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Mislocalization of TDP-43 in the G93A mutant SOD1 transgenic mouse model of ALS

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ABSTRACT

Previous evidence demonstrates that TAR DNA binding protein (TDP-43) mislocalization is a key pathological feature of amyotrophic lateral sclerosis (ALS). TDP-43 normally shows nuclear localization, but in CNS tissue from patients who died with ALS this protein mislocalizes to the cytoplasm. Disease specific TDP-43 species have also been reported to include hyperphosphorylated TDP-43, as well as a C-terminal fragment. Whether these abnormal TDP-43 features are present in patients with SOD1-related familial ALS (fALS), or in mutant SOD1 over-expressing transgenic mouse models of ALS remains controversial. Here we investigate TDP-43 pathology in transgenic mice expressing the G93A mutant form of SOD1. In contrast to previous reports we observe redistribution of TDP-43 to the cytoplasm of motor neurons in mutant SOD1 transgenic mice, but this is seen only in mice having advanced disease. Furthermore, we also observe rounded TDP-43 immunoreactive inclusions associated with intense ubiquitin immunoreactivity in lumbar spinal cord at end stage disease in mSOD mice. These data indicate that TDP-43 mislocalization and ubiquitination are present in end stage mSOD mice. However, we do not observe C-terminal TDP-43 fragments nor TDP-43 hyperphosphorylated species in these end stage mSOD mice. Our findings indicate that G93A mutant SOD1 transgenic mice recapitulate some key pathological, but not all biochemical hallmarks, of TDP-43 pathology previously observed in human ALS. These studies suggest motor neuron degeneration in the mutant SOD1 transgenic mice is associated with TDP-43 histopathology.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease selectively affecting motor neurons of the brainstem and spinal cord, as well as cortical regions. Approximately 5–10% of ALS cases are inherited (fALS), however, the cause of the remaining 90–95% of sporadic ALS (sALS) cases remains unknown. Mutations of superoxide dismutase 1 (SOD1) account for approximately 15–20% of fALS cases (1–2% of all ALS cases), whereas various other gene mutations account for only a small subset of the remaining fALS cases [9]. Nervous system tissue from patients who died with fALS and sALS has been extensively studied to gain insight into the pathological features of ALS. To study the pathogenesis and investigate the details of the progression of ALS, transgenic rodent models harboring fALS-associated mutations in human mutant SOD1 (mSOD) have been developed. These models have proven to be of significant value [8] and replicate several key features observed in both sALS and fALS including progressive loss of motor neurons, neurofilament aggregation, and the accumulation of cytoplasmic ubiquitinated inclusions (UBIs) within degenerating motor neurons [5,20].

Recently, two independent groups [1,14] have reported that TAR DNA binding protein (TDP-43), a nuclear DNA and RNA binding protein proposed to function as a regulator of transcription [16] and alternative splicing [2,13] is present in UBIs in sALS. Furthermore, a number of TDP-43 mutations have been reported in both sALS and fALS cases, suggesting that TDP-43 may play a causal role in the pathogenesis of ALS [19,22,23]. TDP-43 is normally localized to the nucleus, however, in CNS tissue from patients who died with ALS, TDP-43 is redistributed from the nucleus to the cytoplasm, where it appears to be distributed diffusely, or to aggregate as a component of UBIs [1,14]. Although studies of TDP-43 in human ALS cases are generally consistent [1,4,12,14], some aspects of the TDP-43 pathology remain controversial. For instance, Mackenzie et al. [11] suggest that abnormal localization of TDP-43 is present in most sALS and fALS cases but is absent in fALS caused by SOD1 mutations. In contrast, Robertson et al. [17] showed, in two fALS cases carrying SOD1 mutations, that there is mislocalization of TDP-43 to the cytoplasm as well as association with UBIs. Studies of some lines of mSOD mouse models have claimed that there is no TDP-43

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redistribution in these mice [17,21], although very recently, Kiaei et al. [10] reported in abstract form that many neurons from lumbar spinal cords of mSOD mice do show cytoplasmic TDP-43 inclusions similar to those seen in CNS tissue from patients with ALS. Given the discrepancies between previous reports and because transgenic mSOD mice have been employed extensively to study ALS, we have evaluated whether pathological changes in TDP-43 localization are found in G93A mSOD mice.

