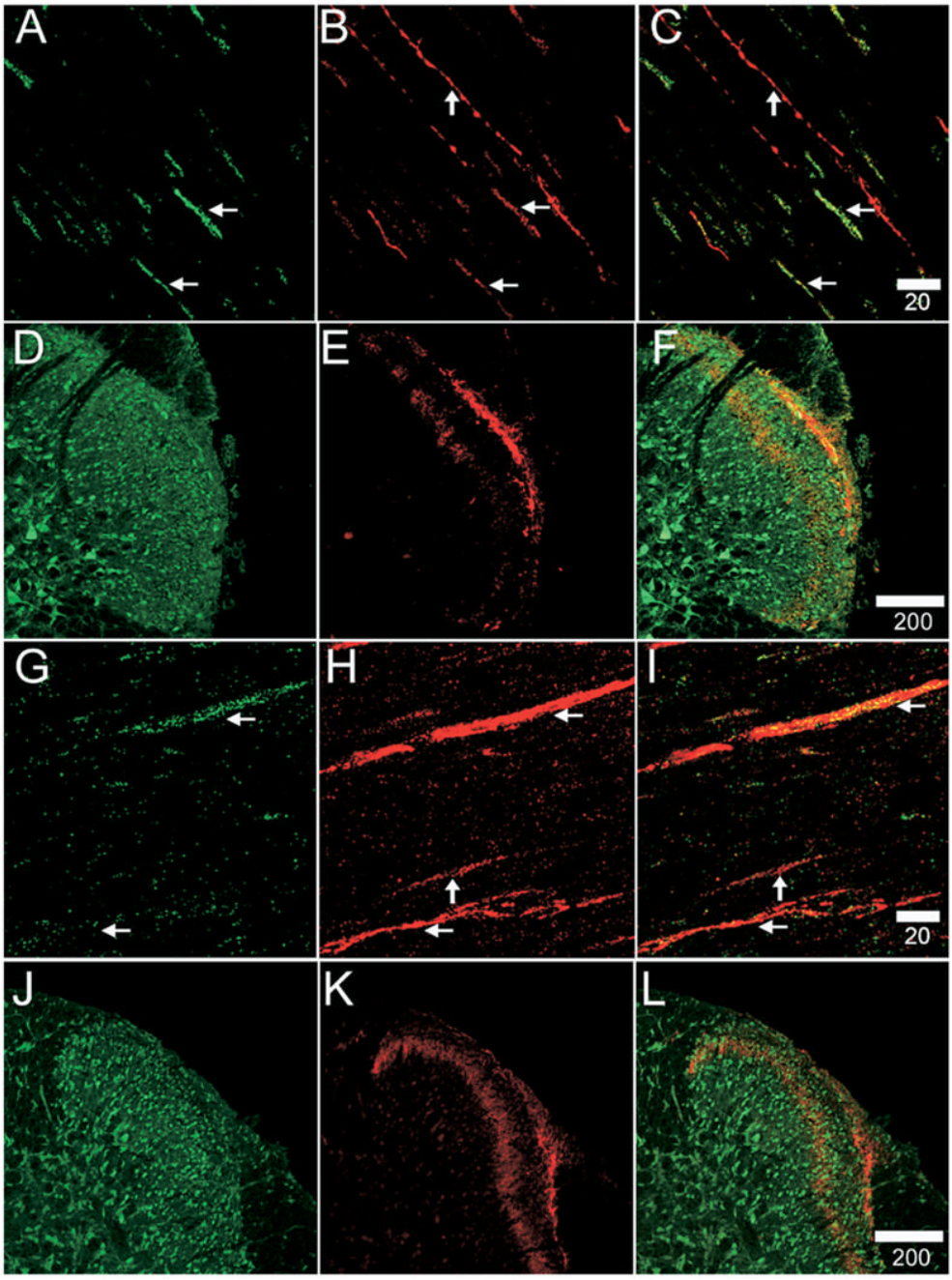

Fig 5.

Stau2 and FMRP immunoreactivities in the dorsal root and spinal dorsal horn. Stau2 (A) and TRPV1 (B and merge in C) immunoreactivities in the dorsal root. Stau2 (D) and TRPV1 (E and merge in F) immunoreactivities in the spinal dorsal horn. FMRP (G) and TRPV1 (H and merge in I) immunoreactivities in the dorsal root. FMRP (J) and TRPV1 (K and merge in L) immunoreactivities in the spinal dorsal horn. Examples of colocalization are illustrated with sidewise arrows. Examples of TRPV1-positive axons that did not colocalize stau2 or FMRP are illustrated with upward arrows. Scale bars=20 μm (A–C and G–I) or 200 μm (D–F and J–L).