Tau mRNA is present in axonal RNA granules and associates with the eukaryotic elongation factor 1A

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**Summary**

The microtubule-associated protein tau is predominantly localized in axons but mechanisms responsible for the localization of tau in the distal part of the axon are not properly understood. Using fluorescence in situ hybridization , we show that tau mRNA is present in the central and distal parts of the axon of cultured rat cortical neurons. In the distal part of the axon, tau mRNA is associated with granules which are distributed throughout the entire length of the axon, including the growth cone. We also show that tau mRNA-containing axonal particles are associated with the eukaryotic elongation factor 1A. The association of elongation factor 1A with axonal tau mRNA suggests that tau is synthesized locally in the axon at a long distance from the cell body. The presence of tau mRNA in the axon might be at least part of the process by which tau is specifically localized to the axon that may be disrupted in neurodegenerative conditions in which tau accumulates in the cell body.

**Introduction**

Tau is a neuronal microtubule-associated protein playing an important role in microtubule assembly and stability. The tau protein is predominantly localized in the axonal compartment over the entire length of the axon. Abnormal perikaryal accumulation of tau is a characteristic feature of several neurodegenerative diseases, including Alzheimer’s disease (Ballatore et al., 2007; Gallo et al., 2007). Understanding the mechanisms responsible for the axonal localization of tau is therefore not only relevant to normal neuronal physiology but also to pathogenic mechanisms in some neurodegenerative conditions.

The mechanisms responsible for the axonal localization of tau are not properly understood. Tau could be synthesized in the cell body only and transported down the axon. Tau can diffuse from the cell body into the axon, however diffusion is only effective over short distances and can only explain tau presence in the proximal part of the axon (Konzack et al., 2007). Over longer distances tau is, at least in part, transported bound to microtubule fragments moving along the axon through a dynein/actin dependent mechanism (Konzack et al., 2007). Another proposed mechanism to explain the specific axonal localization of tau, not mutually exclusive with transport of the protein on microtubules, is that tau is synthesized in the axon following selective transport of its mRNA. In addition to the cell body, tau mRNA has been observed in the proximal axon of rat primary neurons using in situ hybridization (Litman et al., 1993). Thus tau could be synthesized in the proximal part of the axon and transported further down the axon bound to microtubule fragments.

 The targeting of specific mRNAs to subcellular compartments is a mechanism ensuring local synthesis of proteins. This has been well documented in dendrites, which allows local protein translation in particular in response to neurotransmitters or neurotrophic factors (Hirokawa, 2006; Martin and Zukin, 2006). RNA is transported in dendrites as large RNP granules. Kanai *et al.* (Kanai et al., 2004) isolated large RNase-sensitive granules from mouse brain as a binding partner of kinesin KIF5, they contained several different mRNAs and proteins involved in RNA-processing, including the eukaryotic elongation factor 1A (eEF1A). These mRNA-protein complexes were visualized in the dendrites of hippocampal neurons. A body of evidence has emerged showing that mRNAs are present and locally translated in axons in particular during elongation and in response to injury (Donnelly et al.; Sotelo-Silveira et al., 2006; Taylor et al., 2009; Willis et al., 2005).

 Whether endogenous tau mRNA is present in distal axons is still an open question. In this study, we explored the localization of tau mRNA in the axon using fluorescence in situ hybridization (FISH) in primary rat cortical neurons. We show that tau mRNA is not only present in the cell body and proximal axon, but also in granules throughout the axon, including the growth cone. We also show that tau mRNA-containing axonal RNP granules are associated with eEF1A, suggesting that tau mRNA is translated locally in the axon.

**Results and Discussion**

**RNA granules in the axon**

To establish the presence of RNA in the axon, primary rat cortical neurons were stained with the RNA-specific dye, SYTO 14. Neurons were seeded at very low density; hence individual neurons were easily distinguished. Axonal processes were defined by morphological assessment as the longest process in each neuron. Furthermore, to distinguish axons from dendrites, neurons were stained with an antibody against the neurofilament heavy subunit (NF-H), a specific axonal marker. Neurons were brightly labeled by SYTO 14 with the cell body staining intensely (Fig.1A). In addition, SYTO 14 labeled RNA in neuronal processes, dendrites as well as axons, with small granules observed along the length of the axon. To confirm the specificity of the staining, neurons were treated with RNases after labeling with SYTO 14 (Fig. 1B). Treatment with RNases reduced SYTO 14 staining in the cell body and completely abolished granular staining in processes thus confirming that the SYTO 14-positive granules contained RNA.