Transgenic mice expressing human G93A mutant SOD1 were purchased from Jackson Laboratories (B6.Cg-Tg(SOD1-G93A)1Gur/J, stock# 004435) or bred locally with C57BL6 female mice. Mice were genotyped using PCR [6] and wild-type (WT) littermates were used as controls in these studies. Protocols governing the use of animals were approved by the Animal Care Review Committee of Simon Fraser University and were in compliance with guidelines published by the Canadian Council on Animal Care (CCAC). A minimum of 3 mice per group were used in these studies. We defined end stage for mSOD mice as the appearance of a set of behavioral markers including an inability to forage due to paralysis of the hind limbs and an inability of the mice to right themselves within 10 s of lateral recumbency. Mean survival time of the mSOD mice reaching end stage was 178 ± 14 days (mean $\pm$ S.E.M., $n = 9$).

Animals were culled with CO_2, perfused transcardially with PBS, and subsequently with a 4% solution of paraformaldehyde (PFA) in PBS. The spinal cords were dissected out and postfixed in 4% PFA, left overnight in a solution of 20% sucrose in PBS for cryoprotection, and subsequently embedded in Tissue-Tek O.C.T compound (Sakura, Zoeterwoude, Netherlands). Transverse lumbar spinal cord sections of 50 μm were cut using a Leica cryostat. Sections were treated with PBS containing 0.3% Triton X-100 (PBST) for permeabilization, followed by blocking with 5% BSA and 10% NGS. Anti-TDP-43 rabbit polyclonal antibody (Proteintech, 10782-2-AP) was diluted at 1:500 in PBST and incubated with free floating cryo-sections overnight at 4 °C. For double labelling experiments, anti-NeuN mouse monoclonal antibody (Chemicon, MAB378), diluted at 1:1000, anti-ubiquitin mouse monoclonal antibody (Chemicon, MAB1510) diluted in 1:500 were used, and immunoreactivities of interest detected using appropriate Cy3- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Sections were also counterstained for nucleus with 100 ng/ml DAPI (4′-6-diamidino-2-phenylindole, Vector Laboratories). Immunolabelled spinal cord sections were imaged and analyzed using a Leica DM4000B microscope and images captured using a Spot digital camera (DFC350FX, diagnostic Instruments, Sterling Heights, MI) and Leica Application Suite (LAS2.5.0 R1). Labelling of mSOD transgenic mouse tissue was compared to tissues obtained from age-matched WT littermates. Control experiments were carried out in parallel using sections incubated with only secondary antibody and no primary antibody.

Protein lysate was extracted from fresh frozen spinal cord tissue using modified RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing a cocktail of protease/phosphatase inhibitors (1 μg/mL aprotinin; 1 μg/mL leupeptin; 1 μg/mL pepstatin; 1 mM PMSF; 2 mM Na_3VO_4; 1 mM NaF), added to the buffer just before use, to generate a solution containing 0.1 mg tissue/mL. The tissue was homogenized on ice and then the mixture was centrifuged at $17{,}900 \times g$ for 15 min at 4 °C. The resulting supernatants were diluted with Laemmli sample buffer, boiled for 5 min, and separated using 10% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes at 100 V for 1.5 h. Membranes were then blocked for 1 h with 1% BSA or gelatin dissolved in PBS containing 0.1% Tween 20 (PBS-T) and then incubated with the specified primary antibodies overnight at 4 °C. The membrane was washed with PBS-T and blocked for another 0.5 h after which the membranes were incubated with the appropriate HRP-conjugated secondary antibodies for 1 h. Membranes were then washed with PBS-T and immunoreactive proteins visualized using ECL reagents and film (Amersham Bioscience, Piscataway, NJ).

Frozen lumbar spinal cords were weighed and sequentially extracted as previously described with slight modifications [14]. In brief, tissues were extracted at 200 mg/mL in low salt (LS) buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% sucrose, and a cocktail of protease inhibitors) by sonicating twice for 20 s and then centrifuging the resulting mixture at $25{,}000 \times g$ for 30 min at 4 °C. Pellets were sequentially extracted in high salt buffer containing Triton (TX) (LS containing 1% Triton X-100 and 0.5 M NaCl), sarkosyl-containing buffer (SA) (LS containing 1% N-lauroylsarcosine and 0.5 M NaCl) and urea-containing buffer (UR) (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris, pH 8.5), followed by Western blot analysis as described above.

