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Tar DNA-binding protein-43 (TDP-43) regulates axon growth *in vitro* and *in vivo*☆

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ABSTRACT

Intracellular inclusions of the TAR-DNA binding protein 43 (TDP-43) have been reported in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD-TDP). Rare mutations in *TARDBP* have been linked to both ALS and FTD-TDP suggesting that TDP-43 dysfunction is mechanistic in causing disease. TDP-43 is a predominantly nuclear protein with roles in regulating RNA transcription, splicing, stability and transport. In ALS, TDP-43 aberrantly accumulates in the cytoplasm of motor neurons where it forms aggregates. However it has until recently been unclear whether the toxic effects of TDP-43 involve recruitment to motor axons, and what effects this might have on axonal growth and integrity. Here we use chick embryonic motor neurons, *in vivo* and *in vitro*, to model the acute effects of TDP-43. We show that wild-type and two TDP-43 mutant proteins cause toxicity in chick embryonic motor neurons *in vivo*. Moreover, TDP-43 is increasingly mislocalised to axons over time *in vivo*, axon growth to peripheral targets is truncated, and expression of neurofilament-associated antigen is reduced relative to control motor neurons. In primary spinal motor neurons *in vitro*, a progressive translocation of TDP-43 to the cytoplasm occurs over time, similar to that observed *in vivo*. This coincides with the appearance of cytoplasmic aggregates, a reduction in the axonal length, and cellular toxicity, which was most striking for neurons expressing TDP-43 mutant forms. These observations suggest that the capacity of spinal motor neurons to produce and maintain an axon is compromised by dysregulation of TDP-43 and that the disruption of cytoskeletal integrity may play a role in the pathogenesis of ALS and FTD-TDP.

Introduction

Amyotrophic lateral sclerosis (ALS) is a late-onset, relentlessly progressive neurodegenerative disease. TDP-43 is the major protein present in neuronal and glial inclusions in both sporadic and familial forms of ALS and tau-negative frontotemporal dementia (FTD-TDP), (Forman et al., 2007; Mackenzie et al., 2007; Neumann et al., 2006). We, and others, have shown that mutations in the C terminal domain of the gene encoding TDP-43 (*TARDBP*) are causally linked to familial and sporadic ALS and FTD-TDP (Blair et al., 2010; Chio et al., 2009; Corrado et al., 2010; Damme et al., 2010; Drepper et al., 2011; Gitcho et al., 2008; Kabashi et al., 2008; Kwiatkowski et al., 2009; Rutherford et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Vance et al., 2009; Yokoseki et al., 2008). Intriguingly, TDP inclusions are also found in a smaller proportion of patients with Alzheimer's and Parkinson's disease, but their role in the pathogenesis is uncertain (Nakashima-Yasuda et al., 2007).

TDP-43 is a predominantly nuclear protein with DNA and RNA binding properties. It has multiple roles in RNA-processing that are crucial in early development, as genetic deletion of TDP-43 is embryonic lethal between embryonic days 3 and 8 (Chiang et al., 2010; Kraemer et al., 2010; Sephton et al., 2010; Wu et al., 2010). TDP-43 can act as a transcriptional repressor and is implicated in exon skipping/retention, RNA transport and stability and micro RNA biogenesis (Casafont et al., 2009; Strong et al., 2007). In ALS and FTD-TDP, prominent TDP-43 aggregates are present in the cytoplasm of surviving neurons, which often show clearing of the protein from the nucleus (Neumann et al., 2006; Vance et al., 2009). However, it remains unclear whether the gain or loss of TDP-43 function is mechanistic in disease. In cellular and some animal models overexpressing TDP-43, deletion of the RNA binding and C terminal domains was required for toxicity rather than cytoplasmic mislocalisation (Voigt et al., 2010; Wegorzewska et al., 2009). It is possible that both the loss of nuclear TDP-43 and its sequestration in the cytoplasm lead to defective RNA processing (Lagier-Tourenne et al., 2009; Strong MJ, 2010). The extent to which TDP-43 is localised in axons has been uncertain, but a recent study shows its recruitment to motor axons, where it co-localises with a number of

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mRNA-binding proteins (Fallini et al., 2012). Given that TDP-43 binds to nearly one third of all neuronal RNA transcripts, its effects could impact a very wide array of cellular pathways, many of which are critical in neuronal development including components of the cytoskeleton (Polymenidou et al., 2011; Tollervey et al., 2011a, 2011b). For example, TDP-43 interacts directly with the 3′ UTR of the low molecular weight (68 kDa) neurofilament mRNA (NFL) (Strong et al., 2007), stabilising it and preventing its degradation. Conversely, in ALS the expression of the NFL mRNA is suppressed and it is preferentially sequestered to stress granules (Strong et al., 2007; Volkening et al., 2009).

We have previously shown that *in vivo* over-expression of TDP-43 mutant forms in the chick spinal cord analysed over short time intervals (24 h) leads to cell death and developmental delay (Sreedharan et al., 2008). Here we investigate the effects of TDP-43 mutant and wild-type forms on motor neuron integrity and axon outgrowth *in vitro* and *in vivo* over a longer period of time, using the chick embryo model. *In vivo*, we found that spinal motor neurons over-expressing TDP-43 (wild-type and mutant) showed a progressive increase in cytoplasmic and axonal TDP-43 mislocalisation over time. Those neurons expressing mutant TDP-43 were more likely to display prominent nuclear aggregates. A down-regulation of neurofilament-associated antigen (NAA) was observed in axons expressing mutant TDP-43, accompanied by de-fasciculation and truncation of the axon projections. When the same constructs were expressed in dissociated E6 spinal motor neurons, TDP-43 aggregation was observed along with a reduction in axon outgrowth. This was more prominent in cells expressing mutant forms than in those expressing wild-type TDP-43. Taken together, our data suggest that over-expression of TDP-43 (wild-type and mutant) leads to cytoplasmic and axonal mislocalisation of TDP-43 in motor neurons, and an impairment of axon growth.