**Tau mRNA is present in RNA granules in axons**

We next analyzed the distribution of tau mRNA in rat primary cortical neurons using FISH. A digoxigenin (DIG)-labeled RNA probe representing the last 400 3’ bases of the coding region of tau mRNA was synthesized. This sequence encodes the C- terminus of the tau protein, including the third and fourth microtubule-binding repeat domains, a domain not affected by alternative splicing and expressed in rat embryonic neurons. Using this probe, an intense staining for tau mRNA was obtained in cell bodies and proximal axons (Fig. 2A) consistent with previous studies (Litman et al., 1993).

In addition, tau mRNA was also observed in distal parts of the axon. High magnification revealed a granular staining pattern with tau mRNA granules distributed along the length of the axon (Fig. 2B). To validate the specificity of tau mRNA staining a number of control conditions were used (Fig. 2C-E). Importantly, only a weak background signal was detected using a sense probe (Fig. 2C). A weak background signal was also obtained when cells were incubated with the anti-DIG antibody only, without any RNA probe (Fig. 2D). Finally, the tau mRNA signal detected with the antisense probe was reduced to a weak background when cells were treated with RNases before the FISH procedure (Fig. 2E). Thus, endogenous tau mRNA is associated with granules in the axon of rat primary cortical neurons.

These results clearly suggest that neurons target tau mRNA to distal parts of the axon, and are consistent with earlier studies demonstrating that tau mRNA is present as granules in neurite-like processes in P19 teratocarcinoma cells overexpressing tau and differentiated into a neuronal phenotype (Aronov et al., 2001; Aronov et al., 2002). In situ hybridization for tau mRNA in cultured neurons revealed the presence of tau mRNA in the proximal part of the axon only and not in the distal part (Litman et al., 1993). However, in this case, the detection method, alkaline phosphatase, was not as sensitive as fluorescence. Although we found tau mRNA in the axon, tau was not among the proteins synthesized in axons isolated from cultured adult dorsal root ganglion neurons identified in a proteomic study (Willis et al., 2005). However a number of other cytoskeletal proteins were found in this analysis, including β-actin, peripherin, vimentin, that may be required for axonal growth and maintenance.

**Tau mRNA co-segregates with eEF1A**

Dendritic RNP granules are complexes of RNA, RNA processing proteins and transport proteins (Kanai et al., 2004). Among RNA-processing proteins associated with dendritic RNA granules, eEF1A is of particular significance due to its role in translation. The main function of eEF1A is to facilitate the recruitment of aminoacyl-tRNA to the A site of the ribosome in the elongation phase of protein synthesis. Thus the presence of eEF1A in dendritic RNP particles is consistent with local translation. We investigated whether eEF1A was present in tau mRNA granules in the axon. We first determined whether eEF1A was associated with RNA in the axon by combined immunostaining for eEF1A and SYTO 14 staining. eEF1A was mainly localized in the somatodendritic compartment (Fig. 3A). However, eEF1A was also detected in the axon where eEF1A staining co-localized with SYTO 14 in granular material (Fig. 3B).

FISH labeling for tau mRNA was performed in combination with immunostaining for eEF1A (Fig. 3C). Laser scanning confocal microscopy revealed a clear co-localization between tau mRNA and eEF1A in granular material in the proximal as well as in more distal parts of the axon (Fig. 3D, upper and lower panel, respectively). Both eEF1A and tau mRNA was also visualized in the growth cone (Fig. 3E, F), with a high density staining observed for tau mRNA as well as eEF1A at the leading edge of the growth cone (Fig. 3F).

The presence of eEF1A in axonal tau mRNA-containing granules suggests that tau is translated locally in distal axons, including in growth cone, where translation of tau may be essential for growth cone motility. However, other non-canonical roles for eEF1A in RNA-processing have been suggested, such as regulation of mRNA localization (Mateyak and Kinzy).