We examined lumbar spinal cords from 12-week-old mSOD mice for NeuN and TDP-43 histopathology, screening all stained sections visually for abnormal neuronal TDP-43 localization (Fig. 1). NeuN immunolabelling of 12-week-old mSOD ventral horn grey matter showed many healthy neurons resembling age-matched WT mice (Fig. 1a and d). TDP-43 immunoreactivity was observed exclusively in the nucleus in tissues from both mSOD and WT mice of this age (Fig. 1b, c, e and f). To more comprehensively evaluate potential TDP-43 pathology, we examined end stage G93A mSOD mice. Strikingly, motor neurons in lumbar spinal cords from end stage mSOD mice (Fig. 1g–i), but not those of WT animals (Fig. 1j–l) of the same age, showed strong cytoplasmic labelling with anti-TDP-43 antibodies. Furthermore, some of these motoneurons having cytoplasmic TDP-43 immunoreactivity showed axonal TDP-43 immunoreactivity that was sometimes also accompanied by punctate nuclear TDP-43 staining (Fig. 1m). Within the ventral horn of lumbar spinal cord, some TDP-43 immunoreactivity appeared in dystrophic neurite-like structures as well as rounded inclusions resembling Lewy body-like hyaline inclusions (LBHIs) (Fig. 1h), both of which have been previously observed and used to define TDP-43 pathology in human ALS tissues [1,14]. In addition, some surviving motor neurons displaying normal nuclear TDP-43 immunoreactivity are observed adjacent to neurons exhibiting a pathological cytoplasmic distribution of TDP-43 (Fig. 1h). Previous studies of spinal cord tissue from patients who died with ALS have shown that some TDP-43 immunoreactive inclusions also contain ubiquitin, and that TDP-43 immunoreactivity is associated with the periphery of the ubiquitin-containing inclusions [1,3]. It also has been reported that LBHIs are immunoreactive for TDP-43 and ubiquitin in neurons from a sALS patient [15]. To investigate the similarities between G93A mSOD mice and these human ALS cases we analyzed ubiquitin immunoreactivity and its relation to TDP-43-positive structures in end stage mice. Immunohistochemistry using a ubiquitin-specific monoclonal antibody revealed strong ubiquitin immunoreactivity most frequently associated with rounded TDP-43-positive structures (Fig. 1n–s), but this was not associated with other TDP-43-positive structures such as dystrophic neurites. Therefore, the TDP-43 histopathological features of end stage G93A mSOD mice recapitulate those observed in human ALS cases.

These observations suggest TDP-43 redistribution and aggregation occurs in G93A mSOD mice. We therefore assessed whether abnormal TDP-43 species were present in these end stage mSOD mice using Western blotting. First we examined TDP-43 protein expression in spinal cord tissue by Western blot. Full-length TDP-43 and a lower migrating species were detected in lysates obtained from both mSOD and WT littermate mice (Fig. 2a), indicating no difference in expression levels of TDP-43 between mSOD mice and WT controls. The identity of the rapidly migrating species remains unknown. Secondly, spinal cords were sequentially extracted in buffers of increasing ionic and detergent strength, essentially as previously described, and these fractions were then analyzed by