Materials and methods

Plasmids

The N-terminal HA and C-terminal Myc constructs were cloned as described previously (Sreedharan et al., 2008; all cloned into the *pCI-neo* vector). For EGFP-tagged TDP-43 WT and mutant plasmids, the cDNA insert was cloned into the pEFGP-C1 (N-terminal GFP) vector at the XhoI and BamHI sites. Mutant forms of TDP-43 were those containing either the M337V familial mutation, or the Q331K sporadic mutation. Direct sequencing was used to confirm clone alignment and mutagenesis.

Embryos

Pathogen-free white Leghorn hens' eggs were incubated at 39 °C and 70% humidity. Embryos were staged according to Hamburger and Hamilton (1951) and electroporated at HH stage 14 (E2.5).

In ovo electroporation

In ovo electroporation was used to over-express the genes of interest in one half of the spinal cord. On the day of electroporation a small window was cut into the shell to expose the embryo. PBS containing 1% Penicillin–Streptomycin (Invitrogen) was added to keep the embryos moist. Plasmid DNA constructs were dissolved in ddH_2O to give a final concentration of 4.0 μg/μl. In all the experiments, *pEGFPC1* expression vector was used to over-express EGFP as a control. Visualisation of DNA during injection was aided by adding 0.5 μl of 10 mg/ml fast green to 10 μl of DNA solution. A modified method of Momose et al. (1999) was used, as previously described (Sreedharan et al., 2008). Eggs were reincubated for 24–96 h, after which surviving embryos were dissected and fixed using 4% paraformaldehyde for 1–2 h at room temperature.

Cryosections

Fixed embryos were washed with PBST (PBS (Invitrogen) + 0.1% Triton X-100 (Sigma)) 3 times for 30 min each at room temperature and processed through ascending concentrations of sucrose (Sigma) (10%, 20%, 30%) for 30 min each at room temperature. They were finally washed in 1:1 30% Sucrose:OCT and then in OCT (CellPath Ltd.) for 30 min each at room temperature. Embryos were then embedded in OCT, flash-frozen in iso-Pentane (Sigma) using liquid nitrogen and sectioned using a cryostat (Leica Microsystems) maintaining a thickness of 20 μm.

Immunofluorescence

Frozen sections or cells (adhered to glass cover slips) were washed in PBS, permeabilised using 0.5% and 0.05% PBST, respectively (1× PBS + 0.5% Triton X-100). Sections or cells were then blocked using 10% FCS/PBS for 1 h and incubated with primary antibodies overnight at 4 °C (rabbit anti-HA, 1:1000, Sigma; mouse anti-myc, 1:1000, NEB; mouse anti-islet1/2 (4D5), 1:200, DSHB; mouse anti-neurofilament associated protein (3A10), 1:200, DSHB; chicken anti-GFP, 1:1000, Abcam). Sections or cells were washed again with PBS, 3 times for 5 min each, before incubation with fluorescent secondary antibodies for 1–2 h at room temperature (anti-rabbit Alexa Fluor 488; anti-mouse Alexa Fluor 568; anti-mouse Alexa Fluor 488, anti-chicken Alexa Fluor 488, all 1:1000, all Invitrogen). After further washing, sections or cells were mounted using Hardset Vectashield with DAPI (Vector Laboratories). Images were captured using an Axiovert 4.0 (Carl Zeiss) microscope with Image-Pro Express 6.0 (Media Cybernetics). Adobe Photoshop 7.0 (Adobe Systems, Inc.) was used to process the images.

Tunel

Apoptotic cell death was visualized on frozen sections according to the manufacturer's protocol by nuclear DNA fragmentation analysis using a DeadEnd Colorimetric TUNEL System (Promega Corporation, USA). The number of apoptotic cells on the transfected and untransfected sides of the embryo spinal cords was counted and the data were inserted into GraphPad Prism to obtain the graphs. A total of 50–150 embryos were electroporated for each construct and the data were analysed from 10 to 20 sections across at least 6 embryos from 3 sets of experiments.

Neurite length quantification

ImageJ software was used for quantification of neurite length. Cells labelled with anti-GFP and anti-neurofilament antibodies were imaged using an Axiovert camera (Volocity software was used for image processing) at 10× magnification. Images were then used to analyse the total length of the neurites, length of longest and shortest neurites and number of neurite branches using the NeuronJ plugin of ImageJ. A total of ~150 cells per condition were analysed from 3 independent experiments.

Spinal motor neuron cultures

Glass coverslips were coated with poly-D-ornithine and laminin (15 μg/ml and 1 mg/ml, respectively; Sigma). Motor neuron cultures were performed as previously described (Murray et al., 2010). For preparation of spinal motor neurons, we used the ventral portion of the cervical and thoracic spinal cord, which is enriched in motor neurons at HH stages 28–29 (E6). Dissociated neurons were plated on laminin-coated coverslips, in Neurobasal medium containing 2% (v/v) B27 supplement, 2% (v/v) horse serum, 0.1% (v/v) β-mercaptoethanol, 0.35% (v/v) Glutamax, 1% (v/v) penicillin/streptomycin (all from Gibco), 1% (v/v)