In this study, we have demonstrated the presence of tau mRNA in granular structures distributed throughout the axon of primary cortical neurons. The association of the elongation factor, eEF1A, with axonal tau mRNA granules suggests that tau may be synthesized locally in the axon at a long distance from the cell body. Localization of tau mRNA in the axon might be at least part of the process by which tau is specifically localized to the axon. Future investigations will be aimed at identifying proteins associated with tau mRNA-containing RNPs that determine their axonal localization. Irrespective of the precise nature of these proteins, the results presented here are the first to show the presence of tau mRNA in distal axons of cultured neurons. Abnormal sorting of hyperphosphorylated tau to the somatodendritic compartment, where it aggregates into neurofibrillary tangles (NFTs), is a feature of a group of neurodegenerative diseases referred to as the tauopathies, including Alzheimer’s disease (AD) (Ballatore et al., 2007). Aberrant RNA processing is becoming increasingly associated with neurodegenerative disorders, tau mislocalization in tauopathies may be the consequence of mis-targeting of its mRNA to the axon.

**Materials and Methods**

**Antibodies**

Sheep polyclonal anti-digoxigenin antibody was purchased from Roche Applied Science (West Sussex, UK); the mouse monoclonal anti-α-tubulin DM1A was from Sigma-Aldrich (Dorset, UK); the mouse monoclonal anti-eEF1A antibody CBP-KK1 was from Millipore (Durham, UK); rabbit polyclonal anti-NF-H antibody was from Enzo Life Sciences (Exeter, UK).

**Primary neuronal culture**

All culture reagents were purchased from Invitrogen Ltd (Paisley, UK), unless otherwise stated. Cortical neurons were prepared from embryonic day 18 Sprague Dawley rat embryos (Charles River, Margate, UK). Isolated cortices were treated with 0.05% trypsin-EDTA and dissociated into a single cell suspension using gentle trituration. Cells were cultured in Neurobasal medium supplemented with 2% (v/v) B27 serum-free supplement, 2mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) on coverslips coated with 10 μg/ml poly-D-lysine. 3 μM cytosine arabinofuranoside (Sigma-Aldrich) was added to the culture medium after 3 days and neurons were cultured for up to 7 days before further processing. Neurons were maintained at 37˚C in a humidified atmosphere of 5 % CO2/95 % air.

**Preparation of RNA probes**

A cDNA fragment corresponding to the 3’ 400 nucleotides (899-1299) of the coding region of a cDNA encoding the longest isoform of rat tau (Kosik et al., 1989) was subcloned into the pSPT19 vector (Roche) between the *Acc*I and *Eco*RI sites. In vitro transcription was performed with a DIG-labeled nucleotide mixture using a DIG-RNA Labeling kit (Roche) according to the manufacturer’s instructions. Both sense and antisense tau RNA probes were synthesized from the same template by using either the SP6 or the T7 promoter in the pSPT19 vector.

**Fluorescence in situ hybridization**

All steps were performed at room temperature unless otherwise stated. Neurons were washed at 37˚C in PBS containing 5 mM MgCl2 (PBSM) and then fixed for 15 min with 4 % (w/v) paraformaldehyde in PBSM (pre-warmed to 37˚C). Following fixation cells were washed three times in PBSM, permeabilized for 5 min with 0.1 % (v/v) Triton X-100 in PBSM, and then washed three times in PBSM. Before hybridization neurons were prehybridized in the hybridization buffer [30 % (v/v) formamide, 10 mM NaH2PO4, 10 % (v/v) dextran sulphate, 125 µg/ml tRNA, 100 µg/ml salmon sperm DNA (denatured by heating at 95˚C for 3 min before use), 2× saline-sodium citrate (SSC), 10 mM vanadyl ribonuclease and 1× Denhardt’s solution] for 2 hours at 50˚C. Hybridization was then performed using 100 ng of denatured RNA probe per coverslip in hybridization buffer overnight at 50˚C. Following hybridization cells were washed twice in 30 % (v/v) formamide and 2× SSC followed by three times in 2× SSC for 15 min each at 50˚C. Cells were incubated with primary antibodies in 1 % (w/v) BSA in PBSM overnight at 4˚C. Following three washes in PBSM, cells were incubated with appropriate secondary antibodies in 1 % (w/v) BSA in PBSM for 1 hour, followed by three washes in PBSM. Coverslips were mounted in fluorescence mounting medium (DakoCytomation) and slides were viewed under a Zeiss LSM 510 META laser scanning confocal microscope or a Zeiss Axioskop fluorescence microscope with appropriate filters and imaged using LSM 510 imaging software or Metamorph software, respectively. For RNase treatment neurons were incubated with RNase A (10 µg/ml) and RNase T1 (1000 units/ml) in PBSM for 30 min at 37˚C after fixation and permeabilization, but prior to RNA hybridization.