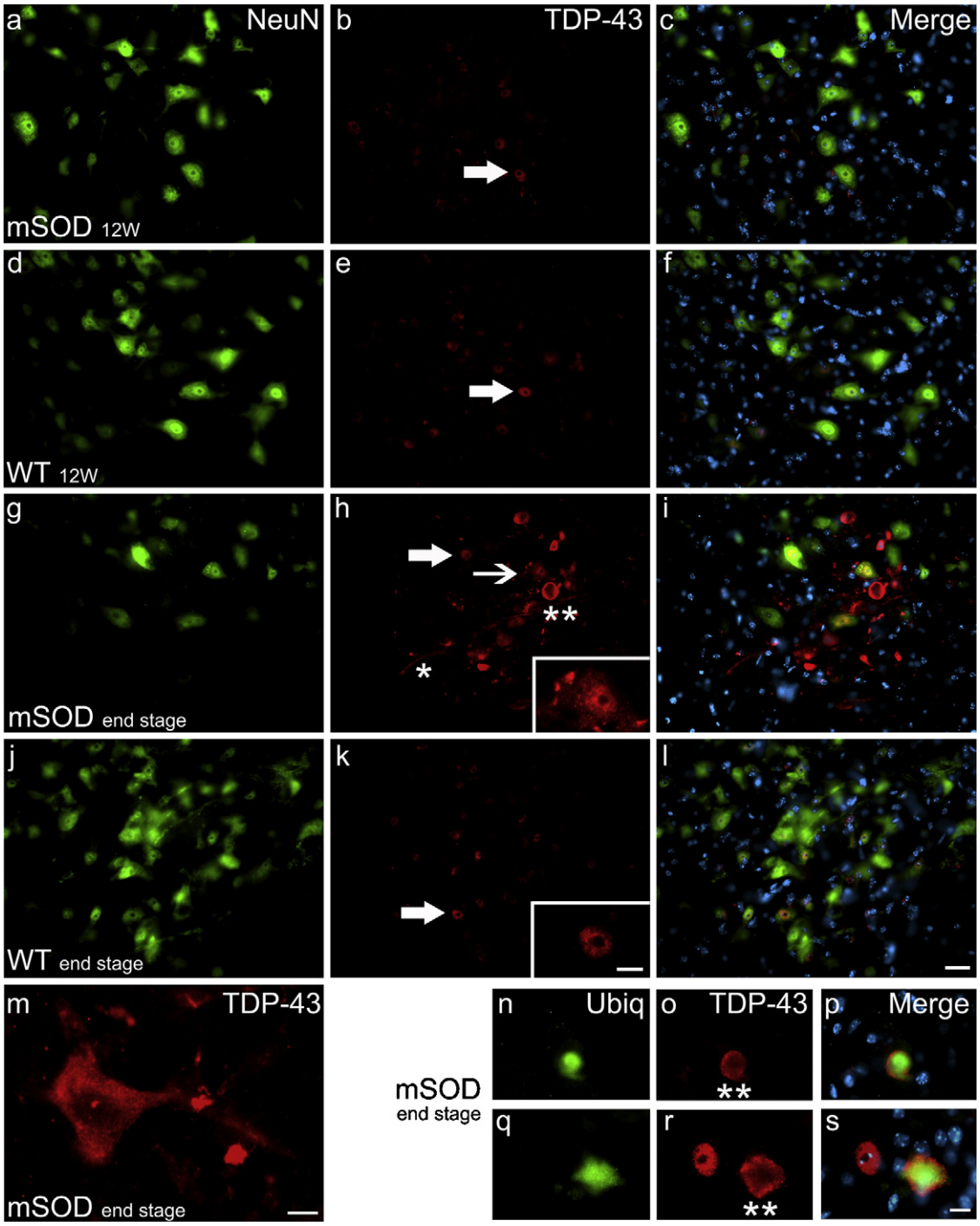

Fig. 1. TDP-43 redistribution in end stage mSOD lumbar spinal cord ventral horn cells. mSOD mice and age-matched wild-type (WT) mice were double labelled with NeuN (green) and TDP-43 (red) antibodies. (a–f) At 12 weeks of age, TDP-43 remains localized in the nucleus (large arrow) of ventral horn cells and shows no distinct aggregates in either mSOD (a–c) or wild-type mice (d–f). (g–s) At end stage, TDP-43 aggregates and redistributes from the nucleus to the cytoplasm (small arrow) in some neurons observed in sections from mSOD mice (g–i). In some ventral horn motor neurons punctate nuclear and axonal staining is observed (m) and dystrophic neurite-like (*) and rounded structures (**) are also labelled (h, o and r). Many TDP-43 positive rounded structures colocalize with ubiquitin (Ubiq, green) (n–s). Mislocalized cytoplasmic TDP-43 is not observed in WT mice of the same age as end stage mSOD mice (j–l). Scale bars: a–l = 25 μm; m–s and inserts = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Western blot. No differences in the abundance or solubility of TDP-43 in different fractions from end stage mSOD and age-matched WT samples could be detected. Furthermore, we observed no pathological ~25 kDa species nor ~45 kDa hyperphosphorylated species, which have been previously detected in human ALS (Fig. 2c).

Recent reports have shown that TDP-43 is mislocalized in affected neurons in CNS tissue from ALS patients, moving from normal localization in the nucleus to the cytoplasm. In addition to TDP-43 mislocalization, both a ~25-kDa C-terminally cleaved fragment and a ~45-kDa hyperphosphorylated TDP-43 species have been reported in urea-soluble protein extracts from ALS tissues [14]. In this work, we have investigated whether similar abnormalities in TDP-43 localization occur in transgenic mice expressing the G93A mutant form of SOD1. In contrast to a previous report on mSOD mice, we observed a redistribution of TDP-43 to the cytoplasm of motor neurons in mutant SOD1 transgenic mice. The differing

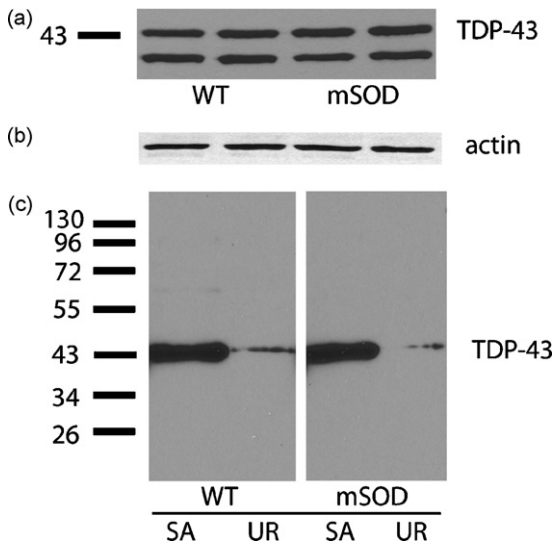

Fig. 2. TDP-43 protein levels in lumbar spinal cord do not change in end stage mSOD mice. (a–b) Western blots of total soluble protein from lumbar spinal cords using rabbit anti-TDP-43 show immunoreactive ∼43 kDa bands. (a) No significant differences between end stage mSOD mice and age-matched WT mice were observed; (b) protein loading is equivalent based on Western blots of lysates using an anti-actin monoclonal antibody; (c) Immunoblot of sequential extracts from lumbar spinal cords. No pathological species detected as ∼45 or ∼25 kDa bands are observed in tissues from mSOD mice (SA, sarkosyl; UA, urea).

results may stem from the stage of the mSOD mice from which spinal cord sections are derived. Consistent with this possibility, it has been suggested that some of the TDP-43 pathological features observed in ALS patients may need time to develop [18]. Previous studies of mSOD mice at 120 days [21], or at 150 days of age [17] have shown normal nuclear TDP-43 localization. The line of mSOD mice studied in this work (B6.Cg-Tg(SOD1-G93A)1Gur/J, stock# 004435) is related to the line studied previously by two other laboratories [17,21]. Turner et al. [21] reported studies using a G93A mSOD line bred with C57BL6 females, providing (B6SJL-Tg(SOD1-G93A)1Gur/J, stock# 002726) transgenic mice, which shows disease onset at ∼90–100 days and a lifespan of ∼110–120 days. Robertson et al. [17] used the same G93A mSOD line bred with C57BL6 providing (B6.Cg-Tg(SOD1-G93A)1Gur/J) transgenic mice, which show disease onset at ∼4.5 months and a life expectancy of ∼5 months. Consistent with these previous studies we also observed only normal TDP-43 localization in mSOD mice of 12 weeks of age but observed TDP-43 pathology in those mice with an extended lifespan of ∼180 days having advanced to end stage disease. Interestingly, despite pathological TDP-43 relocalization, we failed to observe C-terminal TDP-43 fragments (∼25 kDa) and TDP-43 hyperphosphorylated species (∼45 kDa) in these end stage mSOD mice. Furthermore, we also find rounded TDP-43 immunoreactivity (LBHIs) in lumbar spinal cord tissues in end stage mSOD mice associated with ubiquitin inclusions, indicating that at least as far as histopathological features are concerned, all facets of human ALS are present in end stage mSOD mice. One possible interpretation for the lack of apparent biochemical changes in TDP-43 in G93A mSOD mice, including the lack of TDP-43 C-terminal fragments in spinal cords of mSOD mice, could be that brain TDP-43 inclusions are enriched in C-terminal fragments, whereas spinal cord inclusions comprise mostly full-length TDP-43; a suggestion first made by Igaz et al. to account for related observations made using human ALS tissues [7]. Another possibility is that biochemical changes are less prominent in the mice than in human tissue and that it is more difficult to detect these changes, given the small volume of tissue available for study in mice.

In brief, our findings suggest that the mutant SOD1 transgenic mice (G93A) do recapitulate some pathological features, but not all of the biochemical hallmarks, of TDP-43 pathology that have been observed in human ALS. These studies suggest that motor neuron degeneration occurring in mutant SOD1 transgenic mice is associated with TDP-43 histopathology. Given that TDP-43 pathology in G93A mSOD mice of advanced age does recapitulate features observed in human ALS cases, this animal model may be a valuable tool for studying the progression and pathophysiology of ALS and as a useful model to test potential TDP-43-directed therapeutics.

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