**RNA staining**

Neurons were incubated with 500 nM SYTO 14 dye (Molecular Probes) diluted in culture medium for 15 min at 37˚C and washed twice in the same medium before fixation with 4 % (w/v) paraformaldehyde in PBS for 15 min. After fixation, neurons were washed three times in PBS and permeabilized in 0.1 % (v/v) Triton X-100 for 5 min. After three more washes in PBS, cells were incubated with 5 % (w/v) BSA in PBS for 30-60 min, and then incubated with primary antibodies in the same solution for 1 hour. After three additional washes in PBS cells were incubated with appropriate secondary antibodies in 5 % (w/v) BSA in PBS for 1 hour in the dark, followed by three washes in PBS. In some cases fixed and permeabilized neurons were treated with RNase A (10 µg/ml) and RNase T1 (1000 units/ml) in PBS for 30 min at 37˚C before proceeding to immunostaining.

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**Figure legends**

**Figure 1. Presence of RNA granules in axons.** Rat primary cortical neurons were stained for RNA using SYTO 14 before immunostaining. (**A**) Axons were visualized by immunostaining using an NF-H antibody. RNA is present in granules in neuronal processes with RNA granules observed along the length of the axon (arrows). (**B**) Treatment with RNase A and RNase T1 abolishes SYTO 14 staining. Scale bars, 20 μm.

**Figure 2. Tau mRNA is present in RNA granules in axons.** Rat primary cortical neurons were stained for tau mRNA by FISH using an antisense DIG-labeled tau mRNA probe and subsequently immunostained using anti-DIG and anti-α-tubulin antibodies, the latter to visualize the outline of the cells. (**A**) tau mRNA is localized to the somatodendritic compartment as well as the axon (arrow). (**B**) Higher magnification image of the boxed region the axon in A. A granular staining pattern of tau mRNA is observed in the axon (arrows). (**C**) FISH was performed using the sense DIG-labeled tau mRNA probe. (**D**) No RNA probe was used during FISH, only antibodies. (**E**) Fixed and permeabilized neurons were treated with RNase A and RNase T1 before FISH using the antisense tau mRNA probe. No DIG signal was detected in any of the control conditions. Scale bars, (**A**, **C-E**) 20 μm; (**B**) 5 μm.

**Figure 3. Tau mRNA colocalizes with eEF1A in axons.** Rat primary cortical neurons were stained for RNA using SYTO 14 before immunostaining. (**A**) eEF1A is localized to the somatodendritic compartment as well as the axon (white arrow). (**B**) Higher magnification images of the boxed region of the axon in (**A**). A granular staining pattern of eEF1A is observed which colocalizes with SYTO 14-positive RNA granules (arrows). Neurons were stained for tau mRNA using FISH and subsequently immunostained using anti-DIG and anti-eEF1A antibodies (**C**, **D**). (**D**) Higher magnification images of boxed regions of the proximal and distal axon of the neuron in (**C**). Tau mRNA co-localizes with eEF1A in a granular staining pattern in both the proximal and distal axon (arrows). (**E**, **F**) Tau mRNA also colocalizes with eEF1A in growth cones (arrows), enlarged in (**F**). Scale bars, (**A**, **C**) 20 μm; (**B**, **D**, **E**) 10 μm; (**F**) 5 μm.





